Article No. mb982040 J. Mol. Biol. (1998) 282, 495–504

JMB

CAP, the −45 Region, and RNA Polymerase: Three Partners in Transcription Initiation at *lac*P1 in *Escherichia coli*

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The *lac* operon of *Escherichia coli* is positively regulated by the catabolite activator protein (CAP) bound upstream of the −45 region (CAP binding is centered at −61.5; the −45 region extends from −50 to −38). Certain mutations within the −45 region generate sequences that resemble UP elements in base composition and mimic the stimulation by the *rrnB*P1 UP element, yielding up to 15-fold stimulation *in vivo*. These −45 region ''UP mutants'' are compromised in their CAP stimulation. CAP and UP elements do not act in a fully additive manner *in vivo* at the *lac* operon. Transcription assays with the wild-type *lac* promoter and an UP mutant of *lac* indicate that CAP and UP DNA also fail to act in a completely additive manner *in vitro*. RNA polymerase can stabilize CAP binding to promoter DNA with a −45 region UP element against a heparin challenge. This shows that CAP and the UP DNA do not compete for the α-CTD as a mechanism for their lack of additivity. CAP and UP elements both demonstrate decreased stimulation of transcription as RNA polymerase concentration is increased from 0.05 to 10 nM in *in vitro* transcription experiments. In addition CAP also stimulates transcription in a manner that does not decrease as RNA polymerase is varied over this concentration range. This invariable stimulation is by two- to threefold and occurs both *in vivo* and *in vitro*. It is not dependent upon the α-CTD of RNA polymerase and is maintained in the presence of the AR1 CAP mutant HL159. This two- to threefold invariable CAP stimulation appears to depend on the −45 region sequence as our −45 region mutants demonstrate different responses to HL159 CAP stimulation *in vivo*.

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Keywords: RNA polymerase; CAP; *Escherichia coli*; −45 region; activation

Introduction

Transcription initiation in *Escherichia coli* is a multistep process. At the simplest promoters, initiation requires the binding of a five subunit RNA polymerase enzyme to a core promoter, subsequent isomerization to form an open complex, initiation of ribonucleic acid synthesis, and clearance of the promoter. More complex transcription units exist that are controlled by regulatory proteins (activators and/or repressors) and/or upstream DNA sequences (Record *et al.*, 1996). These additional components of promoters, which can affect any step in initiation, allow extensive fine tuning of transcription in response to the needs of the bacterial cell. For example, the *lac* operon is subject to regulation by both a repressor and an activator, the *lacI* and *crp* gene products. These two proteins, in conjunction with RNA polymerase cooperate to provide up to a 50,000-fold range of *lac*P1 transcription (1000-fold from the repressor (Gilbert & Muller-Hill, 1970) and 50-fold from the catabolite activator protein (CAP, product of the *crp* gene) (Beckwith *et al.*, 1972)).

In our work with the *lac* operon, we study the interaction of RNA polymerase with CAP. Another point of interest is to explain how artificially introduced upstream DNA sequences (analogous to the downstream half of the *rrnB*P1 UP element) affect the behavior of RNA polymerase at the *lac*P1 promoter. Ultimately, we are curious about how CAP and UP element DNA work to stimulate

Abbreviations used: CAP, catabolite activator protein; RNAP, RNA polymerase; α-CTD, α-subunit carboxy-terminal domain(s); AR, activating region.

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0022−2836/98/380495–10 $30.00/0

transcription initiation. In particular our aim is to investigate the nature of the stimulation offered by each partner in our *lac*P1 system.

Previous work has established that DNA sequences resembling the UP elements, when located in the −45 region of the *lac*P1 promoter (this consists of the 13 bases from −50 to −38 which fall between the core promoter and the CAP binding site), can increase *lac*P1 transcription in the absence of CAP approximately 15-fold (Czarniecki *et al*., 1997). In effect, by our mutagenesis of the −45 region, we defined a ''half site'' for an UP element at *lac*P1. Our most active mutants bear sequences that are consistent with the consensus UP element sequence defined by S. Estrem & R. L. Gourse (personal communication).

These −45 region mutant *lac*P1 promoters do not respond to CAP stimulation to the same degree as the wild-type *lac*P1 promoter. There is an approximate correlation between increased CAP-independent activity and decreased CAP responsiveness. In all cases, we fail to observe a fully additive effect of CAP and UP elements on the *lac*P1 promoter *in vivo* (Czarniecki *et al*., 1997). The possible interpretations of this lack of additivity are: (1) CAP and UP elements compete with each other in their efforts to make interactions with RNA polymerase; (2) the −45 region sequence affects CAP-dependent stimulation and UP element sequences represent a population that responds poorly to CAP activation; (3) in the presence of both CAP and a −45 region UP element, another step in initiation becomes rate limiting that prevents a full additive effect of CAP plus UP element DNA. Another possibility, which we had not considered before is that in the absense of CAP, both α-subunit carboxy-terminal domains (α-CTD) are bound to the DNA and that CAP displaces one of them in order to bind to its site centered at −61.5. This could lead to decreased −45 region UP element DNA stimulation and result in lack of full additivity. The primary reason that we do not favor this hypothesis is that there is only a half UP element in our constructs. In the absense of a −45 region UP mutation, the DNA upstream of the −35 hexamer at *lac*P1 has not been demonstrated to be an UP element. Thus, the displaced α-CTD would be bound to non-specific DNA in the absence of CAP. An analysis of these questions will shed light on the basic mechanisms involved with these two stimulatory components of some promoters.

