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 catabolism; second, it is the simplest type 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,' RP_c . Then the DNA near the start of transcription is denatured, thus defining the formation of the more stable 'open complex,' RP_o (Kirkegaard et al. 1983). Upon addition of ribonucleotides, the 'initiation complex' forms, RP_i 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 (Amann et al. 1983). Oligonucleotide site-directed mutagenesis was used to remove the *lac* operator from *tac* to create a strong constitutive promoter *conI* (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

Figure 1. Maps of plasmids used in this study. pNN386 is a derivative of pEMBL18, as described in Materials and methods. It contains the *conI* promoter and polylinker, the sequence of which is illustrated in Fig. 2. This plasmid was used as the base vector for the creation of all of the Lac repressor-regulated promoters shown in Fig. 2. pNN386 is a derivative of pNN386 and has the *conII* promoter (Fig. 3) in place of *conI*. pNN387 is a single-copy-number plasmid used to measure the promoter strength of the regulated promoters used in this study. The *LacZY* genes on pNN387 lack a promoter. pNN388 is a single-copy-number plasmid used to measure the ability of transcription to interfere with the expression of the *aadA* gene. The arrows in this diagram represent the direction of transcription of their respective genes. These maps are not drawn to scale.

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^q* backgrounds, as described in Materials and methods (the *lacI^q* 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 mRNA for the repressed and induced states are the same. The induction ratios for the modified *conI* 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, *con* promoters lacking the synthetic *lacO* oligonucleotide still show a twofold in-

quence of each construct is shown in Figure 3B. These promoter constructs were assayed by allowing them to direct β -galactosidase synthesis on pN387 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.

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Table 1. Transcriptional activities of *trpR*-regulated promoters

Promoter	β -Galactosidase units		pRPG9 ^a	Ratios	
	<i>trpR</i> ⁺	<i>trpR</i> ⁻		(R ⁻ /R ⁺)	(R ⁻ /pRPG9)
<i>trp</i>	6.5	792	—	120	—
<i>trm</i> (-19)	22.5	779	—	34	—
<i>conII-1</i> (-5)	226	1382	115	6	12.0
<i>conII-2</i> (-1)	117	1266	—	11	—
<i>conII-3</i> (+2)	120	1755	93	14	18.8
<i>conII-4</i> (+5)	63	637	—	10	—
<i>conII-5</i> (+9)	375	1022	266	3	3.8
<i>conII-6</i> (+16)	122	273	—	2	—
<i>conII</i>	720	605	—	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.

^a 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 Gunslaus 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 *conI* 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 (Hollisghead 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

Figure 4. Schematic representations of promoter arrangements producing transcriptional interference. (A) A map of the relative positions of the *con* promoter derivatives and *aadA*. Arrows indicate the direction of transcription and relative promoter strength. (B) Restriction maps of pNN389 and pNN390 (Table 3). Restriction sites relevant to their construction are shown. (J) A junction created during the cloning in which the restriction sites were destroyed. These maps are not shown to scale.

(IPTG). The presence of the *lac* 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-

Table 2. *cis* and *trans* effects on *aadA* expression

Plasmid(s)	<i>lacI</i> genotype	Efficiency of plating on spectinomycin	
		IPTG	
		(-)	(+)
pNN388	q	1	1
pNN389(<i>conI</i>)	q	10 ⁻⁶	10 ⁻⁶
pNN394(<i>conI</i> -1)	q	1	10 ⁻⁶
pNN394	-	10 ⁻⁶	10 ⁻⁶
pNN395(<i>conI</i> -16)	q	1	10 ⁻⁶
pNN395	-	10 ⁻⁶	10 ⁻⁶
pNN390(<i>tac</i> ⁺)	q	1	10 ⁻⁶
pNN391(<i>tac</i> ⁻)	q	1	1
pRMM61	q	1	1
pNN390, pRMM61	q	1	1
pNN392	q	1	10 ⁻⁴
pNN393	q	<10 ⁻⁷	<10 ⁻⁷
pNN388, pNN393	q	1	1

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*^q and - represents *lacI*⁻. Efficiencies of plating were calculated relative to plating efficiencies on chloramphenicol (40 μg/ml) containing plates for each plasmid except for pRMM61 (40 μg/ml kanamycin) and pNN392 and pNN393 (100 μg/ml ampicillin).

