

# Asynchronous basepair openings in transcription initiation: CRP enhances the rate-limiting step

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**The mechanism of isomerization (basepair openings) during transcription initiation by RNA polymerase at the *galP1* promoter of *Escherichia coli* was investigated by 2-aminopurine (2,AP) fluorescence. The fluorescence of 2,AP is quenched in DNA duplex and enhanced when the basepair is distorted or deformed. The increase of 2,AP fluorescence was used to monitor basepair distortion at several individual positions in the promoter. We observed that basepair distortions during isomerization are a multi-step process. Three distinct hitherto unresolved steps in kinetic terms were observed, where significant fluorescence change occurs: a fast step with a half-life of around 1 s, which is followed by two slower steps occurring with a half-life in the range of minutes at 25°C. Contrary to commonly held expectations, basepairs at different positions opened by 2,AP assays without any obvious pattern, suggesting that basepair opening is an asynchronous multi-step process. cAMP·CRP, which activates transcription at *galP1*, enhanced the rate-limiting step.**

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## Introduction

Both activation and repression of transcription of initiation are most often achieved by DNA-binding gene-regulatory proteins (activators and repressors), which act by modulating the activity of RNA polymerase through contact(s). Such contacts may involve a single activator or repressor bound to a specific DNA site at or near the promoter, as in many prokaryotic genes (Gralla and Collado-Vides, 1996), or one or more components of a conglomerate of proteins assembled at a DNA site that could be kilobase pairs away from the promoter, as is frequently found in eukaryotic systems (Ptashne and Gann, 1997; Carey, 1998). In the latter

examples, the protein–protein contact between a DNA–multi-protein complex and RNA polymerase results in a DNA loop. The core question, which remains unresolved, is how such physical contacts transpire into transcription regulation at the level of RNA polymerase binding, or a later step. A common concept assumes that the contact affects transcription initiation by modifying the structure of RNA polymerase allosterically. Although allosteric mechanisms may contribute to the regulatory processes, a regulator–RNA polymerase contact may act by a different mechanism, that is, by differential energetic stabilization of one or more of the intermediate states and/or transition states of the RNA polymerase–promoter–initiating complex (Roy *et al.*, 1998). Thus, it is important to identify and characterize all the intermediates in the transcription initiation pathway (Chamberlin, 1974; deHaseth and Helmann, 1995; Gussin, 1996; Record *et al.*, 1996).

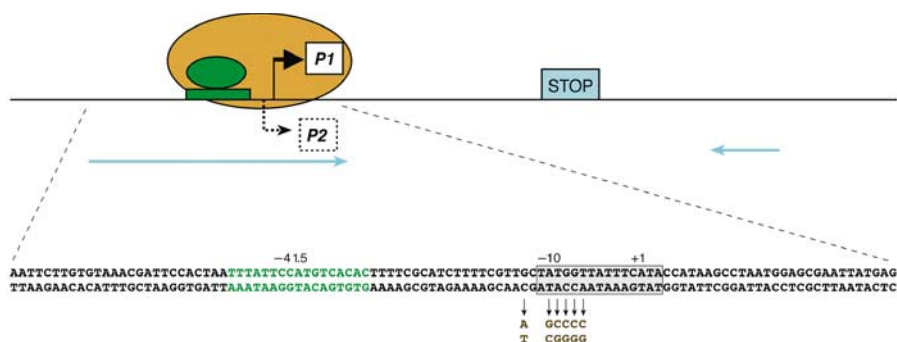
Recently, the determination of structures produced a great deal of information with respect to the RNA polymerase holoenzyme and some complexes with DNA (Murakami *et al.*, 2002a, b). However, a comprehensive identification of all intermediates still remains elusive. The nature of the intervening transition states is even more difficult to study in the absence of direct kinetic methods. A direct real-time method to study the transcription initiation pathway and its regulation is the use of the fluorescent base analog 2,aminopurine (2,AP) to study the basepair-opening steps of the promoter by RNA polymerase (Jia *et al.*, 1996; Újvári and Martin, 1996; Sullivan *et al.*, 1997). In this report, we used a direct real-time spectroscopic method to probe the steps of isomerization by using 2,AP in the *galP1* promoter of *Escherichia coli*. We followed the fluorescence of 2,AP put in different positions of the promoter to dissect isomerization, and studied how its activator, CRP, affects the steps. Our results showed that isomerization of *galP1* is a multi-step process with a rate-limiting one, and that CRP acts by enhancing the rate-limiting step.

