The *Escherichia coli* cAMP receptor protein bound at a single target can activate transcription initiation at divergent promoters: a systematic study that exploits new promoter probe plasmids

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We report the first detailed quantitative study of divergent promoters dependent on the *Escherichia coli* cAMP receptor protein (CRP), a factor known to activate transcription initiation at target promoters by making direct interactions with the RNA polymerase holoenzyme. In this work, we show that CRP bound at a single target site is able to activate transcription at two divergently organized promoters. Experiments using promoter probe plasmids, designed to study divergent promoters *in vivo*

and *in vitro*, show that the divergent promoters function independently. Further *in vitro* experiments show that two holo RNA polymerase molecules cannot be accommodated simultaneously at the divergent promoters.

Key words: bacterial promoters, overlapping promoters, transcription activation.

INTRODUCTION

Bacterial genomes contain many genes that are transcribed from closely spaced promoters. It is very common to find two promoters directing transcription in opposite directions from transcription startpoints that are positioned such that the two transcripts do not overlap. Pairs of promoters such as these are known as divergent promoters; in many cases, the upstream regulatory elements of the two promoters overlap, binding sites for regulatory proteins are centrally located within the control region, and proteins bound there may regulate transcription in both directions. This provides for tightly co-ordinated control of the adjacent genes in response to cellular demands. One well studied transcription regulator is the *Escherichia coli* cAMP receptor protein (CRP), which is involved in the regulation of expression of scores of genes, including many that control the catabolism of sugars, amino acids and nucleotides [1,2]. CRP binds to specific 22 bp DNA sequences at target promoters and activates transcription by making direct interactions with holo RNA polymerase (RNAP). Examples where the DNA target for CRP is located between divergently transcribed genes include the *rhaBAD* operon encoding the enzymes for the catabolism of rhamnose [3], the *cai-fix* operons involved in anaerobic carnitine metabolism [4], the *fucPIK-fucAO* operon encoding the enzymes for utilization of fucose [5], the *paa* catabolic operons responsible for phenylacetic acid degradation [6], the *guaBA-xseA* operon encoding the enzymes involved in the metabolism of guanine nucleotide biosynthesis [7], the *nagE-B* operons that encode enzymes involved in the uptake and degradation of *N*-acetylglucosamine [8], the *papI-B* operon which regulates the expression of adhesive pili [9], and the *hpt-gcd* operon which encodes enzymes for the metabolism of hypoxanthine phosphotransferase and membrane-bound glucose dehydrogenase [10].

The process of CRP-mediated regulation requires initially the

binding of cAMP to form an active CRP homodimer, which then binds to 22 bp target sequences located at a variety of positions within target promoters. Simple CRP-dependent promoters can be grouped into two classes according to the location of the target DNA site for CRP (reviewed in [2]). At Class I promoters, e.g. *lacP1* and the artificial promoter *CC* (–61.5), the CRP-binding site is centred at position –61.5. At Class II promoters, e.g. *galP1* and the artificial promoter *CC* (–41.5), the CRP-binding site overlaps the DNA site for RNAP, apparently replacing the promoter –35 hexamer. Transcription activation at both classes requires direct protein–protein contacts between CRP and RNAP (reviewed in [2]). Thus, for Class I promoters, an exposed-surface loop consisting of residues 158–160 of CRP [activating region 1 (AR1)] in the downstream subunit contacts the C-terminal domain of the α subunit of RNAP [2,11]. At Class II promoters, CRP makes multiple interactions with RNAP [2,12]. AR1 of CRP is functional in the upstream subunit. Additionally, two more activating regions are presented by CRP at the downstream subunit: AR2, composed of residues His-19, His-21 and Lys-101, which contact the N-terminal domain of the α subunit of RNAP; and AR3, composed of residues Lys-52 and Glu-58, which contact the σ subunit of RNAP.

However, since CRP is a homodimer, with each subunit carrying potentially functional activating regions, it is possible that a single CRP dimer is potentially able to drive divergent transcription from two promoters. The main aim of the present work was to investigate the possibility that CRP can support transcription activation at two divergent CRP-dependent promoters, to determine if there is an effect of one promoter on the other divergent promoter, and to study the role of the different activating regions of CRP. Our approach has been to construct model semi-synthetic CRP-dependent promoters, whose overall organization reflects architectures found at naturally occurring promoters. Also, new plasmids were constructed

Abbreviations used: AR1 (etc.), activating region 1 (etc.); CRP, *Escherichia coli* cAMP receptor protein; RNAP, *Escherichia coli* RNA polymerase holoenzyme.

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to facilitate assaying the activity of divergent promoters *in vivo* and *in vitro*.

