Initiation from the Lambda P_{RM} Promoter

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Summary

Wild-type λ repressor activates transcription from the λ P_{RM} promoter by stimulating the rate-limiting isomerization step in the initiation reaction. The positive-control mutants of λ repressor retain the ability to bind operator DNA normally, but fail to activate transcription from the λ P_{RM} promoter in vivo. We have characterized one of these mutants in vitro, and have determined the biochemical nature of the defect. We show that the mutant repressor was deficient in its ability to stimulate the isomerization step in transcription initiation. The initial binding of RNA polymerase to P_{RM} was only slightly reduced by the mutant repressor. We also found that the mutant and wild-type repressors had similar affinities for all three binding sites in the rightward operator. These results provide support for the hypothesis that direct repressor–RNA polymerase interactions are important in the P_{RM} activation mechanism.

Introduction

Activators of transcription initiation bind to DNA sites near promoters. Several hypotheses for the mode of action of transcriptional activators have been proposed. These include activator-induced changes in the DNA (Dickson et al., 1975; McKay and Steitz, 1981) and activator–RNA polymerase interactions (Gilbert, 1976; Ptashne et al., 1980) at the promoter site. Direct evidence to support either class of models has not yet been obtained. We have approached this problem by first studying the kinetics of transcription initiation, because an activator must enhance a rate-limiting step during the initiation process. A functional mechanism (Walter et al., 1967) for transcription initiation that includes the rate-limiting steps can be represented schematically as follows:

$$R + P \underset{}{\overset{K_B}{\rightleftharpoons}} RP_c \overset{k_2}{\rightarrow} RP_o, \quad (1)$$

where R and P represent free enzyme and promoter, respectively; RP_c is an inactive intermediate, the "closed complex"; RP_o is the transcriptionally active "open complex" (Chamberlin, 1974); K_B specifies a reversible binding step; and k_2 is the rate constant of the isomerization between closed and open complexes.

We have recently shown that activation of the λ P_{RM} promoter by the product of the λ *cI* gene, λ repressor, proceeded by a direct enhancement of the isomerization step, and that the initial binding was relatively unaffected (Hawley and McClure, 1982). Lambda repressor binding to O_R2 has been shown to be sufficient for P_{RM} activation (Meyer et al., 1980). This site is near the −35 region of P_{RM} and is thus close enough to the RNA polymerase to allow contact between the enzyme and λ repressor (Ptashne et al., 1980; Figure 1). The involvement of a direct protein–protein contact in P_{RM} activation is consistent with the observation that RNA polymerase bound in an open complex at P_{RM} stabilized the binding of repressor to the rightward operator (Johnson, 1980; Hawley and McClure, 1982).

The properties of the positive-control (*pc*) mutants of the λ and phage P22 repressors described by Hochschild et al. (1983; see also Guarente et al., 1982) provide the most compelling evidence for a direct activator–RNA polymerase interaction. Hochschild et al. have shown that the altered amino acid residues in the *pc* mutants are located on the surface of the DNA-bound repressor that was predicted to contact RNA polymerase. In vivo, these mutant proteins repressed transcription from the P_R promoter normally, but failed to activate P_{RM}. Since repressor binding to the same DNA sites is required for both repressor functions, the simplest interpretation is that the mutations affected only the ability of repressor to activate P_{RM}. However, two of the three λ *pc* repressors actually inhibited P_{RM}, raising the possibility that these mutant repressors failed to activate merely because they interfered with RNA polymerase at P_{RM}. In contrast, the *pc-2* mutant repressor was shown to activate P_{RM} slightly in vivo (Hochschild et al., 1983). The amino acid change in *pc-2*, an Asp to Asn at position 38, is nearly isosteric, suggesting that the mutation was more likely to have removed a favorable contact than to have introduced an unfavorable one. For this reason, we chose the *pc-2* mutant protein for an in vitro characterization of the effect of a positive-control mutation on the P_{RM} activation mechanism. If *pc-2* failed to activate P_{RM} because the mutation had impaired a protein–protein interaction necessary for enhancement of the isomerization rate, then the strong prediction was that k_2 for P_{RM} in the presence of *pc-2* repressor should be only slightly increased, without a significant decrease in K_B.

