Mechanism of CRP-cAMP Activation of lac Operon Transcription Initiation
 Activation of the P1 Promoter

T. Philip Malan†, Annie Kolb‡, Henri Buc‡ and William R. McClure§

Department of Biochemistry and Molecular Biology
Conant Laboratory, Harvard University
Cambridge, MA 02138, U.S.A.

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CRP-cAMP was shown to activate transcription initiation at the Escherichia coli lac promoter in vitro as a result of two separate effects. An indirect component of the activation resulted from an enhancement of the fraction of promoters productively bound by RNA polymerase. This effect was due largely to CRP-cAMP repression of RNA polymerase binding to an overlapping site (lac P2) within the promoter region. In addition, a direct enhancement of RNA polymerase binding at the principal lac promoter (lac P1) was found. The combination of indirect and direct activation by CRP-cAMP was suggested to be responsible for the large activation observed in vivo. Promoter strength parameters were also determined for the L8, UV5 and P4 promoters. The effect of CRP-cAMP on these mutant promoters was shown to be consistent with the activation mechanism deduced for the lac wild-type promoter. DNA supercoiling enhanced the promoter strength of the lac wild-type and UV5 promoters. The combination of supercoiling and CRP-cAMP was necessary for optimal promoter strength for the lac wild-type promoter.

1. Introduction

Transcription initiation from the Escherichia coli lactose operon (lac) promoter is negatively controlled by the lac repressor (see Miller, 1978, for a review), and is positively regulated by cyclic AMP receptor protein, CRP, and cAMP (see de Crombrugghe et al., 1984, for a review). CRP binds cAMP and this complex binds to a specific site in the lac promoter region approximately 60 base-pairs upstream from the transcription start site (Majors, 1975a). The intracellular level of cAMP determines the active concentration of CRP-cAMP. The ratio of fully activated to
unactivated lac expression is about 50 (Beckwith et al., 1972). Most of this activation occurs at the level of transcription initiation.

In addition to CRP-cAMP, DNA sequence and DNA structure have been shown to exert significant effects on lac expression in vivo. Promoter mutations that increase initiation frequency have been selected and sequenced. Two of these, UV5 and P', lie in the -10 region of the lac promoter. Down mutations have been found in regions of the promoter that bind RNA polymerase or CRP-cAMP. For example, the L8 mutation is a single base-pair change at -66 that decreases the effect of CRP-cAMP on lac activation. DNA supercoiling has also been inferred to affect lac transcription in vivo. Sanzey (1979) has shown that DNA gyrase inhibitors have an immediate and specific inhibitory effect on the expression of the lac wild-type promoter.

The mechanism of CRP-cAMP activation has been investigated extensively in vitro. Majors (1975b) and Maquat & Reznikoff (1978) found that CRP-cAMP increased the rate and the extent of lac wild-type promoter utilization by RNA polymerase. The binding of CRP-cAMP to the lac promoter has been characterized with chemical (Majors, 1977) and enzymatic (Schmitz, 1981) protection experiments. The structure of CRP-cAMP has recently been determined by X-ray diffraction methods (McKay & Steitz, 1981; McKay et al., 1982). Finally, several molecular models have been proposed to explain transcription activation by CRP-cAMP (Ebright & Wong, 1981; McKay & Steitz, 1981). Since most of these models are based on unknown structural relationships between CRP-cAMP, the promoter DNA, and the RNA polymerase it is fair to characterize most of them as speculative. Thus, the question, how does CRP-cAMP activate lac transcription? remains unanswered.

We have approached the mechanism of activation by CRP-cAMP from a kinetic standpoint and have attempted to determine the rate-limiting steps in RNA chain initiation at the lac promoter. Our reasoning was based on the notion that CRP-cAMP must in some way increase initiation frequency by enhancing one or more steps during the initiation process. At least two steps participate in the RNA polymerase initiation reaction (Chamberlin, 1974; McClure, 1980):

\[ R + P \overset{k_1}{\underset{k_2}{\rightleftharpoons}} RP_c \rightarrow RP_o. \]  

(1)

The first step involves the reversible binding of RNA polymerase (R) and promoter (P) to form the inactive closed complex (RP_c). In the second step, the closed complex isomerizes to form the transcriptionally active open complex (RP_o). For most promoters reversal of open complex formation is very slow, and initiation of an RNA chain is very fast. Thus, the fractional saturation of RP_c (governed by K_R) and the rate of isomerization (expressed as k_2) combine to determine initiation frequency.

The functional model in equation (1) is useful for studying promoter strength in vitro because K_R and k_2 can be measured (McClure, 1980; Hawley & McClure, 1980). Measurements of several mutant and wild-type promoters have shown that K_R and k_2 vary over a range of at least 100-fold (Hawley et al., 1982). In addition Hawley & McClure (1982) have recently shown that activation of the P_RM
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promoter by cI protein proceeded by a direct 11-fold enhancement of \( k_2 \). The binding of RNA polymerase to \( P_{RM} \) was not significantly affected by the activator.

Our first attempts to interpret \( K_B \) and \( k_2 \) measurements for the lac promoters were complicated by serious quantitative discrepancies. These problems were largely resolved by taking into account the presence of a second RNA start site in the lac promoter region. We have called this promoter lac P2 (Malan & McClure, 1984). Transcription from lac P2 was originally noted by Maquat & Reznikoff (1978). Reznikoff et al. (1982) suggested some explanations for multiple RNA polymerase binding sites in the lac promoter region. Malan (1981) and McClure et al. (1983) proposed a specific role for lac P2 in the CRP-cAMP activation of lac transcription. The essential features of this model are: the binding of RNA polymerase to lac P2 and lac P1 was shown to be mutually exclusive. Moreover, CRP-cAMP represses the binding of RNA polymerase to lac P2. Thus, an essential facet of the lac activation mechanism is the role played by CRP-cAMP to position RNA polymerase optimally at the lac P1 promoter.

The results reported in this paper show that CRP-cAMP activates the lac promoters directly by increasing \( K_B \) and indirectly by repressing lac P2. Therefore, for the lac promoters a third parameter that expresses the probability of filling one of the mutually exclusive sites is required to describe promoter strength. We have defined this probability as the fractional promoter occupancy, \( \phi \). We have used the abortive initiation assay to determine \( K_B \), \( k_2 \) and \( \phi \). We show here which of these parameters are affected by the P\textsuperscript{p} and the UV5 mutations, by DNA supercoiling, and by the CRP-cAMP complex.

2. Materials and Methods

(a) Materials

\textit{E. coli} RNA polymerase was isolated by the procedure of Burgess & Jendrisak (1975). Holoenzyme was separated from core by the method of Lowe et al. (1979). The concentrations of RNA polymerase given in Results were calculated from protein concentrations determined by absorption measurements (\( E_{1%/280\,\text{nm}} = 6.2 \)). Quantitative activity measurements (Chamberlin et al., 1979) and the results of titration experiments have shown that this preparation of RNA polymerase was approximately 50\% active. \textit{E. coli} CRP was a kind gift from J. Krakow (Hunter College, CUNY). The protein was homogeneous as determined by polyacrylamide gel electrophoresis and had a cAMP binding activity of 8000 units/mg. The molar concentration based upon absorption measurements is expressed as dimers of \( M_r = 45,000 \). pMB9 derived plasmids carrying the lac wild-type and L8UV5 promoters were obtained from R. Ogata and W. Gilbert (Harvard University). The corresponding plasmid carrying the P\textsuperscript{p} promoter was obtained from F. Fuller (Harvard). Poly[d(A-T)] and poly[d(I-C)] were synthesized with \textit{E. coli} DNA polymerase I (Klenow fragment.) UTP and CTP were purified to remove contaminating nucleotides as described by McClure et al. (1978). [\( \alpha ^32\text{P} \)]UTP was purchased from New England Nuclear or Amersham, and 3MM chromatography paper was purchased from Whatman.

(b) Plasmid constructions and DNA purification

The 203 base-pair lac promoter-containing DNA fragments were excised with EcoRI from pMB9 based recombinant plasmids constructed as described by Fuller (1981). The
UV5 and L8 lac promoters were constructed in vitro as follows. (5'-32P)-labeled 203 base-pair DNA fragments containing the wild-type and L8UV5 lac promoters were each cleaved with the restriction endonuclease HpaII. The L8 122 base-pair fragment and the wild-type 81 base-pair fragment were ligated using T4 DNA ligase to produce the L8 promoter. Similarly, the wild-type 122 base-pair fragment was ligated to the UV5 81 base-pair fragment to produce the UV5 promoter. (For further details see Schaeffer et al., 1982.) Each promoter-containing DNA fragment was inserted into EcoRI-cleaved pBR322 using T4 DNA ligase. The resulting plasmids were transformed into E. coli strain MM294.

