Kinetics of activation of the P4 promoter of pBR322 by the *Escherichia coli* cyclic AMP receptor protein

James G. HOGGETT† and Ian BRIERLEY*

Department of Biology, University of York, Heslington, York YO1 5DD, U.K.

The activation of transcription initiation from the P4 promoter of pBR322 by the *Escherichia coli* cyclic AMP receptor protein (CRP) has been investigated using a fluorescence abortive initiation assay. The effect of the cyclic-AMP/CRP complex on the linear P4 promoter was to increase the initial binding (K_B) of RNA polymerase to the promoter by about a factor of 10, but the rate of isomerization of closed to open complex (k_t) was unaffected. One molecule of CRP per promoter was required for activation, and the concentration of cyclic AMP producing half-maximal stimulation was about 7–8 μ M. Supercoiling caused a 2–3-fold increase in the rate of isomerization of the CRP-activated promoter, but weakened the initial binding of polymerase by about one order of magnitude. The unactivated supercoiled promoter was too weak to allow reliable assessment of kinetic parameters against the high background rate originating from the rest of the plasmid.

INTRODUCTION

The mechanism of initiation of transcription in prokaryotes has been the subject of extensive structural and kinetic studies. The classical model for describing this process, involving initial association of RNA polymerase (R) with the promoter (P), isomerization of this closed complex (RP_c) to the transcriptionally active open complex (RP_o), followed by promoter clearance [1], is acknowledged to be a considerable oversimplification [2–5], but it remains a useful framework for the interpretation of kinetic assays:

(K_B is the initial binding constant and k_t is the rate of isomerization of the closed to open complex). Abortive initiation assays allow the parameters characterizing the binding and isomerization steps to be determined by analysis of the concentration-dependence of the lag times observed when the reaction is initiated with RNA polymerase [6,7]. The continuous monitoring modification of the assay developed by Bertrand-Burggraf and co-workers exploits the increase in fluorescence which occurs when fluorescent nucleotide analogues labelled with 1-naphthylamine-5-sulphonic acid (AmNS) at the γ -position are incorporated into a transcript, with concomitant release of the label [8]. This modified assay has been used in studies of several promoters, and it has been established that the analogues are good substrates for RNA polymerase in abortive initiation assays [8–13].

Kinetic studies show that cyclic AMP receptor protein (CRP) activation of type I promoters such as *lac*, where the CRP-binding site is centred at –61.5 bp from the transcription start, is due to an increase in K_B [14,15], whereas in type II promoters such as *gal*, where CRP binds around –41.5 bp, the step affected depends on the precise nature of the promoter and the presence or absence of upstream bending sequences [16].

The CRP-dependent P4 promoter of pBR322 is a type II promoter with a CRP-binding site centred at –42.5 bp from the

transcription start [17] (Fig. 1). Binding to the cyclic-AMP/CRP complex to the P4 site is significantly weaker than to the *gal* site, but the DNA bending induced by CRP binding to P4 is at least as pronounced as that occurring at *gal* [18]. The sequence of the P4 promoter deviates considerably from the consensus structure, and in terms of Mulligan & McClure's homology score (47.3) it would, in the absence of CRP, be expected to be the weakest of the identified promoters on pBR322 [19], some of which have been investigated using the abortive initiation approach [8,9,20,21]. The function of the P4 promoter in pBR322, if any, is not known, but the promoter has almost identical counterparts in ColE1 and related plasmids [17], where it has been proposed that it may be responsible either for transcription of some of the *mob* genes or for generating an RNA molecule involved in plasmid mobilization [22].

To provide further information about the mechanism of CRP activation of promoters of the *gal*P1 prototype, we report here a study, using the fluorescence abortive initiation technique, of activation by the CRP from *Escherichia coli* of the P4 promoter

Fig. 1. Sequence of the P4 promoter region

The transcription start site at +1 is indicated by the arrow. The boxed sequences contain the Pribnow box at –10 and an upstream T-tract centred at –65.5. The centre of the CRP-binding site at –42.5 is indicated by a cross; the brackets mark the extent of CRP protection in DNAase footprinting studies, and the 5' TGTGA 3' motif is underlined. The complete DNA fragment used in these studies extended from positions –145 to +136.

Abbreviations used: AmNS, 1-naphthylamine-5-sulphonic acid; CRP, cyclic AMP receptor protein; WASP, water/ammonium sulphate/propan-2-ol (80:18:2, by vol.); K_B , initial binding constant; k_t , rate of isomerization of closed to open complex.

