

Overproduction of the cyclic AMP receptor protein of *Escherichia coli* and expression of the engineered C-terminal DNA-binding domain

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Overproduction of the cyclic AMP receptor protein (CRP) from *Escherichia coli*, up to 25% of the soluble cell protein, has been achieved in an inducible host–vector system under transcriptional control of the λ promoter P_L . This system is ideally suited for large scale production and purification of CRP. In addition, a structural gene for the DNA-binding domain of CRP has been constructed. To this end the nucleotide sequence coding for the C-terminus was fused to the sequence coding for the first 10 N-terminal amino acids and cloned into suitable vectors. Good expression was achieved using the λP_L promoter. The gene product, β CRP, is recognized by anti-CRP antibodies.

INTRODUCTION

In order to understand regulatory processes in detail it is crucial to investigate and analyse the mechanisms by which sequence-specific DNA-binding proteins recognize and interact with their DNA target sites. For some of these proteins one important step forward has been the elucidation of their three-dimensional structure by X-ray crystallography: for example, the cyclic AMP receptor protein (CRP) from *E. coli* (McKay & Steitz, 1981); the *cro* repressor protein from bacteriophage λ (Anderson *et al.*, 1981) and the N-terminal fragment of the *cl* repressor (Pabo & Lewis, 1982). Structure and sequence comparisons have suggested that a common helix–turn–helix motif may play the most important role in sequence-specific recognition, and several models for this interaction have been put forward (Weber *et al.*, 1982; Sauer *et al.*, 1982; Anderson *et al.*, 1982; Steitz *et al.*, 1982). The model for the *cro* repressor was tested using mutant proteins engineered in a synthetic *cro* gene. DNA-binding properties of these mutants were determined, confirming the proposed contacts between *cro* repressor and operator DNA (Eisenbeis *et al.*, 1985). However, the ultimate goal of obtaining structural data on protein–DNA complexes has not been achieved in the above systems to date.

One prerequisite for any structural investigations of protein–DNA complexes is the availability of sufficient amounts of both components. DNA fragments containing specific sites for CRP have been synthesized in large quantity and their solution structures investigated by n.m.r. spectroscopy (Gronenborn *et al.*, 1984; Clore & Gronenborn, 1984*a,b*, 1985). In the present paper we describe the construction of plasmids encoding the *crp* structural gene either under the control of the *tac* promoter (Amann *et al.*, 1983) or the bacteriophage λ promoter P_L (Remaut *et al.*, 1981). These plasmids in the appropriate host strains allow after induction the overproduction of CRP up to levels of approximately

25% of the soluble protein. These constructs therefore provide an ideal source for large scale purification of CRP and of any mutant variants generated by oligonucleotide-directed mutagenesis (A. M. Gronenborn & G. M. Clore, unpublished work). In addition, we report on the design, construction and cloning of the C-terminal part of the *crp* gene coding for the specific DNA binding domain and on the expression of the encoded protein, β CRP.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli DH1 was used for the construction of recombinant plasmids derived from pBR322. *E. coli* CA 7914-3 (*crp*[−], *cya*[−] [*lac*, *pro*], *rpsL*, F'*lacI*^{QZ}+Y⁺pro⁺) was transformed with plasmids containing the *tac* promoter (Amann *et al.*, 1983), and *E. coli* K-12 Δ H1 Δ trp [*rpsL*, *lacZam*, Δ *bio-uvrB*, Δ *trpEA2* (λ Nam7, Nam53, cI857, Δ H1)] (Bernard *et al.*, 1979) was transformed with plasmids derived from pPLc28. XEG·Z (proC::Tn5 Δ gal-165 *thi* Δ *crp*-39 *str* A) was transformed with a variety of *crp* and β *crp* gene containing plasmids to test for *lac* expression.

Molecular cloning

Preparation of plasmid DNA, restriction endonuclease cleavage, isolation of fragments, ligation, and transformation were carried out as described by Maniatis *et al.* (1982).

Antibodies

Anti-CRP antibodies were raised in adult female rabbits by standard immunization procedures: 500 μ g of purified protein in complete Freund's adjuvant was injected intramuscularly at 10 day intervals into the legs and the back of the animals and antiserum was subsequently prepared from weekly bleeds.

Abbreviations used: CRP, cyclic AMP receptor protein of *E. coli* (also known as catabolite activator protein or CAP); β CRP, the genetically engineered protein comprising the C-terminal DNA-binding domain of CRP; *crp* and β *crp*, the structural genes of CRP and β CRP, respectively; IPTG, isopropyl- β -D-thiogalactoside; xgal, 5-bromo-4-chloro-3-indolyl- β -D-galactoside; bp, base pairs; kb, kilobase (pairs).

