

Mutagenesis supports water mediated recognition in the *trp* repressor – operator system

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High resolution crystallographic analysis of the *trp* repressor – operator complex indicates that the principal determinants of specificity are water mediated hydrogen bonds between the helix – turn – helix and the identity elements of the operator. One such hydration site involves a conserved G-C base pair (designated G₆) six nucleotides away from the dyad which, if changed symmetrically to any other pair (e.g. G₆ → A) reduces affinity to non-specific levels. This same water site also contacts the conserved A₅ which, if changed to G (mutation A₅ → G), also diminishes affinity. The stereochemistry of the water mediated hydrogen bonding system predicts that the severe deterioration of *in vitro* binding caused by G₆ → A should be reverted by a second deleterious mutation A₅ → G. This proved to be the case. No other second mutation at conserved operator position 5 or 7 (flanking the G₆ → A) reversed the effect of G₆ → A. *Key words:* gel-retardation analysis/hairpin constructions/second site operator revertant/*trp* operator mutations/*trp* repressor – operator binding

Introduction

The *trp* repressor of *Escherichia coli* represses transcription of genes related to tryptophan biosynthesis by binding specifically to the conserved operator sequences in the promoter of four operons (*trp*, *aroH*, *trpR* and *mtr*) (Gunsalus and Yanofsky, 1980; Heatwole and Somerville, 1991). The protein is a dyad symmetrical dimer in which each subunit contains a helix – turn – helix motif that, in the presence of L-tryptophan, interacts specifically with the surface of successive major grooves of its pseudo-symmetrical target DNA, the *trp* operator (Kelley and Yanofsky, 1982; Schevitz *et al.*, 1985). When two L-tryptophan molecules bind symmetrically to the aporepressor (one per subunit), the two symmetrically disposed DNA binding motifs are oriented so that they support strong specific binding (Otwinowski *et al.*, 1988). The crystal structure of the *trp* repressor – operator complex revealed that the ‘recognition’ of the operator by the repressor is largely the result of stereospecific solvent mediated interactions between the repressor and the bases that specify the operator’s identity (Otwinowski *et al.*, 1988; R.-G.Zhang, C.K.Kundrot, Z.Otwinowski and P.B.Sigler, submitted). The region of the

repressor involved in these water mediated contacts is at the N-terminus of the second helix of the helix – turn – helix motif (Figure 1). Here we report evidence from *in vitro* mutagenesis experiments that supports the participation of water molecules in *trp* repressor – operator recognition. The crystal structure of the repressor – operator DNA complex was used to predict and design a second site revertant of a severely defective operator mutation; that is by changing a second base pair (symmetrically) we reversed the effects of the original operator mutation.

Sequence consensus and mutagenesis experiments (Bennett and Yanofsky, 1978; Bass *et al.*, 1987; Staacke *et al.*, 1990) clearly indicate that the base pairs at positions 4, 5, 6 and 7 from the dyad are the strongest determinants of the operator’s identity. Of these, base pair 6 appears most important; it is absolutely conserved in all *E.coli* operators (Figure 2A) and mutation to any other base pair reduces the stability of the repressor – operator complex by at least 10⁶-fold in the *in vivo* challenge assay of Youdarian and coworkers (Bass *et al.*, 1987). The base pair at position 5 from the operator’s dyad is also conserved, but *in vivo* mutagenesis indicates that a replacement of A₅ with G is better tolerated (only 1.7-fold diminished) than a G₆ to A transition at position 6. Like position 6, a pyrimidine at position 5 results in a complete loss (10⁶-fold) of specific affinity *in vivo* (Bass *et al.*, 1987). The stereochemistry underlying the importance of G₆ and A₅ as identity elements is illustrated in Figure 1. In the crystalline complex, both of these purines interact with the protein through water mediated contacts. In this paper we will focus on the water site that bridges the amide NH of Ala80 to G₆ and A₅ through a stereospecific tetrahedral arrangement of hydrogen bonds.

The premise of this paper is as follows: if the water mediated hydrogen bonds seen in the 1.9 Å crystal structure (R.-G.Zhang, C.K.Kundrot, Z.Otwinowski and P.B.Sigler, submitted) are important in producing strong sequence-specific interactions between the *trp* repressor and operator, then inspection of Figure 1 would suggest that the deleterious effect of replacing G₆ with A₆ might be reverted if A₅ were replaced by G₅. This second site reversion simply swaps the donor and acceptor functions at the 6 position of these two critical purines with preservation of the tetrahedral hydrogen bonding scheme at the bridging water molecule. Using a variation of the gel-mobility retardation assay developed by Haran *et al.* (1992) and a competitive filter-binding assay based on the study of Hurlburt and Yanofsky (1990) we tested this hypothesis and found the predicted reversion.

