

Purification and Characterization of the AAA+ Domain of *Sinorhizobium meliloti* DctD, a σ^{54} -Dependent Transcriptional Activator

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Activators of σ^{54} -RNA polymerase holoenzyme couple ATP hydrolysis to formation of an open complex between the promoter and RNA polymerase. These activators are modular, consisting of an N-terminal regulatory domain, a C-terminal DNA-binding domain, and a central activation domain belonging to the AAA+ superfamily of ATPases. The AAA+ domain of *Sinorhizobium meliloti* C₄-dicarboxylic acid transport protein D (DctD) is sufficient to activate transcription. Deletion analysis of the 3' end of *dctD* identified the minimal functional C-terminal boundary of the AAA+ domain of DctD as being located between Gly-381 and Ala-384. Histidine-tagged versions of the DctD AAA+ domain were purified and characterized. The DctD AAA+ domain was significantly more soluble than DctD_(Δ 1-142), a truncated DctD protein consisting of the AAA+ and DNA-binding domains. In addition, the DctD AAA+ domain was more homogeneous than DctD_(Δ 1-142) when analyzed by native gel electrophoresis, migrating predominantly as a single high-molecular-weight species, while DctD_(Δ 1-142) displayed multiple species. The DctD AAA+ domain, but not DctD_(Δ 1-142), formed a stable complex with σ^{54} in the presence of the ATP transition state analogue ADP-aluminum fluoride. The DctD AAA+ domain activated transcription *in vitro*, but many of the transcripts appeared to terminate prematurely, suggesting that the DctD AAA+ domain interfered with transcription elongation. Thus, the DNA-binding domain of DctD appears to have roles in controlling the oligomerization of the AAA+ domain and modulating interactions with σ^{54} in addition to its role in recognition of upstream activation sequences.

Bacteria often possess a form of RNA polymerase holoenzyme that has σ^{54} as its sigma factor, referred to as σ^{54} -RNA polymerase holoenzyme or σ^{54} -holoenzyme (1, 20). σ^{54} -Holoenzyme regulates the expression of genes involved in a variety of cellular processes, including nitrogen assimilation and fixation, C₄-dicarboxylic acid transport, degradation of aromatic compounds, hydrogen metabolism, flagellar biogenesis, and response to phage infection (13, 20, 39). σ^{54} -Holoenzyme binds to the promoter to form a stable closed complex, but isomerization of this complex to an open complex that can initiate transcription requires a transcriptional activator (21, 30, 37). Activators of σ^{54} -holoenzyme usually bind to sites located 70 bp or more upstream of the promoter, known otherwise as upstream activation sequences (UAS) or enhancers (2, 32). After binding the UAS or enhancer, the activator contacts σ^{54} -holoenzyme bound at the promoter through DNA looping (33, 40). Productive contacts between the activator and σ^{54} -holoenzyme lead to formation of an open complex in a reaction that requires ATP hydrolysis by the activator (46).

Activators of σ^{54} -holoenzyme are modular in structure, generally consisting of an N-terminal regulatory domain, a central domain required for ATP hydrolysis and transcriptional acti-

vation, and a C-terminal DNA-binding domain (39). The central domain is well conserved among σ^{54} -dependent activators and for some activators is sufficient to activate transcription if it is expressed at higher than normal levels (7, 8). This domain belongs to the AAA+ superfamily (ATPases associated with various cellular activities) of ATPases (23, 28).

AAA+ proteins are found in all three domains of life and are involved in a variety of cellular processes, including membrane fusion, proteolysis, DNA replication, and transcription (23, 28). In general, the diverse functions of AAA+ proteins are manifested through chaperone-like activities that reorganize protein-protein or protein-DNA interactions (28, 42). AAA+ proteins undergo conformational changes during the ATP hydrolysis cycle which are thought to exert force on the bound substrate, allowing these proteins to carry out their respective biological activities (4, 36).

AAA+ proteins form oligomeric ring structures that often consist of six subunits but may have from five to eight subunits depending on the protein (28, 42, 47). Crystal structures for AAA+ proteins show that the active site for ATP hydrolysis is located between adjacent subunits within the ring structure (28, 42, 47). The ring structures of AAA+ proteins can be created through the regulated assembly of monomers or dimers, the formation a stable ring upon assembly of the subunits, or the formation of a ring from a single polypeptide chain containing multiple AAA+ domains (42). Activators of σ^{54} -holoenzyme appear to form ring structures through the regulated assembly of subunits (18). Crystal structures were reported recently for the ADP-bound form of the ATPase domain alone and the ATPase and adjacent regulatory do-

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mains of *Aquifex aeolicus* NtrC1, a σ^{54} -dependent activator (18). The protein consisting of the ATPase and adjacent regulatory domains crystallized as a dimer, while the ATPase domain by itself crystallized as a heptameric ring.

C_4 -dicarboxylic acid transport protein D (DctD) activates transcription of *dctA*, which encodes a permease for C_4 -dicarboxylic acids in *Sinorhizobium meliloti* and *Rhizobium leguminosarum* (25, 34). Removal of the N-terminal regulatory domain of DctD was shown previously to result in a constitutively active form of the protein (6, 17). A large collection of mutant forms of DctD that fail to activate transcription has been generated and characterized (5, 43, 44), and further structural information about these proteins will assist in understanding the function of DctD and other σ^{54} -dependent activators.

We report here on the deletion analysis of *S. meliloti* DctD, which allowed us to precisely define the minimal functional C terminus of the AAA+ domain of this σ^{54} -dependent activator. The purified DctD AAA+ domain was shown to bind σ^{54} , hydrolyze ATP, and activate transcription *in vitro*. Interestingly, the DctD AAA+ domain was significantly more soluble than DctD_(Δ 1-142), a truncated DctD comprised of the AAA+ and C-terminal DNA-binding domains, suggesting that it may be more amenable for structural studies than DctD_(Δ 1-142).

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* strain DH5 α [ϕ 80*lacZ* Δ M15 *recA1* *gyrA96* *thi-1* *hsdR17*(r_K^- m_K^-) *supE44* *relA1* *deoR* Δ (*lacZYA-argF*)U169] was used for cloning procedures. *E. coli* strain JM109 (*endA1* *recA1* *gyrA96* *thi* *hsdR17*(r_K^- m_K^-) *relA1* *supE44* Δ (*lac-proAB*) [*F'* *traD36* *proAB* *lacPZ* Δ M15]) was used to assay the *in vivo* activities of the products of the mutant *dctD* genes with 3' deletions. The histidine-tagged DctD AAA+ domains were expressed in *E. coli* strain BL21(DE3) [*F'* *ompT*(*lon*) *hsdS_B*(r_B^- m_B^-) *dcm* *gal* λ (DE3)]. Concentrations of antibiotics in the media for selection were 200 μ g of ampicillin/ml, 20 μ g of kanamycin/ml, and 10 μ g of tetracycline/ml.

