

Second-Site Revertants of *Escherichia coli* *trp* Repressor Mutants

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ABSTRACT

Second-site reversion studies were performed with five missense mutants with defects in the *trp* repressor of *Escherichia coli*. These mutants were altered throughout the gene. The same unidirectional mutagen used in the isolation of these mutants, hydroxylamine, was used in reversion studies, to increase the likelihood that the revertants obtained would have second-site changes. Most of the second-site revertants were found to have the same amino acid substitutions detected previously as superrepressor changes. These second-site revertant repressors were more active *in vivo* than their parental mutant repressors, in the presence or absence of exogenous tryptophan. Apparently superrepressor changes at many locations in this protein can act globally to increase the activity of mutant repressors.

THE *trp* repressor of *Escherichia coli* regulates transcription initiation at the promoter/operator regions of the *trpEDCBA*, *aroH* and *trpR* operons (ROSE *et al.* 1973; SQUIRES, LEE and YANOFSKY 1975; BENNETT *et al.* 1976; JOACHIMIAK *et al.* 1983; ARVIDSON, BRUCE and GUNSALUS 1986; KLIG, CRAWFORD and YANOFSKY 1987). This repressor is a symmetrical dimer composed of identical 107 residue polypeptides (GUNSALUS and YANOFSKY 1980; JOACHIMIAK *et al.* 1983). The *trp* repressor is the tryptophan-activated form of the *trp* aporepressor. The three-dimensional structures of the repressor and aporepressor have been determined (SCHEVITZ *et al.* 1985; ZHANG *et al.* 1987). The repressor polypeptide is 72% helical and its six helical segments (A–F) contain the following residues: A, 10–31; B, 35–42; C, 45–63; D, 68–75; E, 79–92; F, 94–105 (SCHEVITZ *et al.* 1985). All of the helices, except D and E, make extensive contacts with some sections of the second polypeptide subunit (SCHEVITZ *et al.* 1985). The repressor has a helix-turn-helix motif (helices D and E) characteristic of many DNA binding proteins (PABO and SAUER 1984; KELLEY and YANOFSKY 1985; SCHEVITZ *et al.* 1985).

To determine which segments of the molecule are responsible for the DNA binding function, mutants incapable of repressing the *trp* operon were isolated and characterized (KELLEY and YANOFSKY 1985). Most of the defective missense *trp* repressor mutants that were obtained have amino acid substitutions in the helix-turn-helix region (KELLEY and YANOFSKY 1985). A second class of mutants have amino acid replacements at sites known to form the tryptophan binding pocket (SCHEVITZ *et al.* 1985).

Second-site reversion analyses have provided useful structure-function information on several proteins (HELINSKI and YANOFSKY 1963; YANOFSKY 1971; HECHT and SAUER 1985; NELSON and SAUER 1985). In this report we describe the isolation and *in vivo* characterization of second-site revertants of five *trp* repressor mutants. Interestingly, most of the compensatory second-site mutations were previously isolated as superrepressor mutations (KELLEY and YANOFSKY 1985).

MATERIALS AND METHODS

Plasmids and phage: Plasmid pRLK13 contains the wild-type *trpR* gene in the *Bam*HI site of pACYC184. In this construct *trpR* is constitutively expressed from the *tet* promoter of the vector. Plasmids carrying defective mutant *trpR* genes TM44, GS78, TM81, RH84, GR85, and superrepressor *trpR* genes, EK18, DN46, EK49, and AV77, are all derivatives of pRLK13 (KELLEY and YANOFSKY 1985). The single letter amino acid code is used to designate each mutant, with the letter for the original amino acid preceding the letter for its substitute, followed by the number of the residue position at which the substitution occurred. Phage λ TLF1 (YANOFSKY, KELLEY and HORN 1984) is a λ derivative containing a *trpL'*–*lacZ* fusion. Expression of *trp p/o trpL'*–*lacZ* is regulated by the *trp* repressor (the *trp* attenuator is not present). Phage M13mp19 (NORRANDER, KEMP and KLESSING 1983) was used for cloning for sequencing.

