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Further Genetic Analysis of the Activation Function of the TyrR Regulatory Protein of *Escherichia coli*

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Previous reports (J. Cui and R. L. Somerville, J. Bacteriol. 175:1777–1784, 1993; J. Yang, H. Camakaris, and A. J. Pittard, J. Bacteriol. 175:6372–6375, 1993) have identified a number of amino acids in the N-terminal domain of the TyrR protein which are critical for activation of gene expression but which play no role in TyrR-mediated repression. These amino acids were clustered in a single region involving positions 2, 3, 5, 7, 9, 10, and 16. Using random and site-directed mutagenesis, we have identified an additional eight key amino acids whose substitution results in significant or total loss of activator function. All of these are located in the N-terminal domain of TyrR. Alanine scanning at these eight new positions and at five of the previously identified positions for which alanine substitutions had not been obtained has identified three amino acids whose side chains are critical for activation, namely, D-9, R-10, and D-103. Glycine at position 37 is also of critical importance. Alanine substitutions at four other positions (C-7, E-16, D-19, and V-93) caused partial but significant loss of activation, indicating that the side chains of these amino acids also play a contributing role in the activation process.

The TyrR protein of *Escherichia coli* K-12 is a transcriptional regulator which plays a central role in the control of a group of at least eight transcription units (TyrR regulon) encoding proteins involved in biosynthesis and transport of the three aromatic amino acids (for a review, see reference 18). Although the TyrR protein functions primarily as a negative regulator, it can also activate transcription from the promoters (σ^{70}) of the *mtr* and the *tyrP* genes (9, 10, 22, 23). To activate transcription of the *mtr* and *tyrP* genes, the TyrR protein binds to its DNA targets (TyrR boxes), whose centers are located, respectively, 79 and 65 bp upstream from their transcription start sites. In the case of *tyrP*, the TyrR box (box 2) which is responsible for activation is not positioned optimally for activation (1). Repositioning the TyrR box 2 by moving it 3, 4, 13, or 14 bp further upstream, in which case the center of the TyrR box 2 is situated 68, 69, 78, or 79 bp upstream from the transcription start site, results in maximal activation of *tyrP* by TyrR (1). Recently, it has been shown in an in vitro transcription system that purified TyrR protein, in the presence of phenylalanine, can exert a twofold activation of transcription from the *tyrP* promoter. This TyrR-mediated activation occurred with wild-type RNA polymerase but did not occur when a mutant RNA polymerase with a C-terminally truncated α subunit (α-235) was used to carry out the transcription reaction (14). On the basis of this observation, TyrR has been proposed to interact with the RNA polymerase α C-terminal domain and is therefore classed as a class 1 transcription activator.

The TyrR monomer is a polypeptide of 513 amino acid residues, and the protein exists as a homodimer in solution (2, 26). Limited proteolysis studies by Cui and Somerville (7) have indicated that the TyrR protein has three structural domains. The C-terminal domain (amino acids 468 to 513) contains a Cro-like α-helix–turn–helix DNA binding motif (26). The central domain (amino acids 191 to 467), which is homologous to those of the NtrC family of transcriptional regulators, appears to contain an ATP binding site and an ATP-dependent aromatic amino acid binding site (13, 26). These two ligand binding sites are both closely associated with the repression function of TyrR protein. Using UV spectroscopy and a sedimentation velocity meniscus depletion method, Wilson et al. have shown that the TyrR protein contains a separate ATP-independent aromatic amino acid binding site (24). However, the location of this ligand binding site is yet to be determined. The N-terminal domain (amino acids 1 to 190) of TyrR is not homologous to any known procaryotic transcriptional regulators. Functional mapping by deletion mutagenesis has located the activation function to the N-terminal domain (6, 26). By making specific amino acid substitutions, we (25) and others (5) have identified a number of amino acid residues at positions 2, 3, 5, 7, 9, 10, and 16 as playing an important role in activation. As previous functional mapping of TyrR was carried out by site-directed mutagenesis and was limited to the N-terminal region, two questions remained to be answered: (i) is the N-terminal domain the only region which is directly involved in activation, and (ii) are there any other amino acid residues in the N-terminal domain which are essential for activation?

