2.23 CRP purification

CRP was purified using the Ghosaini method.

A 5 ml overnight culture was inoculated with M182Δ*crp* cells harbouring the pDCRP plasmid.

In preparation for the next day, 1.2 ml of ddH_2O was used to rehydrate 60 mg cAMP-agarose, which was mixed for 5 minutes and overnight at room temperature.

The following day, a Bio-rad poly prep column was prepared. This column has an internal reservoir, but is attached to an external reservoir, which is used for pre-loading washes.

The column, and rehydrated cAMP-agarose from the previous day, was moved to a 4 °C cold room, and was half-filled with ddH_2O to equilibrate. cAMP-agarose was then gradually added to the column, and was allowed to settle for two hours (final column volume was 0.8 ml). Two washes were then carried out at high flow rate; first, 10 ml column wash buffer + 1 M NaCl, and then 5 ml dialysis buffer. Zero point two ml of dialysis buffer was kept in the column, on top of the cAMP-agarose; which was left overnight. One ml of the overnight culture was used to inoculate 600 ml of superbroth, supplemented with 100 µg/ml ampicillin, which was incubated with shaking overnight at 37 °C.

The following day, the OD_{650} of this culture was measured using a ten-fold dilution (in superbroth medium) in a Jenway 6300 spectrophotometer. A volume of this (calculated by using the formula $V\ (\mu l) = 2000/OD_{650}$), was saved at -20 °C for the final SDS-PAGE analysis. A 1.5 ml aliquot of the culture was also saved for plasmid extraction.

The remainder of the 600 ml culture was harvested by first aliquoting the culture into two centrifuge pots, followed by centrifugation of at 1600 xg for 15 minutes at 4 °C. One of the resulting pellets was stored at -20 °C, the other was used for the purification. The culture pellet from one pot was resuspended in 6 ml lysis buffer (containing 50 µg/ml PMSF (phenylmethylsulphonyl fluoride in isopropanol) and sonicated. Complete lysis results in a ten-fold drop in OD_{650}, so the success of the sonication was assessed using a 1/500 dilution before,

and a 1/100 dilution after of the culture. The lysate was then transferred to an Oak Ridge tube for centrifuging at 10,000 xg for 30 mins at 4 °C. Two µl of the resulting supernatant was retained for SDS analysis (known as sample '2'), and the remainder was used for the remainder of the purifcation.

The culture supernatant was moved to the cold-room, where the cAMP-agarose column was equilibrated using 5 ml dialysis buffer on a high flow rate. Once the buffer volume in the external resevoir had depleted to 0.2 ml, the supernatant was loaded into internal reservoir (at a flow rate of 5 ml/hr). This wash was kept for SDS-PAGE analysis (known as sample '3'). The first 1 ml of lysate wash from the column, and the remainder of the eluate was also kept for SDS-PAGE analysis (known as sample '4'). When the column buffer volume had receded to 0.2 ml 5 ml dialysis buffer was added to the internal reservoir, with a flow rate of 5 ml/hr, an the wash was kept as SDS-PAGE sample '5'. When 0.2 ml buffer remained in the column, 1 ml column wash buffer containing 5 mM 5'+3' AMP was added. Once the buffer volume had receded to 0.2 ml, another 3 ml was added column wash buffer (containing 5 mM 5'+3' AMP) was added. The wash was kept as sample '6' for SDS-PAGE analysis. When the column buffer volume reduced to 0.2 ml, 1 ml column wash buffer was added. Once the column volume receded to 0.2 ml, another 3 ml of column wash buffer was added. The wash was saved as SDS-PAGE sample'7'.

Once the column buffer volume had receded to 0.2 ml, CRP bound to the column was eluted using 5 ml column wash buffer with 5 mM cAMP. Every 18 minutes, eluate fractions were collected in 0.5 ml aliquots using a slower 2 ml/h flow rate. In total, 8 fractions were collected and used in the SDS-PAGE analysis.

All 8 fractions and the other samples collected were analysed on 4-12 % (*w/v*) SDS-PAGE gels (supplied by Invitrogen). The final volumes of each sample were loaded after boiling for 5 minutes; 5 µl of sample 1 was loaded, 10 µl aliquots of samples 2-7 were mixed with 290 µl

SDS-PAGE sample buffer, 10 µl was then loaded. Five µl SDS-7 standards and 5 µl 0.2 µg/µl wild type CRP standard in sample buffer were run alongside the samples. Fractions were then combined according to the SDS-PAGE gels and were placed in dialysis tubing. The tubing was placed in 500 ml CRP stock buffer at 4 °C and left overnight. The buffer was replenished the next day and left for a 3 hours. CRP preparations stored in microfuge tubes at -20 °C.

CRP concentration was determined by Biorad assays. BSA standard curves were prepared using concentrations ranging from 0 to 10 mg/ml. CRP dilutions were made in 800 µl aliquot (up to 200-fold) using water, and 200 µl Biorad reagent was added to each. After incubated at room temperature for 5 minutes, OD_{595} measurements were taken. CRP concentration (g/L) was calculated using the BSA standard curve, and the Molar concentration was calculated (the molecular weight of CRP is 23.6 kDa).

