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cAMP–CRP Activator Complex and the CytR Repressor Protein Bind Co-operatively to the *cytRP* Promoter in *Escherichia coli* and CytR Antagonizes the cAMP–CRP-induced DNA bend

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Initiation of transcription from the *cytRP* promoter in *Escherichia coli* is activated by the cAMP–CRP complex and negatively regulated by the CytR repressor protein. By combining gel retardation and footprinting assays, we show that cAMP–CRP binds to a single site centered at position −64 and induces a considerable bend in the DNA. CytR binds to a region immediately downstream from, and partially overlapping, the CRP site, and induces a modest bend into the DNA. In combination, cAMP–CRP and CytR bind co-operatively to *cytRP* forming a nucleoprotein complex in which the proteins directly interact with each other and bind to the same face of the DNA helix. CytR binding concomitantly antagonizes the cAMP–CRP-induced bend. This study indicates that the minimal DNA region required to obtain CytR regulation consists of a single binding site for each of cAMP–CRP and CytR. The case described here, in which a protein-induced DNA bend is modulated by a second protein, may illustrate a mechanism that applies to other regulatory systems.

Keywords: DNA bending; CRP-protein; transcription regulation; co-operative DNA binding; heterologous co-operativity

1. Introduction

Sequence-specific DNA-binding proteins have important functions in processes such as initiation of transcription, initiation of replication and site-specific recombination. Many of these processes depend on the assembly of multiprotein complexes in which proteins bound to separated sites on the DNA interact. Moreover, the formation of the complexes often requires that the DNA is deformed into a more bent form (for a review, see Echols, 1986; Raibaud, 1989). Hence, DNA-bending proteins may have crucial roles in these systems by facilitating the interaction between separated DNA-bound proteins.

The importance of both intrinsically bent DNA and protein-induced bends in the initiation of transcription has been clearly demonstrated in many prokaryotic promoters. In several systems the initiation rate depends on the phasing of upstream bend sequences (Bossi & Smith, 1984; Gourse *et al.*, 1986; Bracco *et al.*, 1989; Collis *et al.*, 1989; McAllister & Achberger, 1989). Transcription of the nitrogen fixation genes in *Klebsiella* and the flagellar genes in *Caulobacter* represent examples in which integration host factor (IHF‡), a DNA-bending protein (Robertson & Nash, 1988), is thought to have a structural role by facilitating the interaction between activator proteins bound approximately 100 base-pairs (bp) upstream from the promoter and the RNA polymerase bound at the promoter (Gober & Shapiro, 1990; Hoover *et al.*, 1990). In the *araBAD* promoter, the cAMP–CRP complex is also believed to have a structural role by bending the

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‡ Abbreviations used: IHF, integration host factor; bp, base-pair(s); CRP, cyclic AMP receptor protein.

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DNA in such an orientation that it stimulates the breaking of an inhibitory loop formed by the AraC protein (Lobell & Schleif, 1991).

The cAMP–CRP complex induces a DNA bend estimated from gel-electrophoretic measurements, model-building and crystallographic studies to be 70° to 140° in various promoters (Weber & Steitz, 1984; Kim *et al.*, 1989; Schultz *et al.*, 1991). Although it has not been shown directly that this bend is crucial for activation in promoters in which cAMP–CRP is the only activator, several lines of evidence indicate that bending may contribute to the activation. Bracco *et al.* (1989) found that properly phased A-tract-mediated bends can functionally replace cAMP–CRP in the *gal* promoter *in vivo*. Similarly, Gartenberg & Crothers (1991) showed that cAMP–CRP in the *lac* promoter could be replaced by appropriately phased DNA bending sequences *in vitro*. However, protein–protein interactions between cAMP–CRP and RNA polymerase have also been implicated in activation of transcription, based on the observation that the two proteins interact *in vitro* (Pinkney & Hoggett, 1988) and the isolation of CRP mutants that are defective in activation but still bend the DNA like wild-type CRP (Bell *et al.*, 1990; Eschenlauer & Reznikoff, 1991). Two proposals have been advanced to suggest the function of the cAMP–CRP-induced DNA bend: the bend may promote essential protein–DNA contacts or protein–protein contacts that would not otherwise be sterically possible (Wu & Crothers, 1984; Schultz *et al.*, 1991). Alternatively, the energy stored in the bend may be used in the initiation process (Zinkel & Crothers, 1991; Liu-Johnson *et al.*, 1986). Finally, recent studies on the differential effect of Jun homodimers and Jun–Fos heterodimers imply that distortion of the DNA is also important for regulation of gene expression in eukaryotic cells (Diamond *et al.*, 1990; Kerppola & Curran, 1991).

The CytR repressor protein in conjunction with the cAMP–CRP activator complex regulates initiation of transcription from at least nine promoters in *Escherichia coli* (for a review, see Hammer-Jespersen, 1983). This system illustrates the importance of protein–protein interactions between heterologous gene regulatory proteins in gene expression (Søgaard-Andersen *et al.*, 1991*a*,*b*; Pedersen *et al.*, 1991; Gerlach *et al.*, 1991). Regulation by cAMP–CRP and CytR has been most intensively studied in the *deoP2* promoter, and has revealed that regulation of *deoP2* by CytR *in vivo* is confined to cells containing the cAMP–CRP complex and depends on proper DNA binding of cAMP–CRP to two CRP sites, CRP-1 located around position −40 and CRP-2 located around position −93 (Valentin-Hansen, 1982; Søgaard-Andersen *et al.*, 1990*a*,*b*; Søgaard-Andersen & Valentin-Hansen, 1991). These *in vivo* observations are paralleled by the *in vitro* observation that the CytR repressor protein and the cAMP–CRP complex bind co-operatively to the DNA. CytR binds to *deoP2* with a relatively low affinity in the absence of cAMP–CRP. In the presence of cAMP–CRP, however, the affinity of CytR for *deoP2* is increased 1000-fold and, similarly, the affinity of cAMP–CRP for CRP-1 and CRP-2 is increased 100-fold and 10-fold, respectively (Pedersen *et al.*, 1991). The co-operative DNA binding is mediated by protein–protein interactions between cAMP–CRP and CytR (Søgaard-Andersen *et al.*, 1991*a*; Pedersen *et al.*, 1991). So, in the presence of both protein species, a nucleoprotein complex is formed containing cAMP–CRP at the two CRP sites, CytR in the intervening region and the complex is stabilized by direct interactions between the DNA-bound proteins. The inducer of CytR, while not affecting independent DNA binding of CytR, perturbs the co-operative DNA binding of cAMP–CRP and CytR by interfering with the proper protein–protein interactions (Pedersen *et al.*, 1991; Gerlach *et al.*, 1991).

