Structural basis of transcription activation by Rob, a pleiotropic AraC/XylS family regulator

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ABSTRACT

Rob, which serves as a paradigm of the large AraC/XylS family transcription activators, regulates diverse subsets of genes involved in multidrug resistance and stress response. However, the underlying mechanism of how it engages bacterial RNA polymerase and promoter DNA to finely respond to environmental stimuli is still elusive. Here, we present two cryo-EM structures of Rob-dependent transcription activation complex (Rob-TAC) comprising of *Escherichia coli* RNA polymerase (RNAP), Rob-regulated promoter and Rob in alternative conformations. The structures show that a single Rob engages RNAP by interacting with RNAP α CTD and σ70R4, revealing their generally important regulatory roles. Notably, by occluding σ70R4 from binding to -35 element, Rob specifically binds to the conserved Rob binding box through its consensus HTH motifs, and retains DNA bending by aid of the accessory acidic loop. More strikingly, our ligand docking and biochemical analysis demonstrate that the large Rob C-terminal domain (Rob CTD) shares great structural similarity with the global Gyrl-like domains in effector binding and allosteric regulation, and coordinately promotes formation of competent Rob-TAC. Altogether, our structural and biochemical data highlight the detailed molecular mechanism of Rob-dependent transcription activation, and provide favorable evidences for understanding the physiological roles of the other AraC/XylS-family transcription factors.

INTRODUCTION

In order to survive, bacteria have successfully evolved complex transcription initiation mechanisms to timely and finely evoke adaptive responses to a myriad of environmental threats, through transcribing key genes involved in stress responses by RNA polymerase (1–3). In *Escherichia coli*, RNA polymerase (RNAP) is assembled by a multisubunit RNA polymerase core (αββ′ω) and a principal promoter-specific factor σ70 (alternative σ factors will be required under stress conditions) (4). At transcription initiation stage, in combination with RNA polymerase core, σ70 usually makes specific interactions with consensus −35 element (TTGACA) and −10 element (TATAAT) of canonical promoters by σ70 region 4 (σ70R4) and σ70 region 2 (σ70R2), respectively (5–7). Subsequently, the double strands of promoter DNA melt, allowing the RNAP-promoter closed complex (RPc) isomerizes into a stable RNAP-promoter

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open complex (RPo) which enables transcription initiation without activators (8–12). In contrast, most promoters of bacterial stress response genes do not simultaneously contain the two canonical consensus promoter elements. Thus, transcription activators are required to strengthen the interactions between RNAP and such non-canonical promoters. These interactions remodel promoter DNA, sustain optimal promoter recognition, and finally promote RPo to isomerize into an initiation-competent transcription activation complex (TAC), which is virtually an activator-dependent RPo (13–18).

Rob, originally designated by its binding ability to the right border of the E. coli replication origin oriC (19), is characterized as a representative member of the global AraC/XylS family transcription activators, which regulate expression of diverse subsets of genes involved in multidrug resistance, virulence, and stress response in many clinically important pathogens, such as Vibrio cholerae, Salmonella typhimurium, Yersinia pestis, Pseudomonas putida, Citrobacter rodentium, enteroaggregative E. coli and Shigella spp. (20–23). The common feature of AraC/XylS family transcription activators is the composition of two conserved helix-turn-helix (HTH) motifs that interact with the upstream recognition element A (designated as A site) and the downstream recognition element B (designated as B site) of the highly degenerate corresponding promoters, respectively (20,23). Up to date, a growing number of genetic and biochemical studies have elucidated that Rob is a pleiotropic transcription activator targeting widely distributed Rob regulons. Overexpression of Rob not only causes antibiotics resistance through down-regulating the influx activity, but also leads to adaptive tolerance to organic solvents, superoxide-generating agents and heavy metals, mainly by upregulating the efflux activity to afford increased cellular detoxification (2,24–27).

Though the HTH motif containing Rob N-terminal domain (Rob NTD) shares high sequence and structural similarities with those of the other AraC/XylS family regulators, various studies have proved that Rob also has its own distinct functional and structural characteristics: (i) Unlike SoxS and MarA, which are regulated by certain inducers, Rob is constitutively expressed and maintained at a high level in cell, as can be regulated by itself and other paralogs (19,28). (ii) Rob has a large C-terminal domain (Rob CTD), which was identified as a potential effector binding domain (25,26). Rob CTD also plays physiological roles in the activation of Rob by an effector through a ‘sequestration–dispersal’ mechanism, and in retaining stabilization by protecting Rob from cellular protease degradation by Lon and ClpYQ (29). However, the underlying mechanism of how Rob orchestrates its CTD and NTD to synergistically respond to changing environmental stimuli is still poorly understood. (iii) Comparative analysis of the co-crystal structures of Rob-micF double-stranded DNA (PDB ID: 1D5Y) (30) with MarA-mar double-stranded DNA (PDB ID: 1BL0) (31) shows that Rob displays two major differences in DNA binding from MarA. The second HTH (helix 6) of Rob only binds to the DNA backbone instead of binding to the adjacent DNA major groove as MarA does, and thus orientation of the upstream DNA is un bent while MarA bends DNA with an angle of 35°. Whether this conformation of Rob-micF structure is a functional state is still under dispute. Although previous genetic epistasis experiments and mutational analysis have identified that some key residues of Rob were involved in DNA binding and transcription activation (29,32), the detailed mechanism mediating Rob-dependent transcription activation is still elusive, largely due to the absence of accurate structural information for Rob-dependent transcription activation complex. Furthermore, whether Rob interacts with the C-terminal domain of RNAP alpha subunit (αCTD) and activates transcription of Rob regulons through proposed ‘pre-recruitment’ mechanism for the other AraC/XylS-family transcription factors remain to be elucidated.