Results

A double mutation of the *trp* operator restores specific repressor binding

To test the predictions stated above, a set of DNA oligonucleotides was synthesized containing an idealized wild type

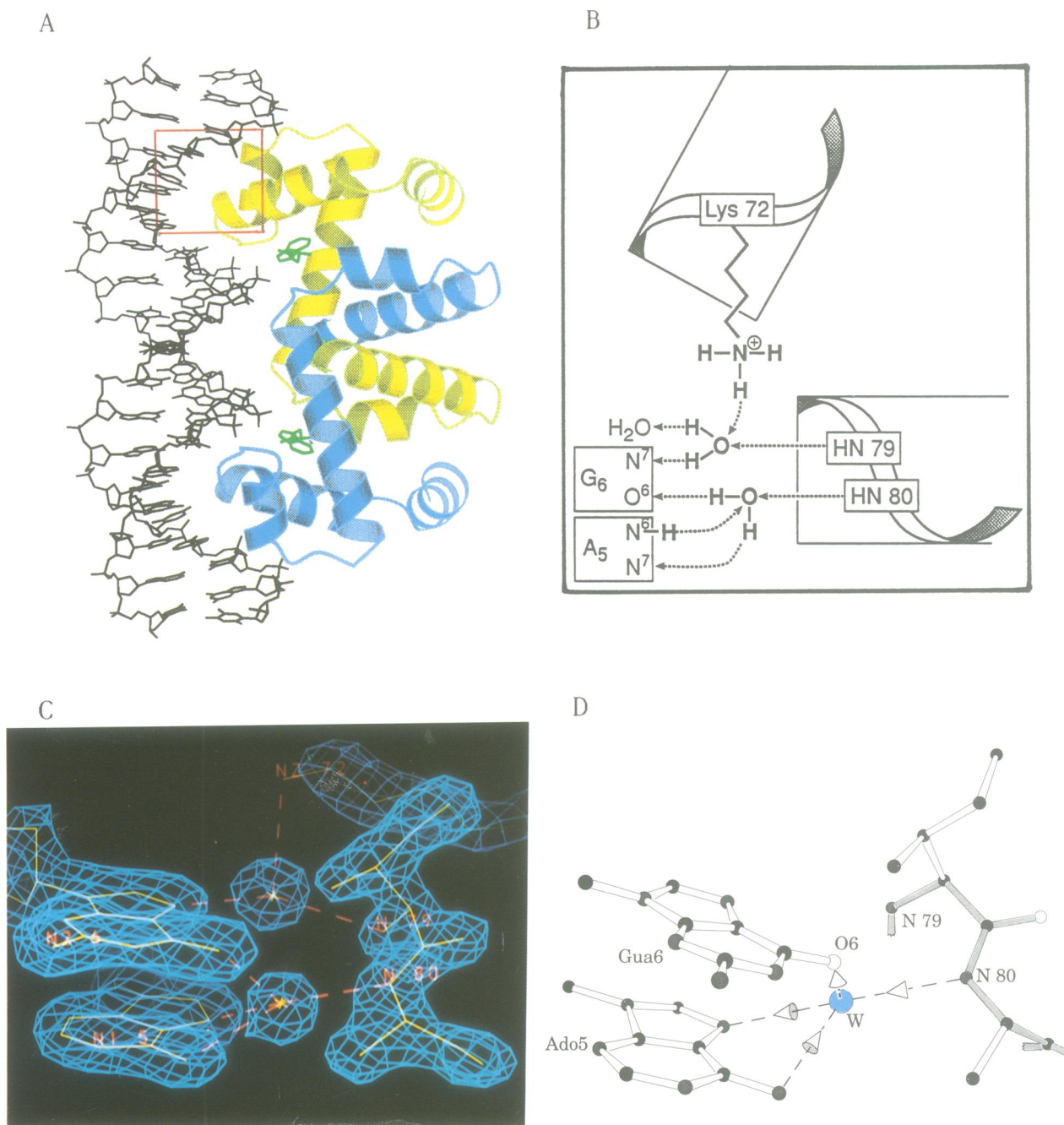


Fig. 1. Schematic representations of interactions addressed in this paper. (A) Ribbon drawing of the crystalline *trp* repressor–operator complex. (B) Schematic representation of water mediated hydrogen bonded interactions observed by Zhang *et al.* (submitted) at the tip of the second helix (helix E) of the helix–turn–helix [outlined in red in (A)]. (C) Electron density of the region schematized in panel (B) showing water mediated interactions. (D) Skeletal drawing of the region in (C) showing a water molecule mediating the interaction between the amido proton of Ala80 and purine bases in sequence positions 5 (adenine N⁶ and N⁷) and 6 (guanine O⁶) of the operator (for numbering see Figure 2).