Construction of 3' deletions of *dctD*. Plasmid pL143 contains codons 143 to 460 of *S. meliloti* *dctD* fused to codon 11 of *E. coli* *lacZ* from pUC13 and expresses DctD_(Δ 1-142), which is a truncated, constitutively active form of DctD (15). Plasmids containing various 3' deletions of *dctD* were generated by digesting pL143 with EcoRV and then treating the linearized plasmid with nuclease Bal31. EcoRV cuts within *dctD* between codons 404 and 405. Following treatment with Bal31, the ends of the DNA were filled in with T4 DNA polymerase and the plasmid was digested with HindIII, which cuts upstream of *dctD*. DNA fragments were isolated from low-melting-point agarose gels and ligated into the HindIII and HincII sites of pUCX, which has translation termination signals in all three reading frames immediately downstream of the HincII site. Plasmid pUCX was constructed by inserting the XbaI linker 5'-CTAGTCTAGACTA G-3' into the SmaI site of pUC13. Underlined sequences indicate the translation termination signals that were introduced in each of the three reading frames. The fusions in all constructs that were analyzed further were sequenced to identify the 3' end of *dctD* and to verify that mutant DctD_(Δ 1-142) proteins of the expected size were expressed.

Construction of plasmids for expression of DctD AAA+ module. Plasmid pHX182 carries a truncated *S. meliloti* *dctD* allele consisting of codons 141 to 390 which encodes the DctD AAA+ domain and is under control of the T7 promoter in vector pET28a (Novagen). A sequence encoding a hexahistidine tag (amino acid sequence of the tag is MGSSHHHHHSSGLVPRGSHM) occurs at the 5' end of the *dctD* sequence in plasmid pHX182. The *dctD* allele in plasmid pHX182 was amplified by PCR using the primers 5'-GGCCGAAGCCCATAT GGAAGGCCTGC-3' and 5'-CAATCTTTCCTCGAGGGTCGCTC4GCTTG AGG-3' with plasmid pYKW3 (44) as the template. Underlined sequences indicate restriction sites for NdeI and XhoI, respectively, and the italics indicate a base change that introduced a translational stop signal. The amplified DNA fragment was inserted into pET28a that had been digested previously with NdeI and XhoI.

Plasmid pHX183 carries a truncated *S. meliloti* *dctD* allele consisting of codons 141 to 394 and is also under control of the T7 promoter in plasmid pET28a. A

sequence encoding a hexahistidine tag (amino acid sequence of the tag is EH-HHHHH) occurs at the 3' end of the *dctD* sequence in plasmid pHX183. The *dctD* allele in plasmid pHX183 was amplified with the primers 5'-CAATCTTT CCTCGAGGGTCGCTC-3' and 5'-CGAAGCCGCATGGAAGGCCTGC-3', with pYKW3 as the template. Underlined sequences indicate restriction sites for XhoI and NcoI, respectively, which were introduced so that the amplified DNA fragment could be cloned into the same sites in plasmid pET28a.

β -Galactosidase assays. The ability of the various mutant forms of DctD to activate transcription was monitored from *dctA*'-'*lacZ* reporter genes carried on pRKRMAZ:+UAS or pRKRMAZ:-UAS (15), which possess or lack the *dctA* UAS, respectively. *E. coli* cultures were grown in Luria-Bertani broth, and expression of the DctD proteins was induced by the addition of 0.5 mM isopropyl- β -D-thiogalactopyranoside to the growth medium as described previously (6). Cells were harvested by centrifugation, cell extracts were prepared by sonication, and spectrophotometric assays of β -galactosidase activity using *o*-nitrophenyl- β -D-galactopyranoside as a substrate were done as described previously (14).

Expression and purification of DctD proteins. The histidine-tagged DctD AAA+ domain proteins were expressed in *E. coli* strain BL21 (DE3) by growing the cells in Luria-Bertani broth to an optical density at 600 nm of \sim 0.4, at which point 0.5 mM isopropyl- β -D-thiogalactopyranoside was added to the growth medium. After 3 to 4 h, cells were harvested by centrifugation, resuspended in buffer A (20 mM N-[2-hydroxyethyl]piperazine-N'-3-propanesulfonic acid [pH 8.0], 65 mM potassium thiocyanate, 5% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and lysed in a French pressure cell at 12,000 lb/in². Crude cell lysates were clarified by centrifugation at 10,000 \times g for 40 min. The resulting supernatant liquid was applied to a nickel-nitrilotriacetic acid resin (Qiagen), and the histidine-tagged DctD AAA+ domain proteins were eluted from the column with 250 mM imidazole as per the supplier's recommendations. Column fractions containing the histidine-tagged proteins were pooled, dialyzed against buffer B (20 mM Tris-HCl [pH 8.8], 20 mM potassium thiocyanate, 5% glycerol, 1 mM dithiothreitol), and applied to a HiTrap Q anion exchange column (5 ml; Pharmacia). The histidine-tagged DctD AAA+ domain proteins were eluted from the anion exchange column with \sim 200 mM KCl in a linear gradient to 1 M KCl in buffer B. DctD_(Δ 1-142) and an N-terminal histidine-tagged version of DctD_(Δ 1-142) were purified as described previously (17, 44).

Expression and purification of σ^{54} proteins. Plasmid pHX185 is a derivative of plasmid pET28a that carries the *S. meliloti* *rpoN* gene (encodes σ^{54}) under control of the T7 promoter in the vector. In plasmid pHX185, *S. meliloti* *rpoN* is joined at its 5' end to a sequence encoding a hexahistidine tag (sequence of the tag is MGSSHHHHHGLVPRGSHN). *S. meliloti* *rpoN* was amplified from plasmid pNtr3.5BE (35) with the primers 5'-GGAGTTTCACACCCATATGG CCTTG-3' and 5'-CCCGTCATCCAAGCTTTTCAGCGACCGG-3'. Underlined sequences indicate restriction sites for NdeI and HindIII, respectively, which were introduced so that the amplified DNA fragment could be cloned into the same sites in plasmid pET28a.

