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# Kinetics of Transcription Initiation at *lacP1*

## MULTIPLE ROLES OF CYCLIC AMP RECEPTOR PROTEIN*

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**The cyclic AMP receptor protein (CRP) acts as a transcription activator at many promoters of *Escherichia coli*. We have examined the kinetics of open complex formation at the *lacP1* promoter using tryptophan fluorescence of RNA polymerase and DNA fragments with 2-aminopurine substituted at specific positions. Apart from the closed complex formation and promoter clearance, we were able to detect three steps. The first step after the closed complex formation leads to a rapid increase of 2-aminopurine fluorescence. This was followed by another rapid step in which quenching of tryptophan fluorescence of RNA polymerase was observed. The slowest step detected by 2-aminopurine fluorescence increase is assigned to the final open complex formation. We have found that CRP not only enhances RNA polymerase binding at the promoter, but also enhances the slowest isomerization step by about 2-fold. Furthermore, potassium permanganate probing shows that the conformation of the open complex in the presence of CRP appears qualitatively and quantitatively different from that in the absence of CRP, suggesting that contact with RNA polymerase is maintained throughout the transcription initiation.**

Most transcription activation occurs during the initiation process. Transcription initiation can be divided into a number of steps (1–4), and theoretically activation can occur by affecting any one of them. Recruitment of RNA polymerase (RNAP)[1] to a promoter is, of course, the first step in transcription initiation, which is followed by a series of isomerization steps. Despite recent availability of a great deal of structural information on RNA polymerase, elucidation of the process of open complex formation remains a major goal. It is difficult to obtain structural information about these intermediates as they are transiently populated during transcription initiation. Most of the information about the intermediates was obtained indirectly, and little is known about the intervening transition states. A direct method of studying the kinetics of isomerization steps would significantly help the dissection of the transcription initiation process.

The cyclic AMP receptor protein (CRP) can activate the transcription of a large number of operons of *Escherichia coli* (5, 6). To activate transcription, CRP binds to a specific DNA site located upstream from a promoter and interacts with RNAP. CRP activates transcription by using two different modes of RNAP interaction. In most cases, CRP activates transcription from a point about 40 bp upstream of the start of transcription, but in others it interacts from a point at least 6 turns of DNA helix upstream from the transcription start site. The interaction points between CRP and RNAP are different depending on whether CRP is activating from the 40-bp upstream site or the other site. CRP was first identified as a transcription activator of the *lac* operon, where CRP binds at a site located 61.5 bp upstream of the start of transcription. From this site, CRP interacts with the $\alpha$ subunit of RNAP and helps recruit RNAP to the promoter. Whether the sole function of CRP at the *lac* promoter is to help RNAP bind to the promoter remains to be seen.

The kinetics of transcription initiation by CRP at the *lac* promoter was examined by Malan *et al.* (7). By using abortive initiation studies, they found that CRP increased the $K_B$ of RNAP binding to the *lac* promoter but had no effect on the subsequent isomerization step. However, some later results suggested effects of CRP beyond the recruitment step, and hence we decided to take a more direct approach and measure the rates of different steps in isomerization by tryptophan fluorescence and fluorescence of the base analog 2-aminopurine (2-AP) incorporated into the DNA of the *lac* promoter. In this article we report kinetics of several isomerization steps and influence of CRP on each of them.

### EXPERIMENTAL PROCEDURES

*Preparation of DNA Templates Containing 2-AP*—Both normal and single 2-AP incorporated DNA templates were amplified by PCR. First, the ~1.1-kb *lac* operon fragment was amplified from *E. coli* MG1655 genomic DNA. By using this DNA fragment as the template with the synthetic oligodeoxynucleotides as forward primers (83 bases, Fig. 1), 778-bp DNA fragments (78 bp upstream sequence and 700 bp downstream sequence of the *lac* operon) containing the *lac* promoter were amplified. To abolish the activity of the *P2* promoter of *lac*, a mutation at −29C→T was introduced at the promoter (8).

*Separation of PCR DNA Fragment from PCR Mixture*—After 4-fold dilution by 1× TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA), the PCR mixture was loaded on a Resource Q column (Amersham Biosciences), which was pre-equilibrated with TE buffer. After loading, the column was washed with 20 ml of TE buffer. Then two gradients (the first from 0 to 0.5 M NaCl in 10 ml of TE buffer and the second from 0.5 to 0.65 M NaCl in 30 ml of TE buffer) were used to elute the PCR DNA fragment from the Resource Q column at 1 ml/min.

*Proteins*—RNAP was purified from *E. coli* K-12 cells (MG1655) as

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[1] The abbreviations used are: RNAP, RNA polymerase; 2-AP, 2-aminopurine; CRP, cyclic AMP receptor protein; dsDNA, double-stranded DNA.

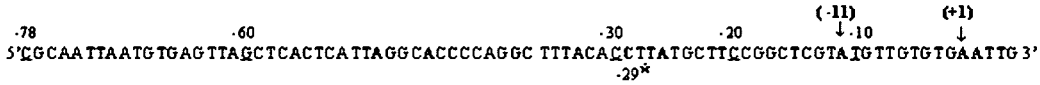


FIG. 1. **Positions of substitution of A to 2-AP at the *lac* promoter.** *29C→T abolishes *P2* promoter activity (8).

described (9). The purified RNAP was found to be >95% pure by Coomassie staining of protein resolved by SDS-PAGE. Protein concentration for RNAP was determined as described (10). CRP was purified to 98% homogeneity by fast protein liquid chromatography (Amersham Biosciences) from an *E. coli* strain carrying the wild type *crp* gene in the multicopy plasmid pHA5 (11).

