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Genetics

Mutations affecting two different steps in transcription initiation at the phage λ P_{RM} promoter

(RNA polymerase/λ promoters)

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ABSTRACT **The abortive initiation assay [McClure, W. R. (1980) *Proc. Natl. Acad. Sci. USA* 77, 5634–5638] was used to study the effects of mutations on the activity of the P_{RM} promoter of phage λ *in vitro*. The transcription initiation properties of four mutant promoters were compared with those of wild-type P_{RM} in the presence or absence of repressor (which activates P_{RM}). Two kinetic parameters were measured: k_2, the rate constant for the transition between *closed* and *open* complexes, and K_B, the equilibrium constant for the initial binding of RNA polymerase to DNA (formation of closed complexes). The primary effect of repressor on wild-type initiation was stimulation of the isomerization reaction: k_2 increased about 7-fold. Both in the presence and in the absence of repressor, *prm*U31 and *prm*E104 (changes at nucleotides −33 and −38, respectively) reduced K_B significantly without affecting k_2, indicating that these mutations affect polymerase binding but not the formation of open complexes. In contrast, *prm*E37 (a change at nucleotide −14) reduced k_2 significantly without affecting K_B. A fourth mutation, *prm*E93 (at nucleotide −39), is phenotypically Prm⁻ primarily because it causes a defect in the O_R2 operator site and, therefore, the mutant promoter is unable to respond normally to repressor. These results are consistent with the idea that the two regions of *Escherichia coli* promoters in which consensus sequences have been identified, the regions at nucleotides −35 and −10, may provide information for two discrete steps in transcription initiation.**

According to current models, there are two main steps in transcription initiation prior to the polymerization of ribonucleoside triphosphates (NTPs) into RNA chains: (*i*) binding of RNA polymerase to DNA to form *closed* complexes, followed by (*ii*) the transition (isomerization) of *closed* complexes to *open* complexes (1, 2). The second step, which appears to involve DNA strand separation (2–4), is followed by very rapid RNA chain initiation in the presence of NTPs (5, 6). A method recently developed by McClure (6) permits the determination of K_B, the equilibrium constant for the binding reaction, and k_2, the forward rate constant for the isomerization reaction, *in vitro*. We have used this method to study the effects of mutations on the P_{RM} promoter of bacteriophage λ. This promoter directs the synthesis of the phage-specific repressor (cI gene product), which is an autogenous positive regulator of transcription initiation at P_{RM} (7, 8).

We have determined K_B and k_2 for wild-type P_{RM} and four mutant promoters in both the presence and absence of repressor. The Prm⁻ phenotypes and nucleotide sequence changes associated with the mutations have been described previously (9, 10). As expected, all four mutations cause defects in transcription initiation *in vitro*. In addition, the properties of the wild-type and mutant promoters are consistent with the idea that the consensus sequence found at −35 in most *Escherichia coli* promoters (11, 12) provides information necessary for the initial binding of RNA polymerase to DNA and the consensus sequence at −10 is required for DNA strand separation and the formation of open complexes.

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MATERIALS AND METHODS

RNA Polymerase, Repressor, and DNA. RNA polymerase holoenzyme was purified by the method of Burgess and Jendrisak (13). Enzyme preparations were 90–95% pure as judged by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, contained 0.7–0.9 mol of σ subunit per mol of polymerase, and were 24–40% active. The activity of purified enzyme was determined by the method of Cech and McClure (14) or by assaying the amount of radiolabeled DNA (containing a single promoter) retained by nitrocellulose filters at saturating polymerase concentrations. Purified λ repressor was a gift from C. Pabo. The DNA template was an 889-base-pair *Hae* III restriction fragment isolated from wild-type or mutant phage according to described procedures (10).

Abortive Initiation. The abortive initiation assay has been described by McClure (6). Standard incubation mixtures included 0.04 M Tris·HCl, pH 8.0; 0.1 M KCl; 0.01 M $MgCl_2$; 1 mM dithiothreitol; 0.05 mM UpA [uridylyl(3′-5′)adenosine]; 0.05 mM [^{3}H]UTP (1 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels); 1 or 2 nM DNA fragment (as indicated); and indicated concentrations of active RNA polymerase. To measure τ_{obs} (the average time required for open complex formation), DNA, enzyme, and substrates were incubated at 37°C. At various times, 20-μl aliquots were removed and assayed chromatographically for UpApU (6). For repressor activation reactions, the DNA fragment was incubated with repressor at 37°C for 10 min prior to the addition of polymerase and NTPs.