The UV5 and L8 promoters were sequenced by the method of Maxam & Gilbert (1980). Digestion of each plasmid DNA with a mixture of HincII and HindIII resulted in the mixture of DNA fragments expected if each plasmid contained one inserted copy of a lac promoter. The sizes of the DNA fragments produced by PvuII digestion of each plasmid (or by a mixture of PvuII and BamHI) revealed that the UV5 promoter was oriented in the same direction as the tet promoter. All the other lac promoters were inserted in orientations opposite to the tet promoter.

Plasmids were purified as described by Maquat & Reznikoff (1978) except that the buffer used for lysis contained 50 mM-Tris-HCl (pH 8.0), 62.5 mM-EDTA, and 1% Triton X-100. Promoter-containing DNA fragments were purified as follows. After digestion of the promoter carrying plasmid with EcoRI, the single stranded ends of the DNA fragments were filled in using E. coli DNA polymerase I (Klenow fragment). The large DNA fragment containing the plasmid vector was precipitated with 8% (w/v) polyethylene glycol (Carbonox 6000) according to Lis (1980). The supernatant was diluted to an ionic strength equivalent to 0.1 M-NaCl and the promoter-containing DNA fragment was precipitated with 10 mM-spermine, washed with 70% (v/v) ethanol, dissolved in 0.5 M-NaCl and dialyzed versus 10 mM-Tris·HCl, 0.1 mM-EDTA as described by Hoopes & McClure (1981). The purified DNA fragments were 211 base-pairs in length. Concentrations were determined from U.V. spectra employing A260nm = 6.5 mM⁻¹ cm⁻¹ DNA phosphate.

The superhelical densities of the plasmids used in abortive initiation experiments were determined as described by Keller (1975). The superhelical density of each plasmid was −0.06 except when specified. When RNA polymerase was tested for nicking activity, more than 90% of the plasmid DNA remained superhelical after 3 h incubation under standard assay conditions.

(c) Abortive initiation assays

The properties and technical aspects of the abortive initiation assay have been described by McClure et al. (1978). The standard buffer conditions used were 0.04 M-Tris·HCl (pH 8.0), 0.10 M-KCl, 0.01 M-MgCl₂, 1 mM-dithiothreitol and 0.1 mg bovine serum albumin/ml. The substrates for the abortive initiation reaction at the lac promoter were 0.5 mM-ApA and 0.04 mM-UTP. [α-32P]UTP was added to 200 to 1000 cts/min per pmol. The products of the abortive initiation reactions were analyzed by spotting 20-μl portions of each reaction mixture onto Whatman 3MM paper, which had been prespotted with 0.1 M-EDTA, and developing with ascending chromatography in WASP solvent (water : saturated ammonium sulfate : isopropanol: 18 : 80 : 2, by vol.) as described by McClure et al. (1978). The ApApUpU product synthesized at the lac promoter migrated with an Rₚ = 0.05 in this chromatography system. The rate of synthesis was expressed in each case as the nmol UTP incorporated/nmol promoter per min.

Control experiments performed with pBR322 DNA showed that an abortive initiation product was synthesized from ApA and UTP (see Fig. 1). This product migrated with ApApUpU on WASP chromatography, but it was not characterized further. Abortive initiation rates for supercoiled lac promoters were determined as follows (i) The total synthesis of abortive initiation products from a supercoiled pBR322 vector carrying an inserted lac promoter was measured; (ii) in the same experiment, the background synthesis from supercoiled pBR322 was measured; (iii) the abortive initiation rate from the inserted lac promoter was obtained by subtraction of pBR322 background synthesis.
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(d) Determination of \( \tau_{obs} \) and promoter strength

\( \tau_{obs} \) was determined using lag assays as described by McClure (1980). RNA polymerase was added following preincubation of nucleotides and template for 10 min at 37°C. Portions of the reaction were sampled at appropriate times and analyzed by WASP chromatography as described above. \( \tau_{obs} \) and the final steady-state rate of the abortive initiation reaction, with 67% confidence limits for each value, were determined using a non-linear least-squares analysis (M. Mulligan and W. R. McClure, unpublished results).

The determination of \( \tau_{obs} \) for the supercoiled lac promoters was complicated somewhat by the pBR322 background synthesis described above. The corrections (described in Results) were relatively small for the UV5 and the activated wild-type promoters. However, the relative corrections in \( \tau_{obs} \) were larger for the wild-type promoter in the absence of CRP-cAMP. These corrections occasionally led to poor computer fits to the data. In these cases, abnormally high steady-state rates were correlated with aberrantly high values of \( \tau_{obs} \) and vice versa. These values were not incorporated in the TAU plot analysis. In general, the steady-state rate of the abortive initiation reaction was independent of the RNA polymerase concentration. Therefore, this parameter was used as an important criterion in judging the quality of the experimental data. In addition we have found that a minimum of 12 to 15 well-chosen time-points were required to determine \( \tau_{obs} \) with an uncertainty less than 20%.

The promoter strength parameters, \( K_B \) and \( k_2 \), corresponding, respectively, to the binding and isomerization steps of equation (1), were determined with a TAU plot analysis. The \( \tau_{obs} \) values obtained from the computer analysis of lag curves were plotted versus the reciprocal RNA polymerase concentration according to equation (2):

\[
\tau_{obs} = \frac{1}{K_B[R]} + \frac{1}{k_2}
\]

From the resulting TAU plot, the isomerization rate constant, \( k_2 \), was obtained as the reciprocal intercept; \( K_B \) was obtained as the ratio, intercept/slope.

(c) Determination of open complex lifetimes

Lifetimes of the RNA polymerase–promoter open complexes were measured using the template challenge technique described by Cech & McClure (1980). Following a 20 min preincubation of polymerase and template at 37°C, a template challenge reagent (e.g., poly[d(A-T)]) was added. At selected times thereafter, portions were withdrawn and added to nucleotide substrates. The fraction of open complexes remaining was analyzed by measuring the abortive initiation rate in a 10 min reaction as described in section (c) above. \( k_{off} \) was calculated from a semilogarithmic plot of the fraction of open complex remaining versus time.

3. Results

(a) RNA polymerase titrations of the lac UV5 promoter

The formation of open complexes at the lac promoter was monitored by measuring the rate of formation of the tetranucleotide ApApUpC from ApA and \([32P]UTP \) (McClure et al., 1978). A titration experiment was performed by following the dependence of this rate on RNA polymerase concentration at a fixed concentration of 211 base-pairs DNA fragment containing the UV5 or L8UV5 promoter. Two RNA polymerase molecules were required to fully occupy this promoter (cf. Fig. 1). Similar titrations on the T7 AI promoter, and quantitative assays performed according to Chamberlin, have shown that this RNA
polymerase preparation was approximately 50% active. The experiment of Figure 1 is therefore consistent with a requirement of one active RNA polymerase molecule per template for full activity. When the L8UV5 promoter was inserted into the supercoiled pBR322 vector, the sharp titration behavior was still seen (cf. curves in Fig. 1). We infer that non-specific binding of RNA polymerase to pBR322 DNA had very little effect on promoter titration. The end point of the titration was, however, displaced. It corresponded to eight molecules of RNA polymerase on a supercoiled vector and to somewhat less on the nicked plasmid. This result suggests that two to three high affinity sites on pBR322 can effectively compete with the L8UV5 promoter for RNA polymerase binding. (Note, however, that an abortive initiation product was synthesized on pBR322 DNA from ApA and UTP; the sites responsible for this activity appear to be weaker than the lac promoters since they are saturated only at higher RNA polymerase concentration.)

(b) The effect of CRP-cAMP on lac promoter occupancy

The rate of synthesis of the abortive initiation product ApApUpU was determined when CRP and cAMP were added first to the DNA followed by addition of RNA polymerase. The reaction was then initiated with the addition of the nucleotide substrates. We have assumed that, under these conditions, the linear accumulation of product is directly proportional to the amount of active
Fig. 2. CRP concentration dependence for activation of wild-type and mutant lac promoters on supercoiled and linear DNA. The activities in the abortive initiation assay are plotted versus the concentration of CRP. Abortive initiation reactions were performed as described in Materials and Methods and in the legend to Fig. 1. Each reaction contained 50 nm RNA polymerase, 1 nm template, 0.20 mM cAMP, and the indicated concentrations of CRP. (a) Wild-type lac promoter on the 211 base-pair DNA fragment. (b) L8 promoter on the 211 base-pair DNA fragment. (c) Wild-type promoter inserted into pBR322. (d) L8 promoter inserted into pBR322. (e) P" promoter on the 211 base-pair DNA fragment. (f) UV5 promoter inserted into pBR322. The background incorporation of UTP from supercoiled pBR322 DNA was subtracted.

complex preformed at the promoter before substrate addition. CRP-cAMP stimulated the rate of synthesis of the abortive initiation product approximately 15 to 20-fold at the wild-type lac promoter (Fig. 2(a)). We have assumed that this increase in synthesis rate reflects the increased probability for a given promoter-RNA polymerase complex to be in the open conformation at lac P1. We have called this probability the occupancy of the promoter. Occupancy is expressed as a fraction: \( 0 < \phi < 1.0 \). The maximal synthesis rate observed for the strongest promoter corresponds to a value of \( \phi = 1.0 \).