Protein gels and immune blots

SDS/polyacrylamide gels for protein separation were prepared and used according to Laemmli (1970). Electrophoretic transfer from the protein gel on to nitrocellulose was carried out for 1 h at 12 V/cm in 24.8 mM-Tris/0.19 M-glycine containing 20% (v/v) methanol at 0 °C. Immunodetection was carried out as follows. The nitrocellulose filter, carrying either the dots of cell lysate or the blotted gel, was washed thoroughly in 10 mM-Tris/0.9% NaCl/0.05% Tween 20 (polyoxyethylenesorbitan monolaurate) and overlaid with the anti-CRP serum at a 1:20 or 1:50 dilution overnight in a humid chamber. After extensive washing the filters were subsequently overlaid for 3–4 h with ^{125}I -labelled goat anti-rabbit antibodies (total globulin fraction) at approx. 5×10^5 c.p.m./ml in 10 mM-Tris/0.9% NaCl/0.05% Tween 20/4% bovine serum albumin/0.2% NaN_3 . These labelled goat anti-rabbit antibodies had been cleaned up by preabsorption onto nitrocellulose filters previously treated with rabbit serum. This treatment removes any non-specific interaction of the labelled goat anti-rabbit antibodies with the anti-CRP serum. Since there is a large excess of goat antibodies over bound rabbit serum, the goat antibodies can be used for

several further experiments. The filters were washed again thoroughly in the wash buffer, air dried and autoradiographed.

DNA sequence analysis

The DNA sequence of the *βcrp* construct was determined by the method of Sanger *et al.* (1977) using α - ^{35}S -labelled ATP after shuttling the insert from pUC9 to M13mp9.

CRP assay

The amount of CRP present in crude cell extracts was assayed using the cyclic AMP binding assay as described by Pastan *et al.* (1984). Cell extracts were prepared as follows. Cultures (10 ml) grown to an A_{600} of 1 were centrifuged, the cells washed with 5 ml of 0.1 M-Tris/HCl, pH 8, centrifuged and resuspended in 1 ml of 0.1 M-Tris/HCl, pH 8, containing 10 mM-EDTA. Lysosyme (50 μl of 4 mg/ml) was then added and the mixture left on ice for 10 min. After this lysis step the mixture was centrifuged and the resulting supernatant used for the cyclic AMP binding assay.

