Position and density effects on repression by stationary and mobile DNA-binding proteins

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We have investigated the effects of two types of DNA-binding proteins on bacterial repression. First, the effects of operator positioning on repression by stationary DNA-binding proteins, the Lac repressor and the Trp repressor, were examined in vivo. Both operator number and positioning play a role in determining in vivo levels of repression. Operators located within a promoter are more efficient regulators than those positioned at the start of transcription. Second, we investigated the effects of DNA-binding protein density on repression using a mobile DNA-binding protein, *Escherichia coli* RNA polymerase. We employed a transcriptional interference assay using convergent transcriptional units. The strong synthetic promoter conI and its derivatives were observed to interfere with expression of the *aadA* gene, which confers spectinomycin resistance upon its host. Transcriptional interference by RNA polymerase occurred only in cis and had a strong dependence on polymerase density that was modulated by varying the promoter strengths. A change in the density of approximately fourfold completely abolished the observed transcriptional interference. Several models are discussed to explain the repression patterns observed for stationary and mobile DNA-binding proteins.

[Key Words: Lac repressor, Trp repressor, *trp* promoter, operating positioning, transcriptional interference; genetic selection]

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The accurate and efficient regulation of gene expression is central to an organism's ability to undergo complex developmental transformations and to adapt successfully to changing environmental conditions. Consequently, organisms have evolved a rich and varied repertoire of mechanisms to control gene expression. These include the ability to modulate the activity of gene expression at every conceivable step in the cascade of expression from the abundance, accessibility, and structural continuity of DNA to the most subtle nuances of catalytic activity and structural integrity of proteins.

The step at which control is most often observed is the level of initiation of transcription. This is true for two reasons. First, it is the most energy-efficient control mechanism for the organism, circumventing the energy cost of transcription, translation, processing, and metabolism; second, it is the simplest step of regulation to detect experimentally and therefore is searched for most frequently and explored most thoroughly. Regulation of transcription is divided into two basic types, positive and negative, classically defined by whether the absence of a functional gene known to provide regulation causes an increase or a decrease in the rate of transcriptional initiation. More recently, this distinction has become blurred with the more detailed inspections of systems of greater and greater complexity.

Negative regulation was first elucidated by Jacob and Monod for the lacZYA operon of *Escherichia coli*. Although more and more complex examples of negative regulation are being discovered, we still lack a thorough basic understanding of precisely how the simplest systems function. For instance, the effect of operator number and position on the initiation of transcription remains largely unexplored. The initiation of transcription is thought to proceed in three steps (Chamberlin 1974; Buc and McClure 1985). First, RNA polymerase binds to the promoter to form the 'closed complex,' RPe. Then the DNA near the start of transcription is denatured, thus defining the formation of the more stable 'open complex,' RPc (Kirkegaard et al. 1983). Upon addition of ribonucleotides, the 'initiation complex' forms, RPc, may actually constitute two steps; the formation of a recycling abortive initiation complex and the subsequent conversion to a stable elongation complex that has been correlated with the loss of the σ-subunit after transcription of 9–11 nucleotides (Carpousis and Gralla 1980; Hansen and McClure 1980). Abortive cycling has yet to be demonstrated in vivo and may be an in vivo artifact. Although most models of repressor action suggest inhibition of polymerase binding as the mechanism of repression (Majors 1975; Squires et al. 1979; Ptashne et al. 1980), there is no reason to think that a repressor could not act by inhibiting any one of the initiation steps mentioned above. In fact, Schmitz and Galas
(1979) have shown that binding of the Lac repressor does not preclude binding of RNA polymerase. Furthermore, Straney and Crothers (1987) have shown that the Lac repressor can actually increase the initial binding of RNA polymerase and that it represses by inhibiting the formation of the open complex when bound at + 1. To address these questions concerning the mode of negative regulation proposed for the lac and trp operons, we performed a detailed analysis of operator-promoter interaction to determine how distance and geometry affect negative regulation.

Recently, much attention has been directed toward the question of how proteins can exert their influence at a distance. This type of regulation is ubiquitous in nature and varied in mechanism. It is involved in dosage compensation in mammals, silencer function in fungi (Brand et al. 1985), and archival DNA in bacteria (Downs and Roth 1987). These are examples of negative regulation that function over long distances, as opposed to the elegant and seemingly simple local mode of negative regulation proposed for the lac operon. Three general classes of mechanisms have been described for such distal actions (Wang and Giaever 1988): tracking of a protein along a DNA, the association of multiple proteins at separate sites to form a DNA loop in between, and distal interactions that are affected by the topology of the DNA. In the second part of our analysis of negative regulation, we examine the role that translocation of RNA polymerase along the DNA plays in repression. We demonstrate that complex regulatory circuitry can be assembled out of simple fundamental regulatory units. These new combinatorial units can now function at a distance and are able to amplify small changes in occupancy into much larger effects.