Here, we have focused on the *cytR* promoter, *cytRP*. It has previously been shown that this promoter is weakly activated by the cAMP–CRP complex (5-fold) and weakly autoregulated (3-fold; Gerlach *et al.*, 1990). We report a more extensive biochemical analysis of protein–DNA and protein–protein interactions in *cytRP*, and we provide evidence that the basic DNA unit required to obtain CytR regulation consists of only a single binding site for each of the two proteins.

2. Materials and Methods

Enzymes for DNA manipulations were purchased from Boehringer-Mannheim, ^{32}P-labeled nucleotides were purchased from NEN-Dupont; all chemicals were analytical grade. Transformation, isolation of plasmid DNA and restriction of plasmid DNA were performed according to protocols of Maniatis *et al.* (1982). ^{32}P-labeled fragments were purified and sequenced as described (Valentin-Hansen *et al.*, 1984).

(a) *Bacterial strains, plasmids and proteins*

pVH002 (Valentin-Hansen *et al.*, 1986), p13-21 and p13-210 were propagated in TG-1 (Δ(*lac-pro*), *supE*, *thi*, *hsdD5*/*F'traD36*, *proA*$^+$*B*$^+$, *lacI*^{q}Δ*M15*). pBend1 and pBend1610 were propagated in MC1000*dam* (*araD139*, Δ*ara*, *leu*)*7697*, Δ*lacZ4*, *galU*, *galK*, *strA*, *dam*) to obtain DNA that could be restricted with *Cla*I.

pVH002 contains the entire *cytR* gene and 250 bp upstream from the start site of transcription; the upstream boundary of the *cytRP* sequence is flanked by an *Eco*RI site. p13-21 and p13-210 are pUC13 (Vieira & Messing, 1982) derivatives containing an *Rsa*I fragment extending from +24 to −180 in *cytRP* cloned in the *Sma*I site; in p13-21, the +24 position is next to the *Bam*HI site in pUC13, and in p13-210 the fragment is in the opposite direction. pBend1610 is a pBend1 (Kim *et al.*, 1989) derivative in which an *Fnu*DII–*Sfa*NI fragment from position −102 to −25 of *cytRP* has been cloned in the *Xba*I site after filling in of all 5'-ends with the DNA polymerase I Klenow fragment (Fig. 5).

CRP protein was purified as described by Ghosaini *et al.* (1988) and CytR was purified as described by Pedersen *et al.* (1991).

(b) *Gel retardation assay*

^{32}P-labeled fragments and proteins were incubated in binding buffer (10 mM-Tris·HCl (pH 7·8), 50 mM-KCl, 1 mM-EDTA, 50 μg acetylated bovine serum albumin/ml, 1 mM-dithiothreitol, 0·05% (v/v) Nonidet-P40, 50 μM-cAMP) containing 20 μg competitor DNA/ml (pGEM4 obtained from Promega) for 30 min at 37 °C in a total volume of 10 μl (Pedersen *et al.*, 1991). Immediately before loading, 2 μl of loading buffer (binding buffer containing 50% (v/v) glycerol and 0·1 mg bromophenol blue/ml) were added to the samples. In all experiments, the samples were loaded with the current on, except in the circular permutation assay; electrophoresis was at 200 V, except in the circular permutation assay in which only 100 V were applied. All gels were 5% (w/v) polyacrylamide gels prepared from a 44 : 0·8 (acrylamide : *N,N'*-methylenbisacrylamide) stock. The electrophoresis buffer employed was 10 mM-Tris·HCl (pH 7·8), 1 mM-EDTA, 50 μM-cAMP. Following electrophoresis, the gels were dried and autoradiographed. The concentrations of proteins and end-labeled fragments are indicated in the legend to each Figure.

(c) *DNase I and hydroxyl-radical footprinting experiments*

^{32}P-labeled fragments (final concentration 1 nM), proteins at the concentrations stated in the Figure legends and competitor DNA were incubated as in the gel retardation assays. Otherwise, DNase I experiments were performed as described by Galas & Schmitz (1978), and hydroxyl-radical experiments as described by Tullius & Dombroski (1986), with the modifications developed by O'Halloran *et al.* (1989).

3. Results

(a) *The binding sites of cAMP–CRP and CytR in* cytRP *overlap*

In order to define the binding sites of cAMP–CRP and CytR in *cytRP* and the stoichiometry of different protein–DNA complexes, gel retardation and DNase I footprinting analyses were performed using purified proteins. In the gel retardation experiment shown in Figure 1, *cytRP* was present on a ^{32}P-end labeled 393 bp fragment containing *cytRP* sequences from position −250 to +133. cAMP–CRP binding to the *cytRP* probe results in formation of a single retarded complex even in the presence of high concentrations of cAMP–CRP suggesting that only one cAMP–CRP complex binds to *cytRP*. In the presence of CytR protein, a single retarded complex is again observed in the gel retardation analysis (Fig. 1(b), lanes 2 and 3), suggesting also in this case that one CytR protein binds to *cytRP*. The use of higher concentrations of CytR protein in this analysis result in unspecific binding of CytR to the probe (data not shown).

