

# Dissecting the functional program of *Escherichia coli* promoters: the combined mode of action of Lac repressor and AraC activator

Rolf Lutz, Tomasz Lozinski, Thomas Ellinger<sup>1</sup> and Hermann Bujard\*

Zentrum für Molekulare Biologie der Universität Heidelberg, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany and <sup>1</sup>ClonDIAG GmbH, Jena, Germany

Received May 29, 2001; Revised and Accepted July 24, 2001

## ABSTRACT

**The mode of action of regulated promoters is largely determined by kinetic parameters which govern the interaction between promoters and proteins involved in induction and repression of transcription. To gain insight into the interplay between positively and negatively acting transcriptional regulators, in this case AraC and LacR, we have generated a panel of promoter sequences derived from P<sub>lac</sub>, the promoter of the *Escherichia coli lac* operon. The function of these promoters is limited at different steps and to various extents within the pathway of RNA polymerase (RNAP)/promoter interaction. Moreover, in all promoters the cAMP receptor protein binding site was replaced by the binding motif of AraC to prevent pleiotropic effects *in vivo* upon activation. Analyzing the activation of these promoters by AraC *in vivo* under conditions of repression by LacR and derepression yielded a three step model of transcription initiation which reveals mechanisms of AraC and LacR action. Our data show three distinct rate limiting steps at which AraC can exert its function. In general, the activator accelerates the formation of the first stable complex between RNAP and promoter. At most promoter sequences, however, its main impact is on the conversion of the closed to the open complex. However, AraC is also capable of eliminating limitations at steps following open complex formation.**

## INTRODUCTION

To start transcription in *Escherichia coli* an RNA polymerase (RNAP) molecule has to follow a multistep pathway. The most prominent steps of this pathway are formation of a closed complex (RP<sub>c</sub>), isomerization of this complex into an open complex (RP<sub>o</sub>), initiation of RNA synthesis and finally, upon formation of a ternary RNA producing complex, clearance of the promoter region (RP<sub>ti</sub>) (reviewed in 1–3). The rate at which

productively transcribing complexes clear the promoter region defines promoter strength (4). In the *E. coli* system promoters differ not only in strength by orders of magnitude (5), their activity is generally regulated by various positively and/or negatively acting elements (reviewed in 6). Promoters, which are controlled by regulatory proteins, have undergone an adaptation process which has made their interaction with RNAP susceptible to regulation. For example, promoters that are controlled by activators must encode one or several rate limiting steps within their functional program in order to be amenable to activation. *A priori*, any step within the transcription initiation process may be rate limiting and thus constitute a target for activator proteins (2). On the other hand, effective control of promoter activity by repressors requires an optimization as well. Thus, repressors that compete directly with RNAP for their mutual binding sites are most effective when the respective promoter sequence is recognized by RNAP relatively slowly (7). As the transcriptional activity of many operons is governed by more than one physiological parameter, the functional program of the respective promoters is optimized accordingly.

The promoter of the *E. coli lac* operon, P<sub>lac</sub>, is controlled negatively by the Lac repressor (LacR) and positively by the cAMP receptor protein (CRP), a principle which conveys a high degree of regulation to the *lac* operon *in vivo*. How is the susceptibility of P<sub>lac</sub> towards these two regulatory proteins brought about? Inspection of the P<sub>lac</sub> sequence reveals interesting deviations from consensus promoter sequences as derived by Mulligan *et al.* (8) and Harley and Reynolds (9). They concern base pair changes in both highly conserved hexamers at –10 and –35 (+1 being the first nucleotide transcribed) as well as in the distance between the two hexamers. Can these sequence deviations be correlated with specific properties that make P<sub>lac</sub> controllable and, if so, which steps in the RNAP promoter interaction are affected?

To approach these questions, we have converted P<sub>lac</sub> stepwise into a sequence with a high homology score [as defined by Mulligan *et al.* (8)], thereby generating a panel of promoters with distinctly altered functional programs. Moreover, we have replaced the CRP binding site of these promoters with the target sequence of the activator protein AraC to avoid the pleiotropic effects characteristic of CRP–cAMP action *in vivo*.

\*To whom correspondence should be addressed. Tel: +49 6221 54 82 14; Fax: +49 6221 54 58 92; Email: h.bujard@zmbh.uni-heidelberg.de

Present address:

Tomasz Lozinski, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

The resulting set of promoters has allowed us to measure *in vivo* the effect of AraC binding on the activity of promoters encoding different functional programs. We were able to discriminate between the influence of the activator on the formation and release of open complexes by analyzing *in vivo* KMnO<sub>4</sub> footprints. Moreover, the impact of the activator on early or late steps of RNAP/promoter interaction was revealed by monitoring AraC stimulation under conditions of repression and derepression of P<sub>lac</sub> via LacR. Our results show that activators like AraC are capable of releasing at least three different rate limiting steps within the pathway of transcription initiation. Moreover, they provide new insights into the role of LacR on the kinetics of RNAP/promoter interaction. In particular, we show that LacR decreases the forward rate constant of all promoters of a *lac*-type architecture by a defined increment. Thus, these studies contribute equally to our understanding of how transcriptional regulator proteins function and how functional programs of regulated promoters are optimized.

## MATERIALS AND METHODS

### DNA, plasmids and *E.coli* strains

The regulatory regions containing promoter/operator sequences of the *lac* operon as well as the binding site for AraC (I<sub>1</sub> and a portion of I<sub>2</sub>) were assembled from synthetic oligonucleotides via the cleavage sites *Xho*I, *Hind*III, *Hinf*I and *Eco*RI, respectively (Fig. 1). After insertion into pDS12Luci (10,11), all sequences were verified according to Sanger *et al.* (12). In pDS12Luci, these regulatory regions control transcription of the *luciferase* gene of *Photinus pyralis* (13). For *in vivo* studies, plasmids were transferred into *E.coli* strains DH5 $\alpha$  (araC<sup>+</sup>, lacI<sup>-</sup>) or DH5 $\alpha$ Z1 (araC<sup>+</sup>, lacI<sup>q</sup>) (14). For footprint analysis with KMnO<sub>4</sub>, *E.coli* strains TR322 (araC<sup>+</sup>, lacI<sup>q</sup>) and TR321 (araC<sup>-</sup>, lacI<sup>q</sup>) (15) were used in addition.

### Determination of *in vivo* promoter activities

Promoter activities were determined by monitoring luciferase activity *in vivo*. Overnight cultures of *E.coli* cells DH5 $\alpha$  or DH5 $\alpha$ Z1 respectively harboring plasmids of the pDS12Luci family which carried the respective promoters were grown at 37°C in LB and diluted 1:100 in the same medium (100  $\mu$ g/ml ampicillin) either in the presence or absence of 0.2% L(+)-arabinose. After 3 h, the OD<sub>600</sub> was measured and the cultures were kept at room temperature for 15–20 min. Aliquots of 100  $\mu$ l were removed from the cultures, 100  $\mu$ l of luciferin solution (125  $\mu$ M) was added and luciferase activity was measured (10 s, delay 0 s) in a Lumat type LB9501 (Berthold, Germany). Activities are given as relative light units (RLU) after subtraction of the instrumental background and normalization to the number of viable cells (10).