The histidine-tagged *R. meliloti* σ^{54} was expressed in *E. coli* strain BL21(DE3) by growing the cells in Luria-Bertani broth to an optical density at 600 nm of \sim 0.4, at which point 0.5 mM isopropyl- β -D-thiogalactopyranoside was added to the growth medium. After 3 to 4 h, cells were harvested by centrifugation, resuspended in buffer A, and lysed in a French pressure cell at 12,000 lb/in². Crude cell lysates were clarified by centrifugation at 10,000 \times g for 40 min. The resulting supernatant liquid was applied to a nickel-nitrilotriacetic acid resin column, and the histidine-tagged σ^{54} was eluted from the column with 250 mM imidazole. Fractions containing histidine-tagged σ^{54} were pooled, dialyzed against buffer C [20 mM N-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid (pH 8.0), 20 mM potassium thiocyanate, 5% glycerol, 1 mM dithiothreitol], and applied to a HiTrap heparin column (5 ml; Pharmacia). The histidine-tagged σ^{54} was eluted with \sim 350 mM KCl in a linear gradient to 1 M KCl in buffer C. Native and N-terminal histidine-tagged *Salmonella enterica* serovar Typhimurium σ^{54} proteins were expressed and purified as described previously (11, 17).

Native gel analysis and σ^{54} -binding assays for DctD proteins. The mobilities of proteins on native gels were analyzed after diluting purified DctD_(Δ 1-142), DctD₍₁₄₁₋₃₉₀₎, DctD₍₁₄₁₋₃₉₄₎, or PspF Δ HTH to a final concentration of 5 μ M monomer in 20 μ l of STA buffer (25 mM Tris-acetate [pH 8.0], 8 mM magnesium acetate, 100 mM KCl, 1 mM dithiothreitol, and 3.5% [wt/vol] PEG 6000). PspF Δ HTH was kindly provided by Chih Lew (University of California at Los Angeles). Where indicated, ADP-aluminum fluoride (ADP \cdot AlF₃, where x is 3 or 4) was prepared *in situ* in assays by mixing 0.2 mM ADP and 5 mM NaF with the proteins in the STA buffer for 5 min at 30°C and then adding 0.2 mM AlCl₃. After 10 min of incubation, samples were loaded onto a 7.5% native polyacrylamide gel and subjected to electrophoresis at 15 V/cm. The electrophoresis and gel buffer contained 25 mM Tris base and 200 mM glycine (pH 8.6). Proteins

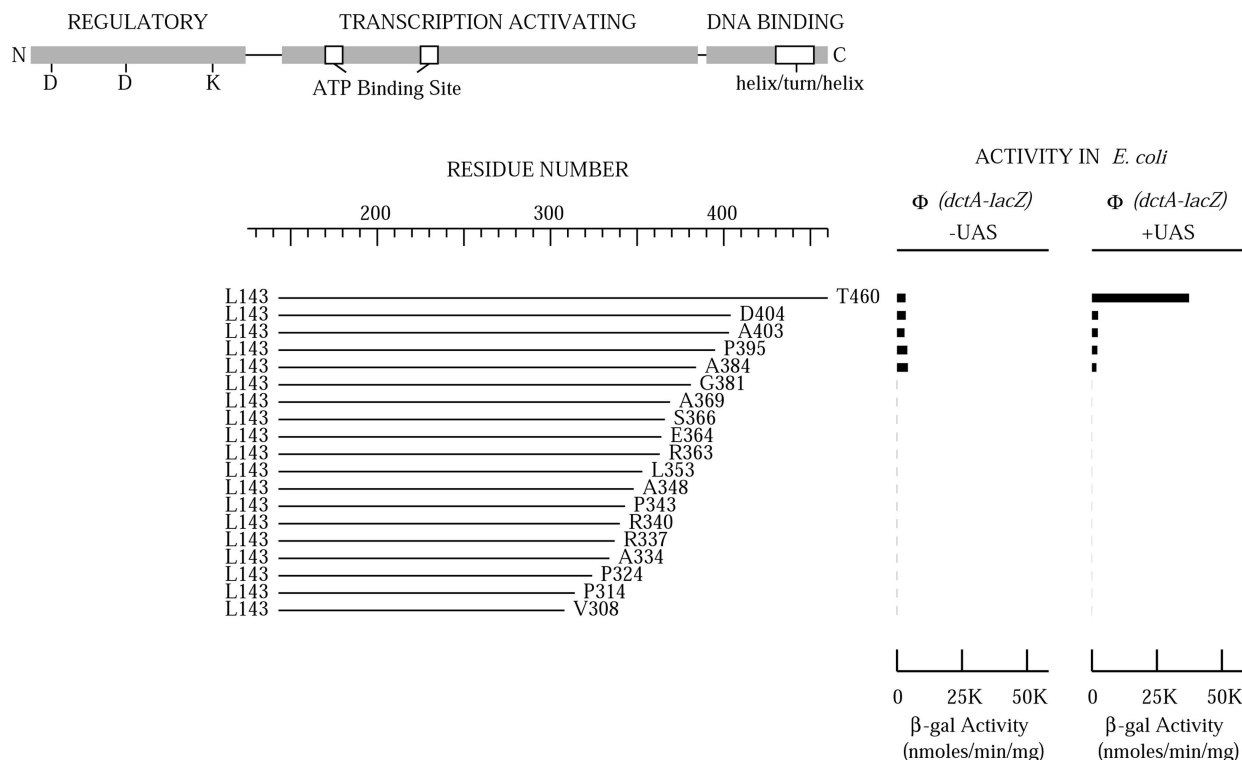


FIG. 1. In vivo activities of C-terminally deleted DctD_(Δ1-142) proteins. The diagram at the top shows the three functional domains of DctD. The relative positions of essential aspartate and lysine residues are indicated in the regulatory (receiver) domain. The relative positions of Walker A and B sequences involved in ATP binding are indicated for the central transcription-activating (AAA+) domain, as is the position of the putative helix-turn-helix motif in the DNA-binding domain. The line diagrams indicate the N-terminal and C-terminal ends of the various DctD deletion mutants generated in this study. Thr-460 is the C-terminal residue of the native protein. The activities of the various mutant forms of DctD in *E. coli* at *dctA'*-*lacZ* reporter genes that either possessed or lacked the UAS are indicated to the right of the line diagrams. None of the DctD protein deletions that extended beyond Ala-384 had activity that was above background levels.

were visualized by silver staining of the resulting gel using a protein silver staining kit (Amersham Pharmacia) as per the supplier's protocol. Assays for complex formation between σ^{54} and DctD proteins were carried out essentially as described previously (3). Proteins were diluted into 20 μ l of STA buffer, and ADP \cdot AlF_x was generated in situ as described above. Final protein concentrations in the reaction mixtures were 1 μ M σ^{54} and 1 μ M DctD monomer. Protein complexes were resolved by native gel electrophoresis as described above and visualized by either silver staining or Western blotting, using antiserum directed against *S. enterica* serovar Typhimurium σ^{54} or *S. meliloti* DctD.