EXPERIMENTAL

Strains, plasmids and promoter fragments

The bacterial strains, plasmids and DNA fragments used in the present study are listed in Table 1, and the primers used during this work are listed in Table 2. Standard methods for manipulating recombinant DNA were used. By convention, the divergent promoters within each fragment are denoted as 'rightward' and 'leftward' promoters, as illustrated in Figure 1. All fragments made in this study contain a single CRP-binding site centred at position -41.5 relative to the transcription start site of each promoter.

Construction of the MSE1, MSE2 and MSE3 promoter fragments

The start point was the *EcoRI*–*HindIII* fragment carrying the *CC* (-41.5) promoter, which has a single consensus DNA site for CRP centred at position -41.5 upstream from the *melR* promoter -10 hexamer and transcription start point (Figure 1). The DNA site for CRP is flanked by a single *XmaI* site (upstream) and a single *Bam*HI site (downstream). The MSE1 fragment, carrying divergent promoters, was constructed by insertion of a -10 promoter element (derived from the *galP1* promoter) upstream of the CRP site (i.e. between *EcoRI* and *XmaI* sites; see Figure 1). To do this, two synthetic oligodeoxynucleotides, D19950 (flanked by an *EcoRI* recognition sequence) and D19947 (flanked by an *XmaI* recognition sequence) (Table 2) were annealed and the single-stranded ends were filled in using Klenow enzyme. The product was purified and ligated into vector generated by digestion with *EcoRI* and *XmaI* of a pAA121

Table 2 Oligo primers used in the present work

Underlining denotes restriction sites.

Primer	Sequence
Construction of MSE series	
D19950	5'-GCAGAAATTCGAGAACTCTCATAATTCGCTCCATTAGGCTTATGGTATGAAATA-3'
D19947	5'-GCACCCGGGCTGCCATAATTATTTTCATACC-3'
D19948	5'-GCACCCGGGCTGCCGTAATTAATTTTCATACC-3'
Construction of plasmid pLSR	
D23542	5'-GCATGAATTCGCTTCGTAATATGACCTAGTATTACTCCCATCCCTCCAG-3'
D23543	5'-GCATGACGTCGCGACGACATGGCTCGA-3'
Construction of pRW70 and pRWX	
D17920	5'-AATTGGATCCCAAGCTTGGGAATTCGC-3'
D17921	5'-AGCTGCGAATTCGCAAGCTTGGGATCC-3'
D21597	5'-GCAGAAATTCGACAACATGAACATATGAAGAGGTGAC-3'
D21598	5'-GCAAGCTTCACAATCTCTGCAATAAGTCGTACC-3'

derivative carrying the *CC* (-41.5) promoter fragment. To construct MSE2, a derivative of MSE1 with the leftward promoter inactivated by a single-base mutation in the -10 hexamer, an identical protocol was used, with D19950 (as before) and D19948, which is the same as D19947 except that it encodes an inactive -10 hexamer element (Table 2). MSE3, a derivative of MSE1 with the rightward promoter inactivated, was constructed by using a derivative of the *CC* (-41.5) promoter fragment carrying the p11G substitution that inactivates the *melR* promoter -10 hexamer (Table 1). The MSE3 fragment, carrying divergent promoters, was constructed by insertion of a -10 promoter element upstream of the CRP site (i.e. between *EcoRI*

Table 1 Bacterial strains, plasmids and promoter fragments used in the present work