### Kinetics of RNAP/promoter complex formation *in vitro*

Kinetics of complex formation between RNAP and promoters were measured in competition assays as described (5). Equal amounts of <sup>32</sup>P-end-labeled DNA fragments (~1000 c.p.m./promoter/binding reaction) of different lengths carrying different promoters were incubated with decreasing amounts of RNAP under standard conditions (25 mM Tris-HCl, pH 7.9, 100 mM KCl, 10 mM MgCl<sub>2</sub>, 2 mM DTT, 0.2 mM EDTA, 2.5% glycerol) for 5 min at 37°C. After addition of 100-fold

excess of M13 ssDNA as competitor for 90 s, specific complexes were monitored by nitrocellulose adsorption followed by electrophoretic analysis of the retained DNA in 6% PAA gels (46% urea, 1 $\times$  TBE). At each RNAP concentration the fraction of stable RNAP/promoter complexes was quantified. The rate constants were determined from the fraction of bound RNAP/promoter complexes as described previously (5). RNAP holoenzyme was prepared by J. Gamer in our group using the method described by Burgess and Jendrisak (16). The concentration of transcriptionally active RNAP was 2 pmol/ $\mu$ l.

### Kinetics of RNAP/promoter complex dissociation *in vitro*

The stability of RNAP/promoter complexes *in vitro* was measured according to Brunner and Bujard (5). End-labeled DNA fragments carrying the synthetic promoters were incubated with a 10-fold molar excess of RNAP in the presence of 50 mM KCl for 10 min at 37°C. At time point 0 a 100-fold excess of competitor M13 ssDNA was added and the incubation was continued for another 90 s before aliquots of the reaction mixture were withdrawn at defined time intervals and subjected to filtration through nitrocellulose filters. The radioactivity retained on the filters was quantified in a scintillation counter and plotted versus time.

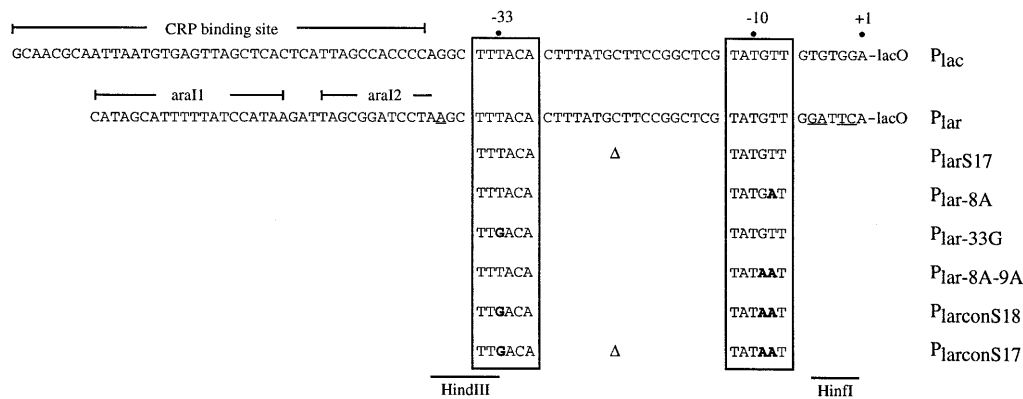
### *In vivo* KMnO<sub>4</sub> footprint analysis

KMnO<sub>4</sub> footprints were carried out essentially as described previously (17,18). *Escherichia coli* strains TR322, TR321 (19) or DH5 $\alpha$  carrying the various plasmids were grown to mid-log phase in M9 minimal medium supplemented with 0.5% glycerol (w/v), 0.2% casaminoacids (w/v), thiamine (0.05 mg/ml) and ampicillin (100  $\mu$ g/ml). Whenever needed, L(+)-arabinose was added to a final concentration of 0.2%. Oxidation reactions were performed by adding 150  $\mu$ l of KMnO<sub>4</sub> (370 mM) to 10 ml of the bacterial culture for 2 min. Whenever required, 40  $\mu$ l of rifampicin (50 mg/ml in methanol) was added 5 min prior to the addition of KMnO<sub>4</sub>. The reaction was terminated by placing the bacterial cultures in Corex-tubes chilled to 0°C. Cells were harvested by spinning the tubes at 5000 g for 5 min. Plasmid DNA was isolated according to Birnboim and Doly (20). Around 0.7  $\mu$ g of DNA was subjected to a primer extension reaction by Klenow enzyme using a <sup>32</sup>P-end-labeled primer (5'-GGATAGAATGGCGCCGGGCC-3'), which annealed ~100 bp downstream of the transcriptional start site. Primer extension products were subjected to gel electrophoresis in a 6% PAA sequencing gel and made visible by autoradiography. The hypersensitive bands were quantitated densitometrically and normalized versus a region located outside the open complex as described (17).

## RESULTS

### Construction of variant sequences of P<sub>lac</sub>

To carry out functional studies with a highly defined set of promoter sequences, where the individual promoters would differ only by defined alterations, we synthesized first the regulatory region of the *E.coli lac* operon spanning from -81 to +20, however, with 5 bp exchanges (Fig. 1). Four alterations at positions -1, -2, -4 and -5 of P<sub>lac</sub> introduced a *Hinf*I cleavage site between the -10 region and the transcriptional start site. A



**Figure 1.** The sequences of  $P_{lac}$  including the CRP binding site and the  $P_{lar}$  variants fused to  $araI_2$ . The sequences are aligned at the conserved hexamers which are boxed and the transcriptional start site (+1). Restriction sites *Xho*I (upstream of the promoters) and *Eco*RI (downstream of the promoters) were used to clone the sequence variants into the pDS12Luci plasmid system (10,11). For exchanging the core promoter sequences (position +1 to -36) and for creating the  $P_{lar}$  derivatives, a *Hind*III cleavage site upstream of the -35 region and a *Hinf*I cleavage site downstream of the -10 region were generated. The base pair exchanges resulting in the respective cleavage sites are underlined. In the core regions of the  $P_{lar}$  variants only the bases of the conserved hexamers are shown. Bases which deviate from  $P_{lar}$  are printed in bold.  $\Delta$  indicates the deletion of a CG base pair in the spacer at position -22. The LacR binding site is designated *lacO*.  $araI_2$  indicates the binding site of AraC. To place the primary AraC binding site  $araI_2$  in an upstream position corresponding to the *araBAD* promoter and to maintain at the same time the sequence of the -35 hexamer of  $P_{lac}$  the  $araI_2$  binding site was shortened by 5 bp.