operator sequence and variants containing one or two pairs of symmetrical changes (Figure 2). Repressor affinity for these oligonucleotides was measured by gel-retardation and filter-binding assays (Figure 3). Symmetrical 18 bp test sequences were incorporated into the 21 bp stem of a generic 'hairpin' construction (Figure 2) similar to that introduced by Haran *et al.* (1992) in the binding experiments that confirmed the true *trp* operator sequence. Such hairpin constructions prevented the dissociation of short two-stranded palindromic duplexes (20 bp or less) and, thereby, eliminated a competing equilibrium which confounded a previously reported *in vitro* binding analysis (Staacke *et al.*, 1990). This

competing equilibrium arises from the fact that the stability of a short conventional two-stranded duplex is bimolecular in the concentration of the component strands. At the nanomolar (or lower) concentration of DNA usually used in *in vitro* binding assays, there is an appreciable dissociation of the individual strands of two-stranded 'palindromic' duplexes. Once dissociated, each strand can fold back on itself to form a small hairpin whose stability is concentration independent. Thus low concentrations (or passage through the gel) favors dissociation of such duplex targets. Tethering the ends of the palindromic duplex strands together with a 5 bp loop converts the construct into a hairpin (Figure

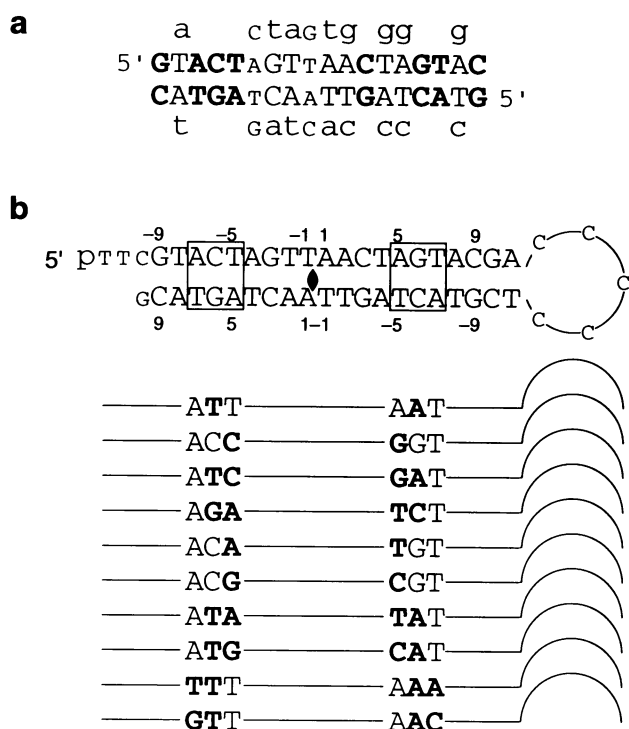


Fig. 2. Mutations introduced into the *trp* operator and subjected to gel-retardation analysis. (a) 18 bp consensus sequence for the four *E. coli* promoters of *trpEDCBA*, *aroH*, *trpR* and *mtr* regulated by *trp* repressor based on the identity of corresponding sequence positions. Large bold capital letters correspond to four occurrences of the same base at that position, large capital letters represent three out of four, small capital letters represent two out of four and lower case letters represent one occurrence at the site. All base pairs of four operators are represented. (b) Hairpin constructions containing the 18 bp consensus operator in the 21 bp stems. Numbering uses the dyad axis (♦) as origin and reflects the symmetry of this idealized sequence. The box indicates the region where symmetrical mutations were introduced. The first five figures are the test sequences whose affinities were also quantified by filter-binding studies.

2). This obviates the second equilibrium and makes feasible the use of short symmetrical targets at the low concentrations required for accurate quantitation of high affinity sequence-specific binding. The five nucleotide loop of the hairpins used here apparently does not interfere with repressor binding when separated by 2 bp from the 18 bp target in the stem of the hairpin (Haran *et al.*, 1992).