Strains: The strain used as transformation recipient in revertant detection and for measuring β -galactosidase activity was CY15075 (W3110 *tnaA2* Δ *lacU169 trpR2*/ λ TLF1) (YANOFSKY and HORN 1981). Tryptophan bradytroph W3110 *trpA46PR9* was used to isolate primary *trpR* superrepressor mutations, as previously described (KELLEY and YANOFSKY 1985). Strain JM101 was used for phage production for DNA sequencing (SANGER, NICKLEN and COULSON 1977).

Media: Following transformation, bacteria were plated on minimal agar (VOGEL and BONNER 1956) supplemented with 0.2% glucose, 0.2% acid hydrolyzed casein, L-tryptophan (20 μ g/ml) and chloramphenicol (20 μ g/ml) and replica

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plated to the same medium with or without tryptophan. Minimal agar plates containing 0.2% glucose, 0.5% NaCl, 1% tryptone, X-Gal (40 $\mu\text{g/ml}$), phenylethylthio- β -D-galactoside (80 $\mu\text{g/ml}$) and chloramphenicol (20 $\mu\text{g/ml}$) were also used. LB broth (MILLER 1972) was used to grow bacterial cultures containing plasmids with putative *trpR* mutations, and to grow cultures infected with M13mp19. Minimal media supplemented with 0.2% glucose, 0.2% acid hydrolyzed casein, chloramphenicol (20 $\mu\text{g/ml}$), with or without tryptophan (20 $\mu\text{g/ml}$), was used to grow cultures for β -galactosidase assays.

Mutagenesis: Plasmid DNA, purified by cesium chloride banding, was mutagenized with hydroxylamine as described by DAVIS, BOTSTEIN and ROTH (1980). DNA was incubated at 37°C for 36 hr in 0.8 M hydroxylamine (pH 6.0), dialyzed versus 10 mM Tris-Cl, pH 7.4, 1 mM EDTA overnight, ethanol precipitated, and resuspended in the same buffer.

DNA sequencing: Plasmid DNAs containing the putative *trpR* mutants were prepared by the method of BIRNBOIM and DOLY (1979). The 440-base pair *Bam*HI fragment containing the entire *trpR* gene was ligated (MANIATIS, FRITSCH and SAMBROOK 1982) into *Bam*HI-digested RF M13mp19 (NORRANDER, KEMP and MESSING 1983). Recombinant plaques were identified, and single-stranded phage DNA was isolated and sequenced by the didexoy chain termination method of SANGER, NICKLEN and COULSON (1977) using ³⁵S-labeled α -dATP (BIGGIN, GIBSON and HONG 1983).

β -Galactosidase assays: All cultures were grown at 31°C because λ TLF1 is a CI857 derivative (temperature sensitive). β -Galactosidase assays were performed as described by MILLER (1972).

RESULTS

Second-site reversion analyses provide the opportunity to detect structural changes in a mutant protein that increase its biological activity. At least two classes might be expected; those in which the second-site amino acid change "corrects" the specific defect in the primary mutant, and those in which the second change increases the inherent activity of the protein. In the present study second-site reversion of five *trp* repressor mutants, TM44, GS78, TM81, RH84 and GR85 (Table 1) was examined. Each of these mutants was generated by treatment of wild-type *trpR* with hydroxylamine (KELLEY and YANOFSKY 1985). These mutants were selected because they had changes throughout the protein, their changes affected the tryptophan binding site or the DNA binding helices (SCHEVITZ *et al.* 1985), and they should not be revertible to wild type by the unidirectional mutagen employed, hydroxylamine.

