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Alterations within the Activation Domain of the σ^{54}-Dependent Activator DctD That Prevent Transcriptional Activation

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Rhizobium meliloti **DctD (C_4-dicarboxylate transport protein D) is a transcriptional activator that catalyzes the ATP-dependent isomerization of closed complexes between σ^{54}-RNA polymerase holoenzyme and the *dctA* promoter to open complexes. Following random mutagenesis of *dctD*, 55 independent mutant forms of DctD that failed to activate transcription from a *dctA′-′lacZ* reporter gene in *Escherichia coli* were selected, and the amino acid substitutions were determined for these mutant proteins. Amino acid substitutions were distributed throughout the central domain of the protein, the domain responsible for transcription activation, but most of the substitutions occurred within three highly conserved regions of the protein. Selected mutant proteins were purified, and their activities were studied in vitro. All of the purified mutant proteins appeared to have normal DNA-binding activity and interacted with σ^{54} and core RNA polymerase, as determined from protein cross-linking assays. Proteins with amino acid substitutions in a region spanning amino acid positions 222 to 225 retained their ATPase activities, whereas proteins with substitutions in other regions had little or no ATPase activity. Taken together, these data suggest that the region that encompasses amino acid residues 222 through 225 probably functions in coupling the energy released from ATP hydrolysis to open complex formation rather than as a major determinant for binding to RNA polymerase.**

Initiation of transcription by the bacterial σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme) appears to occur through a common mechanism that differs markedly from transcription initiation by other forms of RNA polymerase holoenzyme (19, 21, 39). The σ^{54}-holoenzyme can bind the promoter to form a stable, closed promoter complex, but an activator protein is required for the isomerization of this closed promoter complex to an open complex that is transcriptionally competent (27, 30). Where it has been examined in detail, the activator is a dimer in solution that undergoes oligomerization upon binding to two or more specific sites located 100 to 200 bp upstream of the promoter (20, 24, 28, 31). This oligomerization of the activator is required for transcriptional activation (28). To catalyze the isomerization of the closed complex to the open complex, the activator must contact σ^{54}-holoenzyme through a DNA loop (2, 32, 36). Specific contact sites have not yet been identified for either the activator or σ^{54}-holoenzyme, but studies with the σ^{54}-dependent activator DctD showed that this protein could be cross-linked to σ^{54} and the β subunit of RNA polymerase (15). Not only must the activator engage σ^{54}-holoenzyme, but it must also hydrolyze ATP in order to catalyze open complex formation (14, 16, 25, 27, 37, 38).

We have used *Rhizobium meliloti* DctD as a model to study further transcriptional activation with σ^{54}-holoenzyme. DctD activates transcription from *dctA*, which encodes a permease for C_4-dicarboxylates and whose expression is inducible with C_4-dicarboxylates (1, 6, 11, 29). DctD contains three functional domains: an N-terminal regulatory domain, a central domain that is responsible for transcriptional activation and ATP hydrolysis, and a C-terminal domain that is responsible for recognition of the upstream activation sequence (UAS) of the *dctA* promoter regulatory region (11). The N-terminal domain of DctD has homology with response regulators of two-component regulatory systems (22, 33). This regulatory domain inhibits the transcriptional activation and ATPase activities of the central domain of DctD, as its removal yields truncated proteins that constitutively hydrolyze ATP and activate transcription (10, 16).

The transcriptional activation domains are well conserved among σ^{54}-dependent activators and can be subdivided into seven highly conserved regions (designated C1 to C7) (21). Region C1 forms a structural motif known as a phosphate loop or Walker type A sequence that is common to a variety of nucleotide-binding proteins (29, 34). The C4 region is similar to a motif known as a Walker type B sequence that is also common to many nucleotide-binding proteins (29, 34). Region C3 is thought to participate in productive interactions with σ^{54}-holoenzyme (21, 23). Functions have not been proposed for the remaining regions of the domain.

To begin examining structure-function relationships within σ^{54}-dependent activators, we isolated and characterized mutant forms of DctD that failed to activate transcription. Following random mutagenesis of a *dctD* deletion mutant encoding a truncated, constitutively active form of DctD that lacks the entire N-terminal domain [designated $DctD_{(\Delta 1\text{-}142)}$], we isolated over 30 different mutant forms of $DctD_{(\Delta 1\text{-}142)}$ that were unable to activate transcription from a *dctA′-′lacZ* reporter gene in *Escherichia coli*. Selected mutant proteins were purified, and their activities were studied in vitro. Proteins with amino acid substitutions in the C3 region of DctD retained some or all of their ATPase activities but cross-linked normally to σ^{54} and the β subunit of RNA polymerase. We infer that the amino acid substitutions in these proteins disrupt energy transfer from ATP hydrolysis to open complex formation rather than prevent binding of DctD to σ^{54}-holoenzyme.

MATERIALS AND METHODS

Overexpression of *R. meliloti* $DctD_{(\Delta 1\text{-}142)}$ proteins. Plasmid pL143 is a derivative of pUC13 that carries a Φ(*lacZ′-′dctD*)(Hyb) gene in which the first 12 codons of *lacZ* are joined to codons 143 to 460 of *R. meliloti dctD* (13, 16). Plasmid pYKW1 carries a similar Φ(*lacZ′-′dctD*)(Hyb) gene along with the $lacI^q$ gene in the vector pBS+ (Stratagene). Plasmid pYKW2, a derivative of pL143

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that lacks the *Sph*I site immediately downstream of *dctD*, was constructed following partial digestion of pL143 with *Sph*I, filling in the ends with T4 DNA polymerase, and then ligating the blunt ends.

Plasmids bearing chimeric genes that encode histidine-tagged $DctD_{(\Delta 1\text{-}142)}$ proteins were constructed with plasmid pTrcHis-C (Invitrogen). Plasmid pL143 was digested with *Hin*dIII, filled in with T4 DNA polymerase, and then digested with *Eco*RI. The 1.2-kbp *Hin*dIII-*Eco*RI fragment was cloned into pTrcHis-C, which had been previously digested with *Bam*HI, filled in with T4 DNA polymerase, and then digested with *Eco*RI. The resulting chimeric gene had 41 codons (35 from pTrcHis-C and 6 from pUC13) joined to codons 143 to 460 of *dctD*. The chimeric gene was under control of the *lac* promoter and had at its 5′ end six tandem histidine codons that were derived from pTrcHis-C. Selected *dctD* alleles were similarly cloned into pTrcHis-C from derivatives of either pYKW1 or pYKW2.

Construction of P22 challenge phage bearing the high-affinity DctD-binding site. Plasmids pTRH14 and pTRH15 are derivatives of pPY190 (17) that carry the high-affinity site (site B) of *R. meliloti dctA* UAS overlapping the phage P22 *ant* promoter (P_{ant}). The complementary oligonucleotides 5′-ATATGTGCGGA TTTCCGCACAGA and 5′-TCTGTGCGGAAATCCGCACATAT, which bear site B of the *dctA* UAS, were annealed and cloned into the *Sma*I site of pPY190. This placed the insert at −3 relative to the transcriptional start site of P_{ant} and created an *Nde*I site, which made it possible to distinguish between the two potential orientations. Plasmids were chosen in both orientations and designated pTRH14 and pTRH15.