Hairpin constructions bearing symmetrical single and double mutations at positions 5, 6 and 7 of the operator sequence were electrophoresed in the presence of *trp* repressor (Figures 2 and 3). Figure 3A shows the effect of single (symmetrical) mutations at position 5 of the operator on repressor-retarded gel electrophoretic mobility. The only symmetrical mutation at this position which results in a measurable band shift is $A_5 \rightarrow G$. Although the affinity of the repressor towards the operator carrying this mutation is easily detected by a retarded band, it is diminished to 8% of the wild type target affinity when quantitated by the filter-binding experiments described below. Any other base substitution at position 5 diminishes affinity to the point where we cannot detect repressor–DNA complexes in the gel-shift assay even at repressor concentrations as high as $1 \mu\text{M}$ (Figure 3A and data not shown). These results are fully consistent with the results of the *in vivo* challenge assay (Bass *et al.*, 1987). On the other hand, any mutation at

position 6 completely abolishes repressor binding [Figure 3B and *in vivo* analysis of Bass *et al.* (1987)]. In the absence of stereochemical reasons to the contrary, these results with single (symmetric) operator mutations at sequence positions 5 and 6 would cause one to predict that any combination of symmetrical double mutations at positions 5 and 6 would lower the affinity for the repressor beyond that observed for mutation at position 6. However, as outlined above, the crystal structure of the *trp* repressor–operator complex predicts that the symmetrical double mutation that changes both $G_6 \rightarrow A$ and $A_5 \rightarrow G$ should restore specific repressor binding. Figure 3 shows that this symmetrical double mutation does, indeed, restore a level of binding to the operator that is easily detected by the gel-shift assay. Although the affinity is reduced to 5.7% of wild type it represents an essentially complete reversion of the devastating effect of the $G_6 \rightarrow A$ mutation. We tested operators carrying all other double mutations at positions 5 and 6 as well as at positions 6 and 7 (Figures 2 and 3), and none of them can restore detectable repressor binding in the gel-retardation assay. In fact, all such double mutations, when quantitated by filter-binding studies, show an affinity the same as or lower than the $G_6 \rightarrow A$ mutation (Figure 4).

Quantitation of repressor affinity for mutant operators

We have used competition for nitrocellulose filter-binding to determine the relative affinities of the operator mutants. Figure 4 shows the results of competition between a radioactive 43 bp *trp* promoter fragment (Hurlburt and Yanofsky, 1990; Haran *et al.*, 1992) and DNA hairpins containing the wild type and mutated *trp* operator targets. The hairpin containing an idealized wild type operator sequence competes best with the 43 bp promoter fragment; in fact, as shown by Haran *et al.* (1992), it competes as well as an unlabeled 43 bp promoter fragment. The symmetrical mutation $G_6 \rightarrow A$ reduces the affinity for the repressor >500-fold. However, a simultaneous change of $G_6 \rightarrow A$ and $A_5 \rightarrow G$ restores affinity nearly to the level seen with a single $A_5 \rightarrow G$ mutation (5.7% of the wild type). Although wild type affinity has not been completely restored, the severely deleterious effect of the $G_6 \rightarrow A$ mutation has been reversed.

Discussion

Structural basis for the predicted reversion of $G_6 \rightarrow A$

It is appropriate to employ site-directed mutagenesis to test mechanisms inferred from crystallographic studies. This is especially important when the inferences suggest novel and untested biochemical mechanisms. It is for this reason that this paper addresses the crystallographic finding of fixed water molecules as key elements in the stereochemistry of a specific protein–DNA interaction. These observations were unexpected and were challenged by gel-retardation assays that purported to show that an alternative sequence, and not the traditional *trp* operator used in the crystal structure (Figures 1 and 2a), is the target of the *trp* repressor (Staacke *et al.*, 1990). Haran *et al.* (1992) refuted this assertion, showing that the gel-retardation results were anomalies arising from competing equilibria and, once these artifacts were recognized and experimentally addressed, gel-shift and filter-binding experiments confirmed the traditional operator sequence as being the *trp* repressor target. They