Next, the combined binding of cAMP–CRP and CytR to *cytRP* was analyzed (Fig. 1(b)). In the presence of both proteins, a third retarded band is observed (Fig. 1(b), lanes 6 to 10). Most strikingly, the mobility of this complex is higher than that of the cAMP–CRP/*cytRP* complex (Fig. 1(b), compare lanes 4 to 5 and 6 to 10). This anomalous migration

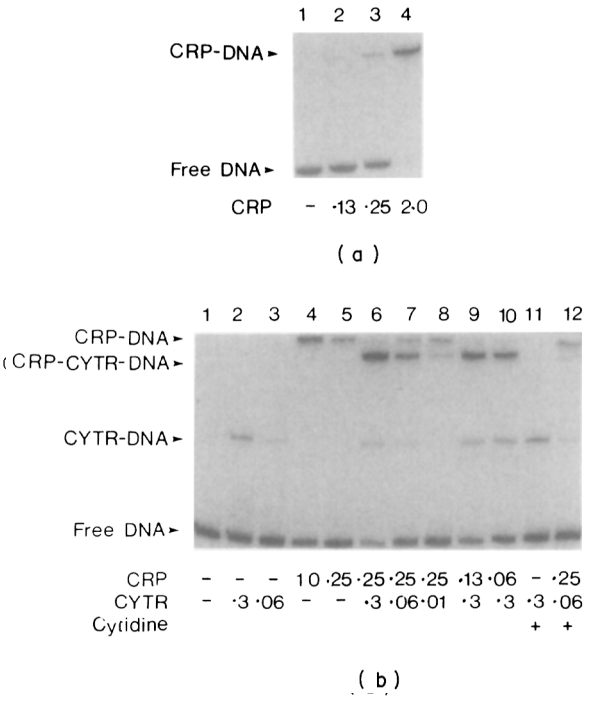

Figure 1. Gel retardation analysis of the binding of cAMP–CRP and CytR to *cytRP*. The ^{32}P-end-labeled fragment used is a 393 bp *Eco*RI–*Ava*I fragment from pVH002 (see legend to Fig. 4 for details) present at a final concentration of 1 ng/μl. The experiments were carried out as described in Materials and Methods in the presence of the amounts of CRP and CytR proteins indicated in ng/μl below each lane. Cytidine was added to a final concentration of 10 mM in the samples in lanes 11 and 12 in (b). The composition of each complex is indicated. Electrophoresis was continued for longer time in (b) than in (a).

is discussed in more detail below. The titration analyses in which either the cAMP–CRP concentration or the CytR concentration is varied, indicate that this complex consists of one cAMP–CRP complex and one CytR protein bound to the *cytRP* probe. Comparison of lane 4 and lane 10 in Figure 1(b) shows that at the conditions of the gel retardation assay, CytR stimulates binding of cAMP–CRP approximately 16-fold and cAMP–CRP stimulates binding of CytR approximately 25-fold (Fig. 1(b), compare lanes 2 and 8).

DNase I footprints of cAMP–CRP bound to *cytRP* (Fig. 2(a)) showed that the protein strongly protects a region extending from position −50 to −80 on the upper strand (Fig. 2(a), lanes 3 to 6), and from position −53 to −80 on the lower strand (data not shown). Previous studies indicate that cAMP–CRP protects a stretch of approximately 30 bp against DNase I digestion (de Crombrugghe *et al.*, 1984). The dimensions of the cAMP–CRP footprint in *cytRP* is, therefore, not compatible with the presence of two DNA bound cAMP–CRP complexes as previously suggested (Gerlach *et al.*, 1990). Furthermore, the DNase I footprint of cAMP–CRP is often characterized by the presence of two hyper-

