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Role of the RNA Polymerase α Subunits in MetR-Dependent Activation of *metE* and *metH*: Important Residues in the C-Terminal Domain and Orientation Requirements within RNA Polymerase

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Many transcription factors activate by directly interacting with RNA polymerase (RNAP). The C terminus of the RNAP α subunit (αCTD) is a common target of activators. We used both random mutagenesis and alanine scanning to identify αCTD residues that are crucial for MetR-dependent activation of *metE* and *metH*. We found that these residues localize to two distinct faces of the αCTD. The first is a complex surface consisting of residues important for α-DNA interactions, activation of both genes (residues 263, 293, and 320), and activation of either *metE* only (residues 260, 276, 302, 306, 309, and 322) or *metH* only (residues 258, 264, 290, 294, and 295). The second is a distinct cluster of residues important for *metE* activation only (residues 285, 289, 313, and 314). We propose that a difference in the location of the MetR binding site for activation at these two promoters accounts for the differences in the residues of α required for MetR-dependent activation. We have designed an in vitro reconstitution-purification protocol that allows us to specifically orient wild-type or mutant α subunits to either the β-associated or the β′-associated position within RNAP (comprising α_2, β, β′, and σ subunits). In vitro transcriptions using oriented α RNAP indicate that a single αCTD on either the β- or the β′-associated α subunit is sufficient for MetR activation of *metE*, while MetR interacts preferentially with the αCTD on the β-associated α subunit at *metH*. We propose that the different αCTD requirements at these two promoters are due to a combination of the difference in the location of the activation site and limits on the rotational flexibility of the αCTD.

In *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, the final step in methionine biosynthesis is the transfer of a methyl group to homocysteine to form methionine. The reaction can be catalyzed by either of two transmethylases encoded by *metE* or *metH* (12). The *metH* gene product is dependent on vitamin B_{12} as a cofactor, whereas the *metE* gene product is not.

Transcription of a number of genes in the methionine biosynthetic pathway, including *metE* and *metH*, is dependent on the MetR activator protein; MetR is a dimer in solution (25) and binds to the consensus site 5′-TGAANNTNNTTCA-3′ (49). Using homocysteine as a coactivator, MetR stimulates expression of the *metE* promoter up to 200-fold (48). MetR protects two adjacent sites, from bp −26 to −49 (site 2) and from bp −50 to −73 (site 1) upstream of the *Salmonella* serovar Typhimurium *metE* transcription start site, from DNase I cleavage (Fig. 1). Within each DNase I-protected region are perfect (site 1) or near-perfect (site 2) matches to the MetR consensus binding site sequence centered at −63 and −42 for sites 1 and 2, respectively (49, 54). Although both sites are necessary for activation of *metE*, genetic data suggest that site 2 is the activation site; MetR at the high-affinity site 1 appears to promote the homocysteine-dependent filling by a second MetR dimer at the lower-affinity site 2 (54). The *Salmonella* serovar Typhimurium *metH* gene is activated up to 19-fold by the presence of MetR (5); however, homocysteine, in contrast to its role at the *metE* promoter, decreases this activation 3-fold, probably by an indirect effect of lowering MetR levels (46, 48). Genetic and biochemical analyses indicate that there is a single MetR dimer binding site at *metH* as well as two alternative start sites separated by 3 intervening bp (Fig. 1) (5, 47); however, regardless of which start site is used, the center of the activation site at *metH* (either at −57 or at −61) is clearly different from the center of the activation site of *metE* at −42. Because of the difference in the number of MetR binding sites and the location of the activation site at these promoters, it is possible that MetR may use different mechanisms to activate these two promoters.

Activation at many promoters results from interactions between an activator protein and RNA polymerase (RNAP) (reviewed in reference 14). The specific subunit of RNAP (α_2, β, β′, or σ) that is contacted by an activator appears to depend at least in part on the location of the activator binding site relative to the RNAP binding site. For many activator proteins that bind upstream of the promoter, such as cyclic AMP receptor protein (CRP), which binds at the *lacP1* promoter, the major contact site on RNAP is the C terminus of the α dimer subunits (αCTD). The α-CRP interaction increases the affinity of RNAP for the *lac* promoter, which, in turn, increases transcription (reviewed in reference 8). Recruitment may be a common mechanism for activators that bind upstream of promoters; it has been shown that activation can occur when the αCTD is replaced with a heterologous protein domain capable of interacting with a specific partner protein bound upstream of the promoter (7). However, the αCTD is not the exclusive target of activator proteins, and activation may also be mediated by RNAP-activator interactions that influence steps of transcrip-

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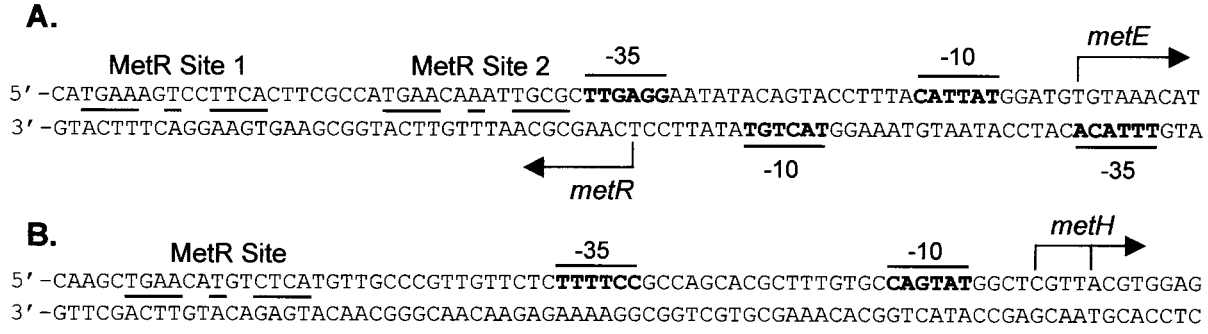

