
On the different binding affinities of CRP at the *lac*, *gal* and *malT* promoter regions

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ABSTRACT

We have determined the stoichiometry of CRP binding to various DNA fragments carrying the *lac*, *malT* or *gal* promoters in the presence of cAMP, using a gel electrophoresis method. In each case, one dimer of CRP binds to the functional CRP site upstream of the transcription start. At the *lac* promoter, a second CRP dimer can bind to the operator region. Direct binding analysis and competition experiments performed at 200 μ M cAMP allow us to measure the affinity of CRP for these different sites and to correlate them with variations in the consensus sequences, already proposed. The order is *lac* > *malT* > *gal* > *lac* operator > *lac* L8 >> non specific sites. No strong coupling exists between the two *lac* sites when on the same fragment. Conversely, we have studied, at constant CRP concentrations, the cAMP levels required to obtain half maximal binding to a particular DNA site: the required cAMP level increases inversely as the affinity for CRP. These variations may account for the differential activation of various cAMP sensitive operons *in vivo*.

Anomalies in the migrations of the 1:1 complexes between CRP and DNA have been analysed and related to the size and to the position of the CRP site in the fragment. The electrophoretic mobility of the complexes depends not only on the size of the fragment but on the position of the CRP site: the mobility is lower when CRP binds near the center of the fragment. This effect is due to a clear change in the persistence length of the DNA induced by CRP binding. We suggest that, upon binding, the protein introduces a local bend (or a kink) in the DNA structure.

INTRODUCTION

When bacteria grown on glucose are shifted to a glycerol medium, the intracellular concentration of cyclic AMP rises from 2 μ M to 6 μ M (1), while the concentration of the receptor protein, CRP, does not change (2). In response to the threefold increase in concentration of the cyclic nucleotide (3, 4), initiation of transcription at specific promoters is turned on (5). Most of the questions related to this process require detailed studies involving all the partners (promoter DNA, RNA polymerase, CRP and cyclic AMP). However, by studying *in vitro* the interaction between the CRP-cAMP complex with DNA fragments carrying various binding sites, one can get some information about

the following questions :

- a) What is the extent of the competition for binding to specific sites due to the large excess of non specific sites present on the DNA genome ?
- b) What is the free energy involved in the association of CRP, at a given concentration of cAMP, with a DNA site of known sequence ? This question has two aspects. First, how does the free energy of association of the cAMP-CRP complex vary as the sequence of the site deviates from consensus sequences proposed in the literature ? Second, how does the concentration of cAMP required for the association of a CRP molecule to its DNA site vary with the DNA sequence ? This later aspect could then be related to reports which indicate that activation of three different operons (lactose, arabinose and tryptophanase) takes place at various cAMP concentrations (6,7).
- c) When there are two sites present on the same fragment, do they interact ? In several cases, a second site exists close to the functional site (in the lactose control region this second site is located within the operator (8)). It has been postulated (9) that this auxiliary site plays a role by enhancing both the rate of access to the main site and the corresponding association constant. We have reconsidered this problem under ionic strength conditions closer to the physiological ones.

In order to tackle these problems we have used the gel electrophoresis method already described by Garner and Revzin (10) and by Fried and Crothers (11). We discuss first how the different complexes migrate in the gel and we determine the stoichiometries of CRP binding at each site. We measure the association constants for the sites at the galactose and maltose promoters and the two sites present in the lactose control region, and compare these values to non specific binding. We describe how these association constants are linked to the concentration of cyclic AMP required in each case for the formation of the cAMP-CRP-DNA complex. We study the coupling existing at equilibrium between the two sites present on various fragments containing the lactose control region and show that anomalies in gel mobility for these fragments are not exceptional ; they do not represent a particular type of conformation of the complex at equilibrium. We suggest an explanation for these migration anomalies after having shown that they correlate well with position of the sites on the fragment.

MATERIALS AND METHODS

By convention *lac*, *gal* and *malT* sequences are numbered from the transcription start point of the cAMP-CRP dependent promoter. Sequences upstream

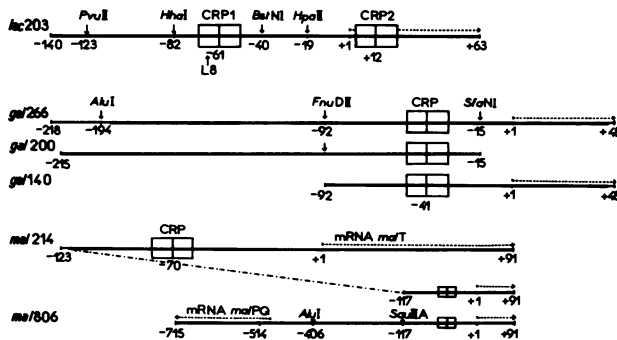


Fig. 1 : SCHEMATIC REPRESENTATION OF THE DIFFERENT DNA FRAGMENTS USED IN THIS STUDY. The *lac*, *gal* and *malT* sequences are represented as heavy lines. The position of a few relevant restriction sites is shown. Numbers refer to the distance in base pairs relative to the start point of transcription (+1). The last two lines show the *mal* fragments on a reduced scale.

of this point are labelled with a "-" prefix. The size of the fragments corresponds to the number of base pairs, matched in a perfect duplex.

Figure 1 depicts the different fragments. The 203 bp *lac* fragment, either wild-type or with the L8 mutation, was isolated from a pBR322 derivative (12). The 186, 144, 121 and 80, 100 and 102 bp *lac* fragments were obtained by cutting the 203 bp fragment by *Pvu*II, *Hha*I, *Hpa*II or *Bst*NI respectively.