In this study, we sought to answer these questions by performing random mutagenesis either on the entire *tyrR* gene or within the region of the *tyrR* gene which includes the promoter and the coding sequence for the first 144 amino acids. In addition, we carried out alanine-scanning mutagenesis to probe if amino acid side chains of the key residues in the N-terminal domain are directly involved in activation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. The bacterial strains used in this study were all derivatives of *E. coli* K-12. The relevant characteristics of the bacterial strains and plasmids used are described in Table 1.

Media and chemicals. The minimal medium used was prepared from half-strength buffer 56 described by Monod et al. (17) and supplemented with appropriate growth requirements. To study regulation, tyrosine or phenylalanine was added to the minimal medium at a final concentration of 1 mM. Trimethoprim was used in nutrient and minimal media at final concentrations of 40 and 10 μg/ml, respectively. Kanamycin was used at a final concentration of 10 μg/ml in both minimal and nutrient media. The chemicals used were all obtained commercially and not purified further. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at a final concentration of 25 μg/ml. [α-^{35}S]dATP

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TABLE 1. *E. coli* K-12 strains, plasmids, and phages used in this study

Strain, plasmid or phage	Characteristics	Source or reference
Strains		
JM101	Δ(*lac-pro*) *thi supE*/F′*traD36 proA*$^+$*B*$^+$ *lacI*^{q} Δ*lacZM15*	15
JP4822	*tyrR366 thr-1 leu-1* Δ*lacZM15* λ$^+$ *thi-1 aroL478*::Tn*10 hsdR gal-351 supE44 gyr A379 tonA2*	26
JP8042	tyrR366 ΔlacU169 recA56	26
XLI-Red	*endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT* Tn*10* (Tet^r)	Stratagene
Phages		
M13tg130	*lacPOZ′*	11
mpMU36	2.2-kb *tyrR* fragment in M13tg130	This study
mpMU37	mpMU36 containing a *Hin*dIII site in the *tyrR*$^+$ gene	This study
Plasmids		
pMU1065	Km^r; 1.77-kb *Eco*RV-*Pvu*II fragment containing the *tyrR*$^+$ gene in the *Hin*dII site of pACYC177 (4)	E. Cornish, this laboratory
pMU2055	Tp^r; *tyrP+4-lacZ* transcriptional fusion; 0.25-kb *tyrP* fragment, with the insertion of 4 bases between the two TyrR boxes, in pMU575 (27)	1
pMU2493	Tp^r; *aroF-lacZ* transcription fusion; *aroF* fragment in pMU575 (27)	B. Dickson
pMU3190	Tp^r; *mtr-lacZ* transcriptional fusion; 189-kb *mtr* fragment in pMU577 (21)	26
pMU6218	Km^r; pSU39 (3) with *Hin*dIII site in polylinker cloning region eliminated	This laboratory
pMU6219	Km^r; 2.2-kb *Eco*RI-*Pst*I *tyrR*$^+$ fragment from mpMU37 in pMU6218	This study

(1,200 Ci/mmol; 10 mCi/ml) for DNA sequencing was obtained from Amersham Corp. Oligonucleotides were synthesized on a Gene Assembler Plus (Pharmacia-LKB, Uppsala, Sweden).

Recombinant DNA techniques. Standard recombinant DNA procedures were carried out as described by Sambrook et al. (19). DNA sequences were determined by the chain termination method of Sanger et al. (20), with T7 DNA polymerase (Pharmacia).

Site-directed mutagenesis. In vitro mutagenesis with synthetic oligonucleotides was carried out with a commercial kit obtained from U.S. Biochemical Corp., and mutations were confirmed by DNA sequence analysis.

β-Galactosidase assay. β-Galactosidase activity was assayed according to the method described by Miller (16). Specific activity is expressed in units defined by Miller (16).

