Transcription Activation by CooA, the CO-sensing Factor from *Rhodospirillum rubrum*

THE INTERACTION BETWEEN CooA AND THE C-TERMINAL DOMAIN OF THE α SUBUNIT OF RNA POLYMERASE

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CoaA, a member of the cAMP receptor protein (CRP) family, is a CO-sensing transcription activator from *Rhodospirillum rubrum* that binds specific DNA sequences in response to CO. The location of the CoaA-binding sites relative to the start sites of transcription suggested that the CoaA-dependent promoters are analogous to class II CRP-dependent promoters. In this study, we developed an in vivo CoaA reporter system in *Escherichia coli* and an in vitro transcription assay using RNA polymerases (RNAP) from *E. coli* and from *Rhodobacter sphaeroides* to study the transcription properties of CoaA and the protein-protein interaction between CoaA and RNAP. The ability of CoaA to activate CO-dependent transcription in vivo in heterologous backgrounds suggested that CoaA is sufficient to direct RNAP to initiate transcription and that no other factors are required. This hypothesis was confirmed in vitro with purified CoaA and purified RNAP. Use of a mutant form of *E. coli* RNAP with α subunits lacking their C-terminal domain (α-CTD) dramatically decreased CoaA-dependent transcription of the CoaA-regulated *R. rubrum* promoter PcooF in vitro, which indicates that α-CTD plays an important role in this activation. DNase I footprinting analysis showed that CoaA facilitates binding of wild-type RNAP, but not α-CTD-truncated RNAP, to PcooF. This facilitated binding provides evidence for a direct contact between CoaA and α-CTD of RNAP during activation of transcription. Mapping the CoaA-contact site in α-CTD suggests that CoaA is similar but not identical to CRP in terms of its contact sites to the α-CTD at class II promoters.

CoaA is a CO-sensing transcription activator from *Rhodospirillum rubrum*, and it activates the expression of the cooSCTJ and cooMKLUXH operons in response to CO (1–3). These two adjacent operons encode proteins that oxidize CO to CO₂ with concomitant reduction of H⁺ to H₂ and allow growth of this organism on CO as a sole energy source (4–6). cooa lies immediately 3’ of the cooSCTJ operon, and its expression does not depend on CO.

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change that allows a dimer of CRP to bind to a specific 22-base pair sequence at target promoters (consensus sequence is 5'–AAATGTGATCTAGCTACATTT-3', in which the most important bases for CRP recognition are in bold) and to activate transcription at those promoters (18). CRP-dependent promoters can be grouped into two classes based on the position of the CRP-binding site relative to the start of transcription as well as on the mechanism for transcription activation (19). At class I promoters, the DNA-binding site for CRP is upstream of that for RNAP and is centered at position −61.5, −71.5, −82.5, or −92.5. At class II CRP-dependent promoters, to which the CooA-dependent promoters are analogous, the binding site for CRP is centered at −41.5, overlapping the −35 region, and the α-CTD binds to DNA upstream of the CRP dimer.

Direct interaction between CRP and RNAP plays a pivotal role in transcription activation at both promoter classes (20, 21). In particular, transcription activation at class II promoters requires two distinct contacts between CRP and the α subunit and a third contact between CRP and σ70. One interaction is between activating region 1 (AR1) of the upstream subunit of the CRP dimer and the α-CTD. This interaction increases initial binding of RNAP to the promoter (22). Recently, residues 285–288 and 317 of σ70 were shown to comprise the surface that interacts with AR1 of CRP at class II promoters (23). The second contact, between activating region 2 (AR2) of the downstream subunit of CRP and the N-terminal domain of the α subunit, facilitates isomerization of the closed complex to the open complex (24). The residues in AR1 and AR2 of CRP are not conserved in CooA, which suggests that there might be certain differences in the interactions of CooA and CRP with α. The third activator region (AR3) in CRP, formed by residues 52–58, interacts with σ70 of RNAP (20). Because the AR3 region in CRP is highly similar to an analogous region in CooA, this region in CooA might serve an AR3-like function.