The *gal* promoter region was obtained either on a 140 bp fragment isolated from pAA187 (13) or on a 266 bp fragment isolated from pAA195 (14). The 200 bp *gal* fragment was obtained by cutting the 1000 bp *gal* fragment isolated from plasmid BdC1 with *Sfa*NI (15). The entire *malA* regulatory region was isolated from plasmid pOM1 on a 806 bp fragment which, cleaved by *Alu*I or *Sau*3AI, produces respectively a 498 or a 208 bp fragment (16). The latter fragment containing the *malT* promoter was cloned into pUR222 to give plasmid pOM50 from which the 214 bp fragment could be isolated (17).

DNA fragments were isolated and 3'end-labelled as already described (18) or 5'end-labelled by kination with γ ³²P ATP (Amersham). Labelled fragments were diluted with excess unlabelled fragment of known concentration. We used fragments with a specific activity of between 5-70 cpm per femtomole.

CRP was purified by B. Blazy (19). For the stoichiometry determinations, it was mildly iodinated using the Bolton and Hunter reagent (20). The specific activity of the labelled protein was determined as in reference 18 (final specific activity of 83 or 123 cpm per femtomole which corresponds to 0.023 or 0.034 ¹²⁵I atom incorporated per CRP dimer). The labelled protein titrates

the *lac* and *gal* promoters as unlabelled protein of the same concentration at 200 μM cAMP.

CRP-DNA complexes were formed at 37°C in 40 mM Tris, 10 mM MgCl_2 , 100 mM KCl, 1 mM dithiothreitol, 100 $\mu\text{g/ml}$ bovine serum albumin and 200 μM cAMP, pH 8.0. They were loaded and run on 7.5% polyacrylamide gels, as previously described (18). Autoradiograms were obtained on pre-flashed Fuji X-ray films. Scanning with a Vernon microdensitometer allowed quantitation of the DNA present in each band after it was checked that exposure was within the linear range of dose response.

In direct titration assays, the CRP concentration was varied and the concentration of the relevant DNA fragment was kept constant. Most experiments were run in duplicate and involved 8 different CRP concentrations ranging from 2 to 500 nM. When the DNA concentration is large with respect to the dissociation constant, the end point of the titration yields α , the number of CRP dimers of a given preparation required to saturate one DNA binding site. This value, as well as the association constant, is also determined from the titration assay using the following relationship :

$$\frac{[C_T](I_b + I_f)}{\alpha I_b} = [D_T] + \frac{(I_b + I_f)}{K \cdot I_f}$$

$[C_T]$ and $[D_T]$ are respectively the total concentration of CRP dimers and DNA fragments, I_b is the intensity of the band containing the 1:1 complex, and I_f the intensity of the free DNA band.

If the fragment contains two binding sites, three bands (free DNA, complex I and complex II) are observed. The first association constant, K_I , is obtained as above. The second one, K_{II} , is obtained by :

$$\frac{K_{II}}{K_I} = \frac{[I_b(II)][I_f]}{[I_b(I)]^2}$$

where $I_b(I)$ and $I_b(II)$ are the intensities of the corresponding bands, containing one or two molecules of CRP. If the sites behave at equilibrium as independent entities K_I and K_{II} would be equal to K_I^0 and K_{II}^0 defined by :

$$K_I^0 = K_{III} + K_{IV}$$

$$K_{II}^0 = K_{III} \cdot K_{IV} / K_I^0$$

K_{III} and K_{IV} are the association constants measured for the same sites present on isolated fragments. Positive (or negative) interactions between the sites are detected if K_I or K_{II} is significantly larger (or smaller) than expected from the above relations.

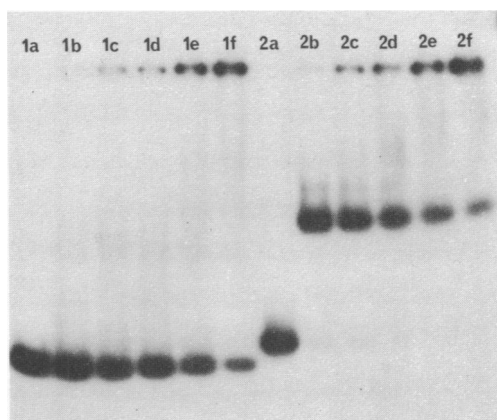


Fig. 2 : VISUALISATION OF SPECIFIC AND NON SPECIFIC COMPLEXES BETWEEN DNA AND CRP USING GEL ELECTROPHORESIS. DNA fragments at 2 nM were incubated with various concentrations of CRP in the presence of 200 μ M cAMP ; 16 μ l of the mixture was applied to the polyacrylamide gel run in TBE buffer with 200 μ M cAMP. In lanes 1a-f, the DNA contains no specific CRP binding site. In lanes 2a-f, we used a 121 bp fragment containing the specific CRP binding site for the *lac* operon. Concentrations of CRP were respectively zero (lanes a), 50 nM (lanes b), 80 nM (lanes c), 100 nM (lanes d), 150 nM (lanes e) and 200 nM (lanes f).

In a competition assay between two labelled DNA fragments A and B, the ratio between K_A and K_B is obtained from :

$$\frac{K_A}{K_B} = \left(\frac{I_b}{I_f} \right)_A \times \left(\frac{I_f}{I_b} \right)_B$$

When a single fragment, A, is labelled, K_B is obtained by observing the apparent change in K_A as the concentration of fragment B is changed.