Calculation of τ_{obs}. The steady-state rate of UpApU synthesis was estimated from the slope of the curve in the period corresponding to 3–5 times τ_{obs}. Starting with estimated values of the slope and τ_{obs}, a least-square computer analysis was used to find the best fit to the equation

$$N = Vt - V\tau_{obs}(1 - e^{-t/\tau_{obs}}), \qquad [1]$$

in which N = total UpApU concentration, V = steady-state rate of UpApU synthesis, and t = time of incubation (6).

Fixed-Time Assays. For some experiments with *prm*E93 (Fig. 5), the "fixed time assay" (15) was used. Enzyme and DNA were mixed at time zero in the absence of substrates. At various times thereafter, aliquots were removed and incubated with substrates for 3 min. The reaction mixtures were then assayed chromatographically for UpApU. Incubation conditions were the same as for abortive initiation assays described above except

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that the specific activity of [^{3}H]UTP was increased to 2.5 Ci/mmol.

Substrates. UpA was purchased from Collaborative Research (Waltham, MA); [^{3}H]UTP (10 Ci/mmol) was purchased from Amersham-Searle (Chicago, IL).

RESULTS

Abortive Initiation at P_{RM}. In a typical abortive initiation reaction, only the substrates corresponding to the first two nucleotides in a particular transcript are supplied. The initial dinucleotide is synthesized repeatedly by each RNA polymerase–promoter open complex without dissociation of the enzyme from the promoter (16). Thus, at any time, the rate of dinucleotide synthesis is a measure of the number of open complexes present. For P_{RM}, the usual substrates for the abortive initiation reaction are ATP and UTP (Fig. 1). However, the DNA template used in these experiments, the *Hae* III 889-base-pair restriction fragment of λ, contains both P_{RM} and P_R. Therefore, to avoid confusion with P_R, which also initiates transcription with pppApU, the dinucleotide UpA is used as a substrate in place of ATP. Hawley and McClure (15) have shown that this substitution limits initiation to P_{RM} because UpA corresponds uniquely to the P_{RM} sequence at $-1/+1$ (Fig. 1).

The results of a typical abortive initiation assay are illustrated in Fig. 2. In the control reaction, RNA polymerase (60 nM) was incubated with the DNA template for 60 min at 37°C to allow complete formation of open complexes prior to the addition of substrates. The addition of UpA and UTP resulted in immediate synthesis of UpApU at the steady-state rate. In a parallel reaction, RNA polymerase, DNA, and substrates were added simultaneously at time zero. In this case, there is a noticeable lag period before the synthesis of UpApU reaches the steady-state rate; the lag period is the average time necessary for open complex formation. Theoretically (6), the limiting rates for the two reactions should be the same; this is indeed the case for the experiment illustrated in Fig. 2. Extrapolation of the time course for the second reaction to the abscissa yields τ_{obs} (the lag time), which was about 20 min at an RNA polymerase concentration of 60 nM.

Determination of τ, k_2, and K_B. Transcription initiation can be diagrammed as follows (1, 2, 6):

$$E + D \overset{K_B}{\rightleftharpoons} E\text{-}D_c \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{-}D_o \xrightarrow{+NTPs} \text{RNA synthesis},$$

in which enzyme (E) is RNA polymerase; DNA (D) is the promoter; E–D_c and E–D_o are closed and open complexes, respectively; K_B is the equilibrium constant for the initial binding reaction; and k_2 and k_{-2} are the association and dissociation rate constants for isomerization. Under the conditions used in these experiments, τ_{obs} is related to the initial RNA polymerase concentration, [RNAP], by the equation (6)

$$\tau_{obs} = \frac{1}{k_2} + \frac{1}{k_2 K_B[\text{RNAP}]}. \quad [2]$$

Therefore, a plot of τ_{obs} versus 1/[RNAP] should be linear with intercept equal to $1/k_2$ and a slope equal to $1/k_2K_B$, in which $1/k_2 = \tau$, the average time necessary for the isomerization reaction.