We show that the only defect in the *pc-2* repressor was a decreased ability to activate the isomerization step; the initial binding interaction was only slightly decreased on the wild-type template. In addition, comparisons between wild-type and *pc-2* repressor revealed no significant differences in their affinities for the three repressor-binding sites in O_R. These results are considered in combination with structural information and in vivo evidence from other laboratories that together suggest a role for protein–protein interactions in the mechanism of transcription activation at λ P_{RM}.

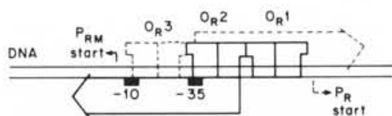


Figure 1. The DNA Sites in the λ O_R Control Region, Shown Schematically under Conditions in Which the λ P_{RM} Promoter Is Maximally Activated

RNA polymerase is represented as a thick arrow. The λ cI protein is shown bound to the O_R1 and O_R2 sites. The region of possible contact between activator and enzyme occurs in the −35 region of P_{RM}. RNA polymerase bound to P_R is shown in dashed outline because repressor bound to O_R1 or O_R2 prevents P_R occupancy. Similarly, cI protein occupancy of O_R3 is shown in dashed outline because this binding normally occurs only at high cI protein concentration and results in repression of P_{RM}. The molecular details of the interaction of repressor with the O_R region of λ are reviewed by Ptashne et al. (1980).

Results

Activation–Repression Characteristics

The pattern of P_{RM} activation and P_R repression in the presence of *pc-2* repressor was compared with that of wild-type repressor in the experiment of Figure 2. The *pc-2* repressor stimulated P_{RM} activity only slightly in vitro at protein concentrations at which P_{RM} was fully activated by wild-type repressor. At higher concentrations, both the wild-type and *pc-2* repressors repressed P_{RM}; the midpoints of repression occurred at approximately the same concentration for both repressors, suggesting that the ability of the mutant repressor to bind O_R3 and to mediate repression of P_{RM} was not altered by the mutation. The midpoint of the P_R repression curve was used to determine the concentration of active *pc-2* repressor. Since the repression of P_R was similar for both wild-type and mutant repressors at all repressor concentrations, we conclude that the cooperative interactions between repressors bound at O_R1 and O_R2 were the same for the two proteins.

Additional evidence for the unaltered binding characteristics of the *pc-2* repressor to O_R was obtained with templates carrying mutations in the three repressor-binding sites of O_R (Figure 3). On these templates, the affinity of the two repressors for single sites could be compared, since the mutations abolished the O_R1–O_R2 cooperativity responsible for both P_R repression and P_{RM} activation on the wild-type template. On the O_R1^- O_R3^- and O_R2^- templates, P_R was repressed by repressor bound to O_R2 and O_R1, respectively, while on an O_R1^- template, P_R repression was a consequence of repressor binding cooperatively to O_R2 and O_R3 (Ptashne et al., 1980). For each of these mutant templates, the patterns of the P_R repression curves were nearly identical for the *pc-2* and wild-type repressors. Thus the individual subsite affinities and the alternate pairwise cooperativity of repressor binding in O_R were not significantly affected by the *pc-2* mutation.

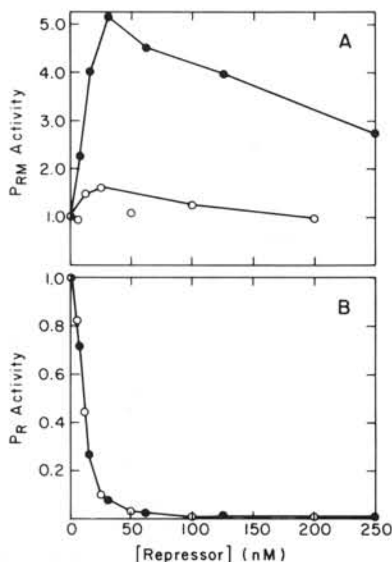


Figure 2. The Effect of the *pc-2* Mutation on Activation of P_{RM} and Repression of P_R