CRP concentrations were compared by eye alongside BSA standards on 4-12 % (*w/v*) SDS-PAGE gel (supplied by Invitrogen). Samples were loaded after being boiled for 2 minutes. Samples loaded were: 0.5 µg, 1.0 µg, and 2.0 µg of CRP preparation in sample buffer (note that these amounts were guided by the Biorad analysis). Ten µl of previously purified CRP sample was also loaded. In addition, 5 µl of SDS-7 standards were loaded.

2.24 *In vitro* multi-round transcription assays

Supercoiled pSR plasmid DNA (containing the desired promoter DNA fragment) was purified using a Qiagen Maxiprep kit, and used as a template for subsequent *in vitro* transcription reactions as described by Kolb *et al.* (1995). Reactions were done in buffer containing 20 mM Tris pH 7.9, 5 mM $MgCl_2$, 500 µM DTT, 50 mM KCl, 100 µg ml^{-1} BSA, 200 µM ATP/GTP/CTP, and 10 M UTP and 5 µCi α-P^{32}- UTP, using 16 µg ml^{-1} template. If required DNA was incubated with purified CRP and 0.2 mM cAMP for 5 minutes (37 °C), before 400 nM RNAP holoenzyme containing σ^{70} (Cambio) was added. The reaction was run for 10 minutes at 37 °C, and stopped with 20 µl of 'STOP' solution. Four µl of each reaction was

Agarose gel electrophoresis:

- **6x Loading dye**: 10 mM Tris pH 7.5, 1 mM EDTA, 20 % (*v/v*) glycerol, 0.025 % (*v/v*) bromophenol blue, 0.025 % (*v/v*) xylene cyanol FF.
- **5x TBE**: 0.445 M Tris borate pH 8.3, 10 mM Na_2EDTA. Supplied by Fisher Scientific. The 5x solution is diluted to a 1x stock using ddH_2O before use.
- **Powdered agarose**: supplied by Bioline.

β-galactosidase assays:

- **13 mM (8 mg/ml) 2-Nitrophenyl β-D-galactopyranoside (ONPG)**: made up to 1 L in 'Z-buffer'.
- **'Z-buffer':** 8.53 g Na_2HPO_4, 4.87 g $NaH_2PO_4.2H_2O$ 0.75 g KCl, 0.25 g $MgSO_4$ per L of ddH_2O, then autoclaved.
- **β-mercaptoethanol**: immediately before use, 271 µl β-mercaptoethanol was added per 100 ml of 'Z-buffer'.
- **1 % (*w/v*) Sodium deoxycholate.**
- **100 % (*v/v*) Toluene.**
- **1 M Sodium carbonate.**

CRP purification:

- **1 M Potassium phosphate (pH 7.5)**: 162.75 ml 1 M K_2HPO_4, 37.26 ml KH_2PO_4.
- **1 M Sodium phosphate (pH 6.8):** 46.3 ml 1 M Na_2HPO_4, 53.7 ml KH_2PO_4.
- **Lysis buffer:** 50 mM potassium phosphate (pH 7.5), 2 mM EDTA, 0.2 M NaCl, 5 % (*w/v*) glycerol. DTT (2 mM) was added after autoclaving.
- **Dialysis buffer:** 50 mM potassium phosphate (pH 7.5), 2 mM EDTA, 5 % (*w/v*) glycerol. DTT (2 mM) was added after autoclaving.

- **Column wash buffer:** 0.5 M potassium phosphate (pH 7.5), 2 mM EDTA, 5 % (*w/v*) glycerol, DTT (2 mM) was added after autoclaving.
- **Column wash buffer with 1 M NaCl:** 0.5 M potassium phosphate (pH 7.5), 2 mM EDTA, 1 M NaCl, 5 % (*w/v*) glycerol, 2 mM DTT (2 mM) added after autoclaving.
- **CRP stock buffer:** 10 mM sodium phosphate (pH 6.8), 0.1 mM EDTA, 0.2 M NaCl, 50 % (*w/v*) glycerol.
- **Superbroth (per litre):** 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 0.19 g KCl.
- **2x SDS-PAGE loading dye.** Purchased from Life Technologies.
- **Pre-cast NuPage 4-12 % (*w/v*) Bis-Tris polyacrylamide protein gels.** Purchased from Life Technologies.
- **1x MES buffer:** 50 mM MES (2-[N-morpholino]ethanesulphonic acid), 50 mM Tris base, 0.1 % (*w/v*) SDS, 1 mM EDTA, pH 7.3. Diluted from 20x MES (purchased from Life Technologies) using ddH_2O.

In vitro transcription:

- **10x Transcription buffer (TNSC buffer)**: 400 mM Tris acetate pH 7.9, 10 mM $MgCl_2$, 1 M KCl, 10 mM DTT.
- **STOP solution**: 97.5 % (*v/v*) deionised formamide, 10 mM EDTA, 0.3 % (*v/v*) Bromophenol Blue/ Xylene Cyanol FF.
- **P^{32} labelled α-UTP (3000 Ci/mmol)**. Supplied by Perkin-Elmer.

Radiolabelling of DNA fragments:

- **G-50 sephadex beads**. Resuspended in a 12 % (*v/v*) slurry with TE.
- **T4 polynucleotide kinase**. Supplied by NEB, used with supplied 10x buffer.
- **P^{32} labelled γ-dATP (7000 Ci/mmol)**. Supplied by MP Biomedicals.