**Table 1.** Kinetic *in vitro* parameters and *in vivo* promoter activities of  $P_{lar}$  variants

Promoter	Homology score (%)	$k_{ON}$ ( $\times 10^7 M^{-1} s^{-1}$ )	$t_{1/2}$ (min)
$P_{lar}$	58	0.08	18
$P_{larS17}$	63	0.20	48
$P_{lar-8A}$	61	0.24	56
$P_{lar-33G}$	64	0.18	54
$P_{lar-8A-9A}$	67	1.5	80
$P_{larconS18}$	74	4.5	27
$P_{larconS17}$	81	20	105

The homology scores were calculated according to Mulligan *et al.* (8). Forward rate constants and dissociation constants between RNAP and promoters were analyzed *in vitro* by filter binding assays as described (5).

single G→A transition at position -39 created a *Hind*III site partly overlapping the -35 region. By utilizing the *Hinf*I and *Hind*III sites the core region of the promoter could be readily replaced by altered sequences. In particular, the *Hind*III site was used to fuse the  $araI_2$  site to  $P_{lac}$  centered around position -53 as found in the  $P_{BAD}$  promoter of the *ara* operon (21). The resulting promoter  $P_{lar}$  exhibited identical *in vitro* and *in vivo* properties ( $k_{ON}$ ,  $t_{1/2}$  and promoter strength) compared to  $P_{lac}$  (data not shown). By substituting 1 bp within the -10 and the -35 region and by deleting 1 bp within the spacer, the sequence of  $P_{lar}$  was stepwise converted towards consensus (Fig. 1). This was reflected by the homology score (8) calculated for the different constructs which increase from 58% for  $P_{lar}$  to 81% for  $P_{larconS17}$  (Table 1). Primer extension analysis of mRNAs produced from the various promoters revealed that the sequence changes did not alter the transcriptional start sites (data not shown), i.e. all  $P_{lar}$  constructs gave rise to identical

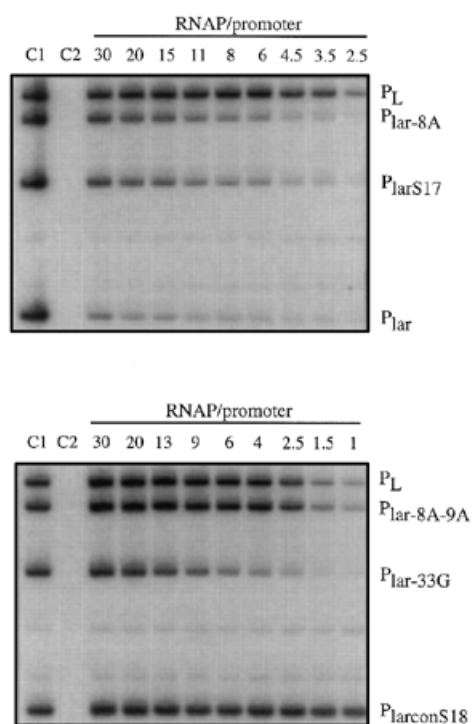
transcripts. Therefore, measurement of luciferase activity *in vivo* directly reflected promoter activity.

#### Effect of sequence alterations on the *in vitro* properties of $P_{lar}$

We have shown previously that rates of complex formation between RNAP and promoters measured *in vitro* reflect well the situation prevailing *in vivo* (7). Therefore, forward rate constants for all promoters under study were determined. As all promoters formed stable complexes ( $t_{1/2} \geq 18$  min; Table 1) the forward rate constants ( $k_{ON}$ ) could be determined under pseudo-first order conditions (Fig. 2 and Table 1). Single base pair changes introduced into the core region of  $P_{lar}$  between +1 and -35 affected the homology scores and the  $k_{ON}$  values in the same sense, which is in good agreement with earlier observations (2,5,18). Thus, generating  $P_{larS17}$ ,  $P_{lar-8A}$  or  $P_{lar-33G}$  increased the homology score of all three sequences by 3–6 U resulting in a 2–3-fold increase in the rate of complex formation. When such single sequence alterations were combined more drastic effects were observed.  $P_{lar-8A-9A}$  exhibits a  $k_{ON}$  value ~14 times higher than  $P_{lar}$  and binds RNAP four times more stably. As expected, the consensus-like promoter  $P_{larconS17}$  shows the highest homology score and exhibits a  $k_{ON}$  value that is close to the highest value of  $30 \times 10^7 M^{-1} s^{-1}$  reported for  $P_{N25}$  (5). The increase in  $k_{ON}$  values at  $P_{larS17}$  compared to  $P_{lar}$  and at  $P_{larconS17}$  compared to  $P_{larconS18}$ , respectively, is due to the differences in spacer length. The same phenomenon was observed at the consensus-like promoters TAC17 and TAC18, respectively (22).

#### Intrinsic promoter strength and activation potential of AraC

We next examined how the individual alterations in our panel of promoters affected (i) the intrinsic strength, i.e. the non-activated and derepressed state of the  $P_{lar}$  variants, and (ii) the activation potential of AraC. To exclude any influence of AraC on promoter activity in the absence of arabinose we first determined the various promoter activities in the  $araC^+$  strain



**Figure 2.** Relative rates of complex formation between RNAP and  $P_{lar}$  variants. A mixture of  $^{32}P$ -end-labeled DNA fragments carrying the various promoters was incubated under standard conditions with RNAP at the molar ratios indicated. Specific complexes were recovered by nitrocellulose filter adsorption and subjected to PAGE. The intensity of the bands was analyzed via a phosphoimager. The relative rate constants for complex formation were derived by plotting the fraction of unoccupied promoters  $1 - F_p$  versus the corresponding fractions of  $P_L$  from phage Lambda which served as a standard promoter as described (5). Lane C1, an aliquot of the reaction mixture that was not subjected to filtration; lane C2, competitor DNA was added prior to RNAP.  $P_{larconS17}$  is not included in this figure but was measured analogously.

TR322 in the absence of arabinose. The data obtained were indistinguishable from those obtained in  $araC^-$  strain TR321 (data not shown; 19) indicating that AraC activation could be completely abolished by omitting arabinose from the growth medium. Therefore, promoter activities were determined in  $araC^+$  strains DH5 $\alpha$  or DH5 $\alpha$ Z1. The data provided in Table 2 show that the intrinsic strengths of the  $P_{lar}$  variants differ over an almost 600-fold range and increase to different degrees when  $P_{lar}$  is mutated towards consensus. Interestingly, the mutations leading to promoters  $P_{larS17}$ ,  $P_{lar-8A}$  and  $P_{lar-33G}$  affect their *in vitro* properties almost identically. By contrast, the *in vivo* promoter strength of  $P_{larS17}$  gives rise to only one-fifth to one-sixth of the other two constructs indicating that  $P_{larS17}$  is limited at a step following RNAP binding. The two consensus-like promoters  $P_{larconS18}$  and  $P_{larconS17}$  exhibit a nearly 600-fold higher promoter activity than  $P_{lar}$  and thus are the strongest promoters in this family. They nevertheless differ 4-fold in their rate of RNAP binding, indicating that  $P_{larconS18}$  is not limited at this level. By contrast,  $P_{lar-8A-9A}$  initiates productive transcription with a 6-fold lower rate than the consensus-like promoters but appears nevertheless unlimited in RNAP binding for reasons addressed in the Discussion. Its limitation must therefore occur at steps following this event. The data

