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Genetics

Mutational studies with the *trp* repressor of *Escherichia coli* support the helix-turn-helix model of repressor recognition of operator DNA

(repressor mutants/operator recognition/negative complementation/superrepressor)

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ABSTRACT **Several classes of *trp* repressor mutants were selected and analyzed *in vivo*. Mutants that produced repressors with either enhanced or reduced activity were obtained. One class of mutants produced inactive or slightly active repressors that were *trans*-dominant to the wild-type repressor. The amino acid substitutions in many of these repressors were clustered in a segment of the polypeptide that is homologous to the DNA recognition domain of the λ*cro* repressor. A second functionally important region of the *trp* repressor was identified; this region could participate in L-tryptophan binding. Observations with *trpR* nonsense mutants suggest that the first 67 residues of the repressor polypeptide are sufficient for subunit association.**

The gene *trpR* of *Escherichia coli* encodes a 108-residue polypeptide that dimerizes to form the *trp* aporepressor (1, 2). This aporepressor, when activated by L-tryptophan, controls three unlinked operons, *trpEDCBA*, *aroH*, and *trpR* (1, 3–5). As expected, the operators of these operons have sequence homologies (1).

We wish to understand how the *trp* repressor recognizes these operators. Mutational, biochemical, and crystallographic studies with several DNA recognition proteins have led to a general model that explains how such proteins bind specific DNA sequences (6–8). Mutational analyses with the *lacI*, *crp*, and λcI genes have identified specific segments of the corresponding polypeptides that influence DNA binding (9–16). X-ray crystallographic analyses have revealed the detailed three-dimensional architecture of λcI, λcro, and crp proteins, and have shown that a homologous segment in each forms two adjacent helices, referred to as helix 2 and helix 3, separated by a sharp bend (17–21). Both model building and mutational studies strongly implicate this homologous segment as the primary DNA recognition region (6, 7, 10, 22, 23). In fact, a mutational change of one residue in the helix 3 region of the crp protein has been shown to permit this mutant protein to recognize an altered DNA sequence (16). Helix 3 is proposed to penetrate the DNA major groove so that its amino acid side chains make contact with specific base pairs. Helix 2 is believed to fold back to make contacts with the phosphate backbone and hold helix 3 in place.

Matthews *et al.* (24) have pointed out that a segment of the trpR polypeptide of *E. coli*, residues 68–86, is homologous to the proposed DNA binding sites of other DNA recognition proteins and have suggested that this segment plays a similar role (7, 24). To test this possibility, we performed a mutational analysis designed to identify the residues in the *trp* repressor that constitute the DNA binding site. Our findings with negative-complementing repressor mutants support the hypothesis that the homologous segment of the *trp* repressor does contribute to the DNA recognition site. Our observations also suggest that all the amino acid residues critical to subunit interaction are contained in the first 67 residues of the polypeptide. Mutants that form repressors with enhanced ability to function at low tryptophan concentrations were also isolated and characterized.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. All strains were isogenic and were derivatives of W3110 *tnaA2* Δ*lacU169* (25). Individual strains carried either the wild-type *trpR*$^+$ allele or the mutant *trpR2* allele. The latter allele contains a frameshift mutation. This conclusion is based on reversion studies using chemical mutagens. This allele does not specify a functional trpR polypeptide and its presence does not result in negative complementation of the wild-type *trp* repressor. The W3110 *trpA46PR9* bradytroph used produces a weakly active tryptophan synthetase α polypeptide (26, 27). The phage derivative λTLF1 encodes a fusion of *trpPOL'* to *lacZ'Y*. This gene fusion specifies a hybrid β-galactosidase that contains the amino-terminal residues of the trp leader peptide (26). The gene fusion removes the *trp* attenuator so that synthesis of the hybrid β-galactosidase encoded in λTLF1 is regulated exclusively by the *trp* repressor (26). Plasmid pRLK13 contains a 440-base-pair *Bam*HI restriction fragment encoding the trp aporepressor polypeptide cloned in the *Bam*HI site of pACYC184. The *trpR* gene in pRLK13 lacks its normal promoter and is constitutively transcribed from the *tet* promoter of the vector. pRLK13 is analogous to pRLK10, which was described earlier (5).