Plots of τ_{obs} versus 1/[RNAP] for wild-type and three mutant promoters incubated with RNA polymerase in the absence of repressor are shown in Fig. 3. In the absence of repressor, the time required for isomerization is about 10 min for wild-type P_{RM}; this corresponds to $k_2 = 1.7 \times 10^{-3}$ sec^{-1} (Table 1). When *prm*U31 or *prm*E104 DNA is used as template, τ is virtually unchanged, but the slopes of the τ plots are significantly al-

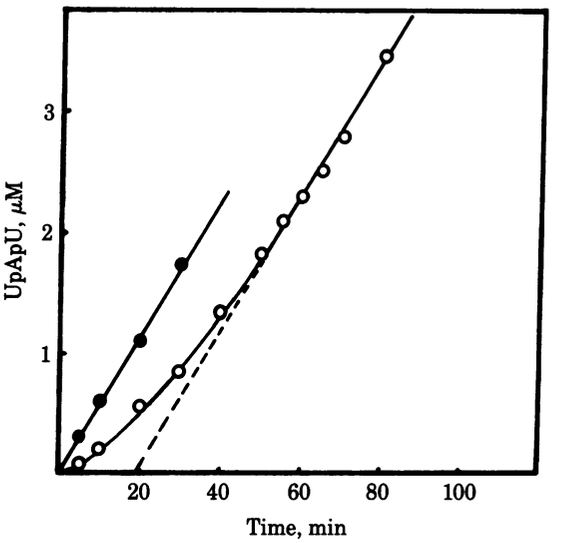

FIG. 2. Determination of τ_{obs}. The *prm*$^+$ 889-base-pair *Hae* III restriction fragment (2 nM) was incubated with RNA polymerase (60 nM) in the presence of substrates. Aliquots were withdrawn at indicated times to assay for the formation of UpApU. In one case (●), enzyme was incubated with DNA for 60 min prior to addition of substrates; in the other case (○), enzyme, DNA, and substrates were added together at time zero. Extrapolation of the slope of the second experiment to the abscissa (----) yields τ_{obs}.

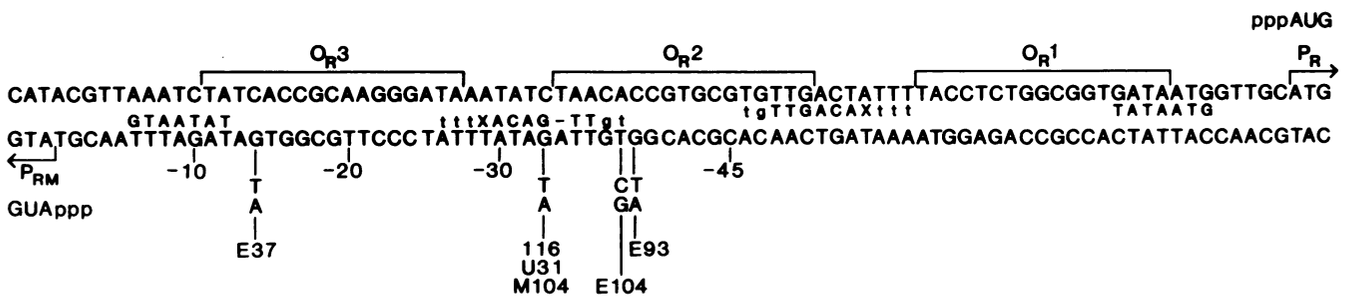

FIG. 1. Nucleotide sequence of P_{RM} and sequence changes associated with *prm*$^-$ mutations. The nucleotide sequence spans the region that includes both P_{RM} and P_R (8); nucleotides are numbered relative to the startpoint of the *cI* message initiated at P_{RM}. Nucleotide changes associated with *prm*$^-$ mutations are also indicated (9, 10). The 5′ termini of the *cI* (P_{RM}) and *cro* (P_R) mRNAs are written parallel to the corresponding DNA sequences. The consensus sequences found at −10 and −35 in *E. coli* promoters (11, 12) are printed between the two DNA strands in the corresponding regions of P_R and P_{RM}. As shown, the consensus sequences include most frequent bases that are found at frequencies greater than 46% (capital letters) or 33–46% (lower-case letters). A one-base gap (hyphen) is necessary to align the −35 consensus sequence with the actual sequence at P_{RM}.