**Table 2.** Promoter activities of  $P_{lar}$  variants in *E. coli* DH5 $\alpha$  in the presence or absence of 0.2% arabinose

Promoter	Promoter activity		Activation factor
	-arabinose	+arabinose	
$P_{lar}$	$1.1 \pm 0.2$	$77 \pm 8$	70
$P_{larS17}$	$5.2 \pm 0.8$	$303 \pm 31$	58
$P_{lar-8A}$	$25 \pm 2$	$478 \pm 81$	19
$P_{lar-33G}$	$29 \pm 3$	$225 \pm 23$	7.8
$P_{lar-8A-9A}$	$88 \pm 10$	$578 \pm 84$	6.6
$P_{larconS18}$	$595 \pm 80$	$605 \pm 122$	1.0
$P_{larconS17}$	$591 \pm 92$	$608 \pm 105$	1.0

Luciferase activities, which were determined as described in Materials and Methods, are given as RLU/cell  $\times 10^4$  and are the mean values of five independent experiments.

provided in Table 2 show that the susceptibility of the various promoters for activation by AraC is highly dependent on the sequence context within the core region. Whereas  $P_{lar}$  is activated  $\sim 70$ -fold by AraC, the activator has no measurable effect on the activity of the two consensus-like promoters ( $P_{larconS17}$  and  $P_{larconS18}$ ). Particularly interesting differences are observed in comparing promoters  $P_{larS17}$ ,  $P_{lar-8A}$  and  $P_{lar-33G}$ , which all bind RNAP with about the same efficiency and with comparable stability (Table 1).  $P_{larS17}$ , which exhibits a 5–6-fold lower promoter strength than  $P_{lar-8A}$  and  $P_{lar-33G}$ , is activated by  $\sim 60$ -fold whereas the activity of  $P_{lar-8A}$  and  $P_{lar-33G}$  is increased only 19-fold and 8-fold, respectively (Table 2). Therefore, AraC must abolish a rate limitation of  $P_{larS17}$ , which follows the formation of the first stable complex. Comparing the stimulation of  $P_{lar-8A}$  and  $P_{lar-33G}$ , two promoters with practically the same intrinsic promoter strength and the same *in vitro* RNAP binding properties, reveals that the latter promoter cannot be activated to the same extent as the former. Like  $P_{lar}$  it must therefore contain a limitation which cannot be overcome by AraC. Interestingly, even if activated by AraC,  $P_{lar}$  reaches only about one-eighth of the promoter strength of the consensus-like promoters demonstrating that AraC cannot fully compensate for all rate limitations encoded in this promoter sequence. The same holds true for  $P_{larS17}$  and  $P_{lar-33G}$  although to a lesser extent (Tables 1 and 2). By contrast, under conditions of activation,  $P_{lar-8A}$  and  $P_{lar-8A-9A}$  nearly reach the activity of the consensus-like promoters.

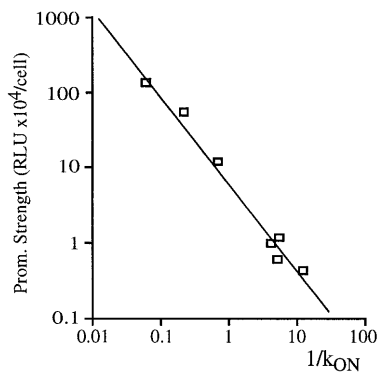
#### Activation of $P_{lac}$ variants under conditions of repression

To determine the impact of AraC on the overall activation potential and in particular on RNAP binding in the presence of LacR promoter activities of the  $P_{lar}$  variants were also measured in strain DH5 $\alpha$ Z1 (Table 3) either in the presence or absence of arabinose. Plotting promoter activities of the repressed but non-activated  $P_{lar}$  variants versus the respective  $k_{ON}$  values obtained *in vitro* revealed an almost perfect inverse correlation (Fig. 3). By contrast, in the non-repressed state, the  $k_{ON}$  values and the activities of the promoters correlate only qualitatively (Table 2), in accordance with previous studies (5,18,23,24). From this correlation we conclude that under conditions of repression  $k_{ON}$  becomes the major rate limiting step at all

**Table 3.** Activitability of  $P_{lar}$  variants in *E.coli* DH5 $\alpha$ Z1

Promoter	Promoter activity		Activation factor
	-arabinose	+arabinose	
$P_{lar}$	0.4 ± 0.1	2.6 ± 0.3	6.5
$P_{larS17}$	0.6 ± 0.1	12 ± 3	20
$P_{lar-8A}$	1.1 ± 0.2	23 ± 2	21
$P_{lar-33G}$	1.2 ± 0.4	10 ± 1	8.5
$P_{lar-8A-9A}$	12 ± 2	85 ± 6	7.1
$P_{larconS18}$	57 ± 9	445 ± 73	7.8
$P_{larconS17}$	145 ± 35	450 ± 92	3.1

All details are essentially as described in Table 2. DH5 $\alpha$ Z1 harbors a chromosomal copy of the *lacI<sup>q</sup>* gene (14).

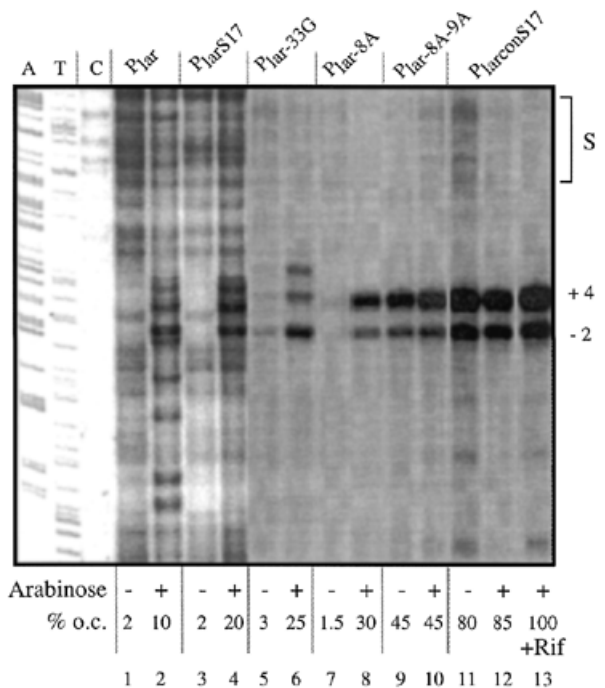


**Figure 3.** Correlation between repressibility, i.e. promoter strength under conditions of repression and forward rate constants ( $k_{ON}$ ) between RNAP and  $P_{lar}$  variants. The promoter activities of the non-activated *lac* promoter variants measured in the repressed state were plotted logarithmically versus the inverse of the respective  $k_{ON}$  values.

promoters. Accordingly, the AraC-mediated promoter activation in the presence of LacR has to be primarily attributed to an increase in  $k_{ON}$ . Thus, unlike in the non-repressed state, AraC is able to increase the rates of complex formation with RNAP at all  $P_{lar}$  variants under conditions of repression by LacR. This becomes most obvious with the consensus promoters  $P_{larconS18}$  and  $P_{larconS17}$  where a 8-fold and 3-fold activation by AraC is observed in the repressed but none in the derepressed state (Tables 2 and 3). Likewise, the activation factors obtained at  $P_{lar}$  and  $P_{larS17}$  differ significantly under conditions of repression and derepression. In the presence of LacR these promoters are activated by only 6-fold and 20-fold, respectively, by AraC compared to a 70-fold and 58-fold activation in the derepressed state. This result implies that besides increasing  $k_{ON}$ , AraC also accelerates at least one subsequent kinetic step at these promoters. Finally,  $P_{lar-8A}$ ,  $P_{lar-33G}$  and  $P_{lar-8A-9A}$  are activated to roughly the same extent either in the presence or absence of LacR (Tables 2 and 3).