P22 challenge phage in which site B of the *dctA* UAS replaced O_{mnt} were constructed by using these plasmids. Phage P22 *mnt*::Kn 9 *arc*(Am) (17) was grown on *Salmonella typhimurium* MS1883 [*leuA414*(Am) *hsdSB*($r^- m^+$) $Fels^-$ *supE40*] (8) bearing either pTRH14 or pTRH15 to allow recombination between the plasmid and phage DNA as described previously (17). The resulting phage lysates were then used to infect *S. typhimurium* MS1582 {*leuA414*(Am) *hsdSB*($r^- m^+$) $Fels^-$ *supE40 ataP*::[P22 *sieA44 16*(Am)*H1455 Tpfr49*} (8). Phage that retained the O_{mnt} were unable to grow lytically on this strain due to the expression of Mnt repressor from the P22 prophage, while recombinant phage that carried the DctD-binding site produced large, clear plaques. Phage were purified from several plaques, and correct phage constructs were verified by DNA sequencing and the challenge phage assay. Challenge phage assays were performed essentially as described previously (17), using *S. typhimurium* MS1883 bearing plasmid pYKW1. In the challenge phage assays, the percentage of lysogeny was calculated as the ratio of lysogens to viable cells multiplied by 100.

Isolation of mutant forms of $DctD_{(\Delta 1\text{-}142)}$ that fail to activate transcription but retain the ability to bind DNA. Plasmid DNA was mutagenized with hydroxylamine essentially as described previously (4). Approximately 5 μg of plasmid pYKW1 DNA was treated for 24 to 48 h at 37°C in a reaction mixture containing 58 mM sodium phosphate (pH 6.0), 0.58 mM EDTA, and 0.4 M hydroxylamine. Reactions were stopped by recovering the plasmid DNA with the plasmid miniprep Wizard resin and columns (Promega). The mutagenized DNA was eluted from the columns with deionized water and stored at −20°C.

Alternatively, the region of *dctD* corresponding to the central domain of the DctD protein was mutagenized by relying on the inherent error frequency of the PCR. Two separate sets of reactions were carried out to mutagenize this region of *dctD*. In one set, the primers were 5′-AGCGGATAACAATTTCACA CAGGA and 5′-TCGTCGAGGAAGAGCGTGCC, which were complementary to sequences within the vector and *dctD*, respectively. From the amplified DNA, a 295-bp *Hin*dIII-*Sph*I DNA fragment was cloned into the same sites of pYKW2, thereby replacing codons 143 to 235 at the 5′ end of the truncated *dctD* gene. In the other set, the primers were 5′-AATGGAGCCGCCGCAGGAC and 5′-AAAGTCTTGCGGGGGATG, both of which were internal to *dctD*. A 576-bp *Sac*I-*Eco*RV fragment was cloned from the amplified DNA into the same sites of pYKW2 to replace codons 215 to 404 of the truncated *dctD* gene. PCR mixtures contained 2 to 5 U of *Taq* DNA polymerase (Promega), 1 ng of template (pYKW2), and 100 pmol of the primers in 50 mM Tris-HCl (pH 9.0 at 25°C), 50 mM NaCl, 15 mM $MgCl_2$, 5% (vol/vol) dimethyl sulfoxide, and 200 μM each of the deoxyribonucleotides. The temperature regimen for PCR was 30 cycles of 2 min at 94°C, 1 min at 42°C, and 3 min at 72°C.

Following mutagenesis of pYKW1 and pYKW2, the plasmids were transformed into *S. typhimurium* MS1883. Transformed cells were grown and infected with challenge phage bearing a DctD-binding site as described above. Plasmids were isolated from the stable, kanamycin-resistant lysogens and used to transform *E. coli* JM109 [*recA1 supE44 endA1 hsdR17 gyr96 relA1 thi* Δ(*lac-proAB*)/F′ *traD36* Δ(*lacZ*)*M15 proA*$^+$*B*$^+$] (40) that carried a *dctA′-′lacZ* reporter gene on plasmid pRMAZ:+UAS (13). Cells were plated onto LB agar that contained 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and the appropriate antibiotics. Plasmids were isolated from white and faint blue colonies, and the mutations on these plasmids were localized by subcloning DNA fragments into pL143. Specific base changes were identified by DNA sequencing of the subcloned regions, which was done at the University of Georgia Molecular Genetic Instrumentation Facility.

Protein purification. *S. typhimurium* σ^{54} and *E. coli* core RNA polymerase were purified as described previously (16). Wild-type and mutant forms of $DctD_{(\Delta 1\text{-}142)}$ were overexpressed in *E. coli* and purified as described previously (16). For purification of the $DctD_{(\Delta 1\text{-}142,\ A225T)}$, the concentration of potassium thiocyanate in the chromatography buffers was reduced from 65 to 20 mM to improve the binding of the protein to the chromatography resins. [In the designation of each of the mutant forms of $DctD_{(\Delta 1\text{-}142)}$, the amino acid substitution is indicated by the subscript. For example, in the mutant $DctD_{(\Delta 1\text{-}142,\ F222L)}$ the F222L designation indicates that Phe-222 is replaced with a Leu residue.]

For histidine-tagged proteins, *E. coli* cells carrying the pTrcHis-C derivatives were purified by nickel-nitrilotriacetic acid agarose chromatography as described by the supplier (Qiagen). Histidine-tagged proteins were eluted from the nickel-nitrilotriacetic acid agarose column with phosphate buffer containing 250 mM imidazole. Peak fractions were pooled, dialyzed against 20 mM EPPS (pH 8.0)–5% (wt/vol) glycerol–0.5 mM dithiothreitol (buffer A), and then loaded onto a DNA-cellulose (Sigma) column (~4 ml). After being washed with several bed volumes of buffer A, histidine-tagged proteins were eluted from the column with a gradient of 0 to 300 mM potassium chloride in buffer A over a volume of 40 ml, with the histidine-tagged proteins eluting at approximately 250 mM potassium chloride.

Transcription assay. Single-round transcription assays were performed as described previously (16), with the following modifications. Reaction mixtures contained 60 nM *E. coli* core RNA polymerase, 100 nM *S. typhimurium* σ^{54}, 400 nM $DctD_{(\Delta 1\text{-}142)}$, and 5 nM pJHL2, which carries the *R. meliloti dctA* promoter regulatory region upstream of the early terminator of phage T7 and yields a transcript of ~430 bp. Proteins and plasmid template were incubated at 37°C for 10 min in buffer containing 80 mM Tris-acetate (pH 8.0), 100 mM potassium acetate, 25 mM ammonium acetate, 5 mM magnesium acetate, 0.1 mM EDTA, and 1.0 mM dithiothreitol (transcription assay buffer). Open complex formation was initiated by adding ATP to a final concentration of 3 mM to each reaction. After 10 min, heparin was added to a final concentration of 0.1 mg/ml to prevent further open complex formation, along with the remaining nucleotides (0.4 mM GTP, 0.4 mM UTP, and 0.1 mM CTP) and 5 μCi [α-^{32}P]CTP to initiate the synthesis of RNA transcripts. Transcripts were precipitated after 10 min, subjected to denaturing gel electrophoresis, and visualized by exposing X-ray film to the resulting gels.