*Transcription in Vitro*—All the *in vitro* transcription reactions in this study were carried out at 37 °C in 25 μl. At first, an initial mixture (10 nM DNA template, 40 nM RNAP, 1 mM ATP, 20 units of RNasin (Promega), 0.1 mM cAMP, 20 mM Tris acetate, pH 7.5, 5 mM magnesium acetate, 200 mM potassium acetate,1 mM dithiothreitol, with or without 50 nM CRP) was pre-incubated at 37 °C for 5 min, and then transcription was initiated by addition of 2.5 μl of NTP mixture (each 1 mM of GTP and CTP, 0.1 mM UTP, and 5 μCi of [α-$^{32}$P]UTP). The reaction was performed for 10 min before 25 μl of 2× stop buffer (80% formamide, 10 mM EDTA, 0.025% xylene cyanol, and 0.025% bromphenol blue) was added to terminate it. Samples were heated at 90 °C for 2 min, and 8 μl of each sample was loaded on a 6% sequencing gel to analyze the transcripts.

*Stopped-flow Kinetics*—The experiments were carried out on an Applied Photophysics SX18MV stopped-flow apparatus in the fluorescence mode. RNAP in buffer (50 mM Tris acetate, pH 7.5, 50 mM sodium acetate, 10 mM magnesium acetate, 0.1 mM cAMP, and 1 mM dithiothreitol) was loaded in one syringe, and the single 2-AP-labeled *lac* promoter DNA, either in the presence or absence of CRP, in the same buffer was loaded in the other syringe. The enzyme and DNA (50 μl each) were rapidly mixed from separate syringes at a flow rate of 6.0 ml/s. The excitation monochromator was set at 315 nm with a slit width of 4 mm, and a photomultiplier tube and a 360-nm cut-off filter (transparent to light with a wavelength greater than 360 nm) were used to measure the 2-AP fluorescence signal. For tryptophan fluorescence experiments, the excitation wavelength was set at 283 nm, and 305-nm cut-off filter (high pass) was used to measure the emission intensity. Experiments were performed at 25 °C. During the progress of the reaction, the time-dependent changes in the fluorescence emission intensity of 2-AP were recorded as 1000 data points. Multiple traces (10–15) were averaged for each experiment to optimize the signal.

*Promoter Clearance*—Promoter clearance experiments were carried out on the same stopped-flow instrument at 25 °C. RNAP was mixed with 2-AP containing DNA either in the presence or the absence of CRP and incubated at 25 °C for 30 min to ensure formation of an open complex (we previously checked that 30 min was sufficient for equilibrium to be established). Then the pre-incubated complex was rapidly mixed with a mixture of four NTPs (each concentration of 0.2 mM) and heparin (50 μg/ml). Measurements of the loss of 2-AP fluorescence signal were upon excitation at 315 nm and emission at ≥360 nm. Similarly, the kinetics traces were averaged from 10 traces.

*Kinetic Data Analysis*—SigmaPlot 2000 was used to analyze the stopped-flow kinetics traces, as reported previously (12). Assuming the kinetics would be pseudo-first-order, the traces were fit using the single exponential Equation 1,

$$F = A[1 - \exp(-k_{obs}t)] + C \quad \text{(Eq. 1)}$$

where $F$ is the fluorescence (observed at ≥360 nm) at time $t$ (seconds); $k_{obs}$ is the observed rate constant; $A$ is the observed amplitude; and $C$ is the fluorescence signal at $t = 0$. The pseudo-first-order rate constants thus obtained in different RNAP concentrations were plotted against RNAP concentration, and the corresponding plots were fit by a hyperbola equation (Equation 2) of non-linear regression analysis in SigmaPlot 2000.

$$k_{obs} = k_2[\text{RNAP}]/(K_d + [\text{RNAP}]) + k_{-2} \quad \text{(Eq. 2)}$$

*Measurement of the Rate by Manual Mixing*—The reaction was carried out on a RTC-2000/S.E. spectrofluorometer from Photon Technology International. Samples were excited at 315 nm (10 nm slit width), and the emission spectra of 370 nm (10 nm slit width) were measured. Prior to the measurement, the temperatures of all the reaction components and instruments were equilibrated at 25 °C. The fluorescence measurement and reaction were executed at 25 °C. Both RNAP and DNA samples were prepared in the buffer as used in stopped-flow assays. The complex formation between the promoter and RNAP was initiated by mixing 50 μl of DNA (70 nM) and 50 μl of RNAP (800 nM). Immediately after the mixing (time 0), the mixture was transferred to a cuvette (Hellma cells, 0.300). As a control, 50 μl of the same DNA was mixed with 50 μl of the buffer. The emission spectra from both samples were recorded every 3 s for more than 300 s.