ATPase and in vitro transcription assays. ATP hydrolysis assays were done essentially as described previously (43), using 5 mM (monomer) concentrations of the DctD proteins. Single-round transcription assays were performed as described previously (10). The supercoiled template used was plasmid pJES534, which carried the *S. enterica* serovar Typhimurium *glnA* promoter and yields a 155-base transcript that contains no uracil (31). Plasmid pJES534 is reported to have a synthetic module of six directly repeated double-stranded oligonucleotides lacking T residues (21 bp each; AGACACCACAGAGACCACACACA) located at position +28 relative to the *glnA* promoter and immediately followed by a T-rich sequence that functions in termination of transcription (31). DNA sequencing of plasmid pJES534, however, showed that there are seven directly repeated oligonucleotide elements (B. T. Nixon, unpublished results). Reaction mixtures contained 0.1 to 5 μ M DctD AAA+ domain, 60 nM *E. coli* core RNA polymerase (Epicentre), 100 nM histidine-tagged *S. enterica* serovar Typhimurium σ^{54} , 20 nM plasmid DNA, 5 U of anti-RNase (Ambion), 4 mM ATP, 400 μ M GTP, 5 μ M CTP, and 7.5 μ Ci of [α -³²P]CTP (3,000 Ci/mmol). Proteins and DNA template were incubated for 5 min at 37°C, after which ATP was added to stimulate open complex formation. After 10 min of additional incubation, the remaining nucleotides were added to allow synthesis of the transcripts. Heparin was added to the reactions to a concentration of 0.1 mg/ml along with the nucleotides to prevent transcription reinitiation. The resulting transcripts were

analyzed on a 6% denaturing polyacrylamide gel, which was exposed to X-ray film to visualize the transcripts.

RESULTS

Identification of the functional C-terminal end of the AAA+ domain of *S. meliloti* DctD. Crystal structures of AAA+ proteins revealed that the AAA+ domain consists of two subdomains, an N-terminal subdomain that has an α/β fold with a nucleotide binding pocket and a C-terminal α -helical subdomain (28). The C-terminal subdomain is variable in size and structurally less conserved than the N-terminal subdomain. To determine the C-terminal boundary of the AAA+ domain of DctD, we generated a series of C-terminal deletion mutants of DctD and examined the activities of these mutant proteins in *E. coli*. A truncated, constitutively active form of DctD that lacks the N-terminal regulatory domain, referred to as DctD_(Δ1-142) (17), was used for this deletion analysis. The abilities of the truncated DctD proteins to activate transcription were assessed in *E. coli* with *dctA'*-*lacZ* reporter genes that either possessed or lacked the UAS.

The C terminus of wild-type DctD is Thr-460. Eighteen different C-terminal deletion mutants of DctD_(Δ1-142) were generated, with the C termini of these mutant proteins ranging from Val-308 to Asp-404 (Fig. 1). Residues Val-416 through

Gln-435 are predicted to form a helix-turn-helix motif that recognizes the *dctA* UAS (8). All of the C-terminal deletions of DctD_(Δ1-142) lacked the helix-turn-helix motif, and as expected, those that retained activity were unable to discriminate between the reporter gene that possessed the UAS and the one that lacked the UAS (Fig. 1). Mutants with deletions that extended up to Ala-384 retained the ability to activate transcription, whereas those with deletions that extended up to Gly-381 or beyond were unable to activate transcription. These data indicated that the minimal functional C-terminal boundary of the AAA+ domain of DctD lies between Ala-385 (the residue adjacent to Ala-384) and Gly-381.

Expression and purification of the DctD AAA+ domain. A DNA fragment bearing codons 141 to 390 of *S. meliloti* *dctD* was cloned into the expression vector pET28a to introduce a sequence encoding a hexahistidine tag at the 5' end of the *dctD* sequence [protein referred to as DctD₍₁₄₁₋₃₉₀₎]. Ser-390 rather than Ala-384 was chosen for the C-terminal end of the DctD AAA+ domain, since this residue corresponds to the C termini of *Helicobacter pylori* FlgR and σ^{54} -dependent activators from several chlamydial species, all of which lack the C-terminal DNA-binding domain found in most other σ^{54} -dependent activators (12, 38). In addition, threading of the DctD sequence onto the *A. aeolicus* NtrC1 structure modeled Ala-384 to Ser-390 as the last helix of the AAA+ α -helical subdomain. A second expression vector for the DctD AAA+ domain was generated by cloning a DNA fragment bearing codons 141 to 394 of *S. meliloti* *dctD* into plasmid pET28a. This second plasmid introduced a methionine start codon in front of codon 141 and a sequence encoding a hexahistidine tag located 3' to codon 394 (protein referred to as DctD₍₁₄₁₋₃₉₄₎). Codon 394 was chosen as the 3' end of the *dctD* sequence, since it was convenient to introduce a XhoI restriction site at this position for the cloning procedure. Both of these proteins activated transcription from a *dctA'*-*lacZ* reporter gene in vivo, and as expected, failed to discriminate between a *dctA'*-*lacZ* reporter that possessed the UAS and one that lacked the UAS (data not shown).

Both DctD₍₁₄₁₋₃₉₀₎ and DctD₍₁₄₁₋₃₉₄₎ were purified by affinity chromatography followed by anion exchange chromatography, which resulted in highly purified preparations of the two proteins (Fig. 2). The predicted sizes of DctD₍₁₄₁₋₃₉₀₎ and DctD₍₁₄₁₋₃₉₄₎ were 29,444 Da and 28,575 Da, respectively, which was consistent with their migration in the sodium dodecyl sulfate-polyacrylamide gel. Purified DctD₍₁₄₁₋₃₉₀₎ was soluble to at least 30 mg/ml, while DctD₍₁₄₁₋₃₉₄₎ was soluble to at least 8 mg/ml. This result was unexpected, since DctD_(Δ1-142) is only soluble to about 2mg/ml (17).