ATPase assay. ATP hydrolysis assays were done essentially as described previously (38). Proteins were diluted to a final concentration of 1 to 2 μM in transcription assay buffer and incubated at 37°C for 5 min. Reactions were initiated by adding [γ-^{32}P]ATP (3 mM; specific activity, ~200 cpm/pmol of ATP). Ten-microliter samples were taken after 1 and 11 min and diluted into 90 μl of ice-cold 1 N formic acid. Five-microliter aliquots of the diluted samples were spotted onto polyethyleneimine-cellulose plates (J. T. Baker), and free phosphate was resolved from ATP on the plates by ascending chromatography using 0.4 M dipotassium hydrogen phosphate plus 0.7 M boric acid. Following chromatography, the plates were air dried and analyzed with a PhosphorImager SI (Molecular Dynamics), using Scanner Control SI (Molecular Dynamics), using Scanner Control and ImageQuant 1.0 software to analyze the data. The identity of each phosphate spot was verified by cochromatography with [^{32}P]phosphate. A standard curve was used to convert the volumes for each phosphate spot into picomoles of phosphate released.

Chemical cross-linking assay. Chemical cross-linking of mutant forms of $DctD_{(\Delta 1\text{-}142)}$ to σ^{54} or the β subunit of core RNA polymerase, using succinimidyl 4-(*N*-maleimidomethyl) cylohexane-1-carboxylate (sulfo-SMCC) as the cross-linking reagent, was performed essentially as described previously (15). The final concentration of each protein in the cross-linking assays was 1 μM. Cross-linking reactions were stopped by the addition of sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and then analyzed by Western blotting as described previously (15).

DNase I footprinting. DNase I footprinting experiments were done with one of two different DNA probes. One probe contained the wild-type *dctA* UAS, while the other probe contained a mutant form of the *dctA* UAS that had a 5-bp insertion between sites A and B (31). The wild-type UAS was present on a 195-bp *Bam*HI-*Eco*RI fragment, while the mutant UAS was on a 200-bp *Bam*HI-*Eco*RI fragment. Both fragments were carried on plasmid pUC19 (31), which was provided by B. Tracy Nixon. The nontemplate strands of the DNA probes were end labeled at the *Bam*HI site with [γ-^{32}P]ATP and T4 DNA kinase as described previously (9). After cutting of the labeled DNA molecules with *Eco*RI, probes were purified by native gel electrophoresis as described previously (9). Various concentrations of wild-type or mutant forms of $DctD_{(\Delta 1\text{-}142)}$ were incubated with the radiolabeled DNA probes (~300 pM; 200,000 cpm) at 30°C for 20 min in 20 μl of the transcription assay buffer supplemented with 1 mM calcium chloride and 2 μg of calf thymus DNA per ml. DNase I was diluted in the transcription assay buffer, and 1 μl was added to each reaction mixture and allowed to digest the DNA for 1 min. The reaction was stopped by adding 20 μl of a solution containing 0.2 mg of tRNA per ml, 5 M ammonium acetate, and 50 mM EDTA. The DNA was precipitated with ethanol, dried under vacuum, resuspended in sequencing loading buffer, and subjected to denaturing polyacrylamide gel electrophoresis. X-ray film was exposed to the resulting gels to visualize the footprints.

RESULTS

Construction of P22 challenge phage that bear a high-affinity DctD-binding site. To develop a genetic system that would allow the selection of mutant forms of $DctD_{(\Delta 1\text{-}142)}$ that re-

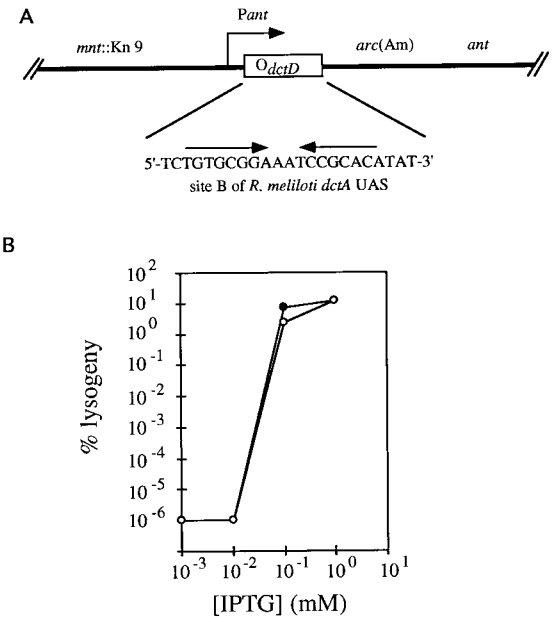

FIG. 1. Overexpression of $DctD_{(\Delta 1\text{-}142)}$ leads to increased frequency of lysogeny for challenge phage bearing a high-affinity DctD-binding site. (A) The *immI* region of the challenge phage bearing the DctD-binding site is illustrated. The DNA sequence corresponds to site B of the *R. meliloti dctA* UAS, which replaced O_{mnt} in the phage and is at position −3 relative to the transcriptional start site of the *ant* promoter (P_{ant}). The arrows above the sequence indicate the dyad symmetry elements within this site. Phage were obtained with the DctD-binding site in either orientation. (B) An *S. typhimurium* strain bearing pYKW1 was grown in the presence of various concentrations of IPTG to induce the expression of $DctD_{(\Delta 1\text{-}142)}$ to different levels. Cells were then infected with challenge phage bearing the DctD-binding site, and the frequency of lysogeny was determined. Challenge phage used in the experiment had the DctD-binding site in either the orientation shown in panel A (closed circles) or in the opposite orientation (open circles).

tained the ability to bind DNA, we constructed challenge phage in which the high-affinity DctD-binding site (site B) of the *R. meliloti dctA* UAS replaced the binding site for the phage P22 Mnt protein (O_{mnt}). In phage P22, the decision between lysis and lysogeny can be influenced by interactions between two phage proteins, c2 repressor and antirepressor. The c2 repressor binds to operator sites that overlap promoters of genes required for lytic growth of the phage. Repression of these genes by c2 repressor channels the phage toward lysogeny. Antirepressor binds to the c2 repressor to inhibit its binding to the operator sites. Expression of *ant*, which encodes antirepressor, is negatively regulated by the products of two other phage P22 genes, *arc* and *mnt*. The products of these genes bind to operator sites that overlap the *ant* promoter, thereby repressing transcription of *ant*. The challenge phage system uses *ant* as a reporter gene and provides a powerful method for studying protein-DNA interactions in vivo (17). Challenge phage are derivatives of phage P22 in which the *mnt* gene is disrupted with a kanamycin resistance cassette and O_{mnt} is replaced with the binding site for another DNA-binding protein. This other protein can then function as a repressor of the *ant* gene, with the kanamycin resistance gene in the phage providing a positive selection for stable lysogens.