* Present address: Division of Virology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, U.K.

† To whom correspondence should be addressed.

on a linear DNA fragment, together with an assessment of the effect of supercoiling on the CRP-activated promoter.

MATERIALS AND METHODS

Protein purification

RNA polymerase was prepared from *E. coli* MRE 600 by the method of Burgess & Jendrisak [23]. Core polymerase was separated from holoenzyme as described by Lowe *et al.* [24]. The molar content of σ -subunit was about 90 %, and the activity of the enzyme assayed on calf thymus DNA was 375 units/mg. The enzyme was about 25 % active in specific promoter binding.

CRP was prepared from *E. coli* M182 Δcrp /pBS $crp2$ essentially as described by Pinkney & Hoggett [25]. Samples were judged by SDS/PAGE to be more than 95 % pure, and the specific activity of cyclic AMP binding was 6000 units/mg. The protein was 30 % active in specific DNA binding [18].

Plasmids and DNA fragments

Plasmid DNA was prepared by a standard alkaline SDS lysis procedure followed by purification on two successive CsCl/ethidium bromide gradients. The *Ap* promoter of pBR322 (which initiates with the same sequence as the P4 promoter) was excised by removing the region between the *Eco*RI and *Sca*I sites to yield pHM1. In the control plasmid (pHM3) the P4 promoter was also deleted by excising the region between the *Nde*I and *Tth*III sites. The P4 promoter region was isolated on a 281 bp *Alu*I fragment and purified from 6 % preparative polyacrylamide gels [18]. DNA concentrations were determined spectrophotometrically.

Nucleotides

Nucleotide triphosphates and dinucleotide monophosphates were obtained from Boehringer and Sigma respectively. AmNS was obtained from Fluka, and UTP- γ -AmNS (i.e. AmNS attached to γ -phosphate of UTP) was prepared as described by Yarbrough *et al.* [26].

Abortive initiation assays

Rates of abortive initiation were determined fluorimetrically by the method of Bertrand-Burggraf *et al.* [8]. Fluorescence measurements were performed at 37 °C using a Perkin-Elmer MPF-3 spectrofluorimeter with excitation and emission wavelengths of 360 nm and 450 nm respectively. The initiating dinucleotide used with the P4 promoter was GpA, which on reaction with UTP- γ -AmNS yielded the trinucleotide GpApU with release of AmNS. The fluorescence change corresponding to complete hydrolysis of UTP- γ -AmNS was determined by treatment with snake venom phosphodiesterase. Standard assay conditions were 40 mM-Tris (pH 8.0), 0.1 M-KCl, 10 mM-MgCl₂, 1 mM-dithiothreitol, 0.5 mM-GpA and 0.05 mM-UTP- γ -AmNS, in total volume of 0.3 cm³. The concentration of DNA template was 2–5 nM (usually 2 nM), and the stoichiometric concentration of RNA polymerase varied from 20 to 200 nM. CRP and cyclic AMP, when present, were at the stoichiometric concentrations indicated. For determination of lag times, the reaction was initiated by the addition of RNA polymerase (thermally equilibrated with an aliquot of buffer) to the equilibrated assay mixture in the fluorescence cell; when CRP was present the assay mixture was preincubated for 15–30 min in the presence of cyclic AMP and CRP before the addition of RNA polymerase. Lag times were evaluated as described [7,8]. Steady-state rates (expressed as nucleotide incorporated/min per promoter) were determined by initiating the reaction with nucleotides after preincubation of RNA polymerase and DNA for a period

greater than five times the lag time; in experiments with CRP and cyclic AMP, these were added to the DNA 15–30 min before the preincubation with RNA polymerase.

RESULTS

Activation of the linear P4 promoter

Transcription from the P4 promoter is known to be initiated (at position 2270 on the pBR322 sequence) with the sequence GAUGCC. The abortive initiation product formed was checked by using GpA and [³H]UTP as substrates in an assay using the procedure of McClure [7]; the product migrated as a single band on water/ammonium sulphate/propan-2-ol (80:18:2, by vol.) (WASP) chromatography with an R_f value of ~ 0.15 (results not shown). Fig. 2 shows the time course of abortive initiation from the P4 promoter on a linear 281 bp fragment monitored by the fluorescence increase which accompanies the cleavage of UTP- γ -AmNS and formation of GpApU. After the initial lag phase, the reaction initiated with RNA polymerase (curve a) reached the same steady-state rate as was observed after preincubating DNA and RNA polymerase (curve b).