Mutagenesis. pRLK13 DNA was treated *in vitro* with 0.8 M hydroxylamine (pH 6.0) for 36 hr at 37°C, dialyzed extensively against Tris EDTA (pH 7) (28), ethanol-precipitated, and then used to transform an appropriate recipient. Strain W3110 *tnaA2*/pRLK13 was grown in the presence of ethyl methanesulfonate or 2-aminopurine essentially as described by Miller (29). Plasmid DNA was prepared from these cells as described (30) and was used directly to transform recipient cells. Only one mutant plasmid was retained from each mutagenized culture. In most cases, the *Bam*HI fragment was subcloned and the phenotype of the original clone was verified.

Isolation of Mutants. *General* trpR *mutants.* The W3110 wild-type strain of *E. coli* will not grow in the presence of appropriate concentrations of DL-5-methyltryptophan (31). However, cells lacking a functional *trp* repressor, such as W3110 *trpR2*, will grow on medium containing this analog, because they produce high levels of the *trp* operon polypeptides. When W3110 *tnaA2 trpR2* is transformed with mutagenized pRLK13 DNA, the transformed cells do not grow on minimal medium/glucose/chloramphenicol/DL-5-methyltryptophan unless the introduced plasmid has an inactive *trpR* gene.

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Negative-complementing trpR *mutants*. Mutant *trpR* genes encoding polypeptides that can inactivate wild-type $trpR^+$ polypeptides are termed *trans*-dominant or negative-complementing. They are analogous to $lacI^{-d}$ mutants (32, 33), which act by forming weakly active heterotetramers composed of wild-type and defective mutant monomers. Some plasmids with *trans*-dominant *trpR* mutations were selected by their ability to confer DL-5-methyltryptophan resistance to bacteria containing a chromosomal $trpR^+$ allele. Others were selected on a different basis; when mutagenized $trpR^+$ plasmid pRLK13 was transformed into W3110 *tnaA2* Δ*lacU169* $trpR^+$ (λTLF1), rare mutant colonies appeared that were deep red on MacConkey lactose/chloramphenicol agar. Transformation with pACYC184 (no *trpR*) or pRLK13 ($trpR^+$) yielded only white colonies on this agar. The mutant *trpR* genes must specify polypeptides that are able to interact with and inactivate wild-type $trpR^+$ monomers produced from the chromosomal $trpR^+$ allele, thereby permitting expression of the *trp–lac* fusion of λTLF1.

Superrepressing mutants. A mutant *trp* repressor that bound operator even under tryptophan starvation conditions might cause auxotrophy by preventing an adequate rate of transcription of the *trpEDCBA* operon. To improve the likelihood of isolating such a mutant, strain W3110 *trpA46PR9*, a Trp bradytroph, was used as a transformation recipient. The *trpA* mutation renders this strain partially starved for tryptophan, hence *trp* operon expression in minimal glucose medium is necessarily increased to compensate for the lesion (26). Even mild repression of the *trp* operon of this strain prevents growth. W3110 *trp46PR9* cells transformed with mutagenized pRLK13 were spread on minimal medium/glucose/tryptophan/chloramphenicol agar. Tryptophan auxotrophs were recognized by their failure to grow when colonies were replica-plated to a medium lacking tryptophan. W3110 *trpA46PR9* cells containing unmutagenized pRLK13 grow on minimal medium despite the presence of large amounts of wild-type repressor.

DNA Sequencing. The entire *trpR* coding region of pRLK13 is on a 440-base-pair *Bam*HI fragment. This fragment from each mutant plasmid was inserted into the single-stranded phage M13mp11 (34) and sequenced by the dideoxy chain termination method of Sanger *et al.* (35) using ^{35}S-labeled α-dATP (Amersham) (36).