## Results

The *gal* operon of *E. coli* is under the control of two promoters, *galP1* and *galP2* (Figure 1), belonging to the so-called ex–10 family, in which the –35 region does not play any role (Bown *et al.*, 1997). We introduced several mutations in the ex–10 region of the *galP2* promoter to eliminate its interaction of the RNA polymerase (Bingham *et al.*, 1986; see Materials and methods). Single basepair substitution experiments demonstrated that each of these mutations severely decrease *galP2* activity while having almost no effect on *galP1* activity (DEA Lewis and S Adhya, unpublished results). A combination of these mutations is expected to abolish completely the *galP2* activity with only a modest down effect, if any, on *galP1*. *In vitro* transcription results using this template were in agreement with this expectation

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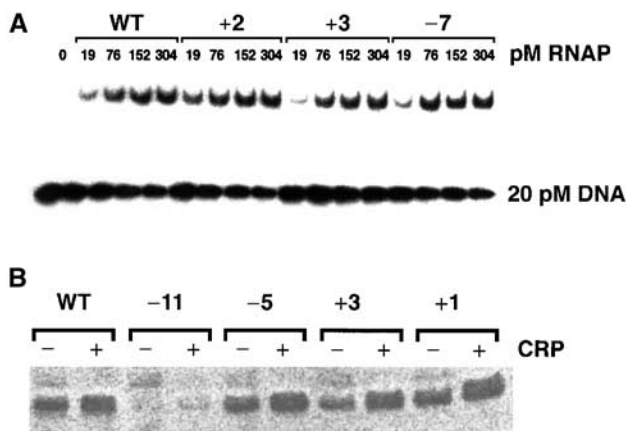


**Figure 1** Top: Relevant region of the *gal* promoter of *E. coli* showing *galP1* and *galP2* (mutated) and depicted by dotted lines, and the CRP-binding site (green). RNA polymerase is schematically drawn in yellow. The two blue arrows are the PCR primers for generating DNA templates for transcription. The blue box indicates a transcription stop signal for the purpose of generating transcript of defined size *in vitro*. Bottom: DNA sequence of the 106-basepair *galP1* promoter fragment. Transcription starts at position +1. cAMP·CRP binding region centered at position -4.5 is shown in green. The shaded segment is the presumed region of basepair distortion. The basepairs indicated by downward arrows around position -10 show mutations in the  $P1^-$  DNA used as a control (see Figure 4). The positions from -19 through -14 have mutational alterations (not shown) that inactivate RNA polymerase binding to the *galP2* promoter.

(see below). *galP1* templates carrying these *galP2*<sup>-</sup> mutations were used in all experiments that follow.

#### Measurement of basepair opening in the *galP1* promoter containing 2,AP

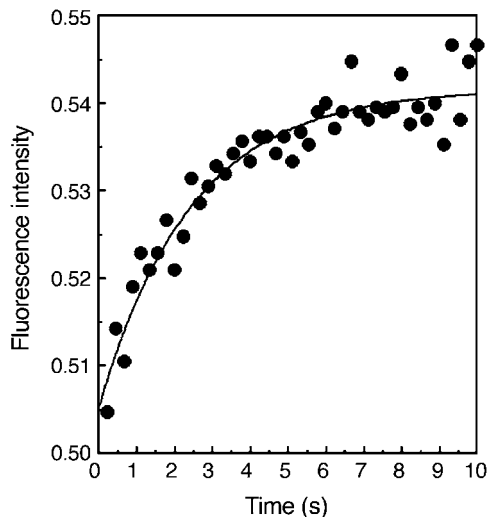
It is generally believed that about a 15-basepair segment becomes single stranded (referred to as 'opened') in the isomerized, that is, open, complex (Gamper and Hearst, 1982; McClure, 1985; Amouyal and Buc, 1987; Attey *et al*, 1994; deHaseth and Helmann, 1995). However, little data exist as to the basepair-opening process and whether the latter is a stepwise process. 2,AP is an adenine analog that pairs with thymine, with only a slightly weaker basepair affinity compared to an A:T basepair in the DNA duplex (Sowers *et al*, 1986; Nordlund *et al*, 1989; Wu *et al*, 1990; Law *et al*, 1996). 2,AP-containing DNA is in the B-form and does not show any major structural alteration (McLaughlin *et al*, 1987; Xu *et al*, 1994). Unlike the normal DNA bases, 2,AP displays fluorescence at neutral pH, which is significantly quenched in the DNA duplex. Consequently, any disruption or unstacking of the 2,AP:T basepair leads to enhancement of fluorescence (Guest *et al*, 1991; Bloom *et al*, 1993; Raney *et al*, 1994; Xu *et al*, 1994), although a correlation of the disrupted structure with fluorescence properties remains difficult because of the potential effect of RNA polymerase on 2,AP quantum yield. However, the rate of fluorescence increase of 2,AP can be quantitatively linked to the rate of opening or distortion of the double helix, and offers a powerful tool to study the process of basepair opening. As the distortion of the helix during the transcription initiation pathway occurs in the -10 region, we prepared templates, each containing 2,AP replacing an adenine in either strand at positions from -12 to +3. Two different-sized templates containing 2,AP were prepared: (i) 106-basepair-long duplexes of two completely chemically synthesized strands, and (ii) PCR-generated 358-basepair-long templates. Both kinds of templates gave similar results, although the quality of fluorescence data appeared to be better in the latter ones. The transcriptional competency of these templates was judged by electrophoretic mobility shift assay (EMSA) of RNA polymerase binding using the 106-basepair long duplexes, as well as by *in vitro* transcription assays using the 358-basepair-long DNA. Figure 2 shows