*Permanganate Probing*—The experiments followed a modification of the protocol described previously (13, 14). Promoter DNA was incubated with RNAP either in the presence or absence of CRP. Samples were then treated with potassium permanganate, which modified thymine residues in single-stranded or distorted regions of the duplex. In the experiments described here, each 100-μl reaction mix contained 20 nM DNA (778-bp PCR fragment) and 200 nM RNAP either in the presence or the absence of CRP (250 nM) in transcription buffer (as above). After incubation at 37 °C (or 25 °C) for 30 min, $KMnO_4$ was added to a final concentration of 12.5 mM. After 2 min, the reaction was quenched by adding 2-mercaptoethanol to 1 M. The DNA was purified by phenol/chloroform/isoamyl alcohol (25:24:1) extraction and passage through a Sephadex G-25 spin column. $^{32}$P-Labeled primer (16-mer) was added, and the sample was heated for 4 min at 90 °C to denature the template DNA and then cooling at 45 °C for 15 min to hybridize. After extension with Klenow DNA polymerase, samples were electrophoresed on an 8% sequencing gel.

## RESULTS

*Positional Effect of 2-AP on the lac Promoter Activity in Transcription in Vitro*—A single 2-AP base was chemically incorporated separately at position −11 or +1 of the *lac* promoter in the non-template strand in place of the adenine base (Fig. 1). To study the effect of 2-AP on the function of the *lac* promoter, the six 778-bp PCR fragments, either with 2-AP at a different position or without 2-AP, were used as the templates for transcription *in vitro* either in the presence or the absence of CRP. As shown in Fig. 2, when a single 2-AP is located at position +1 of the *lac* promoter, compared with normal adenine-containing promoter (Fig. 2, *Control*), no obvious effect of 2-AP could be obtained on transcription *in vitro*, and CRP stimulated the transcription about 5–8-fold (transcript was quantified with a PhosphorImager, data not shown).

*2-AP Destroyed the lac Promoter Activity When Located at Position −11 of the lac Promoter*—The fluorescent base 2-AP is an isomer of normal adenine. It is believed that replacement of normal adenine by 2-AP would not seriously affect the structure of DNA, and 2-AP can be used as a non-perturbing and very sensitive probe to detect conformational changes (14–19). But in our studies, when 2-AP was located at position −11 of the *lac* promoter, in the absence of CRP, the promoter activity was lost both in transcription *in vitro* (Fig. 2) and in stopped-flow kinetics experiments (Fig. 3*A*). In transcription *in vitro*, no obvious *lac* RNA band was seen in the absence of CRP. However, note that CRP could restore activity to the debilitated promoter. In stopped-flow kinetics, no obvious time-dependent increase in 2-AP fluorescence change was obtained in the absence of CRP (Fig. 3*A*). As the control, the DNA template with 2-AP labeled at position +1 of the *lac* promoter indicated a clear signal change (Fig. 3*B*). In a previous article (20), it was observed that substitution of 2-AP at the −11 position blocked enhancement of permanganate activity to all thymines in the promoter region in *galP1*. The binding of RNA polymerase as measured by electrophoretic mobility shift assay, however, appeared to be preserved (20). These results suggest that 2-AP substitution at −11 leads to a very early block in the pathway, perhaps immediately after the closed complex formation.

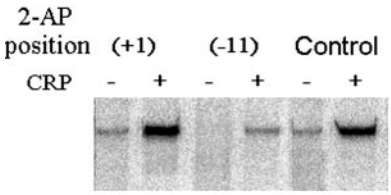


FIG. 2. **The effect of 2-AP labeling on the *lac* promoter activity on transcription *in vitro*.** The templates (778 bp) were made by PCR amplification as described under "Experimental Procedures." Substitution of position −11 from A to 2-AP almost totally abolished promoter activity, but there was no obvious effect of the 2-AP replacement at position +1.

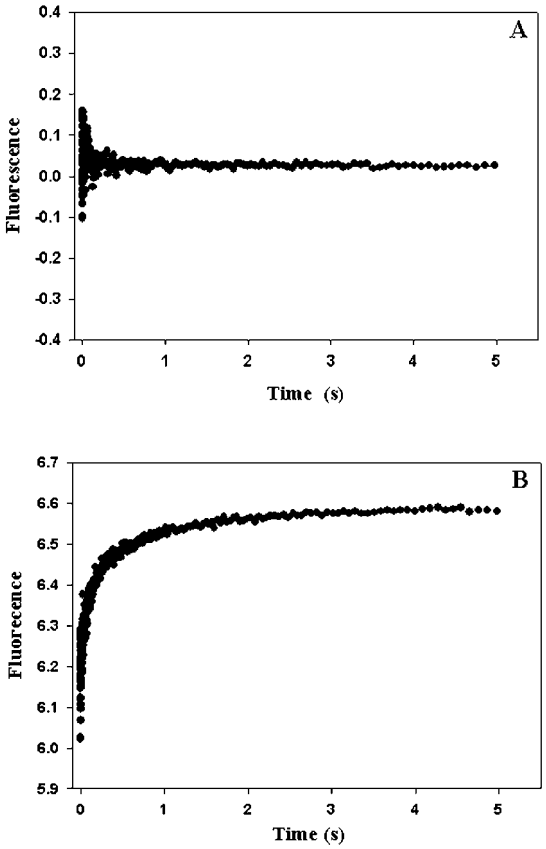


FIG. 3. **2-AP destroyed the promoter activity when located at position −11 of the *lac* promoter.** The interaction between RNA polymerase (100 nM) and 778-bp PCR-amplified 2-AP-containing *lac* promoter DNA (100 nM) was followed by 2-AP fluorescence change (excitation at 315 nm and emission at ≥360 nm) at 25 °C in a stopped-flow system. Shown is the time course. *A*, 2-AP located at position −11 of the *lac* promoter. *B*, 2-AP located at position +1 of the *lac* promoter.