The indicator strain employed, CY15075 (W3110 *tnaA2 trpR2 lac169/λTLF1*) is a lysogen carrying a *trp p/o trpL' - lacZ* gene fusion in which β -galactosidase production is controlled by repressor action at the *trp* promoter-operator. This strain contains a frameshift mutation in *trpR*, therefore strains with this allele do not produce functional repressor or negative complementing repressor. Following transformation of this strain with mutagenized plasmid DNA containing each mutant allele, chloramphenicol resistant colonies were selected on plates containing tryptophan and X-

Gal; colonies with decreased β -galactosidase activity (lighter blue) were identified, picked, purified, and their phenotype confirmed. Approximately 0.5 to 1×10^5 transformants from each mutant were screened. Plasmid DNA was isolated from every presumptive second-site revertant and the entire *trpR* region of each was sequenced.

The positions of the second site substitutions that were detected are indicated in Table I. Regardless of the primary mutant employed, many of the same compensatory second-site changes were obtained. Furthermore, several of the second-site changes, EK18, EK49, and AV77, had been detected previously as primary mutants in studies in which superrepressor (superactive) mutants were selected (KELLEY and YANOFSKY 1985). Since EK18 was detected as a second-site revertant of both GS78 and GR85 (Table 1) and could be readily recombined with distal *trpR* mutations using a *Sal*I site located early in the gene, double mutants EK18-TM44, EK18-TM81, and EK18-RH84 were constructed, and examined (Table 1). None of these double mutants would have been detected in our X-gal screening test for second site revertants.

Of the 27 second-site revertants isolated in this study, 18 were due to the previously detected superrepressor changes, EK18, EK49 and AV77. The other nine isolates had changes at positions 45 and 46 in the repressor polypeptide. To determine whether these changes in an otherwise wild-type protein would give the superrepressor phenotype, an additional set of superrepressors was isolated exactly as before (KELLEY and YANOFSKY 1985). Superrepressor mutant DN46 was obtained in this experiment. This mutation was observed eight times among the second-site revertants of mutant RH84 (Table 1). Like the other superrepressor mutants (Table 2), DN46 is appreciably more active *in vivo* than wild-type *trpR* in media lacking tryptophan, but indistinguishable from wild-type *trpR* in the presence of excess tryptophan.

The relative *in vivo* activities of the various repressors were determined in the presence and absence of exogenous tryptophan by introducing the corresponding plasmids into the repression indicator strain and assaying β -galactosidase levels. As shown in Table 2 each second-site revertant had appreciably more repressor activity than its parental mutant, although none was as active as the wild type repressor or any of the superrepressors. Increased activity over the parental mutant was evident in the presence and absence of exogenous tryptophan. Interpretation of the values obtained with cultures grown in minimal medium is complicated because we cannot assess the indirect effect of each mutant repressor's action on the intracellular concentration of tryptophan. In addition to activating the corepressor, tryptophan feedback inhibits the enzyme catalyzing the initial step in

TABLE 1
Amino acid and codon changes in primary mutants and second-site revertants and constructs

Primary mutation			Compensatory second-site change			No. of isolates
Mutant	Replacement	Codon change	Mutant	Replacement	Codon change	
TM44	Thr44 → Met	ACG → ATG	EK18	Glu18 → Lys	GAG → AAG	C ^a
TM44	Thr44 → Met	ACG → ATG	EK49	Glu49 → Lys	GAA → AAA	3
GS78	Gly78 → Ser	GGC → ACG	EK18	Glu18 → Lys	GAG → AAG	2
TM81	Thr81 → Met	ACG → ATG	EK18	Glu18 → Lys	GAG → AAG	C
RH84	Arg84 → His	CGT → CAT	EK18	Glu18 → Lys	GAG → AAG	C
RH84	Arg84 → His	CGT → CAT	PL45	Pro45 → Leu	CCA → CTA	1
RH84	Arg84 → His	CGT → CAT	DN46	Asp46 → Asn	GAT → AAT	8
RH84	Arg84 → His	CGT → CAT	EK49	Glu49 → Lys	GAA → AAA	6
GR85	Gly85 → Arg	GGA → AGA	EK18	Glu18 → Lys	GAG → AAG	3
GR85	Gly85 → Arg	GGA → AGA	EK49	Glu49 → Lys	GAA → AAA	2
GR85	Gly85 → Arg	GGA → AGA	AV77	Ala77 → Val	GCA → GTA	2

^a Double mutant constructed as described in the text.