**KMnO<sub>4</sub> *in vivo* footprinting of  $P_{lar}$  variants in the presence and absence of AraC**

To distinguish between rate limitations that affect either the formation of open complexes or their release we performed



**Figure 4.** *In vivo* KMnO<sub>4</sub> footprint of the non-template strands of  $P_{lar}$  variants. Promoters  $P_{lar-8A}$ ,  $P_{lar-33G}$ ,  $P_{lar-8A-9A}$  and  $P_{larconS17}$  were footprinted either in strain TR321 (*araC*<sup>-</sup>) in the absence of arabinose or in strain TR322 (*araC*<sup>+</sup>) in the presence of 0.2% arabinose and 1 mM IPTG. Footprints of promoters  $P_{lar}$  and  $P_{larS17}$  were obtained in strain DH5 $\alpha$  in the absence or presence of 0.2% arabinose. Control experiments revealed no difference in the footprint patterns between these strains (data not shown). The hypersensitive signals reminiscent of single-stranded DNA regions indicate open complexes around the transcriptional start site (-4 to +2). To calculate the fraction of open complexes, rifampicin was added to the footprint reaction of  $P_{larconS17}$  in order to trap all available DNA in the open complex (lane 13). This intensity was set as 100% and served as reference for all other footprint signals for the calculation of the fraction of promoters that are open and available to react with KMnO<sub>4</sub> (17,18). The footprint intensities were normalized to an internal standard (S) to allow their direct comparison as described (17). In lanes A and T sequencing reactions of  $P_{lar-8A-9A}$  using the same primer as used for the primer extension reactions are shown. Lane C shows a primer extension reaction of promoter  $P_{lar-8A}$  which was modified *in vitro* in the absence of RNAP.

permanganate footprints of the  $P_{lar}$  variants *in vivo* under steady state conditions either in the presence or absence of arabinose (Fig. 4). In general, KMnO<sub>4</sub> footprints visualize the fraction of promoters where RNAP has formed an open complex during the time of permanganate treatment. The intensity of the footprint signal correlates with the amount of time a promoter is open and available to react with KMnO<sub>4</sub> (18). Strong footprint signals reflect limitations in RNA chain initiation and/or release of open complexes. At the  $P_{lar}$  variants some hyper-reactive bands typically occurring at *lac*-type promoters show up at positions +4 to -2 (Fig. 4). To calculate the fraction of open complexes, rifampicin was added to the footprint reaction of  $P_{larconS17}$  (Fig. 4, lane 13). Due to the high  $k_{ON}$  value and the high activity of this promoter we can assume that all available DNA is trapped in the open complex after 5 min of rifampicin treatment. Compared to  $P_{larconS17}$  footprint intensities at  $P_{lar}$ ,  $P_{larS17}$ ,  $P_{lar-8A}$  and  $P_{lar-33G}$  are very weak in the absence of arabinose. Only 1–3% (Fig. 4, lanes 1, 3, 5 and 7) of

these promoters are engaged in open complexes under steady state conditions. Thus, transcription from these promoters is limited in the formation of open complexes. By contrast, promoters  $P_{\text{lar-8A-9A}}$  and  $P_{\text{larconS17}}$  are engaged in open complexes for ~45 and 85% of the initiation time, respectively, indicating that both promoters are limited on the release of open complexes. Interestingly, the footprint pattern of these two promoters did not change, irrespective of whether AraC was active or not (Fig. 4). In the case of  $P_{\text{lar-8A-9A}}$ , activation by AraC resulted in a ~7-fold activation of the overall transcription process (Table 2) as well as a 7-fold increase in  $k_{\text{ON}}$  (Table 3). The unchanged footprint intensity therefore suggests that the overall activation is equally composed of an acceleration of both formation and release of open complexes. Likewise, we have demonstrated that AraC increases  $k_{\text{ON}}$  at  $P_{\text{larconS17}}$  (Table 3); however, since initial binding of RNAP to this promoter is not the rate limiting step the footprint intensity remains the same in the presence of AraC. In contrast to  $P_{\text{lar-8A-9A}}$  and  $P_{\text{larconS17}}$ , AraC increased the footprint signals at  $P_{\text{lar}}$  (5-fold),  $P_{\text{larS17}}$  (10-fold),  $P_{\text{lar-33G}}$  (8-fold) and  $P_{\text{lar-8A}}$  (20-fold). This demonstrates that AraC accelerates the formation of open complexes (Fig. 4); however, under these conditions a new rate limiting step becomes apparent at the release of the open complex.

#### Calculating the kinetics of transcription initiation

The results obtained in this study allow us to dissect the onset of transcription *in vivo* of  $P_{\text{lar}}$  and its variants into three distinct steps and to estimate the time required to reach each of these steps:  $t_{\text{RPc}}$  for the formation of the closed complex,  $t_{\text{RPo}}$  for the isomerization of the closed into the open complex and  $t_{\text{RPcl}}$  for the release of the open complex followed by mRNA initiation and promoter clearance. We calculate the real times required for RNAP to proceed to the various steps based on the following considerations. (i)  $P_{\text{lacL8UV5}}$ , the core sequence of which is identical to  $P_{\text{lar-8A-9A}}$ , initiates a productive transcript every ~30 s (2). This initiation time was taken as reference point to calculate the time required for starting a productive transcription ( $t_{\text{pt}}$ ) at  $P_{\text{lar}}$  and its variants using their *in vivo* activities as compiled in Tables 2 and 3. (ii) Quantitation of the footprint intensities around the transcription start point enabled us to estimate the time periods that the various promoters were open or closed (17) in the absence or presence of arabinose, reconciling that in the 'unmelted' state promoters might be either unoccupied or occupied by RNAP while engaged in a closed complex (RP<sub>c</sub>). (iii)  $k_{\text{ON}}$  describes the kinetics of stable and transcription-competent RNAP/promoter complex formation which irreversibly removes RNAP from the equilibrium. This complex is capable of prohibiting LacR from binding to its operator site. To calculate the time for RNAP binding to  $P_{\text{larconS17}}$ , we used  $P_{\text{spc}}$ , the promoter of the *spc* ribosomal protein operon, as a reference. The latter promoter binds RNAP every 0.4 s under growth conditions comparable to those used in this study (25). Since  $P_{\text{spc}}$  exhibits a lower homology score and thus presumably lower  $k_{\text{ON}}$  value compared to  $P_{\text{larconS17}}$  we assume that RNAP binding to  $P_{\text{larconS17}}$  occurs within 0.2 s. The times required to form RNAP/promoter complexes at the other  $P_{\text{lar}}$  variants were calculated based on the respective  $k_{\text{ON}}$  values. (iv) Under conditions of repression the activities of the  $P_{\text{lar}}$  variants depend primarily on the rate of complex formation with RNAP indicating that  $k_{\text{ON}}$