RESULTS

Detection of different complexes between CRP and DNA by the method of gel electrophoresis

Figure 2 shows the behaviour of two different DNA fragments, incubated with various concentrations of CRP under the conditions specified in the legend. If the fragment does not contain a specific CRP site (lanes 1, 1a-f), binding of the receptor protein results in a smear trailing behind the free fragment. At higher CRP concentrations, as more dimers are bound, the complex becomes immobilized at the top of the gel. The right hand part illustrates the binding of the complex to the 121 bp fragment carrying the CRP binding site of

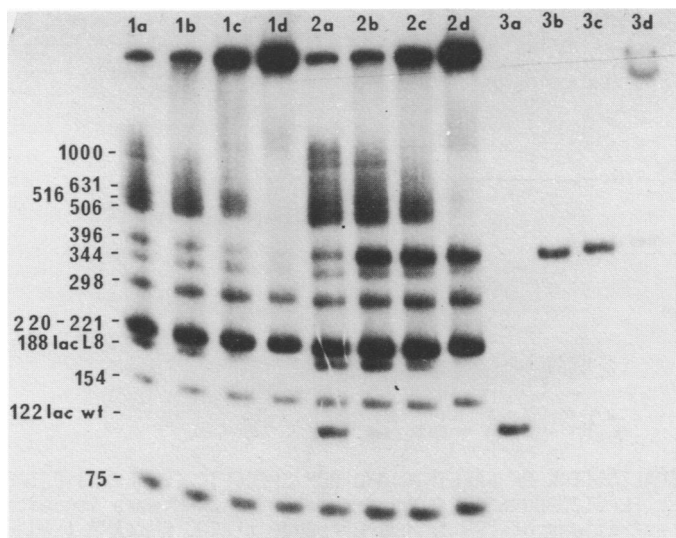


Fig. 3 : COMPETITION FOR CRP BINDING BETWEEN THE *lac* SPECIFIC SITE AND NON SPECIFIC SITES FROM PLASMID DNA. Plasmid pBR203 L8UV5 was cut by *Hinf*I restriction endonuclease and labelled. Sizes of restriction fragments were according to (22). 16 μ l samples containing 1 nM of the labelled hydrolysate and different concentrations of CRP in lanes 1 were applied to a polyacrylamide gel run in buffer containing 200 μ M cAMP. In lanes 3, the samples contained 1 nM of a 121 bp *lac* wild-type fragment. In lanes 2, an equimolecular mixture of the *Hinf*I hydrolysate (1 nM) and of the 121 bp *lac* promoter (1 nM) was applied to the gel. The concentrations of CRP were respectively 0 (lanes a), 20 (lanes b), 100 (lanes c) and 500 nM (lanes d).

the lactose control region. At 50 nM CRP, all the DNA is already displaced in a well defined band. Non specific binding takes then place at the expense of this first complex. The evolution of the pattern is otherwise qualitatively similar to the one described above.

Specific binding clearly takes place before the appearance of the smear corresponding to non specific binding. As one could object that only non specific complexes sufficiently stable during the entry into the gel have been revealed by this assay, we also performed competition experiments. A strong specific CRP binding site was titrated either alone or in the presence of a large excess of non-specific DNA. The displacement of the titration indicates the strength of non specific binding, regardless of whether it leads to stable complexes. Figure 3 shows such an experiment where the competitor DNA, an *Hinf*I hydrolysate of a pBR322 plasmid (22) carrying the *lac* L8UV5 (23) insert, has also been labelled.

TABLE I : STOICHIOMETRIES OF CRP BINDING TO DIFFERENT FRAGMENTS.

DNA fragment	Moles of CRP dimer per mole of DNA fragment	Number of experiments	Standard error
<i>mal</i> 214	0.99	4	0.25
<i>mal</i> 498	1.28	1	-
<i>gal</i> 140	0.84	6	0.24
<i>gal</i> 266	0.94	2	
<i>lac</i> 80	0.83	1	-
<i>lac</i> 121	0.99	2	0.06
<i>lac</i> 203 (first site filled)	1.02	9	0.19
<i>lac</i> 203 (two sites filled)	1.92	4	0.48
<i>lac</i> 203 L8 (first site filled)	1.00	2	0.06
<i>lac</i> 203 L8 (two sites filled)	2.01	2	0.05
<i>lac</i> 203 L8 (non specific sites filled)	11.4	2	1

Lanes 1a-d show the titration of 1 nM of hydrolysate by increasing CRP concentrations. As above, it is in the range 100 nM-500 nM CRP that the bands are titrated, the fragments of larger size disappearing first since they contain more potential non specific sites, the only noticeable exception being the 185 bp *lac* L8UV5 insert. Lane 2a shows the same labelled hydrolysate with an equimolecular amount of a 121 bp *lac* wild-type promoter. In lane 2b with 20 nM CRP, the *lac* wild-type band is strongly attenuated. In lanes 2c and d, with 100 nM or 500 nM it has completely vanished. For comparison, the same experiment was repeated with 1 nM *lac* wild-type DNA alone in lanes 3 : at 20 nM CRP (lane 3b), the free DNA band has disappeared and is replaced by a band moving slower and containing both DNA and CRP (see below). This experiment demonstrates, therefore, that specific DNA binding is poorly affected when more than a 4000 fold excess of non specific sites as defined by McGhee and Von Hippel (24) is present in the mixture.

Stoichiometry of binding

The gel electrophoresis method allows determination of the stoichiometry of the protein specifically bound to each DNA fragment (11,18,26,27) : we used a protein mildly iodinated with ^{125}I which for titration purposes was equivalent to the unlabelled protein (18). Table I indicates the stoichiometry of binding of iodinated CRP to various DNA fragments, determined after cutting