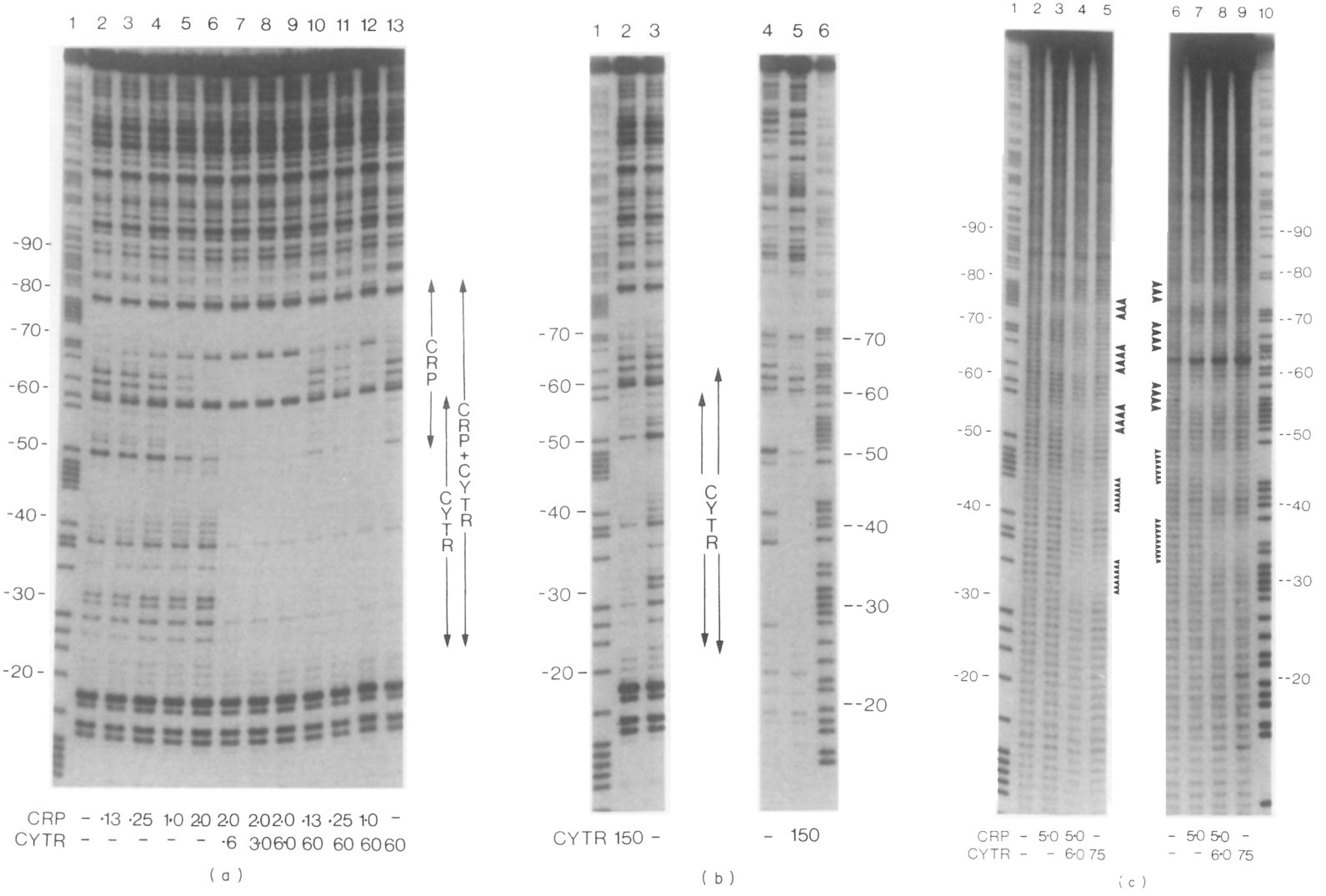

Figure 2. DNase I and hydroxyl-radical footprints of cAMP–CRP and CytR bound to *cytRP*. The fragment used is an *Xba*I–*Pvu*II fragment from p13-21 with the upper strand of *cytRP* 3′-end-labeled with ^{32}P at the *Xba*I site (a), lanes 1 to 3 (b) and lanes 1 to 5 (c) or with the lower strand 5′-end-labeled at the *Xba*I site lanes 4 to 6 (b) and lanes 6 to 10 (c). The final concentrations of cAMP–CRP and CytR are indicated below each lane in ng/μl: the regions protected by the proteins are indicated: the larger arrows in (c), point to the positions cAMP–CRP protects from attack by the hydroxyl radical and the smaller arrows indicate the positions protected by CytR. The sequence is numbered with respect to the start site of transcription (+1). Lanes 1 in (a), 1 and 6 in (b), and 1 and 10 in (c) are the A+G sequences of the probes. Note that CytR at high concentrations has a tendency to form aggregates on the DNA. This can be seen in lane 5 in (c), as a weak protection of 3 regions upstream from the 2 strongly protected regions around positions −33 and −43. Enhanced cleavage at certain positions by the hydroxyl radical is observed in some experiments, however, the cause for this is unknown (see lanes 6 to 9 in (c)).

sensitive DNase I digestion sites that lie within the conserved part of the binding site. In *cytRP* there are two hypersensitive sites in the region encoding the sequence (−72) 5′-TTCAAN$_6$TCACA-3′(−67), that shares homology with the consensus sequence for a cAMP–CRP binding site (5′-TGTGAN$_6$TCACA-3′ (de Crombrugghe *et al.*, 1984). Taken together these data suggest that cAMP–CRP specifically recognizes the region between position −57 and −72 and, thus, the center of the CRP site is positioned at −64.

CytR independently results in protection of a region extending from position −22 to −57 on the upper strand (Fig. 2(b), lane 2) and from position −24 to −64 on the lower strand (Fig. 2(b), lane 5). Hence, cAMP–CRP and CytR have overlapping binding sites on both strands (see Fig. 3 for a schematic drawing).

The combined DNA binding of cAMP–CRP and CytR results in protection of a region covering 59 bp extending from position −22 to −80 on the upper strand (Fig. 2(a), lane 7 to 12). The DNase I digestion pattern in this footprint is identical with the sum of the two independent footprints (Fig. 2(a), compare lanes 6, 13 and 7 to 12), suggesting that the combined footprint is obtained by binding of one cAMP–CRP complex and one CytR protein. Under the conditions of the DNase I footprint, cAMP–CRP stimulates DNA binding of CytR at least 100-fold (Fig. 2(a), compare lanes 7 and 13); by contrast, CytR only stimulates binding of cAMP–CRP minimally (Fig. 2(b), compare lanes 3 to 5 and 10 to 12), contrasting the clear stimulatory role of CytR on cAMP-CRP DNA binding in the gel retardation analysis.

(b) *Cytidine, the inducer of CytR, affects only co-operative DNA binding of CAMP–CRP and CytR*

The inducer of CytR, cytidine, perturbs the co-operative DNA binding of cAMP–CRP and CytR by disrupting the protein–protein contacts between the two proteins (Pedersen *et al.*, 1991; Gerlach *et al.*, 1991). As shown in the gel retardation analysis in Figure 1(b), independent DNA binding of CytR to *cytRP* is unaffected by cytidine, however, the co-operative binding with cAMP–CRP is severely reduced (Fig. 1(b), compare lanes 2, 6, 11 and 12). These data show that cytidine exerts its effect in *cytRP* by perturbing the protein–protein interactions between cAMP–CRP and CytR. Hence, the co-operative DNA binding in each of this type of promoters depends on the same mechanism.