With the assumptions that (i) the formation of the closed complex is a rapid pre-equilibrium, and (ii) $k_t \gg k_b$ (the reverse rate constant for $RP_o \rightleftharpoons RP_c$), and the further condition that $[R] \gg [P]$, the lag time (τ) is related to $[R]$ by the expression [7]:

$$\tau = \frac{1}{k_t} + \frac{1}{k_t K_B [R]}$$

It follows that a plot of τ against $1/[R]$ should yield a straight line with intercept $1/k_t$ and slope $1/k_t K_B$. τ plots for the linear P4 promoter in the presence and absence of cyclic-AMP/CRP are shown in Fig. 3, and the derived values of k_t and K_B are given in Table 1. Within the limits of experimental error, the values of k_t were the same in the presence and the absence of CRP, but CRP produced a 8–9-fold increase in K_B . As has been noted before (e.g. [27]), errors in the estimates of K_B , which is determined as the ratio of intercept to slope, are greater than the errors of either k_t or $k_t K_B$; the latter quantity is equivalent to an overall rate

Fig. 2. Kinetics of abortive initiation from the P4 promoter

Concentrations used: P4 fragment, 2 nM; RNA polymerase, 50 nM; CRP, 50 nM. For curve a the reaction was initiated with RNA polymerase; for b RNA polymerase and promoter were preincubated for 30 min before initiation with nucleotides. The lag time, evaluated from the logarithmic plot of the amplitude of the lag phase (F/F_{max} ; ■), was 110 s.

Fig. 3. τ -plots for linear and supercoiled P4 promoters

Assays at 37 °C were carried out under standard conditions (see the Materials and methods section). Results are shown for the linear P4 promoter on a 281 bp fragment in the absence (Δ) and presence ($\blacktriangle$) of cyclic-AMP/CRP, and for the supercoiled P4 promoter on pHM1 in the presence of cyclic-AMP/CRP ($\blacksquare$). Reliable results for the supercoiled P4 promoter in the absence of cyclic-AMP/CRP could not be obtained, as is discussed in the text.

Table 1. Kinetic parameters of abortive initiation

Values were obtained from the τ plots using stoichiometric concentrations of RNA polymerase. The values in parentheses are corrected for the proportion of RNA polymerase which was active in promoter recognition (25 %).

Promoter	Cyclic-AMP/CRP	$10^3 \times k_t$ (s ⁻¹)	$10^{-6} \times K_B$ (M ⁻¹)	$10^{-5} \times k_t K_B$ (M ⁻¹ ·s ⁻¹)
Linear P4	—	10 ± 1	8.3 ± 0.8 (33)	0.82 ± 0.05 (3.3)
Linear P4	+	10 ± 1	70 ± 10 (280)	7 ± 0.5 (28)
Supercoiled P4	+	25 ± 3	9.2 ± 1 (37)	2.3 ± 0.2 (9.2)

constant for open complex formation, which can be used for comparative purposes as a measure of promoter strength [19,27].

The dependence of steady-state rates on the concentration of RNA polymerase in the presence and absence of cyclic-AMP/CRP is shown in Fig. 4. In both cases the curve reflects tight binding of RNA polymerase to form open complexes, and on the basis that the fragments contain no other independently binding promoter than the P4 promoter, extrapolation indicates that RNA polymerase was 25 % active in specific promoter binding. The difference in the steady-state rates at high concentrations of RNA polymerase in the presence and absence of CRP was about a factor of 3.

The effect of cyclic-AMP/CRP on the steady-state rate at high concentrations of RNA polymerase was exploited for titration purposes, and Fig. 5 shows the dependence of this rate on the concentration of CRP in the presence of saturating concentrations of cyclic AMP. The activity of the CRP preparation used, as determined by its ability to bind specifically to the strong *lac*-binding site in non-denaturing PAGE, was 31 % [18]; when allowance was made for this partial activity, the data in Fig. 5 indicate that one active CRP dimer per promoter is required for complete activation.

Fig. 4. Dependence of the steady-state rates of abortive initiation from the linear P4 promoter on RNA polymerase concentration

Reaction was under standard assay conditions in the absence (Δ) or presence ($\blacktriangle$) of cyclic-AMP/CRP. The concentration of template was 5 nM.

Fig. 5. Dependence of steady-state rates of abortive initiation from the linear P4 promoter on CRP concentration

Concentrations of template, RNA polymerase and cyclic AMP were 5 nM, 100 nM and 0.1 mM respectively, and assays were done under standard conditions.