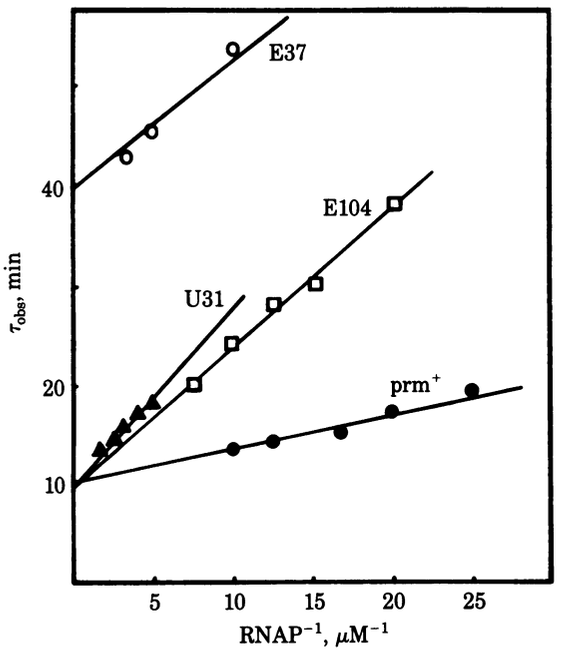

FIG. 3. Determination of k_2 and K_B for wild-type P_{RM} and mutant promoters in the absence of repressor. Values of τ_{obs} obtained in experiments such as the one illustrated in Fig. 2 are plotted as a function of 1/[RNAP] (see Eq. 2). Linear regression analysis was used to determine the slope and intercept of each line. Values of τ, k_2, and K_B determined in this way are listed in Table 1.

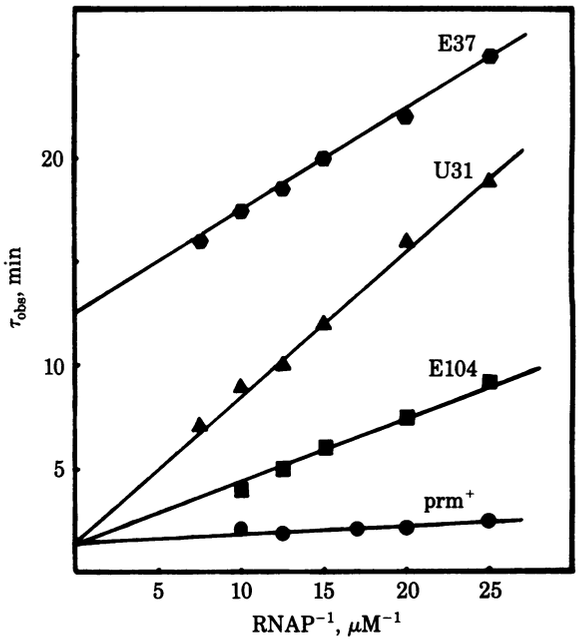

FIG. 4. Determination of k_2 and K_B for wild-type P_{RM} and mutant promoters in the presence of repressor. Values of τ_{obs} were obtained in experiments such as the one illustrated in Fig. 2. Repressor (62.5 nM) was incubated with the DNA template for 10 min prior to the addition of RNA polymerase and NTPs. Calculated parameters are listed in Table 1.

tered. Calculations based on these data (Table 1) show that K_B is reduced to 1/10th and 1/6th by mutations U31 and E104, respectively. Thus, the two mutations affect the binding of RNA polymerase to the promoter but do not affect the transition between closed and open complexes. In contrast, *prm*E37 alters the isomerization rate (k_2 is decreased to 1/4th) but does not affect polymerase binding (K_B is unchanged).