Growth Conditions and Enzyme Assays. Cells were grown in minimal medium (37) supplemented with 0.2% glucose/L-tryptophan (20 μg/ml)/0.2% acid hydrolyzed casein, or chloramphenicol (20 μg/ml), as indicated in the legends to individual tables. All cultures were grown at 30°C because the λTLF1 prophage is a λ*cI857* (temperature-sensitive derivative. β-galactosidase assays were performed as described by Miller (29).

RESULTS

The 82 *trpR* mutants isolated and sequenced in this study and the mutagens used in their isolation are described in Table 1. Nine different nonsense mutations and 19 different missense changes were detected.

Table 1. Characteristics of *trpR* mutants

Mutant	Phenotype	Replacement	Codon change	Mutagen	Independent isolate, *n*
MI1	r	Met-1 → Ile	ATG → ATA	HA	2
Qoc3	r	Gln-3 → stop	CAA → TAA	HA	2
Qoc4	r	Gln-4 → stop	CAA → TAA	HA	4
EK13	S	Glu-13 → Lys	GAA → AAA	HA	1
Qam14	r	Gln-14 → stop	CAG → TAG	HA	3
EK18	S	Glu-18 → Lys	GAG → AAG	HA	1
Wam19	r	Trp-19 → stop	TGG → TAG	HA, 2AP	5
Wop19	r	Trp-19 → stop	TGG → TGA	HA	2
TM44	−d	Thr-44 → Met	ACG → ATG	HA, EMS	4
EK47	−d	Glu-44 → Lys	GAG → AAG	HA	3
EK49	S	Glu-49 → Lys	GAA → AAA	EMS	1
RH54	−d	Arg-54 → His	CGC → CAC	HA, 2AP	5
Qam68	−d	Gln-68 → stop	CAG → TAG	HA, EMS, 2AP	7
AV77	S	Ala-77 → Val	GCA → GTA	HA	1
GS78	−d	Gly-78 → Ser	GGC → AGC	HA	2
GD78	−d	Gly-78 → Asp	GGC → GAC	HA, EMS, 2AP	10
AT80	w	Ala-80 → Thr	GCG → ACG	2AP	3
TM81	−d	Thr-81 → Met	ACG → ATG	HA	1
TM83	−d	Thr-83 → Met	ACG → ATG	HA	1
RC84	−d	Arg-84 → Cys	CGT → TGT	EMS, 2AP	6
RH84	−d	Arg-84 → His	CGT → CAT	HA, EMS	2
GR85	−d	Gly-85 → Arg	GGA → AGA	HA	2
GK85	−d	Gly-85 → Lys	GGA → AAA	HA	1
GE85	−d	Gly-85 → Glu	GGA → GAA	HA, EMS	4
LP96	r/−d	Leu-96 → Pro	CTG → CCG	2AP	1
Qam98	r/−d	Gln-98 → stop	CAG → TAG	HA, 2AP	4
Wam99	r/−d	Trp-99 → stop	TGG → TAG	HA	2
Wop99	r/−d	Trp-99 → stop	TGG → TGA	HA, EMS	2

r, Recessive TrpR; −d, *trans*-dominant TrpR; S, super repressor; w, near wild-type activity. Very weak *trans*-dominant mutants are indicated r/−d. Mutants are named using two letters followed by a number. First letter is the one-letter abbreviation for the wild-type amino acid; second letter is the one-letter abbreviation for the replacing amino acid; number is the codon position. Thus, EK13 and EK49 are glutamic acid to lysine changes at positions 13 and 49, respectively. Nonsense mutations are denoted by am (amber), oc (ochre), or op (opal). HA, hydroxylamine; 2AP, 2-aminopurine; EMS, ethyl methanesulfonate. Since the mutagens used favor G·C → A·T changes, our set of mutants is necessarily restricted.