(c) *cAMP–CRP and CytR bind to the same face of the DNA helix in* cytRP

In order to define the binding sites for cAMP–CRP and CytR more carefully, the contact points between the proteins and the deoxyriboses in the DNA backbone were determined by hydroxyl-radical footprinting experiments (Tullius & Dombroski, 1986) (Fig. 2(c)). In the presence of cAMP–CRP, three clearly defined, regularly spaced, protected patches appeared on both strands (Fig. 2(c), lanes 3 and 7). In the presence of CytR, two clearly defined, protected patches appeared (Fig. 2(c), lanes 5 and 9). Finally, with both proteins, five regularly spaced, protected regions appeared (Fig. 2(c), lanes 4 and 8). For all combinations of proteins, the modified regions were within the protein-binding sites defined by the DNase I footprints and the protection patterns were identical on both strands but offset by two or three nucleotides. The modification pattern observed in the hydroxyl-radical footprints is shown on the DNA helix in Figure 3. This illustration clearly shows that the protected regions all lie on the same face of the helix and suggests that the two proteins bind to the same face of the DNA helix and lie across the minor grooves corresponding to the protected positions. This conclusion is consistent with the observation

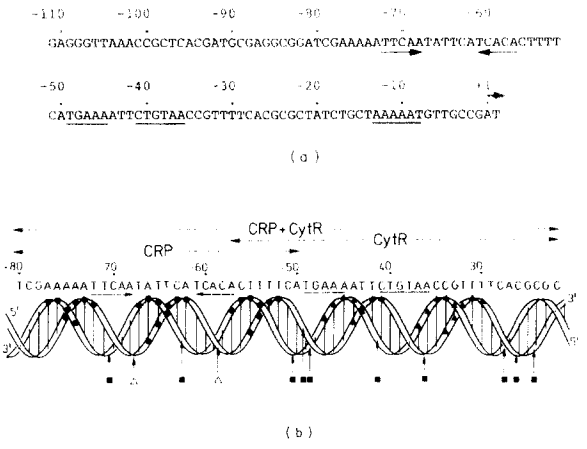

Figure 3. Nucleotide sequence of the upper strand in *cytRP* and schematic representation of the DNase I and hydroxyl-radical footprints of cAMP–CRP and CytR bound to *cytRP*. (a) Sequence of *cytRP*: the numbering is with respect to the start site for transcription (+1), as indicated by the short arrow. The −10 sequence is underlined; the 2 arrows indicate the sequence recognized by cAMP–CRP; the 2 remaining underlined sequences indicate the motif that is, most likely, important for sequence-specific binding of CytR. (b) A helical representation of the positions protected by cAMP–CRP (filled circles) and CytR (filled triangles) from attack from hydroxyl radical. The helix is drawn with 10·5 bp/turn and each vertical line represents 1 bp. The extents of the individual and combined cAMP–CRP and CytR DNase I footprints are indicated above the helix. The nucleotide sequence is that of the upper strand in *cytRP* numbered with respect to the start site for transcription (+1), each nucleotide is written above its corresponding bp. The 2 arrows below the sequence and the 2 underlined regions are equivalent to those in (a). Below the helix, the 2 arrows labeled with open triangles point to the 2 phosphodiester bonds that are hypersensitive to digestion by DNase I in the presence of cAMP–CRP; the arrows labeled with the filled boxes point to the bonds that are not completely protected from digestion by DNase I in the presence of cAMP–CRP and CytR.

that all the regions protected from hydroxyl-radical modification were also protected from digestion by DNase I that cleaves the DNA within the minor groove. Furthermore, the two positions hypersensitive to digestion by DNase I in the CRP site, and the positions that are not fully protected in the combined DNase I footprint, all lie on the opposite face of the helix to that binding the proteins.

(d) *The DNA structure is changed in the cAMP–CRP/CytR*/cytRP *complex compared to the cAMP–CRP*/cytRP *complex*

Two parameters contribute to the retardation of a DNA fragment upon binding of a protein in a gel retardation analysis. First, binding of the protein results in an increased apparent molecular weight of the fragment and, consequently, the mobility is reduced. Second, distortions of the DNA structure, such as bends, impede the movement of the DNA through the gel matrix and, therefore, reduce its mobility (Wu & Crothers, 1984). The effect of a bend on the mobility depends on the relative position of the bend, i.e. for fragments of identical length, a central bend reduces the mobility more than a peripheral bend (Wu & Crothers, 1984). Finally, for central bends the mobility is decreased when the bend angle is varied from 0° to 180° (Liu-Johnson *et al.*, 1986).

As noted above, the cAMP–CRP/CytR/*cytRP* complex migrated faster than the cAMP–CRP/*cytRP* complex in a gel retardation analysis employing a 393 bp fragment (Fig. 1(b)). To analyze in more detail this anomalous migration, we performed a gel retardation analysis with DNA fragments of four different lengths that all contained the binding sites for cAMP–CRP and CytR from *cytRP* in the middle (Fig. 4(a)). With the shortest DNA fragment of 186 bp the cAMP–CRP/*cytRP* complex has a higher mobility than the tri-molecular complex (Fig. 4(b), compare lanes 2 and 3). However, with the three remaining fragments the tri-molecular complex migrates faster than the cAMP–CRP/*cytRP* complex and this effect is more pronounced, the larger the fragment (Fig. 4(b), compare lanes 4 to 12). In this context, it should be emphasized that CytR does not give rise to an independent retarded complex at the concentration employed in these experiments; moreover, the CytR/*cytRP* complexes have mobilities in between that of the free fragments and the cAMP–CRP/*cytRP* complexes (see Fig. 1(b) for data obtained with the 393 bp fragment). Altogether, we take these observations as evidence for two opposing trends that determine the mobility of the tri-molecular complex, i.e. increases in molecular weight and changes in DNA structure. For the smallest fragment, the increased size outweighs the effect of a changed DNA structure, whereas for the larger fragments the change in DNA structure is the predominant effect. Therefore, the simplest explanation for the anomalous migration is that CytR induces a conformational change in the DNA that antagonizes the cAMP–CRP-induced DNA bend in *cytRP*.