Name	Brief description	Origin
Bacterial strains		
M182	<i>E. coli</i> K12 Δ <i>lac</i> strain	M. Casadaban [13]
M182 Δ <i>crp</i>	Δ <i>crp</i> derivative of M182	S. Busby [14]
Bacterial plasmids		
pAA121	pBR322-derived vector for cloning <i>EcoRI</i> – <i>HindIII</i> fragments	A. Kelsall [15]
pSR	pBR322-derived plasmid containing an <i>EcoRI</i> – <i>HindIII</i> fragment upstream of the λ <i>oop</i> transcription terminator	A. Kolb [16]
pLSR	Derivative of pSR carrying two λ <i>oop</i> transcription terminators	This work
pRW50	Broad-host-range <i>lacZ</i> expression vector with <i>EcoRI</i> – <i>HindIII</i> polylinker: <i>lac</i> genes are expressed from promoters directed to <i>HindIII</i> site	J. Lodge [17]
pRW70	Derivative of pRW50 with <i>HindIII</i> – <i>EcoRI</i> polylinker: <i>lac</i> genes are expressed from promoters directed to the <i>EcoRI</i> site	This work
pRWX	Derivative of pRW50 with divergent reporter genes, <i>xylE</i> gene and <i>lacZ</i>	This work
pLG339	Low-copy-number cloning vector encoding <i>Kan^R</i> and <i>Tet^R</i>	N. G. Stoker [18]
pLG339 Δ <i>RS</i>	pLG339 vector with the <i>Tet^R</i> gene deleted	T. Belyaeva [19]
pDW300	Derivative of pLG339 encoding wild-type <i>crp</i> gene and cloned within the <i>Tet^R</i> gene	D. West [20]
pLG339 CRP Leu-159	Derivative of pDW300 encoding CRP with Leu-159	D. West [20]
pLG339 CRP Glu-101	Derivative of pDW300 encoding CRP with Glu-101	D. West [20]
pLG339 CRP Leu-159 Asn-52	Derivative of pDW300 encoding CRP with Leu-159 and Asn-52	D. West [20]
Promoter fragments		
(all <i>EcoRI</i> – <i>HindIII</i> fragments)		
<i>CC</i> (-41.5)	Fragment carrying CRP-dependent promoter with consensus CRP-binding site centred at position -41.5 upstream of the <i>melR</i> transcription start site	K. Gaston [21]
<i>CC</i> (-41.5) p11G	Derivative of <i>CC</i> (-41.5) carrying a point mutation in the -10 hexamer	T. Belyaeva [19]
MSE1	Fragment carrying two divergent CRP-dependent promoters	This work
MSE2	Derivative of fragment MSE1 with inactive -10 hexamer of leftward promoter	This work
MSE3	Derivative of fragment MSE1 with inactive -10 hexamer of rightward promoter	This work

Figure 1 Promoter fragments CC (-41.5), MSE1, MSE2 and MSE3

(a) Diagrams of the CC (-41.5), MSE1, MSE2 and MSE3 fragments, showing the DNA as double lines, the transcription start sites in each direction indicated by bent arrows, the -10 and -35 hexamers as filled boxes and the CRP-binding sites as open boxes. The crosses in certain -10 hexamers represent single base substitutions. All the fragments are flanked by *EcoRI* and *HindIII* restriction sites downstream of the transcription start site of the leftward and the rightward promoters respectively. (b) Top-strand base sequences of the MSE1, MSE2 and MSE3 fragments. The -10 hexamers of promoters are shown in bold, with the inactivating point mutation indicated by the single base that is underlined. The CRP-binding sites are underlined, with the vertical dotted line representing the axial symmetry of the site, and the locations of the *XmaI* and *BamHI* sites are boxed. The bases are numbered above and below the sequences relative to the transcription start sites of the rightward and leftward promoters respectively.

and *XmaI* sites; see Figure 1). As before, the synthetic oligodeoxynucleotides D19950 and D19947 (Table 2) were annealed, the single-stranded ends were filled in using Klenow enzyme, and the resulting fragment was cut with *EcoRI* and *XmaI*. After purification, the product was ligated into vector generated by digestion of a pAA121 derivative carrying the CC (-41.5) p11G promoter fragment with *EcoRI* and *XmaI*.

Construction of plasmid pLSR

The starting point for this was pSR, a cloning vector carrying an *EcoRI*-*HindIII* polylinker and the bacteriophage λ *oop* transcription terminator downstream of the *HindIII* site (Table 1 and Figure 2). pLSR was constructed as a derivative of pSR carrying a second *oop* terminator upstream of the *EcoRI* site (Figure 2). The order of restriction sites within plasmid pSR is *AatII*, *EcoRI*, *HindIII* and *BamHI*, with the *oop* terminator flanked by the *HindIII* and *BamHI* sites. To construct pLSR, an additional *oop* terminator was inserted between the *AatII* and *EcoRI* sites of pSR. The fragment carrying this additional terminator was generated by PCR using pSR as template and the primers D23542 and D23543 (Table 2).

Construction of plasmids pRW70 and pRWX

The starting point for this was pRW50, a broad-host-range *lac* expression vector plasmid carrying an *EcoRI*-*HindIII* polylinker upstream of the *lacZ* gene (Table 1 and Figure 2). pRW50 is organized such that *lac* expression can be driven from cloned

EcoRI-*HindIII* fragments carrying promoters directed towards the *HindIII* site. pRW70 was constructed as a derivative of pRW50 in which the positions of the *EcoRI* and *HindIII* sites were interchanged (Figure 2). To achieve this, two synthetic complementary oligodeoxynucleotides, D17920 and D17921 (Table 2), were annealed and ligated into vector generated by *EcoRI* and *HindIII* digestion of pRW50.