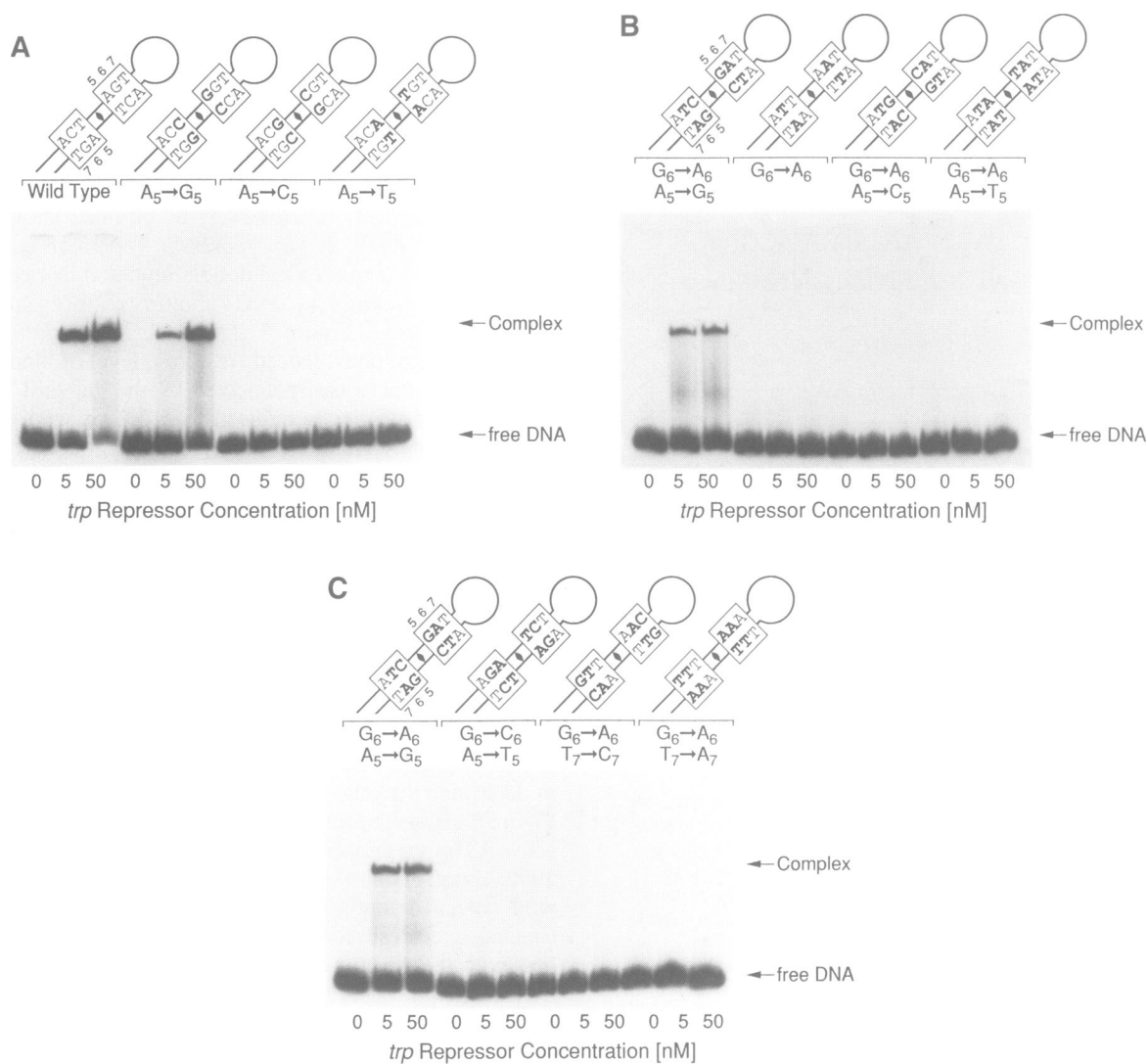


Fig. 3. Gel-mobility retardation analysis of wild type and mutant operator sequences using hairpins whose stems contain the symmetrical duplexes shown in Figure 2. (A) The wild type *trp* operator is compared with single mutations at position 5. Mutations are indicated on the hairpin diagram shown above gels. DNA concentration is 1 nM in all experiments. The stoichiometry of the complex was shown previously to be one dimeric repressor bound to one DNA fragment (Haran *et al.*, 1992). (B) The $G_6 \rightarrow A_6$ mutation plus all four possible base pairs at position 5. (C) The reversion of $G_6 \rightarrow A_6$ by $A_5 \rightarrow G_5$ (first three lanes) plus three examples of mutations flanking changes at position 6 that do not support gel band shift of the *trp* operator.

also pointed out that sequence consensus and mutagenesis support the alignment of the repressor on the operator seen in the crystalline complex. Most recently, the structures of the *trp* repressor-operator complex have been determined in two different crystal forms grown from high salt (Lawson and Carey, 1993; A.Joachimciak, Z.Otwinowski, R.Hegde and P.B.Sigler, personal communication). Both structures show the same interface as that described in the original X-ray study (Otwinowski *et al.*, 1988), and thereby relieves the concern that the solvent of crystallization may have altered the chemistry of the specific interface (Brennan and Matthews, 1989; Carey *et al.*, 1991).