When challenge phage bearing the DctD-binding site were used to infect an *S. typhimurium* strain in which expression from a plasmid-borne *dctD* allele encoding $DctD_{(\Delta 1\text{-}142)}$ had been induced with isopropyl thiogalactopyranoside (IPTG), the frequency of lysogeny increased 7 orders of magnitude (Fig. 1). The orientation of the DctD-binding site did not influence the ability of $DctD_{(\Delta 1\text{-}142)}$ to channel the phage toward lysogeny (Fig. 1B). Overexpression of $DctD_{(\Delta 1\text{-}142)}$ had no effect on lysogeny when cells were infected with P22 phage that lacked the DctD-binding site (data not shown). These data showed that $DctD_{(\Delta 1\text{-}142)}$ could bind to its site on the challenge phage and repress transcription from the *ant* promoter.

Isolation of mutant forms of $DctD_{(\Delta 1\text{-}142)}$ that retain DNA-binding activity but fail to activate transcription. Once we had established that $DctD_{(\Delta 1\text{-}142)}$ could function as a repressor of the *ant* gene in the challenge phage system, we began to isolate mutant forms of $DctD_{(\Delta 1\text{-}142)}$ that failed to activate transcription but retained the ability to bind DNA. Following mutagenesis of a plasmid-borne *dctD* allele encoding $DctD_{(\Delta 1\text{-}142)}$, mutagenized plasmids were introduced into a P22-sensitive *S. typhimurium* strain. Expression of $DctD_{(\Delta 1\text{-}142)}$ was induced with IPTG in these transformed cells, and the cells were then infected with the challenge phage bearing the DctD-binding site to select for $DctD_{(\Delta 1\text{-}142)}$ mutants that retained DNA-binding activity. Plasmids from kanamycin-resistant lysogens were transformed into an *E. coli* strain that carried a *dctA'-'lacZ* reporter gene on a compatible plasmid, which allowed us to screen for mutant forms of $DctD_{(\Delta 1\text{-}142)}$ that were deficient in transcriptional activation by scoring for a Lac^- phenotype.

A total of 55 independent $DctD_{(\Delta 1\text{-}142)}$ mutants were isolated by using this selection and screening procedure. Of these, 49 had single amino acid substitutions at 1 of 24 different amino acid residues. Specific mutations occurred from one to five times in these independent mutants (Table 1). Five of the mutants had two or more amino acid substitutions, and one had a deletion of four amino acids. Most of the mutations occurred at positions that are highly conserved among σ^{54}-dependent activators (Fig. 2), which further suggests the importance of these amino acid residues in the function or structure of σ^{54}-dependent activators. Mutations were distributed throughout the central domain, but most of the substitutions were within either the C1, C3, or C4 region.

The mutant forms of $DctD_{(\Delta 1\text{-}142)}$ fail to activate transcription in vitro. We anticipated that the mutant forms of $DctD_{(\Delta 1\text{-}142)}$ might be defective in one or more steps, including cooperative binding of the protein to the UAS, ATP hydrolysis, interactions with σ^{54}-holoenzyme, or coupling ATP hydrolysis to open complex formation. To determine if the mutant proteins were deficient in any of these activities, we purified and characterized selected mutant proteins. We tried to purify at least one mutant from each of the seven regions, but some of the mutant proteins failed to bind to the chromatography columns under the conditions that we used for the purification of $DctD_{(\Delta 1\text{-}142)}$. The mutant proteins that we purified were $DctD_{(\Delta 1\text{-}142,\ F222L)}$, $DctD_{(\Delta 1\text{-}142,\ T223I)}$, $DctD_{(\Delta 1\text{-}142,\ A225T)}$, $DctD_{(\Delta 1\text{-}142,\ E247G)}$, $DctD_{(\Delta 1\text{-}142,\ D301E)}$, and $DctD_{(\Delta 1\text{-}142,\ R305Q)}$.

All of the purified mutant proteins tested in the single-round transcription assay failed to activate transcription from the *dctA* promoter regulatory region (Fig. 3). These data were consistent with the previous in vivo results in which the mutant proteins failed to activate transcription from a *dctA'-'lacZ* reporter gene in the Lac screen.

DNA-binding activities of mutant forms of $DctD_{(\Delta 1\text{-}142)}$ appear to be unaffected. The challenge phage selection that we used relied on the ability of the mutant forms of $DctD_{(\Delta 1\text{-}142)}$ to recognize the site B from the *R. meliloti dctA* UAS carried by the challenge phage. Therefore, it seemed unlikely that any of the mutants would be defective in recognition of this site. We could not, however, preclude the possibility that some of the mutants were defective in cooperative binding to the UAS. When the two DctD-binding sites of the *dctA* UAS are sepa-

TABLE 1. Summary of *dctD* alleles that disrupt function of $DctD_{(\Delta 1\text{-}142)}$

Amino acid substitution	Codon change	Region	No. of isolates	Phenotype on LB–X-Gal[a]	Mutagenesis method
E174K	GAG→AAG	C1	1	White	HA[b]
G176D	GGC→GAC	C1	1	White	HA
G178S	GGC→AGC	C1	1	White	HA
E180K	GAG→AAG	C1	2	White	HA
V181G	GTC→GGC	Near C1	1	White	PCR
C202Y	TGC→TAC	C2	1	Faint blue	HA[c]
E207K	GAA→AAA	C2-C3	1	Faint blue	HA[c]
E213V	GAG→GTG	C3	1	White	PCR
G220D	GGC→GAC	C3	5	White	HA (4)[d] PCR
A221V	GCC→GTC GCC→GTT	C3	5	White	HA
A221D	GCC→GAC	C3	2	White	PCR
F222L	TTT→CTT TTT→TTA	C3	2	White	PCR
T223I	ACC→ATC	C3	1	White	HA
A225T	GCC→ACC	C3	4	White	HA (3) PCR
G231S	GGC→AGC	C4	1	White	PCR
G239D	GGC→GAC	C4	2	White	HA
E245K	GAG→AAG	C4	2	White	HA
E247K	GAG→AAG	Near C4	5	Faint blue	HA (4) PCR
E247G	GAG→GGG	Near C4	1	White	PCR
E247V	GAG→GTG	Near C4	1	White	PCR
E262K	GAA→AAA	C5	2	White	HA
L269F	CTC→TTC	C5-C6	1	White	HA
D301E	GAT→GAA	C6	1	White	PCR
R305Q	CGG→CAG	C6	1	White	HA
L311P	CTC→CCC	C6	1	White	PCR
G360D	GGC→GAC	C7	1	White	HA
A369V	GCC→GTC	C7	2	White	HA

[a] Determined by introducing the plasmids into *E. coli* DH5α that carried a *dctA'-'lacZ* reporter gene on plasmid pRMAZ+UAS (13) and then growing cells overnight on LB agar containing X-Gal, ampicillin, and tetracycline.
[b] HA, hydroxylamine.
[c] Isolated without the challenge phage selection.
[d] Numbers in parentheses are the numbers of independent isolates obtained for a given mutagenesis method if more than one obtained.

rated, site B shows approximately 50-fold-higher affinity for DctD than site A (13). When the sites are together, however, both sites have the same high affinity for DctD, indicating that DctD binds cooperatively to these sites (13, 31).