Self-association of the DctD AAA+ domain occurs independently of nucleotides. The native gel mobility of *E. coli* PspFΔHTH, a truncated form of phage shock protein F (PspF) that lacks most of the DNA-binding domain, is altered by the addition of ADP · AIF_x (3). The change in native gel mobility of PspFΔHTH caused by ADP · AIF_x is attributed to the self-association of the protein, resulting in the formation of a higher-order oligomer (3). To determine if self-association of the DctD AAA+ domain proteins or DctD_(Δ1-142) is effected similarly by ADP · AIF_x, we examined the behavior of these proteins in a native gel electrophoresis system in the presence or absence of ADP · AIF_x. As reported previously (3), ADP · AIF_x

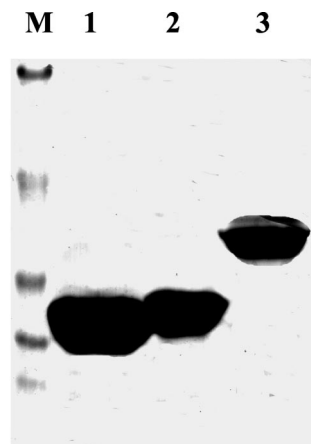


FIG. 2. Purified DctD_(Δ1-142) and DctD AAA+ domain proteins. Purified DctD proteins (20 μ g each) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were visualized in the gel by staining with Coomassie blue. Lane 1, DctD₍₁₄₁₋₃₉₀₎; lane 2, DctD₍₁₄₁₋₃₉₄₎; lane 3, DctD_(Δ1-142). Lane M contains the molecular weight markers: bovine serum albumin (98,000), ovalbumin (54,600), carbonic anhydrase (37,400), soybean trypsin inhibitor (29,600), and lysozyme (20,400).

caused a change in the native gel mobility of PspFΔHTH, which we infer results from an increased association state of the protein (Fig. 3, lanes 1 and 2).

In contrast, the native gel mobility of DctD_(Δ1-142) remained unchanged in the presence of ADP · AIF_x (Fig. 3, lanes 3 and 4). DctD_(Δ1-142) displayed several distinct protein bands on the

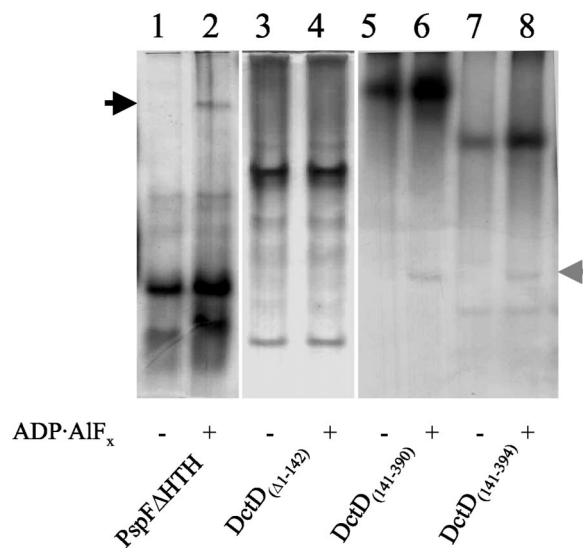


FIG. 3. Native protein gel mobilities of PspFΔHTH, DctD_(Δ1-142), DctD₍₁₄₁₋₃₉₀₎, and DctD₍₁₄₁₋₃₉₄₎ in the presence and absence of ADP · AIF_x. Approximately 3 μ g of purified protein was incubated in the absence (odd-numbered lanes) or presence (even-numbered lanes) of ADP · AIF_x. Following electrophoresis, proteins were visualized in the gel by silver staining. Lanes 1 and 2, PspFΔHTH; lanes 3 and 4, DctD_(Δ1-142); lanes 5 and 6, DctD₍₁₄₁₋₃₉₀₎; lanes 7 and 8, DctD₍₁₄₁₋₃₉₄₎. The arrow to the left of lane 1 indicates the higher-order oligomer of PspFΔHTH that is stabilized by ADP · AIF_x. The arrow to the right of lane 8 indicates forms of DctD₍₁₄₁₋₃₉₀₎ and DctD₍₁₄₁₋₃₉₄₎ that were observed when the proteins were incubated with ADP · AIF_x.

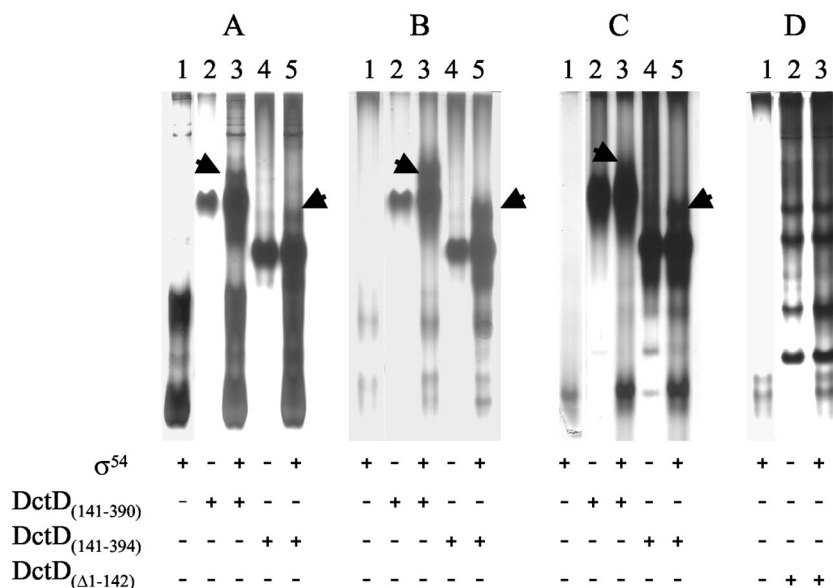


FIG. 4. ADP · AIF_x-dependent complex formation between the DctD AAA+ domain and σ^{54} . Complex formation between DctD proteins and native *S. enterica* serovar Typhimurium σ^{54} (A), histidine-tagged *S. enterica* serovar Typhimurium σ^{54} (B), or N-terminal histidine-tagged *S. meliloti* σ^{54} (C and D) was assessed. DctD₍₁₄₁₋₃₉₀₎, DctD₍₁₄₁₋₃₉₄₎, and a histidine-tagged version of DctD_(Δ 1-142) were included in binding reaction mixtures as indicated at the bottom of each lane. Protein complexes were resolved on a native gel and visualized by silver staining. Arrows in panels A to C indicate the complexes formed between σ^{54} proteins and the DctD AAA+ domain proteins. No complexes were detected between σ^{54} and DctD_(Δ 1-142) under the same reaction conditions. Panel A shows binding reactions done with *S. enterica* serovar Typhimurium σ^{54} , panel B shows reactions with the histidine-tagged *S. enterica* serovar Typhimurium σ^{54} , and panels C and D show reactions with the histidine-tagged *S. meliloti* σ^{54} .