The activity of RNA polymerase bound to P_{RM} (A) or P_R (B) in the presence of increasing amounts of wild-type (●——●) or *pc-2* (○——○) repressor. The DNA fragment containing both promoters was preincubated with either the mutant or wild-type cI protein for 5 min at 37°C. RNA polymerase was then added to initiate an abortive initiation reaction. For P_R, CpApU synthesis from CpA and α-^{32}P-UTP in a 6 min assay was monitored; for P_{RM}, UpApU synthesized in 20 min from UpA and α-^{32}P-UTP was monitored. The promoter activity in each case was normalized to the reaction velocity observed in the absence of repressor. For P_R, this velocity was 500 CpApU per promoter per minute and corresponded to 4400 cpm incorporated into product; for P_{RM}, the velocity was 98 UpApU per promoter per minute, corresponding to 4100 cpm incorporated. Final concentrations in the reaction were: 0.5 nM wild-type 890 bp DNA fragment, 50 nM RNA polymerase, 0.5 mM CpA or UpA, 50 μM UTP labeled to a specific activity of 200 cpm/pmole. The concentration of repressor in each reaction is expressed in terms of active repressor monomers, determined as described in the Experimental Procedures. The reactions also contained 40 mM Tris-Cl (pH 8), 100 mM KCl, 10 mM $MgCl_2$, 100 μg/ml bovine serum albumin and 1 mM dithiothreitol (standard reaction buffer).

In contrast with the similar patterns of repression observed when P_R activity was monitored, the effects of the two repressors on P_{RM} activity differed significantly. Wild-type repressor bound only to O_R2-activated P_{RM} (Figure 3*A*), whereas *pc-2* repressor did not. The requirement for higher repressor concentrations for P_{RM} activation in the absence of O_R1–O_R2 cooperativity was expected from previous studies of P_{RM} activation in vivo (Meyer et al., 1980) and repressor binding in vitro (Johnson et al., 1979).

The activity of P_{RM} on the O_R2^- and O_R1^- templates was determined largely by the inhibitory effect of repressor binding to O_R3. Figure 3B shows that rather high concentrations of repressor were required to saturate the O_R3 site, in agreement with the results obtained with the O_R^+ template (Figure 2). Both wild-type and *pc-2* repressors were similar in this respect.

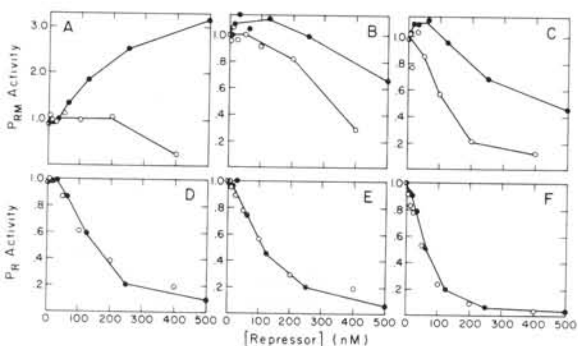


Figure 3. Comparison of the Activation and Repression Characteristics of Wild-Type and *pc-2* Repressors on Templates Carrying Mutant Operators

The fractional activity of RNA polymerase at P_{RM} (A–C) and P_R (D–F) was determined as described in the legend to Figure 2 at the concentrations of wild-type (●——●) and *pc-2* (○——○) repressor shown. (A and D) (O_R1^- O_R3^-) P_{RM} activation and P_R repression result from the binding of cI protein to O_R2. (B and E) (O_R2^-) P_{RM} is repressed by cI protein at O_R3, while P_R is repressed by cI protein at O_R1. (C and F) (O_R1^-) P_{RM} is repressed by cI protein at O_R3, and P_R is repressed by cI protein at O_R2. The mutant templates used in this comparison are described in the Experimental Procedures. The reaction velocities used, corresponding to a fractional activity of 1.0, were 80 (A), 70 (B) or 50 (C) UpApU per promoter per minute, or 300 (D) 340 (E) or 330 (F) CpApU per promoter per minute.

Table 1. Activation and Repression Characteristics of λ cI Protein: Comparisons between the Wild-Type and *pc-2* Repressors

	Template			
	O_R^+	O_R1^- O_R3^-	O_R2^-	O_R1^-
P_{RM} Activation				
cI$^+$	2.4	64	None[a]	None[a]
pc-2	2–3	None	None	None
P_{RM} Repression				
cI$^+$	110	None[b]	270	190
pc-2	~103	~140	134	42
P_R Repression				
cI$^+$	2.2[c]	59	42	21
pc-2	2.2[c]	61	46	18

Data are expressed as the concentrations (in nanomolar) of cI protein (expressed as dimers) required for half-maximal activation of P_{RM} and half-maximal repression of P_{RM} and P_R for the DNA templates indicated. The data from the experiments of Figures 2 and 3 and a cI dimer dissociation constant of 2×10^{-8} M (Sauer, 1979) were used in the calculations.