FIG. 1. The *metE-metR* and *metH* promoters of *Salmonella* serovar Typhimurium. Promoter −10 and −35 sequences are boldfaced; those for *metE* and *metH* are overlined, and those for *metR* are underlined. MetR binding sequences are lined between the DNA strands. Transcription start sites are marked with arrows. The *metE* and *metH* transcription start sites were determined by primer extension (unpublished data). (A) *metE-metR* promoter region; (B) *metH* promoter region.

tion initiation subsequent to RNAP binding. At promoters where the activator binds to sites adjacent to or overlapping the promoter elements, contacts with the N terminus of α (e.g., CRP at *gal*) and with σ^{70} (e.g., λ cI at P_{RM}) have been reported (23, 31). The phage N4 single-stranded DNA binding protein activates N4 late promoters through an interaction with the β′ subunit, and the σ^{54}-dependent activator C_4-dicarboxylic acid transport protein D of *Rhizobium meliloti* interacts in solution with both the β subunit and σ^{54} (22, 26).

In this report, we describe experiments aimed at identifying and understanding the role of activator-RNAP interactions in MetR-dependent activation of *metE* and *metH*. It has previously been shown that removal of the entire αCTD eliminates activation by MetR at both *metE* and *metH* in vitro (15a). Here we describe the effects of various point mutations in the αCTD on the activation by MetR at these two promoters in vivo and in vitro. In addition, we describe a protocol for the reconstitution of RNAP containing oriented α subunits. We have used the oriented α RNAPs to show that MetR-dependent activation at *metE* and *metH* have different requirements for the location of wild-type α subunits within the RNAP enzyme complex in order for activation by MetR to occur, suggesting that the mechanisms of activation by MetR differ at these two promoters.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The strains and plasmids used in this study are listed in Table 1.

Plasmid pREII-Strepα was constructed using site-directed mutagenesis to insert the sequence 5′-GCT TGG AGC CAC CCG CAG TTC GAA AAA GGT GCT-3′ (encoding the *Strep*-tag II amino acid sequence WSHPQFEK [37] flanked by an alanine on the 5′ end and a glycine and an alanine on the 3′ end) between codons 1 and 2 of the *rpoA* gene in plasmid pREIIα. For production of Strep-tagged α protein, plasmid pT7-Strepα was constructed by replacement of the *Xba*I-*Bam*HI fragment of pHTT7f1-NHα with the corresponding fragment from pREII-Strepα. This plasmid did not overproduce the Strep-tagged α as well as plasmid pHTT7f1-NHα overproduces the His_6-tagged α (data not shown); however, we could purify enough Strep-tagged α for reconstitutions of oriented α RNAP. Plasmid pT7-Strep(R45A)α was constructed by site-directed mutagenesis to convert codon 45 of *rpoA* from a CGT (arginine) to a GCT (alanine) codon in plasmid pT7-Strepα. Plasmid pHTT7f1-NH(ΔCTD)α was constructed by site-directed mutagenesis to convert codon 257 of the *rpoA* gene in plasmid pHTT7f1-NHα from GTT (valine) to TAA (stop) and create a *Bam*HI restriction site following the new stop codon. Plasmid pT7-Strep(R45A/ΔCTD)α was constructed by replacement of the *Hin*dIII-*Bam*HI fragment of pT7-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
GS162	*E. coli* K-12 Δ*lacU169*	This laboratory
GS244	GS162 derivative Δ*metR*::Mu	This laboratory
GS972	GS162 derivative carrying chromosomal operator constitutive mutation in *metR* promoter	This laboratory
GS1040	GS162 derivative carrying chromosomal E261K *rpoA* allele	This laboratory
GS1106	GS162 derivative carrying chromosomal *rpoA112* allele	This study
BL21(DE3)	*E. coli* λDE3, which encodes T7 RNAP from the *lacUV5* promoter	Novagen
Plasmids		
pREIIα (and derivatives)	*ori*-pBR322; *lppP-'lacPUV5-rpoA* (and derivatives carrying N268D, L270H, alanine substitutions at residues 275 to 301 and 303 to 329, or N-terminal Strep tag)	3, 10, 18, 53; This study
pHTf1α (and derivatives)	*ori*-pBR322; *ori*-f1; *lppP-'lacPUV5-rpoA* (and derivatives carrying alanine substitutions at residues 255 to 271, 273, or 302)	10, 42
pHTT7f1-NHα (and derivatives)	*ori*-pBR322; *ori*-f1; ϕ10P-*rpoA* with N-terminal His_6 tag (and derivatives carrying R265A, N268D, L270H, or G296A substitutions, or ΔCTD)	41; this study
pT7-Strepα (and derivatives)	*ori*-pBR322; *ori*-f1; ϕ10P-*rpoA* with N-terminal Strep tag (and derivatives carrying an R45A substitution, ΔCTD, or an R45A substitution plus ΔCTD)	This study
pMKSe2	*ori*-pBR322; *lacP-rpoB*	38
pT7β′	*ori*-pBR322; ϕ10P-*rpoC*	56
pRLG593	*ori*-pBR322; *lacPUV5*	35
pGS395	*ori*-pDF41; *tacP-metR*	This laboratory

Strep(R45A)α with the corresponding fragment from pHTT7f1-NH(ΔCTD)α. The plasmids used to express the His_6-tagged N268D, L270H, and G296A α subunits were generated by replacing the *Hin*dIII-*Bam*HI fragments of pHTT7f1-NHα with the corresponding fragments from pREII268Dα, pREII270Hα, and pREII296Aα. The His_6-tagged R265A derivative was generated by replacing the *Hin*dIII-*Bam*HI fragment of pHTT7f1-NHα with the *Hin*dIII-*Bst*YI fragment of pHTf1265Aα. The constructs were confirmed by DNA sequencing.

