Transcriptional repressor CopR acts by inhibiting RNA polymerase binding

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CopR is a transcriptional repressor encoded by the broad-host-range streptococcal plasmid pIP501, which also replicates in *Bacillus subtilis*. It acts in concert with the antisense RNA, RNAIII, to control pIP501 replication. CopR represses transcription of the essential *repR* mRNA about 10- to 20-fold. In previous work, DNA binding and dimerization constants were determined and the motifs responsible localized. The C terminus of CopR was shown to be required for stability. Furthermore, SELEX of the *copR* operator revealed that *in vivo* evolution was for maximal binding affinity. Here, we elucidate the repression mechanism of CopR. Competition assays showed that CopR–operator complexes are 18-fold less stable than RNA polymerase (RNAP)–pII complexes. DNase I footprinting revealed that the binding sites for CopR and RNAP overlap. Gel-shift assays demonstrated that CopR and *B. subtilis* RNAP cannot bind simultaneously, but compete for binding at promoter pII. Due to its higher intracellular concentration CopR inhibits RNAP binding. Additionally, KMnO₄ footprinting experiments indicated that prevention of open complex formation at pII does not further contribute to the repression effect of CopR.

INTRODUCTION

Replication of the streptococcal plasmid pIP501 is regulated by two components that act in concert: the transcriptional repressor CopR (10.6 kDa) and the antisense RNA RNAIII (136 nt) (Brantl & Behnke, 1992). Whereas RNAIII exerts its inhibitory effect by attenuation of the essential *repR* mRNA (Brantl *et al.*, 1993; Brantl & Wagner, 1994), CopR has a dual function: it represses transcription from the essential *repR* promoter pII about 10- to 20-fold (Brantl, 1994), and it prevents convergent transcription from pII and pIII (antisense promoter), thereby indirectly increasing transcription initiation at pIII (Brantl & Wagner, 1997, Fig. 1). Previously, we found that CopR contacts the DNA asymmetrically at two consecutive major grooves that share the consensus motif 5'-CGTG and narrowed down the operator sequence to 17 bp (Steinmetzer & Brantl, 1997). Furthermore, we demonstrated that CopR binds exclusively as a dimer and calculated the equilibrium dissociation constants for the CopR dimers and the CopR–DNA complex to be 0.4 nM and 1.4 µM, respectively (Steinmetzer *et al.*, 1998). A 3D model of the N-terminal 63 amino acids of CopR was built,

allowing the identification of amino acids involved in DNA binding and dimerization (Steinmetzer *et al.*, 2000a, b, 2002a). Furthermore, it was established that the structured acidic C terminus of CopR that forms a β -strand is necessary for stabilization of the protein (Kuhn *et al.*, 2000, 2001). A fluorescence energy study revealed that CopR bends the operator DNA slightly – 20–25° – upon binding (Steinmetzer *et al.*, 2002b). A SELEX analysis showed that *in vivo* evolution of the *copR* operator was directed at maximal binding efficiency (Freede & Brantl, 2004). Plasmid pIP501 belongs, together with pAM β 1 and pSM19035, to the Inc18 family of broad-host-range streptococcal plasmids (Brantl *et al.*, 1990). These plasmids replicate via the theta mechanism in a broad range of Gram-positive hosts, among them *Bacillus subtilis*. The corresponding Cop proteins CopR, CopF (Swinfield *et al.*, 1990) and CopS (Ceglowski *et al.*, 1993) and their binding regions are highly homologous.

Initiation of transcription is a stepwise process (Rojo, 1999), starting with the binding of the RNA polymerase to the promoter and formation of a loose closed complex, which is subsequently rearranged into a tighter closed complex. This is followed by the melting of the DNA around the transcriptional start site, called the open complex. Afterwards, RNA polymerase (RNAP) can form the initiation complex and begin to transcribe the DNA, often producing short abortive transcripts resulting from failed attempts to leave the promoter. Eventually, RNAP escapes the promoter and forms the elongation complex. Virtually every one of these steps can be exploited for

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Abbreviations: α CTD, C-terminal domain of the α subunit of RNA polymerase; EMSA, electrophoretic mobility shift assay; PAA, polyacrylamide; RNAP, RNA polymerase; SELEX, systematic evolution of ligands by exponential enrichment (*in vitro* selection).