**Figure 2** (A) EMSA of RNA polymerase binding to the 106-basepair *galP1* DNA duplex containing 2,AP at the positions indicated. (B) *In vitro* synthesis of *galP1* RNA in the absence and presence of CRP with 358-basepair *gal* DNA templates containing 2,AP at the positions indicated. WT indicates *galP1* DNA containing no 2,AP.

the EMSA and the *in vitro* transcription results for the wild type and several 2,AP-containing DNA templates. The incorporation of 2,AP into the templates made no difference in the binary complex formation as measured by EMSA. Similarly, *in vitro* transcription experiments showed that the *galP1* RNA synthesis and its stimulation by CRP from the 2,AP-containing templates, except the one with the analog at position -11, were comparable to that from the wild-type template. We previously showed that substitution of the adenine by 2,AP at -11 made *galP1* defective (Lim *et al*, 2001).

A distortion in the double helix as a discrete early event during isomerization was suggested recently (Saecker *et al*, 2002). A rapid component of 2,AP fluorescence increase in the *lacP1* promoter *E. coli* was also detected (Liu *et al*, 2003). This observation led us to examine the changes in 2,AP fluorescence by stopped-flow methods. Figure 3 shows the kinetics of fluorescence increase of a 358-basepair DNA containing *galP1* with 2,AP substituted at the +3 position upon RNA polymerase binding. The fluorescence increase showed saturation behavior and can be fitted to a single exponential with an average rate constant of  $0.9\text{ s}^{-1}$ . The 106-basepair

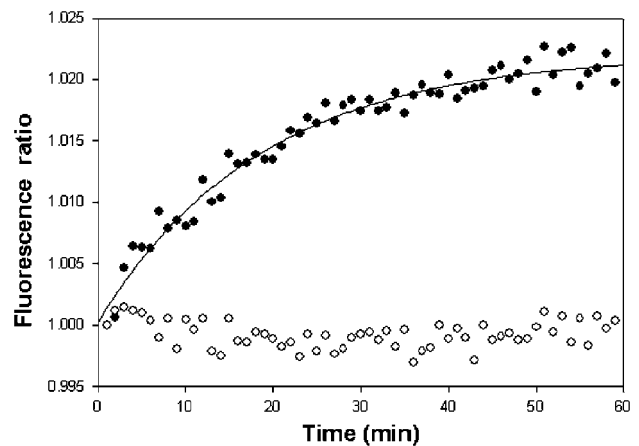


**Figure 3** 2,AP fluorescence intensity changes (in arbitrary units) of the 358-basepair DNA template containing 2,AP at the +3 position of the *galP1* promoter as a function of time (0–10 s) upon rapid mixing with RNA polymerase using a stopped-flow device. A typical run is shown. A derived rate constant for this run may differ from the average rate constant mentioned in the text. See Materials and methods for experimental details.

template containing 2,AP at +3 also gave a similar profile with an average pseudo-first-order average rate constant of  $0.85 \text{ s}^{-1}$  (data not shown). Of the other 2,AP-containing templates tested with 2,AP at  $-6, -5, -4, -3, -2, +1$  or  $+2$ , only the one with 2,AP at position +2 showed significant fluorescence increase by stopped-flow assays. The derived average rate constant was  $0.69 \text{ s}^{-1}$ , suggesting that a helix distortion occurs very early around the  $+2/+3$  region. Given the uncertainties of the stopped-flow measurements, it is unlikely that the values  $0.85$  and  $0.69 \text{ s}^{-1}$  are really different.