The λ^TElac1 phage, a λgt2 derivative containing a *metE-lacZ* translational fusion, is a temperature-resistant derivative of λElac1, which has been previously described (33). The λHlac phage, also a λgt2 derivative but carrying a *metH-lacZ* translational fusion, has been described previously (47).

Media and growth conditions. Tryptone broth (TB), Luria-Bertani broth (LB), and lactose tetrazolium agar were prepared as described previously (27). Glucose minimal medium was Vogel and Bonner minimal salts (51) supplemented with 0.4% glucose. Minimal medium was also supplemented with phenylalanine (50 μg/ml) and thiamine (1 μg/ml), since most of the strains carry the *pheA905* and *thi* markers. Strains carrying α plasmids were maintained in medium supplemented with ampicillin at 100 μg/ml; however, transformants used for the production of RNAP subunits were supplemented with ampicillin at 200 μg/ml. Strains carrying pGS395 were maintained in medium supplemented with kanamycin at 20 μg/ml.

Lysogens were grown at 37°C, except for λHlac lysogens, which were grown at 30°C, since the λHlac phage carries the λ*c*I857, mutation resulting in a temperature-sensitive λcI repressor (32).

β-Galactosidase enzyme assays. For all strains except those carrying the *rpoA112* allele, cells were grown in TB to an optical density at 600 nm (OD_{600}) of approximately 0.5 and then placed on ice for 20 min. The *rpoA112* strain GS1106 was grown as 2- by 2-cm patches on LB agar overnight at the desired temperature; prior to being assayed, the cells were scraped from the plate, resuspended in 1× Vogel and Bonner minimal salts to an OD_{600} of approximately 0.5, and then placed on ice for 20 min. β-Galactosidase enzyme activity was assayed using the chloroform-sodium dodecyl sulfate (SDS) lysis procedure (27). Assays were performed at least twice, with the activity of each sample determined in triplicate.

Mutant isolation. (i) Selection for mutations that affect *metE* expression. Random mutagenesis of the 1-kb *Xba*I-*Bam*HI *rpoA* fragment from pREIIα was performed as described previously (42). The PCR-mutagenized *rpoA* gene was cloned into pREIIα in place of the wild-type *Xba*I-*Bam*HI fragment. The selection strain, GS972λ^TElac1, forms white colonies on lactose tetrazolium agar when transformed with pREIIα. Transformants of GS972λ^TElac1 with decreased expression of the *metE-lacZ* fusion were identified as red colonies on lactose tetrazolium agar. The mutations were further mapped to the αCTD by subcloning the *Hin*dIII-*Bam*HI fragment (codons 230 to 329) from the mutant plasmids into pREIIα.

(ii) Screen for mutations that affect *metE* and/or *metH* expression. Plasmids from an alanine substitution library of the αCTD (residues 255 to 329) were individually transformed into GS162λ^TElac1 and GS972λHlac. As a preliminary screen, a single colony of each transformant was grown for 3 h (OD_{600} of approximately 0.5) in TB and assayed for β-galactosidase activity. Transformants that showed decreases in either *metE-lacZ* or *metH-lacZ* expression were selected for further study. Eight of the alanine substitutions increased *metE* expression in the preliminary screen (maximum *metE-lacZ* expression was 2.3-fold higher than that in wild-type α). However, these mutants were not included in this study.

Preparation of core RNAP containing α homodimers. The His_6-tagged α subunits were prepared under denaturing conditions as previously described (40) with the following modifications: cells were lysed by sonication in buffer B (6 M guanidine-HCl, 20 mM Tris-HCl [pH 7.9], 500 mM NaCl, 5 mM imidazole) instead of a nondenaturing buffer, and the ammonium sulfate precipitation step was eliminated. Inclusion bodies containing β and β′ were prepared as described previously (40). Reconstitutions were performed as described previously (40) with the following exceptions: reconstitution of σ^{70} was not performed at the same time as the core RNAP reconstitution, and activation of the RNAP following dialysis was performed in the absence of σ^{70}. Following activation, the core RNAP mixture was cleared by centrifugation and the reconstituted RNAP was purified by Ni^{2+} ion affinity chromatography as described previously (40). The resulting sample was concentrated to approximately 50 μl with a Microcon YM-100 concentrator (Millipore, Bedford, Mass.), mixed with an equal volume of glycerol, and stored at −20°C.