Results

The Lac repressor shows decreased repression with increasing distance

To determine the precise relationship between promoter function and operator position, a well-characterized promoter lacking all regulation was needed. Because few promoters fall into this category, an existing promoter, tac, was modified to remove its regulatory sequences. tac is a fusion promoter containing the −35 region of the trp promoter and the −10 region of the lac promoter. This hybrid promoter removes the trpR regulatory sequences from the trp promoter and the catabolite-repressing sequences from the lac promoter but leaves the lac operator sequences intact (Aman et al. 1983). Oligonucleotide site-directed mutagenesis was used to remove the lac operator from tac to create a strong constitutive promoter conl [constitutive] (Fig. 1) as detailed in Materials and methods. An EcoRI restriction site was placed at the start of transcription, followed by a polylinker to facilitate operator positioning (Fig. 2A). We constructed new regulated promoters with the 20-bp lac operator spaced approximately every 2 bp from +5 to +40, relative to the start of transcription. The sequence of each construct is listed in Figure 2B. These promoter constructs were assayed by allowing them to direct β-galactosidase synthesis in isogenic lacI + or lacI - backgrounds, as described in Materials and methods (the lacI - mutation results in a 10-fold increase in LacI synthesis). The results of the assays are shown in Figure 2B. Because the precise sequence of the 5' end of each message varies slightly, we cannot strictly control for message stability. Therefore, repressed and induced levels cannot be directly compared from one construct to the next. However, the induction ratio should control for the message stability and can be directly compared if we assume that the structures of the RNA for the repressed and induced states are the same. The induction ratios for the modified conl promoters are listed in Figure 2B. The repression appears to show a tripartite pattern. Operators positioned between +5 and +14 give high levels of repression, ~500-fold. However, positions +17 to +31 give a consistently lower induction ratio of ~100-fold. Finally, induction ratios begin to drop after +30, giving a final ratio of 29-fold at +40. Several points should be emphasized. First, conl promoters lacking the synthetic lacO oligonucleotide still show a twofold in-
**A**

Promoter Sequence

| Position | Sequence |
|----------|----------|
| -35      | GATTCAGTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA |
| -10      | GATTCAGTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA |
| +1       | GATTCAGTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA |
| Leader   | GAATTTGACGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA |

**B**

lac Operator Insertions

| Position | Sequence | β-galactosidase units |
|----------|----------|-----------------------|
| I-1(+5)  | GAATTTATATATAGGCTCCACATTAATTCGAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA | 0.61 270 442 |
| I-2(+7)  | GAATTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA | 0.40 357 853 |
| I-3(+9)  | GAATTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA | 0.64 324 506 |
| I-4(+11)| GAATTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA | 0.80 348 420 |
| I-5(+13)| GAATTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA | 0.60 310 517 |
| I-6(+14)| GAATTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA | 0.82 206 251 |
| I-7(+17)| GAATTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA | 9.3 478 51 |
| I-8(+18)| GAATTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA | 3.2 363 113 |
| I-9(+19)| GAATTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA | 1.5 303 202 |
| I-10(+21)| GAATTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA | 1.8 209 116 |
| I-11(+23)| GAATTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA | 2.1 214 102 |
| I-12(+25)| GAATTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA | 2.1 210 100 |
| I-13(+27)| GAATTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA | 1.8 212 117 |
| I-14(+31)| GAATTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA | 2.7 270 107 |
| I-15(+34)| GAATTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA | 3.2 239 75 |
| I-16(+40)| GAATTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA | 8.2 237 29 |
| conI    | GAATTTGACGCTCCGCAGTATATTCCAGCTGGTACCCGCCCCAGGAGATCCTGTCAGGACCCGCA | 163 346 2 |

**Figure 2.** The construction of lacI-regulated promoters. (A) This shows the sequence of the constitutive promoter conI, the construction of which is outlined in Materials and methods. The base at the start of transcription is indicated in boldface type. The sequence of the polylinker leader region is shown, along with the various restriction enzymes that recognize it. (B) The sequence of the lacI-1 through conI-16 lacI-regulated promoters. Their construction is detailed in Materials and methods. The start of transcription is indicated in boldface type. The positions of the bases contributed by the 20-bp synthetic lac operator are underlined in each promoter. (Right) The values of β-galactosidase activity of the conI derivatives on pNN387 when present in JM107[lacIq] or JM106[lacI-]. The ratio was calculated by dividing the activity in the lacI- background by that in lacIq.

**Position and density effects on repression**

This is probably due to the presence of a lacO sequence within the lacZ gene. When the repressor is bound at this site, it can act as a transcription terminator and thereby partially repress the production of a full-length functional message (Deuschle et al. 1988a). The lac operator within the lacZ gene of pNN387 is 1.3 kb from the start of transcription and whatever contribution it makes to repression should remain constant among the various constructs. Induction ratios listed here should be divided by 2 when considered in absolute terms. The true normalizing factor may be slightly larger if there is an interaction between operators, as suggested by Mossing and Record (1986). Second, it should be noted that the largest contribution to the change in induction ratio can be attributed to an increase in the level of expression in the repressed state because the levels in the absence of repressor are approximately equal for most constructs. Third, a sequence context effect on repressor function exists. Two different constructs, both bearing an operator at position +17 but in a different sequence context, give a difference of twofold in the induction ratio. Therefore, not only is the position of the operator important in determining repressor effectiveness, but its precise context must also play a role. This may be due to an altered operator affinity or to the complex rules governing the interplay of polymerase and repressor that determine the repressed levels of expression. For example, if the polymerase enhances the 'off' rate of the repressor by unwinding the DNA, then repressors whose operators are imbedded in GC-rich sequences may be slightly more efficient repressors than those imbedded in AT-rich sequences (cf. conI-7 and conI-8).

**Operator position is more critical for Trp repressor function**

The Lac repressor is an exceptional molecule. Its dissociation constant has been estimated to be $10^{-13}$ M (Riggs et al. 1969). This tight binding is thought to be responsible for its ability to terminate transcription. It is possible that other repressor molecules with somewhat less extreme properties might not share the pattern of repression observed for the Lac repressor. As a second model, we chose the Trp repressor encoded by trpR. The
Trp repressor was chosen because of its smaller size (24-kD dimer as opposed to a 150-kD tetramer) and its weaker dissociation constant $2 \times 10^{-9}$ M (Klig et al. 1987; Carey 1988).