To determine if any of the mutants were defective in cooperative binding to the *dctA* UAS, we examined the ability of each of these proteins to protect the UAS from digestion by DNase I. For these experiments, we used either a DNA fragment that carried the wild-type *dctA* UAS or one that had a 5-bp insertion between sites A and B of the UAS. Insertion of the 5 bp between the DctD-binding sites had been previously shown to disrupt cooperative binding by $DctD_{(\Delta 1\text{-}142)}$ (31). DNase I footprinting experiments with the DNA fragment bearing the 5-bp insertion indicated that the apparent affinities of the mutant forms of $DctD_{(\Delta 1\text{-}142)}$ for the individual DctD-binding sites were similar to that of the wild-type protein [Fig. 4; data shown only for $DctD_{(\Delta 1\text{-}142)}$ and $DctD_{(\Delta 1\text{-}142,\ T223I)}$]. Likewise, the apparent affinities of the mutant proteins for the native UAS were similar to that observed with the wild-type protein (Fig. 4). Taken together, these data suggest that none of the amino acid substitutions significantly altered the affinity of the proteins for the individual DctD-binding sites or cooperative binding to these sites.

Some of the mutant forms of $DctD_{(\Delta 1\text{-}142)}$ are impaired in the ability to hydrolyze ATP. ATPase activities were determined for each of the purified mutant forms of $DctD_{(\Delta 1\text{-}142)}$. The three proteins with amino acid substitutions in the C3 region, $DctD_{(\Delta 1\text{-}142,\ F222L)}$, $DctD_{(\Delta 1\text{-}142,\ T223I)}$, and $DctD_{(\Delta 1\text{-}142,\ A225T)}$, had ATPase activities that were 13 to 123% of that observed with the wild-type protein (Table 2). ATPase activities were severely impaired, however, for proteins with amino acid substitutions in either the C4 or C6 region [$DctD_{(\Delta 1\text{-}142,\ E247G)}$, $DctD_{(\Delta 1\text{-}142,\ D301E)}$, and $DctD_{(\Delta 1\text{-}142,\ R305Q)}$].

To confirm that the high ATPase activity that we observed with the purified preparation of $DctD_{(\Delta 1\text{-}142,\ T223I)}$ was not due to a contaminating enzyme, we placed a histidine tag at the N terminus of this protein to provide an alternative method for its purification. We also placed a histidine tag at the N termini of other selected mutant proteins to further examine the ATPase activities of these proteins. These histidine-tagged proteins were purified and assayed for ATPase activity. As shown in Table 3, the purified histidine-tagged derivative of $DctD_{(\Delta 1\text{-}142,\ T223I)}$ had reasonably high ATPase activity. The histidine tag apparently inhibited the ATPase activities of the $DctD_{(\Delta 1\text{-}142)}$ and $DctD_{(\Delta 1\text{-}142,\ T223I)}$ proteins, as the activities of the histidine-tagged derivatives of these proteins were 7- to 10-fold lower than the activities of the original proteins (Tables 2 and 3). Despite its reduced ATPase activity, the histidine-tagged $DctD_{(\Delta 1\text{-}142)}$ protein activated transcription in vitro as well as $DctD_{(\Delta 1\text{-}142)}$ (data not shown). For the mutant forms of $DctD_{(\Delta 1\text{-}142)}$ that had little or no detectable ATPase activity [$DctD_{(\Delta 1\text{-}142,\ E247G)}$, $DctD_{(\Delta 1\text{-}142,\ D301E)}$, and $DctD_{(\Delta 1\text{-}142,\ R305Q)}$], we similarly observed very low ATPase activities with their histidine-tagged derivatives.

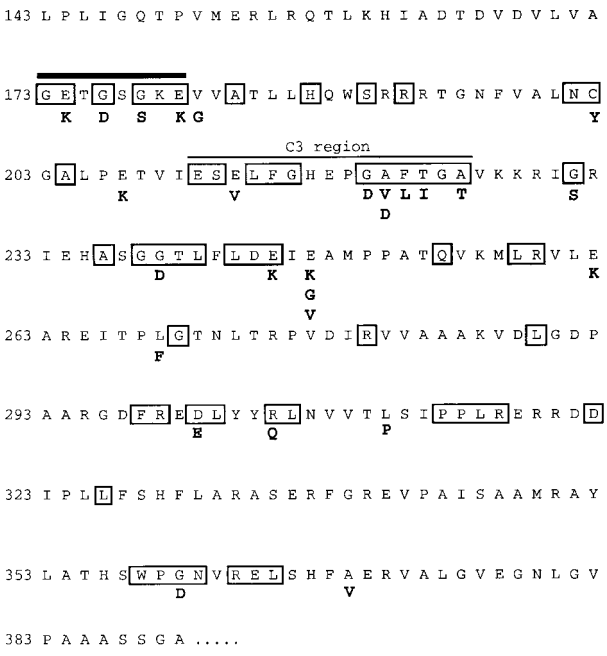

FIG. 2. Amino acid sequence of the activation domain of *R. meliloti* DctD and the locations of amino acid substitutions in mutant forms of $DctD_{(\Delta 1\text{-}142)}$. The numbers to the left of the sequences indicate the amino acid positions in the wild-type *R. meliloti* DctD (11). Amino acid residues that are highly conserved among σ^{54}-dependent activators are indicated in boxes and were determined from an alignment of 15 physiologically distinct σ^{54}-dependent activators that was generated by North et al. (23). The activators used in the alignment were *S. typhimurium* NtrC, *Klebsiella pneumoniae* NifA, *Rhizobium leguminosarum* DctD, *Alcaligenes eutrophus* HoxA, *A. eutrophus* AcoR, *E. coli* FhlA, *E. coli* HydG, *Pseudomonas putida* XylR, *Pseudomonas syringae* pv. *phaseolicola* HrpS, *Pseudomonas aeruginosa* AlgB, *P. aeruginosa* PilR, *Pseudomonas* sp. strain CF600 DmpR, *Caulobacter crescentus* FlbD, *Bacillus subtilis* RocR, and *Bacteroides thetaiotaomicron* RteB. Amino acid residues in *R. meliloti* DctD that are identical to at least 13 of these 15 activators are indicated in boxes. The heavy line above the sequence indicates the phosphate loop (Walker type A sequence) of DctD. The C3 region is also indicated. The amino acid substitutions in the different mutant forms of $DctD_{(\Delta 1\text{-}142)}$ are indicated by boldface letters below the sequence.

We also placed a histidine tag at the N terminus of $DctD_{(\Delta 1\text{-}142,\ G360D)}$, which was one of the proteins that we had been unable to purify. The amino acid substitution in this protein is within the C7 region. The histidine-tagged $DctD_{(\Delta 1\text{-}142,\ G360D)}$, as well as the other histidine-tagged mutant forms of $DctD_{(\Delta 1\text{-}142)}$, failed to activate transcription from the *dctA* promoter regulatory region in the single-round transcription assay [Fig. 3; data shown only for the histidine-tagged derivative of $DctD_{(\Delta 1\text{-}142,\ G360D)}$]. In addition, the histidine-tagged $DctD_{(\Delta 1\text{-}142,\ G360D)}$ showed very little ATPase activity (Table 3).

