

Synergistic transcription activation: a dual role for CRP in the activation of an *Escherichia coli* promoter depending on MalT and CRP

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Activation of the *Escherichia coli* *malEp* promoter relies on the formation of a higher order structure involving cooperative binding of MalT to promoter-proximal and promoter-distal sites as well as CRP binding to three sites located in between. MalT is the primary activator and one function of CRP is to facilitate cooperative binding of MalT to its cognate sites by bending the intervening DNA. It is shown here that CRP also participates directly in *malEp* activation. This function is carried out by the molecule of CRP bound to the CRP site centered at –139.5 (CRP site 3). This molecule of CRP recruits RNA polymerase by promoting the binding of the RNA polymerase α subunit C-terminal domain (α CTD) to DNA immediately downstream from CRP site 3, via a contact between α CTD and activating region I of CRP. The action of MalT and CRP at *malEp* hence provides the example of a novel and complex mechanism for transcriptional synergy in prokaryotes whereby one activator both helps the primary activator to form a productive complex with promoter DNA and interacts directly with RNA polymerase holoenzyme.

Keywords: CRP/*Escherichia coli*/MalT/RNA polymerase α subunit/transcription synergy

Introduction

Prokaryotic transcription activators act either alone or synergistically, depending on promoter architecture. Activation involving only one activator is beginning to be well understood at the mechanistic level for the σ^{70} -dependent promoters (Ishihama, 1992; Lonetto *et al.*, 1998; Rhodius and Busby, 1998; Busby and Ebright, 1999). Most often, transcription activation involves a contact between the regulatory protein and the RNA polymerase holoenzyme (RNAP; subunit composition $\alpha_2\beta\beta'\sigma^{70}$). Schematically, at class I promoters, the activator facilitates RNAP recruitment by interacting with the C-terminal domain of one α subunit (α CTD, 81 amino acids) and by promoting its binding to adjacent DNA. α CTD is linked to the N-terminal domain (α NTD), which is responsible for the main interactions with the other RNAP subunits, via a flexible linker of at least 13 amino acids. The activator binding site can be located at various distances from the –35/–10 RNAP site elements, provided that the activator site is correctly phased with respect to the RNAP site. At class II promoters, the activator binds a DNA motif overlapping the –35 region

and interacts with σ^{70} , α NTD and/or α CTD, the latter interaction promoting α CTD binding to promoter DNA immediately upstream of the activator binding site. While the interaction with α CTD facilitates RNAP recruitment, as observed at the class I promoters, the interaction with σ^{70} or α NTD facilitates isomerization of closed complexes to open complexes.

In contrast, much less is known about the mechanisms underlying synergistic activation. Dissection of a few natural or synthetic co-activation systems has so far revealed two scenarios. According to the first one, synergy results from the independent action of two activators interacting directly with RNAP, as observed at promoters at which they act alone, such as two class I activators, each of them interacting with one α CTD, or one class I activator and one class II activator (Joung *et al.*, 1993, 1994; Busby *et al.*, 1994; Scott *et al.*, 1995; Belyaeva *et al.*, 1998). According to the second scenario, only one activator, the primary activator, interacts directly with RNAP while the second aids the primary activator to form a productive complex with promoter DNA (Richet *et al.*, 1991; Belyaeva *et al.*, 2000).

This paper deals with the mechanism whereby the *Escherichia coli* *malEp* promoter is synergistically activated by MalT, the activator of the maltose regulon, and by the cAMP receptor protein (CRP; also referred to as CAP), a global regulator controlling the utilization of carbon sources (Richet, 1996; Boos and Shuman, 1998). *malEp* is divergent from the *malKp* promoter whose activation also depends on the concerted action of MalT and CRP, both of the promoters directing the synthesis of components of the maltodextrin transport system. While *malEp* and *malKp* display some residual activity in the presence of MalT alone, they are inert in the presence of CRP alone (Schwartz, 1987; Richet and Sogaard-Andersen, 1994; Richet, 1996). MalT is a monomeric protein (103 kDa) that self-associates in the presence of both of its positive effectors, maltotriose and ATP (Schreiber and Richet, 1999), and recognizes a 10 bp asymmetric DNA motif (Vidal-Ingigliardi *et al.*, 1991). CRP is a dimeric protein (2 × 23 600 kDa) that, in the presence of cAMP, specifically binds a 22 bp symmetric DNA site and bends DNA to an angle of ~90° in the complex formed with DNA (Schultz *et al.*, 1991). The activation of *malEp* and *malKp* is a coupled process involving a unique 210 bp regulatory region comprising, from *malEp* towards *malKp*: two MalT sites, three CRP sites and two overlapping sets of three MalT sites staggered by 3 bp (Figure 1). The mechanism whereby MalT and CRP co-activate *malEp* and *malKp* involves a CRP-induced switch between a repressor state and an active state responsible for the repression and activation of both promoters, respectively (Richet *et al.*, 1991; Richet, 1996). Schematically, MalT can form either a repression complex by binding cooperatively the

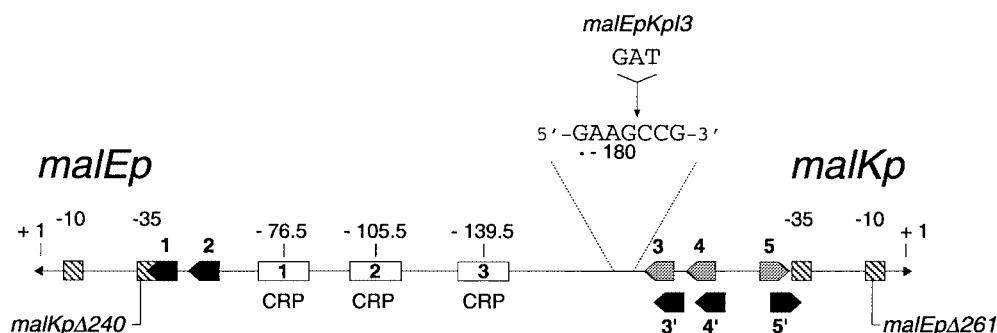


Fig. 1. Structure of the regulatory region of *malEp* and *malKp*. The *malEp*–*malKp* region is drawn to scale with the promoter –10 and –35 elements (hatched boxes), the MalT sites (arrowed boxes) and the CRP sites (open boxes) indicated. The transcription start sites of *malEp* and *malKp* are 271 bp apart. The nucleotide sequence (Raibaud *et al.*, 1989) is numbered with respect to the *malEp* transcription start site. MalT sites 3/4/5 (gray arrowed boxes) are involved in promoter repression while MalT sites 1/2 and 3'/4'/5' (black arrowed boxes) are involved in promoter activation. The 3/4/5 series of sites is staggered by 3 bp from the 3'/4'/5' series of sites. For the exact location of all of the MalT sites, see Richet *et al.* (1991) and Vidal-Ingigliardi *et al.* (1991). The location of the *malEpKpI3* insertion and the end points of deletions *malKp*Δ240 and *malEp*Δ261 are indicated.