Previously, we favored the first hypothesis (Czarniecki *et al*., 1997). Ample evidence exists that CAP and UP elements not only both require the carboxy-terminal domain of the α-subunit of RNA polymerase (α-CTD; Igarashi & Ishihama, 1991; Ross *et al*., 1993), but also require identical or closely located amino acid residues within the α-CTD (Busby & Ebright, 1994). Recent investigation has pinpointed the critical amino acid residues on the α-CTD for UP element use to positions 262, 265, 268, 269, 296, 298, and 299 (Gaal *et al*., 1996). The most critical amino acid residue on the α-CTD for CAP-dependent *lac*P1 stimulation is either position 261 (Tang *et al*., 1994) or position 265 (Murakami *et al*., 1996). The NMR solution structure indicates that position 261 is adjacent to helix 1 and position 265 lies within helix 1 that, in combination with helix 4, is critical to the DNA binding property of the α-CTD (Jeon *et al*., 1995).

Here we address the mechanism responsible for the lack of full additivity in our system. We demonstrate that CAP and UP element DNA fail to act in a fully additive manner *in vitro*; however, they appear to be able to ''share'' the α-CTD, ruling out the first hypothesis that an interference between CAP and the −45 region UP DNA leads to the lack of additivity. Thus, the lack of additivity must be due to an alteration in the rate limiting step for initiation at *lac*P1, a property of the −45 region DNA sequence affecting CAP stimulation and/or displacement of one of the DNA bound α-CTDs.

The existing model for class I CAP-dependent stimulation maintains that a protein-protein contact is responsible for activation of transcription (Busby & Ebright, 1994; Kolb *et al*., 1993). This contact is thought to occur between an amino acid patch on CAP called activating region I (AR1, corresponds to amino acid residues 156 to 162 of CAP) and an activation target on the α-CTD (consisting of amino acid residues 258 to 265) (reviewed by Busby & Ebright, 1994). We present data that a component of CAP-dependent *lac*P1 stimulation is manifested in a manner independent of the concentration of polymerase and independent of interactions between CAP and the α-CTD. This stimulation responds differently to variation of RNA polymerase concentration than does the bulk of wild-type CAP stimulation in *in vitro* transcription assays. *In vitro*, this AR1/α-CTD independent stimulation by CAP covers a range of two- to threefold. *In vivo*, we detect a twofold stimulation by an AR1 deficient CAP (HL159CAP) for the wild-type *lac*P1. These data indicate that class I CAP-dependent transcription is mediated by a mechanism that is more complex than simply the AR1/α-CTD protein-protein contact.

Finally, some of our −45 region mutants display variable stimulation by HL159CAP *in vivo*. Here two are stimulated like the wild-type promoter, one is unaffected, and one is inhibited. These data offer an explanation for the *in vivo* twofold differences in CAP stimulation by certain −45 region mutants described in earlier work (Czarniecki *et al*., 1997). In short, −45 region mutants with similar activities in the absence of CAP stimulation failed to be stimulated equally by CAP *in vivo*. Therefore, the differences in the responses of the wild-type and mutant *lac* promoters to this mode of CAP stimulation (which is maintained in the presence of an AR1 mutant of CAP) is a result of the −45 region sequence. The −45 region thus can be a determinant of the magnitude of a component of CAP stimulation.