pRWX is a derivative of pRW50 carrying the promoter-less *xylE* gene, and is organized such that *xylE* expression can be driven from cloned *EcoRI*-*HindIII* fragments carrying promoters directed towards the *EcoRI* site. To make pRWX, the *Pseudomonas putida xylE* gene, carried by plasmid p71 (a gift from Professor C. M. Thomas), was amplified by PCR using primers D21597 and D21598 (Table 2), which were designed to introduce *EcoRI* and *HindIII* sites respectively at the 5' and 3' ends of *xylE*. The PCR product was purified, restricted with *EcoRI* and *HindIII* and cloned into the pSR vector upstream of the *oop* transcription terminator to give the construct *EcoRI*-*xylE*-*HindIII*-(*oop* terminator)-*BamHI*. The *HindIII* site was removed by filling in with Klenow enzyme to produce an *EcoRI*-*BamHI* fragment carrying the *xylE* gene upstream of the *oop* terminator. This fragment was inserted between the *BglII* and *EcoRI* sites of pRW50 (note that pRW50 carries a single *BglII* site immediately upstream of the *EcoRI* site and that *BamHI* and *BglII* ends are compatible). This resulted in plasmid pRWX (Figure 2), which carries an *EcoRI*-*HindIII* polylinker with the promoter-less *xylE* gene upstream of the *EcoRI* site and the promoter-less *lacZ* gene downstream of the *HindIII* site.

Figure 2 Schematic diagram of plasmids used in the present study

(a) Vectors for *in vivo* studies. pRW70 has the multiple cloning site (MCS) flanked by *Hind*III and *Eco*RI restriction sites, with the *Eco*RI site located adjacent to the *lacZ* expression gene. pRW50 is identical with pRW70, except that the MCS is reversed so that the *Hind*III site is now located adjacent to the *lacZ* expression gene. pRWX contains two reporter genes: *xylE*, located upstream of the *Eco*RI site, and *lacZ*, located downstream of the *Hind*III site. The replication origin (*oriV*) and the tetracycline resistance gene (*tet*^R) are also shown. (b) Vectors for *in vitro* studies. The plasmid pSR has an efficient transcription terminator (*λ oop*) located downstream of an *Eco*RI–*Hind*III MCS, and the plasmid pLSR was constructed by introducing another *λ oop* terminator upstream of the MCS. The replication origin (*oriV*), the ampicillin resistance gene (*Amp*^R) and the location of the RNA I promoter are also shown.

Assays of promoter activity *in vivo*

*Eco*RI–*Hind*III fragments carrying different promoters (Figure 1) were cloned into the *lac* expression vectors pRW50 and pRW70, or into the dual-reporter expression vector pRWX. The resulting plasmids were transformed into the Δlac strains M182 or M182 Δcrp (Table 1), and transformants were grown in L broth supplemented with appropriate antibiotics. β -Galactosidase activities were measured as described by Miller [22], are expressed in Miller units, and are taken to be proportional to the activity of the cloned promoter. Catechol 2,3-dioxygenase (*xylE*) activities were assayed by the method of Zukowski et al. [23]: 1 unit is amount required to convert 1 μ mol of substrate (catechol) into product (2-hydroxymuconic semialdehyde) in 1 min under standard conditions. Protein was estimated by the Biuret method [24].

In vitro transcription assays

Single-round *in vitro* transcription assays were performed as described by Lonetto et al. [25] using DNA templates purified by caesium chloride gradient centrifugation [26]. CRP was purified and checked according to the methods of Ghosaini et al. [27]. The template DNA, CRP, RNAP and nucleotides were prepared and diluted in 1 $\times$ transcription buffer: 5% glycerol, 20 mM Tris/HCl (pH 8.0), 100 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 0.05 mg/ml BSA and 200 nM cAMP. Plasmid DNA (5 nM) was incubated with CRP (100 nM) for 5 min at 37 °C (or with control 1 $\times$ transcription buffer), and then pre-warmed RNAP (100 nM; supplied by Epicentre Technologies) was added for an additional 5 min at 37 °C in a final volume of 12 μ l. Elongation was started by the addition of 4 μ l of pre-warmed mixture containing 200 μ M ATP/CTP/GTP,

10 μ M UTP, 1.5 μ Ci of [α -³²P]UTP and 100 μ g/ml heparin to the template/polymerase mixture, and allowed to proceed for 5 min at 37 °C. Reactions were stopped by the addition of 12 μ l of 20 mM EDTA in formamide containing xylene cyanol and Bromophenol Blue. After heating to 90 °C, samples were subjected to electrophoresis on an 8% sequencing gel and quantified on a Molecular Dynamics PhosphorImager using ImageQuant version 3.3 software. CRP-dependent transcripts were normalized against the control RNA I transcript. Multi-round *in vitro* transcription assays were performed exactly as for single-round experiments, but without heparin.