#### Asynchrony in basepair opening

In order to observe the basepair openings at a later stage of the transcription initiation, we performed manual mixing experiments. In these experiments, the 106-basepair 2,AP-containing DNA duplexes were mixed with RNA polymerase at a final concentration of  $1 \mu\text{M}$  each, which is at least an order of magnitude higher than the dissociation constant of the *galP1* promoter–RNA polymerase closed complex (Goodrich and McClure, 1992). As the on-rates for promoters are very fast, the initial binary closed-complex formation (Knaus and Bujard, 1990) at such concentrations should be over within the mixing time of about 15 s, and hence any change in fluorescence should reflect the step(s) at which significant quantum yield change occurs. Figure 4 shows the fluorescence increase with 2,AP at the  $-2$  position. The rate increase was fitted well to a single exponential, suggesting that the fluorescence increase occurred predominantly in one step. In contrast, the 2,AP fluorescence of the control *galP1*<sup>−</sup> DNA with 2,AP at the same  $-2$  position did not increase upon mixing with RNA polymerase. We assume that the fluorescence increase in the wild type is mostly due to basepair openings at the *galP1* promoter. We conjecture that the observed initial decrease of the fluorescence before reaching a plateau in the mutant template in the absence of specific promoter binding was caused by quenching of fluorescence



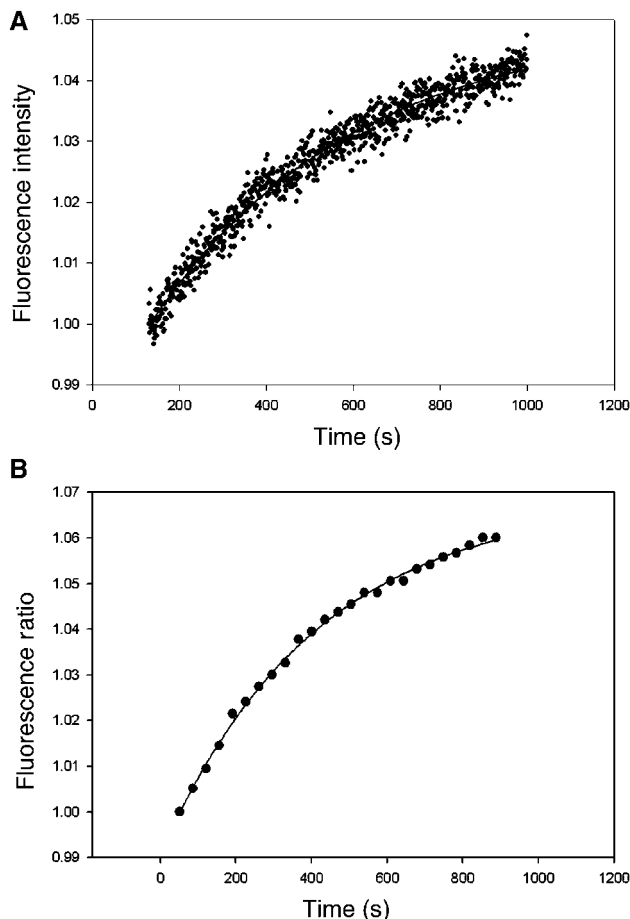
**Figure 4** 2,AP fluorescence changes in 106-basepair DNA templates containing 2,AP at the  $-2$  position of the *galP1* promoter as a function of time upon manual mixing with RNA polymerase in a Perkin-Elmer spectrofluorometer, and expressed as a ratio of two channels. The 2,AP fluorescence increased with time when the promoter was *galP1*<sup>+</sup> (filled circles), and decreased initially and reached a plateau when the promoter was *galP1*<sup>−</sup> (open circles).

when RNA polymerase makes nonspecific interactions with the DNA template that is devoid of any active promoter.

Figure 5 shows the kinetics of 2,AP fluorescence increase by manual mixing when the label was present at the +3 position in both +358-basepair-long PCR-generated (panel A) and the 106-basepair-long synthetic (panel B) templates. The rate constants ( $k_{\text{app}}$ ) were determined to be very similar,  $2.1 \times 10^{-3}$  and  $1.34 \times 10^{-3} \text{ s}^{-1}$ , respectively, justifying the use of the two types of templates interchangeably. Figure 6 lists the rate constants of fluorescence increase (interpreted as basepair openings) obtained by the manual mixing method for *galP1* DNA with 2,AP at different positions from  $-12$  to  $+3$ , as derived from the corresponding single exponential curves (see Materials and methods). It is clear that the forward rate constants for the templates containing 2,AP at different positions vary considerably, in some cases by an order of magnitude. At first inspection, the rate constants determined by manual mixing may be grouped into three different categories: rapid basepair opening at positions  $-12, -10, -4, -2, +1$  and  $+3$ ; moderate openings at positions  $-12, -10$  and  $+1$ ; and slow openings at positions  $-7, -5$  and  $+2$ . It is known that single basepair substitutions at  $-12, -10$  and  $+1$  have moderate down effects on transcription (DEA Lewis and S Adhya, in preparation). Thus, it is very likely that the second group of positions actually belongs to the rapid opening group and shows a moderate rate due to the mutational effects of 2,AP substitutions at these locations. Thus, we classify the basepair openings determined by manual mixing into two classes, pending further exploration of 2,AP mutational effects: rapid ( $-12, -10, -4, -2, +1$  and  $+3$ ) and slow ( $-7, -5$  and  $+2$ ). Two of the slow positions ( $-5$  and  $+2$ ) show no mutational effect in single base substitution experiments (DEA Lewis and S Adhya, in preparation).