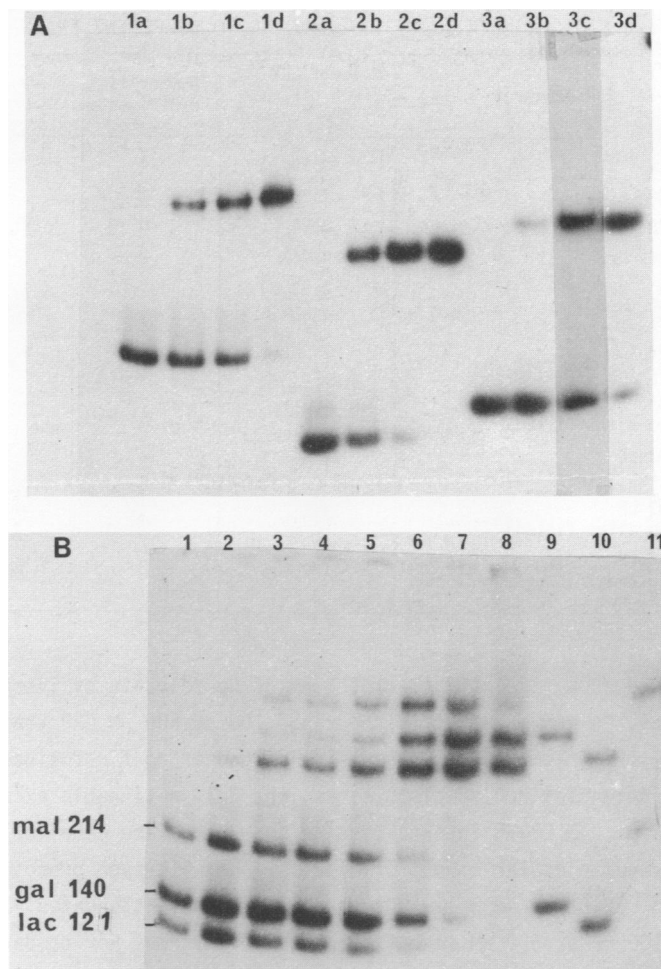


Fig. 4A : BINDING OF CRP TO *malt*, *lac* AND *gal* PROMOTERS. 1 nM of promoter fragment was incubated as described in Materials and Methods, alone (lanes a), with 5 nM CRP (lanes b), with 10 nM CRP (lanes c) or with 20 nM CRP (lanes d). In lanes 1, we used the 214 bp *mal* fragment ; in lanes 2, the 121 bp *lac* wild-type fragment and in lanes 3 the 140 bp *gal* fragment.

Fig. 4B : COMPARISON OF RETENTION OF *malt*, *lac* AND *gal* PROMOTERS BY CRP. A mixture of the three DNA fragments *mal* 214, *gal* 140 and *lac* 121 at 1 nM each was applied to the gel either alone (lane 1) or with increasing concentrations of CRP (from lanes 2 to 8, respectively 2,5,7.5,10,20,100 and 150 nM of CRP). Lanes 9, 10 and 11 indicate the respective mobilities of the *gal* 140 bp fragment, the *lac* 121 bp fragment and the *mal* 214 bp fragment, free and bound.

TABLE II : Relative association constants of CRP binding sites on different DNA fragments in the presence of 200 μ M cAMP.

FRAGMENT	SITE OCCUPIED BY CRP	K_I OR K_{II}	K_I^O OR K_{II}^O (2)
<i>lac</i> 121	CRP site (band III)	100 ⁽¹⁾	
<i>mal</i> 214	CRP site	40	
<i>gal</i> 140	CRP site	10	
<i>gal</i> 262	CRP site	10	
<i>lac</i> 80	Operator site (band IV)	2.5	
<i>lac</i> 121 L8	L8 CRP site (band III)	1.4	
<i>lac</i> 203	CRP + operator sites (band I + band II)	$K_I = 100$ $K_{II} = 5$	$K_I^O = 103$ $K_{II}^O = 2.5$
<i>lac</i> 203 L8	L8 CRP site + operator sites (band I + band II)	$K_I = 2.5$ $K_{II} = 3.3$	$K_I^O = 3.9$ $K_{II}^O = 0.9$
pBR322 digest	Non specific sites	$\sim 1/80$	

(1) We assign a value 100 for the association constant of the CRP complex and the *lac* 121 bp wild-type fragment.

(2) K_I^O and K_{II}^O refer to the association constants expected in the case of independent binding of the cAMP-CRP complex at the two sites carried by the 203 bp fragments.

out the band corresponding to the given complex and counting either for ^{32}P or ^{125}I . We found that a single mole of CRP dimer binds per mole of fragment in each case where a single specific CRP site was present, i.e. all the *malT* and *gal* fragments tested, the *lac* 121 fragment which carries the main CRP site and the *lac* 80 fragment which carries the weaker operator site. In each case a single band of DNA-protein complex is seen in the gel.

The *lac* 203 fragment carries two sites, and shows three bands in the gel. The first band corresponds to a 1:1 stoichiometry and the second one to a 2:1 ratio. This is true for a wild-type as well as for a fragment containing the L8 mutation, which we find does not totally stop CRP binding.

The same method was used to estimate the total number of CRP dimers bound non specifically on a 203 *lac* fragment at very high CRP concentrations where all the DNA was retained close to the top of the gel. A ratio of approximately 12:1 was found ; this corresponds fairly well with the estimated size for a cluster of dimers bound in a non specific fashion, i.e. around 16 bp (28).

The relative affinities of different sites

We have used two methods, direct titration and competition experiments between various sites.

. Fragment with one specific site : Figure 4A compares three fragments *mal* 214 (lanes 1), *lac* 121 (lanes 2) and *gal* 140 (lanes 3) ; *lac* and *mal* fragments bind readily and, at 20 nM CRP (lanes 1d,2d), there is almost no more free DNA. This is not the case for the *gal* fragment which shows a lower affinity (lane 3d). To compare directly the affinities of CRP for the *lac*, *gal* and *mal* fragments, we added increasing concentrations of CRP to an equimolar mixture of the three fragments as shown in figure 4B. At 2 nM CRP (lanes 2), only *lac* is bound ; at 5 nM CRP (lane 3), the maltose complex appears. As expected, the free *gal* fragment which has the lower affinity for CRP is the last to disappear in the titration.

From these competition experiments, we can order the affinities of the three CRP sites as $lac > mal > gal$. After densitometry, the ratio of the corresponding association constants can be deduced (cf. Materials and Methods and Table II). K_{lac} is 2.5 fold higher than K_{mal} which is four times higher than K_{gal} . Similarly we measured the association constants for the *lac* L8 site and for the *lac* operator region. We have repeated this kind of experiment with the CRP sites on different sized fragments (*gal* 242, *gal* 266, *lac* 203) : the ratio of association constants was unchanged.