Fig. 1. Working model with CopR operator sequence. Upper part: working model of copy number control of plasmid pIP501 in *B. subtilis*. Black boxes, promoters; open rectangles, open reading frames; thick arrows, RNAs; grey boxes, proteins; *oriR*, replication origin; filled arrows, activation/positive interactions; horizontal bar, repression; Att, position of premature termination of RNAII transcription. The negative regulation by CopR is transcriptional and is exerted at the *repR* promoter pII. Negative regulation by RNAIII is via induction of transcription termination of RNAII. Repressed RNAII transcription (presence of CopR) permits increased RNAIII transcription (Brantl & Wagner, 1997). Lower part: schematic representation of the DNA sequence containing the CopR target. Minimal binding sites I and II are highlighted by boxes. The -35 and -10 regions of promoter pII are underlined. Dots indicate the transcription start points of RNAII. The fragment used for EMSA comprised nt 190–400, whereas the fragment used for DNase I footprinting and KMnO_4 footprinting comprised nt 160–395. Nucleotide numbering is according to Brantl *et al.* (1990).

transcription repression (Rojo, 1999, 2001). For instance, *Escherichia coli* repressor Fur and *Streptococcus pneumoniae* repressor MalR act by steric hindrance of RNAP binding (Escobar *et al.*, 1998; Nieto *et al.*, 2001) whereas *B. subtilis* Spo0A and *E. coli* Arc inhibit open complex formation at the *abrB* and p_{ANT} promoters, respectively (Greene & Spiegelman, 1996; Smith & Sauer, 1996). By contrast, *B. subtilis* phage $\Phi 29$ protein p4 and architectural protein FIS prevent promoter escape at viral A2c and *E. coli* *gyrB*, respectively (Monsalve *et al.*, 1996; Schneider *et al.*, 1999). Recently, we demonstrated that one repressor employs different repression mechanisms at different promoters: *B. subtilis* CcpN represses abortive transcription at the *gapB* promoter, but promoter escape at the *sr1* and *pckA* promoters (Licht & Brantl, 2009). Interestingly, plasmid pMV158-encoded repressor CopG uses two different mechanisms at the same promoter: it prevents RNAP binding and reverses open complex formation by dislodging RNAP from P_{cr} (Hernández-Arriaga *et al.*, 2009).

To clarify the repression mechanism exerted by CopR, we used electrophoretic mobility shift assays (EMSAs) with CopR and RNAP alone and in combination to analyse steric hindrance. Competition assays with unlabelled operator fragments were applied to calculate the half-lives of the CopR–pII and the RNAP–pII complexes to be 1.2 min and 22 min, respectively. DNase I footprinting

revealed that the binding regions of CopR and RNAP overlap partially. Furthermore, we performed chemical footprinting with KMnO_4 to investigate a possible additional influence of CopR on open complex formation at pII. Taken together, our data show that CopR has only one activity: it inhibits RNAP binding to promoter pII.

METHODS

Enzymes and chemicals. A PCR kit from Roche was used for PCR amplifications. DNA sequencing was performed by the dideoxy chain-termination method with a Sequenase kit from Amersham Bioscience. Chemicals were of the highest available purity.

Overexpression and purification of His₆-CopR. Plasmid pQC60 used for overexpression and purification of a C-terminally His₆-tagged CopR protein from *E. coli* was constructed by cloning of a 307 bp *NcoI/BglII* fragment generated by PCR on plasmid pCOP7 (Brantl & Behnke, 1992) as template with primers SB299 (5'-GAA TTC CCA TGG AAC TAG CAT TTA GAG AA-3') and SB300 (5'-CTG CAG AGA TCT TTA TTA GTG ATG GTG ATG GTG-3') into the *NcoI/BglII*-digested vector pQE60 (Qiagen). The sequence was confirmed. Purification of His₆-CopR from *E. coli* TG1(pQC60) was performed as described previously (Steinmetzer *et al.*, 1998). C-terminally His₆-tagged CopR was verified to function *in vivo* (not shown).