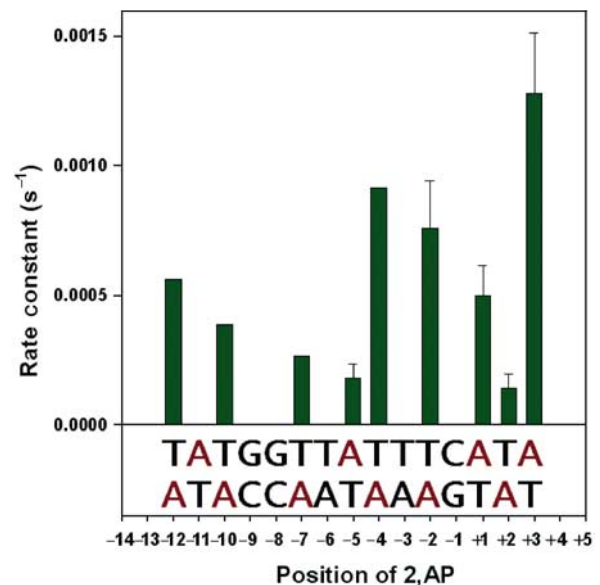
#### Enhancement of basepair opening by CRP

As previously proposed, a regulator of transcription initiation can act by modulating the energetics on one or more steps of the initiation pathway by making differential contact(s) with

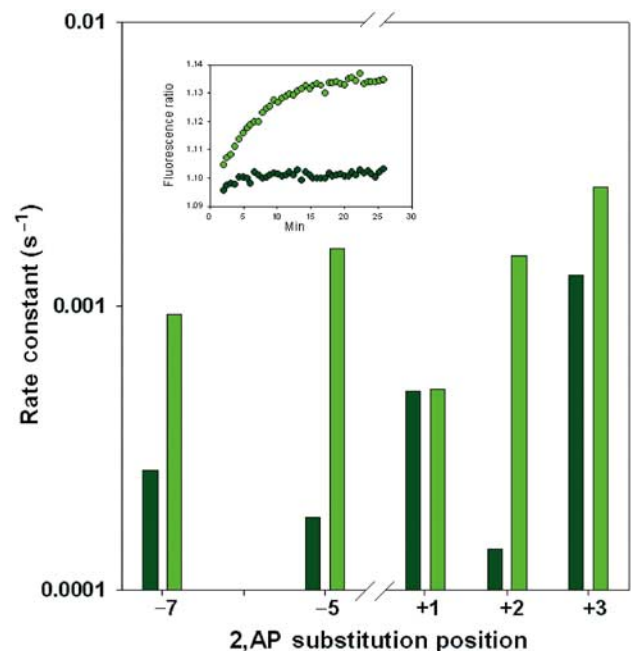


**Figure 5** 2,AP fluorescence increase with (A) 358-basepair PCR-generated templates in a single-channel PTI spectrofluorometer expressed in normalized fluorescence intensity, and (B) 106-basepair synthetic templates in a Perkin-Elmer LS50B spectrofluorometer with alternate scans as described in Materials and methods. Hence, the Y-axis is expressed as a ratio of two channels. In both cases, 2,AP was present at the +3 position. Details are given in Materials and methods.

DNA-bound RNA polymerase, whose conformation, depending on the DNA sequence of the promoter, constantly changes during the course of the reaction (Roy *et al*, 1998). By differential contacts, a DNA-bound regulator lowers the free energy of and stabilizes one or more of the intermediates during the course of the reaction. An activator that establishes contacts during the transition state of the slowest step of isomerization would lower its activation energy and enhance the overall isomerization. From the abortive initiation assays, it was suggested that CRP activates *galP1* by enhancing mostly the isomerization step (Herbert *et al*, 1986; Goodrich and McClure, 1992). We used the 2,AP fluorescence-increase experiments to study the effects of CRP on various basepair-opening rates. We emphasize, as mentioned before, that the rate of fluorescence increase is the important parameter, and not the quantum yield, in assessing the activator effect. Figure 7 shows the effect of CRP on the deformation rate of DNA templates substituted with 2,AP at different positions. It is clear that CRP had little effect on the faster basepair-opening positions as was observed by following 2,AP fluorescence increase at positions +1 and +3. However, CRP accelerated the slower rate constants



**Figure 6** Rate constants of basepair openings at different positions of *galP1* upon RNA polymerase binding as measured by 2,AP fluorescence increase shown in bar graphs. The DNA sequence of the -14 to +5 region of the promoter is shown at the bottom of the bar graphs. The positions of the adenines, which were individually probed by 2,AP in either strand, are shown in red. Error bars are not given for cases in which experiments are carried out only two to three times. Only averages are shown for them.