τ plots for reactions carried out in the presence of repressor (Fig. 4) are qualitatively similar to those in Fig. 3, but τ (the intercept) is reduced in every case. Note first that τ for wild-type DNA decreases from 10 min in the absence of repressor to 1.4 min in the presence of repressor. Thus, activation of P_{RM} by repressor is mediated by about a 7-fold increase in the isomerization rate. On the other hand, repressor has virtually no effect on K_B (Table 1). Fig. 4 also shows that the mutant promoters *prm*U31 and *prm*E104 respond normally to repressor. That is, values of τ for the two mutant promoters are decreased to the same extent by addition of repressor as is the wild-type value. Thus, as in the absence of repressor, the mutations affect only K_B.

Table 1. Effects of mutations on P_{RM} function: Parameters calculated from τ plots

P_{RM} allele	τ, min	k_2, $sec^{-1} \times 10^3$	K_B, $M^{-1} \times 10^{-7}$
	No repressor		
Wild-type	9.8	1.7 ± 0.1	3.0 ± 0.2
E37	*37*	*0.45 ± 0.03*	2.7 ± 0.4
U31	7.3	2.3 ± 0.3	*0.31 ± 0.04*
E104	8.3	2.1 ± 0.1	*0.48 ± 0.02*
E93	9.3	1.7 ± 0.2	2.1 ± 0.4
	Plus repressor (62.5 nM)		
Wild-type	1.4	12 ± 3	2.5 ± 0.4
E37	*13*	*1.3 ± 0.2*	2.5 ± 0.2
U31	1.3	13 ± 2	*0.18 ± 0.02*
E104	1.4	12 ± 1	*0.47 ± 0.01*
E93	*3.4*	*4.9 ± 0.1*	1.2 ± 0.1

Parameters were calculated from τ plots (Figs. 3 and 4) as follows: intercept = $\tau = 1/k_2$; slope = $1/k_2K_B$. Values for k_2 and K_B are mean values of parameters obtained from two independent τ plots for each mutant promoter (three for wild-type) ± deviation from mean. Italicized entries are thought to be significantly different from wild-type.

Finally, in the presence of repressor as in its absence, *prm*E37 is defective in isomerization but not in polymerase binding. However, the effect of the mutation on k_2 is slightly greater (a factor of 10) in the presence of repressor (Table 1) than in its absence.

Effects of the Mutation E93 on Promoter Function. The mutation *prm*E93 is of special interest because it is a change in O_R2, which affects repressor binding to the operator *in vivo* (10). Furthermore, at −39, the mutation lies just beyond the −35 consensus sequence for P_{RM} (Fig. 1). Thus, one must consider the possibility that the Prm$^-$ phenotype of *prm*E93 is due to its inability to be activated by repressor rather than to a defect in the interaction of RNA polymerase with P_{RM}.

Indeed, experiments with *prm*E93 suggest that its Prm$^-$ phenotype is due primarily to a defect in repressor binding at O_R2. In the absence of repressor, the mutation does not alter τ or k_2 (Table 1). However, τ plots for *prm*E93 in the presence of repressor at several concentrations (not shown) yield values of τ significantly greater than the corresponding wild-type values. For example, when the repressor concentration was 62.5 nM, which was chosen to minimize τ for wild-type P_{RM}, τ for *prm*E93 DNA was about 3.4 min (Table 1). As the concentration of repressor was increased, τ decreased to about 3 min at 93.7 nM repressor and about 2 min at 125 nM repressor. These results are consistent with the idea that *prm*E93 causes a defect in repressor binding, which can be overcome to some extent by increasing the repressor concentration.

If this interpretation is correct, then, at suboptimal repressor concentrations, P_{RM} should exist in two states. Those promoters

to which repressor is bound should be capable of undergoing rapid isomerization; those to which repressor has not bound should isomerize very slowly. The two states can be distinguished by examining the instantaneous rate of synthesis of UpApU as a function of time after mixing RNA polymerase and the DNA template. Determination of these rates is accomplished by using a fixed-time assay (15), in which synthesis of UpApU during a fixed 3-min interval is used to calculate the average rate of synthesis during the interval. A plot of $\ln(1 - v/V)$ as a function of time, in which v is the average rate of UpApU synthesis during an interval centered at the indicated time and V is the maximal (steady-state) rate of UpApU synthesis, yields a straight line with slope $= -1/\tau_{obs}$. If a promoter exists in two states, such a plot should be biphasic.