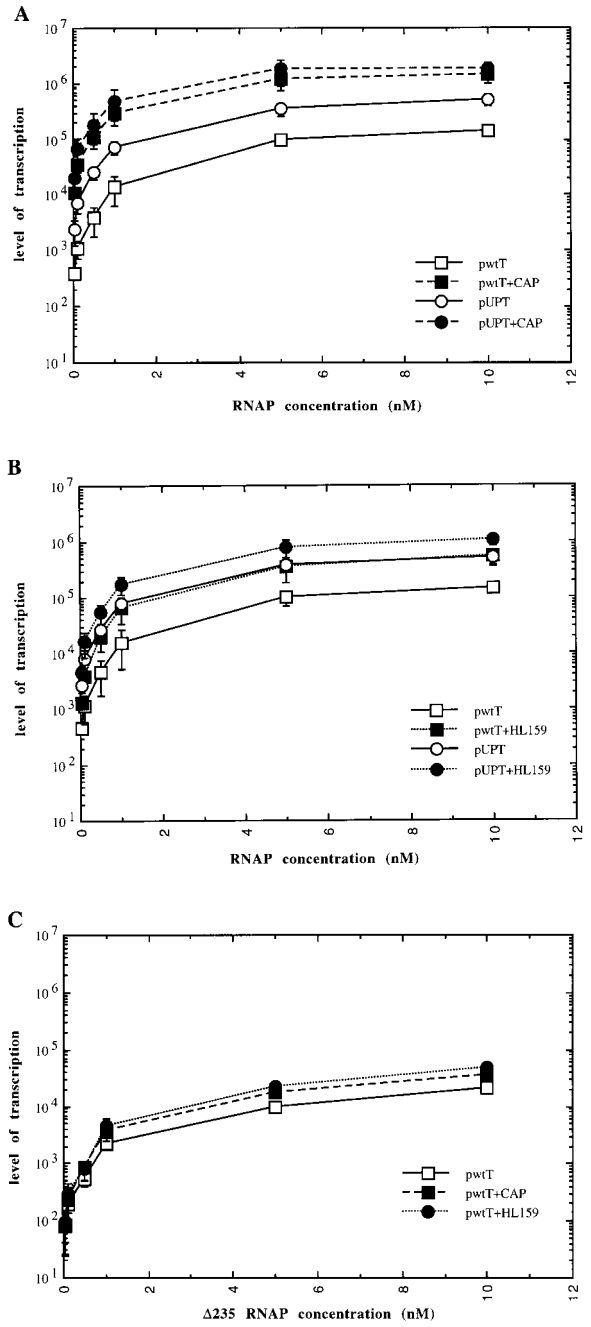

Figure 1. *In vitro* transcription reactions: effect of CAP and UP DNA on RNA polymerase. Multiround transcription reactions were performed for ten minutes at 37°C on supercoiled templates as described in Materials and Methods. DNA was present at 1.0 nM. Level of transcription on the *y*-axis is in arbitrary units and indicates the pixel intensity from a phosphorimager scan. The level of transcription for each assay at each point is an average of three or more independent assays and was corrected for background and normalized (prior to averaging) using the *rna*1 transcript. A, Reactions were in the presence of wild-type RNA polymerase to test effects of CAP and UP element DNA in the −45 region on *lac* transcription. Each point in the graph represents an average of five independent assays. B, Reactions are in the presence of wild-type RNA polymerase and HL159CAP. Transcription levels are the average of four independent assays. C, Reactions are in the presence of Δ235 RNA polymerase at the wild-type *lac*P1 promoter only. Transcription levels are the average of three independent assays.Standard deviations for the assays for each point in each curve are given by the error bars. +CAP indicates +25 nM CAP.

Therefore, the existing model for class I CAP-dependent stimulation, which relies entirely on the AR1/α-CTD contact, is insufficient to explain the full role of CAP at class I promoters (Busby & Ebright, 1994; Kolb *et al.*, 1993). A full explanation must include some component that is responsive to the sequence of the DNA between the CAP and RNA polymerase consensus binding sites and does not involve an AR1/α-CTD contact.

Results

Stimulation by CAP and UP element DNA decreases as the concentration of RNA polymerase is increased *in vitro*

We carried out multiround, *in vitro* transcription reactions on supercoiled templates over a 200-fold range of RNA polymerase concentration to investigate the effects of UP element DNA (UP DNA) and CAP on RNA polymerase activity at *lac*P1 (Figure 1 and Table 1). The −45 region UP DNA serves to stimulate transcription of *lac*P1 *in vitro*. For example, the level of transcription in Figure 1A at 5 nM RNA polymerase using pwtT (wild-type −45 region) is approximately equivalent to that at 0.5 to 1 nM RNA polymerase using pUPT (−45 region UP mutant). CAP also stimulates transcription by RNA polymerase *in vitro*. In Figure 1A, the level of transcription at 5 nM RNA polymerase at pwtT falls within the range of 0.1 to 0.5 nM RNA polymerase at pwtT in the presence of 25 nM CAP.

Of particular interest is that the level of stimulation by either CAP or UP DNA appears to be dependent on the concentration of RNA polymerase; there is a strong inverse correlation between the level of stimulation by CAP or the UP DNA and the concentration of RNA polymerase (Table 1). For example, at 0.1 nM RNA polymerase, CAP stimulation (31.1×) and UP DNA stimulation (6.7×) are maximal in this assay. At 10.0 nM RNA polymerase, the fold stimulations are diminished to 10.1× and 3.6×, respectively. As the RNA polymerase concentration increases, the window of opportunity for stimulation of transcription diminishes and a smaller effect of CAP and/or UP DNA is manifested.

CAP and UP elements fail to act in a fully additive manner *in vitro*

If CAP and UP DNA act in a fully additive manner, the full stimulation of both should be

Table 1. Stimulation of RNAP by CAP and UP DNA at *lac*P1

RNAP concentration (nM)	CAP stimulation	UP DNA stimulation	CAP and UP DNA stimulation of *lac*P1	CAP stimulation of UP*lac*
0.05	28.7	6.1	51.9	8.5
0.1	31.1	6.7	63.9	9.6
0.5	28.0	6.4	47.4	7.4
1.0	20.5	5.2	35.0	6.7
5.0	12.0	3.5	18.2	5.2
10.0	10.1	3.6	12.6	3.5

Data are ratios of the average values graphed in Figure 1A.

observed. This requirement is not met *in vivo* and could be due to a number of possible factors which are worthy of further study (Czarniecki *et al.*, 1997).

Here, we have investigated the possible causes of the lack of additivity *in vivo* by performing *in vitro* transcription experiments. The data in Table 1 clearly indicate that CAP and UP DNA in the −45 region do not act in a completely additive manner at *lac*P1 *in vitro*. The highest stimulation of pUPT by CAP is 9.6× at 0.1 nM RNA polymerase (CAP stimulates pwtT by 31.1× at 0.1 nM RNA polymerase, three times more). At each concentration of RNA polymerase tested *in vitro*, a fully additive effect is not seen. The closest point to full additivity is at 5 nM RNA polymerase where CAP stimulation of pUPT is approximately 43% of CAP stimulation of pwtT.