Water mediated contacts determine base preferences at positions 5, 6 and 7

The water mediated interactions between the N-terminal end of helix E and A_{-7} , G_6 and A_5 provide a stereochemical rationale for the base preferences exhibited in the operator consensus sequence (Figure 2) and in mutagenesis experi-

ments (Bennett and Yanofsky, 1978; Bass *et al.*, 1987; Staacke *et al.*, 1990). In earlier work, the requirement for a purine at operation position -7 was shown to be a water mediated contact between the γ -hydroxyl of Thr83 and the N^7 of the purine (Otwinowski *et al.*, 1988; Luisi and Sigler, 1990). The preference for A over G is due to the arrangement of neighboring polar groups that orient the water molecule so that it will only accept a hydrogen bond from the substituent at the 6 position of the purine. Thus, a hydrogen bond donor, the exocyclic amine of adenine, is preferred over the carbonyl O^6 of guanine (Otwinowski *et al.*, 1988). Here we narrow the focus to the water mediated complementarity between the exposed peptide NH groups at the very tip of the E helix (Ile79 and Ala80) and purines G_6 and A_5 (Figure 1). The water molecule at Ile79 receives two hydrogen bonds from the protein; one from the peptide NH of Ile79 and the other from the ζ -ammonium group of Lys72. The special orienting role of the Lys79 is evident from the fact that other bacterial and phage regulatory

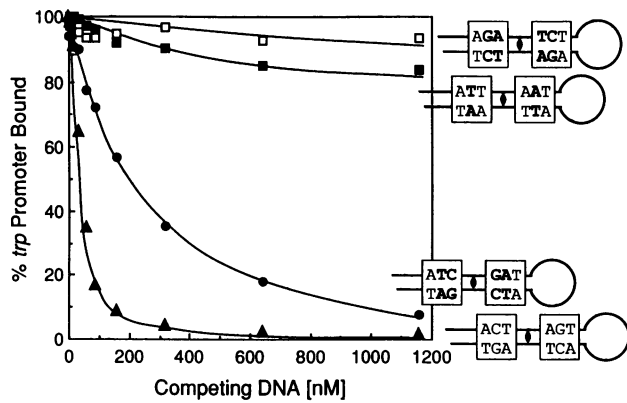


Fig. 4. Filter-binding analysis of *trp* repressor affinity. Assay shows the capacity of a test sequence, in the stem of a hairpin construct (Figure 2), to compete with a [³⁵S]43 bp *trpEDCBA* promoter containing the wild type *trp* operator. Conditions and procedures as in Material and methods. The test sequences containing wild type and mutant operator sequences in the stems of the hairpin. When compared with the effect of the wild type operator sequence (▲), mutation of G₆ → A (■) significantly interferes with the ability to compete. Introducing a second mutation A₅ → G restores to the 'double mutant' (G₆ → A, A₅ → G) much of the ability to compete (●). Typically any other double mutant (G₆ → C, A₅ → T) will compete more poorly than a single mutant at position 6 (□) and is comparable in affinity with that of the *gal* operator (not shown, see Haran *et al.*, 1992).

proteins with a helix–turn–helix have an Ala at the corresponding position, whereas mutagenesis shows that only Lys 72 (or, marginally, the mutation Lys72 → Tyr) supports the function of the *trp* repressor. It is likely that this water molecule is highly polarized as it is fixed to the positive pole of the E helix's dipole as well as the positively charged ammonium group of the Lys72 side chain. Thus, a firmly positioned and oriented water molecule donates a highly polarized hydrogen bond to the electronegative N⁷ of the purine at position 6 of the operator. This means a purine is required at position 6 in the wild type operator, but adenine is not acceptable.

We observe here that changing G₆ symmetrically to any other base, including A₆, dramatically reduces the stability of the complex to 'non-specific' levels. This is consistent with the absolute conservation of G₆ in all *E. coli trp* operators (Figure 2a) and with the results of *in vivo* mutagenesis experiments mentioned earlier (Bennett and Yanofsky, 1978; Bass *et al.*, 1987; Staacke *et al.*, 1990). The reason why G₆ rather than A₆ is required is clear from Figure 1. Tetrahedrally hydrogen bonded water donates two and accepts two hydrogen bonds. Since the amide NH at Ala80 and the exocyclic amine of A₅ are both donors, the water fixed to the amide NH of Ala80 must donate a hydrogen bond to the 6 substituent of this purine. Thus, the O⁶ carbonyl of G₆ is strongly preferred to the exocyclic N⁶ of A₆. The loss of a hydrogen bond (two hydrogen bonds when the symmetrical site is considered) may not account for all of the differences in affinity between G₆ and A₆; the disruptive effect of A₆ may be augmented by steric hindrance between the proton of the exocyclic amine and the firmly fixed water molecule at NH of Ala80. Steric hindrance can be especially disruptive to the complex if the participating groups are otherwise well fixed by their neighbors and the structure cannot adjust locally to accommodate the interference. This may be the case with