Preparation of Strep-tagged α. *E. coli* strain BL21(DE3) transformed with pT7-Strepα (or derivatives) was shaken at 37°C in 100 ml of LB plus ampicillin (200 μg/ml) to an OD_{600} of approximately 0.5, induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to 1 mM, and shaken an additional 6 to 15 h at 22°C. The cells were harvested by centrifugation (4,500 × *g*; 12 min at 4°C) and resuspended in 1.5 ml of buffer W (100 mM Tris-HCl [pH 8.0]–1 mM EDTA) at 4°C. Cells were lysed by sonication, and the lysate was cleared by centrifugation (16,000 × *g*; 15 min at 4°C). The supernatant was treated with 7 μl of a 1-mg/ml avidin solution (prepared in buffer W) for 30 min at 4°C and cleared by centrifugation (16,000 × *g*; 15 min at 4°C). The extract was adsorbed to a 1-ml StrepTactin column (Genosys Biotechnologies, Woodlands, Tex.) pre-equilibrated with buffer W, washed five times with 1 ml of buffer W, and eluted six times with 0.5 ml of buffer E (buffer W with 2.5 mM desthiobiotin). The bulk of the protein eluted in one fraction; the yield was 100 μg of protein as determined by the Bradford assay (4), and the purity was >50% as determined by SDS-polyacrylamide gel electrophoresis (PAGE). The contaminating proteins appeared, based on size, to be the other RNAP subunits; however, native, non-tagged α is distinguishable from the Strep-tagged α on our SDS–15% PAGE gels, and we could not detect any contaminating non-tagged α by staining with Coomassie brilliant blue R.

Preparation of core RNAP containing oriented α subunits. β, β′, and His_6-tagged and Strep-tagged α subunits were prepared as described above. For each reconstitution, 30 μg of each α species was added to make 60 μg of total α. Reconstitution and a first partial purification of core RNAP by Ni^{2+} ion affinity chromatography based on the His_6 tag were performed as described above for the purification of core RNAP containing α homodimers. The eluate from the Ni^{2+}-nitrilotriacetic acid resin (Qiagen Inc., Valencia, Calif.) following this first purification was then applied to a StrepTactin column (bed volume, 1 ml). Adsorption, washes, and elution of the StrepTactin column were performed as described above for the purification of Strep-tagged α. The RNAP typically eluted in one or two fractions that were concentrated as described above for the purification of core RNAP containing α homodimers. The yield was typically 5 to 10 μg, which is much lower than what is recovered from the single purification step for homodimeric RNAP (40); however, these yields were sufficient for several transcription experiments. The concentrated sample contained no traces of native, non-tagged α, as determined by Coomassie brilliant blue R staining of an SDS–15% PAGE gel (data not shown).

In vitro transcription assays. Aliquots of reconstituted core RNAP were incubated in the presence of a 1.5 molar excess of purified σ^{70} subunit to ensure the formation of RNAP holoenzyme for use in single-round runoff transcription assays in the presence of heparin as previously described (16). The activity of each reconstituted RNAP was normalized based on the amounts of *lacUV5* and RNA-I transcripts produced when the supercoiled plasmid pRLG593 was used as the template (35). Based on these initial experiments, the enzymes were used at the concentrations indicated in Fig. 3A and Fig. 4A to test the effect of RNAP containing mutant homodimers or oriented heterodimers of α on *metE* and *metH* transcription in the absence or presence of MetR (final dimer concentration, 135 and 270 nM). Template DNA containing both the *metE* and *metR* promoters and the *metH* template DNA were added simultaneously to reaction mixtures when the RNAPs containing homodimers of α were tested. In transcriptions with the oriented α RNAP, the *metE-metR* and *metH* templates were tested separately. Following electrophoresis, the gels were dried and analyzed by autoradiography. When quantitated, the bands corresponding to the *metE* and *metH* transcripts were analyzed with a Packard InstantImager (Meriden, Conn.). For reporting, the quantitated amount of *metE* and *metH* transcript produced by each RNAP was normalized to the sum of the *lacUV5* and RNA-I transcripts generated from the supercoiled template pRLG593 (35) by the same RNAP. Results for α homodimer RNAP are averages from two transcription experiments. Oriented α RNAP results are averages from two transcription experiments for *metE* and three transcriptions for *metH*.

RESULTS

Selection for α mutations that decrease *metE* expression. A plasmid-borne *rpoA* gene was randomly mutagenized for use in a genetic screen that would identify colonies with decreased *metE* expression. The strain used for the selection, GS972, constitutively expresses MetR. The overexpression of MetR was necessary because the rich medium used for the selection (lactose tetrazolium agar) would repress the wild-type *metR* promoter. A λ lysogen of GS972 carrying a *metE-lacZ* fusion, GS972λ^TElac1, when transformed with plasmid pREIIα expressing wild-type *rpoA*, produces white colonies on lactose tetrazolium agar. Although the chromosomal *rpoA* gene is present in this and all of the transformants used in this study, it has been shown by Western blotting that the α proteins expressed from the high-copy-number plasmids are the predominant α species in the cell (42). Screening of three independent plasmid pools carrying PCR-mutagenized *rpoA* genes yielded two transformants that formed red colonies on lactose tetrazolium agar, indicating decreased *metE-lacZ* expression. The *metE*-down phenotype of one of the mutants was shown to be plasmid associated and could be localized to the αCTD by subcloning of the *rpoA* gene. Sequencing of this mutant revealed a single-base-pair substitution that resulted in a change from asparagine to aspartic acid at amino acid 268 (N268Dα). For the second mutant, a plasmid association test revealed that the *metE*-down phenotype was unstable in GS972λ^TElac1, and

TABLE 2. Effects of point mutations at amino acids 268 and 270 of the α subunit on *metE-lacZ* and *metH-lacZ* expression