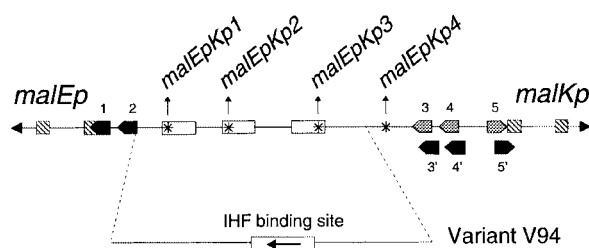
high-affinity sites 3/4/5 or an activation complex by binding cooperatively the two low-affinity sets of sites, 1/2 and 3'/4'/5', from which it activates *malEp* and *malKp*, respectively. MalT is thought to contact RNAP via the MalT protomer bound to the MalT site overlapping the –35 element (Danot *et al.*, 1996). The repression state predominates in the absence of CRP while assembly of the activation complex is favored in the presence of CRP. Negative supercoiling also plays a critical role in the assembly of the activation complex (Richet and Raibaud, 1991). As inferred from bend-swap experiments showing that the three CRP-induced bends can be replaced by a static bend or an IHF (integration host factor)-induced bend without altering the mechanism of *malKp* activation, CRP promotes the cooperative binding of MalT onto sites 1/2 and 3'/4'/5' simply by bending the intervening DNA (Richet and Sogaard-Andersen, 1994). This is the primary role played by CRP in *malKp* activation. Co-activation of *malKp* by MalT and CRP therefore follows the second of the scenarios described above.

In this paper, I investigate the role played by CRP in *malEp* activation and show that *malEp* co-activation by MalT and CRP obeys a new scenario in which CRP, the second activator, both assists MalT, the primary activator, and interacts directly with RNAP.

Results

CRP does not play a purely structural role in *malEp* activation

To test the hypothesis that CRP plays a purely architectural role in *malEp* activation, as found for *malKp*, I examined whether an IHF-stabilized activation complex activates *malEp*. The chimera assayed was the V94 variant in which the three CRP binding sites have been replaced by one IHF binding site (Figure 2) and which exhibits a highly active *malKp* promoter depending on both MalT and IHF and functioning like wild-type *malKp* (Richet and Sogaard-Andersen, 1994). The assays were performed *in vivo* by comparing the amount of β-galactosidase made from various *malEp*–*lac* transcriptional fusions carried by a single-copy-number plasmid in a Δ*lac* strain. The bacteria were grown in minimal medium in the presence of glycerol and maltose, i.e. under conditions in which



Variant	In vivo promoter activity		
	<i>hip</i> ⁺		<i>hip</i> ⁻
	+ maltose	- maltose	+ maltose
	Ep (Kp)	Ep (Kp)	Ep (Kp)
<i>malEpKp</i> ⁺	100 (100)	5 (4)	100 (100)
<i>malEpKp1,-2,-3,-4</i>	8 (2)	-	-
V94	13 (48)	< 1 (< 1)	13 (< 1)

Fig. 2. *In vivo* activity of the chimeric *malEp* variant. The amount of β-galactosidase made by isogenic *hip*⁺ and *hip*⁻ strains (pop2226 and pop4103, respectively) harboring pOM82 (or derivatives thereof) and grown in the presence or absence of maltose was determined as described in Materials and methods. The results are expressed relative to the values obtained for the induced wild-type *malEp* (14 000 and 14 100 U in the *hip*⁺ and the *hip*⁻ strains, respectively). The asterisks symbolize the *malEpKp1*, -2, -3 and -4 mutations that correspond to 3 bp substitutions inactivating CRP sites 1, 2, 3 and 4, respectively (CRP site 4 is a low affinity CRP binding site that does not play any role in *malEp* and *malKp* activation) (Vidal-Ingigliardi and Raibaud, 1991). The values obtained for *malKp* (in parentheses) are from Richet and Sogaard-Andersen (1994).

both MalT and CRP are active. As shown in Figure 2, the *malEp* activity of the V94 chimera represents only 13% of the wild-type level, scarcely higher than observed with a *malEp* variant in which the three CRP binding sites have been inactivated by point mutations (*malEpKp1*, -2, -3). Moreover, while the weak *malEp* activity of the chimera was MalT dependent, as indicated by assays performed in the presence of glycerol only (i.e. under conditions in which the concentration of active MalT is low), it was IHF independent (Figure 2). This contrasts with the high activity of the V94 *malKp* promoter (nearly 50% of the

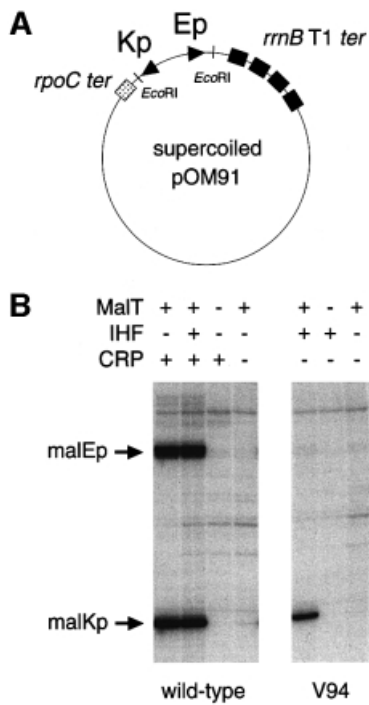


Fig. 3. *In vitro* activity of the chimeric *malEp* variant. *In vitro* transcription assays were carried out in the presence of supercoiled pOM91 variants, 150 nM MalT, 12.5 nM IHF or 150 nM CRP, and 15 nM RNAP. The transcripts made from *malEp* and *malKp* are ~230 and ~180 nucleotides long, respectively.

wild-type level), which is both IHF- and MalT-dependent, and is 24-fold higher than for a *malKp* variant in which the three CRP binding sites have been disrupted by point mutations (Richet and Sogaard-Andersen, 1994; see Figure 2).

Similar results were obtained when the *malEp* chimeric variant was tested in a purified transcription system in which *malEp* and *malKp* are known to behave as *in vivo* (Richet and Raibaud, 1991). The assay used was a single-round transcription assay that monitors the rate of open complex formation. The template was a supercoiled plasmid containing the *malEp*–*malKp* region cloned between two divergent terminators (Figure 3A). As shown in Figure 3B, the V94 chimera displayed little *malEp* activity (<5% of the wild-type level), in contrast to the high activity level of the divergent *malKp* promoter (34% of the wild-type level). Altogether, the *in vivo* and *in vitro* data demonstrate that an IHF-stabilized activation complex does not efficiently activate *malEp*. This result strongly suggests that, besides its structural role in the stabilization of the activation complex, CRP plays an additional role in *malEp* activation that involves critical protein–protein interactions.