From the direct titration curve of each separate fragments, we measured the absolute values of the dissociation constants ; we also measured α , the number of molecules of CRP which, in a given preparation, are required to saturate a given binding site under stoichiometric conditions (cf. Materials and Methods). The observed value of α , between 3 and 5, was independent of the fragment but was consistently higher than 1, the value expected from the stoichiometry of binding. Taking into account this factor, an association constant of $\sim 3.10^9 M^{-1}$ was found for the *lac* CRP site, a value thirty fold lower than that determined by Fried and Crothers in low salt buffer (Tris 10 mM, 1 mM EDTA) (9).

. Fragments with no specific site : We used the competition assay between pBR322 DNA and the *lac* CRP site to estimate K_{ns} , the association constant between CRP and a non specific binding site. The competitor plasmid DNA was present in very large excess with respect to the free protein concentration so that the creation of short lattices of protein bound to vicinal DNA sites was avoided. A value of K_{ns} ranging between 2 and $5.10^5 M^{-1}$ was derived.

. Fragments with two CRP binding sites: As indicated in fig.1, the *lac* 203 fragment possesses two CRP binding sites. Studies on binding to these two sites performed by Crothers and Fried revealed strong coupling at low ionic strength $I = 0.005 M$ and a more complex pattern in the case of the *lac* wild-type. At $I =$

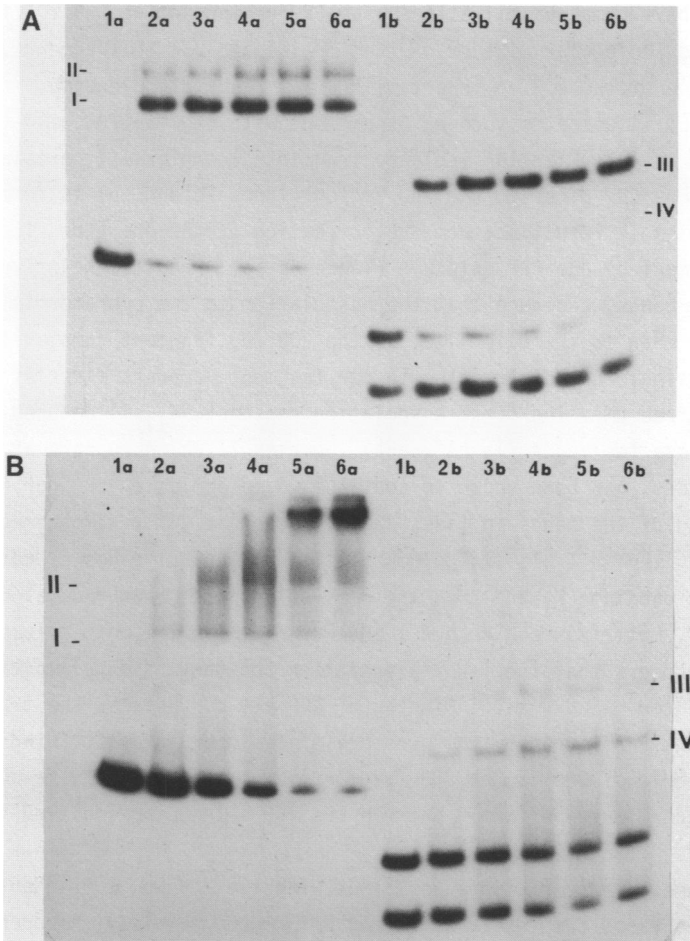


Fig. 5A : BINDING OF CRP TO THE 203 WILD-TYPE FRAGMENT (LANES 1a-6a) AND TO ITS *Hpa*II SUBFRAGMENTS (LANE 1b-6b). Each DNA fragment was used at 1.5 nM alone (lanes 1), with 6 nM CRP (lanes 2), 10 nM CRP (lanes 3), 15 nM CRP (lanes 4), 20 nM CRP (lanes 5) or 25 nM CRP (lanes 6). Complexes with the *lac* 121 bp fragment (CRP site 1) and with the *lac* 80 bp (operator) fragment are numbered III and IV. Complexes with the 203 bp fragment are called I (CRP/DNA ratio = 1) and II (CRP/DNA ratio = 2).

Fig. 5B : BINDING OF CRP TO THE 203 bp L8 FRAGMENT (LANES 1a-6a) AND TO ITS *Hpa*II SUBFRAGMENTS (LANES 1b-6b). DNA concentrations were 4 nM with CRP at 0 nM (lanes 1), 10 nM (lanes 2) 20 nM (lanes 3), 40 nM (lanes 4), 70 nM (lanes 5) and 100 nM (lanes 6).

0.15 M, we have compared titrations with CRP on the entire *lac* 203 fragment and on an equimolecular mixture of the *lac* 121 and *lac* 80 fragments, resulting from cleavage of the 203 bp insert with *Hpa*II restriction enzyme.

Figure 5A illustrates such an experiment with the *lac* 203 wild-type fragment (lanes 1a-6a). With the isolated fragments, complex III, arising from binding of one CRP dimer to the -60 region (lanes 2b and 3b) is formed before complex IV resulting from the occupation of the operator site on the *lac* 80 fragment by one CRP molecule (lanes 4b-6b). From the relative intensities of the bands we deduce that the association to the operator is forty fold weaker than to the main site. On the 203 *lac* fragment, complex I, which contains a single CRP dimer molecule per fragment, appears first at the expense of free DNA. The first association constant, K_I , is deduced from the ratio of these two bands (lanes 2a-6a). Complex II, which appears at higher CRP concentrations, has been shown to contain two molecules of CRP per DNA. From the scanning of the bands in lanes 3a-6a, we obtained K_{II} . The corresponding values are given in Table II, column 3, lines 7 and 8. Values expected for independent binding, K_I^0 and K_{II}^0 , are derived from the measured values of K_{III} and K_{IV} (cf. Materials and Methods) and are given in the next column of the Table. It appears that the first association constant is equal to that found for an isolated *lac* CRP site. Binding of CRP at -60 upstream the transcription start is thus unaffected by the presence of the operator site. Binding of the second molecule of CRP to the 203 fragment is however slightly favoured. Competition experiments performed between the 203 bp and the isolated fragments confirm these conclusions.