Construction of a *Hin*dIII site in the *tyrR*$^+$ gene. The 2.2-kb *Sca*I-*Bam*HI fragment containing the wild-type *tyrR*$^+$ gene from plasmid pMU1065 (26) was cloned into the *Eco*RV-*Bam*HI sites of M13tg130 to form mpMU36. Site-directed mutagenesis was performed on a single-stranded template of mpMU36 by using a synthetic oligonucleotide primer (5′TTATGCGAAGCTTGCGGT3′) to generate a *Hin*dIII site across codons 143 to 145, and this procedure resulted in an aspartate-to-alanine change at position 144 of the TyrR protein. This M13 derivative was named mpMU37.

Construction of deletion mutations of TyrR. The four internal deletion mutations of TyrR (Δ40-49, Δ50-59, Δ60-69, and Δ90-99) were constructed by site-directed mutagenesis with single-stranded mpMU37 as a template and the following four oligonucleotides as primers: 5′CAGACTGCTGAAACTGAT TCGCCCAATGGG3′, 5′AACACCCGCAATACGCTCAAACTCCAGTTC3′, 5′AGGCATCCACGGGACGCGTATTTCGGCCAT3′, and 5′CATATCCACT TTGCTCAACGCCTCCAGTAA3′. Following DNA sequence analysis, each of the 0.7-kb *Eco*RI-*Hin*dIII fragments containing the *tyrR* promoter and the coding sequence for the N-terminal region of TyrR was cloned into the same sites of pMU6219 to replace the corresponding fragment.

PCR random mutagenesis of the TyrR N-terminal region. The PCR mutagenesis was performed on the single-stranded DNA template of the recombinant M13 phage mpMU37. The M13 reverse sequencing primer and the oligonucleotide which was used to generate the *Hin*dIII site in the *tyrR*$^+$ gene were used as primers to amplify a 0.7-kb fragment which contains the upstream region of the *tyrR*$^+$ gene and the coding sequence for the first 147 amino acids of the TyrR protein. Several independent PCRs were carried out in a volume of 100 μl for 30 cycles in the presence of 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 1 mM $MgCl_2$, 0.01% gelatin, 2.5 U of *Taq* DNA polymerase, 100 ng of each of the DNA primers, 10 ng of mpMU37, and the following combinations of nucleotides: 150 μM (each) dA, dT, and dC and 15 μM dG; 150 μM (each) dA, dT, and dG and 15 μM dC; 150 μM (each) dA, dT, and dC and 40 μM dG; or 150 μM (each) dA, dT, and dG and 40 μM dC. The resultant DNA fragments were digested with *Eco*RI and *Hin*dIII, purified on a low-melting-point agarose gel, and ligated into the *Eco*RI and *Hin*dIII sites of pMU6219 to replace the corresponding DNA fragment. The ligation mixtures were transformed separately into JP4822 containing pMU3190 (*mtr-lac* fusion) and plated out on X-Gal minimal plates containing tyrosine (1 mM), trimethoprim, and kanamycin. Plasmid DNA was extracted from the pale colonies. To separate plasmid pMU3190 and the pMU6219 derivatives which contain putative mutant *tyrR* alleles, the plasmid DNA mixtures extracted were each transformed into JM101 and the transformants were plated out on nutrient plates supplemented with kanamycin. The colonies obtained were screened for their sensitivity to trimethoprim. Isolates which were resistant to kanamycin and sensitive to trimethoprim were chosen for preparation of plasmid DNA. Each of the pMU6219 derivatives was then transformed into three strains, JP8042/pMU3190, JP8042/pMU2055, and JP8042/pMU2493.

Each of the 0.7-kb *Eco*RI and *Hin*dIII fragments containing part of the *tyrR* gene was purified from these pMU6219 derivatives and cloned into M13tg130. The DNA sequence of each of the 0.7-kb fragments was determined.

Construction of amino acid substitutions in the N-terminal region of TyrR. Various amino acid substitutions were constructed essentially as described previously (25) with the following modification. The single-stranded form of mpMU37 was used as a template for site-directed mutagenesis, and following DNA sequencing, each of the 0.7-kb *Eco*RI and *Hin*dIII fragments containing part of the *tyrR* gene was cloned into the same sites of pMU6219 to replace the corresponding fragment.