Plasmid	Plasmid *rpoA* allele	Expression in the indicated strain[a] of:					
		metE-lacZ			*metH-lacZ*		
		GS162 (MetR$^+$)	GS244 (MetR$^-$)	GS972 (MetR^{+++})	GS162(MetR$^+$)	GS244 (MetR$^-$)	GS972 (MetR^{+++})
pREIIα	Wild type	487	53	5,046	29	13	143
pREII-N268Dα	N268D	56	56	1,857	32	15	53
pREII-L270Hα	L270H	96	48	5,051	25	11	122

[a] Lysogens were grown in TB to mid-log phase. Results (β-galactosidase activity) are reported as Miller units (27). Standard deviations between assays varied by less than 12% of the reported means.

although subcloning showed that the phenotype was associated with the αCTD, the subclones were also unstable in GS972λ^{T}Elac1. Parallel subcloning of this mutant in GS162λ^{T}Elac1, the wild-type parent of GS972 carrying the *metE-lacZ* fusion, eventually gave rise to a stable phenotype by β-galactosidase assays (data not shown). Sequencing of this stable mutant revealed a single-base-pair substitution that resulted in a change from leucine to histidine at amino acid 270 (L270Hα).

To quantitate the effects of these αCTD mutations on *metE* expression, GS162λ^{T}Elac1 was transformed with plasmids expressing either wild-type α or one of the mutant α alleles. These transformants were grown in TB, and β-galactosidase levels were determined. As expected, the N268D and L270H substitutions strongly decreased *metE-lacZ* expression (Table 2). The *metE*-down phenotype associated with these mutations was lost if the mutant alleles were expressed in GS244λ^{T}Elac1, a *metR* strain, suggesting that these αCTD mutations disrupt MetR-dependent expression of *metE* instead of causing a general defect in transcription (Table 2).

Following multiple manipulations, including plasmid preparation and subcloning, the N268D and L270H *rpoA* alleles were assayed in the retransformed lysogen GS972λ^{T}Elac1. The N268D substitution decreased *metE* expression only threefold, compared to a ninefold decrease in GS162λ^{T}Elac1. Surprisingly, however, the L270H phenotype was completely suppressed (Table 2), so it is unclear how the L270H mutant was isolated as a red colony on lactose tetrazolium plates. We speculate that the original isolate, which exhibited a stronger phenotype on lactose tetrazolium plates than the N268D mutant, may have carried a second mutation (or an alternate substitution at amino acid 270) that was lost because of the detrimental effect it had on overall cellular gene expression. We further speculate that the second mutation was in the αCTD because the phenotype transferred, although unstably, with the αCTD subclones.

The N268D and L270H mutations were also tested for their effects on *metH-lacZ* expression. The same three strains used to test the effects on *metE-lacZ* expression were lysogenized with a phage carrying a *metH-lacZ* fusion. In GS162λHlac, neither α mutation caused a decrease in *metH-lacZ* expression. However, in GS972λHlac, the N268D substitution caused nearly a threefold decrease in *metH-lacZ* expression while the L270H substitution yielded essentially wild-type levels (Table 2). The effect of the N268D substitution on *metH* expression is MetR dependent, because the phenotype was lost in a *metR* background (Table 2).

Screen for alanine substitutions in the αCTD that alter *metE* and/or *metH* expression. To more efficiently identify residues in the αCTD important for activation of *metE* and/or *metH*, we switched to screening an alanine substitution plasmid library of the αCTD (residues 255 to 329). Lysogens GS162λ^{T}Elac1 and GS972λHlac were transformed with the plasmid library to assess the effect of each alanine substitution on both *metE-lacZ* and *metH-lacZ* expression. Transformants that showed decreases in either *metE-lacZ* or *metH-lacZ* expression in a preliminary screen were selected for further study.

Many of the alanine substitutions had modest effects on *metE* expression (Fig. 2A). Among the residues that, when changed to alanine, cause at least a twofold decrease in *metE* expression are the previously characterized DNA binding residues of α: L262, R265, N268, C269, G296, K298, and S299 (10, 17, 28). Consistent with the selection using PCR mutagenesis, L270 was also identified as important for *metE* expression in this screen. In addition, alanine substitutions at residues L260, T263, H276, I278, L281, L289, P293, E302, I303, V306, L307, S309, S313, L314, N320, and P322 all cause at least twofold decreases in *metE-lacZ* expression, with changes at E302, I303, and L314 being more severe than changes at most of the DNA binding residues. The alanine substitution mutants that showed a *metE*-down phenotype in GS162λ^{T}Elac1 were also tested in GS244λ^{T}Elac1 to determine the effects of the alanine substitutions on basal *metE* expression. With the exception of I303A, the alanine substitutions caused at most a 1.3-fold down effect on *metE-lacZ* expression in the absence of MetR (data not shown). These results suggest that the *metE*-down phenotypes caused by these alanine substitutions are MetR dependent. The I303A substitution caused a twofold reduction in basal *metE-lacZ* levels (data not shown). Since this substitution caused a fivefold reduction in MetR-dependent *metE* expression (Fig. 2A), the I303A substitution appears to have an effect on both basal and activated transcription of *metE*. Based on the solution structure of the αCTD (10, 17), all but five of these residues (L270, I278, L281, I303, and L307) are surface exposed; therefore, these residues could contact MetR for activation.