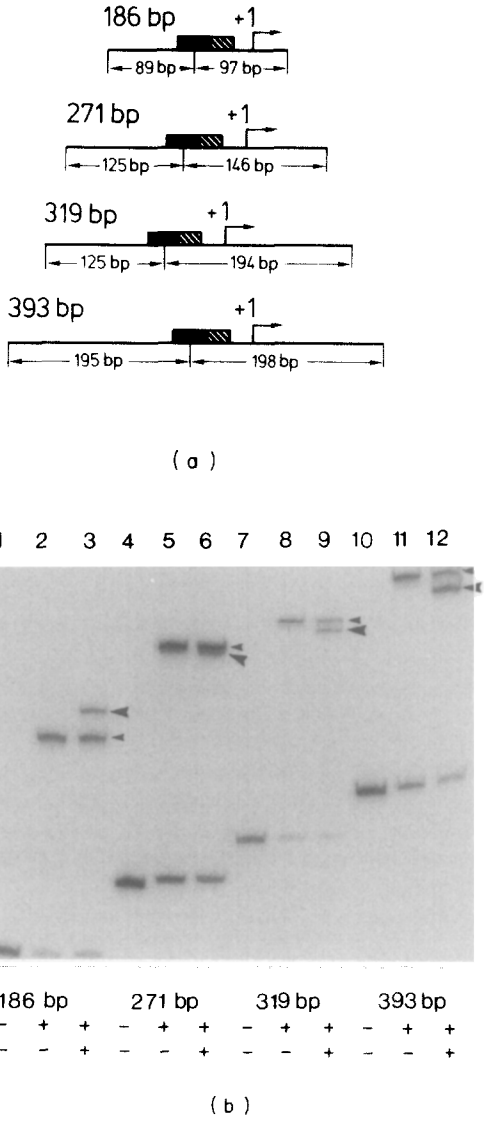

Figure 4. Gel retardation analysis of the binding of cAMP–CRP and CytR to *cytRP* containing fragments of 4 different sizes. (a) A schematic representation of the 4 fragments, the CRP site and the CytR binding site are indicated by a filled and a shaded box, respectively; the start site for transcription (+1) is indicated. The distances indicated are the distances from the ends of the fragments to the center of the CRP site. The 186 bp fragment, is an *Xba*I–*Hpa*II fragment isolated from p13-21; the 271 bp fragment, is an *Xba*I–*Bst*NI fragment isolated from p13-210; the 319 bp fragment, is an *Xba*I–*Pvu*II fragment isolated from p13-210; and, the 393 bp fragment is an *Ava*I–*Eco*RI fragment isolated from pVH002. (b) The fragments indicated below each lane were used in the samples loaded in the lanes. Where indicated, CRP and CytR were added to final concentrations of 1·0 ng/μl and 0·01 ng/μl, respectively. The larger arrow indicates the cAMP–CRP/DNA complex and the smaller arrow indicates the cAMP–CRP/CytR/DNA complex.

In an attempt to describe in more detail the changes in the DNA conformation induced by

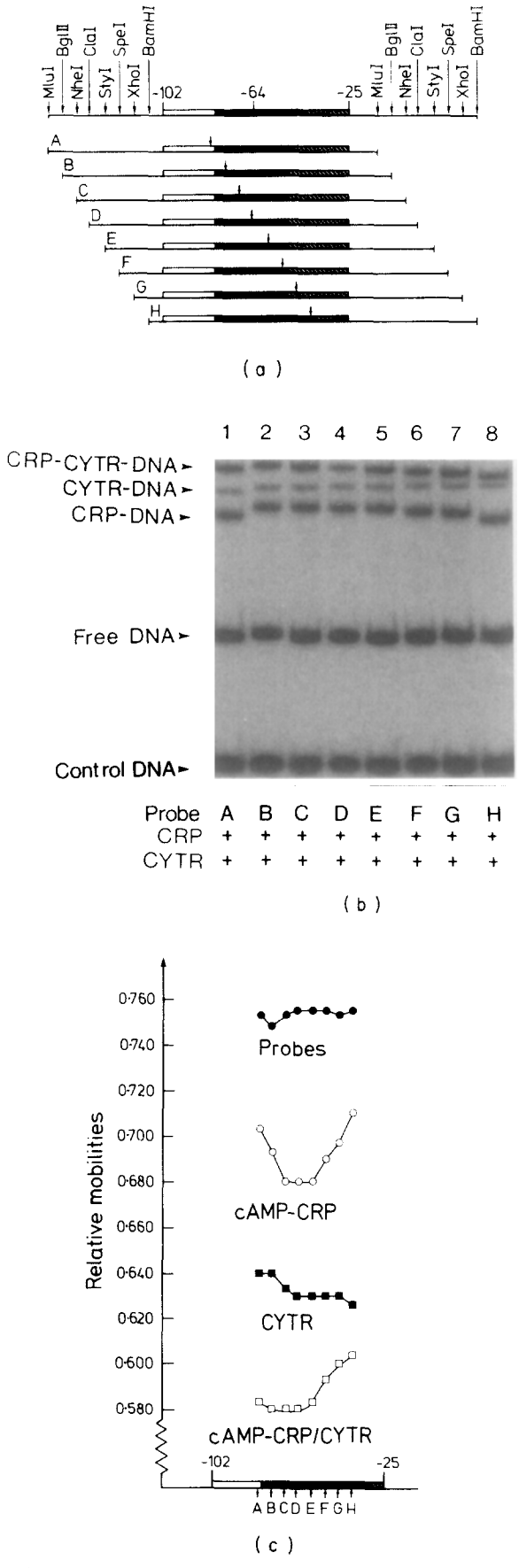

Figure 5. Circular permutation analysis of cAMP–CRP, CytR and cAMP–CRP/CytR induced DNA bends in *cytRP*. (a) The probes used in the circular permutation analysis were generated by restriction nuclease cleavage with 8 different restriction enzymes at sites located in the 2 tandem polylinker sequences flanking the cloned *cytRP* region. All probes are 146 bp in length. The cloned *cytRP* region extends from position −25 to −102. The CRP site and CytR binding site are indicated as a filled and a shaded box, respectively. The small arrow above each probe indicates the center of the probe. CytR binds with the same affinity to the permuted fragments as to the other *cytRP* fragments employed in this work (data not shown). (b) Gel retardation analysis of circularly permuted DNA fragments. CRP and CytR were incubated at final concentrations of 1·0 ng/μl and 0·3 ng/μl, respectively, with the probes shown below each lane. The probes were added to a final concentration of 5 nM. The composition of the different complexes is indicated. The control DNA is a 64 bp *Bam*HI–*Bam*HI fragment isolated from pBend1, which was included to ensure that the small differences in mobility of the permuted complexes was not due to irregularities in the gel. The mobilities of all the complexes are identical in the presence or absence of 10 mM-cytidine (data not shown); however, the combination of all the different retarded bands on 1 gel is most clearly visible in the presence of cytidine, therefore, only a gel in which cytidine was present is shown. (c) Relative mobilities of cAMP–CRP, CytR and cAMP–CRP/CytR complexes as well as the free probes are shown as a function of the position of the center of each probe. The mobility of the complexes was calculated from the bottom of the slots relative to the mobility of the control DNA and corrected for the variations in probe mobilities. The relative mobilities represent the average of 5 to 8 independent experiments, less than 0·5% variations were observed in the mobility of any given complex from gel to gel.

