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Mutant λ phage repressor with a specific defect in its positive control function

(*lacZ* fusions/*in vitro* transcription/protein–protein interaction)

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ABSTRACT **The λ phage repressor is both a positive and a negative regulator of gene transcription. We describe a mutant λ phage repressor that has specifically lost its activator function. The mutant binds to the λ phage operator sites and represses the λ phage promoters P_R and P_L. However, it fails to stimulate transcription from the promoter P_{RM}. The mutation lies in that portion of repressor—namely, the amino-terminal domain—that has been shown [Sauer, R. T., Pabo, C. O., Meyer, B. J., Ptashne, M. & Backman, K. C. (1979) *Nature (London)* 279, 396–400] to mediate stimulation of P_{RM}. We suggest that the mutation has altered that region of repressor which, in the wild-type, contacts RNA polymerase to activate transcription from P_{RM}.**

The λ phage repressor bound to the right operator (O_R) on the phage chromosome simultaneously represses transcription of one set of genes and activates transcription of another. The repressed genes must be active for lytic growth, and one of the genes that is activated by repressor, *c*I, encodes the repressor itself. Thus, the dual role of the repressor (its original name; its activator function has only more recently been recognized) ensures stability of the lysogenic state (for reviews, see refs. 1 and 2).

Fig. 1 schematically depicts our current understanding of the configuration of repressor and RNA polymerase at O_R in a lysogen. Two of the three specific repressor-binding sites (O_R1 and O_R2) are occupied by repressor (3–6). In this state, the promoter P_R, which directs synthesis of the lytic gene *cro*, is turned off, and the promoter P_{RM}, which directs transcription of *c*I, is turned on. O_R1 and O_R2 are each filled by a repressor dimer (7), monomers of which are comprised of two globular domains (8). The amino-terminal domains bind to DNA (9), and the carboxyl-terminal domains contain sites of contact that are important for dimerization (8). Two repressor dimers bind cooperatively to O_R1 and O_R2 because of an interaction between carboxyl termini (10) as suggested in Fig. 1. Repressors bound at these two sites prevent RNA polymerase from binding and initiating transcription at P_R. Also indicated in the figure is a molecule of RNA polymerase bound to the promoter P_{RM}, which directs leftward transcription of *c*I. This promoter is activated by DNA-bound repressor (4–6, 9). How does repressor mediate activation of P_{RM}?

We have suggested that repressor stimulates transcription from P_{RM} by contacting RNA polymerase. This interaction occurs, we believe, between the amino-terminal domain of a repressor bound at O_R2 and polymerase bound at P_{RM}. Several lines of evidence are consistent with this view. First, although, as indicated above, O_R1 and O_R2 are both filled in a wild-type lysogen, it is possible to contrive situations *in vivo* and *in vitro* in which repressor is bound only to O_R2 (4). This suffices to activate P_{RM}. [Thus, in the wild-type situation, repressor bound at O_R1 contributes to positive control primarily by facilitating the binding of a second repressor dimer at O_R2. Interaction between repressor dimers bound at O_R1 and O_R2 results in these sites being filled coordinately even though O_R1 has a 10-fold higher intrinsic affinity for repressor (10).] Second, both *in vivo* and *in vitro*, a fragment of repressor comprising the amino-terminal domain suffices to activate P_{RM} significantly (4, 9). Third, RNA polymerase and repressor bind cooperatively to P_{RM} and O_R2, respectively—binding of one protein enhances the binding of the other (2, 11). Fourth, chemical probe experiments show that repressor bound at O_R2 and RNA polymerase bound at P_{RM} come in close contact with the same phosphate (between bases −36 and −37 from the start of P_{RM} transcription) and, therefore, with each other (2, 11). Given this proximity, it is plausible that the two proteins contact one another.

One picture consistent with the facts outlined above is that one region of repressor recognizes DNA and another contacts polymerase and, thereby, stimulates polymerase binding or initiation. This notion prompted the search for a mutant repressor that failed to activate transcription from P_{RM} but retained its ability to bind to the operator and to repress P_R. We expected that such a mutant repressor also would bind to the second λ phage operator O_L and repress transcription from the second lytic promoter P_L. Here we describe the isolation of a mutant repressor with these properties.