In the present study, we determined two functional cryo-EM structures of Rob-dependent transcription activation complex (Rob-TACI and Rob-TACII). The structures trap two alternative conformations of Rob in complex with E. coli RNAP and micF promoter DNA, whose downstream gene is involved in cellular influx of antibiotics, detergents, and toxins. In Rob-TAC, a single Rob molecule engages RNAP by simultaneously interacting with RNAP αCTD and σ70R4, revealing their versatility and important regulatory roles. Notably, by occluding σ70R4 from binding to promoter -35 element, Rob specifically binds to the conserved Rob binding box through its consensus HTH motifs, and retains DNA bending by aid of the accessory acidic loop (residues 187–193) from Rob CTD. More strikingly, our ligand docking and biochemical analysis demonstrate that the large Rob CTD shares great structural similarity with the global Gyrl-like domains in effector binding and allosteric regulation, and it coordinately promotes formation of competent Rob-TAC along with the DNA-binding Rob NTD. Our results systematically define the key interactions that mediate Rob-dependent transcription activation, provide a new mechanistic framework for bacterial transcription regulation, and may support further exploration on the physiological roles of the other AraC/XylS family transcription factors.

MATERIALS AND METHODS

Plasmid construction

Plasmid pET28a-rob encoding C-terminal His6 tagged E. coli Rob under the control of T7 promoter was synthesized by Sangon Biotech, Inc. Linear micF_mango DNA fragment corresponding to +85 to +50 of E. coli micF promoter followed by Mango III coding sequence was generated by de novo PCR using primers of micF_mango F1, R1 and R2 (Supplementary Table S1) (16,33–36), purified using the QIAquick PCR Purification Kit (Qiagen, Inc.), and stored at −80°C. Two strands of micF DNA fragment corresponding to −63 to +20 of micF promoter were synthesized and annealed in a molar ratio of 1:1. Plasmids carrying rob amino acid substitutions were constructed using site-directed mutagenesis (QuickChange Site-Directed Mutagenesis Kit, Agilent, Inc.). Primers of Rob mutants used in this study are shown in Supplementary Table S1. Sequences of micF_mango DNA and micF DNA are shown in Supplementary Figure S1.
Rob protein purification

*E. coli* strain BL21(DE3) (Invitrogen, Inc.) was transformed with plasmid pET28a-rob or pET28a-rob derivatives. Single colonies of the resulting transformants were used to inoculate 1 L LB broth containing 50 μg/ml kanamycin, and cultures were incubated at 37°C with shaking until OD₆₀₀ reached 0.6. Protein expression was induced by addition of IPTG to 0.5 mM, and cultures were incubated 14 h at 20°C. The cells were harvested by centrifugation (8000 g; 15 min at 4°C), resuspended in 20 ml buffer A (20 mM Tris–HCl, pH 8.0, 0.2 M NaCl, 5% glycerol), and lysed using a high-pressure homogenizer NanoGenizer (Genizer LLC, Irvine, CA, USA). The lysate was centrifuged (13 000 g; 40 min at 4°C), and the supernatant was loaded onto a 3 ml Ni-NTA agarose column (Qiagen, Inc.) equilibrated with buffer A. The column was subsequently washed with 30 ml buffer A containing 0.2 M imidazole and eluted with 15 ml buffer A containing 0.2 M imidazole. The eluate was loaded onto a HiLoad 16/600 Superdex 200 column (GE Healthcare, Inc.) equilibrated in buffer B (20 mM Tris–HCl, pH 8.0, 75 mM NaCl, 5 mM MgCl₂), and the column was eluted with 120 ml of the same buffer. Fractions containing *E. coli* Rob were pooled and stored at –80°C. *E. coli* Rob derivatives were expressed and purified using the same procedure as the wild type protein. The yields were 4 mg/l, and the purities were > 95%.