The HL159 substitution in CRP specifically interferes with *malEp* activation

Several studies have shown that a CRP molecule can operate via a class I mechanism (i.e. via a contact with one of the α CTD) from a DNA site located quite far from the –35/–10 promoter elements (Busby and Ebright, 1999). The CRP surface determinant thereby contacted by α CTD

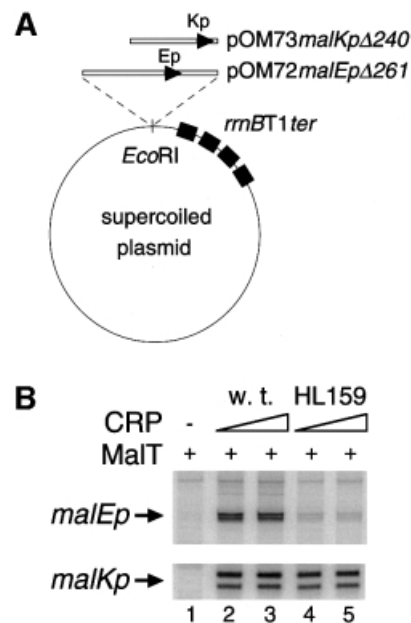


Fig. 4. Effect of the HL159 substitution in CRP on *malEp* and *malKp* *in vitro*. *In vitro* transcription assays were performed in the presence of supercoiled pOM72*malEp*Δ261 (or pOM73*malKp*Δ240), 100 nM MalT, various concentrations of wild-type CRP (or CRP HL159) and 20 nM RNAP. The concentrations of wild-type CRP were 130 nM (lane 2) and 260 nM (lane 3); the concentrations of CRP HL159 were 200 nM (lane 4) and 400 nM (lane 5). The *malEp* and *malKp* transcripts are ~230 and ~120 nucleotides long, respectively.

is activating region 1 (AR1), an exposed β -turn consisting of residues 156–164 (Busby and Ebright, 1999). The HL159 substitution has been shown to impair strongly the interaction between α CTD and CRP without altering the ability of the protein to bind and bend DNA (Zhou *et al.*, 1993, 1994; Attey *et al.*, 1994). To test the hypothesis that the additional role of CRP at *malEp* involves a class I contact with RNAP, the effect of the HL159 substitution on *malEp* activation was analyzed *in vitro* by using *malKp* as a control. In order to avoid indirect effects, the templates used were promoter variants carrying an upstream deletion (*malEp*Δ261 or *malKp*Δ240) that inactivates the divergent promoter without altering the *malEp*–*malKp* regulatory region (see Figure 1) (Raibaud *et al.*, 1989). *In vitro* transcription assays were performed as described above by using supercoiled templates (Figure 4A).

Interestingly, the HL159 substitution in CRP caused a severe defect in *malEp* activation, whereas, as expected, it scarcely affected *malKp* (Figure 4B): *malEp* was only 15% active when the mutant CRP protein was substituted for wild-type CRP while *malKp* retained 83% of the activity level obtained with the wild-type protein. The low *malEp* activity level observed in the presence of the HL159 CRP protein remained unchanged when the concentration of the mutated protein was increased 2- to 4-fold (Figure 4B and data not shown). Both this fact and the absence of a significant effect on *malKp* exclude the possibility that the defect caused by the HL159 substitution results from a reduced occupation of the CRP sites due to the slightly reduced affinity of the HL159 CRP protein for its DNA site (Zhou *et al.*, 1993). Thus, these data strongly suggest that at least one of the three molecules of CRP involved in

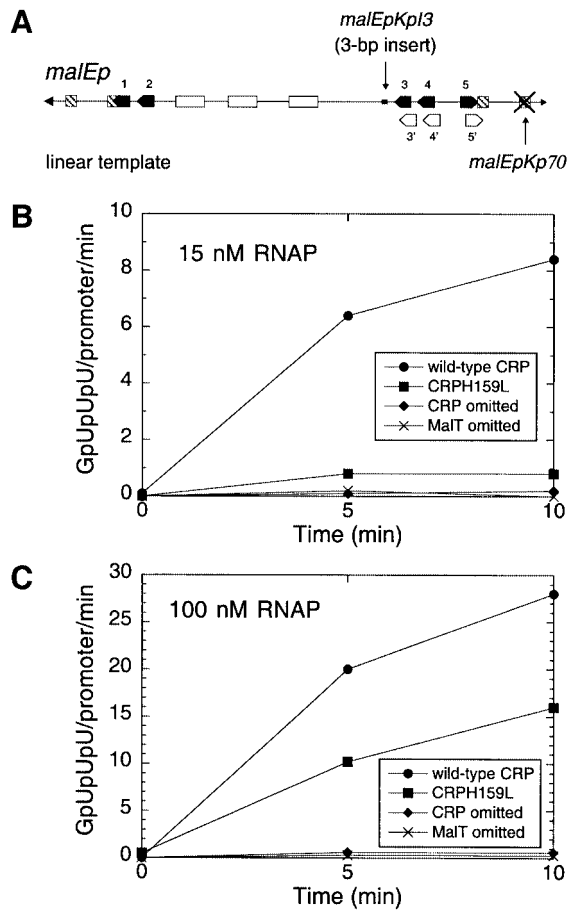


Fig. 5. Effect of the HL159 substitution in CRP on open complex formation in the presence of various concentrations of RNAP. (A) Structure of the linear *malEp* substrate. Owing to the 3 bp insertion at -183 , formation of the activation complex involves cooperative binding of MalT onto sites 1/2 and 3/4/5 and no longer depends on negative supercoiling. (B) Kinetics of open complex formation at *malEp* in the presence of 15 nM RNAP. Open complex formation was monitored by the abortive initiation assay described in Materials and methods using the linear *malEp* model as template. (C) Kinetics of open complex formation at *malEp* in the presence of 100 nM RNAP.

the stabilization of the activation complex also participates in *malEp* activation via a protein-protein contact involving AR1 and possibly α CTD.

Construction of a model *malEp* variant

To make the *malEp* activation process amenable to mechanistic studies, a mutation (*malEpKp13*) was introduced that alleviates the requirement for negative DNA supercoiling at the step of activation complex formation. *malEpKp13* corresponds to a 3 bp insertion in the central region, between CRP site 3 and MalT site 3, which brings the high affinity repressor set of MalT sites (3/4/5) in register with MalT sites 1/2 (Figure 5A) (it should be recalled that the two overlapping sets of MalT sites, 3/4/5 and 3'/4'/5', are staggered by 3 bp). As a result, activation of this *malEp* variant involves cooperative binding of MalT onto sites 1/2 and 3/4/5 instead of 3'/4'/5' (see below). Most likely, both the absence of competing repressor sites and the replacement of the original activator sites by higher affinity sites make assembly of the

activation complex independent of negative supercoiling. A mutation in the *malKp* -10 element (*malEpKp70*) was also introduced to inactivate the *malKp* promoter.

When present on a linear DNA fragment, the *malEpKp13,70* variant functioned like supercoiled wild-type *malEp*. Its activation still depended on the presence of both MalT and CRP, and was strongly depressed by the HL159 substitution in CRP (see Figure 5B). DNase I protection experiments confirmed that MalT binds the 3/4/5 set of MalT sites in the presence of CRP, as expected. The top strand protection pattern of the 3/3' and 4/4' regions (see Figure 6B, lanes 1 and 5 and data not shown) was indeed characteristic of site 3 and 4 occupancy (Richet *et al.*, 1991). In addition, assembly of the activation complex by MalT was still dependent on the presence of CRP. Comparison of the protection patterns obtained in the presence of MalT alone, CRP alone or MalT + CRP shows that MalT sites 1/2 were unoccupied and sites 3/4/5 only partially occupied in the presence of MalT alone, while they were fully occupied in the presence of both MalT and CRP (see Figure 6A and B, lanes 1–6 and data not shown). Reciprocally, MalT stabilized CRP onto its binding sites, which further confirms that MalT and CRP cooperatively bind their cognate sites. The *malEpKp13,70* variant, hereafter referred to as the *malEp* model substrate, was used in all of the experiments described below.