**Table 4.** Kinetics of RNAP/promoter interaction at various  $P_{\text{lar}}$  variants under different conditions of repression and/or activation

promoter	AraC	LacR	$t_{\text{RPc}}$	$t_{\text{RPo}}$	$t_{\text{RPcl}}$	$t_{\text{pt}}$
$P_{\text{lar}}$	i	-	50	2330	50	2430
		+	3700			6080
	a	-	10	20	2	32
	+	778	800			
$P_{\text{larS17}}$	i	-	20	450	10	480
		+	3540			4000
	a	-	1	5	2	8
	+	193	200			
$P_{\text{lar-8A}}$	i	-	15	83	2	100
		+	2315			2400
	a	-	0.8	2	2	5
	+	121	125			
$P_{\text{lar-33G}}$	i	-	20	58	2	80
		+	1940			2000
	a	-	2	7	2	11
	+	236	245			
$P_{\text{lar-8A-9A}}$	i	-	3	14	14	30
		+	172			200
	a	-	0.4	2	2	4
	+	26	30			
$P_{\text{larconS17}}$	i	-	0.2	0.5	3	4
		+	15			18
	a	-	0.04	0.5	3	4
	+	3	6			

'a' indicates that AraC is active, 'i' indicates that AraC is inactive. '+' indicates the repressed state, '-' indicates the non-repressed state.  $t_{\text{RPc}}$ , time required for closed complex formation;  $t_{\text{RPo}}$ , time required for open complex formation;  $t_{\text{RPcl}}$ , time required for RNA initiation and promoter clearance;  $t_{\text{pt}}$ , time required for starting a productive transcription. 'R' denotes RNAP and 'P' denotes promoter. All initiation times and  $t_{\text{RPc}}$  of the unregulated promoters were calculated from the promoter activities and  $k_{\text{ON}}$  values.  $t_{\text{RPcl}}$  equals the time the promoters are open and available to react with  $\text{KMnO}_4$ . It was calculated on the basis of the footprint intensities either in the presence or absence of arabinose. AraC's influence on  $t_{\text{RPc}}$  was derived from the activation factors determined under conditions of repression (+LacR). At  $P_{\text{larconS17}}$  the AraC-mediated increase in  $k_{\text{ON}}$  is ~2-fold higher than its overall impact on promoter activity since the time required to release the open complex contributes significantly to the overall transcription process. All values are given in seconds and are rounded up to their closest power. The error is up to 30%. See Discussion for further details.

becomes the major rate limiting step and the main determinant of the overall promoter activity (Fig. 3). Interestingly, the same correlation can be found at a series of synthetic promoters of the same architecture but different core sequences (7). The observation is also in good accordance with data provided by Schlax *et al.* (26) who demonstrated that LacR acts by decreasing the apparent rate of complex formation between RNAP and  $P_{\text{lacUV5}}$  but leaving later kinetic steps unaffected. Based on this reasoning,  $t_{\text{RPc}}$ ,  $t_{\text{RPo}}$ ,  $t_{\text{RPcl}}$  and  $t_{\text{pt}}$  were calculated for, for example,  $P_{\text{lar-8A}}$  as follows (Table 4).  $P_{\text{lar-8A}}$  gives rise to one functional transcript every 100 s. According to the footprint data the release of the open complex ( $t_{\text{RPcl}}$ ) takes ~2 s.  $t_{\text{RPc}}$ , which we derive from  $k_{\text{ON}}$ , equals 16 s. Given these values,  $t_{\text{RPo}}$  can be calculated according to  $t_{\text{RPo}} = t_{\text{pt}} - t_{\text{RPc}} - t_{\text{RPcl}}$ , which is 83 s. LacR action increases  $t_{\text{pt}}$  to ~2400 s. Activation of  $P_{\text{lar-8A}}$  by

AraC results in a 20-fold acceleration of  $t_i$  (from 100 to 5 s). At the same time the footprint indicates that the promoter is engaged in an open complex for ~35% of the respective time. Taken together these data suggest that  $t_{RP_{cl}}$  remains practically unchanged compared to the non-activated situation. Under conditions of repression AraC's impact on  $k_{ON}$  is 20-fold (accelerating  $t_{RP_c}$  from 2315 to 121 s). We can therefore calculate that AraC accelerates  $t_{RP_c}$  in the non-repressed state from 15 to ~0.8 s. Using the same term as above it follows that AraC accelerates  $t_{RP_0}$  from 83 to 2 s.

## DISCUSSION

Based on the sequence of  $P_{lac}$  of the *lac* operon and the topography of  $P_{BAD}$  of the *ara* operon we have generated a panel of hybrid promoters which were analyzed *in vitro* and *in vivo*. Replacing the CRP binding site with the recognition sequence for AraC enabled us to study promoter activation *in vivo* highly specifically as, unlike CRP, AraC does not produce relevant pleiotropic effects. We are nevertheless confident that the data obtained with the panel of  $P_{lar}$  hybrid promoters describe a paradigmatic situation particularly since a limited set of analogous experiments with  $P_{lac}$  and CRP confirm our findings with  $P_{lar}$  (27). The seven  $P_{lar}$  variants investigated behaved identically with regard to selection of the transcription start point. Moreover, the correlation found between their homology scores and the forward rate constants of polymerase binding was predictable and in good accordance with previously published reports (5,18). By contrast, no correlation was found between the levels of transcription and the match of the conserved hexamers to consensus. This lack of predictability, which has originally been described for  $P_L$  of phage Lambda (23), indicates that the functional program of the  $P_{lar}$  variants has been altered at different levels and to various extents. The set of promoters appeared therefore suited to investigate the impact of regulatory proteins such as LacR and AraC on the RNAP/promoter interaction.