**Figure 7** Effect of CRP on the rate constants at five different positions of adenine as probed by 2,AP. Without CRP (gray); with CRP (green). The inset shows, as an example, the fluorescence data for the +2 position.

significantly. There was a 4–10-fold increase in the rate constants as observed by following 2,AP fluorescence increase at positions -7, -5 and +2. The rates of the +1 and +2 groups were comparable in the presence of CRP. If an activator enhances one or more steps of the initiation

pathway, it must at least enhance the rate-limiting step. This is exactly what was observed. The magnitude of the CRP effect on the isomerization as obtained from abortive initiation assays was similar to that obtained above (Herbert *et al*, 1986; Goodrich and McClure, 1992).

## Discussion

Study of the transient intermediate populations in the process of transcription initiation in real time is crucial for a mechanistic understanding of basal transcription as well as its regulation. The use of 2,AP as a fluorescence probe allowed us to dissect the isomerization step of transcription initiation in greater detail than hitherto possible, both from structural and kinetic viewpoints.

### Isomerization is a multi-step process

Our results discussed above demonstrated different steps of isomerization. It was previously shown that the A:T basepair, called the 'master' basepair, at the  $-11$  position plays a crucial role in initiating the basepair-opening process (Lim *et al*, 2001). Even a small change of shift of the amino group from the 6 (in adenine) to the 2 position (in 2,AP) blocked the basepair opening at  $-11$ . As the 2,AP:T basepair is energetically similar to an A:T basepair, it is likely that this stringent specificity is due to an interaction of  $-11A$  with RNA polymerase. It was suggested that  $-11A$  interacts with aromatic residues of RNA polymerase (Fenton *et al*, 2000; Tsujikawa *et al*, 2002). It was also suggested that the distortion of the duplex at the master position triggers further destabilization of the helix. A significant increase of 2,AP fluorescence, placed at  $+2$  and  $+3$ , determined by stopped flow, indicates, however, that  $+2/+3$  destabilization takes place either simultaneously or immediately following the  $-11A$  interaction. Whether these two steps are identical or consecutive is not known at this point. It was previously proposed that a contact of the RNA polymerase  $\beta$ - $\beta'$  jaw with DNA basepairs occurs relatively early around the start site during isomerization (Saecker *et al*, 2002). The fluorescence change observed with 2,AP labeled at the  $+2/+3$  regions in the stopped flow may be related to this event.

The slower basepair-opening steps in *galP1*, as detected by manual mixing, can be divided into two phases. In the first phase, most of the basepairs in the  $-10$  region became distorted. It appears that in the other phase, basepairs at  $-7$ ,  $-5$  and  $+2$  opened significantly slowly. The slow steps are unlikely to be side reactions because they become faster in the presence of CRP, commensurate with the CRP-mediated enhancement of transcription at *galP1* (see later). Based on the information presented above, we propose the following multi-step kinetic scheme of open complex formation at the *galP1* promoter:

- (i) interaction of  $-11A$  with RNA polymerase and very fast destabilization of the  $+2/+3$  region;
- (ii) fast opening of the basepairs as in positions  $-4$ ,  $-2$  and  $+3$ ;
- (iii) slow opening of the remaining basepairs.

Thus, the basepair-opening process in *galP1* is asynchronous.

It is interesting to compare the two open complexes,  $RP_{O1}$  and  $RP_{O2}$ , demonstrated by Record and co-workers in the  $\lambda P_R$

promoter (Suh *et al*, 1992, 1993).  $RP_{O2}$  showed significantly enhanced permanganate and hydroxy-radical reactivities compared to  $RP_{O1}$  in the  $-4$  to  $+2$  region of  $P_R$ , including a large permanganate reactivity of the  $+2$  base. This may be compared to the fluorescence change in the slowest phase in *galP1* ( $-5$  and  $+2$ ). We also note that  $-7T$  permanganate reactivity is affected inversely between  $RP_{O1}$  and  $RP_{O2}$  (Suh *et al*, 1993).