CRP plays a direct role in RNAP recruitment

To test further the possibility that CRP acts at *malEp* via a class I mechanism, I examined whether the direct role of CRP in *malEp* activation is related to RNAP recruitment, i.e. whether the defect caused by the HL159 substitution in CRP depends on RNAP concentration. The rate of open complex formation at the *malEp* model substrate was measured at two RNAP concentrations in the presence of saturating concentrations of MalT and wild-type CRP or CRP HL159 by performing fixed-time abortive initiation assays (Figure 5B and C). In the presence of CRP HL159, the amount of open complexes formed 5 min after addition of 15 nM RNAP represents 12% of the level reached in the presence of wild-type CRP. In contrast, when 100 nM RNAP was added, the open complex level represented 50% of that achieved with wild-type CRP. The effect of the HL159 substitution on *malEp* activation is thus clearly more pronounced at low RNAP concentration than at high RNAP concentration, thereby indicating that CRP plays a direct role in the formation of closed complexes.

DNase I footprinting studies of the open complexes formed at *malEp*: RNAP-induced protection in the $-120/-125$ and $-155/-164$ regions

To determine whether CRP promotes α CTD binding to upstream positions upon RNAP binding to *malEp*, I used the same approach as Kolb *et al.* (1993) and performed DNase I protection experiments with the *malEp* model substrate under the conditions of the abortive initiation assays described above. Comparison of the top and bottom strand footprints obtained in the absence of RNAP with MalT + wild-type CRP or CRP HL159 shows that the protection patterns were the same, i.e. that, as expected, the CRP HL159 substitution does not impair assembly of the activation complex (Figure 6A and B, lanes 5 and 6). Note also that DNase I hypersensitivity at $-120/-125$ on

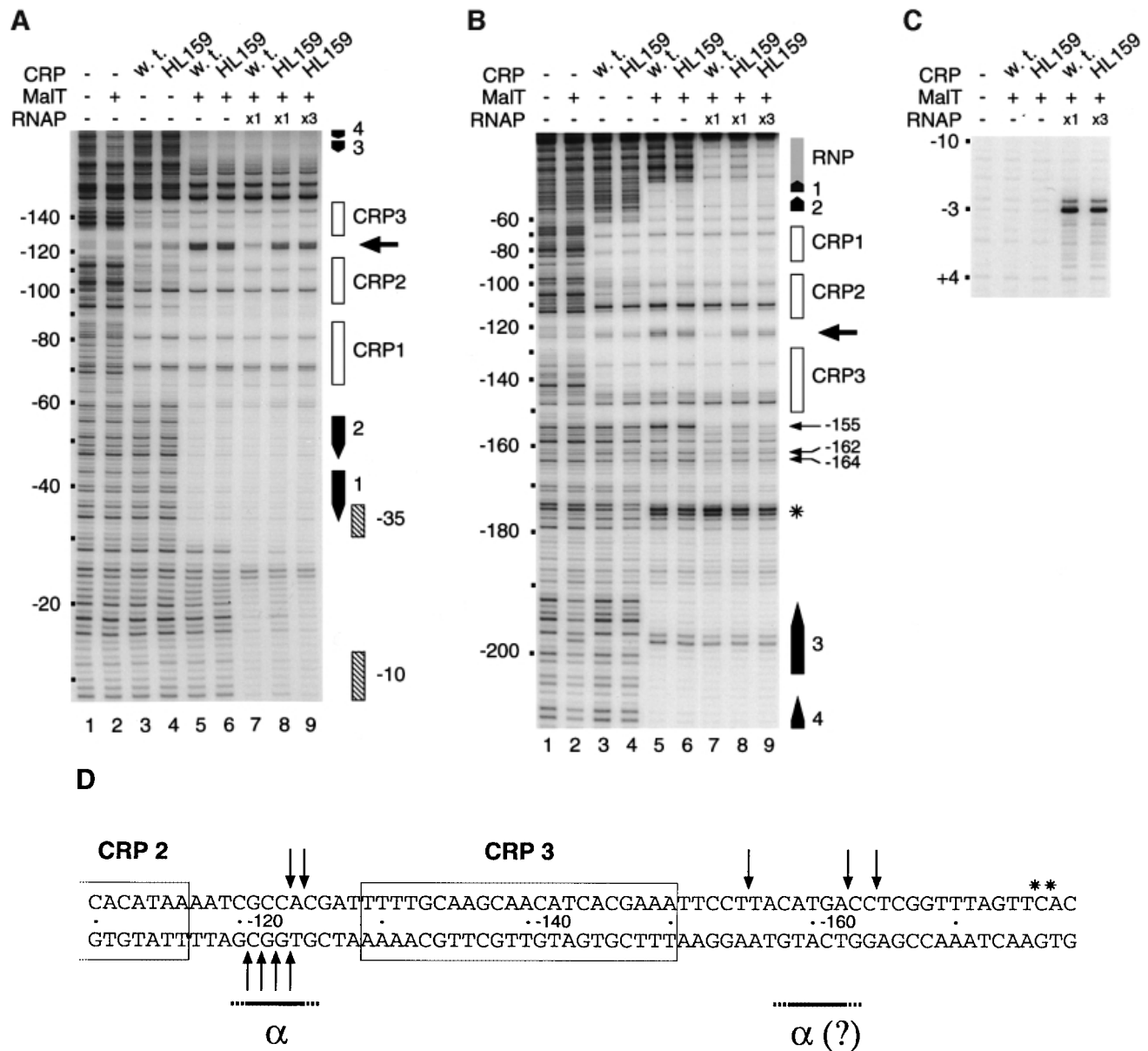


Fig. 6. DNase I and KMnO_4 footprinting of the nucleoprotein complexes formed on the linear *malEp* model promoter. DNase I and KMnO_4 footprinting experiments were performed with the *malEpKpl3 malEp Δ 259* variant. The RNAP concentrations used were 100 nM when noted $\times 1$ and 300 nM when noted $\times 3$. (A) Bottom strand DNase I protection patterns obtained using the pOM53 *EcoRI-KpnI* fragment 3' end-labeled on the bottom strand (non-template strand). (B) Top strand DNase I protection patterns obtained using the pOM53 *PstI-EcoRI* fragment 3' end-labeled on the top strand. (C) KMnO_4 attack patterns obtained using the pOM53 *EcoRI-KpnI* fragment 3' end-labeled on the bottom strand (non-template strand). (D) Location of the RNAP-dependent protections against DNase I cleavage (arrows) and of the enhanced cutting sites that are characteristic of the activation complex and that are not protected upon RNAP addition (asterisks).

both strands as well as at -155, -185 and -186 on the top strand, which characterizes the activation complex, was not altered upon substitution of CRP HL159 for wild-type CRP (enhanced DNase I cleavage most likely reflects minor groove widening due to local DNA bends or structural changes of the double helix accompanying cooperative binding of MaIT onto sites 1/2 and 3/4/5). In the presence of MaIT and wild-type CRP, RNAP binding to the core promoter was accompanied by a clear protection of the -120/-125 region against DNase attack on both strands (Figure 6A and B, lanes 5 and 7). Most interestingly, this RNAP-dependent protection was not observed when CRP HL159 was substituted for wild-type

CRP, even when a higher concentration of RNAP ($\times 3$) was added to achieve the same degree of protection of the core promoter region as in the presence of MaIT and wild-type CRP (Figure 6A and B, lanes 5-9). The simplest interpretation of these data is that RNAP binding to *malEp* involves binding of α CTD to -120/-125 and that α CTD binding depends on an interaction with the AR1 determinant of one of the adjacent CRP, as observed at class I promoters.