### Rate limitations of the $P_{lar}$ variants

The single base pair changes introduced into the spacer, the -10 region or the -35 region of  $P_{lar}$ , generating promoters  $P_{larS17}$ ,  $P_{lar-8A}$  and  $P_{lar-33G}$ , had only minor and rather predictable effects on RNAP binding. Although these promoters bind RNAP relatively slowly our calculations indicate that none of the promoters is limited by  $k_{ON}$ . However, since the rates of complex formation did not correlate with the respective promoter activities, these promoters are obviously limited to different extents at steps following stable complex formation. The permanganate footprints indicate that their major rate limitation occurs at open complex formation. The times required to release the open complexes also differ significantly among these promoters: from ~50 s at  $P_{lar}$  to ~2 s at  $P_{lar-8A}$  and  $P_{lar-33G}$ . These values, however, are somewhat imprecise as the rather weak footprint signals obtained in the absence of AraC were not optimal for quantitation. At  $P_{lar-8A-9A}$  formation and release of open complexes contribute equally to the overall promoter activity. It has been shown previously (28,29) that the closely related  $P_{lacUV5}$  is not limited at the level of RNAP binding but at a step of isomerization from an initial ( $RP_{cl}$ ) to an intermediate ( $RP_{c2}$ ) closed complex and at the onset of mRNA initiation. Although our approach does not permit to

distinguish between two closed complexes our results are in good agreement with these studies. Finally,  $P_{larconS17}$ , and probably also  $P_{larconS18}$ , are primarily limited at the release of open complexes as the permanganate footprints prove unambiguously.

### AraC action at the $P_{lar}$ variants

The impact of AraC on the enzyme's  $k_{ON}$  can be quantified *in vivo* by monitoring the activation level of a given construct under conditions of repression. This analysis reveals that the activator accelerates stable complex formation of all promoters of our collection. However, as demonstrated in Table 4, its main role is to increase the rate of open complex formation, i.e. transition from  $RP_c$  to  $RP_0$ . According to the footprint data the relative rate of open complex release at  $P_{lar}$ ,  $P_{larS17}$  and  $P_{lar-8A-9A}$  is significantly slower compared to the other promoters. They furthermore indicate that AraC can relieve this limitation to a degree corresponding to the rates calculated for the other  $P_{lar}$  variants. Interestingly, the activator seems incapable of increasing  $k_{cl}$  substantially beyond this value ( $t_{RP_{cl}} \approx 2-3$  s; Table 4). At promoters  $P_{larconS17}$ , and presumably  $P_{larconS18}$ , where clearance becomes the kinetic bottleneck, the initiation rate is ~0.25 transcripts/s. This is noticeably below the rate of the very strong *E.coli* promoter *rrnP1* which initiates 1.5-2 transcripts/s during logarithmic growth (2,30). Since we know that the *lacO* sequence, when located in the downstream region, i.e. between +1 and +20, slows down promoter clearance at a variety of promoters without affecting  $k_{ON}$  (7,23,31) we suppose that this sequence is also responsible for the relatively slow release of open complexes of  $P_{lar}$  variants. In this context it is interesting to note that the very strong promoter  $P_L$  of phage Lambda, which is highly optimized towards promoter clearance (23), cannot be activated by AraC (10). Interestingly, when  $P_{lar-8A-9A}$  was activated by AraC, the footprint signals remained unchanged (Fig. 4) regardless of the 7-fold activation of the overall transcription process (Table 2). This result can be explained by assuming that the overall activation observed is composed of an enhanced formation of open complexes as well as of their accelerated release. At the same time, our data indicate that AraC increases  $k_{ON}$  at  $P_{lar-8A-9A}$  7-fold. This activation, however, is qualitatively different from the activation observed under conditions of derepression as here  $k_{ON}$  has become the limiting term. Thus, as with  $P_{lar}$  and  $P_{larS17}$ , AraC is able to accelerate three distinct steps at this promoter. Likewise, the AraC-mediated increase in  $k_{ON}$  at  $P_{larconS17}$  became apparent only under conditions of repression, whereas it had no effect on promoter activity in the non-repressed state. This observation is also in accordance with the unchanged footprint signal (Fig. 4).

### LacR decreases the enzyme's $k_{ON}$ by a defined increment

The data provided in Table 4 show that the impact of LacR on  $k_{ON}$  differs drastically from its effect on the overall promoter activity [compare rows LacR (+) and (-)]. The difference is due to the fact that  $k_{ON}$  is not the major rate limiting step at any of the  $P_{lar}$  variants in the absence of LacR. In order to slow down the overall transcription process significantly LacR has to decrease  $k_{ON}$  to such an extent that it becomes the major rate limiting step of the overall process. Regardless of whether AraC is active or not, the actual quantitative impact of LacR decreases the enzyme's  $k_{ON}$  by a defined increment which is in

the range of 75–150-fold for all promoters examined in this study. Interestingly, it appears to be rather independent of the rate of RNAP/promoter complex formation. Since these observations can also be made at a number of other synthetic promoters of the same architecture (7) we conclude that this mechanism of repression is generally applicable for repressors which directly interfere with RNAP binding.

### Principles of $P_{\text{lar}}$ regulation

$P_{\text{lar}}$  and, accordingly,  $P_{\text{lac}}$  are examples of negatively controlled promoters where the repressor interferes directly with the binding of RNAP. Our data show that the repressor affects the rate of RNAP binding and decreases the apparent  $k_{\text{ON}}$  by a defined increment. However, whether this deceleration of RNAP binding results in an efficient reduction of promoter activity depends on the rate of polymerase binding to the individual promoter in the non-repressed state, i.e. on the competition of the two proteins for their mutual binding site. Moreover, the regulatory range, i.e. promoter activity of the repressed compared to the non-repressed state, is also dependent on the rate of transcript initiation and promoter clearance by RNAP. Interestingly, while  $P_{\text{lar}}$  interacts with RNAP with a low forward rate constant and thus fulfills one criterion for efficient repression, it initiates transcription only slowly. This limits the regulatory range of the Lac repressor/operator system. Also not too obvious is the fact that upon derepression the activity of  $P_{\text{lar}}$  increases only 3-fold even though the rate of closed complex formation increases 150-fold (Table 4) revealing that the derepressed  $P_{\text{lar}}$  is strongly limited at later steps of the promoter/RNAP interaction, namely at the formation and release of open complexes. These limitations are candidates for the action of the activator and indeed AraC, although increasing the rate of complex formation by another factor of 5 has its main impact on accelerating open complex formation and, to a lesser extent, steps following this event. The regulatory range of  $P_{\text{lar}}$  under our *in vivo* conditions is ~200-fold and our data indicate that this range may not be further expanded by activation via AraC. However, the rather modest repression factor may still be increased as it is directly dependent on the occupancy of the operator. The latter may be enhanced by higher intracellular concentrations or, more elegantly, by just increasing the local repressor concentration. In the *lac* as well as in several other operons, nearby secondary operators indeed contribute to higher occupancy of the main operator by this mechanism (32), which at the same time makes the system highly sensitive to low concentrations of inducer. In conclusion, the functional program of  $P_{\text{lar}}$  reflects an interesting optimization for both negative and positive control. Obviously activators like AraC can have an impact on several limiting steps of the enzyme/promoter interaction. On the other hand, optimizing a promoter for efficient repression is primarily directed towards deceleration of the rate of complex formation between promoter and RNAP. Possibly this deceleration can only partially be brought about by modification of the promoter sequence since below a certain threshold the promoter may not become active any more in the derepressed state. Our analysis of the mode of action of AraC as a transcriptional activator and LacR as a directly interfering repressor has revealed an intricate balance between the elements interacting at a start site of transcription. The functional program which governs this finely tuned balance is

embedded in a rather simple promoter sequence. It may give a taste for the great complexity we have to expect when studying quantitative parameters of transcription regulation in more sophisticated systems where a multitude of factors participate in a combinatorial mode.