The sensitivity of different CRP-dependent promoters to cyclic AMP depends on the affinity of the CRP-binding site [28,29]. The CRP site on the P4 promoter is quite weak [17,18], and it would therefore be expected that relatively high concentrations of cyclic AMP would be required to activate the promoter. The effect of varying the cyclic AMP level on the steady-state rate at saturating concentrations of CRP and RNA polymerase is shown in Fig. 6; the mid-point for activation was at a cyclic AMP concentration of 7–8 μ M.

Effect of supercoiling on the P4 promoter

The effect of supercoiling on the strength and activation of the P4 promoter by CRP was investigated using the plasmid pHM1, which was derived from pBR322 by deletion of a region containing the *Ap* promoter which also initiates with the sequence GAU [21]. As a control to estimate background rates, which are much more significant in plasmids than in short DNA fragments, pHM3 was used, in which the P4 promoter was also excised. Attempts to assess the effect of supercoiling in the absence of

Fig. 6. Cyclic AMP-dependence of the activation of the linear P4 promoter

Steady-state rates were determined under standard conditions with the following concentrations: template, 2 nM; RNA polymerase, 100 nM; CRP, 5 nM. The mid-point concentration for cyclic AMP activation was 7–8 μM .

Fig. 7. Steady-state rates of abortive initiation from the supercoiled P4 promoter

Results are shown for pHM1 (2 nM) in the presence (▲) and absence (△) of cyclic-AMP/CRP. Background rates from the control plasmid pHM3 (■) were the same in the presence and absence of cyclic-AMP/CRP.

cyclic-AMP/CRP were unsuccessful because the supercoiled P4 promoter was much less active than the linear form. Steady-state rates of abortive initiation were close to those obtained with the control plasmid lacking the P4 region (Fig. 7), and consequently evaluation of lag times and k_t and K_B parameters was not possible. However, despite this poor activity, the supercoiled promoter was significantly and specifically activated by cyclic-AMP/CRP; Fig. 7 shows the dependence of steady-state rates on RNA polymerase concentration under these conditions, corrected for the background rates. The end point for this titration, obtained by extrapolating the initial part of the curve to the plateau level, is displaced to higher RNA polymerase concentrations than for the linear fragment (Fig. 4). On the basis that RNA polymerase was 25% active in promoter binding, it can be concluded that three or four other promoter sites on pBR322 are competing with P4 for RNA polymerase; this result is consistent with expectation, since the region of pBR322 deleted in pHM1 contained only one of the six promoters on pBR322.

Lag times observed in the presence of cyclic-AMP/CRP were obtained by correcting the reaction progress curves for the control rate on a point-by-point basis. The τ -plot is shown in Fig. 3, and the derived values of k_t and K_B in Table 1. Supercoiling

increased k_t by a factor of 2.5 and decreased K_B about 9-fold; the slope $k_t K_B$ decreased by a factor of about 4, reflecting an apparent weakening effect of supercoiling on the promoter.

DISCUSSION

Strength of the linear P4 promoter

The present studies confirm that the unactivated P4 promoter is weak. The value of $k_t K_B$, corrected for the proportion of active RNA polymerase, was $3.3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, which puts it clearly in the lower range of activity of the promoters considered by Mulligan and co-workers in their empirical relationship between homology score and promoter strength (expressed as the enzyme selectivity $k_t K_B$ [19]). The observed value of $k_t K_B$ is within a factor of 3 of the expected value from their best-fit line for the P4 homology score (47.3), although it is appreciated that there are difficulties in the definition of homology score for promoters such as P4, which lack a clearly defined -35 region. P4 is the weakest of the promoters on pBR322 which have been studied quantitatively; judged by $k_t K_B$ values it appears to be 50–100-fold weaker than *tet* [8,9,21] or *Ap* [21], and considerably weaker than RNA1 (for which there is quantitative disagreement in the literature [20,21]).