To perform a systematic analysis of Trp repression, a second constitutive promoter, conII, was constructed. The sequence of conII is shown in Figure 3A. conII is a derivative of conI that has a KpnI site at $-6$ relative to the start of transcription, allowing a greater range of operator positioning. The trp operator sequence chosen for analysis is illustrated in Figure 3B and was taken from the central region of the trp promoter [$-19$ to $-1$]. This region has been shown to footprint with the Trp repressor in vitro and is the region to which operator constitutive mutations map in the trp promoter (Bennett and Yanofsky 1978). New promoters were created with the synthetic trp operator spaced $-3$--$4$ bp apart from $-5$ to $+16$ relative to the start of transcription. The sequence of each construct is shown in Figure 3B. These promoter constructs were assayed by allowing them to direct $\beta$-galactosidase synthesis on pNN387 in isogenic trpR$^+$ and trpR$^-$ backgrounds, as described in Materials and methods. The induction ratios for the modified conII promoters are shown in Table 1. The ability to repress shows a dramatic distance effect, dropping to barely detectable levels between $+9$ and $+16$. There also appear to be slight positioning effects; operators placed at $-5$ repress at only 50% of the levels of those at positions $-1$, $+2$, and $+5$, suggesting that the precise geometrical relationship between repressor and polymerase may play a role in repression among operators located within a given area. Of course, sequence contest effects as observed for the Lac repressor cannot be ruled out. In general, operator position is more critical for the Trp repressor function and the levels of repression differ significantly from those obtained with the Lac repressor.

![Figure 3. The construction of trpR-regulated promoters. (A) The sequence of the conII promoter present on pNN396. conII is a derivative of conI, and its construction is described in Materials and methods. The start of transcription is indicated in boldface type. (B) (Top line) The sequence of the synthetic trp operator oligonucleotide used to construct the trpR-regulated promoters. (−19 to −2 from trp) Beneath are illustrated the sequences of the conII-1 through conII-6 trpR-regulated promoters. The construction of these promoters is detailed in Materials and methods. The position of the trp operator is underlined in each promoter. (C) The sequence of the trp promoter. The bases important for Trp repressor binding are outlined (Bass et al. 1987). Each set of four bases represents the essential elements of one half-site, and each half-site is numbered below. Kumamoto et al. (1987) suggest that three Trp repressor dimers bind the trp promoter. The first dimer binding half-sites 1 and 2, the second binding 2 and 3, and the third binding 3 and 4. The Trp repressor can share half-sites because the critical bases within a half-site are palindromic. The sequence of tmr, a mutant form of trp, is shown below that of trp. It differs from trp at 6 bases, 3 each in half-sites 1 and 4. The mutational changes are indicated in boldface type.]
Table 1. Transcriptional activities of trpR-regulated promoters

| Promoter | β-Galactosidase units | pRPG9* | Ratios |
|----------|-----------------------|-------|--------|
| trp      | 6.5                   | 792   | 120    |
| trp(−19) | 22.5                  | 779   | 34     |
| conH-1(−5) | 226            | 1382  | 11      |
| conH-2(−1) | 117            | 1266  | 14     |
| conH-3(+)2 | 120          | 1755  | 10     |
| conH-4(+)5 | 63         | 637   | 3      |
| conH-5(+)9 | 375        | 1022  | 2     |
| conH-6(+)16 | 122     | 273   | 0.9   |

β-Galactosidase activities were measured for the promoters listed in Fig. 3 when placed onto pNN387. Activities are calculated according to Miller (1972) and are the average of at least two independent measurements. Activities typically varied no more than 15% between independent measurements.

* pRPG9 represents activities in a trpR+ strain carrying pRPG9, a pBR322-based clone of trpR, which overproduces the Trp repressor.

Repression of the trp promoter is determined by the number and positioning of operators

An outstanding question in prokaryotic regulation is why the trp promoter is so efficiently repressed relative to trpR. The Trp repressor regulates three known loci, trpEDCBA, aroH, and trpR. These operons show differing degrees of regulation in vivo. The aroH and trpR operons are regulated over a four- to fivefold range (Kelley and Yanofsky 1982; Grove and Gunsalus 1987), whereas the trp promoter is regulated over a 70-fold range, excluding attenuation (Kelley and Yanofsky 1982). Evidence has been presented to suggest that these promoters vary in both the number and positioning of Trp repressor-binding sites [Kumamoto et al. 1987]. These results suggest that the trp promoter may have three tandem repressor-binding sites covering ~33 bases (~25 to +7) when footprinted at high concentrations of Trp repressor in vitro. Furthermore, their results suggest that aroH promoter has two tandem binding sites covering ~30 bases (~50 to −20) and the trpR promoter has only one binding site covering 25 bases (~10 to +15) when footprinted in vitro. Kumamoto et al. (1987) suggest that the difference in levels of repression is due to the fact that the trp promoter has three binding sites for the Trp repressor, whereas trpR has only one. So far, only the central operator in trp has been shown to be functional in vivo (Bennett and Yanofsky 1978). Alternatively, the differing positions of the operators within these promoters may determine the efficiency of repression. Differential operator affinities could also be involved. To test these hypotheses and to determine the effect of a single repressor binding site located in the central portion of the promoter recognition elements, a mutant trp promoter trm [trp mutant], was constructed that left the interior Trp repressor-binding site intact (~19 to −1), but destroyed the outer two binding sites. The sequences of the trp and trm promoters are shown in Figure 3C. The mutations used to create trm are shown in boldface type. These changes were designed to combine all of the most severe operator-constitutive mutations for the trp operator, as determined by Bennett and Yanofsky (1978) and Bass et al. (1987) into each of the two exterior half-sites [1 and 4, Fig. 3B]. The repression data for these promoters are in Table 1. The trp promoter is repressed 120-fold, whereas trm is repressed only 34-fold. This evidence suggests that at least one of the other two trp operator sites function in vivo but that the majority of the repression is due to the central operator. Furthermore, it shows that operators positioned deep within the promoter element, overlapping critical RNA polymerase recognition sequences, are more efficient at repression than operators positioned at the start of transcription (cf. trm and −5 or −1 from Table 1). These data also suggest that both positioning and operator number play a role in determining the level of trp repression in vivo.