[a] Indirect or partial activation may have occurred (see text).

[b] Partial repression was observed at cI$^+$ concentrations greater than 0.7 μM (data not shown).

[c] These two values are, by definition, equal because the half-maximal repression of P_R on the O_R^+ template was used to determine the fractional activity of *pc-2* repressor (see Experimental Procedures).

In addition, this experiment confirms previous studies (Meyer and Ptashne, 1980; Hawley and McClure, 1982) that have shown that P_R repression alone is insufficient to activate P_{RM}. If indirect activation occurred at P_{RM}, an increase in P_{RM} activity would have been observed in this experiment. We estimate that less than 10% of the activating effect of wild-type repressor could be due to this indirect effect.

Repression of P_{RM} on the O_R1^- template (Figure 3C) is more complex because of the cooperative binding of repressor to O_R2 and O_R3 on this template. We expected that this cooperativity would result in repression of P_{RM} at lower concentrations of repressor than on the O_R2^- template. This expectation was borne out for the *pc-2* repressor, for which the repression curves for P_R and P_{RM} were similar. However, higher concentrations of wild-type repressor were required for P_{RM} repression on the O_R1^- template. In fact, slight activation was detectable at the lowest concentrations of wild-type repressor. It is likely that this activation resulted from the small fraction of templates to which repressor was bound only at O_R2 and not at O_R3. The mixture of activation and repression would be expected to result in the observed shift of the wild-type repression curve to higher concentrations of repressor relative to the *pc-2* curve. The latter conclusion follows from the finding that *pc-2* repressor is defective in activation.

We conclude that the P_R repression curves determined for the mutant and wild-type proteins reflect similar or identical affinities of the two repressors for the three binding sites in O_R. The differences observed for P_{RM} are most simply explained in all cases by the reduced ability of *pc-2* repressor to stimulate P_{RM} activity. The quantitative comparisons shown in Table 1 support these two conclusions. The wild-type and *pc-2* repressor dimer concentrations required for half-maximal P_R repression were nearly identical on all four templates. The key assumptions in our treatment of the data are that P_R repression on the O_R^+ template was a reliable measure of the activity of the wild-type and *pc-2* repressors, and that the repressor dimer dissociation constant was the same for wild-type and *pc-2*. If either assumption had been in error we would have expected differences in one or more of the P_R repression half-maximal values obtained with the mutant templates. The half-maximal values for repression and activation are not direct measures of the intrinsic binding of repressor to the O_R subsites because of the competition between repressor and RNA polymerase in our activity assays. For this reason, we are more confident of the relative effects of the two repressors on each template than we are of the effect of operator mutations on each activation or repression half-maximal value (see Discussion).

Kinetics of Open-Complex Formation

To define the nature of the defect in P_{RM} activation by *pc-2* repressor, we measured the rates of open-complex formation in the presence of *pc-2* repressor at different RNA polymerase concentrations. The results of the kinetic experiments are shown in a TAU plot

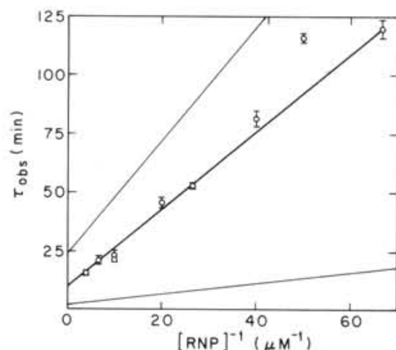


Figure 4. The Effect of *pc-2* Repressor on the Kinetics of P_{RM} Open-Complex Formation

The time required for open-complex formation (τ_{obs}) is plotted against the reciprocal of the RNA polymerase concentration (TAU plot). For each determination of τ_{obs}, the open-complex formation reaction was initiated by addition of RNA polymerase to 0.9 nM O_R^+ DNA template and 50 nM *pc-2* repressor. Portions of this reaction mixture were added to assay buffer containing 0.5 mM UpA and 50 μM α-^{32}P-UTP; the amount of UpApU synthesized in 5 min was determined. The reactions approached a maximum velocity of 565 UpApU per promoter per minute. τ_{obs} ($\pm$ 1 SD) was calculated using a least-squares analysis, as described in the Experimental Procedures. K_B (5.9×10^6 M^{-1}) and k_2 (1.7×10^{-3} sec^{-1}) were obtained from these data as described in the Experimental Procedures. Thin lines: previous determinations of K_B and k_2, obtained in the presence (lower line) or absence (upper line) of wild-type cI protein (Hawley and McClure, 1982).