### ACKNOWLEDGEMENTS

We thank Dr Schleif for *E. coli* strains TR322 and TR321 and Dr Frank and co-workers for the synthesis of oligonucleotides. This work was supported by the Deutsche Forschungsgemeinschaft SFB229 and by the Fonds der Chemischen Industrie Deutschlands.

### REFERENCES

- McClure, W.R. (1985) Mechanism and control of transcription initiation in prokaryotes. *Annu. Rev. Biochem.*, **54**, 171–204.
- Knaus, R. and Bujard, H. (1990) Principles governing the activity of *E. coli* promoters. In Eckstein, F. and Lilley, D.M. (eds), *Nucleic Acids and Molecular Biology*. Springer Verlag, Heidelberg, Germany, Vol. 4, pp. 110–122.
- DeHaseth, P., Zupancic, M.L. and Record, M.T., Jr (1998) RNA polymerase-promoter interactions: the comings and goings of RNA polymerase. *J. Bacteriol.*, **180**, 3019–3025.
- Deuschle, U., Kammerer, W., Gentz, R. and Bujard, H. (1986) Promoters of *Escherichia coli*: A hierarchy of *in vivo* strength indicates alternate structures. *EMBO J.*, **5**, 2987–2994.
- Brunner, M. and Bujard, H. (1987) Promoter recognition and promoter strength in the *Escherichia coli* system. *EMBO J.*, **6**, 3139–3144.
- Collado-Vides, J., Magasanik, B. and Gralla, J.D. (1991) Control site location and transcriptional regulation in *Escherichia coli*. *Microbiol. Rev.*, **55**, 371–394.
- Lanzer, M. and Bujard, H. (1988) Promoters largely determine the efficiency of repressor action. *Proc. Natl Acad. Sci. USA*, **85**, 8973–8977.
- Mulligan, M.E., Hawley, D.K. and McClure, W.R. (1984) *Escherichia coli* promoter sequences predict *in vitro* RNAP selectivity. *Nucleic Acids Res.*, **12**, 789–800.
- Harley, C.B. and Reynolds, R.P. (1987) Analysis of *E. coli* promoter sequences. *Nucleic Acids Res.*, **15**, 2343–2361.
- Berlin, M. (1993) Untersuchung zur Aktivierung von *Escherichia coli* Sigma70-Promotoren durch cAMP/CRP und AraC. PhD thesis. University of Heidelberg, Germany.
- Stueber, D. and Bujard, H. (1982) Transcription from efficient promoters can interfere with plasmid replication and diminish expression of plasmid specified genes. *EMBO J.*, **1**, 1399–1404.
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467.
- De Wet, J.R., Wood, K.V., de Luca, M., Helinski, D.R. and Subramani, S. (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.*, **7**, 725–737.
- Lutz, R. and Bujard, H. (1997) Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I<sub>1</sub>-I<sub>2</sub> regulatory elements. *Nucleic Acids Res.*, **25**, 1203–1210.
- Hahn, S., Dunn, T. and Schleif, R. (1984) Upstream repression and CRP stimulation of the *Escherichia coli* L-arabinose operon. *J. Mol. Biol.*, **180**, 61–72.
- Burgess, R.R. and Jendrisak, J.J. (1975) A procedure for the rapid, large-scale purification of *Escherichia coli* DNA-dependent RNAP involving Polymyxin P precipitation and DNA-cellulose chromatography. *Biochemistry*, **14**, 4634–4638.
- Sasse-Dwight, S. and Gralla, J.D. (1989) KMnO<sub>4</sub> as a probe for *lac* promoter DNA melting and mechanism *in vivo*. *J. Biol. Chem.*, **264**, 8074–8081.
- Ellinger, T., Behnke, D., Bujard, H. and Gralla, J.D. (1994) Stalling of *Escherichia coli* RNAP in the +6 to +12 region *in vivo* is associated with tight binding to consensus promoter elements. *J. Mol. Biol.*, **239**, 455–465.
- Reeder, T. and Schleif, R. (1993) AraC protein can activate transcription from only one position and when pointed in only one direction. *J. Mol. Biol.*, **231**, 205–218.



20. Birnboim, H.C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.*, **7**, 1513–1523.
21. Brunelle, A. and Schleif, R. (1989) Determining residue-base interactions between AraC protein and araI DNA. *J. Mol. Biol.*, **209**, 607–622.
22. Mulligan, M.E., Brosius, J. and McClure, W.R. (1985) Characterization *in vitro* of the effect of spacer length on the activity of *Escherichia coli* RNAP at the *tac* promoter. *J. Biol. Chem.*, **260**, 3529–3538.
23. Knaus, R. and Bujard, H. (1988) P<sub>L</sub> of coliphage lambda: an alternative solution for an efficient promoter. *EMBO J.*, **7**, 2910–2932.
24. Grana, D., Gardella, T. and Susskind, M.M. (1988) The effects of mutations in the *ant* promoter of phage P22 depend on context. *Genetics*, **120**, 319–327.
25. Liang, S.T., Bipatnath, M., Xu, Y.-C., Chen, S.-L., Dennis, P., Ehrenberg, M. and Bremer, H. (1999) Activities of constitutive promoters in *Escherichia coli*. *J. Mol. Biol.*, **292**, 19–37.
26. Schlax, P.J., Capp, M.W. and Record, M.T., Jr (1995) Inhibition of transcription initiation by lac repressor. *J. Mol. Biol.*, **245**, 331–350.
27. Lutz, R. (1996) Untersuchungen zur Aktivierung und Repression von Sigma70 Promotoren in *Escherichia coli*. PhD thesis. University of Heidelberg, Germany.
28. Buckle, M., Pemberton, I.K., Jacquet, M.-A. and Buc, H. (1999) The kinetics of sigma subunit directed promoter recognition by *E. coli* RNA polymerase. *J. Mol. Biol.*, **285**, 955–964.
29. Carpousis, A.J. and Gralla, J.D. (1985) Interaction of RNAP with *lacUV5* promoter DNA during mRNA initiation and elongation. *J. Mol. Biol.*, **183**, 165–177.
30. Zhang, X. and Bremer, H. (1996) Effects of Fis on ribosome synthesis and activity and on rRNA promoter activities in *Escherichia coli*. *J. Mol. Biol.*, **259**, 27–40.
31. Kammerer, W., Deuschle, U., Gentz, R. and Bujard, H. (1986) Functional dissection of *Escherichia coli* promoters: information in the transcribed region is involved in late steps of the overall process. *EMBO J.*, **5**, 2995–3000.
32. Oehler, S., Eismann, E.R., Kraemer, H. and Mueller-Hill, B. (1990) The three operators of the *lac* operon cooperate in repression. *EMBO J.*, **9**, 973–979.