Purification of His₆-RNA polymerase from *B. subtilis*. *B. subtilis* strain 168 containing an *rpoC* gene with a 3' terminal His₆-tag was

grown in 150 ml LB medium containing 50 µg spectinomycin ml⁻¹ at 175 r.p.m. at 37 °C to OD₆₀₀ 2.0–3.0. Cells were harvested by 5 min centrifugation at 8000 r.p.m. for 5 min at 4 °C and the pellet frozen overnight at –20 °C. Cells were resuspended in 10–15 ml lysis buffer (50 mM sodium phosphate pH 8.0, 300 mM NaCl containing 120 µl PMSF added from a 0.1 M stock solution), and sonicated on ice three times for 10 min each. Cell debris was pelleted at 4 °C for 10 min at 13 000 r.p.m., and supernatant loaded onto a Ni²⁺-NTA agarose column (Qiagen) containing 0.5 ml bed volume. The column was equilibrated with 2 vols lysis buffer. After collecting the flow-through, the column was washed twice with lysis buffer, followed by one elution step with 0.5 ml 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 12.5 mM imidazole, a second elution step with 0.5 ml 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 25 mM imidazole, and a third elution step with 0.5 ml 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 37.5 mM imidazole. Afterwards, the column was washed with 5 ml 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 125 mM imidazole. Glycerol was added to a final concentration of 50 % to each elution fraction; fractions were stored at –20 °C and analysed in a 12 % SDS-PAA gel.

Preparation of the labelled CopR target. The 210 bp CopR target for EMSAs was generated by a PCR with [³²P]ATP-labelled oligodeoxynucleotide SB1480 (5'-GTA ACA TTG GAG TTA GAA-3') and unlabelled oligodeoxynucleotide SB1481 (5' GTA TTC TTT ATT CAG TTC-3') on plasmid pPR1 (Brantl & Behnke, 1992) as template.

EMSA. Binding reactions with His₆-tagged CopR or RNAP were performed in a final volume of 20 µl of binding buffer (BB) containing 100 mM potassium glutamate, 10 mM Tris/HCl pH 8.0, 0.1 mM EDTA, 50 mM KCl, 1 mM CaCl₂, 5 mM MgCl₂, 1 mM DTT, 10 mg BSA l⁻¹, 5 % (v/v) glycerol and 1 nM end-labelled DNA fragment. This buffer, which allows only the formation of closed complexes, had to be used to ensure the visualization of both CopR and RNAP complexes with the pII promoter/operator region. Herring sperm DNA (0.05 g l⁻¹) was added as non-specific competitor. After incubation at 37 °C for 30 min, the reaction mixtures were separated on 5 % native PAA gels run at room temperature at 230 V, 25 mA in 0.5 × TBE buffer. Visualization and quantification of the bands were performed on a Fuji-PhosphorImager using the program PCBAS 2.09e (Raytest).

Stability of RNAP–DNA and CopR–DNA complexes. Equilibrium mixtures were prepared by incubating 1 nM labelled DNA fragment and varying concentrations of protein (RNAP or CopR) in BB at 37 °C. Dissociation of complexes between labelled DNA and either protein was made irreversible by addition of 50-fold molar excess of competing unlabelled promoter/operator fragment. Fractions of complexed and free labelled DNA were directly quantified as above. Data were analysed according to the equation for a first-order kinetic process:

$$[\text{complex}] = [\text{complex}]_0 \cdot e^{-k \cdot t}$$

where [complex] represents the concentration of the complex between either RNAP or CopR and the labelled DNA at time *t*, and [complex]₀ is its value at *t*=0. The equation was transformed to allow calculation of complex half-lives:

$$[\text{complex}]_t = [\text{complex}]_0 \cdot (0.5)^{t/t_{1/2}}$$

The half-life (*t*_{1/2}) of the complexes was calculated by fitting the data obtained to the equation using non-linear regression.

DNase I footprinting. The 20 µl reaction mixture contained 1 nM of a 235 bp γ-³²P-labelled DNA fragment obtained by PCR from plasmid pPR1 as template with labelled primer SB6 (5'-CAA CAG AAC CAG AAC CAG AAA-3') and unlabelled primer SB466 (5'-TTA ATT GAT TGG TGG TAA TC-3'), 0.5 × TBE, 0.025 g herring sperm DNA l⁻¹, 6.25 mM MgCl₂ and 4.4 µM His₆-CopR or 90 nM native *B. subtilis* RNAP. Incubation was for 30 min at 30 °C. Subsequently, DNase I cleavage was initiated by addition of 1 µl (0.05 U) of freshly diluted DNase I (Roche) followed by incubation for 2 min at 37 °C. The reaction was stopped by phenol/chloroform extraction. After ethanol precipitation, the pellet was washed with 80 % ethanol and dissolved in 2 µl H₂O and 2 µl formamide loading dye. Samples were denatured at 75 °C for 5 min and separated on a 6 % denaturing PAA gel containing 7 M urea. As size marker, a dideoxy sequencing reaction using plasmid pPR1 and primer SB6 was loaded in parallel. The dried gel was subjected to PhosphorImaging (Fujix BAS 1000).