RNA polymerase stabilizes CAP binding to its DNA site in the presence of −45 region UP DNA

Our earlier work demonstrates that *lac*P1 promoters, which contain an UP element-like sequence in the −45 region, are compromised in their ability to reach full stimulation by CAP *in vivo*. A possible model to explain this phenomenon is that the α-CTD, required for stimulation by both CAP at a class I promoter and UP element DNA (Busby & Ebright, 1994), is unavailable for full implementation of CAP activation when an UP element is present between the CAP and RNA polymerase binding sites at *lac*P1. In order to address this hypothesis, we tested the ability of RNA polymerase to stabilize CAP binding in the presence of a −45 region UP element. We exploited the fact that CAP bound to its site at *lac*P1 is sensitive to a heparin challenge (Tagami & Aiba, 1995). However, in the presence of RNA polymerase, especially at a strong promoter such as *lac*UV5, CAP is able to resist the heparin chase due to interactions with RNA polymerase (Tagami & Aiba, 1995), *via* the α-CTD. The data in Figure 2A (compare lanes three and four) demonstrate that CAP fails to protect its site in the face of a heparin challenge in the absence of RNA polymerase; however, in the presence of RNA polymerase, CAP binding is stabilized. Note the presence of a −61.5 CAP footprint in addition to a strengthened RNA polymerase footprint at the *lac*UV5 promoter (Figure 2A, lanes 5 and 6). The RNA polymerase-dependent stabilization of CAP to a heparin challenge is also manifested at the UP*lac* mutant promoter (Figure 2B, lanes 3 through 6). This indicates that the failure to see an additive effect of CAP and UP DNA *in vivo* is not due to sequestration of the α-CTD by the −45 region DNA preventing an interaction with the CAP protein.

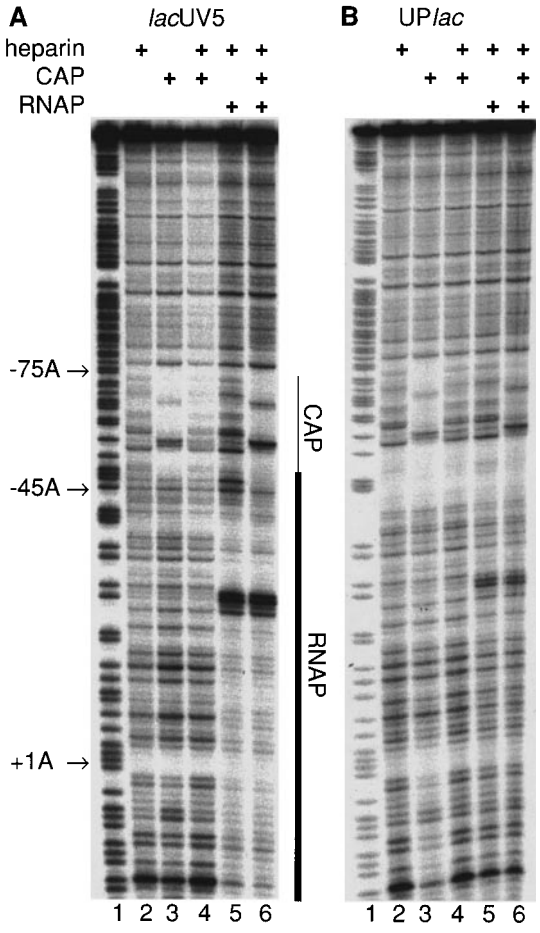

Figure 2. DNase I probing of CAP and RNA polymerase binding. Experimental conditions were as described in Materials and Methods. In A, *lac*UV5 promoter fragment, and B, UP*lac* promoter fragment lane 1 consists of a Maxam-Gilbert G + A sequencing reaction and lane 2 consists of the no protein cleavage control for each promoter. Lanes 2 to 6 are as described in Results.

CAP exhibits a two- to threefold stimulation of *lac*P1 independent of the concentration of RNA polymerase and the presence of an UP element *in vitro*

Even at the highest concentration of RNA polymerase and in the presence of UP element DNA, CAP offers a 3.5-fold stimulation of transcription *in vitro* (Table 1). This is true *in vivo* where we observe that even the most active −45 region mutants (in the absence of CAP stimulation) are stimulated by CAP two- to fourfold (Czarniecki *et al*., 1997). In order to test if CAP can function in a manner independent of the CAP AR1/α-CTD contact, we performed *in vitro* transcription experiments in the presence of the CAP activating region 1 (AR1) mutant, HL159CAP. An AR1 mutant binds to and bends DNA normally at the CAP binding site, but is deficient in the specific protein-protein interaction with the α-CTD (Bell *et al*., 1990; Eschenlauer & Reznikoff, 1991; Zhou *et al*., 1993). At all concentrations of RNA polymerase for pwtT and pUPT, the HL159CAP mutant offers virtually identical stimulation of threefold for pwtT or twofold for pUPT (Table 2 and Figure 1B).

We tested this phenomenon from the standpoint of the RNA polymerase α-CTD (the contact point for AR1 of CAP) by performing *in vitro* transcription reactions with pwtT, an RNA polymerase mutant lacking its α-CTDs (Δ235 RNA polymerase), and CAP or HL159CAP. The results indicate that CAP retains nearly two fold stimulation in the absence of the α-CTD and in the absence of both AR1 and the α-CTD (Table 2 and Figure 1C). This stimulatory contribution of CAP is independent of the RNA polymerase concentration and must not require the protein-protein contact between CAP AR1 and the RNA polymerase α-CTD.