*Studies of the Effect of CRP on Open Complex Formation by Stopped-flow Kinetics and Manual Mixing*—We used 2-AP fluorescence changes upon DNA strand separation to measure the kinetics of DNA binding and formation of open complex in a fluorometer equipped with stopped-flow capability. A 2-AP-modified and PCR-amplified DNA in which the 2-AP was located at position +1 of the *lac* promoter was chosen to use in this stopped-flow kinetics study. The representative kinetic traces are shown in Fig. 4, in which DNA (35 nM) was mixed with RNAP (550 nM) either in the absence (Fig. 4*A*) or presence of CRP (Fig. 4*B*). The fluorescence was excited at 315 nm and measured after passage through a 360-nm cut-off filter; 1000 points were accumulated over the 500-ms time course of the experiment. The data set fits very well in a single exponential, three-parameter equation, as described under "Experimental Procedures." An observed rate constant ($k_{obs}$) was obtained from

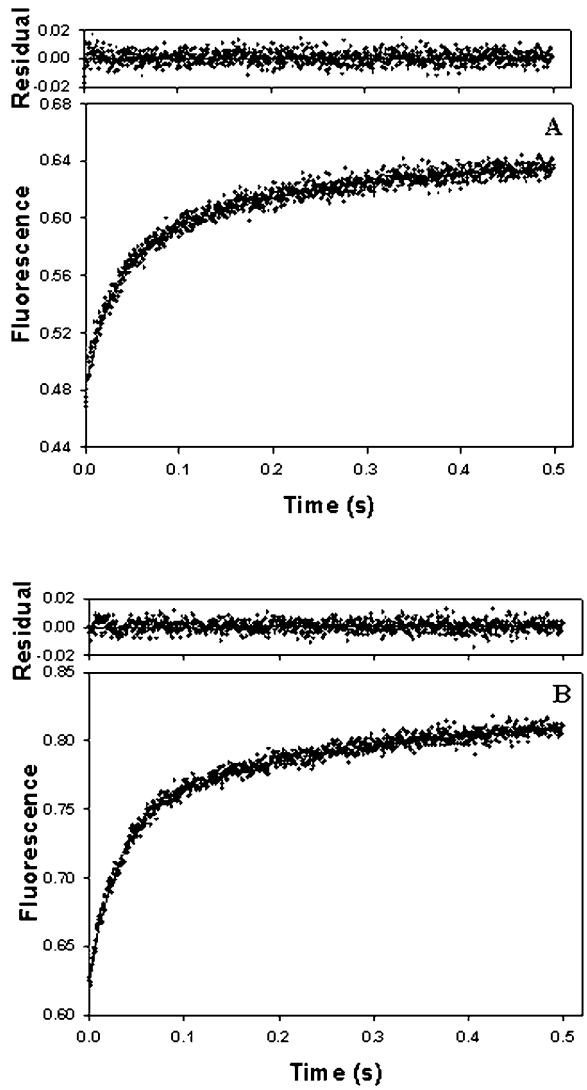


FIG. 4. **Time dependence of the increase in fluorescence upon rapid mixing of RNAP with the 2-AP-labeled *lac* promoter with or without CRP.** Reaction mixture contained 35 nM DNA (778-bp PCR-amplified fragments), 0.1 mM cAMP, 50 mM sodium acetate, 10 mM magnesium acetate, 50 mM Tris acetate, pH 7.5, 1 mM dithiothreitol, and 550 nM RNAP. The fluorescence at λ ≥360 nm was monitored as a function of time with continuous excitation at 315 nm. The kinetic traces obtained under pseudo-first-order conditions from an average of 10 shots in each experiment were fitted to a single exponential, $F = A(1 - \exp(-k_{obs}t)) + C$, which provided a $k_{obs} = 12.39\ s^{-1}$ in the absence of CRP (*A*) and a $k_{obs} = 13.67\ s^{-1}$ in the presence of CRP (*B*). The residuals from the fit are shown at the top.

the curve drawn through the data points. At 550 nM RNAP, CRP had minor effect on the second step, increasing the rate constant slightly from 12.39 ($s^{-1}$) (Fig. 4*A*) to 13.67 ($s^{-1}$) (Fig. 4*B*).

The rate of complex formation at 25 °C on the 2-AP containing template (35 nM) as a function of the RNAP concentration (200–1000 nM) either in the presence or in the absence of CRP is shown in Fig. 5. The corresponding plots fit very well to a hyperbolic equation, suggesting there are at least two steps in the DNA binding pathway, and two *ED* complexes are formed sequentially as shown in Equation 3,

$$E + D \underset{K_d}{\rightleftharpoons} ED_c \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} ED_1 \qquad \text{(Eq. 3)}$$

where *E* is the RNAP; *D* is the dsDNA promoter; $ED_c$ is the closed complex; and $ED_1$ is the intermediate complex. $K_d$, $k_2$, and $k_{-2}$ are the kinetic constants for the above reaction. $K_d$