Mutants Producing Negative Complementing Polypeptides. Many of the nonsense and missense mutants produce negative complementing aporepressor polypeptides, polypeptides that reduce the activity of chromosomally specified wild-type monomers, presumably by forming heterodimers (Table 2). Since the mutant *trpR* genes are present on a high copy number plasmid, there should be a large excess of mutant monomers over wild-type monomers. Thus, if mutant and wild-type subunits could associate randomly, there should be relatively few wild-type homodimers. The strong negative complementation displayed by most of these mutant polypeptides (middle column, Table 2), therefore, suggests that they have a conformation at their subunit contact sites similar to wild type. Yet the lesion in each mutant polypeptide must be at a critical position because mutant repressor homodimers have little or no activity (last column, Table 2), and the resulting heterodimers are, at most, partially active (middle column, Table 2).

Trans-dominant mutants could affect at least two functions of the trpR polypeptide. The most obvious is alteration of the DNA contact site. However, mutations that impair tryptophan binding and hence activation of the aporepressor could also be negative complementing. Many of the *trans*-dominant missense mutations detected were clustered in the region of the gene encoding residues 78–85 of the trpR polypeptide (Table 1; Fig. 1). This is the region Matthews *et al.* pointed out to be homologous to helix 3 of the λ*cro* repressor (24). Fig. 1 shows the sequence of residues in this segment of the trpR polypeptide and compares this sequence with that of the homologous segments of other DNA recognition proteins. Mutations altering residues at positions 78, 80, 83, and 84 of the *trp* repressor are predicted to affect key surface residues that project toward the bases in the major groove.

Table 2. Effect of negative complementing *trpR* mutations

trpR allele on plasmid	Strain background	
	trpR$^+$ (λTLF1)	*trpR* (λTLF1)
pACYC184 (none)	40	12,000
pRLK13 (*trpR*$^+$)	4	4
TM44	1500	5,600
EK47	400	700
RH54	2000	10,000
Qam68	4450	12,000
GS78	1100	2,100
GD78	1000	11,800
AT80	13	60
TM81	900	4,300
TM83	275	7,400
RC84	485	9,800
RH84	360	5,200
GR85	470	1,800
GK85	940	9,500
GE85	1700	10,900
LP96	75	6,400
Wop99	90	3,000

Mutant plasmids were introduced into two related strains, W3110 *tnaA2* Δ*lacU169* (λTLF1) and W3110 *tnaA2* Δ*lacU169* *trpR2* (λTLF1). pACYC184 was the parental vector used for construction of all *trpR* plasmids. All the mutant plasmids are identical to pRLK13 except for the single base-pair change indicated in Table 1. All cultures were grown in minimal glucose medium containing 0.2% acid hydrolyzed casein/chloramphenicol (20 μg/ml)/L-tryptophan (20 μg/ml). β-galactosidase was produced from the resident *trp–lacZ* fusion in the λ prophage. First column presents units of β-galactosidase for strains that also produce the wild-type repressor. Second column presents β-galactosidase levels for strains that only produce the mutant repressor. Assays were performed at least in triplicate. AT80 is grouped with the negative complementing mutations because it affects wild type activity.

While the residues at positions 81 and 85 are not believed to make specific contacts with DNA, amino acid substitutions at these positions might distort helix 3 so that tight operator binding becomes impossible. The most active negative complementing mutant is Qam68. This *trpR* nonsense mutant specifies a 67-residue polypeptide.

Those negative complementing missense mutations falling outside the presumed helix 3 region are localized in the segment from residues 44 to 54. The TM44 and RH54 polypeptides are essentially inactive, while the EK47 polypeptide has slight (6%) repressor activity. Interestingly, an identical change (glutamic acid to lysine) just two residues away, in mutant EK49, has the opposite phenotype—i.e., superrepression (see below).