MATERIALS AND METHODS

Phage Strains. λ112 phage contains a functional immunity region from phage 21 and the P_{RM}–*c*I–*lacZ* operon fusion (6). λ112*c*I26 carries a missense mutation in *c*I. λ112*virC*23 carries a double mutation in O_R2 that prevents repressor from binding to that site (12). λ112*or3-r1* and λ112*or3-c12* bear mutations in O_R3 preventing repressor from binding to that site (5, 6). λ112Δ265*prmup-1or3-r1* bears a deletion of O_R1, a mutation (*prmup-1*) that renders *prm* active in the absence of repressor, and a mutation in O_R3 (*r-1*) that prevents repressor from binding to that site (4). λ200 carries the P_R–*lacZ* operon fusion, has a functional immunity region from phage 21, and does not contain the λ phage *c*I gene (5).

Bacterial Strains. The bacterial strains used, NK5031 (F^-, *lacZ* ΔMM5265, *Su*III$^+$, *Nal*^{R}) and US3 (F^-, *lacZ* ΔM5265, *his*), were lysogenized with each of the above phages. Lysogens were checked for immunity to λ*imm*21 clear phage and λ*imm*21*h*80 clear phage and for sensitivity to λ*imm*434 clear phage. NK5031 (λ112*c*I26), the strain used in the selection for positive control

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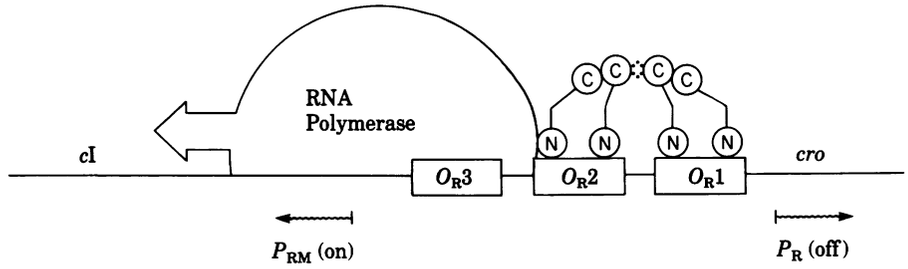

FIG. 1. The configuration of proteins bound to the λ phage rightword operator in a lysogen. Repressor dimers are bound at O_R1 and O_R2. The interaction between carboxyl-terminal domains of the dimers is indicated by the dots. Also shown is a molecule of RNA polymerase bound at P_{RM}. Repressor dimers bound at O_R1 or O_R2 turn off P_R transcription and activate P_{RM} (1, 2, 4, 5).

mutants, forms red colonies on MacConkey lactose indicator plates when β-galactosidase synthesis from the prophage exceeds 400 units. MM294 (*endo* 1^-, r_K^-, m_K^+) was used for repressor purification.

Plasmids. pKB270 (13) is a derivative of pSC101. It contains a fragment of DNA from the λ phage chromosome extending from the *Hin*dIII site in the *rex* gene (past the end of *cI*) to a *Hae* III site in *cro*. (This fragment itself contains an internal deletion that removes O_R1). The *Hae* III site is abutted to two *R*1 fragments that encode *lac* promoters that are oriented toward *cI*. Thus, *cI* expression from this plasmid is under the control of the *lac* promoter.

pKB252 is a derivative of pMB9 (14). It contains the same λ phage fragment and *lac* promoter structure as pKB270 except that O_R1 is intact. Both plasmids bear the tetracycline resistance (tet^R) marker.

Mutagenesis. NK5031/pKB270 was grown to an OD_{600} of 0.5, treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (50 mg/ml) for 15 min at 37°C as described (15), washed, and grown to saturation. The frequency of mutation to rifampicin resistance in the culture was increased by a factor of 1,000–10,000 by the treatment. Plasmid DNA was then prepared.

DNA Constructions. These were performed as described (16).

Construction of a High Copy Number Plasmid Bearing the *cI* Gene. The mutant *cI* gene, *pc-1*, was excised from pKB270 *cIpc-1* on a DNA fragment extending from an *R*1 site 85 nucleotides upstream from the start of the *cI* gene (a converted *Hae* III site in *cro*) to a *Bam*HI site in the tet^R locus. (The *R*1 site was filled in by using DNA polymerase before the plasmid was digested with *Bam*.) This fragment was inserted into a backbone from pKB252 extending from a filled-in *Bgl* II site just downstream from two *lac* promoters to the *Bam*HI site in tet^R. Joining of the flush ends places *cIpc-1* downstream from two *lac* promoters, and joining of the *Bam*HI sites regenerates tet^R. The recombinant plasmid, pGN10, bears the *col*EI replication origin and expresses the *pc-1* repressor under control of the *lac* promoter.