The −45 region sequence plays a key role in AR1/α-CTD independent CAP stimulation of *lac*P1

β-Galactosidase assays were performed using strains lacking chromosomal *crp* and bearing mutant *lac* −45 regions in monocopy (lambda lysogens). These strains were transformed with multicopy plasmids to provide no CAP (pDU9), wild-type CAP (pDCRP), or HL159CAP (pDCRPHL159). The data are presented in Table 3 and indicate that the sequence of the −45 region has an important influence on the magnitude of wild-type CAP stimulation. This influence on stimulation is maintained with the HL159CAP AR1 mutant. The contribution of the −45 region sequence to CAP stimulation (and HL159CAP stimulation) is best demonstrated by a comparison of mutants that behave similarly in the absence of CAP. Mutant 10 and mutant 503 yield similar levels of β-galactosidase activity based on their CAP-independent, UP DNA-stimulated activities (about three times the level of wild-type *lac*P1). Mutant 504 and mutant 506 demonstrate a similar relationship (though they exhibit about seven times the wild-type level). In the presence of wild-type CAP, mutants 503 and 506 are stimulated approximately twofold more than mutants 10 and 504, respectively. In the presence of HL159CAP, mutant 503 is stimulated to a higher level than mutant 10 (1.5-fold for 503 *versus* no stimulation for 10), and mutant 506 is stimulated twofold while mutant 504 is repressed about twofold.

Table 2. AR1-independent CAP (HL159CAP and Δ235 RNAP)

RNAP concentration (nM)	HL159CAP stimulation of pwtT	HL159CAP stimulation of pUPT
0.05	2.4	1.5
0.1	2.9	1.5
0.5	3.9	1.7
1.0	2.9	1.8
5.0	2.6	1.6
10.0	3.0	1.8
Δ235 RNAP concentration (nM)	**CAP stimulation of pwtT**	**HL159CAP stimulation of pwtT**
0.05	1.0	1.2
0.1	1.2	1.5
0.5	1.7	1.5
1.0	1.7	2.0
5.0	1.8	2.4
10.0	1.8	2.3

Data are ratios of the average values graphed in Figure 1B and C.

Discussion

In addition to providing a new way to consider the lack of additivity between CAP and UP element DNA in the −45 region at *lac*P1, this study also provides insights into the nature of the stimulation offered by UP DNA at this promoter and the mechanisms by which CAP acts at class I promoters, of which *lac*P1 is the prototype (Reznikoff,

Table 3. β-Galactosidase assay of −45 region mutants

Mutant	Sequence −50	−45	−40	β-Galactosidase activity in Miller units no CAP	CAP	HL159CAP	Fold stimulation UP DNA	CAP	HL159CAP
Wild-type	t a g g c a c c c c a g g			10.5 (0.9)	215 (27.1)	17.8 (2.4)	x	20.5	1.7
10	t a a t g t a t a a t c g			34.4 (3.7)	182 (15.9)	33.0 (1.7)	3.3	5.3	1.0
503	t t g a t a a a c t a g a			31.5 (6.8)	331 (16.0)	48.9 (2.6)	3.0	10.5	1.5
504	t g c a c g a a t a t a c			80.5 (5.5)	164 (10.1)	33.9 (1.7)	7.7	2.0	0.4
506	t a g a c a c t t t t c g			64.9 (6.9)	308 (17.2)	129 (7.4)	6.2	4.7	2.0

Data represent the average of six points. Standard deviations are in parentheses.

1992). CAP and −45 region UP DNA both stimulate transcription initiation *in vivo* at *lac*P1 (Czarniecki *et al.*, 1997). Here *in vitro* transcription experiments indicate that stimulation by CAP or UP DNA is inversely related to the RNA polymerase concentration. The data do not allow direct analysis of the steps in initiation that are affected by CAP or UP DNA; however, they are consistent with various published work regarding the kinetic effects of UP elements and class I CAP-dependent stimulation as discussed below.

Some evidence exists that UP elements function to stimulate RNA polymerase binding. The full UP element at *rrnB*P1 serves to stimulate binding of RNA polymerase at least 20-fold and perhaps to stimulate a post binding step by as much as fivefold (Rao *et al.*, 1994). The inverse relationship of RNA polymerase concentration with UP DNA stimulation observed in this work is consistent with an interpretation that UP DNA functions in a likewise manner at *lac*P1 (predominantly to stimulate RNA polymerase binding). However, a thorough demonstration of an effect of UP DNA on RNA polymerase binding will require kinetic investigations.

We propose that the predominant effect of CAP is similar or identical mechanistically to the UP DNA in recruiting RNA polymerase to the promoter. This idea is in line with kinetic studies concerning the mechanism of CAP stimulation of the *lac* operon. It was proposed that CAP functions exclusively to stimulate RNA polymerase binding to the *lac*P1 promoter on both supercoiled and linear DNA (Malan *et al.*, 1984). The case for CAP is not completely clear, however, as a competing study indicates that the predominant affect of CAP at *lac*P1 is to increase the rate at which open complexes are formed rather than to increase RNA polymerase binding (Straney *et al.*, 1989). This controversy remains unresolved. The data presented here show that the bulk of CAP stimulation is inversely related to the concentration of RNA polymerase. This is consistent with the model (proposed by Malan *et al.*, 1984) that stimulation of RNA polymerase binding accounts for most of CAP-dependent stimulation.