E. coli RNAP purification

*E. coli* RNAP was prepared from *E. coli* strain BL21(DE3) (Invitrogen, Inc.) transformed with plasmids of pGEMD (37) and plA900 (38). Single colonies of the resulting transformants were used to inoculate 50 ml LB broth containing 100 μg/ml ampicillin, and cultures were incubated 16 h at 37°C with shaking. Aliquots (10 ml) were used to inoculate 1 L LB broth containing 100 μg/ml ampicillin, cultures were incubated at 37°C with shaking until OD₆₀₀ = 0.6, cultures were induced by addition of IPTG to 1 mM, and cultures were incubated 15 h at 20°C. Then cells were harvested by centrifugation (5000 g; 15 min at 4°C), resuspended in 20 ml lysis buffer C (50 mM Tris–HCl, pH 7.9, 0.2 M NaCl, 2 mM EDTA, 5% glycerol and 5 mM DTT) and lysed using a high-pressure homogenizer NanoGenizer (Genizer LLC, Irvine, CA, USA). The lysate was cleared by centrifugation (13 000 g; 30 min at 4°C), and the supernatant was precipitated by addition of 30 g ammonium sulfate, incubated for 30 min at 4°C with stirring, followed by centrifugation (8000 g; 15 min at 4°C). The precipitate was washed with buffer D (20 mM Tris–HCl pH 7.9 and 5% glycerol) containing 0.5 M NaCl, followed by centrifugation (8000 g; 20 min at 4°C). Then the extract is precipitated by addition of 30 g ammonium sulfate, incubated for 30 min at 4°C with stirring, followed by centrifugation (13 000 g; 30 min at 4°C). Subsequently, the pellet was resuspended in buffer D containing 0.5 M NaCl, and loaded onto a 5 ml column of Ni-NTA agarose (Qiagen, Inc.) pre-equilibrated with the same buffer. The column was washed with 25 ml buffer D containing 20 mM imidazole and eluted with 25 ml buffer D containing 0.15 M imidazole. The eluate was diluted in buffer E (20 mM Tris–HCl, pH 7.9, 5% glycerol, 1 mM EDTA and 1 mM DTT) and loaded onto a Mono Q 10/100 GL column (GE Healthcare, Inc.) equilibrated in buffer E and eluted with a 160 ml linear gradient of 0.3-0.5 M NaCl in buffer E. Fractions containing *E. coli* RNAP were pooled and applied to a HiLoad 16/600 Superdex 200 column (GE Healthcare, Inc.) equilibrated in buffer B. The protein was eluted with 120 ml of the same buffer. Fractions containing *E. coli* RNAP were pooled and stored at -80°C. Yields were 2.5 mg/l, and purity was >95%.

Assembly of *E. coli* Rob-TAC

DNA oligonucleotides *micF* scaffold (sequence is shown in Figure 1A) were synthesized (Sangon Biotech, Inc.) and dissolved in nuclease-free water to 1 mM. Template strand DNA and nontemplate strand DNA were annealed at 1:1 ratio in 10 mM Tris–HCl, pH 7.9, 0.2 M NaCl. Then, *E. coli* Rob-TAC was assembled by incubating *E. coli* RNAP, *micF* scaffold, and *E. coli* Rob in a molar ratio of 1:1:1:10 at 4°C overnight. Subsequently, the mixture was applied to a HiLoad 16/600 Superdex 200 pg column (GE Healthcare Life Sciences) equilibrated in buffer B. After identification by SDS-PAGE and electrophoretic mobility shift assay (EMSA), the fractions containing *E. coli* Rob-TAC were concentrated to 18.7 mg/ml using Amicon Ultra centrifugal filters (10 kDa MWCO, Merck Millipore, Inc.).

Cryo-EM data acquisition and processing

The grids with *E. coli* Rob-TAC were imaged using a 300 kV Titan Krios (FEI, Inc.). Images were recorded with Serial EM (39) in counting mode with a physical pixel size of 1.100 Å or 1.307 Å and a defocus range of 1.4–2.2 μm. Data were collected with a dose of 10 e⁻/pixel/s. Images were recorded with a 10 s exposure and 0.25 s subframes. Subframes were aligned and summed using MotionCor2 (40). The contrast transfer function was vacations for each summed image using CTFFIND4 (41). From the summed images, ~10 000 particles were manually picked and subjected to 2D classification in RELION (42). 2D averages of the best classes were used as templates for auto-picking in RELION. Auto-picked particles were manually inspected, then subjected to 2D classification in RELION. Poorly populated classes were removed, resulting in a dataset of 791 759 particles from 4,372 movies for *E. coli* Rob-TAC-I or a dataset of 417 732 particles from 2099 movies for *E. coli* Rob-TAC-II. These particles were 3D-classified in RELION using a map of *E. coli* RPo (PDB ID: 6CA0) (10) low-pass filtered to 40 Å resolution as a reference. For *E. coli* Rob-TAC-I, 3D classification resulted in 4 classes. Particles in Classes 2 and 3...
were combined and further performed 3D classification focused in the region of Rob. After focused 3D classification, particles in Classes 2, 3, 4 were combined and processed by CTF refinement, Bayesian polishing and 3D auto-refinement, and the best-resolved 386,188 particles was post-processed in RELION. The Gold-standard Fourier-shell-correlation analysis indicated a mean map resolution of 4.06 Å of *E. coli* Rob-TACI (Supplementary Figure S3 and S5). Likewise, For *E. coli* Rob-TACIII, 3D classification resulted in 3 classes. Particles in Class 3 were processed by CTF refinement, Bayesian polishing, 3D auto-refinement, the best-resolved 335,021 particles were post-processed in RELION. And the Gold-standard Fourier-shell-correlation analysis indicated a mean map resolution of 4.57 Å of *E. coli* Rob-TACIII (Supplementary Figure S4 and S6).

Cryo-EM model building and refinement

The model of *E. coli* RNAP RPo (PDB ID: 6CA0) (10), the crystal structure of Rob in complex with *E. coli* micF DNA (PDB ID: 1DSY) (30), and the ternary structure of *E. coli* MarA, DNA and RNAP αCTD (PDB ID: 1XS9) (43) were manually fitted into the cryo-EM density maps in Coot (44), followed by adjustment of main- and side-chain conformations in Coot and real-space refinement using Phenix (45). The coordinates were real-space refined with secondary structure restraints in Phenix.