KMnO₄ footprinting. KMnO₄ footprinting was performed according to Sasse-Dwight & Gralla (1989), with a few modifications. A 20 µl reaction mixture contained 1 nM of a γ-³²P-labelled DNA fragment obtained by PCR from plasmid pPR1 as template with labelled primer SB6 and unlabelled primer SB466, 0.5 × TBE, 0.025 g herring sperm DNA l⁻¹, 6.25 mM MgCl₂ and 2.2 µM or 4.4 µM His₆-CopR. Subsequently, native *B. subtilis* RNAP was added to 90 nM final concentration, and incubation was for 30 min at 30 °C. Single-stranded DNA was modified for 2 min at 37 °C by addition of 2.5 µl 80 mM KMnO₄ (freshly prepared from a 0.37 M stock solution). The reaction was stopped with 2 µl 12.8 M β-mercaptoethanol; the DNA was precipitated with ethanol, washed with 80 % ethanol and dissolved in 10 % piperidine followed by a 30 min incubation at 95 °C. The cleavage products were precipitated with ethanol, washed with 80 % ethanol and dissolved in 2 µl H₂O, followed by addition of 2 µl formamide loading dye. After denaturation for 5 min at 75 °C, the samples were separated on a 6 % denaturing PAA gel containing 7 M urea. Visualization and quantification of the bands were performed as for EMSAs.

RESULTS

RNAP–pII complexes are 18-fold more stable than CopR–operator complexes

In the case of the *rep* promoter P_{cr} of plasmid pMV158, which replicates in both Gram-positive bacteria and *E. coli*, a huge difference between the stability of the RNAP–promoter complexes at 37 °C and at 0 °C was found (Hernández-Arriaga *et al.*, 2009). Whereas the 37 °C complex was extremely stable, with a half-life of ≈3 h, the 0 °C complex was very short-lived and no half-life could be determined. To find out whether this is also the case for the complexes of *B. subtilis* RNAP with promoter pII of plasmid pIP501, we performed EMSAs at 4 °C and 37 °C. However, no differences could be observed. Therefore, all subsequent gel-shift experiments were carried out at 37 °C.

Firstly, the kinetics of dissociation of RNAP–pII complexes was investigated. Seven equilibrium mixtures containing 1 nM labelled promoter/operator fragment and 220 nM RNAP were prepared at 37 °C. At different time points, dissociation was initiated by adding a 50-fold excess of competing unlabelled fragment to the single tubes. Total

incubation time for RNAP with the labelled fragment was 70 min, so the incubation with the excess unlabelled promoter fragment varied over time. All samples were loaded after the 70 min time point on a native PAA gel (Fig. 2a). The dissociation of the RNAP–pII complexes appeared to follow first-order kinetics, which yielded a dissociation rate constant k_d of $5.25 \times 10^{-4} \text{ s}^{-1}$, corresponding to a half-life of ≈ 22 min. Experiments with different RNAP concentrations resulted in the same half-life (not shown).

Secondly, the kinetics of dissociation of CopR–operator complexes was measured as above at 37 °C, employing a 50-fold excess of competing unlabelled DNA (Fig. 2b). The equilibrium mixtures contained 1.2 μM CopR and 1 nM labelled promoter/operator fragment. Dissociation was initiated by adding unlabelled competitor. Total incubation time was 25 min. The experiment was performed twice with different CopR concentrations (1.2 μM and 2.4 μM). Both yielded similar dissociation rate constants of $9.6 \times 10^{-3} \text{ s}^{-1}$ (Fig. 2b), which corresponded to a half-life of ≈ 72 s.

From our data we can conclude that the stability of CopR–pII complexes is 18-fold lower than that of RNAP–pII complexes.