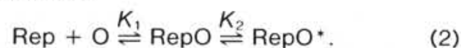
(Figure 4). These data can be analyzed using a simple linear equation (see Experimental Procedures) to obtain K_B and k_2 for P_{RM} in the presence of *pc-2* repressor. Comparison of the values determined for K_B and k_2 in the presence of *pc-2* repressor with those obtained for P_{RM} without added repressor reveals that the *pc-2* repressor increased k_2 by a factor of 2.4 on the wild-type template. K_B was reduced only 40%. In contrast, the wild-type repressor increased k_2 11-fold and had no significant effect on K_B (Hawley and McClure, 1982).

The kinetic data show that *pc-2* repressor is defective in activating P_{RM} initiation frequency. Moreover, the defect is specifically confined to the ability of *pc-2* repressor to enhance the rate of isomerization between the closed and open complex. If *pc-2* repressor had inhibited P_{RM} either by interference with formation of the closed complex or by competition between RNA polymerase at P_{RM} and *pc-2* repressor at O_R3, we would have expected a larger decrease in the apparent K_B. Thus the kinetic results specifically rule out the possibility that the defect in *pc-2* repressor could be an interference with RNA polymerase binding at P_{RM}. In addition, the rather simple in vitro behavior of *pc-2* repressor is in accordance with the prediction that an important contact between cI and RNA polymerase was impaired in the mutant.

Discussion

We favor the hypothesis that cI protein–RNA polymerase interactions participate in the activation of P_{RM}, for the following reasons. First, the proposed structure for the complex between the DNA and the N-terminal domain of repressor places the Asp38 residue relatively far from the DNA helix (Pabo and Lewis, 1982). According to this model, when repressor is bound to O_R2, Asp38 would extend into a space that corresponds to the major groove adjacent to the $P_{RM} - 35$ region. Hochschild et al. (1983) have argued that the amino acid residues altered in all of the *pc* mutants lie on the surface of the O_R2-bound repressor that would closely approach RNA polymerase. The relevance of the proposed structural model to our interpretation is supported by the demonstration that the N-terminal repressor fragment can activate P_{RM} (Sauer et al., 1979). Second, the experiments reported here have provided two key findings: the affinity of repressor for the three sites in O_R was not significantly affected by the *pc-2* mutation, suggesting that the inability of *pc-2* repressor to activate P_{RM} was not the result of an altered interaction with operator DNA (see below); and the ability of RNA polymerase to form an initial closed complex with the promoter was not greatly decreased by the *pc-2* repressor, as would be expected if the mutant repressor simply interfered with RNA polymerase's binding to P_{RM}.

Our conclusion that cI–RNA polymerase interactions are involved in P_{RM} activation does not exclude the participation of DNA conformational changes in this mechanism. However, if a DNA structural change is directly involved in the activation of P_{RM}, our results place limits on possible mechanisms for the putative change(s). For example, the simplest repressor (Rep) binding scheme that also incorporates a conformational change in the operator DNA (O) must include the following steps:



$$\text{Rep} + \text{O} \overset{K_1}{\rightleftharpoons} \text{RepO} \overset{K_2}{\rightleftharpoons} \text{RepO}^* . \quad (2)$$

The unspecified change in DNA conformation in the second step would in this class of models be responsible for transducing the simple binding of repressor into a rate enhancement for open-complex formation. This mechanism follows from our finding that the overall binding affinities ($K_a = K_1 \times K_2$) of the wild-type and *pc-2* repressors were the same at all three operators. If the second step were disfavored in the complex between *pc-2* and the operator, then the first step would have to increase proportionately to compensate precisely for the second-step defect. This explanation appears unlikely, since the *pc-2* mutation apparently did not alter binding to any of the operators, although the overall binding affinities for the three operators differ by a factor of up to 25 (Johnson et al., 1979). Moreover, even if DNA conformational changes are found to occur upon repressor binding to O_R (and none are yet known), these changes can be related to P_{RM} activation if, and only if, they are significantly altered when *pc-2* repressor binds.