Effect of CRP on the linear P4 promoter

The present results show that the effect of cyclic-AMP/CRP on the P4 promoter is confined to K_B and that the rate of isomerization of the closed to open complex is unaffected (Fig. 3 and Table 1). This behaviour resembles that shown by cyclic-AMP/CRP at type I promoters such as *lac* [14,15]. Recent studies on activation of the type II *galPI* promoter show that the kinetic effects of CRP depend critically on the presence or absence of upstream bending sequences [16]. In the presence of three phased adenine-rich sequences centred at -84.5 , -74 and -63 , which cause the DNA to bend by about 60° and to wrap around the CRP/RNA-polymerase complex, CRP does not stabilize the initial attachment of polymerase, but accelerates the isomerization step. In promoters which lack the bending sequences at -84.5 and -74 , so that extensive DNA bending and wrapping does not occur, the effect of CRP is on K_B and not k_t . The interpretation of these findings is that, in the presence of upstream bending sequences, the distortion involved in forming a closed complex with DNA wrapped around CRP and RNA polymerase is energetically costly and thus not assisted by CRP binding, but the distorted structure undergoes isomerization more readily. However, in the absence of upstream bending, a less strained closed complex is formed which is stabilized by CRP, but without any resulting acceleration of isomerization.

The P4 promoter used in the present study was contained on a fragment extending from positions -145 to $+136$. The region upstream of the promoter does not contain multiple phased bending regions as in *gal*, but there is a single T-stretch bending region centred at -65.5 , separated by 55 bp (or 5.25 helical turns, assuming a periodicity of 10.5 bp/helical turn) from the centre of the Pribnow box (Fig. 1). The bending produced by a single A/T-tract (approx. 18° [30]) is not sufficient to allow DNA wrapping around the CRP/RNA-polymerase complex [16], and in addition the separation between the Pribnow box and the T-tract (5.25 turns) would need to be a half-integral number of helical turns (rather than an integral number of turns for an A-tract) for the correct rotational orientation of the curved DNA sequence and efficient activation [31]. The situation for the P4 promoter is therefore analogous to that for *galPI* promoters lacking upstream curved sequences. In agreement with the findings of Lavigne *et al.* [16], we find CRP activation of the P4

promoter to be due to an increase in K_B , with no effect on k_r , although the magnitude of the effect on K_B for P4 (9-fold) is considerably less than that for *gal*/PI (~ 40 -fold).

Sensitivity to cyclic AMP

Individual CRP-dependent promoters are activated at different levels of cyclic AMP concentration both *in vitro* and *in vivo* [29], and there is qualitatively an inverse relationship between the affinity of the DNA-binding site for cyclic-AMP/CRP and the concentration of cyclic AMP required to activate transcription [28,29]. The concentrations of cyclic AMP required to produce half-maximal activation of the *lac*, *gal* and *lac*L8 promoters are about 1.4 μ M, 4 μ M and 10 μ M respectively, paralleling the decrease in CRP binding affinity of the sites (respectively about 1.0, 0.1 and 0.014 relative to the *lac* site). The present data for P4 show that one CRP dimer is required for activation, as in the examples given above (Fig. 4), and the sensitivity of this promoter to cyclic AMP is consistent with the above hierarchy of activation; half-maximal activation occurs at about 7–8 μ M-cyclic AMP, and the relative affinity of CRP binding to the P4 site compared with *lac* is about 0.025 [18].

Effect of supercoiling on the P4 promoter

In the absence of cyclic-AMP/CRP, supercoiling reduced the steady-state rate of abortive initiation from the P4 promoter to such a low level that it could not be analysed reliably. When the promoter was fully activated by cyclic-AMP/CRP, supercoiling reduced the steady-state rate by a factor of 2, but the effect on the unactivated promoter was much more pronounced (Figs. 4 and 7). The explanation for this lack of activity is not clear; supercoiling appears to increase steady-state rates for weak to moderately strong promoters such as *lac*P1, *lac*P2 [32], *lac*P* [33], *tet* and pBR322-RNA1 [9,21], but has little effect on stronger promoters such as *lac*UV5 [32,33] and *lac* Δ 1 [33]. There is a precedent for the observed behaviour of P4, in that linearization of the *bla* (*Ap*) promoter of pBR322 increases the maximal steady-state rate by a factor of 4 [21], but it must be admitted that a reduction in promoter activity on supercoiling (as judged by this criterion) appears unusual.

Supercoiling increases the rate of isomerization (k_r) by a factor of 2.5, but decreases the affinity (K_B) by slightly less than an order of magnitude. By the simple criterion of $k_r K_B$ values, the supercoiled promoter appears to be weaker than the linear form, but this criterion disguises the differential effect of RNA polymerase concentration in the two forms; at higher RNA polymerase concentrations (> 50 nM), the supercoiled promoter forms open complexes more readily than the linear form. Whether such concentrations of free RNA polymerase exist in the cell is unclear; the stoichiometric concentration of RNA polymerase in the *E. coli* cell is about 3 μ M, but the vast majority of this is either actively involved in RNA synthesis or is specifically or non-specifically bound to DNA [34].

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