That CAP and UP elements fail to act in a fully additive manner *in vivo* (Czarniecki *et al.*, 1997) and *in vitro*, as demonstrated here, supports the idea that CAP and UP elements serve to stimulate the same step in transcription initiation at *lac*P1. Our original model was that CAP and UP element DNA compete for the α-CTD and thus could not both maximally act in conjunction; however, we could not rule out that the apparent lack of additivity was due to sequence constraints (good UP DNA −45 region sequences provide poor CAP-dependent stimulation substrates) and/or due to generation of a new rate limiting step. In light of new data, we are forced to reevaluate this model. The DNase I footprinting in Figure 2 indicates that it is likely that RNA polymerase α-CTD can contact the −45 region DNA and CAP simultaneously or that both α-CTDs can function within the −45 region. Due to the stabilization of CAP against a heparin challenge in the presence of RNA polymerase, we have ruled out a competition for the α-CTD as a model to explain the lack of additivity between CAP and UP DNA at *lac*P1. Three models remain to explain the lack of an additive response: (1) in the presence of CAP and −45 region UP DNA, another step in initiation becomes rate limiting; (2) UP DNA responds poorly to CAP stimulation; and (3) CAP displaces an upstream, non-specific α-CTD-DNA interaction and reduces the effect of stimulation by −45 region UP DNA. These models are not mutually exclusive. We disfavor model (3) on the basis that our constructs have only half an UP element and the wild-type sequence does not serve as an UP element. Presumably, there is only a single α-CTD binding site in our −45 region mutants. Thus the second α-CTD, displaced in model (3), would be bound non-specifically to DNA in the absense of CAP.

Previous work demonstrated a twofold −45 region sequence-related effect on CAP stimulation *in vivo* (Czarniecki *et al.*, 1997). The work presented here confirms that the −45 region sequence can affect CAP dependent transcription at *lac*P1. Our data indicate that the −45 region sequence has a role in modulating the residual stimulation present in the absence of the AR1/α-CTD interaction. The −45 region sequence determines the magnitude of the AR1/α-CTD-independent stimulation of *lac*P1 by CAP. *In vivo*, mutants 503 and 506 display twofold greater stimulation by CAP than mutants 10 and 504. This same relationship exists for HL159CAP where mutants 503 and 506 show AR1-independent stimulation, while the sequences in the −45 regions of mutants 10 and 504 are not responsive to activation by HL159CAP.

The AR1 mutant stimulation offered by CAP is an extremely interesting result. Even though a two- to threefold effect is a small contribution in comparison to full CAP stimulation (in our system this appears to be 20 to 30-fold), it constitutes an important part of CAP-dependent stimulation. It serves to demonstrate that CAP is a truly versatile protein, not just in that it stimulates transcription at two classes of promoters (Kolb *et al.*, 1993), but that at class I promoters, *lac*P1 in particular, it operates by at least two mechanisms. The predominant mechanism relies on both AR1 of CAP and the RNA polymerase α-CTD, while the minor contribution by CAP is manifested with an AR1 mutant (*in vivo* and *in vitro*) and/or an α-CTD deletion of RNA polymerase.

AR1 mutant CAP stimulation has been demonstrated previously *in vivo*, even if it has received little mention. For example, in work done with a promoter bearing a CAP site at −72.5 (the CC promoter), β-galactosidase expression was stimulated threefold in a strain containing a plasmid that encodes HL159CAP *versus* a strain bearing an isogenic plasmid lacking the *crp* gene (Savery *et al.*,

1995). A similar observation has been reported for additional AR1 CAP mutants at promoters with CAP binding sites centered at −61.5 (Zhou *et al*., 1994). In addition, there is at least one other case of an α-CTD-dependent upstream activator protein retaining residual stimulation in the absence of the α-CTD. The FIS protein of *E. coli* demonstrates a three-fold stimulation of transcription, *in vitro*, in the absence of the RNA polymerase α-CTD (Bokal *et al*., 1997).

The mechanism by which CAP provides this post-binding, AR1-independent stimulation is elusive. A formal possibility is that the HL159 mutation does not completely knock out AR1. The residual stimulation offered by HL159CAP could be due to a less than optimal protein-protein contact with the α-CTD. We could not test this *in vivo* by using a strain deficient in the α-CTD (this is a lethal mutation when no wild-type α-subunit is supplied). However, the *in vitro* transcription experiments in this study indicate that any residual AR1 activity with the HL159 mutation does not function *via* a contact with the α-CTD because a twofold stimulation is present with either wild-type CAP or HL159CAP when the Δ235 RNA polymerase is used.

An alternative possibility is that the stimulation by HL159CAP is modulated by a contact between CAP and a part of RNA polymerase other than the α-CTD. For example, at class II promoters, such as *gal*P1, CAP binds to a site centered at −41.5 and works through an amino acid patch designated AR2 that makes contact with the amino terminus of the RNA polymerase α-subunit (α-NTD) (reviewed by Busby & Ebright, 1997). AR2 stimulation appears to act by stimulating open complex formation (Niu *et al*., 1996; Rhodius *et al*., 1997). Besides AR2, certain mutants of CAP unmask a third amino acid patch, AR3, that is proposed to make contact with the carboxy terminus of the σ^{70} subunit at class II promoters (Busby & Ebright, 1997). This possibility, an AR3-σ^{70} contact, appears unlikely to account for the AR1-independent CAP stimulation of *lac*. In the wild-type CAP protein, as well as in the HL159CAP used in these studies, AR3 is silent. Further, the CAP binding site at *lac* is located too far upstream to reasonably hypothesize interaction with σ^{70}. In the case of AR2, CAP centered at −61.5 is not in an optimal position to make contact with the α-NTD. However, in our system we detect only two- to threefold AR1-independent stimulation. It may be possible to achieve this level of activation even with a suboptimal AR2/α-NTD contact. We note that it is also possible that some portion of CAP other than AR2 (but not AR1) is making the contact to RNA polymerase. We propose the AR2/α-NTD contact as an example of a possible alternative CAP-RNA polyerase contact at class I promoters.