### CRP enhances the rate-limiting step

In principle, the slow step described above could be a side reaction. However, we assign this last slow step to the generation of the catalytically competent open complex for the following reasons: (i) The step is activated by CRP (Figure 7). (ii) Addition of NTPs clears the promoter of RNA polymerase, which was observed by decrease of the 2,AP fluorescence, presumably because of the restoration of the helix structure (data not shown). The rate constants measured by abortive initiation assays also strongly support that this step is the last rate-determining step of open complex formation (Lavigne *et al*, 1992). A rate constant of isomerization of  $1.4 \times 10^{-3} \text{ s}^{-1}$  was obtained for *P1* by the abortive RNA synthesis measurements with a 113-basepair linear DNA template carrying a mutation in the *P2* promoter ( $-16A$  to C change). The average rate constant (average of the 2,AP experiments at positions  $-7$ ,  $-5$  and  $+2$ ) of the slowest basepair-opening step was about  $8 \times 10^{-4} \text{ s}^{-1}$ . The latter experiments were carried out at  $25^\circ\text{C}$ , whereas the abortive initiation assays were performed at  $37^\circ\text{C}$ . We observed an approximately five-fold enhancement of the slowest 2,AP basepair-opening step at  $37^\circ\text{C}$  when compared to  $25^\circ\text{C}$  (S Roy and S Adhya, unpublished results), making the two rate constants very close.

### Basepairs open asynchronously

Our results suggest that basepair openings at a promoter do not occur uniformly throughout the region but follow different rates depending on the position of the basepair in the  $-10$  region, and imply that individual basepair openings may be somewhat independent events. This is contrary to the commonly held belief that starting with a nucleation point the DNA duplex becomes single stranded in a sequential fashion during isomerization. How can a basepair at a particular position in the helix open up if its two immediate neighbors have not yet done so? Our results of individual 'stand-alone' basepair openings, that is, opening of one basepair without simultaneous opening of one or both neighboring pairs at the same time as reflected by 2,AP fluorescence assays, are consistent with the idea of base flipping (Streisinger and Owen, 1985; Roberts, 1995). In fact, base flipping has been invoked for the interaction between  $-11A$  and aromatic residues in RNA polymerase (Fenton *et al*, 2000; Tsujikawa *et al*, 2002).

## Materials and methods

### Materials

*E. coli* RNA polymerase holoenzyme was purchased from Epicentre Technologies. CRP, purified to 98% homogeneity by FPLC (Pharmacia) from an *E. coli* strain carrying the *crp* gene in a multicopy plasmid (pHA5), was a gift from S Garges (NCI). Synthetic oligodeoxynucleotides containing 2,AP substitutions either as primers or as 106-nt-long chains were purchased from

Sigma-Genosys or Trilink BioTechnologies. The 106-nt-long polymers were purified by polyacrylamide gel electrophoresis.

### Preparations of DNA templates containing 2,AP

DNA duplexes (−75 to +30) for fluorescence measurements were prepared fresh before use. They were made by mixing 10 μl of each complementary oligo (10 μM) with 10 μl of 500 mM Tris-HCl, pH 7.5, containing 100 mM MgCl<sub>2</sub> and 1 M NaCl. The total volume was adjusted to 100 μl with water. Hybridization was carried out by raising the temperature of the mixture to 95°C for 5 min and slowly lowering the temperature (1°C/100 s) to 25°C using a PCR machine. DNA templates for the electrophoretic mobility shift assays were further purified after hybridization by 8% polyacrylamide gel electrophoresis. The 2,AP-containing DNA templates were also prepared by PCR, in which one of the primers was the 2,AP-containing synthetic oligomer. The second primer corresponded to the segment downstream to a transcription terminator (Figure 1). The DNA templates for PCR reactions were plasmid pSA509 containing a 288-basepair segment of the *gal* control region (−197 to +91; Figure 1) followed by a transcription terminator (Choy and Adhya, 1993). After the PCR reaction, the 358-basepair DNA fragments were purified by electroelution (BioRad) followed by ethanol precipitation. The 358-basepair-long PCR DNA templates generated a 125-nt-long RNA transcript from the *galP1* promoter in *in vitro* transcription reactions. All DNA templates contained the following changes to inactivate the P2 promoter: −16A→T, −17G→T, −18T→C and −19G→T.

### In vitro transcriptions

*In vitro* transcription reactions were performed at 25°C in a 50 μl volume. The initial reaction mixture (45 μl) contained 10 nM DNA template, 20 nM RNA polymerase, 20 mM Tris-acetate, pH 7.5, 10 mM magnesium acetate, 200 mM potassium glutamate and 40 U rRNasin (Promega). CRP, when present, was added to the initial reaction mixture at 50 nM. cAMP concentration was 1 μM. The initial mixtures were incubated at 25°C for 20 min, and 5 μl of NTP (2 mM of ATP, GTP and CTP, 0.2 mM UTP and 5 μCi α-<sup>32</sup>P-UTP) was added. The mixtures were further incubated for 10 min at 25°C, and the reaction was terminated by the addition of 50 μl of the STOP solution (BRL). Samples were boiled for 2 min and 3 μl of each sample was loaded onto 8% polyacrylamide-urea sequencing gels to analyze the RNA. Quantitation of the transcripts was done by PhosphorImager.