cAMP–CRP and CytR in *cytRP*, a circular permutation analysis was performed (Wu & Crothers, 1984). Eight permuted fragments, containing the *cytRP* sequence at different positions relative to the ends of the fragment were generated in the vector pBend1 (Kim *et al.*, 1989), by digestion with eight different restriction enzymes (Fig. 5(a)).

As shown in Figure 5(b) and (c), the mobility of the probes varies. The discrete, minimal mobility values are obtained when either of the two sequences (−77) 5′-AAAAA or (−37) 5′-AAAA (sequence for the upper strand) are positioned in the middle of the fragment. Consistently, such A-tracts have been shown to induce a DNA bend of up to 20° (Koo *et al.*, 1986; Levene *et al.*, 1986; Zahn & Blattner, 1987; Koo *et al.*, 1990). In addition, the permuted fragments also contain two runs of four adenine residues on the lower strand centered around positions −31 and −54, respectively. Neither of these two sequences is positioned in the middle of any of the fragments and they do not give rise to discrete minimum values in mobility. However, all the A-tracts are likely to influence the mobility of the fragments.

When any combination of cAMP–CRP and CytR are bound to the fragments, bends are induced in the DNA as shown by the position-dependent alterations in the mobilities of the protein *cytRP* complexes (Wu & Crothers, 1984) (Fig. 5(b) and (c)).

cAMP–CRP causes the largest variations in complex mobilities and, therefore, induces a greater DNA bend than CytR (Thompson & Landy, 1988). The curve presented in Figure 5(c) for the mobility of the cAMP–CRP complexes is nearly symmetrical and indicates that the bend center is located around position −64, i.e. coinciding with the center of the CRP site. On the other hand, the center of the CytR-induced DNA bend cannot be located accurately from the curve in Figure 5(c), although the data indicate that the center is positioned close to the center of permutation H (position −39 in *cytRP*) or downstream from that position. For the cAMP–CRP/CytR complexes, the shape of the mobility curve clearly deviates from that of the individual cAMP–CRP and CytR complexes, indicating that the conformational change in the DNA induced by the combined binding of both proteins is different from those induced by either protein independently. Moreover, the bend center in the combined complex is positioned close to the middle of the cAMP–CRP-binding site.

4. Discussion

Proteins that induce DNA bends are of great importance in the regulation of transcriptional initiation in both prokaryotes and eukaryotes (Gober & Shapiro, 1990; Hoover *et al.*, 1990; Nilsson *et al.*, 1990; Rojo *et al.*, 1990; Claverie-Martin & Magasanik, 1991; Kerppola & Curran, 1991; Lobell & Schleif, 1991; Perez-Martin & Espinosa, 1991) and, most likely, they facilitate the interactions between components of the transcriptional machinery. This makes the DNA-bending protein an obvious target for a gene regulatory mechanism in which the DNA bend is antagonized. Here we have described a regulatory system in which the CytR repressor, a cAMP–CRP antagonist, modulates a DNA bend induced by cAMP–CRP.

Regulation of expression of the *cytRP* promoter involves two DNA-binding proteins, cAMP–CRP and CytR (Gerlach *et al.*, 1990). cAMP–CRP binds to the sequence 5′-TTCAAN$_6$TCACA-3′ centered around position −64. The upstream half of this site deviates strongly from the consensus sequence for a CRP site whereas the downstream half is identical with consensus (de Crombrugghe *et al.*, 1984). This observation in combination with the unusual position of the binding site relative to the +1 position (Gaston *et al.*, 1990; Ushida *et al.*, 1990; Valentin-Hansen *et al.*, 1991) may explain the relatively modest activating effect of cAMP–CRP (5-fold; Gerlach *et al.*, 1990). The presence of only a single CRP site in *cytRP* sets this promoter apart from other CytR regulated promoters that all contain at least two CRP sites (Valentin-Hansen, 1982; Valentin-Hansen *et al.*, 1989; Gerlach *et al.*, 1991; Holst *et al.*, 1992).

CytR binds to a sequence located immediately downstream from, and partially overlapping, the CRP site in *cytRP* (Fig. 3). The sequence responsible for the sequence specific binding of CytR has not been determined. However, it has been speculated that the motif 5′-**TGCAA**ACT**TGTAA** is important for the specific binding of CytR in *deoP2* (Pedersen *et al.*, 1991). (The underlined part of this motif indicates an imperfect inverted repeat and the motif in bold is an imperfect direct repeat.) A degenerate form of this sequence is also present in the CytR-binding site in *cytRP* (Fig. 3). The exact organization of the two motifs is not identical as the spacing between the repeats is one base-pair larger in *deoP2*. Nevertheless, the occurrence of the motif in both promoters emphasizes its importance, although, the exact composition of the binding site and the way in which CytR recognizes the motif remain elusive.

cAMP–CRP and CytR bind simultaneously and co-operatively to *cytRP* covering a region of 57 to 59 bp that corresponds to the two individual protein-binding sites. The co-operativity is more pronounced in the gel retardation assay than in the DNase I footprint. The key to this difference, most likely, lies in the relatively modest degree of co-operativity in combination with the strong salt-concentration-dependent DNA binding exhibited by CytR, i.e. in the gel retardation assay CytR binds several fold stronger to the DNA than in the footprint assay. Therefore, CytR can stimulate binding of cAMP–CRP only minimally under the conditions of the footprint, whereas CytR is able to stimulate binding of cAMP–CRP under the conditions of the gel retardation assay. We are currently pursuing a more detailed analysis of the co-operative binding of cAMP–CRP and CytR to *cytRP*.