The two CooA-regulated promoters, P_cooF and P_cooM, contain 2-fold symmetric DNA sequences that serve as CooA-binding sites and are similar to the CRP consensus sequence. This is consistent with the similarity between CooA and CRP in their helix-turn-helix motives (2, 3). The CooA-binding sites lie at the −43.5 and −38.5 positions relative to the transcription start sites in P_coo and P_cooM, respectively, overlapping with the −35 region (2, 3). This overlap suggests that both CooA-regulated promoters are analogous to class II CRP-dependent promoters. We chose P_cooF for this study because it is the stronger promoter based on the amount of primer extension product and level of coo-encoded proteins synthesized in vivo (1, 2).

Although CooA shares some common features with CRP, such as DNA binding properties and effector-induced activation, it displays striking differences from CRP in the effector-binding domain and in regions AR1 and AR2. We were interested to know whether CooA was necessary and sufficient for CO-dependent activation of transcription and whether the mechanism of activation by CooA was similar to that of CRP. In this work, we used in vivo CooA reporter systems and in vitro transcription assays to examine the properties of CooA in transcription activation. Because of the particular questions we wished to address, we performed the bulk of the work with RNAP from E. coli, so that any differences between CooA and the CRP detected would reflect properties of CooA. The nature of transcription activation by CooA was investigated through the study of the interaction between CooA and RNAP and, in particular, the interaction between CooA and the α-CTD.

**EXPERIMENTAL PROCEDURES**

*Construction of a System for CooA Expression and a Reporter of CooA Activity in E. coli—* CooA was overexpressed from a vector, pYH1A, created as follows. A 1.9-kilobase Poul-BamHI fragment containing P_coo-cod-RNAP promoter, was isolated from plasmid pKK223-3 (8), digested with Poul, mung bean nuclease, and BamHI, and cloned into the EcoRV and BamHI sites of pACY184. A plasmid containing a P_coo-lacZ fusion, pYH4, was constructed by inserting a polymerase chain reaction-amplified EcoRI-HindIII fragment, extending from position +250 to +70 of P_coo, into plasmid pMS61 (25), which created pMSBP_{coo}. The reporter region was recombined from plasmid pMSBP_{coo} into λ phase RS468 (26) in strain DH15a containing pYH1A. Lysogens were screened by the blue color of plaques on Luria broth (LB) + X-gal plates incubated anaerobically in the presence of CO. The promoter region of integrated P_coo-lacZ fusion in the chromosome was confirmed by DNA sequencing.

*In Vitro Transcription Assays—* Polymerase chain reaction-amplified EcoRI-HindIII fragments from positions −250 to −70, −90 to +70, and −60 to +70 of P_coo were cloned into pRG770, which contains transcription terminator rrrrnBT2 downstream of the multicloning site (27), to yield plasmids pYH1F, pYHF2, and pYHF3, respectively. The supercoiled plasmids used as DNA templates for these assays were purified with the Midi Kit from Qiagen. The RNAPs used in these experiments were Er{T} purified from E. coli or a Rhodobacter sphaeroides RNAP preparation enriched for the Er{T} homolog (28). Standard multiple-round transcription assays (13) were modified as described below to accommodate the requirement of CooA for an anoxic environment (28). The tubes contained a 1.7-mm supercoiled plasmid, 3.5 mM RNAP, 40 nM CooA dimer, and a buffer (30 mM KCl, 40 mM Tris acetate, pH 7.9, 10 mM MgCl2, 1 mM dithiothreitol, 100 mM mg bovine serum albumin, 200 mM ATP, 200 mM CTP, 200 mM GTP)) were degassed and filled with argon in the head space. After the addition of dithionite to 1.7 mM to scavenge any free oxygen, CO was added, and the reactions were incubated at room temperature for 15 min. This step served to activate CooA and allow the activated CooA to interact with the promoter and RNAP to form a predicted 21-nucleotide transcript by incorporating ATP, GTP, and CTP. The reactions were then exposed to air, and 10 mM UTP and 5 μCi of 32P-UTP (DuPont) were added to extend the mRNA at room temperature for 20 min. Reactions were terminated and electrophoresed as described (29). The signal intensities of transcripts were quantified using a PhosphorImager (Molecular Dynamics) and ImageQuant software.