parent transcriptionally dependent phenomenon. The first is that transcription by the strong *conI* promoter could be topologically altering the rest of the plasmid and that the *aadA* could produce antisense RNAs that can hybridize to the sense *aadA* promoter is particularly sensitive to topological perturbations. Second, the antisense transcription of *aadA* transcripts and interfere with their normal function. Antisense RNA effects have been documented in both eukaryotic (Izant and Weintraub 1984) and prokaryotic systems (Coleman et al. 1984). A third possible mechanism is that of polymerase interference, whereby RNA polymerase molecules transcribing convergently are thought to impede the progress of each other (Ward and Murray 1979). The first mechanism would predict that transcription of sufficient density 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 transcription by IPTG interfere with *aadA* function. This would tend to rule out the first mechanism, assuming no transient localization of the topological perturbations. 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 introduced into a strain already containing the antisense *aadA* plasmid pNN390. No effect of induction of transcription was observed on resistance to spectinomycin. Growth rates at higher spectinomycin levels, which significantly 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 exaggerate the antisense phenotype, pNN392 was constructed 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 *lacI^q* 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 spectinomycin resistance conferred upon the cell by the plasmid. 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 circumstances, 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.

Promoter occlusion has a strong dependence upon promoter strength

Repression at a distance by convergent transcription may be influenced by a number of factors in vivo, including the distance of separation of promoters, relative promoter strengths, transcription termination and/or transcript stability, and translatability. Many of these factors are poorly understood and are difficult to control properly. However, one factor that can be controlled is the frequency of transcription initiation. To test the effects of varying the level of transcription, the modified *conI* promoters listed in Figure 2 were placed onto the *aadA* assay vector pNN388. The pattern of repression was the same for all the *conI* derivatives *conI*-1 through *conI*-16. The typical result for *conI*-16, pNN395, is listed in Table 2. *conI* alone interfered with *aadA* function, as described above, but every other construct bearing the *lac* operator was able to protect the *aadA* gene in the absence of IPTG. This means that repressing transcription 15-fold (*conI*-16 levels divided by two) is enough to interfere with promoter occlusion. Furthermore, the *conI*-16 construct appeared to have absolutely no phenotypic differences relative to pNN388 in the absence of IPTG.

To define the minimal amount of expression required to interfere with *aadA* function more precisely, a number of *conII* constructs were tested that showed lower levels of regulation. These data are shown in Table 3. The *conII*, *conII*-4, and *conII*-6 promoters were too weak to interfere with *aadA* function even in a

Table 3. Promoter occlusion dependence on promoter strength

Plasmid or promoter	Fold repression ^a	Spectinomycin resistance ^b	
		<i>trpR</i> [−]	<i>trpR</i> ⁺
pNN388	—	R	R
<i>conII</i>	0.9	R	R
<i>conII</i> -1	6.0	S	R
<i>conII</i> -2	11	S	R
<i>conII</i> -3	14	S	R
<i>conII</i> -4	10	R	R
<i>conII</i> -5	3	S	S
<i>conII</i> -6	2	R	R
<i>conII</i> -5, pRPG9	3.8	(R/S)	(R/S)

^a 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 pRPG9 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 RP_o 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 (Schmnitz and Galas 1979; Spassky et al. 1985; calculated after removing 2 bp from each foot-

print to account for the radius of DNase I). Coincidentally, the second repression plateau begins near +17 and extends to +31. It is likely that the second plateau represents a different mechanism of inhibition because the level of repression is reduced fivefold and formation of RP_o should not be sterically hindered. The most reasonable explanation is that repressor binding in this region interferes with formation of RP_i, perhaps by influencing the extent or degree of abortive cycling. This is illustrated in Figure 5 as the transition from C to D. The final plateau consists of only two points, +34 and +40, that show still lower levels of repression. Carpousis and Gralla (1985) have shown that elongating RP_i complexes protect only 10 bases ahead of the next base to be synthesized, i.e., the DNase I footprint extends to +26 when the polymerase is ready to insert base 17. This means that repressors positioned at +34 and +40 will encounter polymerases that have fully initiated transcription. It is likely, therefore, that repressor molecules at these positions are exerting their influence via the process of transcriptional termination identified by Deuschle et al. (1986a) and illustrated as the transition from D to E in Figure 5. Another explanation for the second plateau is that the process of abortive cycling may be more sensitive to transcription termination than are fully engaged polymerases. The test of these hypotheses will require biochemical analysis.

Besse et al. (1986) performed a similar analysis of operator positioning with *lacO*. They used the wild-type *lac* promoter instead of an artificially constructed constitutive promoter. The *lac* promoter has an additional upstream *lac* operator sequence directly preceding the cap-binding site (Fried and Crothers 1981). Because it has been shown that cooperative interactions can occur between properly spaced operators (Mossing and Record 1986; Kramer et al. 1987), their data may be complicated by periodicity effects caused by the phasing requirements for interaction of closely spaced operators. Although they used a system with a different operator, a different promoter, and different repressor levels, results were obtained that do not show major discrepancies with our results and conclusions.