The repression data for trm suggest that a single trp operator has a minimal occupancy of 97% in vivo in the presence of the normal intracellular concentration of Trp repressor (assuming 99% occupancy would be equivalent to 100-fold repression). However, the same operator sequence located at a different position in the same promoter, e.g., −5 or −1, has severalfold weaker repression. Does this mean that they have a significantly lower occupancy than the trm operator or that they have reached the maximal repressing potential at near maximal occupancy for operators located at those positions? One possible model is that when an operator is occupied near the start of transcription, a polymerase is also bound and sits poised, ready to transcribe when the repressor dissociates. In this model, the basal level of the promoter is determined solely by the off rate of the repressor at near full occupancy. This hypothesis would predict that under these conditions, increasing the ‘on’ rate of the repressor by increasing its concentration should have little effect upon the basal level of tran-
scription. With this in mind, an experiment was performed, the results of which are listed in the last two columns of Table 1. pRPG9, a clone of the trpR gene on pBR322, was introduced into strains bearing several of the modified conII promoters. We estimate that pRPG9 overproduces the Trp repressor approximately fivefold. The data from this experiment indicate that increasing the concentration of repressor can increase repression (30–100%) of operators located near the start of transcription significantly. This implies that the simple model as stated above is incorrect. One obvious possibility is that once the repressor falls off, it still has an opportunity to rebind before the polymerase initiates transcription. Two other interesting but more complex models are explored in the Discussion.

**Repression at a distance may be exerted by convergent transcription**

Up to this point, this work and other studies have focused primarily on forms of regulation involving stationary regulatory proteins that exert their influence over very short distances [tens of base pairs]. However, the role played by mobile DNA-binding proteins to provide regulation that functions over longer distances [kilobases] has remained relatively unexplored. One mechanism for repression at a distance could be convergent transcription. To study this phenomenon, we designed the system illustrated in Figure 4A. The conI promoter was placed downstream from the aadA gene in an orientation such that it would transcribe aadA in an antisense direction to determine whether conI could affect aadA expression. aadA [aminoglycoside adenyltransferase] encodes an enzyme that adenylates aminoglycoside antibiotics such as streptomycin and spectinomycin and renders them ineffective [Hollingshead and Vapnek 1984], thus providing resistance for the bacteria bearing this gene. Furthermore, there is some evidence that the adenylated aminoglycosides participate in increasing resistance by blocking transport of the unmodified aminoglycosides to the cell.

The conI promoter is able to interfere with expression of aadA, a weakly expressed gene, and this effect can be monitored by measuring resistance to spectinomycin, as seen in Table 2. Strains containing pNN388 [Fig. 1], which lacks conI, plate with equal efficiency on media containing chloramphenicol or spectinomycin. However, strains bearing pNN389 [Fig. 4B], which contains conI opposing the aadA gene, plate efficiently on media containing chloramphenicol but are extremely sensitive to spectinomycin [i.e., appear aadA-]. To determine whether this effect was due to transcription by conI and not merely the alteration of sequence 3' to the aadA structural gene, a derivative of conI was used that had a lac operator placed at position +5 relative to the start of transcription [Fig. 2]. As shown in Table 2, insertion of a lac operator [pNN394] restored function to the aadA gene in a lacI- background but that this function was again interrupted upon induction of antisense transcription by the addition of isopropyl-β-D-thiogalactoside (IPTG). The presence of the lacI operator had no effect in a lacI- background nor did IPTG. Therefore, we conclude that the induced sensitivity phenotype is due to transcription.

Several different mechanisms could explain this ap-

| Plasmid(s) | lacI genotype | Efficiency of plating on spectinomycin |
|------------|--------------|---------------------------------------|
|            |              |                                       |
| pNN388     | q            | 1                                      |
| pNN389(conI) | q            | 10-6                                  |
| pNN394(conI-1) | q            | 10-6                                  |
| pNN394     | q            | 10-6                                  |
| pNN395     | q            | 10-6                                  |
| pNN390(tac+) | q            | 10-6                                  |
| pNN391(tac-) | q            | 10-6                                  |
| pRM61      | q            | 10-6                                  |
| pNN390, pRM61 | q            | 10-6                                  |
| pNN392      | q            | 10-6                                  |
| pNN393      | q            | 10-6                                  |
| pNN388, pNN393 | q            | 10-6                                  |