*DNase I Footprinting Assays—* A DNA fragment containing the P_{coo} sequence from position −90 to +70 was polymerase chain reaction-amplified with an unlabeled bottom strand primer and a top strand primer labeled with γ-[32P]ATP and polynucleotide kinase. The amplified fragment was purified by polyacrylamide gel electrophoresis, followed by an Elutip Minicolumn (Schleicher & Schuell). The labeled fragment was extended with CooA, or RNAP, or both in the presence of CO and under the stringent anoxic conditions described previously (2), except that 40 mM pure CooA and 3.5 mM RNAP were used in a 20-μl binding reaction. The reactions were treated with 2 units/ml RNaseA, 30 s. DNase I cleavage products were separated on a 6% (w/v) polyacrylamide-urea gel. Neither heparin nor any nucleotides were added into the reactions.

*CO Induction of P_{coo} Expression in E. coli and Measurement of β-Galactosidase Activity in Vivo—* Strains with a P_{coo-lacZ} fusion were grown aerobically in LB medium containing 100 mg/ml ampicillin and 35 mg/ml chloramphenicol for 12 h to reach stationary phase. In stoppered test tubes, 20-μl cultures were diluted into 2 ml of LB medium supplemented with 20 mM glucose and the same antibiotics. The air in the head space was replaced by argon and 2% CO, and the cultures were grown anaerobically to an OD_{600} of approximately 0.45. β-Galactosidase activity was determined according to Miller (30).

*Protein Purification and Reconstitution of RNAP—* N-terminal His-tagged wild-type and mutant α subunits were overexpressed from plasmid pH7T1-NIΔ (31) or derivatives constructed by gene replacement of the EcoRI-BamHI fragment with fragments encoding the desired alanine substitutions (14). Purification of α subunits by Ni^{2+} affinity chromatography was performed as described in Tang et al. (31). Preparation of inclusion bodies of β, β′, and σ70 from strains XL1-Blue (pMKS2), BL21 DE3 (pTTβ), and BL21 DE3 (pLH12 α), respectively, and reconstitution of RNAP were carried out as described (31). CooA was purified from an overproducing strain of R. rubrum (UQ459) by the method of Shelver et al. (7).

**RESULTS**

*CooA Is Necessary for CO-dependent Expression in Different Organisms—* CooA has been shown to be necessary for the CO-dependent expression of the two coo operons of *R. rubrum*
(1, 2), and purified CooA binds DNA in a CO-dependent manner (7). These data are consistent with the hypothesis that CooA is both necessary and sufficient for sensing and activating transcription in response to CO, but they are not conclusive. We therefore examined the requirement for CooA in two heterologous systems. We chose *R. sphaeroides* because it is related to *R. rubrum*, yet does not appear to have the *coo* system, as judged by the absence of CO dehydrogenase activity and a failure to hybridize to probes of the *coo* genes (2). In this organism, a pRK404-based plasmid carrying *cooFSC* was tested against the normal promoter failed to produce detectable CO dehydrogenase activity (2). However, when *cooA* was added to the plasmid in its normal position at the 3′ end of the *cooFSC* operon, exposure of the *R. sphaeroides* strain carrying this plasmid to CO produced easily detectable CO dehydrogenase activity (2).

We then examined CO- and CooA-dependent transcription in *E. coli*, an organism that is less related to *R. rubrum* and also lacks any evidence of a *coo* system. For this test, a reporter system was constructed that contained a *PcooF-lacZ* fusion in the chromosome and a plasmid overexpressing CooA. In this system, we detected a substantial increase of β-galactosidase activity upon CO induction (200 Miller units in the presence of CO and 1.6 units in its absence), suggesting that CooA is sufficient for activating the transcription of *PcooF* in *E. coli*. Similar results with CooA reporters in *E. coli* have recently been reported by others (9). These results establish that CooA is necessary for the transcriptional response to CO and that it is able to associate productively in *vitro* with RNAPs from both *E. coli* and *R. sphaeroides*.