In vitro transcription assay

*In vitro* Mango-based transcription assays were carried out by incubating *E. coli* RNAP, *micF*-mango scaffold, with or without *E. coli* Rob or its derivatives. Transcription assay was performed in a 96-well microplate format. Reaction mixtures (80 μl) contained: 0 or 4 μM Rob or Rob derivatives, 0.1 μM *E. coli* RNAP, 50 nM *micF*-mango scaffold, 1 μM TO1-Biotin, 0.1 mM NTP mix (ATP, UTP, GTP and CTP), with or without ligand (100 μM chenodeoxycholic acid or 5 mM 2,2′-bipyridine), 40 mM Tris–HCl, pH 8.0, 50 mM NaCl, 10 mM MgCl₂, 5% glycerol. First, *E. coli* RNAP, Rob and *micF*-mango scaffold were incubated for 15 min at 37°C, then mixtures were supplemented with 0.030 mg/ml heparin and incubated for 2 min at 22°C. Then NTP mix and TO1-biotin were added into the mixture and incubated for 10 min at 37°C. Finally, fluorescence emission intensities were measured using a multimode plate reader (EnVision, PerkinElmer Inc.; excitation wavelength = 510 nm; emission wavelength = 535 nm). Relative transcription activities of Rob derivatives were calculated using:

\[
A = \frac{(I - I_0)}{(I_{WT} - I_0)}
\]

where \(I_{WT}\) and \(I\) are the fluorescence intensities in the presence of Rob and Rob derivatives; \(I_0\) is the fluorescence intensity in the absence of Rob.

Electrophoretic mobility shift assay

To further clarify whether Rob derivatives affect the formation of Rob-TAC or not, we also carried out electrophoretic mobility shift assays in reaction mixtures (20 μl) containing: 1 μM Rob or Rob derivatives, 50 nM *E. coli* RNAP, 15 nM *micF* DNA in EMSA buffer (40 mM Tris–HCl, pH 8.0, 100 mM NaCl, 10 mM MgCl₂, 5% glycerol). *E. coli* RNAP was firstly incubated with *micF* DNA for 10 min at 37°C, then incubated with Rob or Rob derivatives for 20 min at 37°C. Reaction mixtures were supplemented with 0.050 mg/ml heparin and incubated for 2 min at 22°C. The reaction mixtures were finally applied to 5% polyacrylamide slab gels (29:1 acrylamide/bisacrylamide), electrophoresed in 90 mM Tris-borate, pH 8.0, and 0.2 mM EDTA, and stained with 45 Red Plus Nucleic Acid Stain (Sangon Biotech, Inc.) according to the procedure of the manufacturer, and analyzed by ImageJ (https://imagej.nih.gov/ij/).

Electrophoretic mobility shift assays (EMSA) of *E. coli* Rob and *RNAP* were performed in reaction mixtures (20 μl) containing: 1 μM Rob or Rob derivatives, 2 μM *E. coli* RNAP, 15 nM *micF* DNA in EMSA buffer. Rob was incubated with *E. coli* RNAP for 10 min at 25°C and then incubated with (or without) DNA scaffold for 15 min at 25°C. Reaction mixtures were supplemented with 0.050 mg/ml heparin and incubated 2 min at 22°C. The reaction mixtures were applied to 5% polyacrylamide slab gels (29:1 acrylamide/bisacrylamide), electrophoresed in 90 mM Tris-borate, pH 8.0, and 0.2 mM EDTA, and stained with 4S Red Plus Nucleic Acid Stain (Sangon Biotech, Inc.) or coomassie brilliant blue staining solution (Sangon Biotech, Inc.) according to the procedure of the manufacturer.

Molecular Docking analysis and the burying surface area measurement

All molecular docking studies were performed using Autodock4.2 package (46). Briefly, Rob from the cryo-EM structure of Rob-TACIII was docked with its potential ligands (chenodeoxycholic acid, 2,2′-bipyridine, sodium decanoate and 4,4′-bipyridine). The molecule was added with non-polar hydrogens and assigned partial atomic charges using AutoDockTools (ADT) (46). The coordinates of potential ligands in Rob structure were generated based on the coordinates of rhodamine 6G (R6G) from the crystal structure of the GyrI-like family protein SAV2435 (PDB ID: 5KAW) (53) in combination with CORINA Classic online service. A grid box with 40 × 40 × 40 grid points and 0.2 Å grid spacing centered roughly at the potential ligand binding position was used as the searching space. 100 runs of L Immunarckian Genetic Algorithm were performed to search the protein-ligand interactions. The results were clustered and ranked. Result analyses and figure rendering were performed using PyMOL.

To measure the potential burying surface areas in the Rob-TACI or Rob-TACIII, we used the online web server (http://sts.bioe.uic.edu/castp) and followed the descriptions as illustrated before (54).

Data analysis

Data for *in vitro* transcription assays or EMSA are means of three technical replicates. Error bars represent mean ± SEM of *n* = 3 experiments. Asterisk (***) or (**) indicates highly significant (*P* value < 0.001) or significant (*P* value < 0.01) difference from the wild-type Rob analyzed by one-way ANOVA with Tukey’s multiple comparison test, respectively.
RESULTS

Overall structure of Rob-TAC

In order to obtain the structure of Rob-TAC, we used a nucleic-acid scaffold corresponding to positions –57 to +13 of micF promoter (positions numbered relative to the transcription start site; Figure 1A). MicF encodes an antisense RNA involved in multidrug resistance by repressing expression of the major membrane porin, OmpF (47,48). The micF DNA scaffold is composed of a 20 bp Rob binding box robbox (including recognition element A site with sequence of GCAC and recognition element B site with sequence of CAAA which overlaps with the –35 element), and a 13 bp non-complementary transcription bubble (–11 to +2) with a consensus –10 element, which was positioned at the catalytic center of RNAP for stabilizing core RNAP and allowing RNA synthesis (Figure 1A) (12,16,18,49). Meanwhile, our in vitro Mango-based transcription assay showed that, in the presence of Rob, *E. coli* RNAP efficiently activated transcription of micF-mango promoter, reflecting that these purified proteins are biologically relevant (Supplementary Figure S2A and B). By combing *E. coli* RNAP, Rob and micF DNA scaffold, we successfully assembled Rob-TAC complex in a stoichiometric manner as evidenced from SDS-PAGE and native PAGE analyses (Supplementary Figure S2A, C and D).