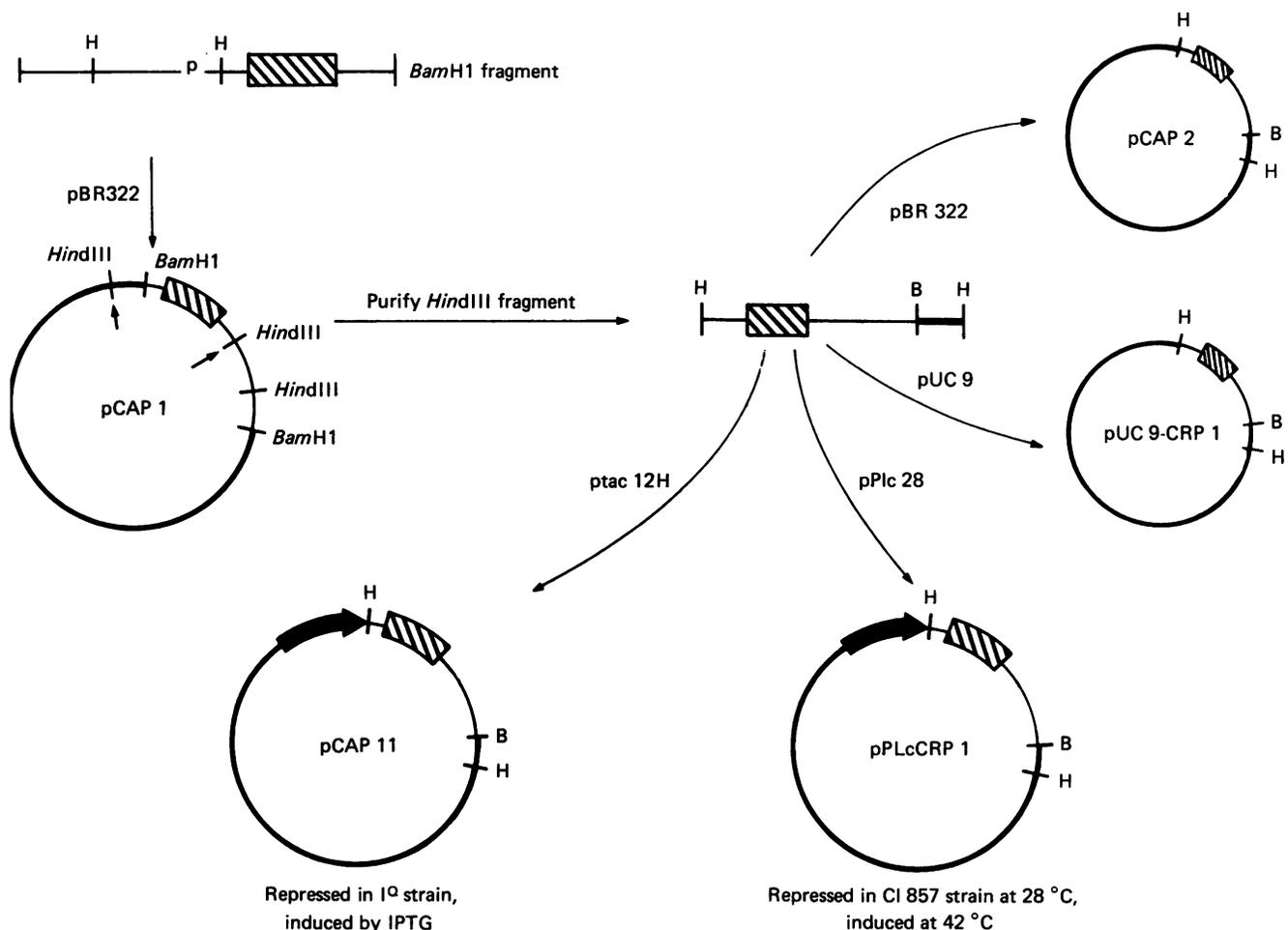


Fig. 1. Construction of expression vectors containing the *crp* gene

Its own promoter (p) is either replaced by the *tac* promoter (pCAP11), the leftward λ promoter P_L (pPLcCRP1) or the *lac* promoter (pUC9-CRP1). B denotes a *Bam*H1 site and H a *Hind*III site. The length of the *Hind*III/*Hind*III fragment containing the *crp* gene is 2.4 kb.

RESULTS

Overproduction of CRP

Fig. 1 outlines the organization of the *crp* gene within the plasmids pCAP1 and pCAP2 which are both derivatives of pBScrp2 (Cossart & Gicquel-Sanzey, 1982), as well as in those plasmids used for the overproduction of CRP. pCAP1 contains the same *Bam*H1 fragment encompassing the *crp* gene which was originally inserted into pBScrp2 but now inserted in opposite orientation. Since it has been shown that CRP can act in an autoregulatory fashion by binding to a site near its promoter (Aiba, 1983; Cossart & Gicquel-Sanzey, 1983), we decided to eliminate the *crp* promoter from all our constructs. This is easily achieved, since the promoter is located about 170 bp upstream of the ATG start of the coding sequence and the CRP binding site lies directly downstream of the RNA polymerase site (Aiba, 1983). Thus one can separate these transcriptional control elements from the coding region using appropriate restriction fragments. A *Hind*III site fortuitously lies between the promoter region and the ribosome binding

site. We therefore used the *Hind*III insert from pCAP2 for insertion into both expression vectors.

The first construction uses the plasmid ptacl2H which contains the *tac* promoter, a fusion promoter derived from the -35 *trp* promoter region and the -10 *lac* UV5 promoter region (Amann *et al.*, 1983) followed by a *Hind*III cloning site. The *Hind*III fragment cut from pCAP2 was inserted into this cloning site and recombinant plasmids containing the gene in both orientations were found after transformation in an *E. coli* strain overproducing the lac repressor (see Fig. 1). CRP production was monitored by a cyclic AMP binding assay (Pastan *et al.*, 1974). Upon induction with IPTG, a 15-fold increase in CRP activity with respect to a wild type strain was found for the strain carrying the plasmid pCAP11 which contains the *crp* gene under transcriptional control of the *tac* promoter.

The second construction uses the plasmid pPLc28 which contains the leftward λ promoter P_L and allows expression to be controlled by the temperature sensitive cI 857 repressor (Remaut *et al.*, 1981). Here too the *Hind*III fragment containing the *crp* gene without its own