Far fewer αCTD alanine substitutions cause at least twofold decreases in *metH* expression (Fig. 2B). Those that do, R265, N268, K298, and S299, are all DNA binding residues of α. However, based on in vitro results, using a twofold decrease in expression as a cutoff for determining whether a residue is important for *metH* expression may be too stringent (see below). Alanine substitutions at D258, L262, T263, V264, C269, L270, L290, P293, N294, L295, G296, I303, and N320 reproducibly cause 1.3- to 1.9-fold decreases in *metH* expression. These 17 alanine substitution mutants were also tested in GS244λHlac to determine the effect of the loss of MetR on the phenotype (data not shown). The *metH*-down phenotype of all of these mutants was lost in the *metR* background, suggesting that these alanine substitutions cause decreases in MetR-dependent expression of *metH*. All but two of these residues (L270 and I303) are surface exposed (10, 17); therefore, these residues could contact MetR for activation.

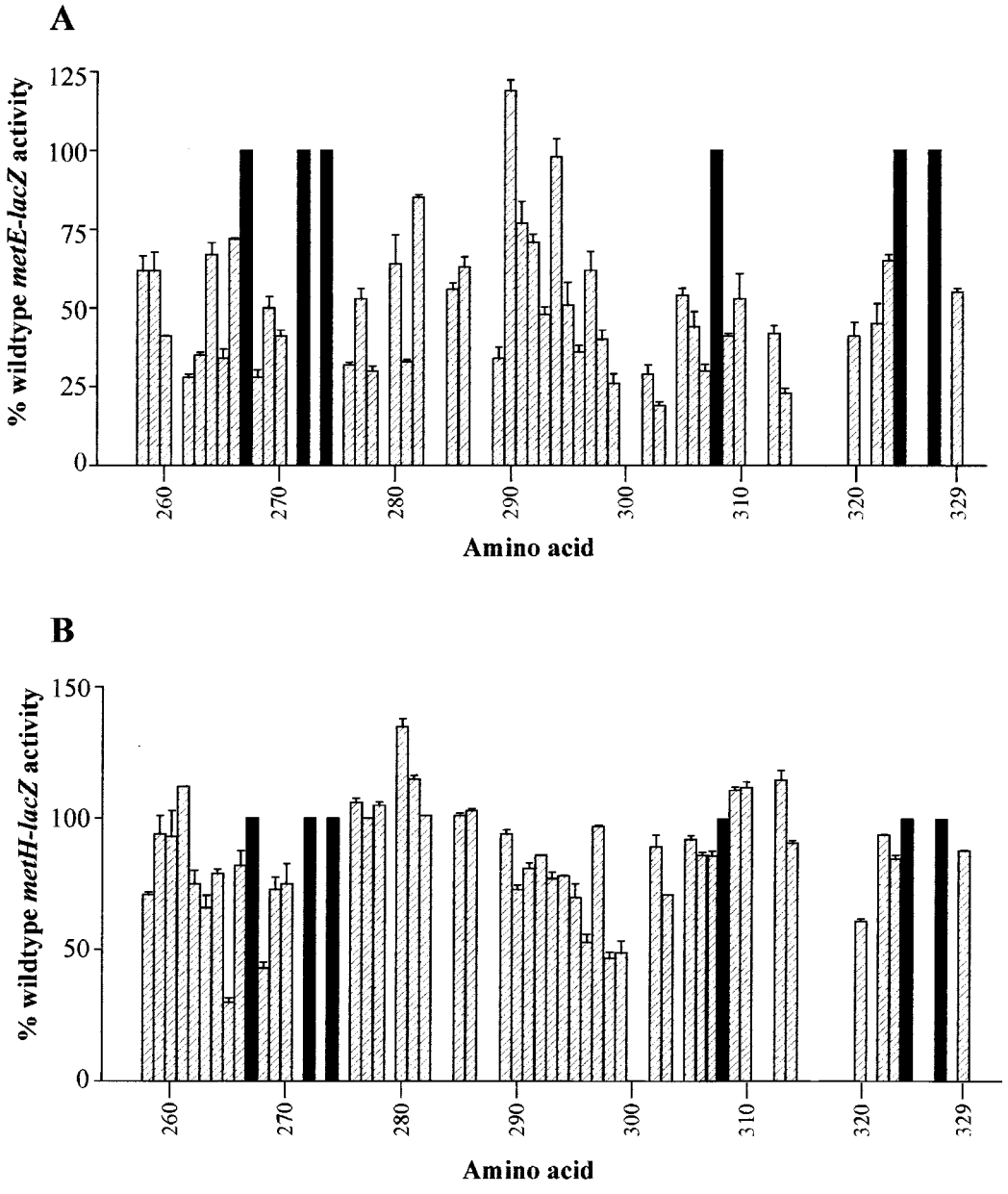

FIG. 2. Alanine scan of αCTD. Plasmids expressing *rpoA* with single alanine substitutions at each residue in the CTD (residues 255 to 329) were assayed for effects on chromosomal *metE-lacZ* (A) and *metH-lacZ* (B) expression. Results are expressed as percentages of the activity in cells carrying plasmids expressing the wild-type *rpoA* gene. Residues 267, 272, 274, 308, 324, and 327 (filled bars) are alanines in the wild-type protein. Residues with no data did not decrease expression of either gene in an initial screen.

In vitro analysis of αCTD mutants. Many of the αCTD mutations show some MetR-dependent phenotype in vivo, especially at *metE*. However, some of these phenotypes may be due to indirect effects, e.g., altering levels of the activator or levels of the enzymes involved in homocysteine metabolism. To determine the direct effect of αCTD mutations on MetR-dependent expression, we reconstituted RNAP in vitro in the presence of either His_6-tagged wild-type or mutant α subunits for use in an in vitro transcription system.