Thus the simplest explanation that encompasses all that is known about the structure and behavior of

the wild-type and positive-control mutants of λ repressor is that cI–RNA polymerase interactions contribute in large part to P_{RM} activation. This conclusion provides a compelling rationale for additional experiments (for example, selection of *pc-2* pseudorevertants in RNA polymerase subunits) and for a more focused consideration of the types of DNA conformational changes that could be expected to participate in activation.

We do not know whether Asp38 actually contacts RNA polymerase or, indeed, whether this residue is only one of several cI amino acid side chains that contact the enzyme. It is nevertheless plausible to imagine that Asp38 plays a direct role in activating the conversion of RNA polymerase closed to open complex at P_{RM}, because single amino acid residues have been shown to be important in other protein–protein interactions. The detailed information available for human hemoglobin mutants provides the best examples. In fact, hemoglobin Kempsey β99 Asp–Asn (Reed et al., 1968) has the identical amino acid substitution found in cI *pc-2*. The Asn side chain in hemoglobin Kempsey cannot make an essential intersubunit hydrogen bond to α42 within the critical α_1–β_2 interface region. As a result, hemoglobin Kempsey is locked preferentially into an oxy-like quaternary structure, and oxygen binding cooperativity is abolished (Perutz et al., 1974). There are, of course, many mechanistic differences between hemoglobin cooperativity and cI activation of P_{RM}. In particular, binding and conformational equilibria are of greatest importance to hemoglobin function, whereas we seek to explain a rate enhancement. Nevertheless, the change in the hemoglobin quaternary structure is not an inappropriate analogy for the conformational change that must occur during isomerization of the ternary complex of DNA, cI protein and RNA polymerase.

The molecular basis for the deleterious effect of losing an Asp or gaining an Asn at position 38 is unknown. Because the substitution is nearly isosteric, it is reasonable to suggest that the carboxyl group on the Asp side chain participates directly in the activation. We have begun to test this idea by studying the effect of reaction conditions on the kinetics of open-complex formation at P_{RM} in the presence and absence of wild-type repressor. For example, we saw no differences in the activation kinetics over a range of pH 7.2–8.0 (Hawley, 1982). Thus, if ionized groups in cI protein or RNA polymerase are involved in the interaction, it is unlikely that the pK_as of these groups fall within the physiological range of pH. Another possibility that we considered is that the carboxyl group could be involved in an interaction with a divalent metal ion required for activation. We found that both repression of P_R and activation of P_{RM} required higher concentrations of repressor in the absence of added Mg^{2+} or Ca^{2+} and in the presence of EGTA and EDTA (data not shown). However, the divalent metal ions were apparently involved only in the binding affinity to the operators, because the repression and activation were still coordinate and the extent of P_{RM} activation was normal. The increased binding affinity of repressor for O_R1 in the presence of divalent cations was described by Johnson et al. (1980).

Our results on *pc-2* repressor can be related to other studies of promoter strength and promoter activation by considering the promoter selectivity map shown in Figure 5. In this schematic representation, promoter strength increases from lower left to upper right. In other words, the strongest promoters have high values of K_B and k_2; the weak promoters have low values of K_B or k_2. The λ P_{RM} promoter is the weakest promoter we have studied. We can also use the selectivity map to relate our in vitro results to RNA-chain-initiation frequency in vivo. The contour lines in Figure 5 correspond to the average times between RNA-chain-initiation events. The calculations depend on K_B, k_2 and an estimate of the concentration of free in vivo RNA polymerase (McClure, 1983). In general, this approach agrees well (±50%) with in vivo estimates of gene expression. For example, λ P_R and cI-activated *prmup-1* each resulted in comparable β-galactosidase activities when these promoters were fused to the *lacZ* gene (Meyer et al., 1980). The in vitro results on λ P_{RM} activation also agree with experiments performed in vivo. Wild-type repressor activated P_{RM} 11-fold in vitro (Hawley and McClure, 1982) and 8 to 10 fold in vivo (Meyer et al., 1980). The *pc-2* repressor is predicted to increase initiation frequency about twofold or less, consistent with the results of Hochschild et al. (1983).