**CooA Is Sufficient to Activate the Transcription of *PcooF* in Vitro**—The above results indicate that CooA is necessary, but only in *vitro* analysis can establish whether it is sufficient for CO-dependent transcriptional activation or whether additional factors are required for this activation. The ability of CooA to activate *PcooF* was studied by monitoring RNA synthesis in a purified system containing only DNA, RNAP, CooA, nucleotide triphosphates, and the proper buffer. To investigate the nature of CooA-mediated activation at the *PcooF*-promoter, we modified the standard *in vitro* transcription assay, because CooA is only able to bind CO when reduced. The modified assay, detailed under “Experimental Procedures,” was kept anoxic in the presence of CO to support activation by CooA until the formation of a 21-nucleotide transcript from *PcooF*. At this point, the reaction was exposed to air and extended aerobically for technical convenience. When the reactions were maintained strictly anoxically throughout the entire experiment, a quantitatively similar result was obtained, indicating that the *in vitro* assay provides sufficient conditions for maximal CooA activity (data not shown).

The *in vitro* transcription assays were performed with a supercoiled DNA template containing the *PcooF* and extending from −250 to +70 of the transcription start site. The reactions were carried out in the presence of CO. As shown in lanes 2 and 4 of Fig. 1, CO-dependent transcripts were detected using RNAP from both *R. sphaeroides* and *E. coli*. The observed size of the transcript from *PcooF* correlated well with the predicted size of 240 nucleotides. In the absence of CO, no transcripts from *PcooF* were seen, whereas transcription of the control (RNA-1) was not affected by CO (Fig. 1, lanes 1 and 3).

These results demonstrate that CooA is sufficient for CO-dependent transcriptional activation and that no other factors are required. The results with the *E. coli* indicate that *PcooF* can be recognized by σ^{70} when activated by CooA.

**CooA-activated Transcription Requires the α-CTD of RNAP**—Because the α-CTD makes specific DNA contacts at some promoters and specific protein contacts with a number of transcription activators including the CooA homolog CRP (13, 20), we wished to test whether there were similar contacts between α-CTD and either CooA or *PcooF*. We first examined whether the C-terminal domain of the RNAP α subunit is required for transcription activation of *PcooF* by CooA. To address this question, we assayed the ability of a reconstituted E. coli RNAP containing a truncation of the C-terminal domain of α to directly transcribe from *PcooF*-dependent promoter *PcooF* in *vitro*. Use of *E. coli* RNAP with the α-CTD truncation resulted in a dramatic reduction of transcription from *PcooF* (Fig. 1, lanes 5 and 6). The enzyme activities of wild-type and mutant RNAPs were similar as demonstrated by transcription from the RNA-1 promoter. With a longer exposure of the x-ray film, we were able to detect a low level of a CO-dependent transcript from *PcooF* using RNA with α-235 (data not shown). Because the α-CTD is known to make contacts with activators and UP elements, the ineffectiveness of the α-CTD-truncated RNAP for *PcooF* transcription suggests that the α truncation disrupts the binding of a to CooA, to a UP element, or to both.