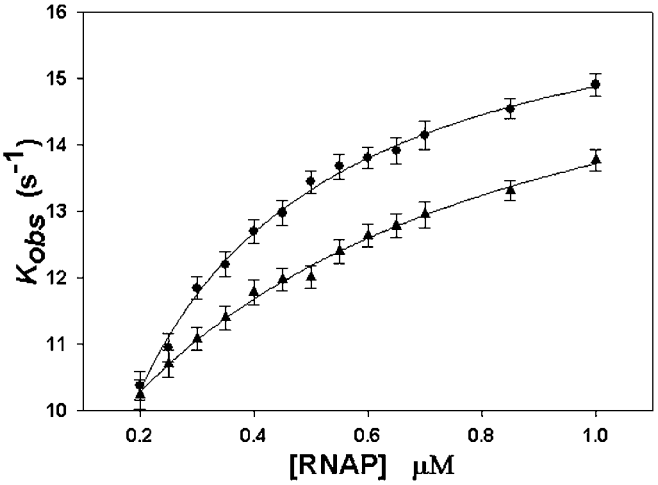


FIG. 5. **Dependence of observed rate constants ($k_{obs}$) of fluorescence increase obtained from stopped-flow experiments at the *lac* promoter on the concentration of RNAP.** The *error bars* represent standard deviations determined from averaging 10 shots at each RNAP concentration. The dependence of $k_{obs}$ on RNAP concentration was fit to the hyperbolic equation $k_{obs} = k_2$ [RNAP]/($K_d$ + [RNAP]) + $k_{-2}$. ▲, without CRP; ●, with CRP.

represents the equilibrium dissociation constant for RNAP ($E$) on the promoter ($D$) that forms the closed complex ($ED_c$); $k_2$ and $k_{-2}$ represent the rate constants for the isomerization reaction and reverse reaction, respectively. The values of the kinetic constants (Table I) were obtained by fitting the dependence to the hyperbolic Equation 2.

As shown in Fig. 5 and Table I, CRP increases RNAP binding on the *lac* promoter about 4-fold, enhances the rate of the immediate isomerization step about 1.7-fold, and decreases the rate of the reverse step reaction about 4.5-fold. The stopped-flow experiments were repeated with unlabeled template while monitoring the tryptophan fluorescence of RNA polymerase. There is a quenching of tryptophan fluorescence, which can be fitted to a single exponential. This rate is significantly slower than 2-AP fluorescence increase rate, suggesting that this step follows the step in which 2-AP fluorescence increase occurs (Fig. 6 and Table I)

We cannot estimate rate constant accurately through a longer time course on the stopped-flow instrument because of bleaching or drift problems occurring at extended times. So 2-AP fluorescence increase at the longer time course was monitored in a manual mixing experiment by a spectrofluorometer. The fluorescence increase occurs over a time frame of minutes indicating a slow step at which a further increase of 2-AP fluorescence occurs. The fluorescence increases both in the absence and in the presence of CRP and can be fitted to a single exponential. The obtained rate constants are $1.75 \times 10^{-3}$ and $3.04 \times 10^{-3}$ $s^{-1}$, in the absence and the presence of CRP, respectively (Fig. 7). This is very likely to be the step that involves formation of open complex for two reasons. 1) After about 5 half-lives of the reaction, addition of NTPs causes promoter clearance as assayed by quenching of 2-AP fluorescence. 2) The rate constant of this step is very similar to that obtained from abortive initiation data by Malan *et al.* (7) ($3 \times 10^{-3}$ $s^{-1}$). The relative amplitudes are estimated to be about 44% of total fluorescence change for the fast phase and 56% of total fluorescence change for the slow phase. These values were obtained from the extended time stopped-flow experiments, in which we used the template with 2-AP labeled at position +1 and a control experiment to correct for the machine drift and bleaching (data not shown).

*No Effect of CRP on Promoter Clearance*—As an assay for promoter clearance, we also used the stopped-flow apparatus to measure the rate of promoter annealing upon addition of four NTPs to preformed open complexes. The open complex has a higher fluorescence than the free dsDNA. After RNAP was cleared from the promoter, a time-dependent decrease in fluorescence was observed either in the presence or the absence of CRP (Fig. 8). By using the single exponential Equation 1, two similar values of $k_{obs}$ were obtained either with CRP or without CRP, suggesting no effect of CRP on promoter clearance. But the observed amplitude is different. In the presence of CRP, $A = 0.052$ (Fig. 8*B*); in the absence of CRP, $A = 0.041$ (Fig. 8*A*), indicating more open complex available in the presence of CRP.

*Using Permanganate to Detect Open Complexes*—Potassium

TABLE I
*Kinetics constant for interaction of RNAP and the lac promoter*

| Rate constants | +CRP | −CRP |
|---|---|---|
| $K_d$ (μM)[a] | 0.15 ± 0.03 | 0.56 ± 0.09 |
| Determined by stopped-flow | | |
| $k_2$ ($s^{-1}$) | 15.14 ± 0.14 | 9.10 ± 0.23 |
| $k_{-2}$ ($s^{-1}$) | 1.78 ± 0.16 | 7.86 ± 0.32 |
| $k_3$ ($s^{-1}$)[b] | 2.5 | 3.3 |
| Determined by manual mixing | | |
| $k_4$ ($s^{-1}$)[b] | $3.04 \times 10^{-3}$ | $1.75 \times 10^{-3}$ |

[a] The dissociation constant was calculated from RNA polymerase concentration dependence of 2-AP fluorescence increase in the stopped-flow experiment.
[b] See Equation 6 for definition.