Random mutagenesis with a mutator strain. Random mutations in the *tyrR*$^+$ (*Hin*dIII) gene were generated by transforming pMU6219 into competent cells of XLI-Red, obtained from Stratagene, according to the manufacturer's instructions. Kanamycin-resistant colonies were selected; individual colonies were then cultured overnight in rich medium containing kanamycin, subcultured, and again grown overnight; and these cultures were used to prepare DNA.

RESULTS

Construction of a *Hin*dIII site in *tyrR*. To facilitate the cloning and sequencing of a fragment which contains the *tyrR* regulatory region and the coding sequence corresponding to the N-terminal region of the TyrR protein, we have introduced a *Hin*dIII site across amino acid codons 143 to 145 by site-directed mutagenesis (see Materials and Methods). This procedure resulted in substitution of alanine for aspartate at position 144 of the TyrR protein. The resultant TyrR protein was shown to have a wild-type phenotype with respect to both repression and activation. The DNA fragment carrying the *tyrR* gene with the *Hin*dIII site was subsequently used as a template for mutagenesis.

Deletion mutations of *tyrR*. Previously we have made three internal deletions within TyrR which removed residues 10 to 19, 20 to 29, or 30 to 39, and we showed that proteins with each of these deletions are defective in phenylalanine- and tyrosine-mediated activation of *mtr* and *tyrP+4* (25). To further characterize the activation domain of TyrR, we used site-directed mutagenesis to construct four new internal deletions of residues 40 to 49, 50 to 59, 60 to 69, and 90 to 99 (Fig. 1; See Materials and Methods). The 0.7-kb *Eco*RI-*Hin*dIII fragment carrying each of the mutations was cloned into plasmid pMU6219 to replace the corresponding fragment of the wild-

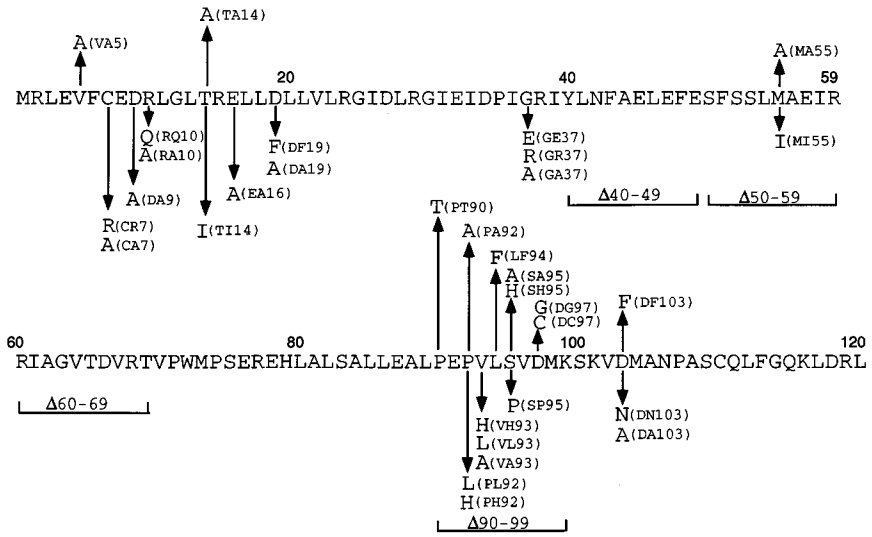

FIG. 1. Description of various amino acid substitutions and deletions in the N-terminal region of the TyrR protein. The first 120 amino acid residues of the TyrR protein of *E. coli* K-12 are shown. The amino acid substitutions and deletions which affect the ability of TyrR to activate transcription are shown below the TyrR sequence. The substitutions which do not significantly affect transcription activation are shown above the TyrR sequence.

type *tyrR*$^+$ gene. Each of the pMU6219 derivatives was transformed into strain JP8042 (*tyrR366* Δ*lacU169 recA56*) carrying one of the three *lacZ* fusion plasmids, pMU3190 (*mtr-lacZ* fusion), pMU2055 (*tyrP+4-lacZ* fusion), or pMU2493 (*aroF-lacZ* fusion). Strains which contained the *mtr-lacZ* or *tyrP+4-lacZ* plasmid were used to analyze TyrR-mediated activation, whereas the strain with the *aroF-lacZ* fusion was used to measure TyrR-mediated repression. β-Galactosidase activities of these strains were assayed, and the effects of these deletion mutations on activation and repression are shown in Table 2. All the deletions caused abolition of TyrR-mediated activation at both the *mtr* and *tyrP+4* promoters. The TyrR-mediated repression of *aroF*, however, was unaltered by these deletions.