The top strand footprints also revealed RNAP-dependent protection at three positions (-155, -162 and -164) upstream from CRP site 3 (Figure 6B, lanes 5-7). The most striking protection affects the phosphodiester bond

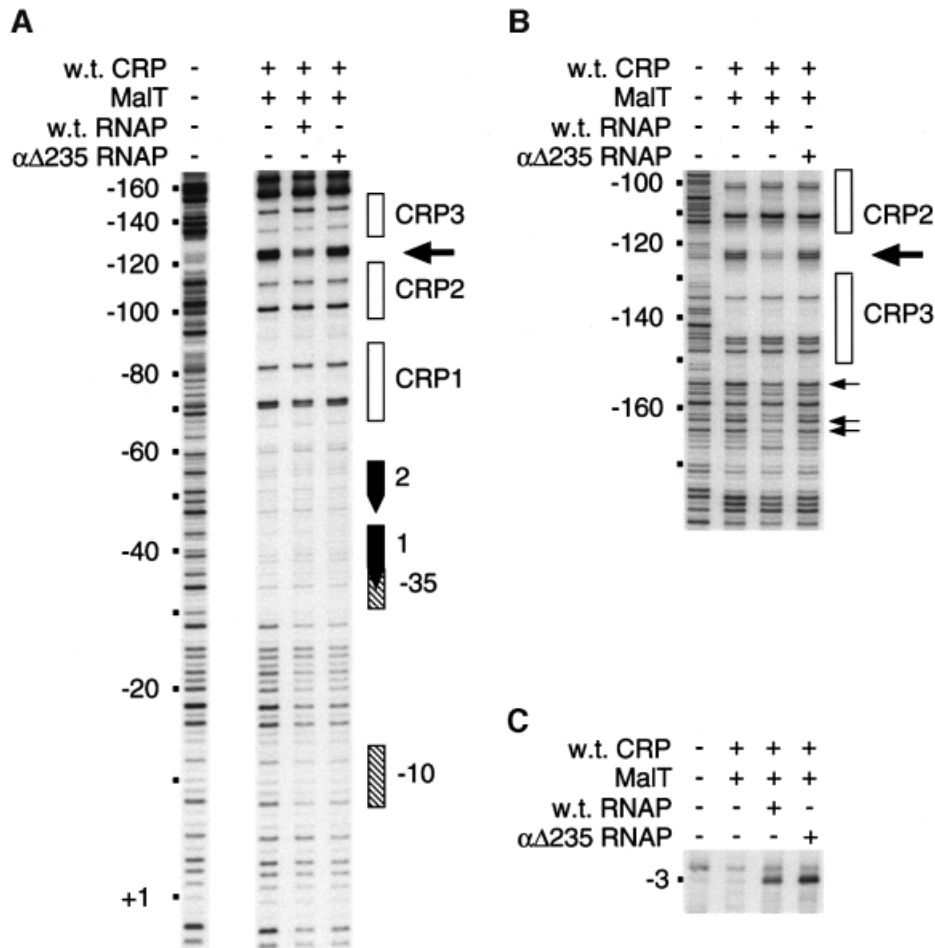


Fig. 7. DNase I and KMnO_4 footprinting of the complexes formed at *malEp* in the presence of reconstituted wild-type or mutant RNAP. (A) Bottom strand DNase I protection patterns obtained using the pOM53 *EcoRI-KpnI* fragment 3' end-labeled on the bottom strand. (B) Top strand DNase I protection patterns obtained using the pOM53 *PstI-EcoRI* fragment 3' end-labeled on the top strand. (C) KMnO_4 attack patterns obtained using the pOM53 *EcoRI-KpnI* fragment 3' end-labeled on the bottom strand.

between nucleotides -155 and -156. Interestingly, these RNAP-dependent protections were still observed when CRP HL159 was substituted for wild-type CRP (Figure 6B, lanes 5–9). These results are best explained by proposing that the other α CTD binds the -155/-164 region and that this binding does not involve any interaction with the AR1 surface element of CRP bound to site 3.

To verify that the complexes formed at *malEp* upon RNAP addition are open complexes regardless of the CRP variant present, the nucleoprotein complexes were probed with KMnO_4 , a reagent that reacts preferentially with unpaired thymine. The assays involving CRP HL159 were performed in the presence of a 3-fold higher concentration of RNAP to ensure equal protection of the core promoter (see Figure 6A, lanes 7 and 9). As shown in Figure 6C, RNAP binding to *malEp* resulted in the same KMnO_4 hypersensitivity of the thymine at position -3 irrespective of whether *malEp* had been pre-incubated in the presence of MaIT + wild-type CRP or MaIT + CRP HL159. Thus, these results clearly indicate that the RNAP complexes analyzed were open complexes in both cases and that the open complexes formed in the presence of CRP HL159

have the same characteristic as those formed in the presence of wild-type CRP.

α CTD is involved in the RNAP-dependent protection observed upstream and downstream from CRP 3

To determine whether the protection observed at -120/-125 and -155/-164 depends on α CTD, DNase I protection experiments were performed with reconstituted RNAPs containing wild-type α or an α variant lacking the C-terminal domain ($\alpha\Delta 235$) (Hayward *et al.*, 1991; Blatter *et al.*, 1994). The concentration of $\alpha\Delta 235$ RNAP was adjusted so as to give the same protection (~50%) of the core promoter region and the same amount of open complexes as reconstituted wild-type RNAP (Figure 7A and C). The bottom and top strand patterns obtained when MaIT and wild-type CRP were incubated in the absence or presence of the reconstituted holoenzymes showed that, unlike wild-type RNAP, $\alpha\Delta 235$ RNAP binding did not lead to protection of the -120/-125 region (Figure 7A and B). Interestingly, protection of the -154/-165 region on the top strand also disappeared when $\alpha\Delta 235$ RNAP was