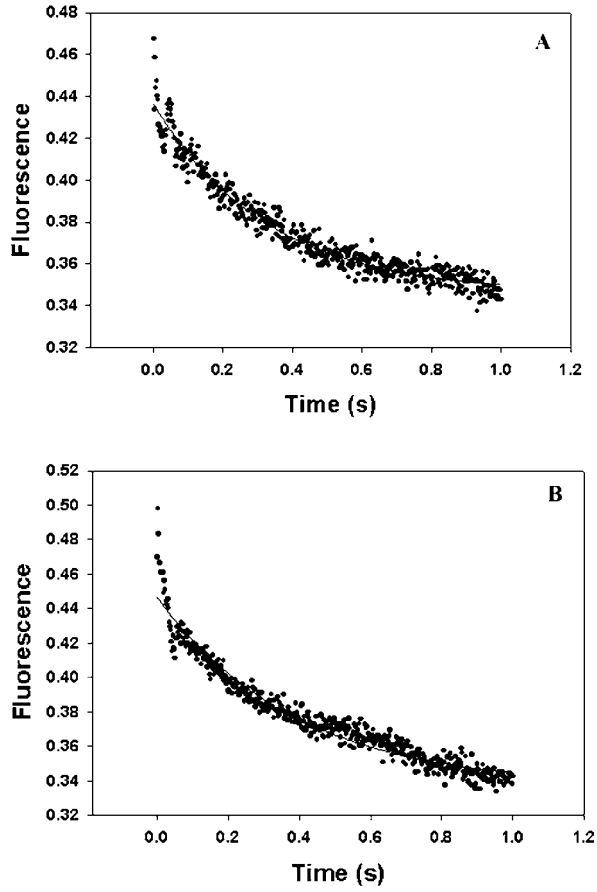


FIG. 6. **Time dependence of fluorescence quenching upon rapid mixing of RNAP with the unlabeled *lac* promoter with or without CRP.** Reaction mixture contained 35 nM DNA (778-bp PCR-amplified fragments), 0.1 mM cAMP, 50 mM sodium acetate, 10 mM magnesium acetate, 50 mM Tris acetate, pH 7.5, 1 mM dithiothreitol, and 500 nM RNAP. The fluorescence at λ ≥305 nm was monitored as a function of time with continuous excitation at 283 nm.

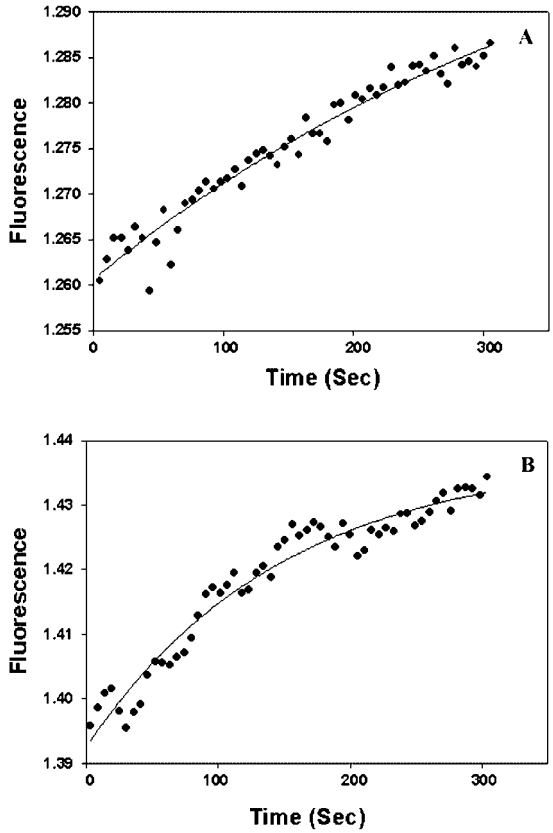


FIG. 7. **Fluorescence increase of +1 2-AP-labeled promoter DNA upon manually mixing with RNA polymerase in the absence (*A*) and in the presence (*B*) of CRP.** Samples were excited at 315 nm (10 nm slit width), and the emission spectra of 370 nm (10 nm slit width) were measured at 25 °C. Experimental details are described under "Experimental Procedures."

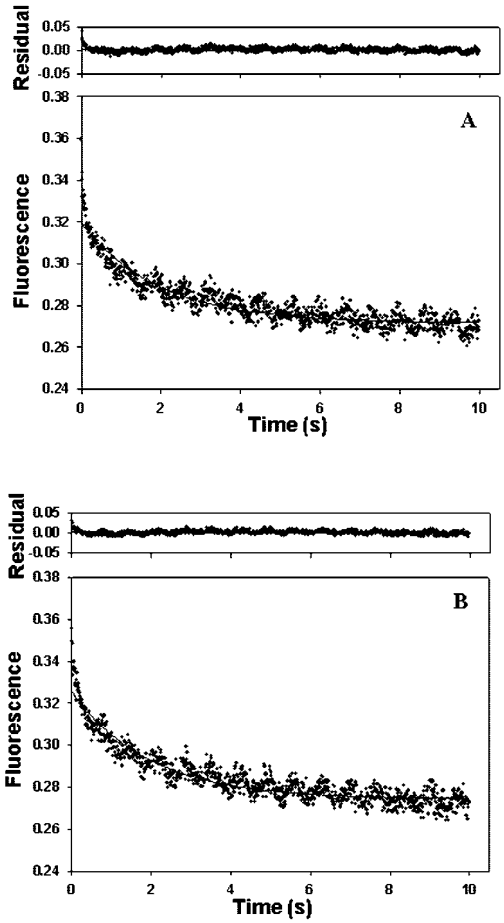


FIG. 8. **Time-dependent loss of the fluorescence signal upon mixing NTPs with preformed open complexes without or with CRP.** The preformed open complex (35 nM DNA (778-bp PCR-amplified fragments), 500 nM RNAP ± 250 nM CRP) was reacted with a mixture of NTPs at 200 $\mu$M each and 50 $\mu$g/ml heparin at 25°C. The fluorescence was excited at 315 nm and measured after passage through a 360-nm cut-off filter. One thousand data points were accumulated over the 10-s time course of the experiment. The kinetic traces from an average of 10 shots in each experiment were fit to a single exponential $F = A\ (1 - \exp(-k_{obs}t)) + C$. *A*, without CRP, the $k_{obs}$ is 0.5100 $s^{-1}$. *B*, with CRP, the $k_{obs}$ is 0.5198 $s^{-1}$.