In the following we consider how the occupancy of the P1 promoter depends on (i) the nature of the lac promoter mutants; (ii) the presence of CRP and cAMP; (iii) the degree of supercoiling of the template.
For the UV5 lac promoter the occupancy of Pl was high in the absence of CRP-cAMP. Addition of CRP did not affect the rate of synthesis at Pl (these high values are associated with an occupancy of 1). The experiment of Figure 2(f) was performed with the UV5 promoter (and a wild-type CRP binding site). Comparable results were obtained with the L8UV5 promoter (data not shown).

The P' promoter was partially occupied in the absence of activator and full occupancy was observed at saturating concentrations of CRP-cAMP. For the L8 and wild-type promoter fragments, the stimulation factor was much larger, but full occupancy was not reached even in the presence of a large excess of CRP-cAMP.

In conclusion, the occupancy values observed in the absence of CRP-cAMP have been found to be insensitive to the presence of the L8 mutation. However, they did depend strikingly on the nature of the sequence in the -10 region. The effect of the corresponding "up" mutants is more evident in the absence of the CRP-cAMP complex than at saturation. The L8 mutation decreased the apparent affinity of CRP-cAMP for the promoter. The incomplete occupancy observed for the L8 promoter at high CRP-cAMP concentrations may be due to the binding of CRP-cAMP to other sites in the promoter, which could interfere with polymerase binding. At high concentrations (>500 nM), CRP-cAMP also inhibited open complex formation at the wild-type promoter (data not shown).

(c) CRP-cAMP dependence of the activation process

For the wild type, the P' and the L8 promoters, the rate of synthesis of the abortive product synthesized at Pl can be used to titrate the promoter fragment with CRP-cAMP in the presence of RNA polymerase (cf. experiments of Fig. 2, that were performed at saturating concentration of cAMP). CRP stimulation of the linear wild-type promoter exhibited essentially titration behavior. Tight binding was also observed in CRP interaction with the supercoiled wild-type promoter and P' promoter. In each case approximately 2.5 dimers of CRP were required to fully activate the promoter. Hence, titration was unaffected by the DNA present in the pBR322 vector (approx. 5 m-DNA phosphate). Binding of the CRP-cAMP complex to non-specific DNA or to the CRP binding site located on pBR322 (Queen & Rosenberg, 1981) must be relatively weak. We estimate that the apparent association constant between CRP-cAMP and those lac promoters is greater than $10^8$ M$^{-1}$ in the presence of RNA polymerase.

The L8 mutation markedly reduced this apparent association constant. The stimulation of the linear L8 promoter was half-maximal only at 25 nM CRP-cAMP (cf. Fig. 2). Half-maximal activation occurred at a lower CRP concentration on the insert (9 nM) than on the linear template. This increased affinity for the supercoiled template was expected because of the modest unwinding that results from CRP-cAMP binding in the lac region (Kolb & Buc, 1982). We will show elsewhere that supercoiling also decreased the concentration of cAMP required for half-maximal stimulation on the wild-type promoter (Malan & McClure, 1984).
(d) *The effect of supercoiling on the abortive initiation rates of lac promoters*

In order to investigate the effect of supercoiling on promoter occupancy, we measured the abortive initiation rates on supercoiled and linearized portions of the same DNA preparation. The experiment was to cleave each promoter-carrying plasmid with EcoRI and to measure the abortive initiation rate of each promoter before and after cutting without any further purification of the promoter-containing fragment. This direct method avoided errors which were inherent in the comparison of abortive initiation rates between different DNA preparations. The results in Table 1 show that supercoiling significantly increased the occupancy of the wild-type lac promoter, primarily when it is non-activated. The rates observed for the L8UV5 promoter were within experimental error, unaffected by EcoRI cleavage.

These results are consistent with the data obtained from several separate experiments (for example, Fig. 2(a) to (f)). Linearization of the supercoiled unactivated wild-type promoter reduced the rate by approximately tenfold. Linearization of the CRP-cAMP-activated wild-type promoter had a smaller (approx. 40%) effect.

We conclude, therefore, that the UV5 and L8UV5 promoters are characterized by abortive initiation rates that are independent both of supercoiling and of the presence or absence of CRP. Their common specific abortive initiation rate (~120) is taken as characteristic of a fractional occupancy of the P1 promoter equal to 1. The low occupancies of the linear wild-type and L8 promoters ($\phi = 0.03$) in the absence of CRP were increased 15 to 25-fold by the presence of the CRP-cAMP complex and also about tenfold by inserting them into a negatively supercoiled template ($\sigma = -0.06$). The combined effect of supercoiling and of the presence of the activator resulted in an occupancy nearly equivalent to that observed on the UV5 promoter.

### Table 1

<table>
<thead>
<tr>
<th>Template</th>
<th>Supercoiled</th>
<th>RI-cleaved</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Wild-type (5)</td>
<td>30 ± 10</td>
<td>3 (2–8)</td>
</tr>
<tr>
<td>(2) Wild-type + CRP-cAMP (6)</td>
<td>120 ± 20</td>
<td>80 ± 10</td>
</tr>
<tr>
<td>(3) L8UV5 (2)</td>
<td>120</td>
<td>135</td>
</tr>
<tr>
<td>(4) L8UV5 + CRP-cAMP (3)</td>
<td>115</td>
<td>120</td>
</tr>
</tbody>
</table>

Supercoiled pBR322 plasmids containing inserted lac promoters were linearized with EcoRI and their activities in the abortive initiation assay were compared to those of intact plasmids. Abortive initiation assays were performed as described in Materials and Methods. Each reaction contained 50 nM RNA polymerase and 1 nM supercoiled or EcoRI-cleaved template. EcoRI cleavage was performed in the abortive initiation reaction buffer for 30 min at 37°C. Abortive initiation assays were performed immediately upon completion of the EcoRI digestion reaction. The number of separate assays performed for each template is shown in parenthesis. The abortive initiation assay activities are expressed as nM-UTP incorporated/nM-promoter per min. Standard deviations from the mean are shown for the wild-type ± CRP-cAMP, except for the RI-cleaved wild-type where the range of values is shown in parenthesis. Completion of RI cleavage was verified by polyacrylamide gel electrophoresis.
(e) The effect of CRP and cAMP on the kinetics of open complex formation at the lac promoter

We used the TAU plot analysis of McClure (1980) to evaluate the kinetic contributions of CRP-cAMP and supercoiling to the activation of the lac promoters. The concentrations of CRP, cAMP and RNA polymerase were chosen based on the results of the titration experiments reported above. In order to measure $\tau_{obs}$ for the supercoiled lac promoters, the background synthesis of abortive initiation products from the pBR322 was subtracted, and the data processed as explained in Materials and Methods. The lag curves determined in this way followed the predicted exponential approach to the final steady-state rate, as shown by comparison of experimental data with the theoretical curve (Fig. 3(b)). TAU plots showing the effects of CRP-cAMP on the rates of open complex formation at the linear and supercoiled wild-type lac promoters are shown in Figure 4. The promoter strength parameters calculated from the TAU plot analysis are summarized in Table 2.

CRP-cAMP dramatically increased the rates of open complex formation (decreased $\tau_{obs}$) at all of the lac promoters, particularly at low RNA polymerase concentrations. This effect was due to a large (greater than tenfold) increase in $K_A$ (Table 2). In fact, only a minimum estimate can be given for this effect, since in
CRP-cAMP activation of lac P1 promoter.

**Figure 4.** The effects of CRP-cAMP and supercoiling on the kinetics of open complex formation at the wild-type lac P1 promoter. $t_{obs}$ is plotted versus the reciprocal of the RNA polymerase concentration. $t_{obs}$ was determined at several RNA polymerase concentrations on the linear and on the supercoiled wild-type promoters in the presence and in the absence of CRP-cAMP. Each reaction contained 0.1 to 5.0 nM DNA template and the indicated concentration of RNA polymerase. Reactions were initiated with the addition of RNA polymerase, following a 15 min preincubation of DNA, CRP (20 nM), cAMP (2000 M) and nucleotide substrates at 37°C. Portions of the reaction were removed at various times. The incorporation of UTP was determined as described in Materials and Methods. $t_{obs}$ and the final steady-state rate of ApApUpU synthesis were determined for each reaction using a non-linear least-squares computer program as described in Materials and Methods. Lag curves were constructed for promoters inserted into pBR322 as described in the legend to Fig 3. (a) $t_{obs}$ on the linear wild-type promoter in the absence (○) and in the presence (●) of CRP-cAMP. (b) $t_{obs}$ on the supercoiled wild-type promoter inserted into pBR322 in the absence (○) and in the presence (●) of CRP-cAMP. Error estimates (1 s.d.) in $t_{obs}$ are shown as vertical bars as described in Materials and Methods.