Two lines of evidence suggest that the co-operative DNA binding is achieved through direct interactions between cAMP–CRP and CytR. First, the inducer, cytidine, specifically perturbs co-operative DNA binding of cAMP–CRP and CytR without affecting independent DNA binding of CytR, and, as previously argued for *deoP2*, this observation points to the importance of direct interactions between the two proteins (Pedersen *et al.*, 1991). Second, CytR regulation of *cytRP* is interrupted in the presence of CRP mutants that contain amino acid substitutions in the domain interacting with CytR (Søgaard-Andersen *et al.*, 1991*a*).

The co-operativity between cAMP–CRP and CytR, determined by both gel retardation and footprinting assays, is much larger in *deoP2*, *tsxP2* and *cddP* (Gerlach *et al.*, 1991; Pedersen *et al.*, 1991; Holst *et al.*, 1992) than in *cytRP*. Two observations are important in this context: first of all, in *deoP2*, *tsxP2* and *cddP* formation of the cAMP–CRP/CytR/DNA complex involves binding of tandem cAMP–CRP complexes; second, the binding sites for cAMP–CRP and CytR in *cytRP* are partially overlapping and, thus, the proteins may not be optimally positioned to make protein–protein interactions and one protein may sterically interfere with binding of the second protein. These structural differences are paralleled by a functional difference as *deoP2* is regulated tenfold by CytR whereas

cytRP is only regulated threefold (Valentin-Hansen, 1985; Gerlach *et al.*, 1990).

(a) *CytR antagonizes the cAMP–CRP-induced DNA bend*

The circular permutation analysis clearly indicated that both cAMP–CRP as well as CytR induce DNA bends in *cytRP* and that the bend in the combined complex deviates from that induced by either protein alone. This observation, taken together with the anomalous migration of the cAMP–CRP/CytR/*cytRP* complexes in gel retardation assays strongly indicate that the overall DNA bend in the combined complex is smaller than the bend induced by cAMP–CRP independently. It should be mentioned that we cannot strictly rule out the possibility that the bend in the combined complex is larger than the bend induced by cAMP–CRP, however, several lines of evidence argue against this interpretation. First, as the mobility of a DNA fragment decreases when the bend angle is increased from 0° to 180° for a central bend (Liu Johnson *et al.*, 1986), CytR would have to bend the DNA more than 180° in order to explain the anomalous migration of the combined complexes. Second, the absence of regularly spaced DNase I hypersensitive sites in the CytR and combined footprints argues against the idea that CytR induces a large bend. Finally, the introduction of a bend of this size within 57 to 59 bp would be energetically very costly. In this context, Snyder *et al.* (1989) observed that DNA fragments containing IHF bound to two sites migrated faster than fragments containing IHF at one site if the IHF induced bends were in phase. In this case, however, the two IHF binding sites were separated by more than 50 bp contrasting the close relationship between the protein-binding sites in *cytRP*. The only other example we are aware of in which two neighboring proteins modulate a DNA bend, is the combined binding of cAMP–CRP and RNA polymerase at the *lac* promoter, which induces a "hyper"-bend during the formation of an open complex (Zinkel & Crothers, 1991). In this system, the interaction between the two proteins is productive and ultimately leads to initiation of transcription, whereas the cAMP–CRP/CytR system illustrates a regulatory mechanism that could apply to any regulatory system in which activity depends on a protein-induced DNA bend.

How does CytR antagonize the cAMP–CRP-induced DNA bend? The bends in the different complexes in *cytRP* are the *sum* of intrinsic DNA bends and protein-induced bends. Furthermore, the permutation analysis does not provide exact information about the path of the DNA helix in a protein–DNA complex, and, therefore, the mobility curve for the combined complex cannot be interpreted in terms of the topology of the DNA. Also, any model must take into account that cAMP–CRP and CytR bind on the same face of the helix, that cAMP–CRP and CytR interact directly (Søgaard-Andersen *et al.*, 1991*a*) and that cAMP–CRP in the combined complex still contacts the DNA in the region of the CRP site at which most of the bending has been shown to occur (Schutlz *et al.*, 1991). Based on these considerations, several not mutually exclusive models can be imagined: (1) as the protein-binding sites are overlapping, cAMP–CRP may have reduced DNA contacts in the combined complex and thereby bend the DNA less; (2) cAMP–CRP and CytR may both retain their DNA contacts, however, a sharp bend is introduced between the two binding sites; (3) cAMP–CRP and CytR retain their DNA contacts but CytR bends the DNA away from the protein; and, (4) cAMP–CRP and CytR retain their DNA contacts but the bends are out of phase. We are presently not able to distinguish between these possibilities, but clearly phasing analyses may provide more detailed information about the path of the DNA in the combined complex (Zinkel & Crothers, 1987).

From the analysis of cAMP–CRP/CytR/DNA complexes in different CytR regulated promoters it appears that each promoter has its own characteristics. In *deoP2*, CytR fits exactly between two cAMP–CRP complexes (Pedersen *et al.*, 1991); in *cddP*, one of the two cAMP–CRP complexes is repositioned by 2 bp upon binding of CytR (Holst *et al.*, 1992); in *tsxP2*, one of the two cAMP–CRP complexes is, most likely, repositioned by 20 bp (Gerlach *et al.*, 1991); and in *cytRP*, only one cAMP–CRP complex is involved in formation of the repression complex. This diversity in the co-operative binding of two heterologous proteins with antagonistic effects might be a way to provide each promoter with its own particular regulatory characteristics.

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