PCR-mediated random mutagenesis of *tyrR*. To isolate random positive-control (PC) mutations in the N-terminal region of TyrR, PCR mutagenesis with *Taq* DNA polymerase was carried out (see Materials and Methods). A number of possible *tyrR* PC mutants were obtained. The plasmids carrying each of the putative PC mutant *tyrR* alleles were transformed into JP8042 carrying the *aroF-lacZ* fusion to check for repression. These PC mutants were classified into two groups based on their phenotype with respect to repression, the first group being mutants which were defective in *aroF* repression and the second group being mutants with normal repression of the *aroF* promoter. Sequencing analysis of six mutants from group 1 revealed that all had mutations which caused major perturbations in the TyrR protein or its formation (four had single-base deletions or insertions which caused a frameshift within *tyrR*, and two had single-base changes which led to the substitution of either isoleucine for methionine at position 1 or a stop codon for arginine at position 10). Ten independent isolates from group 2 were sequenced, and amino acid changes caused by these mutations are shown in Fig. 1. Four of them produced an identical single-base change, changing arginine to glutamine at position 10 (RQ10). Three others had identical mutations changing threonine to isoleucine at position 14 (TI14). Two had mutations changing proline to leucine at position 92 (PL92), and one had an aspartate-to-asparagine change at position 103 (DN103).

The effects of these four different mutations on transcription from the three promoters, *mtr*, *tyrP+4*, and *aroF*, were quantified by β-galactosidase assay. As expected, all four mutants were defective in transcription activation at both the *mtr* and *tyrP+4* promoters (Table 2), and they all exhibited about the same degree of repression at the *aroF* promoter as the wild-type *tyrR*$^+$ control (Table 2). With regard to mutants RQ10, TI14, and PL92, the defect in activation was complete or almost complete. The defect in activation for mutant DN103, however, was less severe and was influenced by the amino acid used as the effector. At the *mtr* promoter, we observed 12- and 3-fold reductions in activation in the presence of tyrosine and phenylalanine, respectively. At the *tyrP+4* promoter, the defect was about threefold with tyrosine but was only marginal with phenylalanine as the effector.

Site-directed mutagenesis of *tyrR*. To further characterize the role in activation of the region between residues 90 and 103, we made one or more point mutations at positions 90, 92, 93, 94, 95, 97, and 103 (Fig. 1). This was carried out by site-directed mutagenesis using mixed oligonucleotides which allow the change of one amino acid residue to a variety of other residues (see Materials and Methods). Although this method has the potential to produce a number of substitutions at each position, in most of our cases, single substitutions have been isolated. These mutations are listed in Table 2. Functional analysis in vivo showed that all mutants that were isolated maintained wild-type repression of the *aroF* promoter (Table 2). Mutant SP95 was completely unable to activate transcription, and mutants PH92, VL93, and VH93 showed major decreases in activation from either the *mtr* or *tyrP+4* promoter (Table 2). The levels of activation at the *mtr* promoter were largely unchanged with mutants SH95 and DG97, which showed slight increases in activation (1.5- to 2-fold) at the *tyrP+4* promoter (Table 2). Mutants PT90, DC97, and DF103 showed enhanced activation from both the *mtr* and the *tyrP+4* promoters (Table 2). In the case of mutant LF95, the activation efficiency was slightly decreased at the *mtr* promoter and unchanged at the *tyrP+4* promoter (Table 2).