The frozen Rob-TAC complex was subsequently analyzed on a Titan Krios cryo-EM, and the data was sequentially processed, modelled and refined. Fortunately, we finally trapped two structures of Rob-TAC, namely Rob-TACI and Rob-TACII, at nominal resolutions of 4.06 and 4.57 Å, respectively (Figure 1B and C; Supplementary Figures S3–S7; Table 1). Local resolution calculation exhibits two central core RNAP at 3.5–4.5 Å and peripheral RNAP αCTD and Rob at 5.5–7.0 Å (Supplementary Figure S5C and 6C). There is only one Rob molecule present in each complex, simultaneously engaging RNAP αCTD and robbox of promoter DNA, as is different from the reported dimeric transcription activators (13,14). Though the two structures superimpose onto each other well at the densities of *E. coli* RNAP core enzyme, promoter DNA, and Rob NTD, the obvious extra density of...
Rob CTD in Rob-TACII exhibits significant differences from that of Rob-TACI, with a rotation of ~35° (Supplementary Figures S7D). In Rob-TACI, Rob CTD retains in close proximity to RNAP αCTD and helix 1 from Rob NTD, possibly playing a potential role in RNAP recruitment and facilitates DNA binding (Supplementary Figures S7B and C). While in Rob-TACII, Rob CTD moves away from RNAP αCTD and helix 1 of Rob NTD, mainly interacts with the C-terminal portion of Rob NTD and the long linker connecting Rob NTD and Rob CTD, rendering the acidic loop of Rob CTD close to the downstream promoter (Figure 1A and B). Since the density and occupancy of Rob CTD in Rob-TACII seem to be significantly weaker and lower than that in Rob-TACI, we infer that the non-stable Rob-TACI is most probably a pre-state of the stable Rob-TACII. Therefore, we choose Rob-TACII to be described in the following section.

Comparative structural analysis shows that E. coli RNAP containing σ^70 in Rob-TAC structures maintains in a similar conformation to that of E. coli RNAP-promoter open complex (PDB ID: 6CA0) (10) with root mean square deviation (RMSD) of 1.410 Å (3237 Cα aligned) and 1.492 Å (3237 Cα aligned) in Rob-TACI and Rob-TACII, respectively. By aid of β' coiled coil, σ^70R2 contacts the consensus −10 element. RNAP accommodates the transcription bubble and downstream dsDNA in the same way as those in RPo (10) (Supplementary Figures S8 and S9). One distinguishing feature of Rob-TACII is that Rob occludes σ^70R4 from binding to the -35 element and specifically binds rob-box. Furthermore, the co-crystal structure of Rob complexed with mifF double stranded DNA could be well fitted into the upstream promoter density map, except for the obvious DNA bending towards Rob CTD (Supplementary Figure S7E), especially the acidic loop of Rob. Such DNA conformational change shares some similarities with that of MarA-mar binary complex (PDB ID: 1BL0) (31) (Figure 1B and C; Supplementary Figure S7B and C).

**RNAP αCTD is required for Rob-dependent transcription activation of class II promoters**

Distinct from previous in vitro transcription assay that inferred RNAP αCTD was not required for transcription activation of class II promoters (50), in Rob-TACII, there exists abundant interactions between Rob NTD and RNAP αCTD, burying a surface area of 149.39 Å^2 (Figure 2A-B). Evidently, residues Q3, G5, D9 and W13 from helix α1 of Rob form hydrogen bonds with residues P293 and N294 in the N-terminal loop of helix α1, and helix α4 of RNAP αCTD, respectively. Residues G5 and G32 of Rob form van der Waals contacts with residues V264 and L295.
of RNAP αCTD (Figure 2C). Moreover, residue Y33 in the loop connecting helix α2 and α3 of Rob generates hydrogen bonds with residues from the DNA binding ‘265 determinant’ (R265 and K298) of RNAP αCTD (Figure 2C), indicative of DNA binding deficiency of RNAP αCTD targeting at promoter UP elements (AT-rich DNA regions locate approximately from −40 to −60 of a promoter) (51). Consistent with this, mutation of the key residues of Rob-RNAP αCTD interaction led to obvious decrease in both transcription activation activities by Mango-based transcription assay and Rob-TAC formation activities evidenced by EMSA (Figure 2D, Supplementary Figure S10A and B), suggesting the necessity of these residues to Rob-dependent transcription activation.

In comparison, structural analysis shows that interactions between Rob and RNAP αCTD in Rob-TACII are similar to those between MarA and RNAP αCTD (MarA-RNAP αCTD, PDB ID: 1X39) (43) (with DNA omitted for clarity, Figure 2E) as well as between the class II transcription activator protein TAP (T. thermophilus TTHB099) and RNAP αCTD from TAP-dependent transcription activation complex (TAP-TAC, PDB ID: 5I2D, Figure 2F) (13), with buried surface areas being 123.81 Å² and 182.54 Å², respectively. In Rob-TACII and MarA-RNAP αCTD complexes, each activator uniquely contacts the DNA binding ‘265 determinant’ of RNAP αCTD by the homologous helices and loops. Likewise, in TAP-TAC, activating region 4 (AR4) of one TAP monomer interacts with a cluster of residues in the N-terminus of helix α4, the loop connecting helix α3 and α4, and ‘265 determinant’ of RNAP αCTD (Figure 2F). These implies the important regulatory role of the ‘265 determinant’ of RNAP αCTD.