The binding sites of CopR and RNAP at the *repR* promoter pII overlap

DNase I footprinting was used to investigate the binding of RNAP and CopR at the promoter/operator region of pII. To this end, a 235 bp labelled DNA fragment was incubated with either *B. subtilis* RNA polymerase (90 nM) or His₆-CopR (4.4 μM), or simultaneously with both proteins, and the complexes were subsequently treated with DNase I. As shown in Fig. 3, the RNA polymerase protected the region between –58 and +15 (+1 is the transcription start site). This region includes the binding site for the α CTD between –40 and –60. Here, additionally, strong enhancements of DNase I reactivity on the opposite side of the double helix, probably originating from distortion of the upstream region as a result of an α CTD interaction, are seen. The region protected by RNAP clearly overlaps the CopR binding site between –62 and –37. Although the minimal recognition region of CopR comprises 17 bp, earlier ethylation and methylation interference footprinting had shown that up to five more nucleotides are contacted – albeit weakly – upstream and downstream of these 17 bp (Steinmetzer & Brantl, 1997). This was confirmed later by hydroxyl radical footprinting studies (Steinmetzer *et al.*, 2002a), where a 29 bp region was protected and contacts were found

Fig. 2. Kinetics of dissociation of RNAP–pII and CopR–pII complexes at 37 °C. (a) EMSA analysis of the stability of the RNAP–pII complexes. Equilibrium mixtures contained 220 nM His₆-RNAP and 1 nM labelled DNA. The total incubation time was 70 min, but at the indicated time points, a 50-fold excess of unlabelled DNA was added. Analysis was on a 5% native PAA gel. (b) EMSA analysis of the stability of the CopR–operator/pII complex. Conditions were as in (a), except that 1.2 μM CopR was used and total incubation time was 25 min. R, RNAP–DNA complex; C, CopR–DNA complex; F, free target DNA. Below the autoradiographs of the gels in (a) and (b) linear fits of the corresponding data are shown.

between positions -60 and -36 on the top strand and between positions -65 and -40 on the bottom strand. Three distinct areas of protection – I, II and III – visible on the top and on the bottom strand are marked by grey lines in Fig. 3. The footprint pattern of the bottom strand was shifted by 4–5 nt in the 3' direction compared with the top strand. The additional footprint III compared with the data from chemical interference footprinting (Steinmetzer & Brantl, 1997) resulted from non-base-specific contacts between CopR and DNA (Steinmetzer *et al.*, 2002a). The DNase I footprinting data presented here corroborate the previous finding that CopR interacts with a few more nucleotides upstream and downstream of its minimal binding region. Therefore, we can deduce that the binding region of CopR overlaps partially with the binding region of RNAP (see Fig. 3). The concurrent addition of both proteins yields a mixture of both single footprints. No modified bands indicative of simultaneous binding of CopR and RNAP are visible.

From the footprinting data one would expect a competition between RNAP binding and CopR binding. However, DNase I footprinting does not allow us to determine unequivocally whether CopR and RNAP can bind at the same time. To answer this question, we resorted to EMSAs.

CopR and RNAP compete for binding to the target DNA

In EMSAs performed by addition of both CopR and RNAP at the same time or consecutively, no supershifted bands could be detected (e.g. Fig. 4), demonstrating that binding of these molecules at pII is mutually exclusive. This result is in agreement with the overlapping binding regions of the two proteins. To investigate whether CopR and RNAP can dislodge each other at promoter pII, a time-dependent EMSA analysis was applied (Fig. 4). Recent calculations from *E. coli* showed that in both exponential and stationary phase, on average ≈ 2600 molecules of RNAP are present in one bacterial cell (Piper *et al.*, 2009). These are either engaged in ongoing transcription or bound to low-affinity sites, i.e. only a few free molecules are available. For wild-type *B. subtilis* strain DB104 containing low-copy-number pIP501 derivative pCOP4, the number of CopR monomers per cell was calculated to be $\approx 15\,000$, corresponding to an intracellular CopR concentration of roughly 20–30 μM (Steinmetzer *et al.*, 1998). To simulate the *in vivo* situation where CopR is in large excess over free RNAP, and to be still able to detect protein–DNA complexes, we used 4.7 μM CopR and 0.22 μM RNAP. First, the time-dependent displacement of RNAP by CopR was analysed. To this end, RNAP and a labelled 210 bp promoter/operator fragment were incubated at 37 °C, and CopR was added at different time points. Total incubation time was 70 min. As shown in Fig. 4(a), already after 0.5 min, a strong CopR–DNA complex was visible, and after 70 min, only a weak RNAP–DNA complex could be detected, comprising $\approx 20\%$ of the initially bound RNAP.