The same experiments were performed with the 203 bp L8 fragment (cf. Fig. 5B). In this case, complex IV is formed before complex III, the operator exhibiting a slightly better affinity for CRP than the L8 site. With the 203 L8 fragment, the first association constant K_I is equal to the value found for the operator site (cf. fragment *lac* 80) and lower than expected. The second association constant K_{II} is better than the first one and almost four times better than expected.

We conclude therefore that the strong coupling measured by Crothers and Fried at low ionic strength on the same 203 bp fragments is not observed here. On the contrary, in the L8 case, it seems that the first CRP dimer binds only at the operator site. A small positive coupling is however observed in both cases, as the second site is filled.

Fixation of CRP at various cAMP concentrations

We have repeated titration experiments with fixed CRP concentrations and

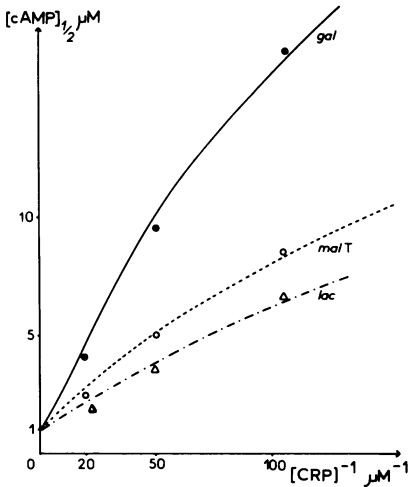


Fig. 6 : cAMP concentrations required to complex half of the *gal* 140 (●), *mal* 214 (○) and *lac* 121 (△) fragments as the free concentration of CRP is changed.

different cAMP concentrations. Saturation curves representing the formation of the corresponding complex versus cAMP concentration have the same shape in the case of the *lac*, *gal* and *mal* sites. The curves show positive cooperativity. The concentration of cAMP required to reach a given value of saturation varies according to the site and with the concentration of CRP present. The better the site, the lower is this nucleotide concentration. Additionally, the concentration of cyclic nucleotide required for half saturation decreases as the free protein concentration is increased. These results are shown in fig.6 where the value of $[cAMP]_{1/2}$ is plotted versus the reciprocal of the free protein concentration for the three sites tested. At infinite concentration of protein, the curves extrapolate to the same value of $[cAMP]_{1/2}$, 0.8 μM . Thus, under our experimental conditions, even at very high CRP concentration, cAMP is still required to obtain the formation of a specific complex migrating as a defined band in the gel. Dissociation of cAMP from the complex is characterized by a constant which does not depend on the fragment tested. This suggests that once the protein is bound to its specific site, the sequence of the binding site does not significantly alter the dissociation constants of the cAMP molecules, bound to the nucleic acid protein complex.

Takahashi *et al.* (29) have measured the adsorption isotherms of cAMP for free CRP or for CRP complexed with the *lac* DNA. From these isotherms, and from the values of K_I given in Table II, one can calculate the probability of forming complexes between a given DNA fragment and a molecule of CRP containing either one or two molecules of cAMP as the free cAMP concentration and

TABLE III : MOBILITIES OF THE CRP-DNA COMPLEXES. RELATION BETWEEN MOBILITY OF THE 1:1 COMPLEX AND THE POSITION OF THE BINDING SITE IN THE FRAGMENT. Mobilities are measured with respect to the *lac* 203 bp fragment which we arbitrarily assigned a mobility of 1. The gel system was calibrated using the mobilities of the above DNA fragments and the mobilities of fragments in an *Hinf*I pBR203 digest (Fig. 3). The calibration curve, used to determine the values of N', was derived by applying a least square analysis to these data (for the last two points, however, the curvature of the calibration graph was taken into account).

Fragment bp	Mobility of free DNA	Mobility of first complex	Mobility of second complex	N' bp	N'/N	l bp	L bp	$1-e = \frac{l}{L}$
<i>lac</i> 80	1.45	0.84	-	282	3.53	28	52	0.54
<i>lac</i> 100 wt or L8	1.27	0.83	-	288	2.88	21	79	0.27
<i>lac</i> 102	1.26	0.73	-	359	3.52	48	54	0.89
<i>lac</i> 121 wt or L8	1.22	0.70	-	381	3.15	42	79	0.53
<i>lac</i> 144	1.14	0.76	0.48	335	2.33	20	124	0.16
<i>lac</i> 144 L8	1.14	0.65	0.48	425	2.95	52	92	0.27
<i>lac</i> 186	1.07	0.49	0.34	596	3.21	62	124	0.50
<i>lac</i> 186 L8	1.07	0.495	0.34	591	3.18	52	134	0.39
<i>lac</i> 203	1	0.40	0.30	721	3.55	79	124	0.64
<i>lac</i> 203 L8	1	0.48	0.30	610	3.00	52	151	0.34
<i>gal</i> 140	1.18	0.60	-	473	3.38	51	89	0.36
<i>gal</i> 200	1.02	0.66	-	417	2.085	27	173	0.14
<i>gal</i> 242	0.92	0.31	-	865	3.57	89	153	0.58
<i>gal</i> 266	0.85	0.31	-	865	3.25	89	177	0.50
<i>mal</i> 214	0.95	0.46	-	635	2.97	53	161	0.33
<i>mal</i> 498	0.58	0.18	-	1700	3.41	161	337	0.48
<i>mal</i> 806	0.39	0.09	-	2240	2.78	161	645	0.25

the free CRP concentration are varied. The calculations will be given elsewhere. The three curves given in figure 6 result from such a computation.