Localization of the pc-1 Mutation to a *Hin*dIII Fragment. pGN10 was digested with *Hin*dIII. A fragment extending from a naturally occurring site internal to *cI* (480 nucleotides from the 5' end of *cI*) (17) to a site past the end of the gene was deleted. A host bearing the deleted plasmid was no longer immune to virulent λ phages. The deleted plasmid was digested with *Hin*dIII, and a fragment from pKB252 extending from the site internal to *cI* to the site past the end of the gene was inserted. Recombinants that had regenerated *cI* intact were recognized by immunity to virulent λ phage. However, the recombinant plasmid was still unable to activate P_{RM}. This result localizes *pc-1* to the first 480 nucleotides of the *cI* gene.

Protein Purification. λ phage wild-type and *pc-1* repressor were purified from 294/pKB252 and 294/pGN10 according to method 2 of Johnson *et al.* (18). These strains synthesize about 0.2% of their protein as repressor. Final samples were judged to be greater than 90% pure by gel analysis.

***In Vitro* Transcription.** *In vitro* transcription was carried out in the presence of 6 μM [α-^{32}P]UTP as described (19). Samples were analyzed by acrylamide/urea gel electrophoresis, followed by autoradiography and, in some cases, excision of gel bands and counting of Cerenkov radiation.

DNA Sequence Determination. The method of Maxam and Gilbert was used (20). 3'-End labeling was accomplished by filling in cohesive ends using *E. coli* DNA polymerase I large fragment and 2 μM [α^{32}-P]dXTPs. 5'-End labeling was achieved by using T4 polynucleotide kinase in the presence of 1 μM [α-^{32}P]ATP.

Media and Assays. Media and use of MacConkey plates were as described (16). β-Galactosidase assays and units were as described by Miller (15).

RESULTS

Isolation of Repressor Mutants Specifically Deficient in Positive Control. We devised a screen for λ phage repressor mutants that are unable to stimulate P_{RM} but are able to repress P_R and P_L (Fig. 2). Our *Escherichia coli* tester strain contains a prophage that carries the *lacZ* gene fused to P_{RM}. In this strain, *lacZ* transcription initiates at P_{RM}, and the level of β-galactosidase is a measure of P_{RM} activity. The prophage does not encode an active λ phage repressor; its essential functions are repressed by the repressor of phage 21. In the absence of added λ phage repressor, this strain directs synthesis of only 100 units of β-galactosidase, which results from the unstimulated P_{RM} activity, and forms white colonies on MacConkey lactose indicator plates. If a plasmid, which directs synthesis of λ phage repressor (pKB270), is introduced into this strain, β-galactosidase levels are stimulated to 1000 units and, therefore, the strain forms red colonies on the indicator plates. The *cI* gene on the plasmid is under control of the *lac* promoter, and the plasmid, which is present in a few copies per cell, directs synthesis of repressor to a level 5 times that found in a single λ phage lysogen (13). [These levels are not high enough to repress P_{RM} substantially by binding of repressor to O_R3 (6)].

We anticipated that a plasmid encoding a mutant *cI* gene specifically deficient in positive control (pc^-) would be recognized as follows: tester bacteria bearing the mutant plasmid would form white colonies on indicator plates (because P_{RM} would not be stimulated) but would be immune to superinfecting λ phages (because P_R and P_L would be repressed). Therefore, our strategy was to treat pKB270 with mutagens, to transform the tester strain with this DNA, and to plate on MacConkey lactose indicator plates that had been seeded with λ phages that kill nonimmune hosts. (The plates also contained tetracycline as an additional selection for the plasmid.) White