native gel which represent either different conformations or different oligomeric forms of the protein. In contrast to DctD_(Δ 1-142), DctD₍₁₄₁₋₃₉₀₎ and DctD₍₁₄₁₋₃₉₄₎ migrated predominantly on the native gel as single species in the absence or presence of ADP · AIF_x with mobilities that were similar to that of the PspF Δ HTH higher-order complex (Fig. 3, lanes 5 to 8). PspF Δ HTH was shown previously to self-associate at high concentrations in the absence of nucleotides by native gel electrophoresis (9), but the results shown in Fig. 3 suggested that DctD₍₁₄₁₋₃₉₀₎ and DctD₍₁₄₁₋₃₉₄₎ have a greater tendency to form higher-order oligomers in the absence of ADP · AIF_x than PspF Δ HTH. ADP · AIF_x did appear to stabilize the higher-order oligomers of DctD₍₁₄₁₋₃₉₀₎ and DctD₍₁₄₁₋₃₉₄₎, however, since the intensities of the bands corresponding to these complexes appeared to increase when ADP · AIF_x was present. In addition, a minor, faster-migrating form of DctD₍₁₄₁₋₃₉₀₎ and one of DctD₍₁₄₁₋₃₉₄₎ were observed in the presence of ADP · AIF_x, which we infer to be different oligomeric forms or conformations of these proteins. Interestingly, the predominant oligomeric form of DctD₍₁₄₁₋₃₉₄₎ migrated faster than that of DctD₍₁₄₁₋₃₉₀₎. The DctD₍₁₄₁₋₃₉₀₎ monomer is slightly larger than that of DctD₍₁₄₁₋₃₉₄₎ due to its larger histidine tag. Differences in size and amino acid composition between the two DctD AAA+ domain proteins may have accounted for the different mobilities of these proteins in the native gel. Alternatively, these proteins may differ with respect to either their conformations or oligomeric states.

DctD AAA+ domain proteins form a stable ternary complex with σ^{54} and ADP · AIF_x. Previous studies with PspF, PspF Δ HTH, and the *Azotobacter vinelandii* NifA AAA+ domain showed that these σ^{54} -dependent activators formed a

stable complex with σ^{54} in the presence of ADP · AIF_x (3). Therefore, we examined DctD_(Δ 1-142) and the DctD AAA+ domain proteins to see if these proteins would similarly form a stable ternary complex with σ^{54} and ADP · AIF_x. Three σ^{54} proteins were tested in this assay, native *S. enterica* serovar Typhimurium σ^{54} , an N-terminal histidine-tagged *S. enterica* serovar Typhimurium σ^{54} , and an N-terminal histidine-tagged *S. meliloti* σ^{54} .

The *S. enterica* serovar Typhimurium σ^{54} proteins migrated on the native gel as two species (Fig. 4A and B, lanes 1 and 3), indicating self-association of the protein. Reaction mixtures in which native or histidine-tagged *S. enterica* serovar Typhimurium σ^{54} was incubated with DctD₍₁₄₁₋₃₉₀₎ or DctD₍₁₄₁₋₃₉₄₎ and then ADP · AIF_x was generated in the mixture revealed bands that migrated immediately above the DctD AAA+ domain and were not present in the control lanes (Fig. 4A and 4B). These new bands were not observed in the absence of ADP · AIF_x (data not shown). In some assays a protein band that migrated immediately below the major DctD AAA+ band was also apparent, but this band was not consistently observed. Western blot analysis confirmed that the new complexes observed when σ^{54} and one of the DctD AAA+ domain proteins were incubated together in the presence of ADP · AIF_x contained both σ^{54} and DctD (Fig. 5). As reported previously with PspF (3), these protein complexes were not observed if the DctD AAA+ domain was incubated with ADP · AIF_x before the protein was mixed with σ^{54} (data not shown). Unique complexes were also observed when DctD₍₁₄₁₋₃₉₀₎ or DctD₍₁₄₁₋₃₉₄₎ was incubated with *S. meliloti* σ^{54} in the presence of ADP · AIF_x (Fig. 4C). We assume that these complexes contain σ^{54} , but we could not verify this by Western blotting since *S.*

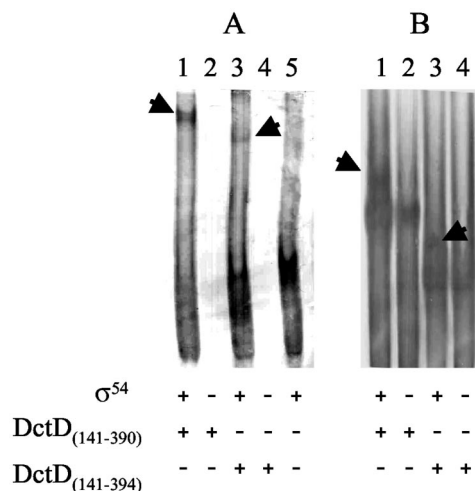


FIG. 5. Western blot analysis of ternary complexes. Complexes formed between *S. enterica* serovar Typhimurium σ^{54} and DctD₍₁₄₁₋₃₉₀₎ or DctD₍₁₄₁₋₃₉₄₎ in the presence of ADP · AlF_x were analyzed by Western blotting using antiserum directed against *S. enterica* serovar Typhimurium σ^{54} (A) or *S. meliloti* DctD (B). The proteins included in the binding reaction mixtures are indicated below each lane. The arrows in panel A indicate the presence of σ^{54} in the ternary complexes, while the arrows in panel B indicate the presence of the DctD AAA+ domain proteins in the ternary complexes. The gel used for panel B was subjected to electrophoresis for a longer period of time to achieve better separation of the ternary complex from the DctD AAA+ domain complex.

meliloti σ^{54} did not cross-react with the antiserum directed against *S. enterica* serovar Typhimurium σ^{54} . In contrast to the results with the DctD AAA+ domain, we did not observe complexes between DctD_(Δ 1-142) and any of the σ^{54} proteins that we tested (Fig. 4D; data shown only for *S. meliloti* σ^{54}).