All of the mutant forms of $DctD_{(\Delta 1\text{-}142)}$ interact with σ^{54} and the β subunit of RNA polymerase. We had previously shown that $DctD_{(\Delta 1\text{-}142)}$ could be cross-linked to σ^{54} and the β subunit of RNA polymerase with the heterobifunctional cross-linking reagent sulfo-SMCC (15). These observations suggested that $DctD_{(\Delta 1\text{-}142)}$ may engage these subunits to activate transcription. We wanted to determine if any of the mutant forms of $DctD_{(\Delta 1\text{-}142)}$ failed to cross-link with either σ^{54} or the β subunit. All of the purified mutant proteins cross-linked to σ^{54} and the β subunit (Fig. 5; data shown only for proteins with amino acid substitutions in the C3 region). All of the histidine-tagged proteins likewise cross-linked to σ^{54} and the β subunit, but not as efficiently as the proteins without the histidine tag (data not shown). We do not understand why the histidine-tagged proteins cross-linked less efficiently, but the histidine tag does introduce a cysteine residue that may compete with cysteine residues in σ^{54} and the β subunit for reaction with the maleimide group of sulfo-SMCC.

The data presented above suggested that none of the mutant proteins with single amino acid substitutions that we tested were defective in binding to σ^{54}-holoenzyme. Given that the C3 region of DctD has been suggested to be involved in protein-protein interactions with σ^{54}-holoenzyme (21), we wanted to determine if a mutant form of $DctD_{(\Delta 1\text{-}142)}$ that lacked an intact C3 region could be cross-linked with either σ^{54} or the β subunit. One of the mutant forms of $DctD_{(\Delta 1\text{-}142)}$ that we isolated following random mutagenesis with PCR had a deletion of Phe-222 through Ala-225 in the C3 region. This protein was purified and found to cross-link normally with σ^{54} and the β subunit (data not shown). These data further support the idea that the C-terminal segment of the C3 region (the GAFTGA motif) is not required for strong interactions with σ^{54}-holoenzyme.

DISCUSSION

Most amino acid substitutions in mutant forms of $DctD_{(\Delta 1\text{-}142)}$ affect conserved residues. The *R. meliloti* $DctD_{(\Delta 1\text{-}142)}$ protein provides an excellent model for the genetic analysis of structure-function relationships in σ^{54}-dependent activators. Because $DctD_{(\Delta 1\text{-}142)}$ lacks the N-terminal regulatory domain, amino acid substitutions that result in loss of activity of the protein are likely to directly affect catalysis of open complex formation rather than interfere with phosphorylation or the subsequent conformational change of the protein.

We isolated 27 unique mutant forms of $DctD_{(\Delta 1\text{-}142)}$ that carry single amino acid substitutions within the activation domain (Table 1; Fig. 1). These mutations occurred at 24 different positions within this domain. Most of the substitutions (16 of 24) affected residues that are identical in a majority of phylogenetically and functionally diverse σ^{54}-dependent activators. Many of the other substitutions affected residues that are reasonably well conserved or are in the vicinity of identical amino acid residues. This extensive collection of mutant forms of $DctD_{(\Delta 1\text{-}142)}$ will be a valuable resource for further biochem-

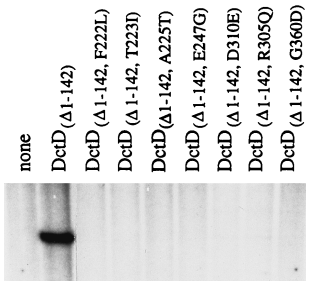

FIG. 3. Mutant forms of $DctD_{(\Delta 1\text{-}142)}$ fail to activate transcription in vitro. Purified mutant forms of $DctD_{(\Delta 1\text{-}142)}$ were tested for the ability to activate transcription from the *dctA* promoter regulatory region in a single-round transcription assay. Transcription assay reactions contained plasmid template, *E. coli* core RNA polymerase, *S. typhimurium* σ^{54}, and either $DctD_{(\Delta 1\text{-}142)}$ or one of the mutant forms of $DctD_{(\Delta 1\text{-}142)}$ as indicated above each lane. For $DctD_{(\Delta 1\text{-}142,\ G360D)}$, the histidine-tagged derivative of the protein was used in the transcription assay. Open complexes were formed by adding ATP. The remaining deoxynucleoside triphosphates and [α-^{32}P]CTP were then added to allow synthesis of transcripts, along with heparin, which was added to terminate open complex formation. Transcripts were visualized by subjecting the reaction mixtures to denaturing gel electrophoresis and then exposing X-ray film to the resulting gel.