Four α mutants were tested in vitro: three substitutions in α DNA binding residues (R265A, N268D, and G296A) and L270H, since this substitution affects *metE* but not *metH* expression in vivo. The activity of each reconstituted RNAP was normalized based on its ability to make the αCTD-independent *lacUV5* and RNA-I transcripts from plasmid pRLG593 (Fig. 3A) (34).

In a purified transcription system containing template DNA, homocysteine, ribonucleoside triphosphates, and wild-type RNAP, both *metE* and *metH* expression were dependent on the addition of MetR (Fig. 3B, lanes 7 to 9). However, when the mutant RNAPs were used, all four α mutations caused severe decreases in MetR-dependent *metE* expression (Fig. 3B [compare lane 8 to lanes 11, 14, and 17, and compare lane 20 to lane 23] and 3C). Quantification of *metE* levels in the absence of MetR indicates that all four of these mutations in the αCTD caused less than 2.5-fold decreases in basal *metE* ex-

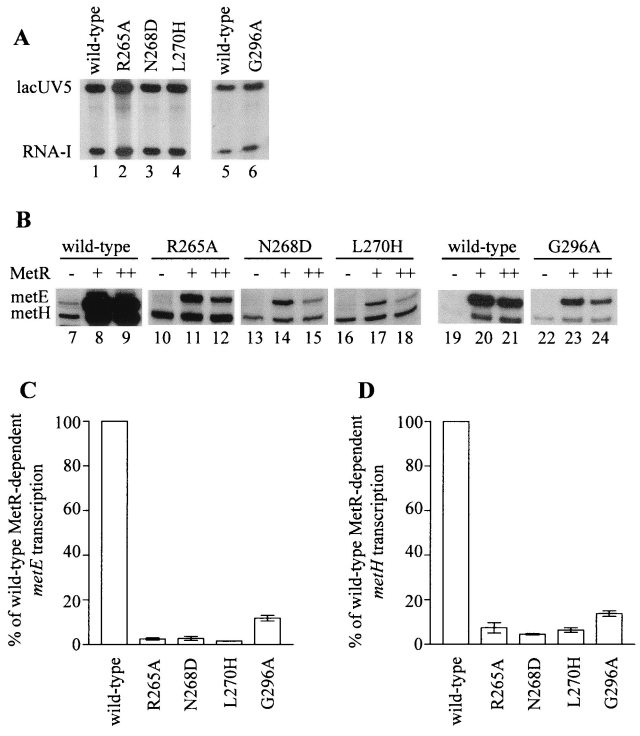

FIG. 3. In vitro transcriptions using RNAP with αCTD substitutions. The RNAP is designated by the α subunits in the holoenzyme. (A) Transcripts from single-round transcription experiments with the supercoiled plasmid pRLG593 as a template to equalize the activity of each RNAP. Indicated are the positions of the αCTD-independent transcript initiated at the *lacUV5* promoter and the plasmid-derived RNA-I transcript. The activity of the G296A RNAP was determined in a separate experiment, so it is shown with the wild-type RNAP from the same experiment. RNAPs were used at the following concentrations in these experiments: 20 nM wild-type, R265A, and N268D RNAPs, 60 nM L270H RNAP, and 50 nM G296A RNAP. (B) Transcripts from single-round transcription experiments using a linear template carrying the *metE-metR* control region as well as a linear template with the *metH* promoter in the absence (−) and the presence of 135 nM (+) or 270 nM (++) MetR dimer. The transcripts initiated at the *metE* and *metH* promoters are indicated. (C and D) Effects of αCTD substitutions on *metE* (C) and *metH* (D) activation. The *metE* transcript produced by each RNAP in the presence of 135 nM MetR and the *metH* transcript produced by each RNAP in the presence of 270 nM MetR from two independent in vitro transcription experiments were quantitated and normalized to the amounts of *lacUV5* and RNA-I transcripts produced by the same RNAP. The normalized amount of transcript produced by each mutant RNAP is reported as a percentage of the expression by wild-type RNAP (± 1 standard deviation).

pression (Fig. 3B, lanes 7, 10, 13, 16, 19, and 22); however, this quantification is subject to large errors because the *metE* basal levels are so low. As indicated above, the in vivo *metE*-down phenotype of most of the αCTD substitutions, including R265A, N268D, L270H, and G296A, was lost in the absence of MetR, suggesting that these mutations primarily affect activated *metE* transcription.

The effects of the αCTD mutations on *metH* were also tested in the same transcription reactions. All four α mutations resulted in severe defects in MetR-dependent activation of *metH* expression (Fig. 3B [compare lane 9 to lanes 12, 15, and 18, and compare lane 21 to lane 24] and 3D). The L270H substitution had only a minor effect on *metH* expression in vivo (Table 2); however, in the in vitro system, the effect of this mutation on *metH* expression was as severe as those of the other point mutations (Fig. 3D). As is the case for *metE*, quantification of the basal *metH* levels is subject to large errors; however, if we measure these levels, none of the substitutions caused more than a 1.6-fold decrease in basal *metH* expression. These in vitro results are consistent with our in vivo analysis of basal *metH* levels, where we found that none of the αCTD substitutions that affected *metH* levels in the presence of MetR had any effect on *metH* levels in the absence of MetR.