Superrepressing *trpR* Mutants. The basis of the selection for these mutants was repression of the *trpEDCBA* operon of the bradytroph W3110 *trpA46PR9* at low intracellular tryptophan concentrations, thereby preventing growth on minimal medium. The wild-type repressor, even when overproduced from a plasmid, does not inhibit growth of the bradytroph under comparable conditions. Although in the absence of exogenous tryptophan the mutant plasmids were lethal to the strain in which they were selected, they were tolerated by cells with a wild-type *trpEDCBA* operon. To evaluate the *in vivo* function of these mutant repressors, mutant plasmids were introduced into the repressor-deficient indicator strain W3110 *trpR2* Δ*lacU169* *tnaA2* (λTLF1). Production of β-galactosidase from the *trp–lac* fusion in the prophage in this strain is controlled by the *trp* repressor specified by the introduced plasmid. It can be seen from the β-galactosidase levels in Table 3 that each of the four mutant repressors decreased expression from the λTLF1 fusion prophage in tryptophan-free medium more than the wild-type repressor. However, all the strains responded to exogenous tryptophan (Table 3), indicating that none of the mutant repressors was totally tryptophan independent.

DISCUSSION

Although the helix-turn-helix model for DNA recognition was proposed on the basis of detailed structural information for the λcI, λcro, and crp proteins, it has been extended to many DNA recognition proteins for which three-dimensional structural information is not yet available (reviewed in ref. 8). All of these proteins have in common a homologous 20-residue segment that corresponds to the second and third helices of the DNA binding site of the λcro protein. The three known three-dimensional structures reveal that the most highly conserved residues in this 20-residue segment serve structural roles, while the residues at the variable positions, particularly in helix 3, contact bases that are exposed in the major groove of DNA (40). The proteins are symmetric dimers of identical subunits, and the DNA segments recognized have an appropriate dyad symmetry (8).

To examine the applicability of the helix-turn-helix model to the *trp* repressor, we isolated repressor mutants and identified the amino acid change in each. An important conclusion from our findings is that the model is supported by many of the mutants that were isolated. Thus, 10 of the 14 different missense mutations that decrease or eliminate repressor activity affect residues in the suspect region homologous to helix 3 of the λ*cro* repressor (Table 1; Fig. 1). Most of these mutant *trp* repressors have a *trans*-dominant effect when present in a strain that also produces the wild-type repressor, indicating that mutant subunits readily associate with and inactivate wild-type subunits. Negative complementation of this type has also been observed with *lacI* mutants (31, 32), most of which have alterations in the first 60 residues of the *lac* repressor, the segment that comprises the DNA binding domain (refs. 14, 22, 32, and 41; see Fig. 1).