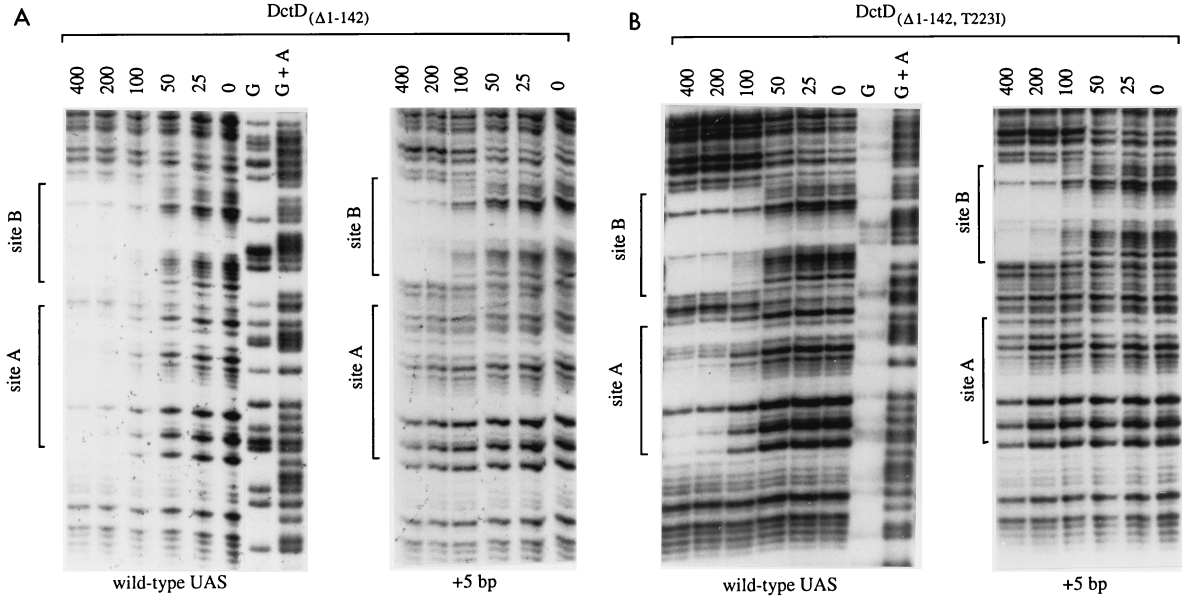

FIG. 4. DNase I footprinting of $DctD_{(\Delta 1\text{-}142)}$ and $DctD_{(\Delta 1\text{-}142,\ T223I)}$ at either the *R. meliloti dctA* UAS or a mutant form of the UAS. DNase I footprinting assays were performed with a DNA fragment that carried either the *dctA* UAS (wild-type UAS) or a mutant form of the UAS that had a 5-bp insertion between sites A and B (+5 bp). The nontemplate strand of the DNA fragment was end labeled in both cases. The regions containing the footprints were aligned with chemical sequencing reactions (18) of the DNA fragment that carried the wild-type UAS (lanes G and G+A). The regions corresponding to sites A and B are indicated. (A) DNA fragments bearing either the *dctA* UAS or the mutant UAS were incubated with different concentrations of $DctD_{(\Delta 1\text{-}142)}$ and then treated with DNase I. The number above each lane indicates the concentration (nanomolar) of $DctD_{(\Delta 1\text{-}142)}$ used in the assay. (B) DNA fragments were incubated with various concentrations of $DctD_{(\Delta 1\text{-}142,\ T223I)}$ and then treated with DNase I. The number above each lane indicates the concentration (nanomolar) of $DctD_{(\Delta 1\text{-}142,\ T223I)}$ used for the assay. DNase I footprints were visualized by subjecting the reactions to denaturing gel electrophoresis and then exposing X-ray film to the resulting gels. The other mutant forms of $DctD_{(\Delta 1\text{-}142)}$ showed similar patterns of protection in the DNase I footprinting assay.

ical characterization of mechanism of transcriptional activation by σ^{54}-dependent activators.

One difficulty in working with mutant forms of an enzyme is distinguishing between amino acid substitutions that cause major structural changes within the protein and those that specifically affect catalysis. Some of the mutant proteins did not behave the same as $DctD_{(\Delta 1\text{-}142)}$ in the purification protocol, suggesting that these amino acid substitutions caused major structural changes within these proteins. Most of the mutant forms of $DctD_{(\Delta 1\text{-}142)}$ that we characterized in vitro, however, behaved like $DctD_{(\Delta 1\text{-}142)}$ in the purification protocol, suggesting that the amino acid substitutions in these mutant proteins did not drastically alter the structure of the protein. In addition, all of the purified mutant proteins bound cooperatively to the *R. meliloti dctA* UAS. The determinants for cooperative binding in NtrC likely lie within the activation domain (7), and we infer that such determinants for cooperative binding are also likely to reside in the activation domain of DctD. The fact that all of the purified mutant forms of $DctD_{(\Delta 1\text{-}142)}$ bound cooperatively to the UAS further suggested that none of the amino acid substitutions in these mutant proteins caused major structural changes in the activation domain of the protein.

Substitutions in the C3 region of $DctD_{(\Delta 1\text{-}142)}$ uncouple ATP hydrolysis from transcriptional activation. About one-third of the single amino acid substitutions (20 of 55 independent mutants) were within the C3 region, whereas only a few mutations occurred in some of the other well-conserved regions of the protein (Table 1; Fig. 1). The C3 region is thought to be involved in productive interactions between the activator and σ^{54}-holoenzyme, functioning in either contact with σ^{54}-holoenzyme or coupling ATP hydrolysis to open complex formation (21, 23). This hypothesis was inferred from two different lines of evidence. First, *S. typhimurium* $NtrC^{repressor}$ proteins with single amino acid substitutions in the C3 region (Ala-216 to Val and Ala-220 to Thr; these amino acid residues correspond to Ala-221 and Ala-225 of DctD, respectively) bind DNA nor-

TABLE 2. ATPase activities of mutant forms of $DctD_{(\Delta 1\text{-}142)}$

Protein	Phosphate released[a] (pmol/10 min)	% of activity of wild-type protein
$DctD_{(\Delta 1\text{-}142)}$	9,260	100
$DctD_{(\Delta 1\text{-}142,\ F222L)}$	1,200	13
$DctD_{(\Delta 1\text{-}142,\ T223I)}$	11,400	123
$DctD_{(\Delta 1\text{-}142,\ A225T)}$	3,100	34
$DctD_{(\Delta 1\text{-}142,\ E247G)}$	120	1.3
$DctD_{(\Delta 1\text{-}142,\ D301E)}$	<15	<0.16
$DctD_{(\Delta 1\text{-}142,\ R305Q)}$	<15	<0.16

[a] Assay mixtures contained 1.5 μM $DctD_{(\Delta 1\text{-}142)}$ protein (dimer). Each value is the difference in phosphate released following incubation for 1 and 11 min and represents an average of at least two independent sets of assays. For a given protein, the values for the individual assays varied from 6 to 40%.

TABLE 3. ATPase activities of histidine-tagged proteins

Protein	Phosphate released[a] (pmol/10 min)	% of activity of wild-type protein
$DctD_{(\Delta 1\text{-}142)}$	862	100
$DctD_{(\Delta 1\text{-}142,\ T223I)}$	1,550	180
$DctD_{(\Delta 1\text{-}142,\ E247G)}$	130	15
$DctD_{(\Delta 1\text{-}142,\ D301E)}$	120	14
$DctD_{(\Delta 1\text{-}142,\ R305Q)}$	38	5
$DctD_{(\Delta 1\text{-}142,\ G360D)}$	80	9

[a] Assay mixtures contained 1.5 μM histidine-tagged $DctD_{(\Delta 1\text{-}142)}$ protein (dimer), and values are from a single determination.

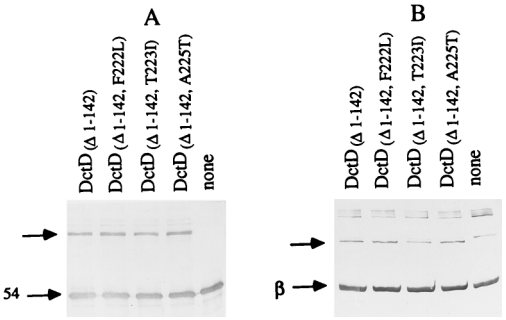

FIG. 5. Cross-linking mutant forms of $DctD_{(\Delta 1\text{-}142)}$ to either σ^{54} or the β subunit of RNA polymerase. Mutant forms of $DctD_{(\Delta 1\text{-}142)}$ were treated with the cross-linking reagent sulfo-SMCC, dialyzed to remove excess cross-linking reagent, and then mixed in an equimolar ratio with either *S. typhimurium* σ^{54} or *E. coli* core RNA polymerase. Portions of the cross-linking reactions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, after which proteins were transferred to nitrocellulose for immunoblotting with either antiserum directed against *S. typhimurium* σ^{54} (provided by S. Kustu) (A) or monoclonal antibody specific for the β subunit (provided by K. Severinov) (B). Membranes were then treated with an alkaline phosphatase conjugate of either anti-rabbit immunoglobulin (A) or anti-mouse immunoglobulin (B). Cross-reacting bands were visualized by incubating the membranes with the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolylphosphate. The lower arrows indicate σ^{54} and the β subunit, while the cross-linked products are indicated with the upper arrows. The different mutant forms of $DctD_{(\Delta 1\text{-}142)}$ are indicated at the top. As a control, sulfo-SMCC was added to buffer that contained no $DctD_{(\Delta 1\text{-}142)}$ protein (none), and the solution was dialyzed and mixed with either σ^{54} or core RNA polymerase as described above. Note that in the control involving core RNA polymerase, a cross-reactive band that migrates more slowly than the β-$DctD_{(\Delta 1\text{-}142)}$ cross-linked species is present. This band is also present in the cross-linking reactions that included $DctD_{(\Delta 1\text{-}142)}$ proteins, and we infer that it is a cross-linked product of β and some other subunit(s) of core RNA polymerase that results from carryover of unreacted sulfo-SMCC.