Dilutions of cells were plated on LB plates supplemented with spectinomycin (80 µg/ml), with or without 3 mM IPTG. With respect to the lacI genotype, q represents lacI+ and — represents lacI-. Efficiencies of plating were calculated relative to plating efficiencies on chloramphenicol (40 µg/ml) containing plates for each plasmid except for pRM61 [40 µg/ml kanamycin] and pNN392 and pNN393 [100 µg/ml ampicillin].
growth of pRMM61 alone, were also observed. This was seen best in a "laclq
BcII site in the coding region of aadA to create pNN393. This frameshift mutation completely eliminated any spec-
tic transcripts and interfere with their normal function. Antisense RNA effects have been documented in both eukaryotic [Izant and Wein-
traub 1984] and prokaryotic systems [Coleman et al. 1984]. A third possible mechanism is that of polymerase interference, whereby RNA polymerase molecules trans-
scribing convergently are thought to impede the progress of each other [Ward and Murray 1979]. The first mechanism would predict that transcription of sufficient den-
sity anywhere on the plasmid should suffice to produce topological perturbations. We tested this possibility by constructing two plasmids containing the tac promoter in the same location, 3' to the aadA gene but in opposite orientations. Only when tac is transcribing toward the aadA gene, as in pNN390, does the induction of tran-
scription by IPTG interfere with aadA function. This would tend to rule out the first mechanism, assuming no transient localization of the topological perturba-
tions. We can distinguish between the second and third possible mechanisms if the effects are seen in trans because polymerase interference can only function in cis. A test was set up to make a cis/trans determination. A second low-copy-number plasmid bearing an intact aadA gene, pRMM61 [Mulligan and Long 1985], was intro-
duced into a strain already containing the antisense aadA plasmid pNN390. No effect of induction of trans-
scription was observed on resistance to spectinomycin. Growth rates at higher spectinomycin levels, which sig-
ificantly show the growth of pRMM61 alone, were also checked and no difference in growth between pRMM61 alone and pRMM61 plus pNN390 [S.J. Elledge and R.W. Davis, unpubl.] was found. This suggested that the effect was cis. However, we could not control rigorously for equivalent copy numbers of the two plasmids. To exag-
gerate the antisense phenotype, pNN392 was con-
structed by placing a tac—aadA construct from pNN390 onto pBR322. Even at this high copy number, an effect of transcription upon spectinomycin resistance was readily observed. This was seen best in a lacIq background, and higher levels of spectinomycin [1 mg/ml] were needed to observe sensitivity. A mutation was introduced into the aadA gene on this construct by filling in a BclI site in the coding region of aadA to create pNN393. This frameshift mutation completely eliminated any spec-
tinomycin resistance conferred upon the cell by the plasm.

The mutant plasmid was introduced into a strain containing the low-copy-number plasmid pNN388 [Fig. 1] which carried an intact aadA gene. Under these cir-
cumstances, it would be expected that pNN393 would be producing massive amounts of nearly perfectly homologous antisense transcripts to aadA and that if there were antisense interference, we would observe it upon induction of transcription. The results are shown in Table 2. The presence of the plasmid pNN393 made no difference upon the level of spectinomycin resistance whether or not transcription was induced. Therefore, we conclude that the effect we observe occurs only in cis and is due to a form of promoter occlusion. This does not exclude a role for strictly cis antisense RNA effects, i.e., RNA–RNA hybrid formation among transcripts from the same template.

**Table 3. Promoter occlusion dependence on promoter strength**

| Plasmid or promoter | Fold repression* | Spectinomycin resistanceb |
|---------------------|------------------|--------------------------|
|                     | trpR− trpR+      |                          |
| pNN388              | —                | R R                      |
| conII               | 0.9              | R R                      |
| conII-1             | 6.0              | S R                      |
| conII-2             | 11               | S R                      |
| conII-3             | 14               | S R                      |
| conII-4             | 10               | R R                      |
| conII-5             | 3                | R S                      |
| conII-6             | 2                | R R                      |
| conII-5, pRPG9      | 3.8              | (R/S) [R/S]              |

*The fold repression data is reproduced from Table 1 [ratios; R+/R−] for clarity.

b (R) Resistance to spectinomycin [80 μg/ml]. Under these conditions, the strains plated with an equal efficiency on both chloramphenicol and spectinomycin plates. (S) Sensitivity to spectinomycin, indicating an efficiency of plating of <10−4.
trpR− background. The reason for this is unclear, but they do produce considerably less β-galactosidase than the other constructs shown in Table 1. However, conII-1, conII-2, conII-3, and conII-5 all showed the ability to interfere with aadA function in a trpR− strain, but conII-1, conII-2, and conII-3 are all resistant to spectinomycin in a trpR+ background. This argues that the sixfold repression of conII-1 conferred by the Trp repressor is sufficient to allow full phenotypic expression of aadA. The most interesting construct is conII-5. conII-5 interferes with the phenotype of aadA even in a trpR+ background indicating that a threefold reduction in transcription is not sufficient to prevent transcriptional interference. However, introduction of pRP9 into the strain containing the conII-5 construct produced an intermediate phenotype, microcolonies grew on the spectinomycin plates. Thus, the degree of repression of promoter function needed to prevent promoter occlusion of aadA has been narrowed down; threefold is insufficient, fourfold allows some degree of aadA phenotypic expression, and sixfold gives full expression. In further support of these numbers, we found that the lac promoter was unable to produce promoter occlusion in our assay. The lac promoter is only three times weaker than tac in vivo [Deuschle et al. 1968b]. This shows that promoters that are threefold weaker than tac, either due to repression or to a lower constitutive level of transcription, are insufficiently powerful to inactivate aadA.

Discussion

Repressors bound at different positions may repress by different mechanisms

Operator sequences have been found at virtually every location in prokaryotic promoters, but the significance of their positioning has remained largely unexplored. We have examined the position effects of two different repressor proteins, the Lac and Trp repressors. The Lac repressor shows both distance and sequence context effects upon its ability to repress and gave a tripartite pattern of repression. The fact that lac operator placement within a given region tended to produce similar levels of repression suggested that the repression in those regions acts through the same mechanism, distinct from mechanisms employed in adjoining regions. We have constructed a model in Figure 5 to summarize our observations.