**Table 2**

Promoter strength parameters for the lac P1 promoters

<table>
<thead>
<tr>
<th>Promoter</th>
<th>$K_B$ (M$^{-1}$)</th>
<th>$k_2$ (s$^{-1}$)</th>
<th>$k_{-2}$ (s$^{-1}$)</th>
<th>$K_o$ (M$^{-1}$)</th>
<th>$\phi$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>$1.5 \times 10^7$</td>
<td>0.003</td>
<td>$9.9 \times 10^{-5}$</td>
<td>$5 \times 10^8$</td>
<td>0.03</td>
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<tr>
<td>Wild-type (S.C.)†</td>
<td>$9.0 \times 10^6$</td>
<td>0.025</td>
<td>N.D.</td>
<td>$22.0 \times 10^6$</td>
<td>0.04</td>
</tr>
<tr>
<td>Wild-type + CRP-cAMP</td>
<td>$2 \times 10^8$</td>
<td>0.003</td>
<td>$6.0 \times 10^{-5}$</td>
<td>$5 \times 10^9$</td>
<td>0.66</td>
</tr>
<tr>
<td>Wild-type (S.C.) + CRP-cAMP</td>
<td>$3 \times 10^8$</td>
<td>0.012</td>
<td>N.D.</td>
<td>$23.0 \times 10^8$</td>
<td>0.012</td>
</tr>
<tr>
<td>L8 (S.C.) + CRP-cAMP</td>
<td>$1 \times 10^8$</td>
<td>0.025</td>
<td>N.D.</td>
<td>$9.0 \times 10^7$</td>
<td>0.110</td>
</tr>
<tr>
<td>UV5</td>
<td>$9.0 \times 10^6$</td>
<td>0.110</td>
<td>$9.0 \times 10^{-5}$</td>
<td>$1 \times 10^{10}$</td>
<td>1.0</td>
</tr>
<tr>
<td>L8UV5 (S.C.)</td>
<td>$1 \times 10^8$</td>
<td>0.016</td>
<td>$5.0 \times 10^{-5}$</td>
<td>$5 \times 10^{10}$</td>
<td>1.0</td>
</tr>
<tr>
<td>UV5 + CRP-cAMP</td>
<td>$2 \times 10^8$</td>
<td>0.12</td>
<td>N.D.</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>P⁺</td>
<td>$1 \times 10^7$</td>
<td>0.005</td>
<td>N.D.</td>
<td>0.30</td>
<td>1.0</td>
</tr>
<tr>
<td>P⁺ + CRP-cAMP</td>
<td>$2 \times 10^8$</td>
<td>0.016</td>
<td>N.D.</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

$K_B$, $k_2$ and $k_{-2}$ were determined as described in Results. The overall equilibrium constant, $K_o$, was calculated by: $K_o = K_B k_2 / k_{-2}$.

† The abbreviations used are: S.C., supercoiled template; N.D., not determined; $\phi$, the occupancies were calculated from the steady-state abortive initiation velocities assuming that 120 UTP/promoter per min corresponded to $\phi = 1.0$; see the text.
the presence of CRP-cAMP, \( K_B \) was too large to be measured accurately. CRP-cAMP had little effect on \( k_2 \). The final steady-state rates were constant at all RNA polymerase concentrations tested. Therefore, the kinetic constants that we obtained were not functions of variable promoter occupancy.

Supercoiling the wild-type promoter resulted in an eightfold increase in \( k_2 \). In the absence of CRP-cAMP, supercoiling affected \( K_B \) only slightly. The effect of supercoiling on \( K_B \) could not be quantified accurately in the presence of CRP-cAMP, since this parameter was too large to be determined. In contrast, supercoiling the L8UV5 promoter resulted in a dramatic increase in \( K_B \) (~100-fold) and a decrease in \( k_2 \). TAU plots for the linear and superhelical L8UV5 promoters are shown in Figure 5 (cf. also Table 2). TAU plots were also obtained for the linear UV5 promoter and for the L8UV5 promoter on a nicked plasmid. In both cases, the results were comparable to the linear L8UV5 promoter (data not shown). Promoter strength parameters determined for the UV5 promoter at an intermediate superhelical density (\( \sigma = -0.03 \)) were intermediate between those of the linear promoter and those of the fully supercoiled promoter (Table 2).

(f) Open complex lifetimes

The rate of dissociation of RNA polymerase from preformed open complexes at the linear lac wild-type promoter was determined using the poly[d(I-C)] challenge assay described in Materials and Methods. The results of this experiment are shown in Figure 6. The \( t_{1/2} \) for dissociation in the absence of CRP-cAMP was 120 minutes. In the presence of CRP-cAMP the \( t_{1/2} \) was 190 minutes. The initial promoter occupancy was about ten times greater in the presence of CRP-cAMP, but the dissociation of open complexes was only about two times slower. Because this difference was small, we considered the possibility that poly[d(I-C)] could bind all of the added CRP-cAMP or even displace CRP-cAMP in some fashion leaving an unactivated wild-type open complex to dissociate at nearly the characteristic unactivated rate. We therefore challenged the CRP-cAMP-activated promoter with a DNA fragment containing the strong T7 Al promoter. The lifetime determined with the T7 Al challenge was indistinguishable from that determined with poly[d(I-C)] (Fig. 6). We do not know whether CRP-cAMP can dissociate frequently from open complexes. However, the concentration of free CRP-cAMP in the experiment of Figure 6 was high enough to completely saturate the lac CRP binding site, even if there were several adventitious CRP-cAMP binding sites on the T7 DNA fragment. We conclude, therefore, that CRP-cAMP has only a modest stabilizing effect on open complexes at the wild-type lac promoter. In other words, the TAU plot experiments showed that \( K_a \) was increased by CRP-cAMP and the dissociation rates are consistent with the idea that only \( K_B \) is affected by CRP-cAMP.

4. Discussion

Three parameters (\( \phi \), \( K_B \) and \( k_2 \)) are required to describe the strength of the lac promoters and the increase in strength when they are activated by CRP-cAMP or
Fig. 5. The effect of supercoiling on the kinetics of open complex formation at the lac L8UV5 promoter. $\tau_{\text{obs}}$ is plotted versus the reciprocal of the RNA polymerase concentration. $\tau_{\text{obs}}$ was determined using the lag assay described in Materials and Methods and in the legend to Fig. 3. The template concentration was 1 nM. (○) $\tau_{\text{obs}}$ measured on the linear L8UV5 promoter. (□) $\tau_{\text{obs}}$ measured on the L8UV5 promoter inserted into supercoiled pBR322 DNA.

Fig. 6. The effect of CRP-cAMP on open complex lifetimes at the lac wild-type promoter. The fraction of open complex remaining following template challenge is plotted logarithmically versus time. The standard reaction conditions described in Materials and Methods were employed; the abortive initiation assay was used to determine the fraction of open complexes as described in Materials and Methods. In the absence of CRP-cAMP, the RNA polymerase-promoter complex was challenged with 0.10 mM-poly[d(I-C)] (●); in the presence of CRP (200 nM) and cAMP (100 µM), the RNA polymerase-promoter complex was challenged with 0.10 mM-poly[d(I-C)] (○) or 6 nM-DNA fragment containing the T7A1 promoter (□).
by negative supercoiling. The specific steady-state rate of synthesis of the abortive initiation product, ApApUpU, is related to the probability of RNA polymerase reaching the correct open complex after completion of the lag phase (called here the occupancy of the P1 promoter). The duration of this lag phase reflects the kinetics of open complex formation and has been analyzed according to the two-step model in equation (1) (Introduction). TAU plot analysis allows the separate quantitation of the binding, $K_B$, and isomerization rate constant, $k_2$. In the following sections we consider the activation mechanism from several points of view. We argue that the results obtained with the mutant lac promoters and with the supercoiled lac promoters all point toward a unified view of CRP-cAMP action in the lac operon, in which the probability and the kinetics of open complex formation are both responsible for the overall effect of the activator.

The linear lac wild-type promoter is a very inefficient promoter in vitro ($\phi = 0.03$; $K_B = 1.5 \times 10^7 \text{ M}^{-1}$; $k_2 = 3 \times 10^{-3} \text{ s}^{-1}$). Figure 7 shows schematically how these parameters were improved by the presence of CRP-cAMP and by negative supercoiling so as to confer on the wild-type promoter an efficiency that was comparable to the UV5 (or L8UV5) mutants.

The presence of CRP-cAMP significantly increased the rates of lac P1 open complex formation. It did so by improving $K_B$ without affecting $k_2$. This simple generalization applies to all of the promoters that have been tested here. In each case the direct activating effect was of the same order of magnitude with the result that the rate of formation of open complex at P1, at saturating CRP-cAMP and for most RNA polymerase concentrations ($>5 \times 10^{-9} \text{ M}$) was uniquely

![Diagram](image)

**Fig. 7.** The effect of CRP-cAMP on the strength of lac promoters. Each promoter has been placed on the promoter selectivity map above according to the values for $K_B$ and $k_2$ listed in Table 2. The effect of CRP-cAMP on each promoter is shown schematically by the arrows. In all of the cases tested, the major effect of CRP-cAMP was an increase in $K_B$. 
determined by $k_2$. Negative supercoiling is a differential effector of these promoters. It increased $k_2$, the rate of open complex formation fourfold for the wild-type lac promoter, and it decreased this parameter about sevenfold on the L8UV5 template (cf. Table 2).