permanganate ($KMnO_4$) has been shown previously (21) to oxidize pyrimidine residues, and the oxidation preferentially targets thymine residues in single-stranded DNA or distorted regions of the duplex. Therefore, permanganate probing is an efficient tool to detect various transcription complexes and study the mechanism of transcription factors (13).

The primer extension pattern obtained when $KMnO_4$ was used to modify the *lac* promoter either in the presence or the absence of CRP is shown in Fig. 9. Specifically, bands at −11, +1, +13, +15, and +16 along the bottom strand (Fig. 9*A*) become reactive both in the presence of RNAP and RNAP + CRP, but the intensities of these bands in the presence of CRP (Fig. 9*A*, *lane 7*) are about 2–8-fold for those without CRP (Fig. 9*A*, *lane 6*). The thymine residue at +2 appeared to be specifically reactive when CRP was present. In the top strand (Fig. 9*B*), compared with the control (Fig. 9*B*, *lane 5*), the thymine residues at −36, −35, −34, −28, −8, −7, −3, +3, and +4 exhibited hyperreactivity to permanganate either in the presence (Fig. 9*B*, *lane 7*) or the absence of CRP (Fig. 9*B*, *lane 6*), but the stops at these positions are stronger in the presence of CRP. In addition, bands at −29, −27, −22, −21, −15, −12, −10, and −5 occurred specifically or much more strongly in the presence of CRP. A similar result has been obtained at 25 °C (Fig. 9*C*). The results suggest that, in the presence of CRP, more transcription complexes are formed, and the conformation of those complexes might be different from those formed in the absence of CRP. The conformation of the promoter DNA might be changed by binding of CRP, which resulted in some of the bases becoming more easily accessible to $KMnO_4$.

## DISCUSSION

We have re-examined the kinetics of transcription initiation of the *lac* promoter with and without CRP using 2-AP fluorescence increase kinetics, a direct measure for binding and isomerization. We emphasize here that fluorescence increase amplitudes of the steps are functions of quantum yields of the initial and final states, which may be affected by presence of CRP. Hence, it is difficult to relate the amplitudes to reaction progress or structure. The base analog 2-AP substituted for adenine at +1 position in the *lac* promoter without affecting promoter activity. The exception was substitution at −11 where the *lac* promoter activity was completely obliterated. Lim *et al.* (20) found the same phenomenon when substituting for the adenine at −11 of the *gal* promoter. The (+1) 2-AP-substituted template was used for the detailed kinetic study here. This template was used for stopped-flow experiments to obtain information about the binding step and an early isomerization step. Manual mixing experiments were used to obtain information about a slower step. Tryptophan fluorescence of RNA polymerase was used to obtain information about another early isomerization step, probably involving major conformational change in the polymerase. Finally, by addition of NTPs after open complex formation we were able to assay the promoter clearance step.

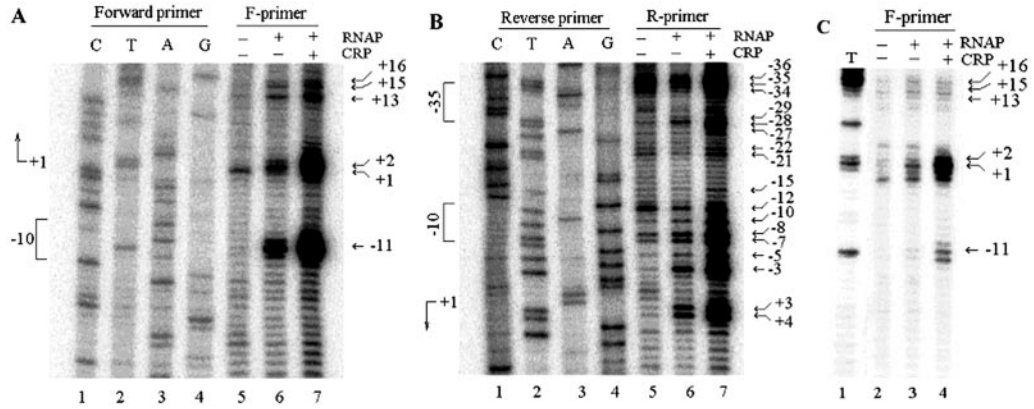


FIG. 9. **$KMnO_4$ probing of the region of the *lac* promoter opened by RNAP in the presence or the absence of CRP.** *A*, primer extension analysis of the bottom strand at 37 °C. *B*, primer extension analysis of the top strand at 37 °C. *C*, primer extension analysis of the bottom strand at 25 °C. *Lanes 1–4* in *A* and *B* and *lane 1* in *C* are the sequences of the *lac* promoter; *lanes 5–7* in *A* and *B* and *lanes 2–4* in *C* represent the determination of the positions of modification of the promoter without protein, with RNAP, and with both RNAP and CRP, respectively. Positions of the modification are labeled. The DNA template used is 778-bp PCR-amplified fragments.