Fig. 5 illustrates results of fixed-time assays of UpApU synthesis with either *prm*$^+$ or *prm*E93 DNA as template at a polymerase concentration of 60 nM. As expected, at 62.5 nM repressor, a plot of $\ln(1 - v/V)$ as a function of time is monophasic for *prm*$^+$ DNA; on virtually every DNA molecule in the reaction mixture, bound repressor facilitates the rapid formation of open complexes (Fig. 5*A*). In contrast, the corresponding plot for *prm*E93 DNA is biphasic even at 93.7 nM repressor and is monophasic only at a repressor concentration of 125 nM, twice the concentration required for the wild-type template (Fig. 5*B*). Moreover, by lowering the repressor concentration to 31.2 nM, it is possible to produce a biphasic curve for *prm*$^+$ DNA (Fig. 5*A*).

These results are consistent with the idea that repressor facilitates the transition between closed and open complexes by binding to the template at O_R2 (15, 17) and that *prm*E93 is phenotypically Prm$^-$ primarily because it is defective in repressor binding to the operator. However, the calculated value of K_B for *prm*E93 DNA in the absence of repressor (Table 1) indicates that the mutation may also cause a slight defect in polymerase binding.

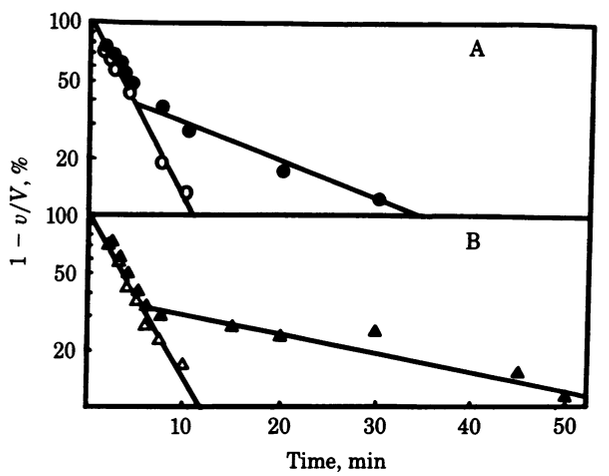

FIG. 5. Fixed-time assays of abortive initiation from *prm*$^+$ and *prm*E93 DNA. Enzyme (60 nM) and DNA template (1 nM) were incubated together beginning at time zero. At various times, substrates were added to separate reaction mixtures. After incubation for 3 min, the reactions were stopped and the amount of UpApU synthesized was assayed chromatographically to determine the average rate of synthesis during each 3-min interval. The data are transformed to fit the equation $\ln(1 - v/V) = -t/\tau_{obs}$, which is obtained by differentiation and rearrangement of Eq. 1. Indicated times are midpoints of 3-min incubation periods. (*A*) *prm*$^+$ DNA preincubated for 10 min with repressor at 31.2 nM (●) or 62.5 nM (○). (*B*) *prm*E93 DNA preincubated with repressor at 93.7 nM (▲) or 125 nM (△). Slopes of the slowly initiating (repressor-free) phases yielded values of τ_{obs} of 22 min (*A*) and 38 min (*B*).

DISCUSSION

Our data (Table 1) permit the following conclusions: (*i*) Repressor facilitates the transition between closed and open complexes at P_{RM}; k_2 is increased by a factor of about 7, which is similar to the value of 11 reported by Hawley and McClure (15). (*ii*) Two mutations in the −35 region, U31 and E104, affect the binding of RNA polymerase to the promoter to form closed complexes, but do not affect the transition from closed to open complexes. (*iii*) The mutation *prm*E37 at −14 (close to the −10 region) affects the transition step but does not affect polymerase binding. These results are consistent with the idea that information for these two steps in transcription initiation is partitioned between the two regions in which consensus sequences have been identified (11, 12).