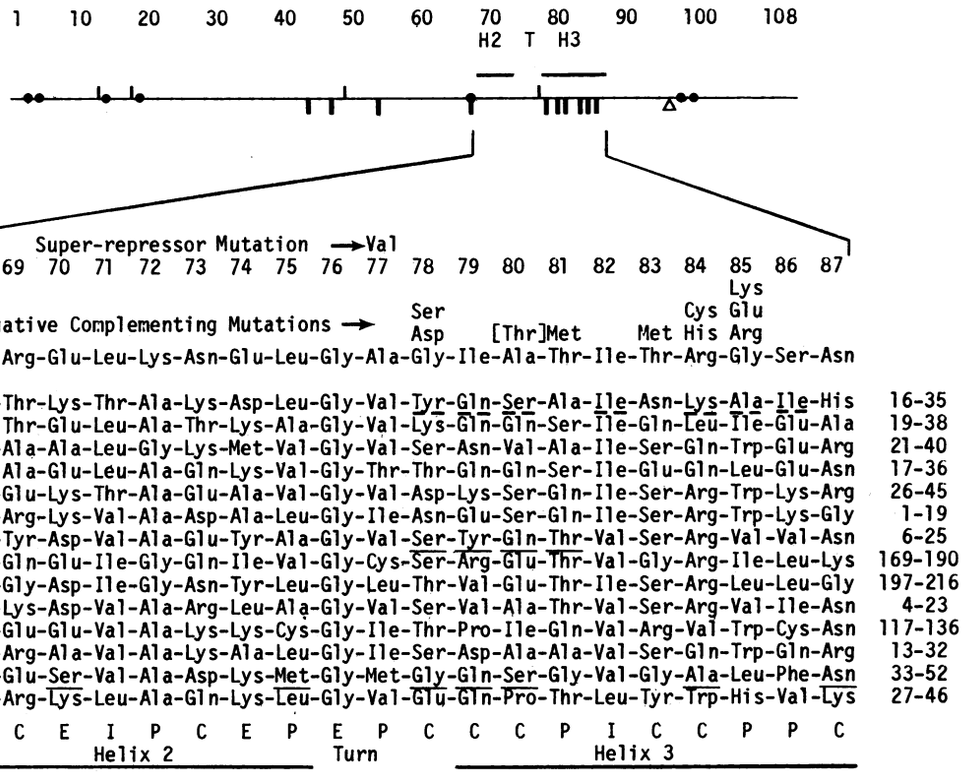

FIG. 1. Comparison of a segment of the *trp* repressor with homologous segments of other sequence-specific DNA recognition proteins. Line at top of the figure represents *trpR*, and scale above it shows the residues in the trpR polypeptide. Dots on the line indicate positions of nonsense mutations. Vertical bar above the line designates position of a superrepressor mutation; vertical bar below the line marks position of a *trans*-dominant negative-complementing mutation. Triangle marks position of a change that results in a leucine to proline substitution. H2 T H3 indicates the segment of the trpR polypeptide that is homologous to the helix 2-turn-helix 3 DNA binding domain of λ*cro* repressor. This region is expanded below, with residue number and identity in the trpR polypeptide indicated. Replacements in negative complementing and superrepressing mutants are given. Mutant AT80 affects wild type repressor activity. Its amino acid replacement is in brackets. Below are segments of other DNA binding proteins that are homologous to DNA recognition sequences of λcI and λcro proteins, aligned to maximize matches (taken from refs. 6, 7, 24, 38 and 39). Numbers indicate codon positions. Underlined residues are those changed in other *trans*-dominant mutant repressors (10, 23); dashed underlines indicate recessive mutations (A. Pakula and R. Sauer, personal communication). Capital letters at bottom of the figure indicate whether an amino acid side chain is exposed to solvent (E), partially exposed (P), totally interior (I), or makes contact with DNA (C), based on analyses with the λcro protein (7, 24). λCro, repressor of bacteriophage λ; 434Cro, repressor of bacteriophage 434; P22R, repressor of bacteriophage P22; 434R, repressor of bacteriophage 434; λcII, repressor of bacteriophage λ; P22cI, repressor of bacteriophage P22; lacI, lactose repressor of *E. coli*; CAP, catabolite repressor protein of *E. coli*; fnr, anaerobic regulatory protein in *E. coli*; galR, galactose repressor of *E. coli*; Matal, al mating-type protein of *Saccharomyces cerevisiae*; P22Cro, repressor of bacteriophage P22; λcI, repressor of bacteriophage λ; tetR, tetracycline resistance repressor of Tn*10*.

Mutations in this region (Fig. 1) also destroy operator binding in the λcI (10) and λ*cro* repressors (Andrew Pakula and Robert Sauer, personal communication), and may be negative complementing in λcI, when the mutant repressor is overproduced (42).

Mutant Qam68 encodes only the first 67 residues of the *trp* aporepressor, yet the inactive polypeptide it produces is a potent negative complementing subunit (Table 2). This finding indicates that the truncated polypeptide formed by this mutant possesses most, if not all, of the essential subunit contact sites. Mutant *lac* repressors have been described that display the same phenotype (9). Similarly, the carboxyl-terminal fragment of the λcI repressor will poison wild-type repressor polypeptides *in vitro* (43). This domain contains the oligomerization contacts. The DNA binding domain in both the *lacI* and λcI repressors is near the amino terminus, whereas in the *trp* repressor this domain appears to be closer to the carboxyl terminus. It has been pointed out by de Crombrugghe *et al.* (44) that most characterized repressors that bind operator in the absence of a cofactor have their helix-turn-helix region near the amino terminus. The three proteins known or suspected to require cofactors for operator binding, CAP, trp repressor, and fnr repressor, have the DNA binding domain near the carboxyl terminus (44). The significance of this is not clear.