As mentioned in the introductory section, Straney and Crothers [1987] have shown that the Lac repressor acts by interfering with the formation of the open complex when bound at +1. It is very likely that the repression plateau we observe for operator positions +5 to +14 works in the same fashion, not by inhibition of RNA polymerase binding, but by interfering with another step such as RP0 formation, step B to C [Fig. 5]. Further support for this model stems from the fact that the DNase I footprints for the Lac repressor and RNA polymerase open complex overlap until the operator is located at approximately +17 [Schmitz and Galas 1979; Spassky et al. 1985; calculated after removing 2 bp from each foot-
Figure 5. A model for the operator position effects on repression, i.e., the interference with specific steps in the initiation of transcription. The open rectangles represent promoter DNA. The large ellipse represents RNA polymerase, and the smaller ellipse represents the α-subunit. The filled rectangles of the promoter represent regions of DNA that prevent the progression to the next step of transcription initiation (indicated by a letter) when bound by the repressor protein. The filled regions are not meant to represent a footprint of a repressor, but the position where the leftmost edge of an operator must be located to effect repression. The stippled regions are shown to indicate the inexact nature of the boundaries. Boundaries may vary, depending upon both the properties of the promoter and the nature of the repressor protein.

Multiple operators in the trp promoter function in vivo

The repression data for trm suggest that at least one of the two exterior operator sites in the trp promoter is occupied in vivo and contributes to repression of the promoter. This work is consistent with the in vitro work of Kumamoto et al. (1987), which suggested that the Trp repressor could bind to four successive major grooves in the trp promoter. However, the $34 \times$ fold repression of trm shows that the majority of the repression is due to the central operator. This explains the observations of Bennett and Yanofsky (1978) that all operator constitutive (O°) mutations isolated in the trp promoter map to the central operator. Their observations suggest that mutations specifically removing the outer binding sites would not produce a severe regulatory phenotype. Our data concur and demonstrate that the outer two operator sites contribute only fourfold to the regulation of trp. The most severe O° mutations isolated by Bennett and Yanofsky would remove two operator sequences simultaneously leaving one exterior operator sequence intact. Coincidentally, the most severe O° mutations in each half-site of the central operator left a residual twofold repression. An individual twofold repression for the exterior operators would explain precisely the fourfold difference in repression between trp and trm if we assume that the operators act independently. It should be noted that it has not yet been proven that multiple Trp repressors interact with the trp promoter. It is possible but unlikely, given the crystal structure, that one molecule of Trp repressor could interact with all of the sequences important for repression.

Our data suggests that operator number and positioning can explain the difference in regulation of the trp and trpR promoters. Operators in the central region of a promoter, such as found in trp, can confer strong regulation, whereas operators near the start of transcription,
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such as in *trpR*, confer weaker regulation. Our data do not address the levels of repression of the *aroH* operon because we have not investigated repression in the ~50 to ~20 promoter region. Differences in operator affinities among the three promoters appear to be very slight in vitro [Klig et al. 1988] and may only play a significant role when active repressor concentrations approach the $K_d$. Differences in promoter strength may also play a role in precisely defining repression levels. It is possible that weaker promoters are more efficiently repressed by different repression mechanisms that are strong promoters. This question must be addressed in the future to further our understanding of the interaction of repressors and RNA polymerases on the surface of promoter DNA.

**Negative regulation at a distance by promoter interference**

We have shown that a strong promoter, conI, can act to silence the *aadA* gene at a distance. This form of repression occurs only in cis. It shows a directionality dependence and appears to be mediated via transcription. Although general topological interference does not appear to play a role, we cannot rule out a role for transient local superhelicity effects observed during convergent transcription [Wu et al. 1988]. We feel that the most likely explanation for this phenomenon is one of transcriptional interference, first described by Ward and Murray (1979), for *pL* interference with expression of the *trp* operon on a *htp*-transducing phage. Their work preceded the discovery of antisense RNA effects, so that control was missing from their study. Nevertheless, it is likely that they were observing the same phenomenon. Adhya and Gottesman (1982) also observed promoter interference among promoters transcribing in the same direction. Although similar in principle, it differs mechanistically from our convergent interference because it does not involve RNA polymerase collisions. The problem of polymerase collisions must be a common one in evolution and may be one of the major constraints on the evolution of gene arrangements. Perhaps the cell has evolved special machinery to allow polymerases to bypass one another during transcription. This does not appear to be the case for RNA and DNA polymerases during replication [Brewer 1988], but that involves different circumstances because the DNA replication complex must access both strands of the DNA. Due to its effectiveness as a repression mechanism and the frequency with which it must occur by chance in vivo, one might expect that organisms would exploit this form of repression more frequently than has been observed. In fact, it may be that the effects of convergent transcription have been overlooked in many cases due to the prevailing paradigm that most prokaryotic regulation is exerted through the promoter of a given gene. This is especially true in the cases when the effects are subtle and are coupled with more conventional types of regulation. It is, however, an energetically expensive mechanism of repression and may find selective advantages only when used for limited times such as a short period during a complex developmental program. Circumstances when homeostatic energy considerations are less important, such as during the developmental program of a lytic phage infection, may be another example. It might also find use as a switching mechanism between convergently transcribed genes with antagonistic functions such as must exist among the myriad of regulatory networks controlling gene expression in bacteriophage.

The repressing potential of promoter occlusion is large. It can change sensitivity to spectinomycin over a 100-fold range [S.J. Elledge and R.W. Davis, unpubl.] when used to repress *aadA*. We have found a strikingly strong dependence upon promoter strength for this repression. Changes in promoter initiation rates of two- or threefold can have profound effects $[10^6]$ upon plating efficiencies of strains on selective media. The mechanism appears to have the capacity to function as a polymerase density-dependent switch. One possible explanation for this capacity is, at subrepressing transcription rates, RNA polymerase collisions are occurring that slow down the progress of successful transcription from each promoter in a linear fashion. If one promoter becomes sufficiently strong so as to initiate transcription at near maximal rates, it could literally cover the DNA template with polymerase molecules [Kammerer et al. 1986; Brunner and Bujard 1987]. Under these conditions, polymerases from the strong promoter could eventually overcome the opposing polymerases by mass action to the point where they began to arrive at and transcribe through the weaker promoter. Once this occurs, the weaker promoter is essentially repressed by the competing polymerases and no longer initiates opposing RNA polymerase molecules, thus enhancing the dominance of the strong promoter and leading to a sigmoidal repression pattern. This model can be broken down into two components: [1] a mutual 'transcriptional interference' due to translational interference between polymerases, and [2] classical 'promoter occlusion,' where opposing polymerases cover and repress a promoter as described by Adhya and Gottesman (1982). This model is attractive because it explains the strong dependence on promoter strength, as well as requiring the presence of a strong promoter such as *tac*. Careful measurements of in vivo initiation rates of these promoters while varying the relative promoter strengths will be required to test this and other models. Experiments must also be devised to determine whether opposing RNA polymerase molecules actually interfere with each other during transcription in vivo. They are primarily accessing one strand after initiation. This is a question of fundamental importance.