Numerous observations make an AR2/α-NTD contact more attractive as a hypothesis for the source of class I AR1-independent CAP stimulation. First, CAP causes a severe bend in the DNA upon binding (Schultz *et al*., 1991). This bend is toward the CAP dimer and would serve to bring AR2 of CAP nearer to the polymerase if both proteins are bound to the same face of the DNA helix. Second, HL159CAP stimulation occurs in the absence of the α-CTD (Figure 1C and Table 2), but in our studies, the α-NTD is not altered. Thus, both the RNA polymerase assembly function and the AR2 binding function of the α-subunit are maintained. Third, the AR2/α-NTD contact is thought to stimulate isomerization of the open complex rather than RNA polymerase binding (Niu *et al*., 1996; Rhodius *et al*., 1997). A possible interpretation of the observation that the stimulation by HL159CAP is independent of the concentration of RNA polymerase in our *in vitro* transcription assays is that the two- to threefold stimulation takes place after RNA polymerase has bound the promoter. This interpretation is consistent with the nature of AR2 stimulation at a class II CAP dependent promoter.

Finally, the −45 region sequence effect on the AR1-independent CAP stimulation indicates that this residual CAP stimulation at class I promoters may be very sensitive to slight alterations in the DNA structure of the −45 region. Different −45 region sequences will have slightly different local helical structures. The already tenuous ''interaction'' we have proposed between CAP AR2 and the α-NTD in this hypothesis may not permit the resulting slight changes in the relative orientation CAP to RNA polymerase. The −45 region sequence may not be the sole determinant of the magnitude of the AR1-independent stimulation offered by CAP; however, it is certainly an important participant in this partnership between CAP and RNA polymerase at *lac*. A good way to test this hypothesis would utilize a double mutant of CAP that destroys both AR1 and AR2 and see if CAP stimulates RNA polymerase at *lac*P1. Use of α-NTD mutants which fail to respond to CAP at class II promoters would complement such an investigation.

Regardless of the nature of the AR1 and/or α-CTD-independent CAP stimulation of *lac*P1 reported here, a number of very important observations have been made. Foremost, the current model for CAP activation at class I promoters is certainly insufficient. Although the bulk of class I CAP stimulation is manifested through the AR1/α-CTD protein-protein contact, CAP retains two- to threefold stimulation at *lac*P1 in the absence of this contact. In particular, this residual stimulation remains in the absence of the α-CTD which has not been reported for a class I promoter. The model should be updated to reflect this versatility of CAP.

Second, the stimulation by CAP and −45 region UP DNA are not fully additive at *lac*P1 either *in vivo* or *in vitro*. Formerly we had three models to describe this phenomenon. However, the stabilization of CAP against a heparin challenge in the presence of RNA polymerase has eliminated a

Table 4. Strains used in this study

Strain	Relevant genotype	Reference
RZ201a	F- Δ(*lac-proAB*)x111 *ara thi rpsL* Δ*crp tet*	This study
RZ201awt	RZ201a::λ*plac5*	This study
RZ201a10	RZ201a::λ*plac5*(P10)	This study
RZ201a503	RZ201a::λ*plac5*(P503)	This study
RZ201a504	RZ201a::λ*plac5*(P504)	This study
RZ201a506	RZ201a::λ*plac5*(P506)	This study

P10, P503, P504, and P506 refer to the −45 region sequence icorporated into λ*plac5*. The sequences are listed in Figure 3.

competition for the α-CTD as a model to describe the lack of additivity. This study presents further evidence for a minimum twofold −45 region sequence-dependent effect on CAP stimulation. This modest effect could explain the lack of additivity for a number of mutants from our previous study, and for mutant 503, which is used in this study as well. However, some −45 region UP DNA mutants reported previously displayed a greater defect than a twofold defect in their response to CAP. The participation of the −45 region DNA in the CAP-RNA polymerase partnership in transcription at *lac*P1 is insufficient to explain these more dramatic examples of incomplete additivity. Thus, both of the remaining models, a −45 region sequence effect on CAP stimulation and an alteration of the rate limiting step in the presence of CAP and a −45 region UP element could still be responsible for the lack of additivity in our system.

Materials and Methods

Bacteria, DNA, and proteins

The strains used in this study are listed in Table 4. The promoter constructs used in these studies are given in Figure 3. The radiolabeled DNA fragments used in the DNase I footprinting experiments were derived by restriction digestion of plasmids bearing the *lac*UV5 promoter (a *lac*P1 mutant with a perfect −10 hexamer sequence) and the UP*lac* mutant promoter (the wild-type *lac*P1 with the downstream half of the *rrn*BP1 UP element sequence inserted from −50 to −38). The fragments, representing DNA from +57 to −186 of the *lac*P1 promoter, were labeled on the non-template strand using AMV super reverse transcriptase (Molecular Genetics) and [α-^{32}P]dATP (DuPont). Fragments were purified by electrophoresis on 5% (w/v) polyacrylamide gels. Plasmids pwtT and pUPT were generated by cloning the *Hin*dIII/*Eco*RV fragment of pRLG770 (which contains the *rrnB* terminator) into the *Hin*dIII/*Nar*I sites of pUC119 and a derivative of pUC119 bearing the UP mutation in the −45 region. RNA polymerase was purchased from Epicentre Technologies, Madison, WI. RNA polymerase lacking the carboxy-terminal 94 amino acid residues (Δ235 RNA polymerase) was a kind gift from Sarah Aiyar (R.L. Gourse laboratory, University of Wisconsin, Madison, WI). DNase I was supplied by Sigma. CAP and HL159CAP (CAP with an AR1 mutation consisting of an histidine to leucine change at position 159) were purified using cAMP affinity chromatography (Zhang *et al.*, 1991). Plasmids pHA5 (Aiba *et al.*, 1982) and pDCRPHL159 (Bell *et al.*, 1990) were used for expression of CAP and HL159CAP, respectively. Plasmids pDU9 (no CAP), pDCRP (wild-type CAP) and pDCRPHL159 (Bell *et al.*, 1990) provided no CAP, wild-type CAP, and HL159CAP in the strains used in the β-galactosidase assays.