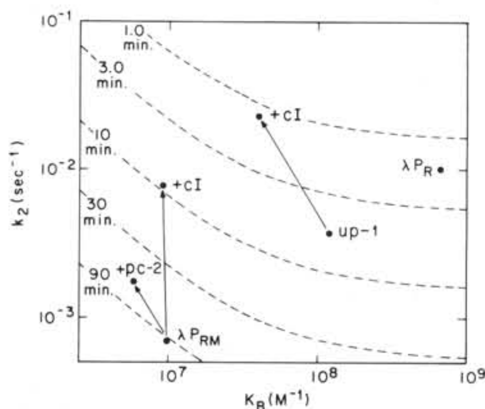


Figure 5. Comparison of the Kinetics of Activation of P_{RM} by Wild-Type and *pc-2* Repressor

The K_B and k_2 values determined from the TAU plot of Figure 4 for *pc-2* activation of P_{RM} (+ *pc-2*) are shown on this promoter selectivity map. The K_B and k_2 values reported previously (Hawley and McClure, 1982) for the unactivated P_{RM} (λP_{RM}) and mutant *prmup-1* (up-1) promoters and for the activation of both promoters by wild-type repressor (+ cI) are also shown. The values for λ P_R were reported previously (Hawley and McClure, 1980). Arrows: magnitude of the activation by the wild-type and *pc-2* repressors. Dashed curves correspond to equal initiation frequencies calculated using an estimate of the in vivo concentration of free RNA polymerase of 30 nM (McClure, 1983). At the left, the average time between RNA-chain-initiation events is shown for each contour line.

The interpretation of the repression–activation curves of Figures 2 and 3 is somewhat complicated, because the extent of repression or activation at each repressor concentration is determined primarily by the kinetics of open-complex formation and not simply by the equilibrium binding characteristics. Thus the midpoints of the curves can be altered by changing the time allowed for open-complex formation. Relatively short incubation times, such as we used in these experiments, would be expected to resolve more sensitively any small differences in repressor function. In fact, repression–activation curves are a highly sensitive and convenient assay for determination of fractional activity of different protein preparations and for comparison between wild-type and mutant repressors, in which an understanding of the reaction mechanism is unimportant to the comparison. Where such an understanding is required, as in the interpretation of the effects of operator mutations on repression and activation, the mechanistic details must be obtained by additional methods, such as the direct determination of K_B and k_2.

Experimental Procedures

Materials

UTP was purchased from P-L Biochemicals and purified as previously described (McClure et al., 1978). CpA and UpA were purchased from Sigma. α^{32}-P-UTP (600 Ci/mmole) was from Amersham. 3MM chromatography paper (19 cm × 100 m roll) was from Whatman.

DNA Fragments and Enzymes

The wild-type P_R and P_{RM} template was the 890 bp DNA fragment obtained following Hae III–EcoR I digestion of the plasmid pKB252 (Backman et al., 1976). The templates containing the operator mutations *vs326* (O_R1^-), *vN* (O_R2^-) and *vs326 or3-r3* (O_R1^- O_R3^-) were isolated as 675 bp DNA fragments following Hind III–Eco RI digestion of DNA purified from derivatives of λ 112 bacteriophage (Meyer et al., 1980). The fragment purification procedure has been described (Hawley and McClure, 1982).

Escherichia coli B RNA polymerase was purified according to the procedures of Burgess and Jendrisak (1975) and Lowe et al., (1979). The RNA polymerase was 60%–70% active holoenzyme, as determined by activity measurements on intact T7 D111 DNA (Chamberlin et al., 1979) and by titrations with a DNA fragment containing the T7 A1 promoter (Cech and McClure, 1980).

Wild-type and *pc-2* mutant λ repressor were gifts from R. T. Sauer and A. Hochschild, respectively. Except where indicated, the concentrations of wild-type repressor are reported in terms of active monomers, based on a molecular weight of 2.6×10^4 (Sauer and Anderegg, 1978) and an activity of 30%, determined by the method of Johnson et al. (1980). The reported concentrations of *pc-2* repressor were based on a repression activity assay in which the concentration of repressor dimers required for half-maximal activity of P_R was assumed to be the same for the wild-type and *pc-2* repressors (see Results; Figure 2). The activity determined in this assay (40% active monomers) was within the range of activity (30%–80%) typically observed for preparations of wild-type repressor.