Table 3. Properties of superrepressing plasmids

trpR allele on plasmid	With Trp	Without Trp
EK13	4	220
EK18	4	115
EK49	3	60
AV77	4	130
trpR+ (pRLK13)	5	800

Recipient strain was W3110 *tnaA2* Δ*lacU169 trpR2* (λTLF1). All cultures were grown in minimal medium/0.2% glucose/0.2% acid-hydrolyzed casein/chloramphenicol (20 μg/ml), with or without L-tryptophan (20 μg/ml). Numbers represent units of β-galactosidase.

Both LP96 and Wop99 produce relatively inactive proteins and yet only weakly disable wild-type subunits (Table 2). Based on our previous interpretations, we believe that each of these mutant proteins has a complete helix 2–helix 3 domain and the necessary subunit contact sites (Fig. 1). LP96 substitutes a proline for a leucine near the end of the polypeptide, and we are unable to predict how the resulting structural change might affect remote segments of the mole-

cule. Wop99 is missing the last 10 amino acid residues and yet seems to generate moderately active heterodimers. It would appear that whatever function the carboxyl-terminal portion of the molecule serves, this segment is needed in only one subunit of the dimer.

Not all of the mutations we have studied decrease the activity of the *trp* repressor. A change in the trpR polypeptide in the presumed hinge region connecting helix 2 to helix 3 (in mutant AV77) appears to have exactly the opposite effect—i.e., enhancement of repression. Examination of Fig. 1 reveals that, in most other repressors, there is a somewhat bulky hydrophobic residue immediately adjacent to the invariant glycine at the bend between the helix 2 and helix 3 regions. The *trp* repressor is exceptional in having alanine at this position. In the superrepressor mutant AV77 valine replaces alanine, making the *trp* repressor more like the tighter binding phage repressors.

The other three superrepressing alleles have glutamic acid to lysine substitutions at positions 13, 18, or 49 of the *trp* repressor. We cannot exclude nonspecific binding to DNA as the explanation for their action. However, we think this is unlikely because such mutations within *lacI* are paradoxically $LacI^-$ *in vivo* (11, 12). The phenotype observed here could result from enhanced binding of L-tryptophan, which would activate the aporepressor at abnormally low intracellular tryptophan concentrations. Alternatively, some of these mutant repressors may be capable of assuming a partially active conformation in the absence of tryptophan. Analyses with pure mutant repressors should distinguish among these possibilities. Mutant EK49 deserves special attention because it produces the most active repressor (Table 3), yet its amino acid change is located in the same polypeptide segment as those in the three negative-complementing mutants TM44, EK47, and RH54. Obviously, this segment of the polypeptide also plays a major role in repressor function.

The mutations reported here are consistent with the proposal that the *trpR* repressor recognizes specific operator sequences via a helix-turn-helix structure. The first two-thirds of the repressor polypeptide appears to be sufficient for dimer assembly *in vivo*. The amino-terminal segment of the protein is susceptible to superrepressor mutations. This may indicate that this region of the polypeptide is involved in L-tryptophan binding. It will be interesting to reexamine these interpretations when the three-dimensional structure of the *trp* repressor is known (45).

We must point out that not all bacterial DNA binding proteins have segments with strong homology to the sequences shown in Fig. 1 (7, 46, 47). However, it would not be surprising if such a structure does exist in these proteins but is formed from a more divergent primary sequence. In this regard, the recent observations of Laughon and Scott (48) are noteworthy. They have shown that the highly conserved homoeo box of the Ubx, Antp, and ftz proteins of *Drosophila* has weak homology to both the yeast mating-type proteins and the DNA recognition domain of bacterial repressors. These homeo proteins are believed to control entire batteries of developmentally expressed genes. Perhaps the helix-turn-helix motif of DNA recognition will prove to be common to all DNA sequence-recognizing proteins.

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