Operators located within a promoter are more efficient than those positioned near the start of transcription

Repressors bound near the start of transcription repress not by preventing RNA polymerase from binding but by interfering with some other step in promoter activation. Repressors bound farther upstream in promoters at positions overlapping the recognition sequences for RNA polymerase can repress by preventing polymerase binding. We wished to determine which form of repression was intrinsically more efficient. A priori, one might expect that occlusion would be the most efficient. However, the possibility exists that a bound polymerase and repressor may be able to interact to form a stable repressing complex that has a greater stability than either protein alone. We observed that a single *trp* operator located internally between -19 and +1 was a more effi-

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 σ -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.

cient repressor in vivo than any *trp* operator positioned near the start of transcription. A Trp repressor bound in this internal regions is thought to preclude binding of RNA polymerase (Squires et al. 1975). Why do the repressing potentials differ if the operators are identical, having the same affinity for repressor and potentially the same occupancy in vivo? One explanation is the 'polymerase as repressor' model. Once a promoter initiates transcription, the polymerase will cover temporarily the repressor-binding site of operators located near or after the initiation of transcription. A time window will exist in which binding of the repressor is occluded while a second polymerase is free to load and form an open complex. This gives the polymerase a slight temporal advantage for rebinding. However, if the operator is located within the promoter, both repressor and polymerase will have equal access to it once the promoter is cleared. A second explanation is the 'polymerase as bouncer' model. When both polymerase and repressor are bound simultaneously to a promoter, the polymerase can effectively increase the off rate of the repressor, perhaps by melting the DNA in the vicinity of the repressor or by physically bumping it. Both models predict a lower occupancy for operators positioned near the initiation of transcription, the first by decreasing the on rate of the repressor and the second by increasing the off rate. We favor the polymerase as repressor model because increasing the concentration of Trp repressor significantly increases repression of operators near the initiation of transcription, a fact that may be inconsistent with the second model. These models are not mutually exclusive and may function concurrently in vivo. Furthermore, the effectiveness of each mechanism may vary, depending on the position and nature of the repressor and which step in initiation of transcription is being blocked, e.g., B to C or C to D in Figure 5.

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-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^c) 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^c mutations isolated by Bennett and Yanofsky would remove two operator sequences simultaneously leaving one exterior operator sequence intact. Coincidentally, the most severe O^c 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 regulation.

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,

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 λ *trp*-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. Cir-

cumstances 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 (10^5) 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 translocational 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*, *hsdR17*, *supE44*, *relA1*, Δ (*lac-proAB*)] was used as the *lacI*⁻ host for measuring unrepressed levels of β -galactosidase activity. JM107 [*endA1*, *gyrA96*, *thi*, *hasR17*, *supE44*, *relA1*, Δ (*lac-proAB*), (F', *traD36*, *proAB*⁺, *lacI*^q Δ M15; Yanisch-Perron et al. 1985) was used as the *lacI*^q*lacZ*⁻ host for measuring repressed levels of β -galactosidase activity. CY 15076 (W3110 *tna2*, Δ *lacU169*) and CY15075 (W3110 *tna2*, Δ *lacU169*, *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 (Gunslaus 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 OD₆₀₀ of 1 at 37°C in M9 minimal media supplemented to 40 μ g/ml L-proline, 1 μ l 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'. Measurements of β -galactosidase activity of the *trp*, *trm*, and *conI* derivatives in the CY backgrounds were performed in M9 media supplemented with 0.1% glucose, 40 μ g/ml chloramphenicol, and 100 μ g/ml L-tryptophan.

When drug sensitivities were being measured on plates, LB medium was supplemented with either chloramphenicol (40 μ g/ml), spectinomycin (80 μ g/ml), kanamycin (40 μ g/ml), or ampicillin (50 μ g/ml). *E. coli* is not sensitive to spectinomycin in minimal media. All plates contained an additional 100 μ g/ml L-tryptophan. 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 T4 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 (Russel 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 pEMBL18 (Dente et al. 1983) that was not in the *lacZ*' gene to form pEMBLNot. Secondly, a *NotI* site was inserted into the *ClaI* site of *ptac11* (Amann et al. 1983). The *NotI*-*EcoRI* *tac*-containing fragment was then cloned into *NotI*-*EcoRI* digested pEMBLNot to create pEMBL*tac*. Single-stranded (ss)DNA was prepared from this construct by superinfection with R408 (see above). The ssDNA of pEMBL*tac* was annealed with an oligo-

nucleotide of sequence 5'-CGAGCTCGAATTCCACACATTA-TACGAGCCG-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 μ g 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^r, Cm^r plasmid was deleted across its *HincII* 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).