The Abortive Initiation Reaction

Details of the abortive initiation reaction have been described (McClure et al., 1978). Standard reaction conditions were 40 mM Tris–Cl (pH 8); 100 mM KCl, 10 mM $MgCl_2$, 100 μg/ml bovine serum albumin, 1 mM dithiothreitol (standard reaction buffer); 0.5 mM UpA or CpA; 50 μM UTP; α-^{32}P-UTP was added to a specific activity of 200 to 500 cpm/pmole. The abortive initiation reaction products (UpApU or CpApU) were separated from labeled UTP by ascending chromatography on Whatman 3MM paper in WASP solvent (water, saturated ammonium sulfate–isopropanol; 18:80:2 by volume). The radioactivity in the product peak was normalized to the total radioactivity on the chromatograph.

Repression–Activation Curves

The rates of UpApU synthesis from P_{RM} and CpApU synthesis from P_R were measured at different concentrations of wild-type and *pc-2* repressor as described in the legend to Figure 2. The rate observed at each repressor concentration was converted to a fractional activity, with the rate observed in the absence of repressor assigned a value of 1.0. In several cases, in which low repressor concentrations did not systematically affect the rate of trinucleotide synthesis, the average value of these rates was used as the $F = 1.0$ value.

Determination of τ_{obs}

The average time required for open-complex formation (τ_{obs}) at P_{RM} in the presence of *pc-2* repressor was measured using the fixed-time assay described previously (Hawley and McClure, 1982). The open-complex formation reaction was initiated by mixing RNA polymerase with the 890 bp DNA fragment containing the wild-type O_R region in standard assay buffer at 37°C. Portions of this reaction mixture were removed at various times and added to nucleotide substrates for the P_{RM} abortive initiation reaction (0.5 mM UpA, 0.05 mM α-^{32}P-UTP labeled to a specific activity of 200 cpm/pmole). After 5 min, a portion of this reaction mixture was streaked onto the origin of a chromatograph prespotted with 0.1 M EDTA and processed as described above. The fractional extent of open-complex formation, F, was determined by comparing the rate measured at each time with the maximum rate measured at long time ($>4 \times \tau_{obs}$). These data were plotted as $\ln(1 - F)$ versus time. τ_{obs} was obtained from the reciprocal slope of a line fitted by linear least-squares regression. The bars shown in the TAU plot of Figure 4 correspond to the standard deviations in the slopes of these least-squares lines. A few of the τ_{obs} values in Figure 4 were determined by a different method, called a lag assay. In this procedure, the nucleotides were present in the open-complex formation reaction from the time of addition of RNA polymerase. A lag preceded the final steady state rate of trinucleotide synthesis. This lag was analyzed with a computer program described elsewhere (Hawley and McClure, 1982) to yield τ_{obs}. The values for τ_{obs} obtained with either method agreed within experimental error. (For example, in the experiment of Figure 4, τ_{obs} at 100 nM RNA polymerase was determined with both assays; both values are shown in the figure.)

Determination of K_B and k_2

τ_{obs} is related to the apparent equilibrium constant (K_B) and isomerization rate constant (k_2) in equation (1) as follows:

$$\tau_{obs} = \frac{1}{k_2 K_B [R]} + \frac{1}{k_2}, \tag{3}$$

where [R] is RNA polymerase concentration (McClure, 1980). τ_{obs} measured at different concentrations of RNA polymerase was plotted against the reciprocal of the RNA polymerase concentration (TAU plot) to yield values for k_2 (reciprocal of the intercept) and K_B (intercept to slope ratio). An alternative way to determine K_B and k_2 is by rearrangement of equation (3) to yield the expression

$$\frac{1}{\tau_{obs}} = k_2 - \frac{1}{\tau_{obs} K_B [R]}. \tag{4}$$

From a plot of $1/\tau_{obs}$ versus $1/\tau_{obs}[R]$, k_2 is obtained as the intercept and K_B as the negative reciprocal slope. An unweighted least-squares analysis of the data plotted in this fashion was used to obtain the K_B and k_2 values reported in the legend to Figure 4.

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