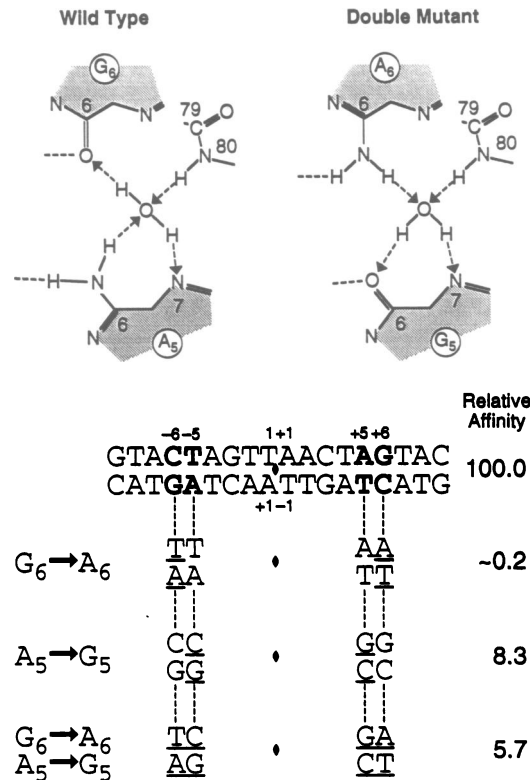


Fig. 5. Diagram and table summarizing results of gel-shift and filter binding assay with mutant operators. The relative affinity of mutant operators was evaluated from competition filter-binding data by a multiple non-linear regression method (FFit program by David I.Hoyer). Data points were fitted to the equation that related the amounts of [³⁵S]43 bp promoter–repressor complex retained on the filter to the concentration of competing DNA. (%B = 100/(K_d/K_c) × (D_c/RD_c) - 1) + 1), where %B is the percent of [³⁵S]43 bp promoter–repressor complex retained on the filter, K_d is the dissociation constant for the 43 bp promoter (4 × 10⁻¹⁰ M), K_c is the dissociation constant for competing DNA, D_c is the concentration of competing DNA and RD_c is the concentration of the competing DNA–repressor complex. K_d, K_c and RD_c are adjustable parameters to optimize the fit. K_d by quantitative gel shift analysis is 2 × 10⁻¹⁰ M (A.Joachimiak and T.E.Haran, unpublished).

purine 6 for three reasons. First, the DNA is tightly anchored to the protein through an extensive array of phosphate contacts; second, the purine at position 6 is sandwiched between base pairs that have 'specific' interactions with the protein; and third, the position of the water molecule is stabilized by three other interactions.

It is also clear from Figure 1 that a purine is required at position 5. Substituting a pyrimidine for A₅ provides no basis for specific interactions and hence results in severe deterioration of binding (Figure 3A). The change A₅ → G, which replaces the exocyclic amine with a carbonyl, also decreases affinity, but by only 10-fold, which is considerably less than the reciprocal change at G₆. One explanation of the relative tolerance for O⁶ in the purine at operator position 5 is that the range of hydrogen bond acceptance angles for the carbonyl group is much larger than for the amino group, thus providing the intervening water molecule more degrees of freedom to adjust. Also, when replacing an amine with a carbonyl, no hydrogen atoms are introduced to cause steric hindrance. In general, the hydrogen bonding patterns of carbonyl oxygens are less stereo-restrictive, as evidenced by the preference of oxygen over nitrogen atoms

for surface hydration in proteins (Baker and Hubbard, 1984). Other factors unrelated to hydrogen bonding, such as sequence-dependent deformability, may also influence the affinity of the mutant operators. Clearly, a complete structural explanation for the disruptive role of any change in functional groups must await the crystal structure of the complexes containing mutationally altered operators.

The second site mutation: $A_5 \rightarrow G$ reverses the deleterious effect of $G_6 \rightarrow A$

We have shown that introducing a second symmetrical mutation, $A_5 \rightarrow G$, reverses the severe deterioration of specific binding caused by the symmetrical change, $G_6 \rightarrow A$. This result was predicted from the tetrahedral pattern of water mediated hydrogen bonds between the operator's conserved purines at sequence positions 5 and 6 and the peptide NH at position 80. No other mutation at position 5 will revert the $G_6 \rightarrow A$ mutation; indeed, all other symmetrical double mutations involving positions 6 and 5 or 6 and 7 are reduced in their affinity for the repressor by a factor of at least 500.