β-Galactosidase assays

Assays were done as described (Czarniecki *et al.*, 1997).

DNase I footprinting

Reactions were carried out in 20 μl in 1 × R buffer (10 mM Tris-acetate (pH 7.9), 10 mM MgCl2, 1 mM DTT, 100 μg/ml bovine serum albumin, 100 μM cAMP, 100 mM potassium glutamate). DNA fragments were present at 1 nM, CAP was present at 25 nM and RNA polymerase was present at 200 nM total protein (2.5 units as defined by Epicentre Technologies; 1 unit of RNA

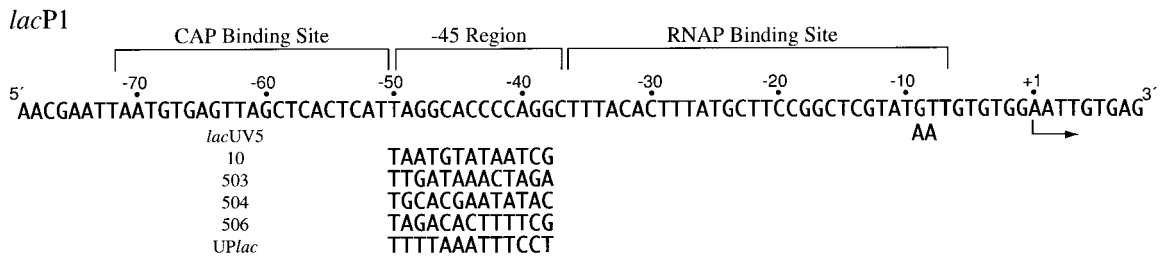

Figure 3. Non template strand of the *lac*P1 promoter. The promoter region consists of the RNA polymerase binding site, i.e. the core promoter, the −45 region, and the CAP binding site. The top sequence represents the wild-type promoter. Variants used in this study include *lac*UV5, 10, 503, 504, 506, and UP. The sequence changes of these mutants are listed below the wild-type promoter.

polymerase incorporates 1 nM of radioactive NTP into RNA in ten minutes at 37°C using bacteriophage T7 DNA template). Glycerol was added to a final concentration of 5%. Reactions were incubated with CAP for five minutes at 37°C. RNA polymerase was added and reactions were incubated for an additional 30 minutes at 37°C. Heparin was added to a final concentration of 60 μg/ml and tubes were transferred to 30°C for one minute, then 10 ng of DNase I was added and the reaction was stopped after an additional minute at 30°C by addition of 25 μl of stop solution (1.5 M sodium acetate, 50 mM EDTA, 0.2 mg/ml glycogen) and 100 μl ethanol. Reactions were precipitated, washed with 70% ethanol, resuspended in 4 M urea loading buffer (4 M urea, 0.5 × TBE (Sambrook *et al.*, 1989), 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol), boiled for one minute, loaded onto a 6% polyacrylamide (19:1, acrylamide/bis)/8 M urea sequencing gel and electrophoresed at 30 W, constant power. Gels were dried and exposed to autoradiography film.

In vitro transcription

Reactions were carried out in 25 μl of 1 × R buffer supplemented with 50 mM potassium glutamate (final 150 mM). Templates were supercoiled plasmids bearing rho-independent terminators downstream of the *lac*P1 (pwtT) or the −45 UP mutant promoter (pUPT). Reactions contained 1 nM template; 25 nM CAP or HL159CAP; 200 μM each ATP, CTP and GTP; 10 μM UTP (plus 1 μl [α-^{32}P]UTP, 3000 Ci/mmol), 2% (v/v) glycerol, and RNA polymerase as indicated for Figure 1, Table 1, and Table 2. Reactions were incubated with or without CAP or HL159CAP for five minutes at 37°C. Upon addition of RNA polymerase, reactions were incubated (37°C) for an additional ten minutes prior to addition of 25 μl of stop solution and 100 μl of ethanol. Reactions were precipitated and electrophoresed as described for DNase I footprinting. Gels were scanned on a phosphorimager and bands were quantitated using ImageQuant software (Molecular Dynamics). Bands were corrected for background and normalized to the *rna*1 transcript. The data are graphed in Figure 1. Standard deviations are indicated with error bars.

Acknowledgements

We extend thanks to R. L. Gourse for communicating results prior to publication and to P. J. Kiley for helpful comments on the work and manuscript. This work was supported by grant MCB-9419784 from the N.S.F. R.J.N. was supported in part by N.I.G.M.S. training grant T32GM07215. W.S.R. is the Evelyn Mercer Professor of Biochemistry and Molecular Biology.

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Edited by J. H. Miller

(Received 30 March 1998; received in revised form 9 June 1998; accepted 24 June 1998)