The in vivo analyses provided in this work have given us insights into the interactions of regulatory molecules on the surface of promoter DNA. The spacing analysis has shown that regulatory molecules can have significant effects when placed much farther away than their normal positions in vivo. This has interesting implications for the pathway of evolution of regulatory sequences in general. The models generated for both local and distant repression will serve to guide future in vitro
experiments and enhance our understanding of the phenomenon of repression.

Materials and methods

Bacterial strains

E. coli JM107 was used as a transformation recipient for all plasmid constructions unless indicated otherwise. JM106 [endA1, gyrA96, thi, hisD17, supE44, relA1, Δlac-proAB] was used as the lacI+ host for measuring unrepressed levels of β-galactosidase activity. JM107 [endA1, gyrA96, thi, hisD17, supE44, relA1, Δlac-proAB], [F-, tuaD36, proAB+, lacIΔM15, Yanisch-Perron et al. 1985] was used as the lacI+ lacZ- host for measuring repressed levels of β-galactosidase activity. CY 15076 [W3110, ΔlacU169] and CY15075 [W3110, ΔlacU169, trpR2] [Kelley and Yanofsky 1985] were used as the trpR hosts for measuring repressed and unrepressed levels of β-galactosidase activity. W3110 is a wild-type strain used for measuring Lac repressor titrations. pRPG9 [Guslans and Yanofsky 1980] was a gift from M. Kuroda.

Media, enzymes, assays, and genetic methods

β-Galactosidase assays were performed as prescribed in Miller (1972). For measurements of the conI derivatives, plasmids in JM106 backgrounds were grown to an O.D600 of 1 at 37°C in M9 minimal media supplemented to 40 µg/ml l-proline, 1 µl B1, 0.1% glucose, and 40 mg/ml chloramphenicol. Plasmids in JM107 backgrounds were grown under the same conditions, except that the proline was left out of the supplements to select for the F' host. Measurements of β-galactosidase activity of the trp, trm, and conII derivatives in the CY backgrounds were performed in M9 media supplemented with 0.1% glucose, 40 µg/ml chloramphenicol, and 100 µg/ml X-gal t-lytrophan.

When drug sensitivities were being measured on plates, LB medium was supplemented with either chloramphenicol [40 µg/ml], spectinomycin [80 µg/ml], kanamycin [40 µg/ml], or ampicillin [50 µg/ml]. E. coli is not sensitive to spectinomycin in minimal media. All plates contained an additional 100 µg/ml X-gal t-lytrophan. When necessary, IPTG was added to a final concentration of 3 mM.

Restriction endonucleases, E. coli DNA polymerase I large fragment, T4 polynucleotide kinase, T4 DNA polymerase, and DNA ligase were purchased from New England Biolabs. Deoxyribonucleotides and ATP were purchased from P-L Biochemicals. Drugs were purchased from Sigma.

DNA sequencing

Plasmid DNAs were sequenced by the method of Sanger et al. (1977). Single-stranded plasmid DNAs were prepared by the method of Zagursky and Berman (1984), using R408 [Russe1 et al. 1986] as a helper phage.

Plasmid constructions

pNN386 was constructed in several steps. First, a NotI linker [New England Biolabs] was placed into the PvuII site of pEMBLL18 [Dente et al. 1983] that was not in the lacZ gene to form pEMBLLNot. Secondly, a NotI site was inserted into the ClaI site of ptacl1 [Amann et al. 1983]. The NotI–EcoRI tac-containing fragment was then cloned into NotI–EcoRI digested pEMBLLNot to create pEMBLLtac. Single-stranded ssDNA was prepared from this construct by superinfection with R408 [see above]. The ssDNA of pEMBLLtac was annealed with an oligonucleotide of sequence 5′-CGAGCTCGAATTCACACTA-TACGAGGCCGCCACAATT-3′ and extended with the Klenow fragment of DNA polymerase I and transformed into W3110. This oligonucleotide creates a deletion of the leader region of the tac promoter that removes the lac operator and because high copy number plasmids bearing lacO can induce the chromosomal lacZ gene by repressor titration, we screened the transformants on 40 µg X-Gal/ml ampicillin plates containing 0.1% glucose for transformants that remained white. Several transformants were chosen and sequenced by Sanger dideoxy sequencing and a plasmid bearing the proper deletion to form conI [Fig. 2] was named pNN386 [Fig. 1].

pNN388 was constructed in two steps. First, pDPT427 [Taylor and Cohen 1979] a single-copy-number, Sp+, Cm+ plasmid was deleted across its HindII sites to create pSE150. pSE150 has two closely spaced HindIII sites. A HindIII partial was done and a NotI linker inserted to create pNN388 [Fig. 1].

pNN379 was constructed by ligating the 8.2-kb HindII–BamHI promoterless lacZ fragment from pMC279 [Casadaban et al. 1980] into HindIII–BamHI cut pNN388. Very light blue, Cm+, Sp+ colonies were chosen for analysis. A strain in which the lacZ gene had replaced the adaA gene was chosen and named pNN387 [Fig. 1].