Permanganate footprinting

Permanganate footprinting experiments were performed as described by Savery et al. [28] using fragments from plasmid DNA purified by caesium chloride gradient centrifugation [26]. Fragments were end-labelled with phage T4 polynucleotide kinase and [γ -³²P]ATP (7000 Ci/mmol), and were purified on Sephadex G-50 columns (Bio-Rad). The *Pst*I–*Hind*III fragments used in the experiment shown in Figure 5 were specifically labelled at the *Hind*III end by prior treatment of the *Hind*III ends with calf alkaline phosphatase. The *Eco*RI–*Hind*III fragments used in the experiment shown in Figure 7 were labelled at both ends after treatment of fragments with calf alkaline phosphatase. Complexes with the labelled promoter fragments (4 nM final concentration) were incubated for 30 min at 37 °C in a total volume of 20 μ l containing CRP (100 nM), RNAP (200 nM) and 1 $\times$ binding buffer (5% glycerol, 0.1 M NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 0.05 mg/ml BSA, 20 mM Tris/HCl, pH 8.0, and 200 nM cAMP). After addition of 1 μ l of potassium

permanganate (0.2–2 M) and incubation for 4 min at 37 °C, the reaction was stopped by the addition of 50 µl of a mixture containing 3 M ammonium acetate, 0.1 mM EDTA and 1.5 M β -mercaptoethanol. Samples were phenol extracted, precipitated with ethanol, resuspended in 50 µl of piperidine (1 M), heated at 90 °C for 30 min and, after removal of the piperidine by evaporation, resuspended in 8 µl of formamide containing 1 mM EDTA, 5 M urea, 5 mM NaOH, xylene cyanol and Bromophenol Blue. Samples were analysed by electrophoresis on 8 % sequencing gels, calibrated with Maxam-Gilbert 'A + G' sequencing reactions [29].

RESULTS AND DISCUSSION

New plasmids for the study of divergent promoters

In our previous studies [17], we measured promoter activities *in vivo* by using pRW50, a broad-host-range *lac* expression vector plasmid carrying an *EcoRI*–*HindIII* polylinker upstream of the *lacZ* gene (Table 1 and Figure 2). pRW50 is organized such that *lac* expression can be driven from cloned *EcoRI*–*HindIII* fragments carrying promoters directed towards the *HindIII* site. pRW70 was constructed as a derivative of pRW50 in which the positions of the *EcoRI* and *HindIII* sites were interchanged, such that *lac* expression would be driven from cloned *EcoRI*–*HindIII* fragments carrying promoters directed towards the *EcoRI* site (Figure 2).

Another derivative of pRW50, pRWX, was constructed that carried the *xylE* gene as well as the *lacZ* gene. pRWX is organized such that *lacZ* expression can be driven from cloned *EcoRI*–*HindIII* fragments carrying promoters directed towards the *HindIII* site, and *xylE* expression can be driven from cloned *EcoRI*–*HindIII* fragments carrying promoters directed towards the *EcoRI* site (Figure 2). During the construction, stop codons and an efficient transcription terminator were inserted downstream of *xylE*. Thus pRWX is a promoter probe vector that allows the assay of the *in vivo* activity of divergent promoters carried on any fragment that can be cloned between the *EcoRI* and *HindIII* sites.

In order to monitor promoter activity *in vitro*, plasmid pLSR was constructed, starting with pSR, a cloning vector carrying an *EcoRI*–*HindIII* polylinker upstream of the bacteriophage λ *oop* transcription terminator element (Table 1 and Figure 2). To construct pLSR, a second λ *oop* transcription terminator was inserted upstream of the *EcoRI* site. Thus the activity of divergent promoters carried on any fragment that is cloned between the *EcoRI* and *HindIII* sites of pLSR can be detected by *in vitro* transcription assays. RNAP molecules that initiate at the cloned divergent promoters run to the *oop* terminators and thus create discrete-sized transcripts that can be resolved by gel electrophoresis. In order to ensure that divergent promoters result in different-sized transcripts, pLSR was organized such that rightward transcription terminates 87 bp beyond the *HindIII* site and the leftward transcript terminates 111 bp beyond the *EcoRI* site.

CRP can support transcription activation of divergent promoters

We constructed an *EcoRI*–*HindIII* fragment (MSE1) carrying a single DNA site for CRP, flanked by promoter elements that create divergent Class II CRP-dependent promoters. The rightward and leftward promoter sequences are derived from the *E. coli* *melR* and *galP1* promoters respectively, and were constructed with identical –10 hexamer elements but different –35 elements. Derivatives (MSE2 and MSE3) carrying point mutations in either –10 element were also constructed (Figure 1).