Rob occludes σ70R4 from binding to promoter -35 element and synergistically redirects RNAP to Rob regulons

Despite the critical interactions between σ70R2 and promoter consensus –10 element, interactions between σ70R4 and promoter consensus –35 element have also been extensively proved to be essential for RPo formation and transcription activation (3,5,10,12). While in case of class II promoters that contain activator binding box overlapping with –35 element to some extent, σ70R4 remodeling is required to maintain successful transcription initiation. As expected, in Rob-TACII, Rob forms several types of interactions with the long helix α5 of σ70R4, with a buried surface area of 40.46 Å² (Figure 3A-C). Thus, helix α5 and the adjacent loop of Rob in Rob-TACII occlude σ70R4 from binding to –35 element, rendering it makes weaker interactions with DNA (Figure 3C and D). Residues S84, Q85, Q86 in the loop connecting helix α5 and α6 of Rob form three hydrogen bonds with residues Q589, R588 and E585 in σ70R4, respectively. Residues D75 and D83 of Rob simultaneously make ionic bonds with residues R596, R599, K593 and R586 from σ70R4. Furthermore, residues of L74 and L78 from Rob form van der Waals contacts with the hydrophobic residue A592 from σ70R4. In accordance with the above interactions (Figure 3E), our transcription and EMSA analyses of mutants also showed that the key residues are functionally relevant (Figure 3F; Supplementary Figure S10B). This observation is consistent with the previous genetic and biochemical experiments which suggested that Rob possibly occludes σ70R4 from binding to promoter -35-hexamer and synergistically redirect RNAP to specific robbox-containing promoters (32).

Rob specifically interacts with two DNA major grooves of robbox and the acidic loop facilitates such interaction

As a representative example of the AraC/XylS family transcription activators, Rob harbors two highly conserved DNA-binding HTH motifs (designated as HTHA and HTHB, respectively) in Rob NTD (Figure 4A). Unlike MarA, which can insert its two conserved HTH motifs into the corresponding DNA major grooves of mar promoter and binds DNA by 35°, the co-crystal structure of Rob–nicF showed that Rob could only insert helix α3 of HTHA into the A site of robbox, with helix α6 of HTHB contacting the phosphate backbone of B site, and thus rendering pro-
Figure 4. The interactions between Rob and the micF promoter DNA. (A) Domain architecture of Rob, SoxS and MarA (top panel); the sequences of the micF promoter for Rob protein with the A- and B-box sequences highlighted in red and green, respectively (bottom panel). (B) Rob in complex with the micF promoter DNA. Rob-NTD and Rob-CTD are represented as blue or orange cartoon, respectively. (C, D) Detailed interactions between Rob and the micF promoter DNA. Residues from Rob involved in interacting with A- or B-site sequences of micF promoter DNA are shown in green sticks. (E) Zoom-in view of the interactions between the acid loop and the micF promoter DNA. The acidic loop (residues 187-193) connecting strands β3 and β4 in the extra C-terminal domain of the Rob protein is highlighted in magenta. Residues from the acidic loop that contact DNA are shown in green sticks. (F) Substitutions of residues involved in Rob-DNA interactions suppressed in vitro transcription activity. Data for in vitro transcription assays are means of three technical replicates. Error bars represent ± SEM of n = 3 experiments. Asterisk (***) or (**) indicates highly significant (P value < 0.001) or significant (P value < 0.01) difference from the wild-type Rob analyzed by one-way ANOVA with Tukey’s multiple comparison test, respectively.
moter DNA unbent (Supplementary Figures S11A) (30). However, some other in vivo and in vitro assays on Rob held different opinions (32,52) and it needs to be further clarified. Surprisingly, both helix α3 of HTHα and helix α6 of HTHβ in Rob-TACII insert into the DNA major grooves of robbx and make the DNA bent with an orientation similar to that of MarA (Figure 4A–D; Supplementary Figures S11B), revealing a general promotor remodeling mode for the AraC/XylS family transcription activators. By using helix α3 of HTHα and helix α6 of HTHβ, Rob makes abundant interactions with the corresponding DNA major grooves of robbx (Figure 4C and D; Supplementary Figures S11C). As to the A site of robbx, residues W36 and S34 of Rob form hydrogen bonds with –45A and -47G of the non-template strand DNA, respectively. Residues Y33, H37 of Rob make contacts with the DNA phosphate backbone of the non-template strand DNA. Both of residues Q39 and R40 form two hydrogen bonds with the template strand DNA, indicating their critical roles in stabilizing DNA. Besides, several residues (D25, N26, K35, K43, D44, A49, G51) from helix α3 and the adjacent loops interacts with the DNA phosphate backbone of the template strand DNA. In respect to the B site of robbx, residues Q86 and R90 of Rob insert into the major groove and specifically recognize bases of –34T, –35T from the template strand DNA, and –37T, –38G from the non-template strand DNA (Figure 4D). In addition, residues of R55, D83, S84, T87, K94 make extensive contacts with the non-template strand DNA, while residues of Q86, K93, T89, T99 contact template strand DNA. Another striking feature of Rob-TACII is that the acidic loop of Rob (spanning from S187 to E193) moves in close proximity to the bent nicF promoter DNA. Structural analysis shows that residue K190 makes obvious ionic bonds with the DNA phosphate backbone of –31G and -32T from the template strand DNA (Figure 4E). In good agreement with these observations, substitutions of the above key residues involved in Rob-DNA interactions (Y33A, Q39A and R90A) and acidic loop (K190A, del187-193) confer significant defects in transcription activation with Rob-DNA and promoter DNA.