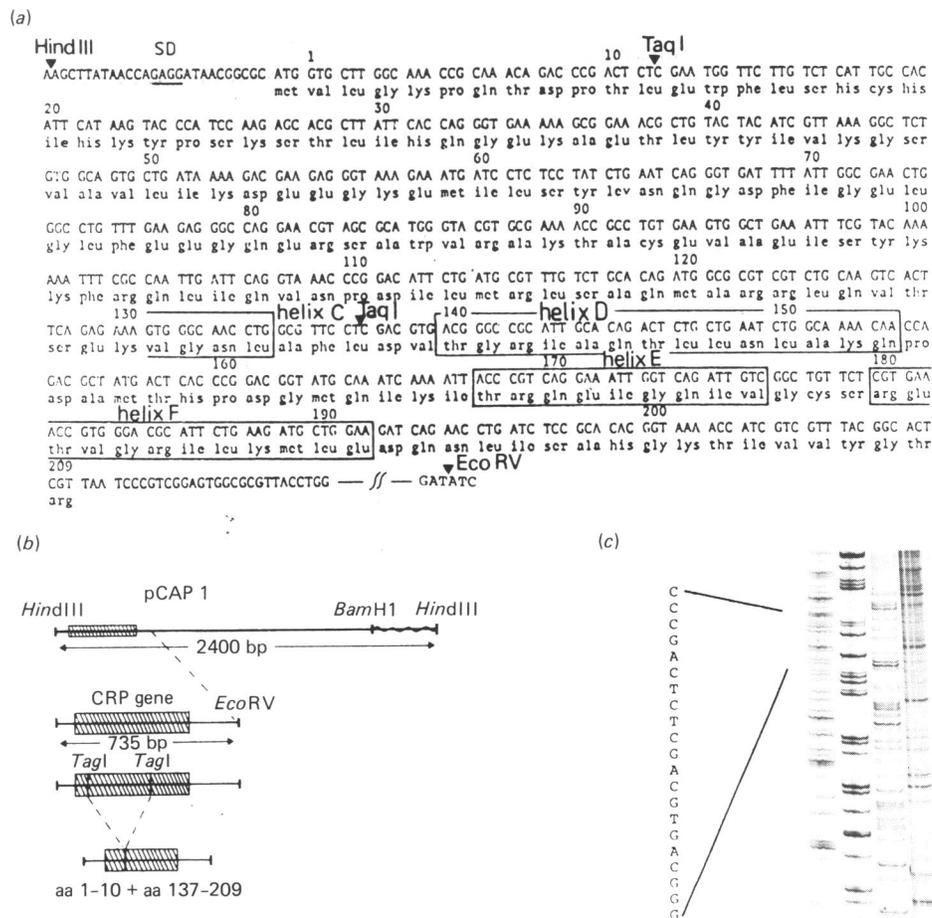


Fig. 2. (a) Nucleotide sequence of the *crp* structural gene (Aiba *et al.*, 1982; Cossart & Gizquel-Sanzey, 1982), (b) strategy for the construction of a gene coding for the C-terminal DNA-binding domain of CRP, and (c) sequencing gel comprising the sequence of the β CRP gene

In (a) structural elements are indicated (helices C to F) as well as restriction endonuclease cleavage sites. SD, Shine-Dalgano (ribosome binding) sites. In (c) the region across the *Taq*I joint is indicated.

promoter was inserted into the *Hind*III cloning site and, after transformation of *E. coli* K-12 Δ H1 Δ trp, recombinant plasmids with the gene in both orientations were found. The plasmid pPLcCRP1 (Fig. 1), which contains the *crp* gene under λ P_L control, can be induced by a temperature shift from 29 °C to 42 °C in mid-exponential phase, thereby switching CRP production on up to levels approx. 100 times that of a strain harbouring the same plasmid in a non-induced state.

Construction of synthetic gene for the C-terminal DNA-binding domain of CRP

Fig. 2 outlines the scheme used for the construction of a synthetic gene coding for the C-terminal DNA binding domain of CRP. The idea for the construction of such a gene was born from the requirement of a small size protein in order to determine the structure of a protein-DNA complex by n.m.r. methods. As far as can be judged from the X-ray structure (McKay *et al.*, 1982) the C-terminal domain is folded independently from the rest of the protein being connected to the N-terminal domain via a hinge region. This hinge region is formed by amino acids 135-138. The nucleotide sequence for Leu-137 and Asp-138 reads CTCGAC (Cossart & Gicquel-Sanzey, 1982; Aiba *et al.*, 1982). Thus it contains a cutting site for *Taq*I, which should therefore allow the separation of the DNA sequence coding for the C-terminal domain from the rest of the coding sequence (Fig. 2a). We decided to construct a fusion gene by joining this C-terminal part of the sequence to the very first stretch

of the coding sequence. This approach was chosen since there is a second *Taq*I site in the *crp* coding sequence, namely at the position coding for amino acids Leu-11 and Glu-12 (Fig. 2b). Both cutting sites occur at equivalent positions in the reading frame. Thus, upon ligation the reading frame should be left intact in such a manner that the nucleotide sequence now encodes a protein which consists of the first 10 amino acids of CRP followed by the C-terminal domain with Leu-11 being replaced by Leu-137.