Fig. 3. The binding sites of CopR and RNAP overlap partially. DNase I footprinting analyses of the interaction of CopR and RNAP with promoter pII were performed as described in Methods. On the left margin, nt positions around pII are shown. The regions protected by CopR and RNAP are indicated by thick black lines. Grey lines indicate the protected regions I to III from previous hydroxyl radical footprinting assays (Steinmetzer *et al.*, 2002b). C, control without DNase and without proteins. Left panel, Sanger sequencing reaction on plasmid pPR1 with primer SB6. CopR was added to a final concentration of 4.4 μM , RNAP to 90 nM. In the last two lanes, 4.4 μM CopR and RNAP (90 or 180 nM) were applied simultaneously.

The time-dependent displacement of CopR by RNAP was analysed in the same way, but here, CopR was added first, and RNAP was added after the same time points (Fig. 4b). Although virtually all operator sites were already saturated with CopR after 0.5 min, a faint RNAP–DNA complex appeared, which became stronger with increasing incubation time. Apparently, the rapid dissociation of the CopR–operator complexes compared to the 18-fold more stable RNAP–DNA complexes accounts for the displacement of the repressor by the polymerase: the addition of RNAP to a previously equilibrated CopR–DNA binding mixture

Fig. 4. CopR and RNAP compete for binding at promoter pII. (a) Time-dependent displacement of RNAP by CopR. RNAP (220 nM) was mixed with labelled DNA in binding buffer, and, at different time points, CopR was added to 4.7 μ M. Total incubation was for 70 min at 37 °C, followed by separation on a 5 % native PAA gel. (b) Time-dependent displacement of CopR by RNAP. CopR (4.7 μ M) was mixed with labelled DNA in binding buffer, and, at different time points, RNAP was added to 220 nM. Incubation and separation were as in (a). R, RNAP–DNA complex; C, CopR–DNA complex; F, free target DNA. The incubation times with the corresponding competitor are indicated above the gels.

passively displaces the equilibrium in the direction of the dissociation of the CopR–DNA complexes, by sequestering free DNA. Although – after 70 min incubation – CopR bound 98.8 % of the labelled DNA fragment whereas RNAP bound only 1.2 %, our data show that CopR and RNAP can displace each other at the *repR* promoter pII, i.e. they can compete for binding.

CopR inhibits RNAP binding at promoter pII

To investigate whether CopR acts by steric hindrance, i.e. prevention of binding of the RNAP at promoter pII, we studied the concentration-dependent displacement of RNAP by CopR using EMSAs. First, a labelled pII/operator DNA fragment was incubated with different concentrations of CopR in the absence of RNAP (Fig. 5a). At 4.7 μ M CopR, all DNA was bound by CopR, and at 19 μ M, both specific and unspecific CopR–DNA complexes could be observed. Such complexes were detected previously (Steinmetzer *et al.*, 1998) and most probably also exist *in vivo* at the 20–30 μ M intracellular CopR concentration. In the second experiment, 0.22 μ M RNAP was mixed with different concentrations of CopR and competition was

started by adding the reaction mixture containing the labelled target DNA (Fig. 5b) followed by 30 min incubation at 37 °C. Already at 0.3 μ M CopR, a clear displacement of RNAP could be observed, but even at 19 μ M CopR, about 20 % of the DNA molecules originally bound by RNAP were still present in complex with pII. The competition experiment was repeated with two other RNAP concentrations (0.44 μ M and 0.88 μ M), but the results were the same. Therefore, we can conclude that CopR inhibits *in vitro* binding of the RNAP to promoter pII approximately fivefold.