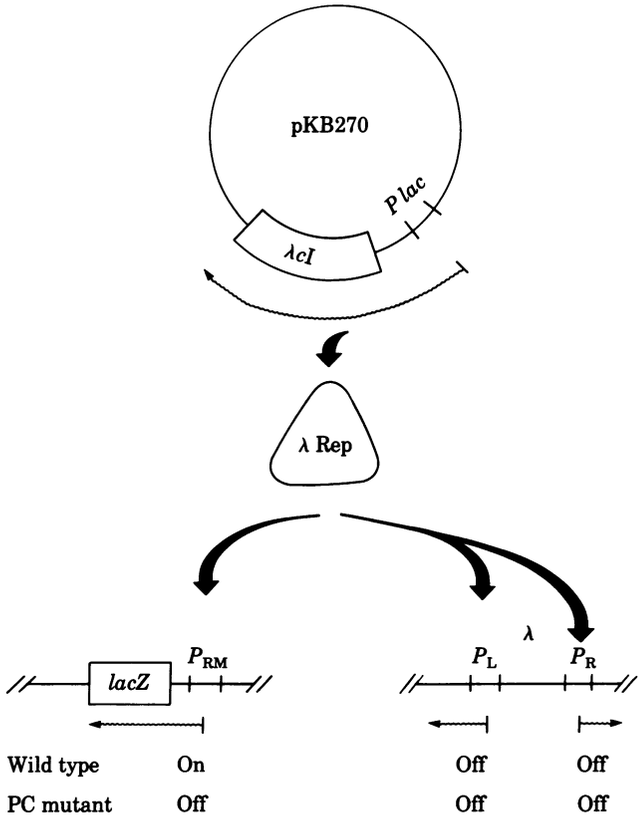

FIG. 2. Isolation of *pc* mutants. Plasmid pKB270 directs synthesis of the λ phage repressor under the control of the *lac* promoter (13). The tester strain bears a *lacZ* gene whose transcription is directed by λ phage's P_{RM} (6). In this strain, repressor stimulates β-galactosidase synthesis and renders the bacterium immune to superinfecting λ phages by repressing transcription from P_R and P_L. *pc* mutants fail to stimulate β-galactosidase synthesis.

colonies appeared at a frequency of about 0.1%. One such clone bore the mutation, *pc-1*, which is the subject of this report.

Properties of *pc-1 in Vivo*. The ability of the *pc-1* repressor to stimulate P_{RM} and to repress P_R was quantitated by adding the mutant plasmid to various tester strains and assaying β-galactosidase (Table 1). The tester strains contained prophages bearing *lacZ* fused *either* to P_{RM} or to P_R. The salient results are as follows. First, *pc-1* repressor was as efficient as wild type in repressing P_R. Second, whereas wild-type repressor stimulated P_{RM} activity 10-fold, *pc-1* repressor did not stimulate and, in fact, repressed P_{RM} basal levels by about 30%. Third, the same result was obtained if the prophage bore an O_R3 mutation that prevents repressor from binding to that site. Thus, the low levels of P_{RM} activity in strains with the *pc-1* repressor were not due to repression of P_{RM} by the binding of the mutant repressor to O_R3. Fourth, the repression of P_{RM} basal activity by the *pc-1* repressor was abolished by a mutation in O_R2 that prevents repressor from binding to that site. A more sensitive measure of this negative effect of repressor on P_{RM} basal transcription was made with a prophage bearing the mutation *prmup-1* (5). This mutation increased the P_{RM} basal activity 10-fold (Table 1), and the template bearing it was also mutant in O_R1 and O_R3, so that O_R2 was the only functional repressor binding site. *pc-1* repressor resulted in a 75% reduction in this activity, whereas wild-type repressor stimulated the activity of the *prmup-1* template 3-fold.

These results indicate that, when bound to O_R1 and O_R2, *pc-1* repressor turns off P_R but, unlike the wild-type repressor, fails to stimulate P_{RM}. Unexpectedly, *pc-1* repressor lowers the basal

Table 1. Effect of *pc-1 in vivo* on β-galactosidase levels*

Strains	Repressor: None	Repressor: Wild-type	Repressor: *pc-1*
P_{RM}-*lacZ*, O_R1^+, O_R2^+, O_R3^+	100	1000	70
P_{RM}-*lacZ*, O_R1^+, O_R2^+, O_R3^-	100	1500	70
P_{RM}-*lacZ*, O_R1^+, O_R2^-, O_R3^+	100	400	110
P_{RM}*upl-lacZ*, ΔO_R1, O_R2^+, O_R3^-	1000	3500	250
P_R-*lacZ*, O_R1^+, O_R2^+, O_R3^+	4000	50	30