The construction was carried out in the following way. The 738 bp *Hind*III/*Eco*RV fragment was prepared by digestion of pCAP1 or pCAP2 DNA with *Hind*III and *Eco*RV and purified by preparative agarose gel electrophoresis. This purified fragment DNA was subsequently cut with *Taq*I, yielding three fragments: a 60 bp *Hind*III/*Taq*I fragment, a 378 bp *Taq*I/*Taq*I fragment and a 300 bp *Taq*I/*Eco*RV fragment. The 60 bp fragment and the 300 bp fragment were again purified by preparative agarose gel electrophoresis. These were subsequently joined by ligation and inserted into *Hind*III and *Eco*RV digested pBR322. Transformants were analysed in order to detect the desired insert by small scale plasmid DNA preparation and digestion of this DNA with *Hind*III/*Eco*RV and *Taq*I. The latter can be used for the unambiguous identification of the arrangement within the insert. The correct plasmid was designated p β CRP and used for subsequent work (see Fig. 3). In addition to the analysis of the insert by restriction mapping, it was shuttled into M13mp9 and the nucleotide

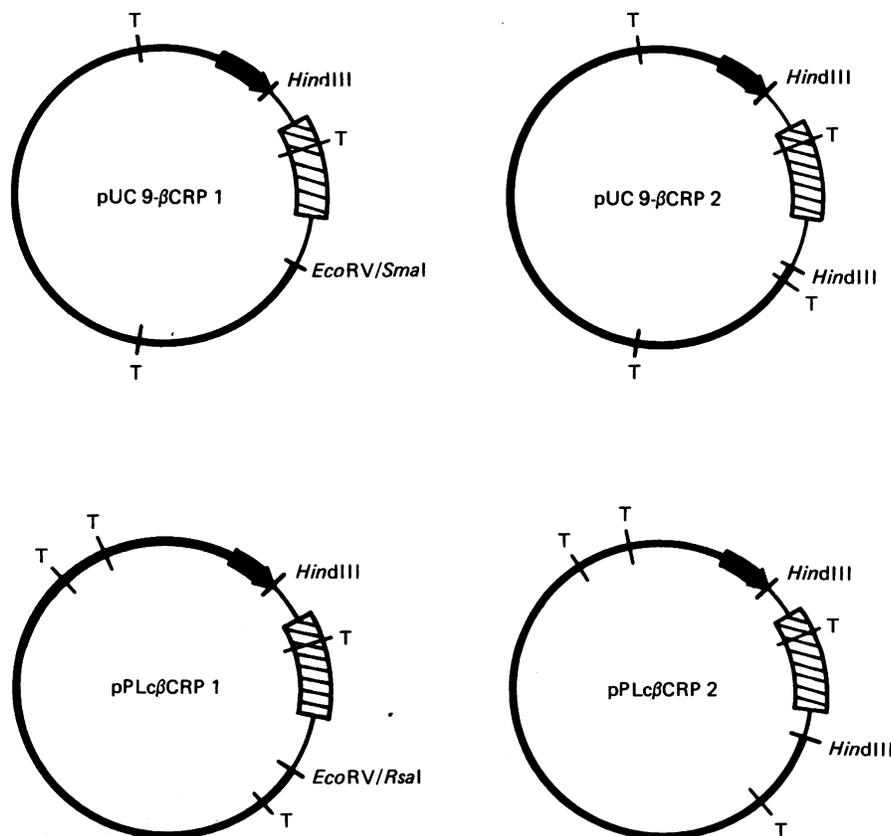


Fig. 3. Plasmids containing the β *crp* gene

The length of the *Hind*III/*Eco*RV insert is 360 bp. T denotes *Taq*I sites.