By using (+1) 2-AP-substituted *lac* promoter DNA, we were able to measure the dissociation constant for the closed complex formation, which is $5.6 \times 10^{-7}$ M in the absence of CRP. Record and co-workers (22) have determined a value of $3.7 \times 10^{-8}$ M at 28 °C for the λ-$P_R$ promoter under similar conditions. In the presence of CRP, the dissociation constant reduces about 4-fold to $1.5 \times 10^{-7}$ M. Clearly, CRP strongly augments the recruitment step, and in its presence the dissociation constant becomes comparable with that of a strong promoter like λ-$P_R$. Malan *et al.* (7) obtained $K_B$ values for the *lacP1* from abortive initiation data at 37 °C, which are $\sim 7 \times 10^{-8}$ and $5 \times 10^{-9}$ ($K_B^{-1}$), without and with CRP, respectively. These values differ from our current estimate by approximately an order of magnitude. The two sets of values are difficult to compare as our templates contained T29C mutation and was conducted in 25 °C. In addition, no error estimate was given for the kinetic parameters obtained by abortive initiation. Because our values are derived by a direct assay on a template containing a *lacP2*$^-$ mutation, it is likely to be more reliable.

The stopped-flow experiments also indicate the presence of a rapid isomerization step in which the fluorescence of 2-AP increases with a forward rate constant of 9.1 and 15.1 $s^{-1}$, without and with CRP, respectively. When the stopped-flow experiments were followed by tryptophan fluorescence of RNA polymerase, we observe a rapid quenching of tryptophan fluorescence with a rate constant of 2.4 and 1.76 $s^{-1}$, with and without CRP, respectively. A fourth step can be measured from increase in 2-AP fluorescence by manual mixing experiments, which is assigned to the attainment of the catalytically competent open complex. The rate constants for this step in the absence and in the presence of CRP are $1.75 \times 10^{-3}$ and $3.04 \times 10^{-3}$ $s^{-1}$, respectively. A complete kinetic scheme is shown in Equation 4.

$$E + D \underset{K_d}{\rightleftharpoons} ED_c \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ED_1 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ED_2 \overset{k_4}{\rightleftharpoons} ED_0 \qquad \text{(Eq. 4)}$$

The most interesting part of the kinetic profile of open complex formation at the *lac* promoter is the detection of an early step in which an increase of 2-AP fluorescence occurs. It is generally believed that fluorescence of 2-AP is significantly quenched in the stacked conformation within B-DNA and some deformation of the double helix is a pre-condition for relief of the quenching. This would suggest a significant deformation of the double helix, at least around (+1), occurs at a very early step of the pathway. Based on current structural data it has been proposed that a significant DNA distortion around the −10 region is necessary for binding of polymerase to both upstream and downstream regions during the early part of the pathway (22). It is possible that this distortion is propagated to the +1 region. It is interesting that the substitution of 2-AP at −11 leads to a very early block. Recent work from deHaseth and co-workers (23) suggests that 2-AP at that position may be immobilized in an RNA polymerase pocket. One possibility is that flipping of −11 adenine and interaction with RNA polymerase is necessary for the DNA distortion that is blocked by −11 2-AP substitution. However, other models of early promoter-polymerase interaction may explain this early conformational change in the promoter DNA as well.

The potassium permanganate results show that there are increases in reactivity in the presence of CRP and RNAP compared with RNAP alone. Importantly, the differences are not only in magnitude but show that bases at −11, −3, −5, +1, +2, +3, and +4 have disproportionately more reactivity in the presence of CRP and RNAP. There are changes detected by the potassium permanganate probing in between the −10 and the −35 seen in the presence of CRP that are not seen in its absence. Thus, the increase in band intensities are not merely due to the presence of a greater amount of open complex in the population but likely to be due to CRP-polymerase contact preserved in the open complex. Several authors including Straney *et al.* (24) found stabilization of the open complex at *lac* by CRP; our results are consistent with theirs. Previously, we have proposed that activators that increase the $K_B$ act by establishing contact between the activator and polymerase during the first ternary complex, and this contact may be preserved throughout or may even be strengthened (25). This appears to occur in CRP activation of the *lacP1* promoter. $ED_c$ is stabilized relative to the unbound form, and $ED_1$ is also stabilized relative to the $ED_c$ form indicating further enhancement of CRP-polymerase interaction. Although we do not know if any further stabilization occurs, the great deal of enhancement of permanganate sensitivity cannot rule that out. It is also instructive that CRP can partially overcome the block that results from substitution of −11 adenine by 2-AP, consistent with a broad role in the isomerization process.

A previous report (7) on measuring the kinetic parameters of transcription initiation at the *lac* promoter found that CRP affected only the binding of RNAP to the promoter. In our study, we have found that at 25 °C CRP indeed increases the binding of RNAP to the *lac* promoter but also affects the promoter isomerization step. The effect of CRP on RNAP binding is to decrease the $K_d$ value about 4-fold. The effect of CRP on

the slowest step of isomerization is about 2-fold. However, a great deal of stabilization of the open complex occurs in the presence of CRP. A simulation with kinetic parameters suggests that these multistep changes may lead to a total 15–20-fold enhancement of promoter activity. Thus, it is possible now to account for a large part of the 40-fold activation level obtained in *in vivo* stimulation. Because all of our studies were conducted at 25 °C, in a strict sense the mechanism is valid at 25 °C.

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