Figure 3 Activities of the MSE1, MSE2 and MSE3 rightward and leftward promoters

The bar charts show β -galactosidase and catechol 2,3-dioxygenase activities measured in M182 cells carrying the expression vector pRWX containing the MSE1, MSE2 or MSE3 fragment. Cells were grown aerobically in L broth containing 35 µg · ml^{–1} tetracycline and were harvested during exponential growth. β -Galactosidase and catechol 2,3-dioxygenase activities were assayed by the standard Miller [22] protocol and the method of Zukowski et al. [23] respectively. Each value is the mean $\pm$ S.D. of three independent assays.

Table 3 Activity of different *crp* genes in the MSE1 fragment

β -Galactosidase levels (in Miller units) in M182 Δ *crp* cells containing the MSE1 promoter cloned into pRW50 (to measure rightward promoter activity) or pRW70 (to measure leftward promoter activity) are shown. Cells also carried pLG339 derivatives encoding wild-type or mutant CRP as indicated (or the control 'empty' plasmid, pLG339 Δ RS). Cells were grown aerobically in L broth containing 35 µg · ml^{–1} tetracycline and 25 µg · ml^{–1} kanamycin, and were harvested during exponential growth.

CRP gene	Expression (units)	
	Left	Right
No CRP	51	26
CRP	960	2284
CRP Leu-159	357	633
CRP Glu-101	51	258
CRP Leu-159/Asn-52	5132	8138

The MSE1, MSE2 and MSE3 *EcoRI*–*HindIII* fragments were cloned into the expression vector pRWX, and the resulting recombinants were transformed into M182 host cells. The activities of the cloned rightward and leftward promoters were measured by determining the activities of β -galactosidase (encoded by *lacZ*) and catechol 2,3-dioxygenase (encoded by *xylE*) respectively. The results (Figure 3) show that both the leftward and the rightward promoters in the MSE1 fragment were active in the M182 *crp*⁺ genetic background. In contrast, in the M182 Δ *crp* genetic background, β -galactosidase and catechol 2,3-dioxygenase activities were close to background levels (results not shown), indicating that expression from both promoters is dependent on CRP.

Mutation of the leftward –10 hexamer in MSE2 results in complete loss of activity of the leftward promoter, confirming that the measured activity of the leftward promoter in fragment MSE1 is not an artefact. Similarly, mutation of the rightward –10 hexamer in MSE3 results in complete loss of activity of the rightward promoter. Inactivation of the leftward –10 hexamer in MSE2 results in very little change in the activity of the rightward promoter. Inactivation of the rightward –10 hexamer in MSE3 results in very little change in the activity of the leftward promoter. Thus, while CRP can activate transcription at both

Figure 4 Transcription from the rightward and leftward promoters of the MSE1, MSE2 and MSE3 fragments

The Figure shows gel analysis of labelled RNA made in multi-round (a) and single-round (b) *in vitro* transcription assays with supercoiled pLSR plasmid carrying the MSE1, MSE2 or MSE3 fragment as template, as shown. Transcripts initiating at the leftward and the rightward promoters run to a strong terminator and give the bands marked 'Left' and 'Right' respectively. Also shown is the RNA I transcript from the pLSR vector that is used as an internal control. To make the transcripts, 4 nM template DNA was incubated with 100 nM RNAP (and 100 $\mu\text{g} \cdot \text{ml}^{-1}$ heparin in single-round assays only) either with 100 nM wild-type CRP (+) or without CRP (—). The lower panels show quantification of the transcript bands, normalized to the RNA I transcript, from the experiment in the upper panels (open bars, leftward transcripts; shaded bars, rightward transcript). In an independent experiment, data were obtained that fell within 10% of the values shown.

divergent promoters in the MSE1 fragment, the activity of each promoter is unaffected by the activity of the other promoter.

Role of the activating regions of CRP

At Class II CRP-dependent promoters, three activating regions of CRP can contact RNAP: the principal contacts involve AR1 and AR2, and AR3 plays a minor role, although its function can be enhanced by the K52N (Lys-52 → Asn) substitution [2]. To investigate the role of the different activating regions at the divergent promoters carried by the MSE1 fragment, the effects of the H159L (His-159 → Leu) and K101E (Lys-101 → Glu) substitutions, that disrupt AR1 and AR2 respectively, and the K52N substitution, that enhances AR3, were investigated. Here, to assay the rightward and the leftward promoters, the MSE1 fragment was cloned into pRW50 and pRW70 respectively. The recombinant plasmids were transformed into M182 Δcrp cells, carrying plasmids encoding different *crp* mutants, and β -galactosidase activities were measured. The results (Table 3) show that, as expected, both the leftward and rightward MSE1 promoters were barely active in the absence of the *crp* gene, but active in its presence. Expression from both promoters was greatly reduced with the *crp* genes encoding the H159L or K101E substitutions; in contrast, the K52N substitution resulted in a large increase in promoter activities. Thus, at the MSE1 divergent promoters, the functions of the three activating regions of CRP are exactly as expected for typical Class II CRP-dependent promoters.