TABLE 2
Repressor activity of *trpR* mutants with second-site changes, and superrepressor mutants

Mutant plasmid ^a	Second-site change	Units β -galactosidase activity ^b		Superrepressor plasmid ^a	Units β -galactosidase activity ^b	
		-Trp	+Trp		-Trp	+Trp
TM44		9,000	5,900	(wild type) ^a	500	10
TM44	EK18	3,700	1,600	No plasmid ^c	12,000	12,000
TM44	EK49	2,100	400	EK18	179	4
GS78		6,000	2,300	EK49	68	4
GS78	EK18	3,500	500	DN46	167	12
TM81		8,300	4,000	AV77	188	7
TM81	EK18	7,800	2,400			
RH84		10,000	5,300			
RH84	EK18	7,500	1,200			
RH84	PL45	7,100	1,000			
RH84	DN46	5,300	400			
RH84	EK49	2,100	500			
GR85		600	2,000			
GR85	EK18	1,800	500			
GR85	EK49	400	400			
GR85	AV77		200			

^a Each *trpR* gene was present in pACYC184, in the repression indicator strain, CY15075. This strain contains the *trpR2* allele, a frameshift allele with no repressor activity.

^b Cultures were grown in the presence or absence of exogenous tryptophan (see MATERIALS AND METHODS) and assayed for β -galactosidase activity.

^c The recipient strain. When this strain contained the parental plasmid pACYC184, identical values were obtained.

tryptophan biosynthesis. In second-site revertants in which the second-site change was EK18 or EK49, EK49 generally restored greater repressor activity. This finding is consistent with the behavior of the corresponding superrepressors, namely that the EK18 superrepressor is somewhat less active than the EK49 superrepressor (KELLEY and YANOFKY 1985).

DISCUSSION

Five repressor mutants that produce defective proteins were employed in second-site reversion studies. Of the five, TM44, RH84 and GR85, have substitu-

tions of amino acid residues that comprise the tryptophan binding site (SCHEVITZ *et al.* 1985; ZHANG *et al.* 1987). In addition, four of the mutants, GS78, TM81, RH84 and GR85, have amino acid changes in helix E, the segment of the polypeptide believed to be responsible for sequence-specific recognition of operator DNA (SCHEVITZ *et al.* 1985; ZHANG *et al.* 1987). The second-site reversions could have corrected the original defects in a mutant-specific manner or they could have acted globally by increasing the activity of the repressor. The results obtained clearly show that the second-site compensatory changes that were most common in the mutants examined acted globally, *i.e.*,

each was able to restore activity to several of the primary repressor mutants. Furthermore, all but one of these second-site changes exhibits a superrepressor phenotype when it is present in an otherwise wild type repressor protein (KELLEY and YANOFSKY 1985; this study). These findings suggest that the second-site changes we detected increased the activity of the repressor by mechanisms unrelated to the basis of repressor inactivation in each mutant.

It is perhaps surprising that all of the superrepressor changes were not observed among the revertants obtained with each of the mutants. The limited second-site reversion spectra of some of the mutants could be due to sample size, ease of primary detection of second-site revertants of some mutants, or constraints imposed by the parental amino acid changes. Of the superrepressors previously identified only EK13 was not recovered as a second-site compensatory change in this study. Since EK13 is the weakest of the superrepressors (KELLEY and YANOFSKY 1985) perhaps its absence indicates that this change would be insufficient to increase repressor activity to a level that would have been detected in our screening procedure.