Electrophoretic mobilities of the CRP-DNA complexes

Table III gives a summary of the mobilities of complexes obtained with fragments of different sizes. Two points are evident. First, even for very long DNA fragments, the change in mobility due to the binding of one molecule of CRP is still very significant. Second, for fragments of similar size a great variability in mobility is observed. For instance, *lac* 203 wild-type, *lac* 203 L8 and *mal* 214, complexed with a single molecule of CRP, migrate with mobilities of 0.4, 0.48 and 0.46. Explanations other than changes in the charge of the polyelectrolyte or in the molecular weight must be found.

We have analyzed these effects in figure 7 and in the five last columns of Table III. For each complex we define the excentricity of the site, e : $1 - e$ is the ratio between the distance, l, of the center of the CRP site from the nearest end to the distance, L, from the opposite end ($0 < e \leq 1$).

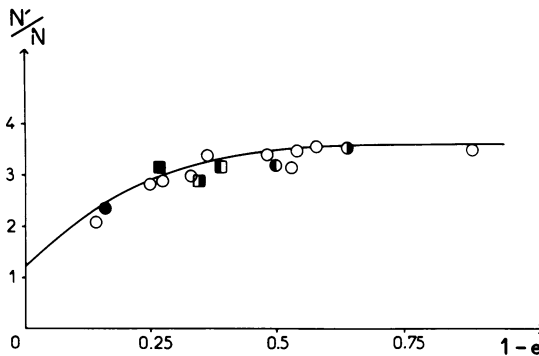


Fig. 7 : APPARENT SIZE INCREASE OF THE CRP-DNA (1:1) COMPLEX VERSUS THE POSITION OF THE BINDING SITE IN THE FRAGMENT. ○ : single site fragment ; ●, ○, ○ : *lac* wild-type fragments (respectively 144, 186, 203 bp) ; ■, ■, ■ : homologous L8 fragments.

We then characterize the perturbation in mobility due to the binding of the CRP dimer by the ratio N'/N , N being the length in base pairs of the fragment, N' being the length of a free DNA fragment which would have the same mobility as the complex in the same gel system. Fig. 7 shows that N'/N does not appreciably depend on the size of the fragment and is a smooth function of $1 - e$.

We have also plotted the mobilities of the free DNA fragments and of their 1:1 complexes with CRP versus the logarithm of N . Due to the position effect described in the preceding paragraph, the points corresponding to the complexes are more scattered than those for free DNA. We noticed, however, after a linear regression analysis of the data, that the average slopes of the two sets of results were identical.

These two observations (average slope identical and effect of the position of the site on the mobility) strongly suggest an explanation consistent with the present theories of mobilities of DNA fragments in those types of gels (30,31,32). Perturbations similar to the one discussed here have been observed when the only parameter affected was the end to end distance (30) or the persistence length, a , of the DNA fragment (32) ; a characterizes the tendency of the axis of the DNA to remain straight during the migration (strictly speaking, a is the average projection of the vector which joins its two ends on the original direction of the DNA axis). From these theories we deduce that the empirical factor used in fig. 7, N'/N , should be equal to a/a' , the relative change of the persistence length due to CRP binding. The fact that this parameter is more affected when the site is at the center of

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
	Ⓐ	A	A	.	T	G	T	G	A	C	A	
	Ⓑ	A	A	.	T	G	T	G	A	C	A	C	A	.	T	.	.	ΔG_{kcal}
Non specific site		0
<i>lac</i> operator	→ +4	A	A	t	T	G	T	G	A	g	c	g	g	a	t	a	a	c	A	A	t	T	t	-3
<i>gal</i> site	← -34	A	A	g	T	G	T	G	A	c	a	T	g	g	a	a	t	A	A	A	t	T	t	-3.9
<i>malT</i> site	→ -78	A	A	t	T	G	T	G	A	c	a	c	a	g	t	g	C	A	A	A	t	T	c	-4.9
<i>lac</i> site	→ -69	t	A	a	T	G	T	G	A	g	t	T	a	g	c	t	C	A	C	t	c	a	t	-5.4
<i>lac</i> L8 site	→ -69	t	A	a	T	G	T	a	A	g	t	T	a	g	c	t	C	A	C	t	c	a	t	-2.7

Fig. 8 : COMPARISON OF THE SEQUENCES OF THE VARIOUS CRP SITES WITH THE PROPOSED CONSENSUS SEQUENCES. The 22 bp stretches are written so that the TGTGA motives are at positions +4 to +8. Positions which agree either with the sequence proposed by Ebright (A, (37)) or with that deduced by de Crombrughe *et al.* (B, (5)) are written in capitals. The arrow shows the direction of transcription and the numbers immediately following each arrow give the position of the first T of the TGTGA motif relative to the transcription start. ΔG represents the free energy of stabilization at each site, relative to non specific binding.

the fragment than when it is close to the end suggests that the perturbation in mobility could be due to a kink or a bend at the CRP site.

DISCUSSION AND CONCLUSIONS

We have compared three functional CRP binding sites, at the *lac*, *gal* and *malT* promoters, two non functional ones, the *lac* operator sequence and the L8 mutant, and the non specific sequences. Each specific site binds one molecule of CRP dimer but with a different affinity. The stoichiometry is always the same, one dimer per site. This confirms independent determinations made previously on the *lac* and *gal* control regions by the gel electrophoresis method (17,18,26,27), by ultracentrifugation (26,33) and by spectroscopy (33,34).