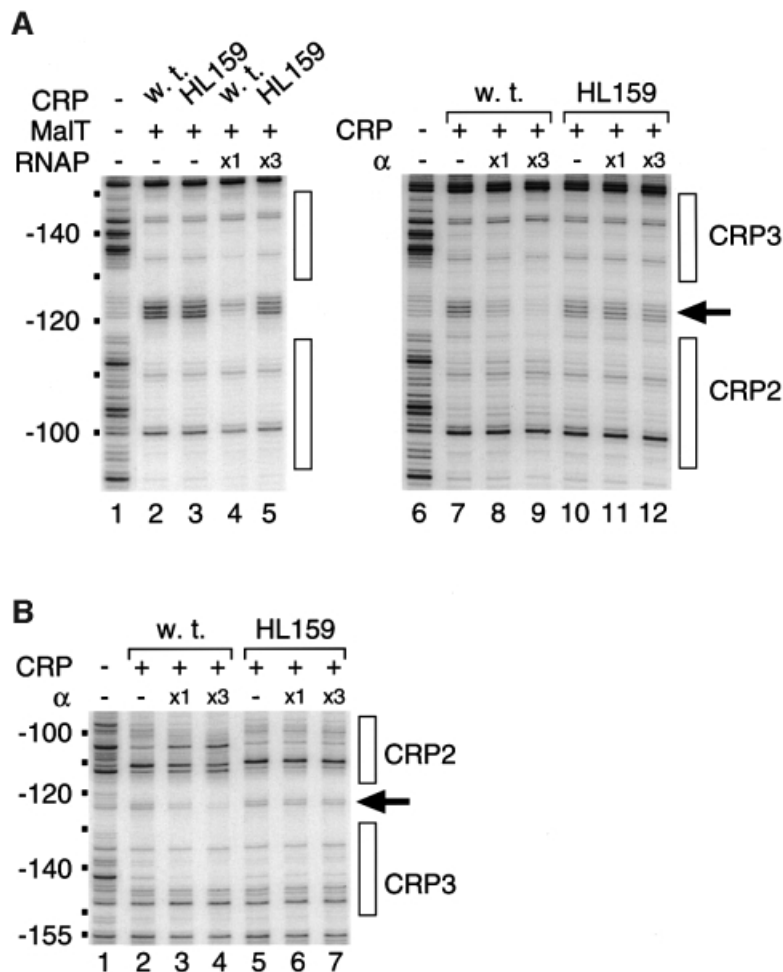


Fig. 8. Recruitment of purified α by CRP. (A) Bottom strand DNase I protection patterns obtained using the pOM53 *PstI-EcoRI* fragment 3' end-labeled on the bottom strand. Lanes 1–5: the DNase I footprinting assays were performed as described in Materials and methods in the presence of 75 nM wild-type CRP or 300 nM CRP HL159. Lanes 6–12: the assays were performed in a low ionic strength buffer (36 mM HEPES-KOH pH 8.0, 40 mM potassium acetate, 10 mM magnesium acetate, 1 mM CaCl₂, 1 mM DTT, 100 μ g/ml acetylated BSA, 0.2 mM cAMP) in the presence of 150 nM wild-type CRP or 460 nM CRP HL159. (B) Top strand DNase I protection patterns obtained using the pOM53 *PstI-EcoRI* fragment 3' end-labeled on the top strand. The assays were performed as described in Materials and methods in the presence of 75 nM wild-type CRP or 300 nM CRP HL159. In both (A) and (B), the concentrations of purified α used were 1.2 μ M when noted $\times 1$ and 3.7 μ M when noted $\times 3$.

substituted for wild-type RNAP (Figure 7B). Altogether, these data thus indicate that the RNAP-dependent protection observed on both sides of CRP 3 involves α CTD.

Purified α and CRP cooperatively bind region -120/-125 and CRP site 3, respectively

As shown by Attey *et al.* (1994), cooperative binding of CRP and purified α can be detected by DNase I footprinting. To obtain direct evidence that protection of the -120/-125 region is due to α CTD binding to -120/-125 and depends on an interaction between α CTD and one of the adjacently bound CRP molecules, I examined whether CRP alone, at a saturating concentration, promotes the binding of purified α to region -120/-125. As shown by Figure 8A (lanes 6–9), α -dependent protection of region -120/-125 was observed on the bottom strand when the *malEp* model substrate was incubated in the presence of wild-type CRP and purified α . The protection pattern was similar to that induced by RNAP in the presence of MalT and wild-type CRP (Figure 8A, compare lanes 1–4 with lanes 6–9). Moreover, protection of region

-120/-125 was strongly reduced when purified α was incubated in the presence of CRP HL159 (Figure 8A, lanes 10–12). Similar protection experiments were also performed in the presence of subsaturating concentrations of CRP. Figure 8B shows the top strand patterns obtained. As observed for the bottom strand, purified α protected region -120/-125 in the presence of wild-type CRP but not in the presence of CRP HL159. Most importantly, α binding to -120/-125 was accompanied by a better protection of CRP site 3 and a lower occupation of CRP site 2, an effect that was not observed with CRP HL159. This observation provides clear evidence that α binding to -120/-125 does not involve CRP 2, but involves a direct contact between α and the AR1 region of the CRP 3 downstream subunit. The lower occupancy of CRP site 2 observed in the presence of wild-type CRP and purified α is most likely due to the fact that CRP 1 promotes α binding to DNA upstream of CRP site 1, as suggested by the observation that addition of purified α to wild-type CRP led to clear protection of the -97/-100 region, i.e. the proximal half of CRP site 2 (Figure 8B, lanes 1–4) [note that CRP affinity for CRP

site 1 is 6-fold higher than its affinity for CRP site 2 (Vidal-Ingigliardi and Raibaud, 1991)].

Discussion

The work presented here provides a clear understanding of the mechanism whereby MalT and CRP co-activate *malEp*. It establishes that, besides its structural role in the stabilization of the activation complex formed by MalT, CRP participates directly in *malEp* activation via a class I mechanism and that this function is carried out by the molecule of CRP bound to site 3. Two observations support the conclusion that the role of CRP involves direct protein-protein interactions in addition to its architectural function. First, both *in vivo* and *in vitro*, an IHF-stabilized activation complex activates *malEp* only very weakly while it activates the *malKp* promoter almost as effectively as a CRP-stabilized activation complex. Secondly, the HL159 substitution in CRP, which is located at the surface of the protein and does not affect its ability to bind and bend its DNA recognition motif (Zhou *et al.*, 1993), strongly impairs *malEp* activation *in vitro* while it scarcely has any effect on *malKp* activation. Several lines of evidence indicate that this additional role of CRP is carried out by the molecule of CRP bound to site 3 and that it consists of the recruitment of α CTD to the -120/-125 site via a direct contact between α CTD and the AR1 region of CRP 3: (i) RNAP binding to the activation complex leads to protection against DNase I attack of the -120/-125 region; (ii) the HL159 substitution in CRP or deletion of the C-terminal domain of the α subunit cause the disappearance of the RNAP-dependent protection of region -120/-125; (iii) wild-type CRP and purified α bind CRP site 3 and region -120/-125 cooperatively in the absence of any other protein; and (iv) the HL159 substitution in CRP suppresses this cooperative effect. Thus, the action of CRP 3 has all of the characteristic features of a class I mechanism (Busby and Ebright, 1999): CRP 3 operates from a distal site; it promotes α binding to a DNA sequence located downstream from the CRP binding site via a contact between the AR1 determinant of CRP and the α subunit; and it facilitates RNAP recruitment, as inferred from the fact that disruption of the AR1 region causes a stronger defect in *malEp* activation at low RNAP concentration than at high RNAP concentration.

It might seem surprising that the molecule of CRP bound to site 3, which is centered at -139.5, can act as a class I activator although its binding site is not located on the proper face of the DNA helix and is much further away from the RNAP site than classically observed for a class I mechanism. Class I activation by CRP indeed requires that the CRP site be located on the same face of the DNA helix as the core RNAP site, i.e. its binding site has to be centered near positions -61.5, -72.5, -82.5, -92.5. In addition, the most distal position producing some stimulatory effect is -92.5 (Gaston *et al.*, 1990; Ushida and Aiba, 1990). However, the path followed by the DNA double helix within the higher order structure formed by MalT and CRP at *malEp* most likely allows CRP 3 to interact productively with one of the α CTD. In particular, the two CRP-induced bends interposed between CRP site 3 and the RNAP site probably bring CRP site 3 closer to the RNAP site. As shown by Belyaeva *et al.* (1998), the

presence of a proximal CRP binding site at -41.5 indeed increases the distance from which CRP can act as a class I activator by two helix turns. The CRP-induced DNA unwinding (Douc-Rasy *et al.*, 1989) might also allow for a reorientation of the DNA segment carrying region -120/-125 and CRP site 3 with respect to the core promoter, and compensate for the improper phasing between CRP site 3 and the core promoter sequence.