pNN387 was constructed by ligating the 8.2-kb *HindIII*-*BamHI* promoterless *lacZ* fragment from pMC279 (Casadaban et al. 1980) into *HindIII*-*BamHI* cut pNN388. Very light blue, Cm^r, Sp^s colonies were chosen for analysis. A strain in which the *lacZ* gene had replaced the *aadA* 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 *ptac11* 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^r, *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^rKm^r colonies. Restriction endonuclease analysis allowed us to distinguish the two possible orientations of inserts. A plasmid with the *tac* promoter transcribing toward *aadA* 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* *tac*-*aadA*-containing fragment from pNN390 into *BamHI*-*HindIII*-cleaved pBR322 and selecting for Ap^r Sp^r colonies. This construct gives resistance to high levels of spectinomycin (1 mg/ml) but becomes Sp^s in the presence of 3 mM IPTG. pNN393 was made by Klenow filling in the *BclI* site in the *aadA* 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'-AATTGTGAGCGCGACAATT-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 *NotI*–*HindIII* *con*-containing fragments into *NotI*–*HindIII*-cleaved pNN387 to assay promoter strength with β -galactosidase. *conI*-1 was made by ligating *lacO* into *EcoRI*-cleaved, Klenow filled-in pNN386. *conI*-2 was made by ligating *lacO* into *SacI*-cleaved, T4 DNA polymerase-flushed pNN386. *conI*-5 was made by ligating *lacO* into *KpnI*-cleaved, T4 DNA polymerase-flushed pNN386. *conI*-3 was derived from *conI*-5 by deleting the internal 4 bp of the *SacI* site by cleaving with *SacI*, making flush with T4 DNA polymerase, and ligating. *conI*-4 was derived from *conI*-2 by filling in an *EcoRI*-cleaved *conI*-2 with the Klenow fragment of DNA polymerase. *conI*-9 was made by ligating *lacO* into *SmaI*-cleaved pNN386. *conI*-6 was derived by deleting the internal 4 bp of the *SacI* site from *conI*-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). *conI*-7 was made by ligating the *lacO* into *Asp718*-cleaved, Klenow filled-in pNN386. *conI*-10 was made by ligating the *lacO* into *XmaI*-cleaved, Klenow filled-in pNN386. *conI*-8 was derived from *conI*-10 by deleting the internal 4 bp of the *SacI* site by cleaving with *SacI*, making flush with T4 DNA polymerase, and ligating. *conI*-11 was derived from *conI*-9 by filling in an *EcoRI*-cleaved *conI*-9 with the Klenow fragment of DNA polymerase I. *conI*-12 was derived from *conI*-10 by filling in an *EcoRI*-cleaved *conI*-10 with the Klenow fragment of DNA polymerase I. *conI*-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. *conI*-13 was derived by deleting the internal 4 bp of the *SacI* site from *conI*-14 by cleaving with *SacI*, making flush with T4 DNA polymerase, and ligating. *conI*-15 was derived by filling in an *EcoRI*-cleaved *conI*-14 with the Klenow fragment of DNA polymerase I. *conI*-16 was made by ligating *lacO* into *PstI*-cleaved pNN386 made flush by the action of T4 DNA polymerase.

conII was derived from *conI* 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-TACGAGCCG-3' and extended with the Klenow fragment of DNA polymerase I, cleaved with *EcoRI*, and transformed into JM107. This oligonucleotide creates a deletion of the *conI* promoter from -5 to +12, including the *EcoRI* site. Transformants were sequenced and a clone was chosen with the sequence shown for *conII* in Figure 3 and designated pNN396. *conII*-1 was made by ligating the blunt *PstI* *trp* operator oligo(*trpO*) of Figure 3 into *KpnI*(blunt)–*PstI*-cleaved pNN396. *conII*-2 was made by ligating *trpO* into *Asp718*(blunt)–*PstI*-cleaved pNN396. *conII*-3 was made by ligating *trpO* into *SmaI*–*PstI*-cleaved pNN396. *conII*-4 was made by ligating the *trpO* oligonucleotide into *EcoRI*(blunt)–*PstI*-cleaved pNN386. *conII*-4 is actually a derivative of *conI*. *conII*-5 was made by ligating *trpO* into *BamHI*(blunt)–*PstI*-cleaved pNN396. *conII*-6 was made by ligating *trpO* into *XbaI*(blunt)–*PstI*-cleaved pNN396. All uses of the word blunt after a restriction enzyme site refers to filling in with T4 DNA polymerase. All constructions were sequenced as described above, and their sequences are listed in Figure 3. Each *conII* promoter derivative was transferred to pNN387 and pNN388 by ligating the *NotI*–*HindIII* promoter-containing fragment to *NotI*–*HindIII*-cleaved pNN387 or pNN388.

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

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