As pointed out by Takahashi *et al.* (34), selective binding at specific sites can be obtained without much non specific binding, provided the ionic strength is high enough. This was the case in our previous study which showed that CRP binding did not unwind DNA (36). One should however caution that, in some cases, non specific aggregation arises at concentrations of CRP lower than expected. This was the case here for the 214 bp fragment carrying the *malT* control region. Perhaps in this case, the main site serves as a nucleation center for the aggregation process, a behaviour not observed with the other specific sites.

Using the competition assay, we report a difference in free energy of association of 5.4 kcal/mole between the functional *lac* site and random non

specific sequences, 17 bp long. Taking non specific DNA as a reference, we have compared the values of free energies of association observed between sites of known sequence and have tried to correlate them with variations from the consensus sequences (5,37). In figure 8, all these sequences have been aligned on the most conserved motif 5'TGTGA 3'. Binding at the *lac* operator is stabilized by 3 kcal/mole with respect to non specific DNA. In this case the left hand part of the sequence fits the consensus sequence whilst the right hand part does not. The galactose site is functional. A consensus A appears at position 17 and we observe an extrastabilization of 0.9 kcal/mole. Binding to the *malT* site (where a consensus C appears at position 16) is further stabilized by 1 kcal/mole over binding at the galactose site. Hence a gradual stabilization occurs as one obtains a better fit with the consensus sequences and, from the *lac* operator site to the maltose site, a gain in free energy of 1.9 kcal/mole is added to the stabilization of 3 kcal previously attributed to the TGTGA sequence. These observations support our proposal (5,18) that one subunit of the dimer is strongly anchored at the TGTGA sequence while the other subunit ensures progressively tighter contacts with the opposite part of the binding site. An alternative explanation with only one CRP subunit interacting with the whole DNA consensus sequence has been proposed by Ebright (37).

The lactose site is only moderately stabilized with respect to the more asymmetrical *malT* site (0.5 kcal). As the two sequences differ considerably, it is difficult to guess which elements cause this stabilization. Strikingly, however, the L8 point mutation, once introduced in the consensus sequence of *lac* causes a loss of 2.7 kcal in the free energy of stabilisation. This loss, close to that attributed to the whole TGTGA sequence, represents half the total stabilization of *lac* with respect to non specific binding.

In summary, functional sites bind better than non functional sites but by a small margin. The TGTGA motif brings a large part of the free enthalpy of association but other "consensus" motives present in the functional sites and probably recognized by the other subunit provide another significant contribution to the stabilisation. The drastic effect due to the L8 mutation suggests to us that local free energies of association are perhaps not strictly additive, at least in the TGTGA sequence.

The same hierarchy in site recognition is found when the cAMP concentration is varied during the binding assay. From the analysis of the data shown on figure 6, we have argued that the dissociation constant of cAMP bound in the CRP-DNA complex does not significantly depend on the primary sequence of

the site. Once bound to DNA by its C-terminal domain, the cAMP site present in the N-terminal domain does not seem to be very much affected by the nature of the nucleic acid sequence. However, as expected, the probability of the CRP being bound at a given cAMP concentration strongly depends on the nature of the site provided the CRP concentration is not too high. Note that these differences arise in a range of cAMP concentration close to the physiological one. At least for this reason, it is therefore expected that different CRP sensitive operons will be activated at different critical cAMP concentrations.

These results have been obtained with the electrophoretic technique which is simple but subject to several criticisms. The most serious is that conditions which are used to form the complexes are not identical to the ones used to isolate them. During migration within the gel, complexes are stabilized by the so-called "cage effect" (11) so that the critical step is the entry into the gel. Thus, only those complexes for which the rate of dissociation is slower than the rate of penetration into the gel can be detected. We have always worked under the same conditions, and further have checked that strong perturbations in the entry time do not significantly affect the results. We have also confirmed all the data given here by competition experiments. Therefore we do not believe that kinetic effects strongly alter the values of the relative association constants reported here (compare our data with those reported by Baudras *et al.* (35)). However further work is required to clarify two discrepancies. Firstly, association constants measured by the gel technique are consistently higher than the values obtained at equilibrium. Secondly, we and others (10,11) have observed that preparations of CRP which are fully active as judged by other criteria form stable complexes with DNA fragments with a low efficiency (α ranging between 3 and 5).

Despite these limitations, the gel technique offers two advantages. Firstly, it allows separate estimates of the various association constants when several sites are present on the same fragment. Secondly, it gives the mobility of the complexed fragment. From the analysis of these data, we concluded that CRP, once bound, decreases the length over which the axis of the DNA chain persists in the same direction as its initial orientation. The mean end-to-end distance of the chain decreases because the chain loses its stiffness and has a more random orientation. A bend or a kink would easily explain why this effect is larger for a site located near the center of the fragment. This explanation neatly agrees with independent evidence derived from similar measurements (38) or from a recent refinement of the crystal structure (39).

Using the calibration shown in figure 7, one can also exploit the changes

in mobility of different complexes to determine where a given CRP molecule binds. As an illustration, consider the case of fragments bearing the *lac* operator CRP site and the mutated L8 functional site. From the value of K_1 (which was equal to the association constant at an isolated operator site) we supposed that the first complex corresponded to a CRP dimer uniquely associated at the operator. This assignment was used in Table III for computing the values of e : assuming that CRP binds first at the main site for the wild-type fragment and at the operator site for the L8 mutant, we obtained a good fit with the curve. On the contrary, if one assumes that the first molecule of CRP binds first at the L8 site on the *lac* 203 L8 and *lac* 144 L8, the fit is either poor or bad. In this case, the values of N'/N remain the same since they arise from the same mobility measurements but $1 - e$ takes now the value assigned to the corresponding wild-type fragments in Table III. Taken together, the migration values and the association constants suggest therefore that on L8 fragments, it is predominantly the operator site which is first occupied. This deduction is at variance with the explanations proposed by Crothers and Fried who attributed the difference in migration of the first complexes between CRP and either *lac* L8 or *lac* wild-type to a particular DNA conformation specifically stabilized in the wild-type complex (9).

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