We ascribe the reversion to the restoration of the water mediated hydrogen bonding pattern at the peptide NH of Ala80 where the polarity of the two hydrogen bonds has been reversed, i.e. an acceptor (the O^6 of guanine 6) has been replaced by a donor (the exocyclic N^6 of adenine 6). Conversely the donor, the N^6 of adenine 5, has been replaced by an acceptor, the O^6 of guanine 5.

Why has the reversion of the $G_6 \rightarrow A$ not fully restored wild type affinity to the operator? Although formally the hydrogen bonding system has been restored, its stereochemistry has not. The oxygen atom of the intervening water is much closer to the plane of the purine at position 5, i.e. considerably below the plane of purine 6. Since the directionality of hydrogen bonds from the exocyclic aromatic amine is more constrained to the aromatic plane, the hydrogen-bonds donated to the out-of-plane water by A_6 are weakened. The O^6 of G_6 is preferred, as noted earlier, because the carbonyl oxygen is more tolerant of out-of-plane interactions. Here again there may be additional factors in the 'double mutant' that alter the stability of the complex without affecting the hydrogen bonding scheme. A crystallographic analysis of the *trp* repressor complex containing this doubly altered operator is called for.

Materials and methods

Purification of *trp* repressor

The *trp* aporepressor protein was overexpressed in the strain pETG13atrpr3/BL21DE3 kindly provided by Dr C. Lawson (Brookhaven National Laboratory). The protein was purified using the procedure of Joachimiak *et al.* (1983). In an alternative accelerated purification protocol the protein was chromatographed on a Mono Q (16/10) column and eluted with a linear 100–300 mM NaCl gradient. This was followed by separation on a Mono S (10/10) column and the aporepressor was eluted with linear 0–250 mM NaCl gradient. Aporepressor preparations were shown to be homogeneous by polyacrylamide gel electrophoresis under denaturing conditions and silver staining. The protein is fully active, upon addition of L-tryptophan, in DNA binding assays and in calorimetric titrations.

Preparation and labeling of DNA fragments

All oligodeoxynucleotides used in this study were synthesized on an automated DNA synthesizer (Applied Biosystems) using reagents from Peninsula. The oligonucleotides were purified by electrophoresis on polyacrylamide gels containing 7 M urea. DNA fragments were eluted from the gel with 0.5 M sodium acetate pH 5.2 and ethanol precipitated. Excess salt was removed by washing the precipitate with 70% ethanol and the DNA was vacuum dried.

Small amounts (2–4 pmol) of oligonucleotides were labeled with ^{32}P or ^{35}S . The 5' termini of hairpin duplexes (Figure 2) were labeled with [γ - ^{32}P]ATP (specific activity ~5000 Ci/mmol) and T4 polynucleotide kinase. The 3'-recessed ends of the *trpEDCBA* promoter fragment used in filter-binding competition experiments (Figure 4) were filled in with [α - ^{35}S]dATP (specific activity ~600 Ci/mmol), dTTP and the Klenow fragment of DNA polymerase I. The hairpinned radiolabeled oligonucleotides were purified by polyacrylamide gel electrophoresis in the presence of 7 M urea, while the *trpEDCBA* promoter was purified on native polyacrylamide gels. All fragments were extracted from the gel as described earlier. After precipitation the radiolabeled DNA samples were dissolved in buffer (10 mM Tris-HCl, 1 mM EDTA pH 7.6) and 5 mM DL-dithiothreitol. Hairpin DNA were annealed by heating the DNA samples to 95°C and shock cooling on ice. All DNA samples were stored at -80°C. DNA solutions of known concentration and specific radioactivity were prepared by adding the labeled DNA to a 50:1 molar excess of unlabeled DNA of known concentration (Haran *et al.*, 1992).

Gel mobility retardation assay

The gels used in Figure 3 were native 16% polyacrylamide gels (acrylamide/bisacrylamide ratio = 75:1) cast and run with 10 mM phosphate buffer (pH 6.5) and 0.25 mM L-tryptophan. The gels were prerun at 12.5 V/cm for 1.5 h, and run at 25 V/cm for 3 h, with recirculation of the buffer. The gel temperature was kept at 15°C by using a constant temperature gel apparatus (Hoeffer) and a circulating refrigerated water bath (VWR). Samples (10 μ l) comprising 1.0 nM DNA, 5.0 nM or 50 nM *trp* repressor, 10 mM phosphate buffer (pH 6.5), 0.5 mM L-Trp, 25 mM NaCl and 0.1% Nonidet P40 (Sigma), were incubated for 30 min at room temperature. Samples were loaded while the gels were running. After electrophoresis the gels were vacuum dried at 80°C. The bands were localized by autoradiography.

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