CopR obstructs open complex formation at promoter pII

The EMSAs revealed that even at high concentrations of CopR the formation of the RNAP–pII complex is not completely inhibited (Fig. 5). For pMV158-encoded CopG two functions were found: it prevents access of RNAP to the promoter and it actively dissociates open complexes (Hernández-Arriaga *et al.*, 2009). To investigate whether as well as inhibiting RNAP binding, CopR also dissociates open complexes, we studied the effect of CopR on the

Fig. 5. CopR inhibits binding of RNAP to promoter pII. (a) EMSA analysis of CopR binding to its operator. Increasing amounts of CopR were added to labelled DNA (1 nM) and incubation was for 30 min at 37 °C, followed by separation on a 5% native PAA gel. (b) EMSA analysis of concentration-dependent displacement of RNAP by CopR. RNAP (220 nM) was mixed with increasing amounts of CopR, and subsequently a reaction mix containing binding buffer and 1 nM labelled DNA was added. Incubation and gel separation were as in (a). R, RNAP–DNA complex; U, unspecific CopR–DNA complex; C, CopR–DNA complex; F, free DNA target.

formation of open RNAP–pII complexes by KMnO_4 footprinting. KMnO_4 preferentially oxidizes the 5,6 double bond of pyrimidine bases in distorted or denatured regions of DNA or at their junction with native duplex DNA. Therefore, it has been used to examine the extent of the melted region of an open RNAP–promoter complex (Sasse-Dwight & Gralla, 1989). A labelled 235 bp DNA fragment containing the CopR operator and promoter pII was incubated with *B. subtilis* RNAP (90 nM) and His₆-CopR (2.2 μM or 4.4 μM) and subjected to KMnO_4 treatment as described in Methods (see Fig. 6). In the absence of CopR, the DNA region between positions –12 and –3 (+1 is transcription start point) at promoter pII is partially melted by formation of an open transcription initiation complex. A cleavage of the DNA occurred at thymidines at positions –12, –10, –7 and –3. In the presence of 4.4 μM CopR, the formation of this single-stranded region was inhibited by 84%. This result demonstrates that CopR impedes the formation of an open RNAP–promoter complex. However, since CopR already inhibits an earlier step, namely access of RNAP to promoter pII, and this inhibition was calculated to be about 80%, no significant extra contribution of the obstruction of open complex formation was found. Hence, in contrast to CopG, no second activity of CopR could be established.

DISCUSSION

In this study, we investigated the complexes formed by the *B. subtilis* RNAP at the *repR* promoter pII and elucidated the mechanism by which CopR represses transcription from this promoter.

Fig. 6. CopR obstructs open complex formation at promoter pII. KMnO_4 footprinting was performed as described in Methods, using 90 nM *B. subtilis* RNA polymerase and 2.2 or 4.4 μM His₆-CopR. The left panel displays a sequencing reaction on plasmid pPR1 with primer SB6. On the left margin, nt positions around pII are shown. The detected single-stranded positions in the open complex are indicated on the right.

Evidence for the repression mechanism used by CopR was provided by competition assays in which the order of addition of RNAP and repressor was inverted. No specific ternary complexes were observed in any case (Figs 4 and 5), demonstrating the mutually exclusive binding of CopR and RNAP to the promoter/operator region. In the presence of both proteins, the apparent equilibrium extent of formation of RNAP–pII complexes depended on the concentration of both proteins (Fig. 5 and unpublished data). Since the intracellular concentration of CopR is much higher than that of freely available RNAP, CopR can successfully compete with RNAP for binding to the same region of the DNA, where promoter and operator overlap (Fig. 3). CopR hinders the access of RNAP to the promoter region by steric exclusion, probably by competing with the α CTD of the polymerase. This constitutes a classical model of action of a transcriptional repressor, which also applies to e.g. λ cI repressor binding to the O_{R1} operator of the p_R promoter (Hawley *et al.*, 1985), the LexA repressor at the *uvrA* promoter (Bertrand-Burggraf *et al.*, 1987), the Fur repressor of *E. coli* (Escobar *et al.*, 1998) and the MalR repressor of *S. pneumoniae* (Nieto *et al.*, 2001). Although in these conventional cases, steric hindrance occurs by binding of a repressor to sequences that overlap those recognized by RNAP, binding of the polymerase can also be inhibited by proteins like CytR or DnaA whose binding sites do not overlap that of RNAP: *E. coli* CytR repressor binds at the *deo* promoter at position –70 (Valentin-Hansen *et al.*, 1996) and for DnaA, a nucleation site allows binding of additional repressor molecules finally leading to a nucleoprotein complex that impedes RNAP binding (Lee & Hwang, 1997).

For CopG of plasmid pMV158 and *E. coli* RNAP also a partial overlap of the contacted regions has been found, and a simultaneous binding of CopG and RNAP was excluded (Hernández-Arriaga *et al.*, 2009). Furthermore, these authors could show that different sides of the promoter/operator region were contacted. In the case of CopR, at least in the upper strand, the CopR and RNAP binding sites are located on the same face of the DNA (Fig. 3).