In vitro activity of the DctD AAA+ domain. The purified DctD AAA+ domains were examined for their ability to hydrolyze ATP. The measured ATPase activities of DctD₍₁₄₁₋₃₉₀₎ and DctD₍₁₄₁₋₃₉₄₎, which were determined at a single concentration (2.5 μ M monomer), were 228 and 175 pmol of ATP hydrolyzed/min/pmol of DctD monomer, respectively. These values were comparable to that of DctD_(Δ 1-142), which was 251 pmol of ATP hydrolyzed/min/pmol of protein, and somewhat higher than that of an N-terminal histidine-tagged DctD_(Δ 1-142) protein, which was 107 pmol of ATP hydrolyzed/min/pmol of protein.

DctD₍₁₄₁₋₃₉₀₎ and DctD₍₁₄₁₋₃₉₄₎ were also tested for their ability to activate transcription in vitro. Titration of DctD₍₁₄₁₋₃₉₀₎ and DctD₍₁₄₁₋₃₉₄₎ in a single-round transcription assay showed that increasing concentrations of these proteins resulted in increased transcript levels (Fig. 6A). A supercoiled plasmid DNA template that carried the *S. enterica* serovar Typhimurium *glnA* promoter region upstream of a uracil-free tract was used for the transcription assay. This DNA template was used because σ^{54} -holoenzyme has a higher affinity for the *glnA* promoter than it does for the *S. meliloti* *dctA* promoter.

In transcription assays that contained the higher concentrations of DctD₍₁₄₁₋₃₉₀₎, a ladder of transcripts that were shorter than the full-length transcript was observed in addition to the full-length transcript (Fig. 6A). The pattern of the shorter transcripts was somewhat regular, with a repeat phase of about

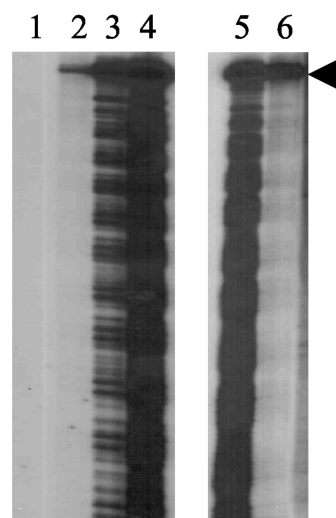


FIG. 6. In vitro transcriptional activation by DctD AAA+ domain proteins. Transcriptional activation by DctD₍₁₄₁₋₃₉₀₎ and DctD₍₁₄₁₋₃₉₄₎ from the *S. enterica* serovar Typhimurium *glnA* promoter was assessed in a single-round in vitro transcription assay. Lanes 1 to 4 contained 100 nM, 500 nM, 1 μ M, and 5 μ M DctD₍₁₄₁₋₃₉₀₎ monomer, respectively, and show increased transcript levels with increasing concentrations of DctD₍₁₄₁₋₃₉₀₎. Lane 5 contained 5 μ M DctD₍₁₄₁₋₃₉₀₎ monomer, and lane 6 contained 5 μ M DctD₍₁₄₁₋₃₉₄₎ monomer. The arrow indicates the full-length transcript, which is ~155 bases in length.

11 nucleotides. This pattern of transcripts was not seen for DctD₍₁₄₁₋₃₉₄₎ (Fig. 6A), nor was it observed previously for DctD_(Δ 1-142) (17, 43, 44). Several lines of evidence argue against these shorter transcripts resulting from contaminating RNase activity. First, DctD₍₁₄₁₋₃₉₀₎ and DctD₍₁₄₁₋₃₉₄₎ were purified in the same manner and both protein preparations were very homogeneous (Fig. 2), yet the shorter transcripts were evident only in assays in which transcripts were initiated with DctD₍₁₄₁₋₃₉₀₎. Second, the phasing pattern of the shorter transcripts is not typical of nuclease digestion. Third, both the full-length and shorter transcripts were stable in the transcription assay when the incubation time following transcription initiation was extended from 10 to 60 min (Fig. 7A). Analysis of these data with a PhosphorImager showed that there were no appreciable differences in the levels of the full-length transcripts at the various incubation times, which strongly argued against RNase accounting for the shorter transcripts. Finally, adding DctD₍₁₄₁₋₃₉₀₎ to assay mixtures in which open complexes were formed with DctD₍₁₄₁₋₃₉₄₎ did not result in the shorter transcripts (Fig. 7B), which argues further against the DctD₍₁₄₁₋₃₉₀₎ preparations being contaminated with RNase. We infer from these observations that the shorter transcripts result from DctD₍₁₄₁₋₃₉₀₎ interfering with the transcription elongation complex when it is present at high levels.

DISCUSSION

The α -helical subdomain of the DctD AAA+ domain plays an important role in transcriptional activation, since certain substitutions in this region interfere with DctD_(Δ 1-142) function (43; H. Xu, M. T. Kelly, B. T. Nixon, and T. R. Hoover, submitted for publication). Similarly, mutations in the α -heli-

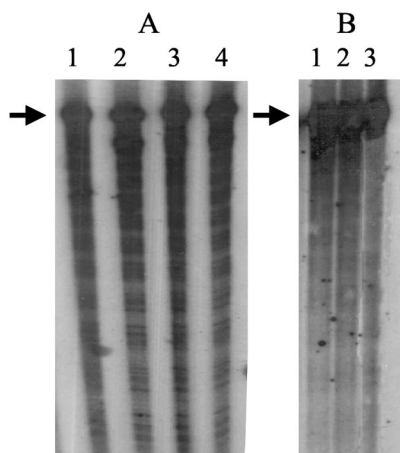


FIG. 7. Stability of the transcripts formed in vitro. (A) In vitro transcription assays with $5 \mu\text{M}$ DctD₍₁₄₁₋₃₉₀₎ were incubated for various periods of time after the initiation of transcription by the addition of nucleotides to the reaction mixtures. Heparin was included to prevent transcription reinitiation. Assay mixtures were incubated for 10 min (lane 1), 20 min (lane 2), 30 min (lane 3), or 60 min (lane 4) after the addition of the nucleotides. Levels of the full-length transcript in lanes 1 to 4 as analyzed with a PhosphorImager SI (Molecular Dynamics) were virtually identical (volumes were 1.51×10^6 , 1.49×10^6 , 1.51×10^6 , and 1.49×10^6 , respectively). (B) Formation of open complexes at the *glnA* promoter were initiated with $5 \mu\text{M}$ DctD₍₁₄₁₋₃₉₄₎, after which DctD₍₁₄₁₋₃₉₀₎ was added to the reaction mixture at various times. In lane 1, no DctD₍₁₄₁₋₃₉₀₎ was added to the assay mixture, and the reaction was stopped 10 min after the addition of nucleotides and heparin. In lane 2, $5 \mu\text{M}$ DctD₍₁₄₁₋₃₉₀₎ was added immediately after adding the nucleotides and heparin, and the reaction was stopped 10 min later. In lane 3, $5 \mu\text{M}$ DctD₍₁₄₁₋₃₉₀₎ was added 10 min after the addition of nucleotides and heparin, and the reaction was stopped after an additional 10 min of incubation. Transcript levels in the three lanes were essentially the same as determined by PhosphorImager analysis (volumes were 3.26×10^4 , 3.01×10^4 , and 3.10×10^4 , respectively).