**CooA Utilizes Protein-Protein Contacts with α-CTD to Facilitate RNAP Binding to *PcooF***—To analyze potential protein-protein interaction between CooA and RNAP and to specifically address interactions between α-CTD and CooA, we performed DNase I footprinting experiments using wild-type RNAP or the α-CTD-truncated RNAP in the presence or absence of CooA. As shown in Fig. 2, lanes 2–4, CooA alone protected *PcooF* on the top strand from positions −55 to −31 relative to the start site of transcription, in agreement with our previous observations (2, 7). In contrast, neither the wild-type nor the α-CTD-truncated RNAPs alone protected *PcooF* from DNase I (lanes 5 and 7). This result indicates that RNAP does not form a stable complex at *PcooF* in the absence of CooA. When both CooA and wild-type RNAP were incubated together with the DNA fragment, the protected region extended from the CooA-binding site in both directions, upstream to −68 and downstream to at least −1 (lanes 8 and 9). A similar result was obtained when *R. sphaeroides* RNAP was used, indicating that the RNAPs from
these two different organisms function similarly in interacting with CooA at P_{cooF} (lanes 10–12). In contrast to the results obtained with the wild-type RNAP, the α-CTD-truncated E. coli RNAP showed no evidence of forming a stable complex even in the presence of CooA (lane 6). This suggests that CooA activates transcription by enhancing the initial and stable binding of RNAP to P_{cooF} through direct protein–protein contact with the α-CTD.

**Analysis of CooA-regulated Promoter P_{cooF}**—As noted above, the presence of CooA and wild-type RNAP, but not the α-CTD-truncated variants, caused DNase I protection to extend upstream and downstream of that region protected by CooA alone. The region upstream of the CooA-binding site is A+T-rich (Fig. 3) relative to most of the *R. rubrum* genome, reminiscent of the A+T-richness of UP elements in *E. coli* promoters that contact the α-CTD to increase transcription (13, 38). To test whether α interacts with this region in a sequence-specific manner, we created P_{cooF} constructs differing only in the extent of that region. P_{cooF} sequences from positions −90 to +70 and −60 to +70 were cloned into the transcription assay vector pRLG770, resulting in the constructs pYHF2 and pYHF3, respectively. These plasmids were tested for CooA-dependent in *vitro* transcription activity with wild-type RNAP. The level of transcripts from those constructs was quantitatively compared with that of the construct pYHF1 containing the P_{cooF} sequences from −250 to +70. As shown in Fig. 4, these three templates yielded similar amounts of CooA-dependent transcripts, indicating that the specific sequences upstream of position −60 in P_{cooF} do not contribute significantly to promoter activity. This also suggests that the upstream A+T-rich sequence of P_{cooF} does not act as a UP element. Although we cannot exclude the possibility that some portion of the protection upstream of the CooA-binding site is due to a conformational change in CooA induced by the presence of RNAP, the interaction of α-CTD with DNA upstream of CooA is consistent with the sequence-nonspecific interactions between DNA and α-CTD observed at class II CRP-dependent promoters (23).

**Identification of Residues in α-CTD That are Critical for CooA-dependent Activation at P_{cooF}**—We wished to determine whether CooA made similar contacts with the α-CTD as seen with its homolog, CRP, and also to test whether the extended DNase I protection upstream of CooA reflected the direct contact of α-CTD with DNA. To address these questions, we measured CooA-activated transcription using a CooA reporter system containing a P_{cooF}-lacZ fusion integrated into the *E. coli* chromosome, a plasmid expressing CooA, and a library of plasmids encoding single alanine substitutions throughout the entire α-CTD (23). The effects of the α-CTD mutants in this screen were small and somewhat variable (data not shown), but they did identify a few potential mutants that were then assayed for their effects on CooA-dependent transcription in *vitro*.

We tested reconstituted RNAPs in *vitro* containing single alanine substitutions for Thr285, Val306, Glu288, and Arg317 (the patch on α that interacts with CRP at class II CRP-dependent promoters (23)), Arg265 (the residue most important for interaction of α with DNA (14)), and a few additional mutants suggested by the *in vivo* screen, including Val286, Leu312, and Ser213.