### EMSA

A volume of 1 μl of appropriately diluted RNA polymerase was mixed with 2 μl of <sup>32</sup>P-labeled DNA fragments (200 pM) and 2 μl of the binding buffer (100 mM Tris-acetate, pH 7.5, 50 mM magnesium acetate and 1 M potassium glutamate) in a total reaction volume of 10 μl. The reaction mixtures were incubated at 37°C for 10 min, followed by the addition of 1 μl of heparin (0.5 mg/ml). The entire reaction volume was loaded onto 4% polyacrylamide gel for electrophoresis.

### 2,AP fluorescence measurements by stopped-flow mixing

For rapid fluorescence measurements of base opening, a stopped-flow spectrometer with fluorescence detection (Applied Photophysics, SX 18MV) was used. A volume of 500 μl each of RNA polymerase (200 nM) and DNA template (600 nM) in 2 × transcription buffer was set up in separate syringes. In all, 25 μl of each reagent was rapidly mixed (6.0 ml/s) in the reaction chamber at a time. Upon the loading of each reagent onto the stopped-flow device, 10 measurements were performed consecutively. The

reaction was excited with 315 nm and emission at 370 nm was measured for 10 s. Reactions were prepared and measured at 25°C. The kinetic data of fluorescence were automatically processed, analyzed and saved onto a hard disk of the computer connected to the spectrofluorometer.

### 2,AP fluorescence measurements by manual mixing

Fluorescence measurements were performed in a Perkin-Elmer Luminescence Spectrometer LS 50B and a, RTC-2000/SE spectrofluorometer from Photon Technology International. Samples were excited at 320 nm (10 nm slit width) and the emission spectra of 370 nm (15 mm slit width) were measured. Prior to the measurements, the temperatures of all the reaction components and instruments were equilibrated at 25°C. The fluorescence measurement and the binary complex formation were executed at 25°C. The binary complex formation between the promoter and RNA polymerase was initiated by mixing 50 μl DNA template (1 μM) and 10 μl RNA polymerase (5 μM). The buffer was 0.05 M Tris-HCl, pH 7.5, containing 0.1 M NaCl and 10 mM MgCl<sub>2</sub>. As a control, 50 μl of the same DNA (1 μM) was mixed with 10 μl of the RNA polymerase storage buffer. Immediately after the mixing (time 0), both reactions were transferred to cuvettes (Hellma Cells, 0.300). The emission spectra from both samples were recorded alternately every 15 s for 2000 to 4000 s by employing a motorized 4-position turret cuvette holder. To compensate for the machine error, such as machine drift, the fluorescent values of the experiment (DNA + RNA polymerase) were divided by those of the control (DNA + RNA polymerase storage buffer) to calculate the fluorescence ratios. We assumed that the kinetics of binary complex formation is very rapid at the concentrations used (Knaus and Bujard, 1990). This is likely to be the case as the concentration of RNA polymerase and DNA is several-fold greater than a  $K_D^{-1}$  value of  $2.5 \times 10^{-7}$  obtained from abortive initiation experiments (Lavigne *et al.*, 1992), and the on-rate measured for most promoters is around  $10^8 \text{ M}^{-1} \text{ s}^{-1}$  (Knaus and Bujard, 1990). Under these conditions, there is a rapid formation of the initial complex that then isomerizes by the first-order kinetics. Sigma plot 4.0 was used to fit the fluorescence data. The rate constants of the open-complex formation were calculated by fitting the fluorescence ratio to the equation  $f = y^0 + a(1 - e^{-bt})$ , where  $f$  is the ratio at time  $t$ ,  $y^0$  is the initial ratio,  $a$  is the observed amplitude and  $b$  is the apparent first-order rate constant ( $k_{app}$ ) of basepair opening. The rate constant  $k_{app}$  is related to the true first-order rate constant ( $k$ ) by the relationship  $k_{app} = kK_a D_T / (1 + K_a D_T) + \beta k$ , where  $K_a$  is the association constant and  $D_T$  is the total DNA concentration. This relationship was obtained because of a rapid equilibrium between free DNA and the closed complex, the total DNA concentration being greater than that of total RNA polymerase. Although the formal concentrations of DNA and RNA polymerase were the same, the active fraction of RNA polymerase used was only 20% fulfilling the above condition. The value of  $\beta$  was determined by carrying out the reaction at two different DNA concentrations for three different templates. The obtained values for different templates were remarkably close. The average value and standard error obtained was  $0.295 \pm 0.057$ . This value for  $\beta$  was used to obtain the value of the forward rate constant for all the templates from  $k_{app}$ .

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