pNN390 and pNN391 were constructed as follows. First, a portable promoter fragment carrying the Neo gene of Tn5 and ptacl on an EcoRI fragment was obtained from pSE185. This fragment has both the Neo gene and the tac promoter reading in the same direction, with the tac promoter downstream of Neo transcribing into the EcoRI site. We ligated this 1.8-kb Km+, tac-containing EcoRI fragment made blunt by filling in with the Klenow fragment of DNA polymerase I to NotI–HindIII [filled in by the Klenow fragment of DNA polymerase I], cleaved pNN388, and selected Cm+Km+ colonies. Restriction endonuclease analysis allowed us to distinguish the two possible orientations of inserts. A plasmid with the tac promoter transcribing toward adaA was named pNN390 [Fig. 4]. The plasmid with the tac promoter placed in the opposite orientation was named pNN391.

pNN392 was made by ligating the BamHI–HindIII tacaadA-containing fragment from pNN390 into BamHI–HindIII-cleaved pBR322 and selecting for Ap+ Sp+ colonies. This construction gives resistance to high levels of spectinomycin [1 mg/ml] but becomes Sp+ in the presence of 3 mM IPTG. pNN393 was made by Klenow filling in at the BclI site in the adaA gene of pNN392.

pNN394 was constructed by ligating the NotI–HindIII fragment containing the conI-1 promoter (see below) into NotI–HindIII-cleaved pNN388. pNN395 was the identical construction, only using the conI-16 promoter fragment.

Construction of regulated promoters

All of the promoters shown in Figure 2 are derivatives of conI. The conI promoter was designed with an EcoRI restriction site placed at the start of transcription followed by a polylinker to facilitate operator positioning. Into the various restriction sites illustrated in Figure 2A on pNN386, we placed an oligonucleotide containing a synthetic lac operator sequence. The lac operator used was the 20-bp symmetrical sequence of Sadler et al. [1983] of sequence 5′-AAATGGACGCCGCCACAATT-3′ (referred to here as lacO). In general, we identified clones by transformation into W3110 and screened for induction of the chromosomal lacZ gene, as described above. lacO-positive
clones were sequenced as described above and then transferred to pNN387 by ligating the NolI–HindIII-con-taining fragments into NolI–HindIII-cleaved pNN387 to assay promoter strength with β-galactosidase. conl-1 was made by ligating lacO into EcoRI-cleaved, Klenow filled-in pNN386. conl-2 was made by ligating lacO into SacI-cleaved, T4 DNA polymerase-flushed pNN386. conl-5 was made by ligating lacO into KpnI-cleaved, T4 DNA polymerase-flushed pNN386. conl-3 was derived from conl-5 by deleting the internal 4 bp of the SacI site by cleaving with SacI, making flush with T4 DNA polymerase, and ligating. conl-4 was derived from conl-2 by filling in an EcoRI-cleaved conl-2 with the Klenow fragment of DNA polymerase. conl-9 was made by ligating lacO into Smal-cleaved pNN386. conl-6 was derived by deleting the internal 4 bp of the SacI site from conl-9 by cleaving with SacI, making flush with T4 DNA polymerase, and ligating. In the T4 reaction, an extra C at position +11 was deleted from the SacI site (see sequence in Fig. 2). conl-7 was made by ligating the lacO into Asp718-cleaved, Klenow filled-in pNN386. conl-10 was made by ligating the lacO into Xmal-cleaved, Klenow filled-in pNN386. conl-8 was derived from conl-10 by deleting the internal 4 bp of the SacI site by cleaving with SacI, making flush with T4 DNA polymerase, and ligating conl-11 was derived from conl-9 by filling in an EcoRI-cleaved conl-9 with the Klenow fragment of DNA polymerase I. conl-12 was derived from conl-10 by filling in an EcoRI-cleaved conl-10 with the Klenow fragment of DNA polymerase I. conl-14 was made by ligating the lacO into XbaI cleaved, Klenow filled-in pNN386. In this construct, a base was deleted from each side of the filled XbaI site. conl-13 was derived by deleting the internal 4 bp of the SacI site from conl-14 by cleaving with SacI, making flush with T4 DNA polymerase, and ligating. conl-15 was derived by filling in an EcoRI-cleaved conl-14 with the Klenow fragment of DNA polymerase I. conl-16 was made by ligating lacO into PstI-cleaved pNN386 made flush by the action of T4 DNA polymerase.

conl-1 was derived from conl by oligonucleotide site-directed mutagenesis. ssDNA was prepared from pNN386 by superinfection with R408 (see above). This ssDNA was annealed with an oligonucleotide of sequence 5'-ATCCCCGGGTACCATTA-3' and extended with the Klenow fragment of DNA polymerase I. conl-12 was derived from conl-10 by filling in an EcoRI-cleaved conl-10 with the Klenow fragment of DNA polymerase I. conl-14 was made by ligating the lacO into XbaI cleaved, Klenow filled-in pNN386. In this construct, a base was deleted from each side of the filled XbaI site. conl-13 was derived by deleting the internal 4 bp of the SacI site from conl-14 by cleaving with SacI, making flush with T4 DNA polymerase, and ligating. conl-15 was derived by filling in an EcoRI-cleaved conl-14 with the Klenow fragment of DNA polymerase I. conl-16 was made by ligating lacO into PstI cleaved pNN386 made flush by the action of T4 DNA polymerase.

The trp and trn promoters were synthesized as double-stranded oligonucleotides with NolI and HindIII overhangs and cloned directly into NolI–HindIII-cleaved pNN386 for sequencing. They were subsequently subcloned from these sequencing templates onto pNN387 using NolI and HindIII.

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