Rob CTD activates transcription through allosteric coordination with Rob NTD

In E. coli, Rob is postulated as a global regulator of multidrug resistance. This is mainly attributed to the distinct Rob CTD which forms a global GyrI-like domain capable of binding small molecules (25,26,53). However, the detailed molecular mechanism is poorly determined. To gain insight into the ligand binding properties and the evoked conformation changes of Rob, we compared Rob from Rob-TACII with other GyrI-like domains in complex with ligands from other transcription factors involved in bacterial stress response (18,53,55). Intriguingly, comparative structural analysis shows that Rob CTD superimposes well with the evolutionarily conserved GyrI-like domain family regulators, such as Staphylococcus aureus SAV2435 (PDB ID: 5KAW) with an RMSD of 2.21 Å (133 Ca aligned) (Figure 5A) (53). These GyrI-like domains are mainly composed of antiparallel β-strands, connecting loops and long characteristic helices, and have been proved to be adaptive for broad selectivity of ligand binding and biological signaling. These ligand binding pockets exhibit similar stereochemical properties and some connecting loops were also verified to play regulatory roles in the allosteric regulation process. Consistent with the above points, our molecular docking analysis of Rob with rhodamine 6G (R6G) and other four potential ligands (chenodeoxycholic acid, 4,4′-bipyridine, sodium decanoate and 2,2′-bipyridine) exhibits well fitted ligand binding pockets (Figure 5A–C; Supplementary Figure S12), indicating similar allosteric regulation mode. In accordance with the well-defined nature of ligand binding pockets, all the four potential ligands are mainly surrounded by cluster of aromatic and heterocyclic amino acids (Figure 5C and D; Supplementary Figure S12B and D), and mutation of the involved conserved residues largely compromises Rob-dependent transcription activity and Rob-TAC formation, especially Rob W164A and E262A (Figure 5E; Supplementary Figure S12E and F; Supplementary Figure S10E). This implies their biological significance in maintaining stereochemical conformation of Rob CTD and activating Rob-dependent transcription activity. As allosteric regulation model has been proposed for GyrI-like domain containing factors upon effector binding, it is therefore tempting to speculate that Rob may allosterically activate transcription through synergistical coordination with its effector sensor CTD and effector responder NTD.

**DISCUSSION**

Bacteria has established diverse transcription regulation strategies to respond to a variety of environmental stresses and transcription activation is a well-known strategy. Though both in vivo and in vitro experiments have elucidated that the AraC/XylS family transcription activators play fundamental roles in multidrug resistance, metabolism of carbohydrate, heavy metals sensing, and virulence (20,22,23), long-standing questions regarding to their molecular mechanism still remain. Based on our structural and biochemical analysis of Rob-TAC, several key fea-
agreement with the mutational analysis that different determinants of RNAp αCTD are required for CRP-dependent transcription activation (13,43,56,57). The Rob-σ70R4 interactions occlude σ70R4 from binding to canonical promoter elements, make it acting as a co-sigma factor. This also resembles the efficient σ appropriation mechanism mediated by MotA and AsIA (49). Sequence alignment of Rob and its homologs (MarA, SoxS, TetD and RamA) shows that the key residues (D9, W13, Y33 and D75) of Rob involved in the above interfaces are highly conserved (Supplementary Figure S13), implying similar significant protein-protein interactions for the AraC/XylS-family transcription factors to remodel RNAp. (iii) Rob remodels promoter DNA not only by its two conserved HTH motifs, but also by aid of the acidic loop, which possibly facilitates DNA bending and promotes formation and stability of Rob-TAC. (iv) The global Gyrl-like domain containing Rob CTD serves as an environmental stimuli sensor, which structurally stabilizes and activates transcription through allosteric coordination with the stress responder Rob NTD.