A second important effect of CRP-cAMP, which is specific to the wild-type and P\textsuperscript{a} promoters, must be considered, namely promoter occupancy. The occupancy of the UV5 (or L8UV5) promoter was not affected by supercoiling or by the presence or absence of CRP-cAMP. We have therefore assigned a value of 1 to the UV5 promoter occupancy corresponding to a steady-state rate of 120 UTP incorporated per template per minute. In contrast, the occupancy of the wild-type P\textsubscript{1} promoter was increased from 0.03 in the absence of any activator to about 0.65 by CRP-cAMP alone, or to about 0.3 by supercoiling alone. In the presence of both of these activators the occupancy of the wild-type promoter was in the range of 0.8 to 0.9. The occupancy values for the wild-type promoter were more variable than those obtained for P\textsuperscript{a} or UV5. The values reported above correspond to the titration experiments of Figure 2. The occupancy values for some of the kinetic experiments summarized in Table 2 were lower.

In summary, it is only when the CRP-cAMP is bound at the proper site on a negatively supercoiled template that the wild-type lac promoter reaches a strength comparable to that of the UV5 promoter in our transcription assay ($\phi = 1$, $K_p = 3 \times 10^8$ M\textsuperscript{-1}, $k_2 = 0.012$ s\textsuperscript{-1} for the wild-type; $\phi = 1$, $K_p = 1.5 \times 10^8$ M\textsuperscript{-1}, $k_2 = 0.016$ s\textsuperscript{-1} for the UV5 mutant) (cf. Fig. 7).

(a) A plausible explanation for the change in occupancy values

We have assumed that the catalytic activity of RNA polymerase is proportional to the fraction of open complexes at the promoter. If this is so, the relative rates will depend neither on the nature of the enzymatic assay, nor on the concentration of substrates which are used. The second prediction was verified since Michaelis constants for ApA and UTP were found to be identical within experimental errors for the wild-type lac promoter in the presence as well as in the absence of CRP-cAMP (results not shown). A more direct measure of promoter occupancy relies on the quantitation of the number of transcripts produced from promoter-bound RNA polymerase when transcription was initiated following the addition of heparin. Such an approach has already been used by Maquat & Reznikoff (1978). In their study, the promoter occupancy measured in a single round of synthesis experiment directly paralleled the relative abortive initiation rates reported here. We cannot rule out the possibility that promoter mutations could affect the catalytic activity of RNA polymerase in some cases, but such effects appear to be small for the lac promoters we have examined.

It has also been shown that the lac control region contains two competitive overlapping promoters (Malan & McClure, 1984). The second promoter, P\textsubscript{2}, initiates transcription poorly. CRP-cAMP increased the occupancy of P\textsubscript{1} and coordinately decreased the occupancy of P\textsubscript{2} (Malan & McClure, 1984). We propose that competition between the two promoters is primarily responsible for the different final occupancies observed at lac promoters under different conditions.
We have therefore analyzed the present data by considering competing branched pathways of the type:

$$\begin{align*}
R + P & \xrightarrow{k_1} R_P \\
R_P & \xrightarrow{k_2} R_P'
\end{align*}$$

Here $K_1$ and $K_1'$ replace $K_B$ (association constants during the first step); $k_2$ and $k_2'$ define the isomerization rates for the two pathways.

(b) The mechanism of CRP-cAMP action

The CRP-cAMP complex increased $K_B$ without affecting $k_2$, regardless of whether the occupancy of the P1 promoter was maximal (in the L8UV5 or UV5 cases) or not (in the wild-type, F$^\lambda$ or L8 cases). In the simplest cases (namely, L8UV5 and UV5), scheme 1, the two-step model described above, is appropriate for our interpretation. CRP-cAMP activates RNA polymerase positioned at the correct site by affecting only the initial binding step.

In the case of the other lac promoters, the situation was more complex since occupancy at P1 increased when CRP-cAMP was present. We used scheme 2 to analyze this situation. The constants derived from the TAU plots are now apparent constants, which are called $\text{App}K_B$ and $\text{App}k_2$.

A complete quantitative analysis of the lac case is not yet possible because the current data are not extensive enough or, in some cases, sufficiently accurate to challenge the more detailed models considered in the Appendix. However, the following general conclusions can be made.

(i) In all these models, the closed complexes $R_P$ and $R_P'$ are assumed to be in rapid equilibrium with the free species. Under these conditions, $K_B$ evaluated from TAU plots is an apparent association constant, $\text{App}K_B$, that is simply the sum of the association constants $K_1$ and $K_1'$ related to each closed complex. $\text{App}K_B$ increases when CRP-cAMP is present, as the relative occupancy of the P1 promoter increases and as the P2 pathway is shut off. This can only occur if $K_1$ (the microscopic constant related to the P1 pathway and the term which becomes predominant as the activator is added) increases in the presence of CRP-cAMP. Hence, as in the UV5 case, CRP must affect positively the formation of the closed complex corresponding to the P1 pathway. In contrast, if there were no direct effect on binding, all of the competitive RNA polymerase binding models predicted a decrease in $\text{App}K_B$ due to CRP-cAMP (and an increase in $\text{App}k_2$).

(ii) Because the two paths are coupled, both forward rates, $k_2$ and $k_2'$, contribute to the lag observed when one measures the rate of the products synthesized at P1. As shown in the Appendix, the forward rate constant, $\text{App}k_2$, is a weighted average of the isomerization rate constants $k_2$ and $k_2'$ for P1 and P2. Thus, $\text{App}k_2$ can vary from $k_2'$ to $k_2$. 
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\[ \text{Appk}_2 = \frac{k_2 K_1 + k_2' K'_1}{\text{App} K_B} \]

and the relative occupancy at P1 measures the flux through the P1 pathway

\[ \phi = \frac{k_2 K_1}{k_2 K_1 + k_2' K'_1} \]

When most molecules are engaged in the P2 pathway (low value of \( \phi \)) \( \text{Appk}_2 \) is approximately equal to \( k_2' \); when the P1 occupancy is high, it tends towards \( k_2 \). Experimentally, we have shown that, upon addition of CRP-cAMP, \( \text{Appk}_2 \) does not change significantly. Hence \( k_2 \) should not be appreciably affected by the addition of CRP-cAMP. \( k'_2 \) should be approximately equal to \( k_2 \).

(iii) We therefore suggest that the CRP-cAMP complex acts directly to enhance the initial binding of RNA polymerase and indirectly to position RNA polymerase at the proper start site. The closed complexes formed in the presence of CRP-cAMP all lead to the P1 pathway.

(c) Role of mutations

The L8 mutation did not directly affect any kinetic characteristic of the pathway. It only resulted in a poorer binding of the CRP-cAMP complex, as expected. A total of 2-5 dimers of our preparation of CRP were required to fully activate the wild-type promoter. However, it has been shown recently (Garner & Revzin, 1982; Fried & Crothers, 1983; Kolb et al., 1983) that a single dimer of CRP binds in the -60 lac region, another one binding downstream at the operator site with a decreased (20 x) affinity. We must conclude that either the CRP protein was only 40% active or that a complex mechanism involving two CRP dimers is involved in activation. In a UV5 background, the L8 mutation did not have any effect on the kinetics of open complex formation, or the occupancy of the promoter.

Mutations in the -10 region (P\(^s\), UV5) only affected \( \text{Appk}_2 \) (cf. Fig. 7). The effect was stepwise, being more marked in the UV5 case. Furthermore, these mutations increased the relative occupancy of the P1 promoter even in the absence of CRP-cAMP. The increases in occupancy were also greater for UV5 than for P\(^s\). The simplest interpretation is that each of these base-pair changes results in an increase in \( k_2 \). The P1-P2 competition model then predicts the stepwise increase in occupancy.

(d) Role of negative supercoiling

Negative supercoiling affects the wild-type and the UV5 promoters in opposite ways. This result is in agreement with in vivo experiments. But the fact is obviously puzzling.

For the UV5 promoter, where the kinetic scheme is simple, the data can be analyzed semi-quantitatively. Since \( K_B \) is increased on the supercoiled template this means that some unwinding (in the topological sense) takes place at this step.
Quantitation of this angular change has been performed following the method proposed by Davidson (1972). The difference in free energy between a particular topoisomer and the relaxed DNA has been estimated according to the equation proposed by Pulleybank et al. (1975). An unwinding of one-third of a turn taking place during the formation of the closed complex on the UV5 promoter accounts for the experimental data reported here. This is a crude but minimal estimate since we cannot take into account the variation of the number of superhelical turns of the pBR322 template as other RNA polymerase molecules reach their target promoters on this vector.

A similar theoretical treatment can be applied to the variation of the isomerization rate constant with supercoiling density, assuming that the transition state leading to RP does not change. Since $k_2$ decreased with increases in supercoiling, this may mean that the DNA template rewinds. The magnitude of the change leads to an estimate of the rewinding of 0.1 of a helical turn.