The degree of inhibition by CopR determined in EMSAs and in KMnO₄ footprinting was, at 80 % and 84 %, nearly the same. Consequently, an additional contribution of the obstruction of open complex formation to the repression mechanism is unlikely. Therefore, a second activity of CopR can be excluded. This is in contrast to what has been found for CopG of plasmid pMV158 and for IclR, an *E. coli* repressor of the *aceBAK* operon, where in addition to steric hindrance, the repressors were able to actively dissociate open complexes (Hernández-Arriaga *et al.*, 2009; Yamamoto & Ishihama, 2003). In the case of IclR, however, this second activity was achieved by binding to a region far upstream of the primary binding site, whereas binding of four CopG molecules to its single operator was responsible for its dual repression mechanism.

Furthermore, we determined that the stability of the CopR–pII complexes ($t_{1/2} \approx 1.2$ min) is 18-fold lower than that of the RNAP–pII complexes ($t_{1/2} \approx 22$ min). This is 8-fold less than the determined 771-fold stability difference (Hernández-Arriaga *et al.*, 2009) between the RNAP–P_{cr} ($t_{1/2} \approx 180$ min) and the CopG–P_{cr} complexes (14 s). The half-life of the CopR- and CopG-operator complexes was 5-fold different, but still in the same range. Due to the different sources of RNAP (*B. subtilis* RNAP for pII and *E. coli* RNAP for P_{cr}) a direct comparison of the stability of the RNAP–promoter complexes is not feasible. However, the much shorter half-life of the Cop–DNA complexes could explain how in both cases, the repressor can be displaced by the RNAP from its operator. The half-lives of the complexes provide the time frame within which regulation can occur. Since CopR is synthesized constitutively, repression can be achieved quickly, although the half-life of the RNAP–pII complex is quite long. However, when cells divide and the CopR concentration decreases, transcription of the *repR* mRNA can – due to the short half-life of the CopR–DNA complexes – be resumed immediately.

The fact that even at very high concentrations of CopR, no complete inhibition of RNAP binding was observed (Fig. 5b) is in accordance with the data from previous transcriptional pII–*lacZ* fusions, where at the enormous concentration of 300 μ M CopR provided by the multicopy plasmid pCOP9, only a 10- to 15-fold decrease in β -galactosidase activity was measured, but no complete repression of *repR* mRNA transcription was observed (Brantl, 1994). Two earlier hypotheses for the lack of complete shut-off of *repR* transcription could be refuted: (i) insufficient levels of CopR from its native promoter

were excluded by determining the intracellular CopR concentration to be 20–30 μ M and (ii) a low binding constant for CopR was ruled out by calculating the k_d of the CopR–operator complex to be 0.4 nM (Steinmetzer *et al.*, 1998). We suggest that the displacement of CopR by RNAP observed here is responsible for the inability of CopR to totally repress pII. This result accords with the previous observation that the P_{cr} promoter of plasmid pMV158 is also never totally blocked by CopG (del Solar *et al.*, 1990), which forms an even 771-fold less stable complex with its target than RNAP (Hernández-Arriaga *et al.*, 2009). By contrast, a third plasmid-encoded Cop repressor, CopB from *E. coli* plasmid R1, can totally silence its cognate *repA* promoter, which is activated only when the plasmid copy number drops dramatically (Nordström *et al.*, 1984). However, for CopB/RNAP and the *repA* promoter, no reciprocal displacement studies have been conducted.

The CopR protein of streptococcal plasmid pIP501 is highly homologous to the Cop proteins of the related plasmids pSM19035 (CopS, Ceglowski *et al.*, 1993) and pAM β 1 (CopF, Swinfield *et al.*, 1990). Their amino acid sequences diverge only at the C terminus, which is solely responsible for protein stability (Kuhn *et al.*, 2000, 2001). Furthermore, the three Cop operator sites are almost identical, differing only in the spacer region between binding sites I and II and in the flanking region. Our previous SELEX experiments showed that these minor differences are negligible as regards efficient binding and regulation *in vivo* (Freede & Brantl, 2004). Therefore, we conclude that all three Cop repressors use the same classic mechanism – steric hindrance – to exert their inhibitory effects.

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