It is worthwhile to mention several observations during our experiments. (i) Rob protein we purified exhibits obvious transcription activity; (ii) Rob CTD deletion mutant (residues 1–133) was mostly expressed as inclusion bodies and reduced greatly in solubility; (iii) The two alternative conformations of Rob-TAC we obtained show active ‘dispersal’ state, which may include an effector molecule during expression and purification. In agreement with this, the effectors play an insignificant role in enhancing transcription activities and Rob-TAC formation (Supplementary Figure S12E, S10A, B and E), and mutation of the potential ligand binding residues of Rob CTD apparently cause defects on transcription activities (Figure 5E; Supplementary Figure S12F). These data demonstrate that Rob CTD is essential for the stability and activity of Rob, and ligand is required for Rob-dependent transcription activity through binding to Rob CTD. Consistent with previous in vitro results, this also provides favorable evidence for the ‘sequestration-dispersal’ mechanism of Rob in vitro, which proposes Rob CTD as a novel off-on switch for the regulation of Rob’s activation activity upon ligand binding (25,26,29,47). Thus, it is probably that in the ‘sequestration’ state, the residues involved in Rob-RNAP αCTD and Rob-σ70R4 interfaces might be enclosed by the large Rob CTD (and/or a cofactor) and retained in an inactive manner; while ligand binding is able to change the ‘sequestration’ conformation of Rob into a ‘dispersal’ one, and the above enclosed residues of Rob NTD will be exposed to engage RNAp and finally initiate transcription. (iv) To identify the two alternative conformations of Rob-TAC, further single-molecule FRET assay was performed on the scaffold with a Cy5 fluorophore labelled on template strand of micF DNA2 and a Cy3B fluorophore on the 281-Cystine of Rob mutant (Supplementary Methods). The results showed only one FRET peak in each assay, and the FRET values were comparable and consistent with a distance of 5.5 nm from Rob-TACII rather than 7.7 nm from Rob-TACI (Supplementary Figure S14). Considering on this, we propose that the conformation of Rob-TACII is physiologically relevant and may have dominant significance for Rob-dependent transcription activation.
Figure 6. Proposed models for class-II-activator-dependent and Rob-dependent transcription activation. (A) Proposed working model for class-II-activator-dependent transcription activation. (B) Proposed working model for Rob-dependent transcription activation. Rob shows weak transcription activation activity in the absence of Rob-RNAP interactions, and activates the transcription of most genes with the promoter containing Rob binding box under stress.

In summary, a model for Rob-dependent transcription regulation can be proposed (Figure 6B). When environmental stimuli are absent, Rob CTD keeps in inactive sequestration state, and thus transcription cannot be initiated. Upon ligand binding, Rob CTD undergoes conformation change, turns Rob into de-sequestration active state, and relocates itself in cells. If Rob encounters a robbox-containing promoter, Rob CTD along with its acidic loop plays critical roles in the coordination with Rob NTD, promote two HTH motifs to insert into the corresponding DNA major grooves, render DNA bent to form Rob-TACII and finally initiate transcription efficiently. Consistent with the fact that Rob interacts with both RNAP αCTD and σ70R4 as revealed in the structure of Rob-TAC, Rob was found to form larger complex with RNAP in the absence of promoter DNA (Supplementary Figure S10C), suggestive of a ‘pre-recruitment’ mechanism, implying Rob possibly also activates transcription through a ‘pre-recruitment’ mechanism like SoxS (58). However, whether Rob activates transcription at the promoter clearance stage yet needs to be further clarified.

Comparative structural analysis shows that Rob CTD endows the evolutionarily conserved GyrI-like domain as the other GyrI-like domain containing transcription regulators (53). These GyrI-like domains possess similar residue composition, stereochemical properties, and potential allosteric regulation mechanism, implying their significant physiological roles. This resembles the recently reported Bacillus subtilis BmrR (PDB: 7CKQ) (18) and E. coli EcmrR (PDB: 6XL5) involved in multidrug-sensing (55). However, there exists some big differences among them. Unlike the monomeric Rob, both BmrR and EcmrR function as dimers, sit on the distal face of promoter DNA, show no or few interactions with RNAP, and activate transcription mainly through promoter-distortion mechanism. This reveals that it is the two small conserved HTH motifs and the accessory acidic loop that accomplishes similar promoter remodeling functions to the above-mentioned dimers, which may reflect evolutionary significance to the AraC/XylS-family transcription factors.

By performing Pfam search using Rob sequence as a query, 2650 homologous protein sequences from various bacteria show the same architecture of ‘HTH_18 plus GyrI-
like’ as Rob and are classified as transcription regulators, including AfrR and Caf1-R, revealing the global distribution and diverse functions of these ‘Rob-like regulators’. Further sequence analysis shows that critical residues from HTH motifs of Rob NTD and aromatic residues in Rob CTD are highly conserved in these Rob-like regulators, which is in good agreement with the key residues defined in our Rob-TAC, implying their general regulatory roles during the physiological adaptation processes. However, new molecular transcription activation mechanism about them is yet to be explored experimentally. Meanwhile, it also unveils the potential role of Rob in providing a novel target for the antibacterial drug discovery.

During the review process of this manuscript, Hao et al. published the structure of RamA-class II activator complex, which also visualized a homologous activator interacts with RNAP αCTD as well as σ70R4 (59), exhibiting similar interfaces as those in TAP-TAC and our Rob-TACII. Since CAP shares great similarity with TAP, though RNAP αCTD was invisible in CAP-TACII due to its high flexibility (18), it might probably engage RNAP in a similar dynamic manner. These data collectively reveal a general regulation mode for class II activator-dependent transcription activation. Considering that RNAP αCTD is conserved among all types of organisms, it could be generally applicable to other transcription factors in other organisms. However, not all class II activators remodel σ70R4 of RNAP by occluding the interactions between σ70R4 and -35 consensus element (such as TAP), this regulation mode of Rob might be an accessory way to efficiently remodel RNAP. As to class I promoters containing an activator binding site upstream of the −35 element, the corresponding activator is too far in distance to contact σ70R4, and it may variably activate transcription by combination with the highly distinctive determinants of RNAP αCTD (one or two), σ70R4 and promoter DNA.

DATA AVAILABILITY

Accession number for cryo-EM density map: EMD-32166 for of Rob-TACII and EMD-32165 for Rob-TACIII (Electron Microscopy Data Bank). Accession number for atomic coordinates: 7VWZ for of Rob-TACIII and 7VWW for Rob-TACII (Protein Data Bank).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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