In the case of the wild-type promoter, most of the unwinding takes place during the isomerization step since this rate constant is significantly affected by negative supercoiling. No quantititative treatment can be given here since the scheme is complex. A shift in relative promoter occupancy indicated that the P2 promoter is depopulated in favor of the P1 promoter, as if more unwinding was taking place along the P1 pathway than on the P2 path. The main conclusion is that CRP-cAMP and supercoiling act on two successive steps of the reaction pathway.

(e) Comparison between the in vitro properties of the lac promoters and the expression of the lac operon in vivo

Our in vitro results with the supercoiled lac promoter agree well with the properties of the promoter in vivo. The fully activated wild-type lac promoter is a very strong promoter in vivo. Estimates of the initiation frequency at the fully activated promoter range from one to ten RNA chains per minute (Contesse et al., 1970; Kennel & Riezman, 1977). The kinetic properties of the fully activated linear lac promoter in vitro predict an initiation rate of only 0.1 RNA chain per minute. However, $\tau_{obs}$ values measured for the fully activated supercoiled wild-type promoter in vivo correspond to an initiation frequency of approximately one RNA chain per minute, in good agreement with the in vivo data.

The supercoiled L8UV5 promoter also behaves as a strong promoter in vivo and in vitro, as illustrated by comparison with the wild-type promoter. In addition, the relative activities of the wild-type and L8UV5 promoters in vivo were most accurately reproduced in vitro when the promoters were supercoiled, in which case they functioned nearly identically. Sanzey (1979) showed that transcription from the fully activated wild-type lac promoter was inhibited fivefold in vivo by the addition of 100 µg nalidixic acid/ml, a drug that inhibits the activity of DNA gyrase. The $\tau_{obs}$ values measured in vitro at saturating concentrations of CRP-cAMP predict a fivefold increase in initiation frequency on the supercoiled template.

In contrast, the addition of nalidixic acid increased expression from the lac L8UV5 promoter approximately twofold (Sanzey, 1979). The $\tau_{obs}$ values measured
in vitro at concentrations of RNA polymerase greater than 20 nm also predict an increase in transcription from the L8UV5 promoter as the DNA is relaxed.

CRP-cAMP stimulates the wild-type lac promoter about 50-fold in vivo (Beckwith et al., 1972). In order to account for this effect, we must consider both the occupancy and the kinetic effects of CRP-cAMP. The occupancy effect on the supercoiled template would result in a fourfold indirect activation by CRP-cAMP. Since the effect of CRP-cAMP is primarily on the binding constant, the magnitude of the kinetic effect depends on the concentration of RNA polymerase in the cell; this value is not known. The behavior of several lambda and lac promoters in vitro is best reconciled with in vivo data by assuming a concentration of free RNA polymerase in the cell of approximately 15 to 60 nm (McClure, 1983). At this concentration of RNA polymerase, the TAU plot analysis predicts a three- to fivefold kinetic activation by CRP-cAMP. The overall CRP-cAMP activation is predicted to be 20-fold, compared to 50-fold in vivo. We can suggest several reasons for this discrepancy: (i) Our calculation does not account for the fact that RNA polymerase has a very long clearance time at P2, whereas the clearance time at P1 is very fast (for discussion see Malan & McClure, 1984); (ii) the in vivo RNA polymerase concentration may have been overestimated; (iii) our reaction conditions in vitro may not accurately reproduce the environment of the DNA in vivo; (iv) the torsional tension of our supercoiled DNA in vitro may be higher than the torsional tension of intracellular DNA (Pettijohn & Pfenniger, 1980; Sinden et al., 1980); (v) finally, other specific factors may contribute to CRP-cAMP activation.

(f) Structural implications

Our data do not suggest a molecular model for CRP-cAMP action, nor do they favor any of the published models. In addition, our data do not suggest whether the direct activation of P1 by CRP-cAMP takes place through a protein–protein interaction (Gilbert, 1976) or through an alteration in promoter structure (Dickson et al., 1975). However, these results do provide important functional information about CRP-cAMP action at the lac promoter and provide constraints which may be applied in judging future models of CRP action. In particular the dual function of CRP-cAMP to position RNA polymerase properly and to increase \( K_a \) for lac P1, suggests an explanation for the diverse positions of CRP-cAMP binding sites at several promoters. Both effects result in activation of lac transcription. In other operons it is possible that only the positioning effect is required for activation.

The mechanism of CRP-cAMP activation of the lac promoter is strikingly different from the activation at lambda PrM by lambda cI protein, the other well-characterized transcriptional activator. The principal effect of cI activation is to increase \( k_2 \) approximately tenfold (Hawley & McClure, 1982, 1983). Only small changes were observed in \( K_B \) or in maximum promoter occupancy. Both CRP-cAMP and cI affect only one step in open complex formation, but CRP-cAMP affects \( K_B \) while cI affects \( k_2 \). Clearly, positive control can be accomplished through a variety of mechanisms. The properties of the activator–RNA
polymerase–DNA interaction which determine the mechanism of activation are largely unknown and will be an interesting problem for further study.

We have shown that promoter strength can be studied in vitro with supercoiled templates using the abortive initiation reaction. Transcription experiments, which often rely on quantitation of run-off transcripts when performed on DNA fragments, are not practical to perform when the promoter being studied is inserted into a circular plasmid vector. In contrast, the same easily quantitated abortive initiation products are synthesized from the lac promoters when they are located on a DNA fragment or when inserted into supercoiled pBR322. In those cases where we can make a comparison, the properties of the lac promoter on a DNA fragment are nearly identical, when measurements using the abortive initiation reaction were compared to transcription experiments (Stefano & Gralla, 1982a,b). We believe that the results obtained using the abortive initiation reaction on the supercoiled lac promoter also accurately reflect the properties of the promoter.

Appendix

The lac promoter region contains two competitive, overlapping promoters (Malan & McClure, 1984). CRP-cAMP, promoter mutations, and supercoiling all affect both the final occupancies of the two promoters and the rate of open complex formation at the lac P1 promoter. A priori, both the final occupancies of P1 and P2 and the kinetics of open complex formation at P1 might be affected through a direct effect on the P1 promoter or through an indirect effect on P1 resulting from a change in the properties of P2. In order to distinguish between these possibilities, we have derived equations that relate observable parameters to the microscopic constants in several plausible models. This analysis has allowed us to suggest criteria for assigning a particular effect to a specific step in open complex formation at P1 or P2, and to propose a model for regulation of lac transcription. This analysis may also be applicable to the study of the regulation of the gal promoters and other dual promoter systems.

McClure (1980) has shown that open complex formation takes place in two discrete kinetic steps:

\[ R + P \rightleftharpoons R P + k_2 P_h \]

Usually, the first step is a rapidly reversible binding step. The second step is a slower isomerization of the kinetic intermediate (the closed complex, RPH). The reverse of the second step is very slow and may usually be neglected. In this case, the average time required for open complex formation \( \tau_{\text{obs}} \) is described by the equation:

\[ \tau_{\text{obs}} = \frac{1}{k_2 K_B[R]} + \frac{1}{k_2}. \]

Studies with the lac L8UV5 promoter and several other promoters support this two-step functional mechanism, at least at temperatures above 25°C (McClure,
unpublished results). TAU plots (in which \( \tau_{\text{obs}} \) is plotted versus reciprocal RNA polymerase concentration, according to equation (A2)) for lac P1 are linear and yield apparent values for \( K_B \) and for \( k_2 \) (App\( K_B \) and App\( k_2 \), respectively). However, these values are not the true microscopic constants for P1; they also reflect the presence and properties of P2. Since RNA polymerase binding at lac P2 results in anomalous TAU plot parameters (see below) and because this recently discovered promoter is somewhat less well characterized, we have considered the overall effects of different models of open complex formation at P2 on the rate and extent of open complex formation at both lac promoters. These models are considered in order of increasing complexity. The final model is consistent with all observed properties of the two lac promoters. These models also allow us to consider more generally the effects of RNA polymerase binding at sites overlapping with promoters, and of RNA polymerase exclusion by repressor molecules. Any final model describing P1–P2 competition must take into account the qualitative correlation between the rate of open complex formation at P1 and the final occupancy of P1, and the two distinct TAU plots for P1 and P2. On linear DNA, \( \tau_{\text{obs}} \) values measured at P2 were independent of RNA polymerase concentration and were much longer than the \( \tau_{\text{obs}} \) values measured at P1 over the range of RNA polymerase concentrations used.

**Model 1: Rapidly reversible single-step binding at P2**

We consider first the effect of an additional competitive site, at which RNA polymerase binds rapidly and reversibly, on the rate of open complex formation at the P1 promoter.

**Model IA**

\[
R + P \xrightarrow{k_r} R^P \xrightarrow{k_i} R^P_o
\]

In this scheme, \( R^P \) could represent many different sites of interaction between RNA polymerase and the promoter. If competitive binding takes place at multiple sites, \( K_B \) is the sum of the corresponding association constants.