Possible involvement of aspartate 19. The first 20 amino acids of the TyrR protein contain 5 negatively charged amino acids, E-4, E-8, D-9, E-16, and D-19. We have reported pre-

TABLE 2. Activation and repression of various *lacZ* fusions by different TyrR mutants

TyrR mutant	% Activation[a] of:				Fold repression[b] of *aroF*[c] with Tyr
	mtr		*tyrP+4*		
	Tyr	Phe	Tyr	Phe	
tyrR$^+$ control[d]	100	100	100	100	696
tyrR null mutant[e]	0	0	0	0	1
Deletion mutagenesis					
Δ40-49	<0	<0	<0	<0	425
Δ50-59	<0	1	<0	<0	546
Δ60-69	<0	4	<0	<0	347
Δ90-99	<0	0	<0	<0	510
PCR mutagenesis					
RQ10	<0	<0	<0	<0	850
TI14	<0	<0	<0	<0	638
PL92	<0	<0	6	16	450
DN103	8	29	28	74	696
Site-directed mutagenesis					
DF19	4	21	14	8	306
PT90	162	114	173	172	589
PH92	4	9	18	42	403
VH93	27	31	24	64	589
VL93	18	27	12	25	850
LF94	80	82	92	119	696
SH95	112	102	142	140	696
SP95	<0	3	0	<0	450
DC97	132	129	151	190	546
DG97	103	114	146	203	638
DF103	152	156	169	209	546
Random mutagenesis					
GE37	9	4	<0	<0	957
GR37	34	10	14	12	850
MI55	8	8	<0	<0	957

[a] Activation by the mutant as a percentage of that by *tyrR*$^+$ (*Hin*dIII) (values for the *tyrR* null mutant strain were subtracted from both mutant and *tyrR*$^+$ [*Hin*dIII] values before this calculation was made). Tyr, growth in minimal medium supplemented with 10^{-3} M tyrosine, Phe, growth in minimal medium supplemented with 10^{-3} M phenylalanine.

[b] Specific activity of β-galactosidase of the *tyrR* null mutant strain divided by that of *tyrR*$^+$ (*Hin*dIII) or *tyrR* mutant strains.

[c] β-Galactosidase units expressed from the *aroF* promoter are 7,654 when derepressed in the *tyrR* null mutant background and 11 when fully repressed by the *tyrR*$^+$ gene (multicopy) in the presence of tyrosine.

[d] Specific activity of β-galactosidase in the *tyrR*$^+$ control which represents 100% activation is 7,601 U for *mtr* and 995 U for *tyrP+4*.

[e] Specific activity of β-galactosidase in the *tyrR* null control which represents 0% activation is 438 U for *mtr* and 127 U for *tyrP+4*.

viously that substitutions of E-4 and E-8 with neutral amino acids such as in EL4 and EF8 showed practically no change in activation (25). On the other hand, substitution of a positively charged arginine for either aspartate 9 (DR9) or glutamate 16 (ER16) resulted in a significant reduction in the activation function of TyrR. As part of a continuing analysis of these charged residues, doped oligonucleotides were used to introduce changes at position 19. To date, only one mutant has been isolated and studied in detail. In this mutant, phenylalanine is substituted for aspartate (DF19) (Fig. 1). In contrast to what was observed at positions 4 and 8, this substitution at position 19 significantly reduced activation without significantly affecting repression (Table 2).

Random mutagenesis of *tyrR* using a mutator strain. To overcome the possibility that PCR mutagenesis using *Taq* DNA polymerase with low levels of dC or dG was producing a biased sample of mutants and to confirm that only the N-terminal domain was involved in activation, we used the XLI-Red mutator strain of Stratagene, which is deficient in three of the primary repair genes (*mutS*, *mutD*, and *mutT*). In this strain the random mutation rate is about 5,000-fold higher than in a wild-type strain (8). Plasmid pMU6219 was transformed into XLI-red, and Km^r colonies were selected. After two cycles of overnight growth in rich medium, these cultures were used for preparation of DNA. Transformations were carried out using JP8042 containing pMU3190 (*mtr-lac* fusion plasmid) as the recipient, and transformants were plated on X-Gal indicator plates in the presence of 1 mM tyrosine. Pale blue colonies appeared at a frequency of about 1 to 2%. Eight such colonies, from separate transformations, were subsequently shown to have decreased activation of *mtr-lac* and *tyrP+4-lac* but normal repression of *aroF-lac*. An equal number of isolates which were defective in both activation and repression were also obtained. Sequencing analysis of the eight mutants defective in activation but not repression was carried out for the region corresponding to the first 200 amino acid residues. This indicated that they included one RQ10, one CR7, two with a change of glycine to arginine at position 37 (GR37), one with a change of glycine to glutamate at position 37 (GE37), one with a change of methionine to isoleucine at position 55 (MI55), and one with two mutations, GR37 and a change of aspartate to valine at position 9 (DV9). The mutations RQ10 (this paper) and CR7 (25) had already been identified as causing loss of activation, and position 9 was also known to be important (25), so these three mutants (RQ10, CR7, and DV9-GR37) were not studied further. The three new mutants, GR37, GE37, and MI55 (Fig. 1), were sequenced in their entirety to establish that there were no further mutations. The effects of these mutations on activation and repression measured by β-galactosidase assay are shown in Table 2.