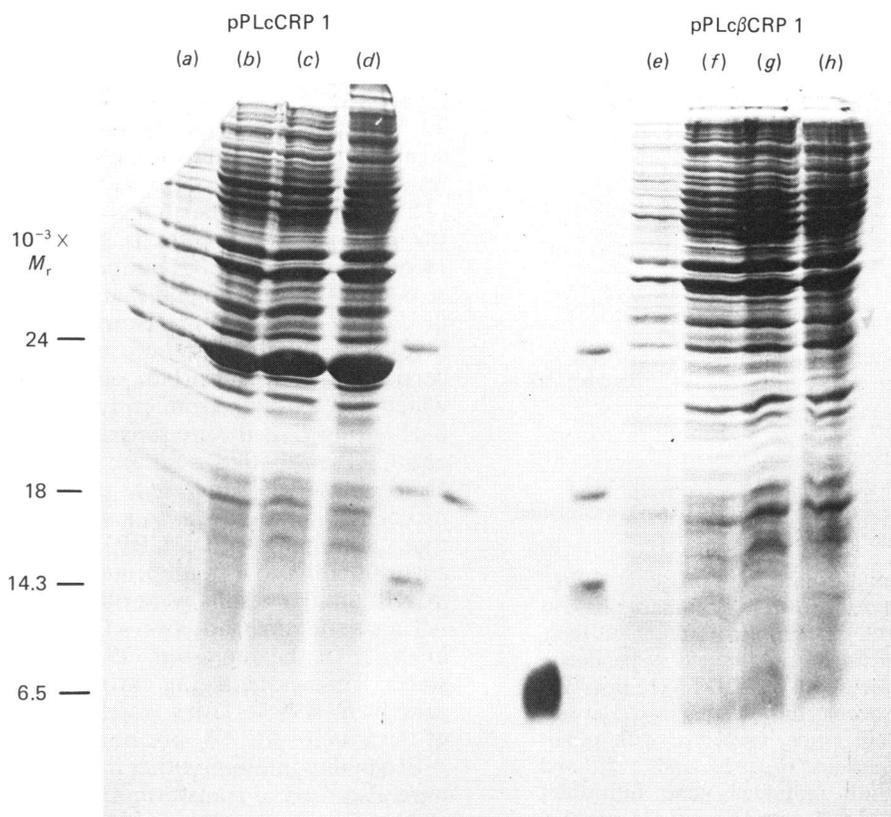


Fig. 4. SDS/polyacrylamide-gel (15%) of cell extracts of *E. coli* $\Delta H1\Delta trp$ transformed either with pPLcCRP1 (lanes *a-d*) or pPLc β CRP1 (lanes *e-h*) at different time points

(*a*) and (*e*), time of induction (i.e. temperature shift from 29 °C to 42 °C approx. mid-exponential phase; (*b*) and (*f*) 42 °C, 2.5 h after induction; (*c*) and (*g*), 42 °C, 5.5 h after induction; (*d*) and (*h*), 42 °C, 17.5 h after induction. A 250 μ l sample of each culture was taken and centrifuged. The supernatant was then removed and the pellet was boiled in Laemmli buffer and loaded directly onto the gel. The middle lanes contain protein standards: trypsinogen (M_r 25000), β -lactoglobulin (M_r 18400), lysozyme (M_r 14300) and bovine pancreatic trypsin inhibitor (M_r 6500).

sequence determined by dideoxy-sequencing (Sanger *et al.*, 1977). Fig. 2(c) shows part of the sequencing gel comprising the *TaqI* junction at the triplets coding for Thr¹⁰-Leu¹¹-Asp¹³⁷, demonstrating the correct sequence.

Expression of β CRP

In order to determine if the corresponding protein β CRP was produced in a strain harbouring the βcrp gene, we transferred the 360 bp *HindIII/EcoRV* insert from p β CRP to pPLc28 in the same way as outlined previously for the other plasmids. Since pPLc28 does not contain an *EcoRV* site and *EcoRV* leaves blunt ends, we used the *RsaI* site in pPLc28 which also leaves a flush end. Ligation of the 360 bp *HindIII/EcoRV* fragment into *HindIII/RsaI* cut pPLc28 followed by transformation into *E. coli* K-12 $\Delta H1\Delta trp$ led to a large number of transformants, most of which contained the envisaged insert, as judged by restriction enzyme mapping. One of these was called pPLc β CRP1 and was used later to examine protein synthesis (Fig. 3). Subcloning the 360 bp *HindIII/EcoRV* fragment into *HindIII/SmaI*-digested pUC9 (Viera & Messing, 1982) resulted in two different clones containing the insert in the correct orientation (i.e. behind the *lac* promoter), namely pUC9- β CRP1 in which the *EcoRV* site is fused to the *RsaI* site, and pUC9- β CRP2 which contains the insert flanked by

HindIII sites (Fig. 3). This *HindIII* insert, excised from pUC9- β CRP2, was shuttled across to *HindIII*-cut pPLc28, yielding recombinant plasmids with the insert in either orientation. The plasmid with the correct orientation (i.e. containing the *crp* gene behind the λP_L promoter), known as pPLc β CRP2, was also used in the expression experiments.

Fig. 4 shows a polyacrylamide gel with cell extracts from *E. coli* $\Delta H1\Delta trp$ harbouring pPLc β CRP1 and pPLcCRP1 taken at different time points before and after induction. All tracks containing pPLcCRP1 extracts taken after the temperature shift from 29 °C to 42 °C (*b*, *c* and *d*) show as their most prominent band that of CRP with an M_r of 22500, thus demonstrating the large overproduction of CRP after induction. For those lanes containing pPLc β CRP1 extracts (*f*, *g* and *h*), such a clear cut demonstration of an overproduced protein is not observed. Closer inspection, however, shows a somewhat diffuse band at the very bottom of the tracks which is not present in the lanes containing pPLcCRP1. In addition, examination of cell extracts of *E. coli* $\Delta H1\Delta trp$ containing pPLc β CRP1 and pPLc β CRP2 on urea gels revealed the presence of a protein band corresponding to an M_r of approx. 9000 after induction.