mally and have ATPase activities that should be sufficient to activate transcription, but they are unable to catalyze open complex formation (23). The second line of evidence comes from comparison of amino acid sequences of σ^{54}-dependent activators and other transcriptional activators. Some regulatory proteins, such as *E. coli* TyrR and *Rhodobacter capsulatus* NtrC, have domains that share homology with the activation domain of σ^{54}-dependent activators, but they regulate transcription with a form of RNA polymerase holoenzyme other than σ^{54}-holoenzyme (3, 12, 21, 26). These regulatory proteins, however, lack all or part of the region that corresponds to the C3 region of σ^{54}-dependent activators.

The three mutant forms of $DctD_{(\Delta 1\text{-}142)}$ with substitutions in the C3 region that we studied [$DctD_{(\Delta 1\text{-}142,\ F222L)}$, $DctD_{(\Delta 1\text{-}142,\ T223I)}$, and $DctD_{(\Delta 1\text{-}142,\ A225T)}$] had properties that were similar to those of the $NtrC^{A216V}$ and $NtrC^{A220T}$ proteins. These mutant forms of $DctD_{(\Delta 1\text{-}142)}$ bound cooperatively to the *dctA* UAS and also retained the ability to hydrolyze ATP. The ATPase activity of one of these mutant proteins, $DctD_{(\Delta 1\text{-}142,\ T223I)}$, was greater than that of $DctD_{(\Delta 1\text{-}142)}$, while the other two mutant forms of $DctD_{(\Delta 1\text{-}142)}$ retained significant levels of their ATPase activity. Despite the fact that the ATPase activities of $DctD_{(\Delta 1\text{-}142,\ F222L)}$ and $DctD_{(\Delta 1\text{-}142,\ A225T)}$ were lower than that of $DctD_{(\Delta 1\text{-}142)}$, their ATPase activities were greater than that of the histidine-tagged $DctD_{(\Delta 1\text{-}142)}$ protein, which was capable of activating transcription both in vivo and in vitro (data not shown). This result suggested that the ATPase activities of these mutant forms of $DctD_{(\Delta 1\text{-}142)}$ should have been sufficient for transcriptional activation.

One advantage of using $DctD_{(\Delta 1\text{-}142)}$ as a model for structure-function studies of σ^{54}-dependent activators is that interactions between $DctD_{(\Delta 1\text{-}142)}$ and σ^{54} or core RNA polymerase can be examined with chemical cross-linking reagents (15). All three of the mutant forms of $DctD_{(\Delta 1\text{-}142)}$ with substitutions in the C3 region cross-linked to σ^{54} and the β subunit of RNA polymerase (Fig. 5). When considered with the ATPase activities of these proteins, these cross-linking data suggest that the region that encompasses positions 222 to 225 of DctD probably functions in coupling the energy released by ATP hydrolysis to open complex formation rather than as a major determinant for binding to σ^{54}-holoenzyme. We cannot, however, rule out the possibility that this part of the C3 region must interact with σ^{54}-holoenzyme during open complex formation, but these interactions occur only transiently during the transcriptional activation cycle and are too weak to be detected by the cross-linking assay.

The failure to identify mutant proteins that do not cross-link with either σ^{54} or the β subunit in this study could imply that the cross-linking assay is not sensitive enough to identify amino acid substitutions that interfere with protein-protein interactions between $DctD_{(\Delta 1\text{-}142)}$ and σ^{54}-holoenzyme. Recent work from our laboratory, however, argues against this interpretation. We have isolated additional mutant forms of $DctD_{(\Delta 1\text{-}142)}$ following localized mutagenesis of the C3 region. Some of these mutant proteins with substitutions in the N-terminal half of the C3 region failed to cross-link with the β subunit and cross-linked poorly with σ^{54}, suggesting that the cross-linking assay is able to distinguish mutant forms of $DctD_{(\Delta 1\text{-}142)}$ that have reduced affinities for σ^{54}-holoenzyme (35).

Substitutions in other regions of $DctD_{(\Delta 1\text{-}142)}$ disrupt one or more functions needed for transcriptional activation. Several of the amino acid substitutions in the mutant forms of $DctD_{(\Delta 1\text{-}142)}$ were within the GXXXXGK nucleotide-binding motif of the protein. In our initial attempts, we were unable to purify two different proteins with substitutions in this region [$DctD_{(\Delta 1\text{-}142,\ G178S)}$ and $DctD_{(\Delta 1\text{-}142,\ E180K)}$], and histidine-tagged derivatives were not constructed for either of these mutant proteins. Substitution of the penultimate residue of this nucleotide-binding motif in the σ^{54}-dependent activators NtrC (Gly-173 to Asn) and XylR (Gly-268 to Asn) results in loss of ATPase activity (24, 38), and we infer that substitutions in this motif similarly interfere with the ATPase activity of $DctD_{(\Delta 1\text{-}142)}$.

The remaining mutant forms of $DctD_{(\Delta 1\text{-}142)}$ that we tested [$DctD_{(\Delta 1\text{-}142,\ E247G)}$, $DctD_{(\Delta 1\text{-}142,\ D301E)}$, $DctD_{(\Delta 1\text{-}142,\ R305Q)}$, and the histidine-tagged $DctD_{(\Delta 1\text{-}142,\ G360D)}$ protein] had little or no ATPase activity (Tables 2 and 3). The amino acid substitutions in these proteins were in the C4, C6, and C7 regions of the activation domain. Similar results were reported for the $NtrC^{repressor}$ proteins $NtrC^{S207F}$, $NtrC^{R294C}$, $NtrC^{G355V}$, $NtrC^{R358C}$, and $NtrC^{R358H}$, all of which lacked detectable ATPase activity (23). The amino acid substitutions in these $NtrC^{repressor}$ proteins were in the C3, C6, and C7 regions. The mutational analyses of these two σ^{54}-dependent activators suggest that several regions within the activation domain are involved in ATP binding and hydrolysis.

It is unclear if the low levels of ATPase activity observed for some of these mutant forms of $DctD_{(\Delta 1\text{-}142)}$ [e.g., $DctD_{(\Delta 1\text{-}142,\ E247G)}$] are responsible for the failure of these proteins to catalyze open complex formation, or if these proteins are deficient in other activities needed to catalyze open complex formation. All of these proteins bound cooperatively to the *dctA* UAS and all could be cross-linked to σ^{54} and the β subunit of RNA polymerase, suggesting that these proteins interact normally with themselves and with σ^{54}-holoenzyme.

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