This work also revealed RNAP-dependent protections just upstream from CRP site 3, at -155/-164. While this effect clearly depends on the presence of the C-terminal domain of α , it does not depend on the AR1 region of CRP. These protections might result from the binding of the second copy of α CTD to region -155/-164. The sequence of this region does not display any obvious similarity to the consensus of UP element subsites (Estrem *et al.*, 1999), but, as suggested by the DNase I hypersensitivity at -155 that characterizes the activation complex, the DNA double helix might present some local structural features that favor α CTD binding. Binding of the second copy of α CTD to DNA just upstream from the CRP site has also been observed at the *lac* promoter, a simple class I promoter, by Kolb *et al.* (1993) and Naryshkin *et al.* (2000), but in this case it is not known whether α CTD binding to upstream DNA is independent of the AR1 determinant of the upstream CRP subunit.

In conclusion, the mechanism underlying the synergistic action of MalT and CRP at *malEp* follows a complex and new scenario in which the second activator both assists the primary activator and acts as a class I activator. Such a scheme corresponds to a combination of the two scenarios so far described. A similar strategy might also be at the basis of *araBAD* promoter co-activation by AraC and CRP: CRP indeed both facilitates the formation of an activation complex by AraC by breaking the repression loop and participates directly in the activation of the *araBAD* promoter, most likely as a class I activator (Lobell and Schleif, 1991; Zhang and Schleif, 1998). Given the flexibility conferred by the flexible linker tethering α CTD to RNAP, one might predict the existence of even more complex combinations of the two basic synergistic strategies already described, such as one in which two activators acting independently upon RNAP are each assisted by another regulatory protein, helping them to form productive complexes with promoter DNA.

The large size of the higher order structure responsible for *malEp* and *malKp* activation, which is made of at least five MalT protomers and three molecules of CRP, i.e. ≥ 640 kDa of protein, makes *malEp* and *malKp* unique among the regulated prokaryotic promoters so far described. Through direct contacts with RNAP mediated by MalT 1 (Danot *et al.*, 1996) and CRP 3 (this work), the activation complex recruits RNAP to *malEp* and promotes initiation of transcription, turning the inert *malEp* promoter into a promoter stronger than the synthetic *tac* promoter (my unpublished data). It is worth noting that *malEp* activation represents a highly integrated process wherein the functions of the components of the activation complex are multiple and interrelated, e.g. CRP 3 both stabilizes the activation complex and interacts with the RNAP α subunit, while CRP 1 and 2 stabilize the activation complex and most likely also facilitate the interaction between CRP 3 and α CTD. Finally, one should

emphasize that, in some respects, the activation complex operating at *malEp-malKp* resembles the eukaryotic enhanceosomes whose assembly also involves cooperative binding of several transcriptional activators as well as architectural proteins and that recruit general transcription factors and RNA polymerase II holoenzyme to the promoter through specific protein-protein contacts (Giese *et al.*, 1995; Kim and Maniatis, 1997; Kim *et al.*, 1998).

Materials and methods

Strains and plasmids

pop2150 is a derivative of MC4100 (Casadaban, 1976) containing the Δ *malA510* deletion, which removes the beginning of the *malT* gene (Raibaud *et al.*, 1983). pop2226 is a derivative of MC4100 carrying *malPp201*, a promoter-down mutation of the *malPQ* operon (O. Raibaud, unpublished). pop4103 is a Δ [*hip*]::cat derivative of pop2226 (Richet and Sogaard-Andersen, 1994).

All of the *malEp-malKp* variants used here are derivatives of the 478 bp *EcoRI-EcoRI* fragment of pOM18 that carries the wild-type *malEp-malKp* region (Raibaud *et al.*, 1989). M13malEK1 is an M13mp11 derivative containing the 478 bp *EcoRI-EcoRI malEp-malKp* fragment from pOM18 (Vidal-Ingigliardi and Raibaud, 1991) inserted into the *EcoRI* site of M13mp11. pOM53, a pSB118 derivative containing the *malEp* Δ 259 *malEpKp13* variant (from +38 to -259 with respect to *malEp*), was constructed as follows. A DNA fragment extending from +38 (*malEp*) to +8 (*malKp*) was obtained by PCR using the M13malEK1*malEpKp13* variant as template and primers EK1 (5'-GGTCCT-TGTTGGTGAAG-3') and EK70 (5'-TTCATGATGGGCTGGCTAGC-GTCATCAGGAGAT-3'), which replaces the '-10' sequence of *malKp* by an *NheI* site. The PCR DNA fragment was made blunt ended, cut by *NheI*, and cloned between the *HincII* and *XbaI* sites of pSB118, yielding pOM53. The construct was verified by sequencing. pOM72*malEp* Δ 261 and pOM73*malKp* Δ 240 contain the *malEp* Δ 261 variant of *malEp* and the *malKp* Δ 240 variant of *malKp*, respectively, inserted upstream of the *rrnBT1* terminators of pOM70 (Richet and Raibaud, 1991). pOM82 is a derivative of pJEL126 (Valentin-Hansen *et al.*, 1986) containing the wild-type 478 bp *EcoRI-EcoRI malEp-malKp* fragment inserted into the *EcoRI* site of pJEL126, with *malEp* fused to the promoterless *lacZYA* operon present on the vector (Richet, 1996). pOM91 is a derivative of pOM90 containing the wild-type *EcoRI-EcoRI malEp-malKp* fragment inserted into the *EcoRI* site between the divergent *rpoC* and the *rrnBT1* terminators, with *malEp* upstream of the *rpoC* terminator and *malKp* upstream of *rrnBT1* (Richet and Sogaard-Andersen, 1994). Derivatives of pOM82 and pOM91 containing the *malEpKp1*, -2, -3, -4 mutations or the V94 variant (Vidal-Ingigliardi and Raibaud, 1991; Richet and Sogaard-Andersen, 1994) were obtained by cloning the mutated *EcoRI-EcoRI malEp-malKp* fragment into pJEL126 and pOM90, respectively.

β -galactosidase assays

Strains pop2226 and pop4103 harboring derivatives of pOM82 were grown in M63 minimal medium (Miller, 1972) supplemented with 1 μ g/ml thiamine, 30 μ g/ml ampicillin, 0.5% glycerol and, when indicated, 0.4% maltose. The cultures were inoculated to a low cell density ($A_{600} \sim 4-9 \times 10^{-4}$) and grown at 30°C up to an A_{600} of 0.5-0.9. β -galactosidase was assayed at 28°C as described by Miller (1972), using chloroform and 0.002% SDS to disrupt the cells. Enzyme activities, expressed in Miller units, are the average of assays performed in duplicate on at least two independent cultures and corrected for the background level [324 U, as determined by measuring the amount of β -galactosidase made by strain pop2150 (pOM82)]. The variations observed between two independent experiments were <20%.