Cells were grown to exponential phase in M9 minimal medium and then β-galactosidase was assayed as described by Miller (15). Wild-type and *pc-1* repressor were provided by pKB270 (tet^R). Cultures of strains containing this plasmid always had >95% of its cells tet^R even in the absence of selection. P_{RM}-*lacZ*, O_R1^+, O_R2^+, O_R3^+ data were compiled from three strains: NK5031 (λ112*cIsus*34), NK5031 (λ112*cI*26), and US3 (λ112*cIsus*34). P_{RM}-*lacZ*, O_R1^+, O_R2^+, O_R3^- data were compiled from four strains: US3 (λ112*cIsus*34, O_R3c12), US3 (λ112, O_R3r1), NK5031 (λ112, *cIsus*34, O_R3r1), and NK5031 (λ112, O_R3r1). In all cases, consistent results were obtained. In the above experiments, we observed that *pc-1* inhibition of P_{RM} basal activity was greater in NK5031 than in US3 and that the value of 70 units of β-galactosidase activity is the average of activity found in NK5031 (60 units) and US3 (80 units). (Strains with wild-type repressor and an O_R3^+ prophage displayed one-third lower enzyme levels than the corresponding O_R3^- strains because of partial turn off of P_{RM} by repressor bound at O_R3.) P_{RM}-*lacZ*, O_R2^- data were obtained using strain 5031 (λ112, $O_R2virC23$). In this strain, the O_R2 mutation prevents full stimulation of P_{RM} by wild-type repressor. P_{RM}*upl-lacZ*, ΔO_R1, O_R2^+, O_R3^- data were obtained using US3 (λ112, *cIsus*34, Δ265*prmup-1*,O_R3-*r1*). P_R-*lacZ* results were obtained using NK5031 (λ200).
* Expressed as units of β-galactosidase activity as defined in ref. 15.

level of P_{RM} transcription, and this negative effect is mediated by binding to O_R2.

Properties of *pc-1 in Vitro*. The *pc-1* mutant gene was transferred to a high copy number plasmid (pGN10) to facilitate purification of the mutant repressor. Wild-type and mutant repressors were then purified and tested for their ability to bind DNA and to regulate transcription *in vitro*. The *pc-1* repressor failed to stimulate P_{RM} under conditions that resulted in a 5-fold stimulation by wild-type repressor. Both repressors turned off P_R transcription at roughly the same concentration (50 nM for 50% repression) (Fig. 3). As measured in a DNase protection experiment, both repressors filled O_R1 and O_R2 coordinately at about 10 nM, and they filled O_R3 at about 100 nM (A. Johnson, personal communication). Thus, we did not detect any

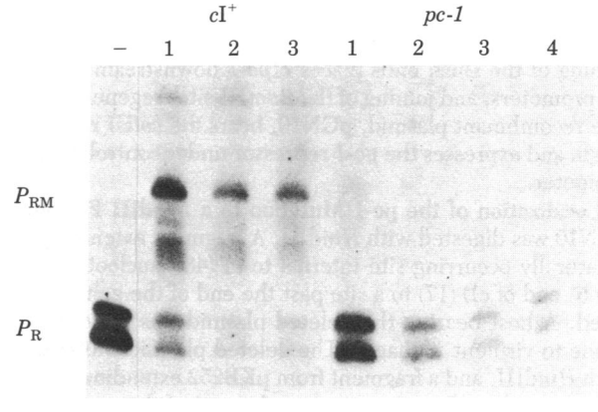

FIG. 3. Repression of P_R and activation of P_{RM} *in vitro*. Transcription was performed either in the absence of added repressor (–) or in the presence of wild-type or *pc-1* repressor added at 50 nM (lane 1), 100 nM (lane 2), and 200 nM (lane 3). P_{RM} stimulation and P_R repression were quantitated by cutting out the relevant bands from the gel and counting. At concentrations of wild-type repressor that repressed P_R 95%, P_{RM} was stimulated 5-fold.