The classes of second site revertants that were recovered were limited by the specificity of the mutagen employed. Hydroxylamine causes C → T changes exclusively. This mutagen was chosen for the present study to avoid reverting the primary mutants, which were also hydroxylamine-induced, to wild type. Nevertheless, hydroxylamine treatment could have caused changes of several of the primary mutant residues; Met44 could be converted to Ile (ATG → ATA); Ser78 could be replaced by Asn (AGC → AAC); Met81 could change to Ile (ATG → ATA); His84 could be replaced by Tyr (CAT → TAT); and Arg85 could mutate to Lys (AGA → AAA). None of these changes was detected, suggesting that substituting these amino acid residues at the respective protein positions would not give highly active repressors.

Relatively little can be said with certainty about how each superrepressor change increases the activity of the mutant proteins. Only one of the superrepressor proteins has been characterized biochemically, EK49, and it has been shown to decrease the rate of dissociation of repressor from operator DNA 10-fold (KLIG and YANOFSKY, 1988). Since EK49 and three of the other superrepressor changes increase the net positive charge of the repressor, it is possible that each of these changes increases activity similarly, by decreasing repulsion of the repressor from the negatively charged phosphate backbone of the operator. Three of the second-site changes, those at positions 45, 46 and 49, are in the central core region of the repressor (SCHEVITZ *et al.* 1985). This region of the protein spans the DNA minor groove in between the consecutive major grooves that contain the four symmetrical base pairs

that are thought to serve as the repressor recognition sequence (SCHEVITZ *et al.* 1985; BASS *et al.* 1987). The PL45 superrepressor change does not introduce an additional positive charge in the central region of the repressor, however replacement of Pro-45 could increase the number of residues in helix C (SCHEVITZ *et al.* 1985), thereby increasing repressor contact with operator DNA.

There are many positions in the protein other than 13, 18 and 49 at which hydroxylamine mutagenesis could lead to a Glu → Lys (GA^Δ → AA^Δ) replacement. Glu residues are located at positions 13, 18, 47, 49, 59, 60, 65, 70, 74, 95, 101, 102. That Lys replacements of only three of these Glu residues were detected suggests that changes of the others do not appreciably increase the activity of the repressor. Alternatively, Lys substitution of some of these Glu residues could destabilize the protein, resulting in its degradation.

It is likely that superrepressor AV77 functions by a totally different mechanism than the other superrepressors. Located in the turn of the helix-turn-helix motif of the wild-type repressor, the small Ala side chain may allow the repressor's DNA reading heads to shift out of the active DNA binding conformation in the absence of bound tryptophan (ZHANG *et al.* 1987). Substitution of Val for Ala could maintain the DNA reading heads in a conformation more closely resembling that of the tryptophan-activated native repressor. In this regard it is interesting that the AV77 change was detected as a second-site reversion only in mutant GR85. The GR85 mutation totally eliminates tryptophan binding *in vitro*, and eliminates the *in vivo* effect of tryptophan on the repressor's low level of activity (MARMORSTEIN *et al.* 1987; GRADDIS *et al.* 1988). It is conceivable that the AV77-GR85 protein is able to assume an active conformation despite being incapable of binding tryptophan. An alternative, although unlikely, explanation is that the second-site revertant repressor has regained tryptophan binding ability. Equally interesting is the fact that the same defective mutant, GR85, is reverted by second-site changes EK18 and EK49. Assuming that the EK18-GR85 and EK49-GR85 proteins do not bind tryptophan, it would appear that the EK18 and EK49 changes can increase the activity of the GR85 repressor despite loss of what is considered to be an essential activating step of this repressor, namely tryptophan binding. It is important to note, however (Table 2), that the GR85 protein does have some activity *in vivo*.

The principal conclusion from this study is that the *trp* repressor of *E. coli* appears to have evolved as a protein with less-than-maximal activity. This conclusion has also been reached for the lambda repressor in similar reversion studies (NELSON and SAUER 1985; HECHT and SAUER 1985); global suppressors of pri-

many mutations were isolated and these second-site reversions were identified as superrepressors. It is likely that these less active states endow these proteins with the ability to accomplish their *in vivo* functions more effectively. Perhaps operator recognition and strength of DNA binding are distinct features of repressor molecules that may be separately optimized in each repressor.

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