In order to address the question whether the βcrp gene is indeed expressed and a protein of the correct size can

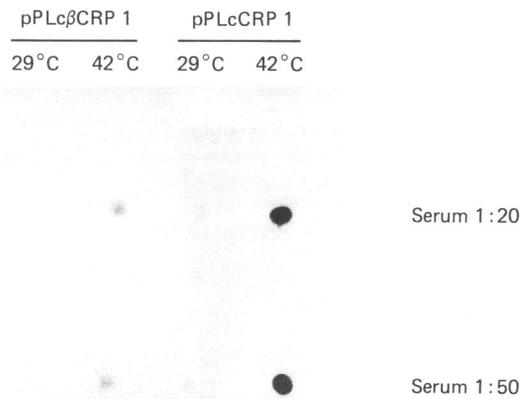


Fig. 5. Dot-blot of cell extracts of *E. coli* $\Delta H1\Delta trp$ transformed with pPLcCRP1 and pPLc β CRP1

Cells were grown to mid-exponential phase at 29 °C, divided, and one culture was grown to stationary phase at 29 °C, the second at 42 °C. Samples (500 μ l) of these cultures were taken, centrifuged and the cells suspended in lysis mix (50 mM-glucose/10 mM-EDTA/25 mM-Tris, pH 8.0, and 5 mg of lysozyme/ml). Of this mix, 5 μ l was dotted onto nitrocellulose strips, overlaid with rabbit anti-CRP serum at dilutions of 1:20 and 1:50 and subsequently probed with iodinated goat anti-rabbit antibodies. Radioactivity was detected by autoradiography at -70 °C overnight.

be found, we used antibodies directed against CRP to probe for such a β CRP protein. The underlying basis for this experiment is that a variety of different antibodies recognizing different parts of the protein will be present in a polyclonal serum. Thus, if the isolated C-terminal domain of CRP is folded in the same way as it is in the intact protein, some of the antibodies in the serum should

be able to recognize the new β CRP protein. Fig. 5 shows the result of a dot-blot assay for two *E. coli* strains harbouring the *crp* gene and the β *crp* gene, respectively, on a plasmid. Lysed cells taken from cultures grown at 29 °C (i.e. in a repressed state) show only a very weak signal for both plasmids when challenged with anti-CRP antibodies, whereas cells from the induced cultures (42 °C) show a distinct signal. Thus, upon induction of the β *crp* gene, a protein is synthesized which can be recognized by anti-CRP antibodies.

In order to determine the size of the protein expressed in *E. coli* $\Delta H1\Delta trp$ harbouring pPLc β CRP1 and to investigate the specificity of the dot-blot experiment, we carried out a Western blot of a polyacrylamide gel on which cell extracts from pPLc β CRP1 and pPLcCRP1 harbouring *E. coli* were separated (Fig. 6a). Here again anti-CRP antibodies were used for detection. As can be clearly observed (Fig. 6b), the strain containing the pPLcCRP1 plasmid shows a strong CRP band whereas that containing pPLc β CRP1 shows a diffuse band corresponding to a small protein of the size expected for β CRP. Similar results were obtained with pPLc β CRP2. All lanes contain in addition a CRP band exhibiting equal intensity in the repressed (29 °C) and induced (42 °C) states. This is due to the chromosomal copy of the *crp* gene in *E. coli* $\Delta H1\Delta trp$ which is expressed irrespective of the state of the λP_L promoter.

Plasmids containing either the *crp* gene or the β *crp* gene were also used to transform an *E. coli* host in which the chromosomal *crp* gene is deleted, XEG-Z. Western blot analysis and immunodetection again demonstrated the expression of β CRP for the pPLc β CRP plasmids, but now as the sole band on the autoradiograms. However, somewhat lower levels of expression were seen, as no control of transcription is possible. In order to investigate any possible function of β CRP *in vivo*, a large variety of β *crp* gene containing plasmids were tested on *lac* indicator plates. In contrast to all the *crp* gene containing plasmids, no shift from the wild type Lac⁻ phenotype to a Lac⁺ phenotype was found on either EMB or xgal/IPTG plates for plasmids containing the β *crp* gene.