In vitro transcription experiments

To investigate the MSE1 promoters further, *in vitro* methods were adopted. To perform *in vitro* transcription, the MSE1, MSE2 and MSE3 fragments were cloned into plasmid pLSR and both multi- and single-round experiments were performed. In all experiments, template DNA was pre-mixed with CRP and RNAP.

In single-round transcription experiments, transcripts produced are due to one round of transcription, as heparin is included with the nucleoside triphosphate mixture (see the Experimental section). In multi-round experiments, heparin is omitted, and RNAP can initiate several cycles of transcription activation. Results from each set of experiments are shown in Figure 4: transcription initiation at the leftward promoter resulted in a 149-base transcript, while transcription initiation at the rightward promoter resulted in a 122-base transcript. Production of both of these transcripts was dependent on CRP. The interpretation of the experiment is facilitated by the appearance of the 107-base RNA I transcript that is encoded by the vector pLSR plasmid sequence. Previous studies [16] have shown that the RNA I promoter is independent of CRP, and thus we used the RNA I transcript as an internal marker to quantify CRP-dependent transcript formation. The results show that, as expected, the leftward promoter is inactive in the MSE2 fragment and the rightward promoter is inactive in the MSE3 fragment.

The lower panels of Figure 4 show the quantification of transcripts originating at the MSE leftward and rightward promoters (expressed in arbitrary units normalized to the RNA I transcript). In the multi-round experiment (Figure 4a), the activity of the leftward promoter was barely affected by the rightward promoter, and vice versa. This is similar to the situation *in vivo*. The results from the single-round experiment (Figure 4b) are more difficult to interpret. Expression from the leftward promoter appeared to be reduced by expression from the rightward promoter, but expression from the rightward promoter did not appear to be affected by expression from the leftward promoter. The reason for performing the single-round experiment was to investigate whether any one DNA fragment could accommodate only one RNAP molecule, fixed either to the rightward promoter or to the leftward promoter, or two RNAP molecules fixed simultaneously at both promoters. If only one RNAP could be accommodated, then inactivation of one promoter in the MSE2 and MSE3 fragments might lead to an

Figure 5 Permanganate footprint analyses

Shown is an autoradiograph of a sequence gel run to analyse products after treatment of end-labelled MSE1, MSE2 or MSE3 fragments with different concentrations of KMnO_4 . A 4 nM concentration of each fragment (end-labelled at the *Hind*III site) was incubated with 100 nM purified wild-type CRP and 200 nM RNAP prior to treatment with permanganate. Lane M shows a Maxam–Gilbert sequencing reaction specific for A + G residues used to calibrate the gel. Lanes 1, 2 and 3 represent a control experiment for MSE1, MSE2 and MSE3 respectively without treatment. The numbers beside the gel are positions of bands relative to the rightward transcription start site. The different final concentrations of KMnO_4 used were 10 mM for lanes a, e and i, 20 mM for lanes b, f and j, 40 mM for lanes c, g and k, and 100 mM for lanes d, h and l. The permanganate-sensitive bands around the -10 hexamers of the leftward and rightward promoters are indicated by the thin and thick arrows respectively.

increase in the occupancy of the divergent promoter. Since the single-round transcription experiment gave an ambiguous result, other strategies were adopted to investigate the problem.

Open-complex formation at the MSE promoters detected by reactivity to permanganate

Transcriptionally competent complexes at bacterial promoters can be detected by their reactivity to permanganate, which can modify thymines in the single-stranded 'bubble' that forms in open complexes [28,30]. Thus ternary CRP–RNAP–DNA complexes were formed with end-labelled MSE1, MSE2 and MSE3 fragments, the complexes were treated with permanganate (see

Figure 6 Ratios of open complexes formed at the rightward and leftward promoters of the MSE1 fragment

The intensity of open complexes formed at the rightward and the leftward promoters of fragment MSE1 shown in Figure 5 was measured, and the ratios of open complexes formed were then calculated for each permanganate concentration. In an independent experiment, data were obtained that fell within 10% of the values shown.