A single relaxation time will be observed characterized by the equation:

\[
\tau^{-1} = k_{-2} + k_2 \frac{K_B[R]}{1 + (K_R + K'_R)[R]}.
\]  

(44)

If \( k_{-2} \) is negligible, as it is for many promoters,

\[
\tau = \frac{1}{K_B[R]k_2} + \frac{K_B + K'_B}{K_R k_2}.
\]

(A5)

The apparent isomerization rate, calculated from the intercept of the TAU plot, is given by:

\[
\text{App}k_2 = \frac{k_2 K_B}{K_B + K'_B}.
\]

(A6)
The apparent binding constant, calculated from the quotient of the TAU plot intercept and slope, is given by:

$$\text{App}K_B = K_B + K'_B.$$  \hfill (A7)

Thus, the introduction of such competitive binding sites results in a decrease in $\text{App}k_2$ and an increase in $\text{App}K_B$, their product remaining constant. This stronger binding takes place at the expense of efficient channeling toward the open complex, and the apparent isomerization rate is decreased accordingly.

**Model I B**

This case is kinetically indistinguishable from a case in which $RP'$ results from an isomerization of $RP_c$:

$$R + P \xrightleftharpoons{k_s}{k_2} RP_c \xrightleftharpoons{k_2}{k_{-2}} RP_0.$$  \hfill (A8)

These models display identical TAU plot effects because $P$, $RP_c$ and $RP'$ are all in rapid equilibrium in each case.

If $RP'$ were an open complex (e.g. the open complex for P2), and could be detected, it would form first and then decay as $RP_0$ is formed (characteristic relaxation time 2). The final relative occupancies of P1 ($\phi$) and P2 ($\phi'$) for the simple competitive models are given by:

$$\frac{\phi}{\phi'} = \frac{K_B k_2}{K'_B k_{-2}} \quad \text{or} \quad \frac{\phi}{\phi'} = \frac{k_2}{K' k_{-2}}.$$  \hfill (A9)

Model I can be ruled out in the case of the lac promoters because open complex formation at P2 is not rapid, but rather takes place via a long lag.

We have used this analysis to predict the effect of a repressor molecule on the kinetics of open complex formation, if the repressor binds rapidly and reversibly and prevents formation of the closed complex. In this case, the repressor does not alter $\text{App}k_2$, but it decreases $\text{App}K_B$ by a factor equal to $1 + K_i[I]$, where $[I]$ is the repressor concentration and $K_i$ its association constant to the operator.

**Model II: Competition between overlapping two-step promoters**

$$R + P \xrightleftharpoons{k_s}{k_2} RP_c \xrightleftharpoons{k_2}{k_{-2}} RP_0$$  \hfill (A10)

This model is a more realistic suggestion for lac P1–P2 competition, since both open complexes are formed with multi-step pathways. The above scheme is formally equivalent to the three-state system:
C'RP-CAMP ACTIVATION OF lac PI PROMOTER

\[ \text{RP}_0^\prime \xrightarrow{k_2} M \xrightarrow{k_k} \text{RP}_e. \]  

(A11)

where M is a pool of species in rapid equilibrium, and the rate constants, \( \lambda \), correspond to the overall rate constants for interconversion between the three principal species. For Model II,

\[ \lambda = \frac{k_2 K_B}{K_B + K_B + [R]^{-1}}, \quad \lambda_{-1} = k_{-2} \]

\[ \lambda' = \frac{k_2' K_B'}{K_B + K_B' + [R]^{-1}}, \quad \lambda'_{-1} = k'_{-2}. \]  

(A12)

The relaxation kinetics of this three-state system have been thoroughly investigated (Bernasconi, 1976). Two relaxation times should be observed. In general, the kinetic behavior of such a system is rather complex. However, two limiting cases have interesting properties and are worth considering.

**Limiting case IIA:** This case assumes that \( \lambda \) and \( \lambda_{-1} \) are much greater than \( \lambda' \) and \( \lambda'_{-1} \). This model is interesting because it predicts a longer \( \tau_{\text{obs}} \) at P2 than at P1. The characteristic feature of this case is that open complexes are first formed at P1 and then open complexes are formed at P2 as the P1 open complex dissociates. The first relaxation time is therefore detected by following open complex formation at P1. The initial lag in open complex formation at P1 is predicted, as in model I, by the equation:

\[ \tau_1^{-1} = k_{-2} + \frac{k_2 K_B [R]}{1 + (K_B + K_B') [R]}. \]  

(A13)

The second relaxation time characterizes open complex formation at P2 coupled with partial dissociation of the P1 open complex:

\[ \tau_2^{-1} = k'_{-2} + \frac{k_2' K_B' [R] k_{-2}}{1 + (K_B + K_B') [R]} \times \tau_1. \]  

(A14)

Assuming \( \tau_1^{-1} \gg k'_{-2} \)

\[ \tau_2^{-1} = k'_{-2} + \frac{k_{-2} k_2' K_B'}{k_2 K_B}. \]  

(A15)

This model predicts short, RNA polymerase concentration-dependent \( \tau_{\text{obs}} \) values for P1 and longer, RNA polymerase concentration-independent \( \tau_{\text{obs}} \) values for P2, both of which are observed in lac. However, it also predicts that P1 is fully occupied during the first step, the second step corresponding to a slow increase in P2 occupancy at the expense of P1. Neither of these two last predictions is observed in lac.

**Limiting case IIB:** This model assumes that \( \lambda \) and \( \lambda' \) are much greater than \( \lambda_{-1} \) and \( \lambda'_{-1} \). These assumptions correspond in part to the properties of the lac P1 and P2 promoters, since the rates of open complex formation at P1 and P2 are much faster than the rates of dissociation of the open complexes \( \text{RP}_0 \) and \( \text{RP}_e' \). The shortest relaxation time is equal to the reciprocal of the sum of the forward rate constants.


The rate associated with the second relaxation time is an average of the reverse rate constants given by:

$$\tau_2^{-1} = \frac{k_{-2}[k_2'K_B'] + k_{-2}'[k_2K_B]}{k_2'K_B' + k_2K_B}.$$  \hspace{1cm} \text{(A17)}$$

The first relaxation time corresponds to the initial formation of RP, and RPA.

TAU plots for P1 and for P2 would each measure $\lambda_1$ and should be identical. The apparent binding and isomerization constants are described by:

$$\text{App}K_B = K_B + K'_B,$$  \hspace{1cm} \text{(A18)}$$

$$\text{App}k_2 = \frac{k_2K_B + k_2'K_B'}{K_B + K_B'}.$$  \hspace{1cm} \text{(A19)}$$

While the apparent rate constants for P1 and P2 are identical, the absolute rates of open complex formation may be different. The ratio of occupancies of P1 and P2 after the first relaxation step is equal to the ratio of the absolute rates of formation of P1 and P2. The relative occupancies of P1 and P2 are given by:

$$\frac{\phi}{\phi'} = \frac{k_2K_B}{k_2'K_B'}.$$  \hspace{1cm} \text{(A20)}$$

Thus, during this first step, the distribution of RNA polymerase between P1 and P2 is determined by the relative second-order rate constants for open complex formation at P1 and P2.

The second relaxation time corresponds to a slow redistribution of P1 and P2 occupancy. The final equilibrium occupancy is given by:

$$\left[ \frac{\phi}{\phi'} \right]_e = \frac{k_2k_{-2}'K_B}{k_{-2}'k_2'K_B}.$$  \hspace{1cm} \text{(A21)}$$

The second relaxation time is not dependent on the concentration of RNA polymerase.

The kinetic behavior of the lac P1 and P2 promoters does not fit model II B either, since the first lag (relaxation time) is observed only through the formation of RP, at P1 while the second one manifests itself only at P2. During the first step P1 is poorly occupied while P2 is not yet active. The inclusion of an additional, stable intermediate complex in the pathway to open complex formation at P2 resolves this paradox. Although only two limiting cases of model II have been considered in detail, no limiting or intermediate case of model II predicts all of the observed properties of the lac promoters.

Model III: A model for promoter–promoter competition in the lac control region

The absence of P2 activity during the first relaxation time can be explained by assuming that open complex formation at P2 takes place in three steps:
The box enclosed by broken lines above corresponds to the pool of species in rapid equilibrium. These three species are the same as those in the model II and are designated as "M" in equation (A1I). The only difference between model II and model III is that in the latter case $R_{P}^{t}$ is formed by two isomerization steps following the formation of $R_{P}^{c}$. The first, relatively rapid, isomerization step results in the formation of the stable intermediate complex $R_{P}^{i}$. Since $k_{-1}$ is small, formation of $R_{P}^{i}$ essentially commits the RNA polymerase molecule to binding at $P_{2}$. The same rate and occupancy arguments apply to $R_{P}^{i}$ as to $R_{P}^{t}$ in model IIIB. $R_{P}^{i}$ should be formed with the same relaxation time as $R_{P}^{t}$. In addition, since the formation of $R_{P}^{i}$ commits RNA polymerase to binding at $P_{2}$, the occupancies of $P_{1}$ and $P_{2}$ are determined by the rate competition between the formation of $R_{P}^{t}$ and $R_{P}^{i}$. The occupancy expressions are therefore identical to those derived for $R_{P}^{t}$ and $R_{P}^{i}$ in model IIIB.