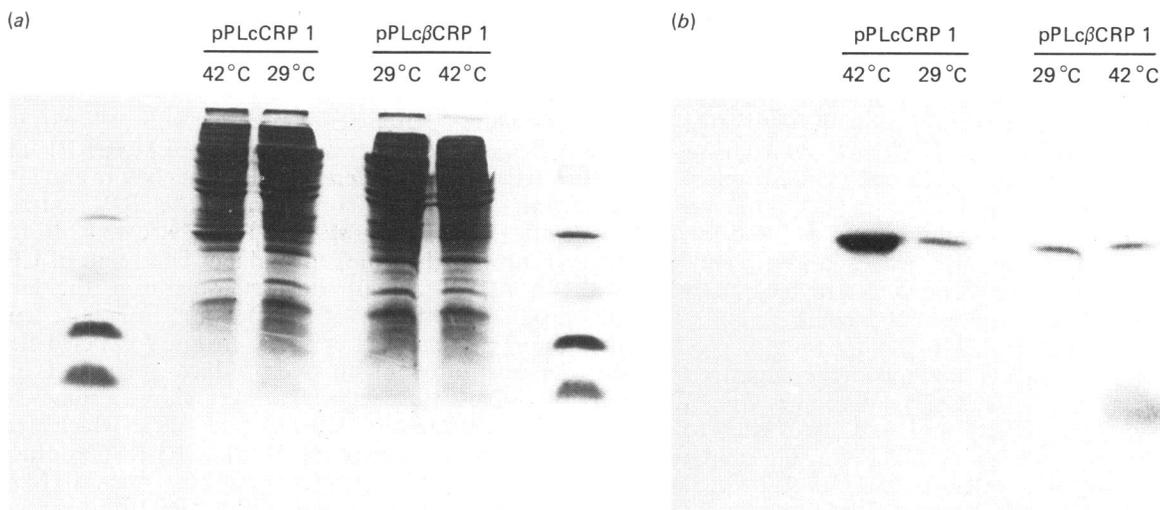


Fig. 6. Western blot of cell extracts of *E. coli* $\Delta H1\Delta trp$ transformed with pPLcCRP1 and pPLc β CRP1

(a) 15% SDS/polyacrylamide gel, stained after blotting. (b) Autoradiograph of the blot after incubation with rabbit anti-CRP serum (1:20 dilution) and detection with iodinated goat anti-rabbit antibodies.

DISCUSSION

Overproduction of DNA binding proteins is a prerequisite for any biophysical studies on the molecular mechanisms of protein-DNA recognition. Using the λP_L promoter in combination with an inducible host system (Remaut *et al.*, 1981) has allowed CRP production up to levels of approx. 100 times that of an *E. coli* wild type strain. Using the expression vector pPLcCRP1 it is very easy to produce and purify CRP in large quantities. The λP_L promoter system seems a somewhat better system than the *tac* promoter system in this case for two reasons. First, repression of the *tac* promoter in the absence of IPTG is not complete, at least not for our host strain which carries the *I^q* gene on the F'. Second, and most importantly, the level of CRP production achieved with the λP_L promoter is a factor of 10 higher than that with the *tac* promoter. Furthermore, heat induction is cheaper and easier than IPTG addition. One point worth mentioning is the fact that these extremely high levels of CRP do not interfere with cell growth and viability. Growth curves for the induced pPLcCRP1 harbouring strain are almost indistinguishable from those of a strain harbouring only pPLc28 and cell growth continues to an A_{660} value of around 4 in both cases.

Protein design by genetic engineering seems to be the method of choice to study structure-activity relationships. Using oligonucleotide-directed mutagenesis a variety of systems are presently under investigation, such as the enzymes tyrosyl-tRNA synthetase (Winter *et al.*, 1982; Way *et al.*, 1983), dihydrofolate reductase (Villafranca *et al.*, 1983) and triosephosphate isomerase (Petsko *et al.*, 1984). DNA-binding proteins such as the *cro* repressor have also been investigated (Eisenbeis *et al.*, 1985). We have constructed at the DNA level a novel protein which contains the DNA binding domain of CRP. This protein, β CRP, however, cannot fulfill the role of CRP *in vivo*, namely transcriptional enhancement of the *lac* gene as judged on *lac* indicator plates. This may be due to several reasons. First, β CRP might not interact with the specific CRP site on the DNA in the same manner as the CRP-cyclic AMP complex does. Second, since β CRP is a monomer, it is likely that its DNA-binding constant is much lower than that of the dimeric CRP so that a much higher protein concentration may be required to achieve the transcriptional effect. Or third, it might not be a DNA-binding protein at all. We think the latter highly unlikely, since all the structural features commonly found in DNA-binding repressors are preserved. Experiments are now necessary to test the DNA-binding properties of β CRP and to attempt its purification for structural studies. This should open the way to study contact points between the DNA-binding helix in β CRP and DNA fragments by n.m.r. spectroscopy.

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