This conclusion may not be true in all cases. Indeed, *prmup*-1, an "up" promoter mutation at −31 in P_{RM} (17), and *x*3, a P_R^- mutation at −32 (18), appear to affect both k_2 and K_B (15, 18). These results suggest either that information in the −35 region can influence both steps in the formation of open complexes or that the binding and isomerization steps in initiation each may represent a series of (two or more) reactions (see ref. 15).

The phenotype of *prm*E93 appears to be due primarily to an effect on repressor activation of P_{RM}. The results of the fixed-time assays (Fig. 5) agree with calculations based on τ plots (Table 1 and data not shown), which suggested that, at a repressor concentration optimal for rapid initiation at wild-type P_{RM}, the *prm*E93 template exists in two states: a repressor-bound state, which permits RNA polymerase to form open complexes rapidly ($\tau = 1.4$ min), and a repressor-free state, in which the template forms *open* complexes more slowly ($\tau = 10$ min). Based on this interpretation, a value of τ of about 2 min (which was obtained for *prm*E93 DNA at a repressor concentration of 125 nM) indicates that the fraction of repressor-bound templates is about 93%. Because of the limited sensitivity of the fixed-time assay, this fraction would be expected to produce the monophasic curve drawn in Fig. 5*B*.

A series of fixed-time assays at several repressor concentrations can be used to estimate the equilibrium constant for the binding of repressor to either wild-type or mutant DNA. On the basis of these assays (not shown), we estimate that the repressor monomer concentration for which 50% of the template is in the rapidly isomerizing state is about 25–30 nM for *prm*$^+$ DNA and about 70–80 nM for *prm*E93 DNA. The equilibrium constant for the formation of repressor dimers from monomers is 20 nM (see ref. 19). Therefore, the repressor dimer concentrations necessary for 50% binding were 6–8 nM and 24–28 nM for wild-type and *prm*E93 DNA, respectively. These values cannot be used to calculate actual values of K_d because the fraction of repressor active in binding has not been determined. Nevertheless, these calculations suggest that the affinity of repressor dimers for *prm*E93 DNA is about 1/4th to 1/3rd the affinity for wild-type DNA. [The published value of K_d for wild-type DNA is 6 nM (19).]

In addition to its effect on O_R, it is possible that *prm*E93 directly affects RNA polymerase binding to P_{RM}. We observe a small difference between values of K_B obtained for wild-type and mutant DNA in the absence of repressor (Table 1). This is also reflected in a difference between the slopes of the second (repressor-free) phase of plots of fixed-time assays (Fig. 5). The biological significance of a small difference in K_B is difficult to assess. However, it is worth noting that *prm*E104, which affects the last nucleotide of the P_{RM} consensus sequence, reduces K_B to 1/6th, whereas *prm*E93, which lies just outside the consensus sequence (Fig. 1), decreases K_B in the absence of repressor by less than 1/2.

The assumptions on which the abortive initiation analysis is based have been discussed in detail elsewhere (6) and have been shown to be valid for P_{RM} and P_R (15, 18, 20), but they probably are not valid for all promoters (21). In addition, one of the assumptions (that $k_2 \gg k_{-2}$) may not be valid for mutant promoters for which k_2 is reduced. Therefore, estimates of k_2 and K_B for *prm*E37 in the absence of repressor may be subject to error. However, the fact that the effects of *prm*E37 on k_2 in the presence and absence of repressor are similar suggests that such error is minimal.

Finally, one advantage of this method is worth emphasizing. The sensitivity of the abortive initiation assay permits it to be used even in the study of weak promoters. The prime examples of this are P_{RM}, which is very weak in the absence of repressor (22), and its mutant forms, which are even weaker.

A preliminary account of abortive initiation analysis of two of the mutants, based on experiments performed at somewhat different substrate concentrations, has been published elsewhere (23).

We are very grateful to Drs. D. Hawley and W. McClure for communicating detailed information about the abortive initiation assay prior to publication, for discussing unpublished results of their experiments with wild-type P_{RM}, and for suggesting important modifications of our experimental methods. We thank Ms. K. Matz for help in preparation of the manuscript and Dr. C. Pabo for a gift of purified repressor. This research was supported in part by National Institutes of Health Grant AI17508.

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