The second isomerization step is slower. The relaxation time for this step (estimated to be 30 min for $t_{obs}$ for $P_{2}$) should be approximately equal to $\frac{1}{k_{-1} + k_{-3}}$. This relaxation time is independent of RNA polymerase concentration.

This model also predicts a third relaxation time corresponding to a redistribution of $R_{P}^{b}$ and $R_{P}^{i}$. This redistribution is similar to that described for model IIIB. Independent measurements indicate that the rate constants for the dissociation of $R_{P}^{b}$ and $R_{P}^{i}$ are nearly identical and that both are slow ($t_{1/2} = 120$ min). Therefore, the final redistribution step should also be slow. In fact, in experiments involving very long times of incubation, we have observed a slow loss in $P_{1}$ activity ($t_{1/2} > 50$ min) at the wild-type promoter. No loss of activity is observed at the $LSUV5 P_{1}$ promoter in similar experiments. Perhaps this apparent loss of activity is actually the result of the predicted slow redistribution of RNA polymerase occupancy.

The three-step model proposed for open complex formation at $P_{2}$ is not without precedent. Buc & McClure (unpublished results) have found that a second intermediate in open complex formation at the $lacUV5$ promoter is detectable under certain conditions. Model III makes testable predictions about the behavior of the $lac P_{2}$ promoter. First, it predicts that the TAU plot for $P_{2}$ should be very flat, since $t_{obs}$ is dominated by the RNA polymerase concentration-independent term $\frac{1}{k_{3} + k_{-3}}$. Second, it predicts that formation of the putative stable complex $R_{P}^{c}$ might be detected using a template challenge technique. Model III
also predicts that the formation of RP₁ should take place with the same relaxation
time as the formation of RP₂.

The kinetic and occupancy effects of the L8UV5 mutation are consistent with
the predictions of model III. Since the UV5 mutation lies in the −10 region of P₁,
it is likely that this mutation simply increases k₂ for P₁. The magnitude of this
increase in k₂ is sufficient to quantitatively account for the increase in occupancy
resulting from the UV5 mutation.

We have presented several models describing competition for RNA polymerase
binding between two overlapping promoters (see Table A1 for summary). Two
important conclusions result from this analysis. First, it is impossible to prove the
existence of a second promoter by kinetic measurements alone. The existence of a
second RNA polymerase binding site must be independently demonstrated.
(Occupancy effects can suggest the existence of a second RNA polymerase binding
site, however.) Second, for models II and III, it is not possible to assign values to
each microscopic constant by measuring only the apparent binding constant, the
apparent isomerization rate, and the relative occupancies of the two promoters. It
is possible, however, to assign changes in the kinetic behavior of the two-promoter
system to changes in an individual microscopic constant, assuming that only one

\[
\text{Table A1}
\]

<table>
<thead>
<tr>
<th>Model</th>
<th>Step</th>
<th>( Appk₂ )</th>
<th>( AppK_B )</th>
<th>( \phi )</th>
<th>( \phi' )</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA</td>
<td>1</td>
<td>( \frac{k₂K_B}{K_B + K_B'} )</td>
<td>( K_B + K_B' )</td>
<td>0 → 1</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>1</td>
<td>( \frac{k₂K_B}{K_B + K_B' + k₋₂} )</td>
<td>( K_B + K_B' )</td>
<td>0 → 1</td>
<td>0</td>
</tr>
<tr>
<td>IIIB</td>
<td>1</td>
<td>( \frac{k₂K_B + k₃K_B'}{K_B + K_B'} )</td>
<td>( K_B + K_B' )</td>
<td>0 → ( \frac{k₂K_B}{k₃K_B + k₂K_B'} )</td>
<td>0 → ( \frac{k₂K_B}{k₂K_B + k₂K_B'} )</td>
</tr>
<tr>
<td>IIIB</td>
<td>2</td>
<td>( \frac{k₋₂k₂K_B' + k₋₂k₂K_B}{k₃K_B + k₂K_B'} )</td>
<td>–</td>
<td>( 1 \rightarrow \phi_e' )</td>
<td>( 0 \rightarrow \phi_e' )</td>
</tr>
<tr>
<td>III</td>
<td>1</td>
<td>( \frac{k₂K_B + k₃K_B'}{K_B + K_B'} )</td>
<td>( K_B + K_B' )</td>
<td>0 → ( \frac{k₂K_B}{k₂K_B + k₂K_B'} )</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>2</td>
<td>( k₃ + k₋₃ )</td>
<td>–</td>
<td>Unchanged</td>
<td>( \frac{k₂K_B'}{k₂K_B + k₂K_B' + k₃ + k₋₃} )</td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>( \frac{k₋₃k₂K_B' + k₋₃k₂K_B}{k₂K_B + k₂K_B'} )</td>
<td>Negligible</td>
<td>( \phi_e )</td>
<td>( \phi_e' )</td>
</tr>
</tbody>
</table>

The 3 models, the kinetic constants and the observable parameters are described in the text. The
occupancies of P₁ and P₂ are each expressed as a fraction between 0 and 1. \( \phi_e \) and \( \phi_e' \) refer to the final
equilibrium occupancies described in the text.

\* Assuming \( k₋₂ \) is much less than \( k₂K_B/(K_B + K_B') \).
\*\* \( \alpha \) is approximately equal to \( k₋₃/(k₃ + k₋₃) \).
Qualitative and quantitative criteria for assigning observable changes in promoter behavior to changes in individual rate constants

<table>
<thead>
<tr>
<th>Increase in $k_2$</th>
<th>App$k_2$</th>
<th>App$K_B$</th>
<th>$\phi$</th>
<th>Quantitative diagnostic remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increase in $k_2$</td>
<td>+</td>
<td>Unaffected</td>
<td>+</td>
<td>$Appk_2^{-1} = \frac{AppK_B}{k_2 K_B^{\prime}} (1 - \phi)$</td>
</tr>
<tr>
<td>Increase in $K_B$</td>
<td>+ for $k_2 &gt; k_2^{\prime}$</td>
<td>+</td>
<td>+</td>
<td>$AppK_B - K_B^{\prime} = k_2^{\prime} = \frac{k_2}{k_2^{\prime} - 1} (1 - \phi)$</td>
</tr>
<tr>
<td>- for $k_2 &lt; k_2^{\prime}$</td>
<td>$Appk_2^{-1} = k_2^{\prime} + \phi(k_2^{\prime} - 1)$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increase in $K_B$</td>
<td>for $k_2 &gt; k_2^{\prime}$</td>
<td>+</td>
<td>-</td>
<td>$AppK_B - K_B^{\prime} = k_2^{\prime} - \frac{1}{k_2^{\prime} - 1}$</td>
</tr>
<tr>
<td>- for $k_2 &lt; k_2^{\prime}$</td>
<td>$Appk_2^{-1} = k_2^{\prime} + (1 - \phi) (k_2^{\prime} - 1)$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These criteria were derived based upon the assumptions of model IIB, but the relationships between $\tau_{obs}$ at $P_1$, and $P_1$ occupancy also apply to model III. An isolated change in an individual microscopic constant results in a characteristic pattern of changes in observable parameters. A $+$ denotes an increase and a $-$ denotes a decrease in the indicated TAU plot parameter. The quantitative diagnostic remarks describe the quantitative relationships between $AppK_B$, $Appk_2$, and promoter occupancy as an individual microscopic constant is changed. Equations (18), (19) and (20) have been converted into relations which yield convenient linear plots between $Appk_2^{-1}$ versus $\phi$ or $AppK_B$ versus $\phi$. From these plots the individual constants can be estimated.

The effects of changes in individual constants on observable properties of the promoters are summarized in Table A2. It can be seen that a change in a particular microscopic constant results in a unique pattern of changes in App$k_2$, App$K_B$ and $\phi$. This simplistic analysis is limited, however, due to the limited accuracy of measurements of App$k_2$ and App$K_B$. For example, if $K_B^{\prime}$ is much larger than $K_B$, an increase in App$K_B$ may not be detected.

A more powerful approach, if feasible, would be to use a series of conditions (for example, a gradual change in the superhelical density of the template, or in the CRP-cAMP concentrations) over which promoter occupancies change significantly. If App$k_2^{-1}$ and $\tau_{obs}$ are then plotted versus occupancy, the shapes of the two curves specify which microscopic constant is changed. Table A2 lists equations specifying the quantitative relationships between $\tau_{obs}$ or App$k_2$ and $P_1$ occupancy. This approach allows a more quantitative, and more secure, evaluation of changes in microscopic constants. The use of a wide range of conditions makes detection of small changes in App$k_2$ and App$K_B$ more likely.

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