Figure 7 Permanganate footprint analyses of complexes formed at fragments labelled at both ends

Shown is an autoradiograph of a sequence gel run to analyse products after permanganate (100 mM) treatment of complexes formed between 4 nM MSE1, MSE2 or MSE3 fragments (end-labelled at both the *Eco*RI and *Hind*III sites), purified wild-type CRP (100 nM) and RNAP (200 nM) as indicated. The lanes marked M show Maxam–Gilbert sequencing reactions specific for A + G residues used to calibrate the gel. The numbers beside the gel are the positions of some bands relative to the *Hind*III site. The groups of short fragments and of long fragments are indicated by the arrows.

the Experimental section), and the resulting products were analysed on sequence gels. Figure 5 shows a typical gel: with the MSE1 fragment, open complexes at both the leftward and rightward promoters can be distinguished. The open complex at the rightward promoter results in opening of bases between positions +1 and -10 (i.e. near the start point of the rightward promoter). The open complex at the leftward promoter results in opening of bases near position -80 with respect to the rightward promoter (i.e. near position +1 for the leftward promoter). With the MSE2 fragment, as expected, open complexes at the rightward promoter are formed, but complexes at the leftward promoter are greatly decreased. With the MSE3 fragment, open complexes at the leftward promoter are formed, but complexes at the rightward promoter are greatly decreased.

The experiment was performed with different concentrations of permanganate reagent and, for the MSE1 fragment, the ratios of signals corresponding to the leftward and rightward promoters were calculated at each concentration. Data in Figure 6 indicate that this ratio did not change significantly over a 10-fold range of permanganate concentrations. Since the DNA fragment was end-labelled at the *Hind*III site downstream of the leftward promoter, this argues that each fragment can accommodate only one RNAP molecule in a CRP-dependent open complex, and not two RNAP molecules. In these experiments, more than 90% of the fragments were modified at the highest permanganate concentrations, while less than 25% were modified at lower concentrations. Thus the observed constant ratio of signals corresponding to the leftward and rightward promoters is due to the ratio of distribution of RNAP between the leftward and rightward promoters. If individual fragments could accommodate two RNAP molecules simultaneously in open complexes at both promoters, then the signal due to the promoter that is closer to the labelled end of the MSE1 fragment would be increased with respect to the signal from the other promoter at higher permanganate concentrations.

The experiment with the MSE1 fragment at the highest permanganate concentration was repeated with DNA fragments

Figure 8 Model of CRP-dependent activation at the MSE1 promoters

The disposition of different CRP and RNAP subunits during transcription activation at either divergent promoter is shown. We suggest that activation of the two promoters is sequential, i.e. only one RNAP binds to one promoter at any time, during which the other divergent promoter is turned off. The -10 and -35 hexamers are represented by filled boxes. The small arrows represent the direction of transcription. α -CTD and α -NTD denote the N-terminal and C-terminal domains respectively of the α subunit of RNAP.

labelled at both the *EcoRI* and *HindIII* ends. If individual fragments can accommodate just one RNAP, at either one or the other promoter, then permanganate should generate both long and short labelled fragments. The results in Figure 7 confirm that this is the case. Further data in Figure 7 confirm that open-complex formation is dependent on CRP, although some CRP-independent signals and protein-independent background signals are observed under our conditions.

Conclusions

As far as we know, this is the first attempt to make a detailed study of divergent promoters activated by the binding of a single transcription activator. To facilitate our studies both *in vivo* and *in vitro*, some new plasmids were constructed. Divergent promoters carried on a DNA fragment can be assayed *in vivo* either by parallel cloning into the *lacZ* expression vectors pRW50 and pRW70, or by cloning into pRWX, which carries two reporter genes (*lacZ* and *xylE*) organized divergently from a polylinker (Figure 2).

CRP is functional as a homodimer that binds to an inverted repeat sequence at target sites [1]. Because both CRP subunits carry potentially functional activating regions that can interact with RNAP, it is possible that a single bound CRP dimer could activate transcription from divergent promoters. Our present study, which focused on Class II CRP-dependent promoters, has shown that this is indeed the case. Our results show that, *in vivo*, the activity of the rightward promoter is not affected by the activity of the leftward promoter, and vice versa. However, the *in vitro* experiments indicate that the rightward promoter and the leftward promoter cannot be occupied simultaneously by RNAP. This is perhaps not surprising, since it is known that

RNAP binds both upstream and downstream of CRP at simple Class II CRP-dependent promoters [2]. Our observation that the two divergent Class II CRP-dependent promoters in the MSE1 fragment do not interfere with each other *in vivo* argues that the time occupancy of each promoter by one RNAP molecule must be low, and that the two promoters are activated sequentially (Figure 8).

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