The results of the *in vitro* transcription analysis with a subset of the variant RNAPs are shown in Fig. 5. The activity of each RNAP preparation was normalized to the transcription from the RNA-1 promoter. The R265A, L307A, and V287A RNAPs were defective in CooA-dependent transcription of P_{cooF}, providing 13, 34, and 37% of wild-type RNAP activity, respectively (Fig. 5, lanes 2, 3, and 7). Because R265A (14) and L307A affected UP element-dependent transcription and RNAP extended the DNase I protection upstream of the CooA-binding site, this strongly suggested that α-CTD makes contacts with DNA upstream of CooA and that these contacts are important for CooA-mediated transcription activation. V287A was also defective in CooA-dependent transcription, although the other α-CTD variants important for class II CRP-dependent transcription (T285A, E288A, and R317A) had little or no effect. These results suggest that the CooA contact site in the α-CTD of RNAP shares some determinants of, but is not identical to, the site for CRP contact at class II-type promoters. RNAPs containing α mutants V306A and S313A, which were also suggested by the *in vivo* screen, were not defective in CooA-dependent transcription in *vitro* (data not shown).

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S. Bales, M. Burgess, S. Aiyar, and R. L. Gourse, unpublished data.
**Interaction between RNAP and CooA**

![Diagram](image)

**Fig. 3. Sequence of PcooF region.** The figure shows the upper strand sequence of PcooF. The transcription start site is at +1. The underlined sequences represent the putative -10 region, the 2-fold symmetrical CooA-binding site, and the A+T-rich sequence upstream of CooA-binding site as marked, respectively. The region protected by CooA in DNase I footprinting experiments is indicated by the filled bar. The open bar represents the region protected by wild-type RNAP plus CooA.

**DISCUSSION**

Many transcription activators directly contact the α subunit of RNAP (16, 17). In this study, we determined that CooA is sufficient for directing RNAP to initiate transcription of PcooF and that CooA-activated transcription of PcooF requires the C-terminal domain of the RNAP α subunit. Consistent with these observations, CooA facilitates the binding of wild-type RNAP to PcooF but not that of α-CTD-truncated RNAP. These observations suggest that direct protein-protein contact between CooA and the α-CTD of RNAP plays an essential role in transcription activation of PcooF.

We suspect that the α-CTD/CooA interaction plays a role similar to that between AR1 of CRP and α-CTD at class II CRP-dependent promoters. In class II CRP-dependent promoters, α-CTD makes nonspecific contacts with the DNA segment immediately upstream of the CRP site (16, 23). In this study, we found that wild-type RNAP, but not RNAP with the α-CTD truncation, extends DNase I protection upstream of that protected by CooA alone. Furthermore, we determined that α R265A and L307A, which decrease UP element-dependent transcription, also affect PcooF activity. Because the specific DNA sequence upstream of the CooA-binding site is not critical for PcooF activity, we propose that α-CTD makes nonspecific contacts with the DNA immediately upstream of the CooA site and that this protein-DNA interaction is important for CooA-activated transcription of PcooF.

Val287 is also important for CooA-activated transcription, although the other tested α residues that contact CRP at class II promoters are not critical for CooA-dependent transcription of PcooF. Therefore, a different set of side chains, but probably the same region of α-CTD, might be important for activation by CRP and CooA. This hypothesis is consistent with the low similarity between CRP and CooA in activating region 1 (22).

In the DNase I footprinting analysis, RNAP does not completely protect the DNA downstream of PcooF +1, even in the presence of CooA and in the absence of heparin (Fig. 2). In addition, the majority of the CooA-RNAP-PcooF complexes detected in gel-shift assays are heparin-sensitive (data not shown). This partial downstream protection and the heparin sensitivity suggest that the detected ternary complex (CooA-RNAP-PcooF) may be a mixed population of closed and open complexes. Although most CRP-dependent promoters, such as lacP1, mefT, goIP1, form stable open complexes with RNAP in the presence of CRP (32–35), other promoters, such as rnrB P1 even in the presence of its activator protein Fis (36), form an unstable open complex with RNAP (37).

Because basal level transcription of PcooF in the absence of active CooA is not detectable, it is formally possible that the deletion of α-CTD affects basal level transcription of PcooF and not its activation by CooA. However, the requirement for at
least one residue in α-CTD (Val287) that has no effect on DNA binding (23) suggests that the α-CTD requirement involves, at least in part, a protein-protein interaction between CooA and α-CTD.

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