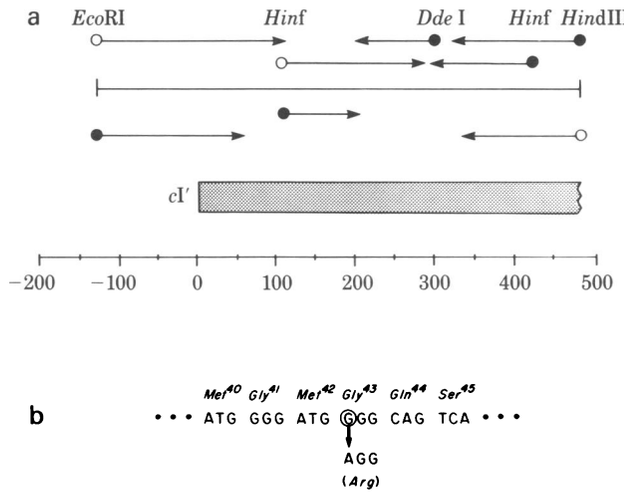

FIG. 4. The DNA sequence change caused by the *pc-1* mutation. (*a*) Strategy to determine the sequence change caused by the *pc-1* mutation. Sites labeled for sequence determination: ●, ends that were labeled by radioactive triphosphates using DNA polymerase; ○, ends that were labeled by polynucleotide kinase. Arrows show the direction of sequence determination. (*b*) The *pc-1* sequence change. The *pc-1* mutation is a G·C→A·T transition at nucleotide 130 of *c*I, which results in a glycine-to-arginine change at amino acid 43 of the encoded protein. This was surmised by comparing the sequence determined above with that previously reported (17).

major difference between *pc-1* and wild-type repressor in their binding affinities to the rightward operator or in cooperative interactions between dimers bound at O_R1 and O_R2.

Location of the *pc-1* Mutation. The location of the *pc-1* mutation was first shown to be in the amino-terminal 480 nucleotides of the *c*I gene. This determination was made by replacing the *Hin*dIII fragment of pGN10 encoding the carboxyl terminal third of the *c*I gene with the homologous fragment of a wild-type *c*I gene (from pKB252). The recombinant plasmid was unable to stimulate P_{RM} when introduced into an appropriate tester strain, showing that the *pc-1* lesion lay within the amino-terminal *Hin*dIII fragment of *c*I. The DNA sequence of this fragment was determined (Fig. 4). The only change from wild type (17) was a G·C→A·T change in codon 43 of *c*I, which results in a glycine to arginine change in the protein.

DISCUSSION

The λ phage repressor mutant *pc-1* binds to the λ phage operators and mediates negative control about as efficiently as does wild-type repressor. It is, however, defective in its ability to stimulate transcription of P_{RM}. The mutation lies in the amino-terminal domain of the repressor (amino acid residue 43). Formation of dimers and cooperativity between adjacent dimers, both of which are essential for normal repressor function, are apparently not affected by the mutation.

Our results are consistent with the idea that positive control of P_{RM} results from a contact between RNA polymerase and the amino-terminal domain of a repressor bound at O_R2. The *pc-1* change may define the region of repressor that contacts RNA polymerase. Structural studies of λ phage repressor suggest that on a repressor bound at O_R2, the glycine at position 43 would very closely approach RNA polymerase bound to P_{RM} (C. Pabo and M. Lewis, personal communication).

The *pc-1* repressor not only fails to stimulate P_{RM} but, when bound to O_R1 and O_R2 (or to O_R2 only), represses basal transcription from P_{RM}. We consider two hypotheses that might explain this negative effect. (*i*) The glycine-to-arginine change at amino acid 43 has modified repressor so that it aberrantly excludes polymerase from P_{RM}. (*ii*) The interaction between repressor and RNA polymerase inherently involves both positive and negative components, with a net positive effect on transcription. According to this idea, the *pc* mutation has eliminated the major stimulatory interaction between repressor and RNA polymerase and, thereby, has unmasked a negative interaction. Recent results of Hawley and McClure provide support for the second hypothesis. They have studied *in vitro* the two stages of RNA polymerase binding to promoters: the initial binding to form the so-called closed complex, followed by isomerization to an open complex competent to initiate transcription (21). They find that the primary role of repressor in stimulating P_{RM} is to facilitate isomerization of RNA polymerase to the open complex at that promoter, and that closed complex formation at P_{RM} is in fact partly inhibited by repressor bound to O_R2. By using the mutant promoter, *prmup-1*, this inhibition is about 3-fold (W. McClure and D. Hawley, personal communication). This is about the extent of repression of this promoter that we observe with *pc-1 in vivo*. These results, then, are consistent with the notion that the only aberrance in *pc-1* is that it is unable to stimulate open complex formation because it fails to make a positive contact with RNA polymerase that is made by wild-type repressor.

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