cal subdomains of other σ^{54} -dependent activators, including *S. enterica* serovar Typhimurium NtrC, *Pseudomonas putida* XylR, and *E. coli* PspF Δ HTH, have been reported to result in a loss of activity (19, 27, 29, 43). Because the α -helical subdomains of AAA+ proteins are variable in length, we undertook a deletion analysis of the 3' end of *dctD* to define the minimal functional C-terminal end of this subdomain in DctD. The results of the deletion analysis indicated that the minimal functional C-terminal end lies somewhere between Gly-381 and Ala-385. This result was somewhat surprising, since threading the DctD sequence onto the *A. aeolicus* NtrC1 structure (18) modeled the DctD segment Ala-384 to Ser-390 as the last helix of the AAA+ α -helical subdomain, suggesting that DctD does not require the last helix of the α -helical subdomain to function in vivo.

With the exception of the loss of DNA-binding activity, the DctD AAA+ domain was functional in vivo and in vitro. Analysis of the DctD AAA+ domain on the native gel suggested that it migrated predominantly as a single oligomeric species, while DctD_(Δ 1-142) migrated as several different species. These data suggest a role for the DNA-binding domain in preventing assembly of DctD into a functional oligomeric complex in the absence of binding to the UAS, implying that the DNA-binding domain interacts with the AAA+ domain. DNase I foot-

printing of DctD_(Δ 1-142) at the *dctA* UAS revealed changes in the footprint in response to ADP \cdot AIF_x, consistent with the idea that the DNA-binding domain can communicate with the AAA+ domain, at least in the transition state during ATP hydrolysis (45). Alternatively, intermolecular interactions between the DNA-binding domains of DctD monomers may interfere with assembly of a functional oligomeric complex, which may be supported by the observation that introduction of three alanine substitutions in the enhancer recognition helix of *S. enterica* serovar Typhimurium NtrC eliminates DNA-binding activity and stabilizes the dimeric state of the protein (26).

AAA+ proteins often form hexamers (28), although the crystal structure of the AAA+ domain of *A. aeolicus* NtrC1 with ADP bound indicated that this protein forms a heptamer (18). We could not ascertain the oligomeric state of the DctD AAA+ domain from the native gel, but future studies will address this issue as well as determining if the predominant oligomeric forms of DctD₍₁₄₁₋₃₉₀₎ and DctD₍₁₄₁₋₃₉₄₎ differ from each other. An unexpected result was that the DctD AAA+ domain is significantly more soluble than DctD_(Δ 1-142). The increased solubility of the DctD AAA+ domain and its more uniform oligomeric nature appear beneficial for structural studies of DctD, since we have recently obtained crystals of DctD₍₁₄₁₋₃₉₀₎ that diffract X rays.

Unlike DctD_(Δ 1-142), the DctD AAA+ domain formed a stable complex with σ^{54} in the presence of ADP \cdot AIF_x, suggesting that the DNA-binding domain interferes with formation of the ternary complex. Inhibition may have resulted from steric hindrance by the DNA-binding domain of interactions between the AAA+ domain and σ^{54} . Alternatively, since not all of the DctD_(Δ 1-142) species observed on the native gel are likely to be capable of forming a complex with σ^{54} in the presence of ADP \cdot AIF_x, the inhibition of ternary complex formation by the DNA-binding domain may have been an indirect effect.

In the in vitro transcription assay with DctD₍₁₄₁₋₃₉₀₎, a large number of transcripts which were shorter than the full-length transcript were produced, which we infer to result from DctD₍₁₄₁₋₃₉₀₎ interfering with transcription elongation. The pattern of termination appeared remarkably regular, suggesting that the transcription elongation complex was more susceptible to termination at regular intervals along the DNA template. This phasing may have been influenced by periodicity of the DNA template in the transcription vector, which was constructed using a synthetic module of directly repeated double-stranded oligonucleotides of 21 bp in length (31). DctD₍₁₄₁₋₃₉₄₎ did not generate the shorter transcripts in the in vitro transcription assay. This may have been because either the DctD segment from Ser-390 to Leu-394 or the histidine tag at the C terminus of DctD₍₁₄₁₋₃₉₄₎ prevented the AAA+ domain from interfering with transcription elongation.

Inhibition of transcription elongation by DctD₍₁₄₁₋₃₉₀₎ suggests that it contacts the transcription elongation complex. These interactions could lead to either stalling of the transcription elongation complex on the DNA or the premature release of transcripts from the complex. The failure to observe the regular pattern of shorter transcripts when DctD₍₁₄₁₋₃₉₀₎ was added to assay mixtures after open complexes were formed with DctD₍₁₄₁₋₃₉₄₎ suggests that DctD₍₁₄₁₋₃₉₀₎ must be present

before transcription initiation to inhibit elongation. One possible explanation for this is that DctD₍₁₄₁₋₃₉₀₎ does not always disengage σ^{54} -holoenzyme following open complex formation and remains associated with RNA polymerase upon promoter clearance. If DctD₍₁₄₁₋₃₉₀₎ can associate with the transcription elongation complex, to which subunit(s) of RNA polymerase might it bind? As is the case with other σ factors, σ^{54} is thought to dissociate from RNA polymerase following promoter clearance (41), but it is unclear if σ^{54} could remain associated with RNA polymerase in the transcription elongation complex if DctD₍₁₄₁₋₃₉₀₎ was also part of the complex. Recent studies have provided evidence that σ^{70} can remain associated with transcription elongation complexes (22, 24), which at least raises the possibility that σ^{54} could likewise. Alternatively, DctD₍₁₄₁₋₃₉₀₎ could engage the β subunit of RNA polymerase, since DctD can be chemically cross-linked to this subunit even in the absence of σ^{54} (16).

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