TABLE 3. Activation and repression of various *lacZ* fusions by different alanine-substituted TyrR mutants[a]

TyrR mutant	% Activation of:				Fold repression of *aroF* with Tyr
	mtr		*tyrP+4*		
	Tyr	Phe	Tyr	Phe	
tyrR$^+$ control	100	100	100	100	696
tyrR null mutant	0	0	0	0	1
DA9	3	3	2	<0	957
RA10	<0	0	<0	<0	402
GA37	<0	12	0	0	765
DA103	0	13	20	42	765
CA7	20	30	50	67	150
EA16	33	68	48	64	696
DA19	40	80	52	76	696
VA93	48	95	27	71	850
VA5	43	85	101	99	589
TA14	97	94	95	100	638
MA55	64	87	91	167	696
PA92	91	82	82	106	765
SA95	55	70	145	142	696

[a] See Table 2, footnotes *a* to *e*.

Amino acid substitutions GE37 and MI55 both caused complete loss of activation of *tyrP+4* and a reduction of activation of *mtr-lac* to low levels, and the amino acid substitution GR37 resulted in a significant but incomplete loss of reduction in activation of both *mtr-lac* and *tyrP+4-lac*. In contrast, all three mutations showed near wild-type levels of repression of *aroF-lac*.

Alanine-scanning mutagenesis of *tyrR*. Results of previous studies (5, 25) and those described above have shown that the amino acid substitutions at positions 2, 3, 5, 7, 9, 10, 14, 16, 19, 37, 55, 92, 93, 95, and 103 can significantly affect TyrR-mediated activation while having no significant effect on TyrR-mediated repression. In order to probe further whether the side chains of these particular amino acids play a critical role in the activation reaction, we substituted each one with alanine. We did not include the RA2 and LA3 substitutions, as previous work (5, 25) had already shown that such mutants had a reduced ability to activate gene expression. The alanine-substituted mutants were tested for their ability to activate expression from the *mtr* and *tyrP+4* promoters and also for their ability to repress *aroF* expression.

The results are presented in Table 3. Apart from mutant CA7, in which *aroF* repression was reduced from 696- to 150-fold, the other mutants showed only marginal or no effects on TyrR-mediated repression.

Three alanine substitutions, namely, DA9, RA10, and GA37, showed an almost complete loss of ability to activate expression of *mtr* and *tyrP+4*, and a fourth, DA103, had greatly reduced ability to activate *tyrP+4* but had lost virtually all ability to activate *mtr*. An intermediate group of mutants, CA7, EA16, DA19, and VA93, showed some impairment of activation, with the greatest changes seen in tyrosine-mediated activation. As previously noted, one of these, CA7, showed a marked decrease in repression which may indicate some instability of the protein. The last group of mutants, VA5, TA14, MA55, PA92, and SA95, showed only marginal changes in activation, indicating that the side chains of amino acids at these positions do not play significant roles in activation.