Proteins

ATP-free MalT was prepared as described (Schreiber and Richet, 1999). Pure IHF from *E. coli* was a gift from H. Nash (NIH, Bethesda). Wild-type CRP and CRP HL159 were purified as described (Richet and Raibaud, 1991). Although both preparations of CRP were 30% active in titration assays with a DNA fragment containing CRP site 1, a 3- to 4-fold higher concentration of CRP HL159 had to be used in abortive initiation assays and footprinting assays so as to have the CRP sites occupied to the same extent by both proteins. This difference is most likely due to the fact that

the HL159 substitution slightly decreases the affinity of CRP for its DNA site (Zhou *et al.*, 1993).

Native RNA polymerase holoenzyme was purified as described by Hager *et al.* (1990) except that the cells were broken with a French press and the chromatography on a MonoQ column replaced by chromatography on a Poros HQ/M column (Boehringer Mannheim). Reconstituted RNAP containing His-tagged wild-type α or $\alpha\Delta$ 235 was prepared as described by Tang *et al.* (1995) and further purified by chromatography on a MonoQ PC 1.6/5 column (Pharmacia Biotech). As indicated by transcription assays performed with the *lacUV5* promoter, the reconstituted $\alpha\Delta$ 235 RNAP was \sim 2.5-fold less active than the reconstituted wild-type enzyme.

Purified His-tagged α subunit was obtained by preparing denatured recombinant protein from strain BL21 (DE3) (pHTT7f1-NH α) as described (Tang *et al.*, 1995). After chromatography on Ni²⁺-NTA agarose, the α subunit was renatured by dialysis using the same procedure as for RNA polymerase reconstitution (Tang *et al.*, 1995) and further purified by anion-exchange chromatography on a Poros HQ/M column (Boehringer Mannheim) to remove contaminating nucleases. The protein was concentrated by dialysis against a buffer containing 20 mM Tris-HCl pH 8.0, 0.2 M NaCl, 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT) and 50% glycerol, and stored at -20°C.

Single-round transcription assays

Supercoiled plasmid DNA (0.25 nM), purified in a CsCl-ethidium bromide gradient, was pre-incubated at 20°C for 20 min in the presence of MalT and CRP (or IHF) in 18 μ l containing 36 mM HEPES-KOH pH 8.0, 200 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT, 100 μ g/ml acetylated bovine serum albumin (BSA), 1 mM maltotriose, 0.1 mM ATP and 0.2 mM cAMP. Two microliters of native RNAP were added and open complex formation was allowed to proceed for 2 min. Two microliters of a solution containing 1 mg/ml heparin (Sigma, H-0880), ATP, GTP and CTP (2 mM each), and 0.1 mM [α -³²P]UTP (5 Ci/mmol) were then added to block open complex formation and to allow RNA synthesis. The reaction was quenched 10 min later by adding 80 μ l of a solution containing 0.375 M sodium acetate, 12.5 mM EDTA and 12 μ g of sonicated plasmid DNA. RNA was deproteinized and ethanol precipitated before electrophoresis through a denaturing polyacrylamide gel. After drying, the gel was autoradiographed using Kodak XAR-5 film without a screen. The relative amounts of transcripts were quantified by scanning the gel on a phosphorimager. Under these conditions, the amounts of open complexes made at *malEp* and *malKp* after 2 min incubation in the presence of RNAP represented 50-75% of the maximum.

Abortive initiation assays

ATP-free MalT (175 nM) and CRP (75 nM wild-type protein or 300 nM CRP HL159) were pre-incubated at 30°C for 20 min in 18 μ l of 36 mM HEPES-KOH pH 8.0, 100 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT, 100 μ g/ml acetylated BSA, 1 mM maltotriose, 0.1 mM dATP, 0.2 mM cAMP, 1 mM DNA fragment [dATP is as effective as ATP in promoting activation of transcription by MalT in the presence of maltotriose (Richet and Raibaud, 1989)]. Two microliters of a RNAP solution were then added and the mixture incubated for varying times at 30°C. The synthesis of abortive products (GpUpUpU) was initiated by adding 2 μ l containing 5 mM GpU, 0.5 mM [α -³²P]UTP (0.6 Ci mmol⁻¹) and 500 μ g/ml heparin, and allowed to proceed for 30 min. The reaction products were separated from free [α -³²P]UTP by chromatography on Whatman 3MM paper as described by McClure (1980). The chromatograms were dried and scanned on a phosphorimager to quantify the amount of GpUpUpU formed. The DNA template used was a 355 bp *malEp-malKp* DNA fragment containing the *malEpKp13 malEpKp70* mutations and extending from +73 (*malEp*) to +8 (*malKp*). It was synthesized by PCR on the M13malEK1*malEpKp13* template using primers EK9 (5'-GGATGCGTGCACCTGTT-3') and EK70 (see above). The DNA fragment was then purified using the PCR product purification kit from Boehringer Mannheim and its concentration was determined as described (Richet and Raibaud, 1989).

DNase I footprinting

Footprinting experiments were performed with the *EcoRI-KpnI* or *PstI-EcoRI* fragment from pOM53, which contains the *malEpKp13 malEp* Δ 259 derivative of *malEp*. The DNA fragments were purified by electrophoresis on a polyacrylamide gel and specifically end-labeled at their *EcoRI* end by filling in with the Klenow fragment of DNA polymerase I in the presence of dTTP and [α -³²P]dATP, followed by ethanol precipitation.

In experiments involving native RNAP or purified α , the labeled DNA fragment (<1 nM) was pre-incubated for 15 min at 30°C in the presence of 175 nM MalT and 75 nM wild-type CRP (or 300 nM CRP HL159) in 18 μ l of the reaction mixture used for the abortive initiation assays and supplemented with 1 mM CaCl₂. Two microliters of native RNAP or purified α were then added and the mixture incubated for 15 min. DNase I attack was performed by adding 2 μ l of a DNase I solution and further incubating the mixture for 1 min. Digestion was stopped and the samples processed as described for *in vitro* transcription assays. After electrophoresis, the gel was dried and autoradiographed on Kodak XAR-5 film without an intensifying screen except for the gel shown in Figure 7A.

The assays involving reconstituted RNAP were performed with a low ionic strength buffer (7 mM Tris-HCl pH 7.7, 18 mM HEPES-KOH pH 8.0, 5 mM potassium acetate, 40 mM NaCl, 3 mM magnesium acetate, 1 mM CaCl₂, 1 mM DTT, 300 μ g/ml acetylated BSA, 1 mM maltotriose, 0.1 mM dATP, 0.2 mM cAMP) supplemented with 7.5% polyethylene glycol 6000. Labeled DNA was pre-incubated for 10 min at 30°C in the presence of 20 nM CRP and 30 nM MalT; reconstituted RNAP (75 nM wild-type form or 600 nM α Δ 235 form) was added and the mixture further incubated for 5 min before adding DNase I.

KMnO₄ attack

The assays were performed under the same conditions as for DNase I footprinting, except that after complex formation with RNAP, 2 μ l of 66 mM KMnO₄ were added and the mixture further incubated for 30 s at 30°C. The reaction was quenched by adding 2 μ l of 2.3 M β -mercaptoethanol and 80 μ l of the stop solution used for *in vitro* transcription assays. After deproteinization and ethanol precipitation, DNA was resuspended in 100 μ l of 10% piperidine, heated for 30 min at 90°C and vacuum dried. DNA was then resuspended in 50 μ l of water, vacuum dried, resuspended in 50 μ l of water and vacuum dried before electrophoresis through a denaturing polyacrylamide gel.

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