DISCUSSION

In this study, we have used a variety of genetic approaches, such as PCR random mutagenesis, random mutagenesis using the *E. coli* mutator strain XLI-Red, and oligonucleotide site-directed mutagenesis, to identify a number of new amino acid substitutions which render the TyrR protein defective in transcriptional activation. We conclude that the residues at positions 14, 19, 37, 55, 92, 93, 95, and 103 are important for transcriptional activation at both the *mtr* and the *tyrP+4* promoters. In addition, we isolated by both PCR mutagenesis and random mutagenesis new isolates with the substitution of glutamine for arginine at position 10 (RQ10). This position has previously been shown to be critical for activation (25). Residues 10, 14, and 19 fall into a previously identified region, whereas amino acids at positions 37, 55, 92, 93, 95, and 103 appear to define additional regions important for activation. The fact that those PC mutations obtained by random mutagenesis of the entire *tyrR* gene were all found in the N-terminal part of the protein suggests that the N-terminal domain is the only domain which is associated with the activation function of TyrR.

The inability of these mutant proteins to activate does not result from the loss of DNA binding activity or from instability of the mutant proteins as, with one exception, they can repress the *aroF* promoter to about the same extent as the wild-type TyrR protein.

In alanine-scanning experiments we have substituted alanine for the amino acids at all the newly identified positions and also made alanine-substituted derivatives at positions 5, 7, 9, 10, and 16, where the amino acid substitutions had previously been shown to affect transcriptional activation (25). Results of these alanine-scanning experiments indicated the importance of side chain interactions beyond β-carbon of amino acids D-9, R-10, D-103, and, to a lesser extent, C-7, E-16, D-19, and V-93. Previous work, including alanine scanning, indicated that side chains of amino acids 2 and 3 may also be critical in activation, whereas various substitutions at positions 4, 6, and 8 had no effect on activation (5, 25). Protein-protein interactions between TyrR protein and the α-subunit of RNA polymerase in activation have been proposed, as a result of in vitro transcription studies (14). Of the various amino acids where substitution results in impairment of activation (5, 25), those within the regions between positions 2 and 19 and between positions 92 and 103 appear to be likely to participate directly in protein-protein interactions. One possibility is that the two regions form separate contact sites which interact independently with RNA polymerase. Alternatively, these two regions could be folded close together, in a tertiary structure, to form a single contact site. Recent evidence by Wilson et al. has suggested the existence of an ATP-independent aromatic amino acid binding site in the TyrR protein, and this effector binding site has been proposed to be responsible for TyrR-mediated activation of the *mtr* and *tyrP* promoters (24). The location of this effector binding site has not yet been determined, and we cannot rule out the possible involvement of these regions of the protein, encompassing amino acids 2 to 19 and 92 to 103, in the binding of one or more of the aromatic amino acids. It is noteworthy that substitutions at positions 16, 19, 92, 93, and 103 which impair activation have a two- to threefold greater effect on tyrosine-mediated activation than on that mediated by phenylalanine. This may indicate a role of these residues in effector binding.

The respective roles of glycine 37 and methionine 55 which are implicated by the substitutions GE37, GR37, and MI55, each of which significantly impairs activation, have been

probed by alanine substitutions. The lack of an activation defect in MA55 indicates that the side chain of M-55 plays no role in activation. In contrast, the complete loss of activation function of the TyrR protein as a consequence of replacing a glycine residue with alanine at position 37 suggests that the structural flexibility of the protein at this point is absolutely required for the activation function of TyrR. In the case of D-103, the observation that a phenylalanine substitution at this position causes increased activation clearly indicates that the side chain interaction does not require the carboxyl group of the aspartate. A similar observation has been reported with λ cI repressor, in which the substitution of aspartate 38 by asparagine destroyed activation of the P_{RM} promoter and substitution of the same aspartate by phenylalanine improved the activation function of the wild-type repressor (12). Failure to observe major effects on activation with substitutions PA92 and SA95 in comparison with the major effects seen in PH92 and SP95 suggests that the side chains of these amino acids do not play a critical role in activation, but the tertiary structure of the protein does not tolerate particular substitutions at these positions.

With regard to the deletion mutants, it is apparent that the PC defect caused by the deletion mutations Δ50-59 and Δ90-99 may, at least in part, be due to the elimination of crucial amino acid residues (e.g., methionine 55, proline 92, valine 93, and serine 95) from these regions. The observation that deletion mutations Δ40-49 and Δ60-69 also destroy activation could indicate that they disrupt the tertiary structure of the activating domain or could imply that further residues between 40 and 69 are necessary for activation.

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