In vivo, the *metE*-down phenotypes of all four of these mutations could be at least partially suppressed by the high constitutive MetR levels in GS972 (Table 2 and data not shown). This is not the case in vitro, however. As more MetR was added to reaction mixtures (270 nM dimer) with wild-type RNAP, the amount of *metE* transcript decreased (Fig. 3B; compare lanes 8 and 9). The mutant RNAPs also produced less *metE* transcript in the presence of 270 nM MetR dimer than with 135 nM MetR (Fig. 3B; compare lanes 11 and 12, 14 and 15, 17 and 18, and 23 and 24). These results suggest that the suppression seen in vivo is due to the presence of chromosomally derived wild-type α that is incorporated into RNAP.

A wild-type αCTD in the β′-associated α subunit is sufficient for activation of *metE* in vivo. The in vivo and in vitro experiments show significant differences in the magnitudes of the effects of the mutant αCTDs on activation, the effect of the L270H substitution on *metH* expression, and the suppression of the *metE* phenotypes by high concentrations of MetR. We considered the possibility that these differences were due to the nature of the mutant RNAPs used in vivo and in vitro. In vivo, even though the mutant *rpoA* genes expressed from high-copy-number plasmids should be the predominant form of α in RNAP, the chromosomal *rpoA* is intact, so wild-type α can also be incorporated into RNAP either as homodimers of wild-type α or heterodimers of wild-type and mutant α. In contrast, the in vitro purification and reconstitution protocol produces wild-type or mutant RNAP having α dimers of homogeneous subunits. In an attempt to reproduce in vivo the severe loss of MetR activation observed in vitro, we constructed a derivative of the wild-type GS162λ^{T}Elac1 lysogen, GS1106λ^{T}Elac1, in which the chromosomal copy of *rpoA* was replaced with the *rpoA112* allele encoding an α subunit reported to have a temperature-sensitive lethal defect for assembly of core polymerase (20). Strains carrying *rpoA112* on the chromosome cannot grow at the nonpermissive temperature (42°C) unless an alternative functional *rpoA* gene is also present, such as a plasmid-borne *rpoA* gene. However, certain αCTD substitution mutations are unable to complement the *rpoA112* allele at 42°C, including R265A, N268A and G296A (10). The L270Hα allele can complement *rpoA112*, so the effect of L270Hα on *metE-lacZ* was examined in the *rpoA112* background. Since expression of the *metR* promoter is severely reduced at high temperatures (data not shown), the cells used for the assay also carried plasmid pGS395, which constitutively expresses MetR from a heterologous promoter.

As expected based on the results in GS972λ^{T}Elac1, when tested in the *rpoA112* background at a permissive temperature (37°C) that allows the RpoA112 α protein to participate normally in the assembly of RNAP, the L270H substitution did not affect *metE-lacZ* expression (Table 3). Surprisingly, when tested at the nonpermissive temperature (42°C) that inhibits normal assembly by the RpoA112 α protein, the cells expressing the L270Hα did not mimic the severe loss of MetR activation predicted from the in vitro results if all of the cellular RNAP at 42°C had incorporated homogeneous L270Hα dimers; instead, these cells expressed *metE-lacZ* as well as did the wild type (Table 3).

The *rpoA112* assembly defect at the nonpermissive temperature is due to an arginine-to-cysteine substitution at position 45 of α (R45Cα) (15). An alanine substitution at the same α position (R45Aα) can still dimerize but can no longer interact

TABLE 3. Effect of the L270Hα mutation on *metE-lacZ* expression in an *rpoA112* background

Lysogen[a]	Plasmid	Plasmid *rpoA* allele	β-Galactosidase activity[b] at:	
			37°C	42°C
GS1106λ^{T}Elac1	pREIIα	Wild type	12,016	7,724
GS1106λ^{T}Elac1	pREII-L270Hα	L270H	17,652	7,767

[a] A λ^{T}Elac1 lysogen carrying plasmid pGS395 (which overexpresses MetR) was transformed with the indicated *rpoA*-expressing plasmid.

[b] Units of β-galactosidase activity from cultures grown in TB are reported as Miller units (27). Standard deviations between assays varied by less than 14% of the reported means.

with the β subunit even at 37°C to assemble the $\alpha_2\beta$ precursor required for core polymerase formation (21). However, mixtures of R45Aα and wild-type α can form α heterodimers in vitro that successfully interact with the β subunit via the wild-type moiety and can thus form core polymerase having oriented α subunits: wild-type α contacting the β subunit (α^I) and R45Aα contacting the β′ subunit (α^{II}) (29). If the consequence of the *rpoA112* mutation (R45Cα) at the nonpermissive temperature is the same as that of the R45Aα substitution, then cultures of the *rpoA112* lysogen GS1106λ^{T}Elac1 expressing the plasmid-encoded L270Hα and grown at 42°C should produce a fraction of RNAP that contains L270Hα homodimers, as well as some RNAP that contains mixed L270Hα^I–R45Cα^{II} heterodimers. The homodimeric L270Hα RNAP should be equivalent to the purified His_6-tagged L270Hα RNAP used in the in vitro transcription experiments and would thus contribute very little to *metE-lacZ* expression (Fig. 3C). According to this hypothesis, the nearly wild-type level of expression of *metE-lacZ* in the *rpoA112* background cell must be due principally to the heterodimeric L270Hα^I–R45Cα^{II} RNAP. Furthermore, these results would predict that MetR activation of *metE* is insensitive to the residue at position 270 of α^I provided that the wild-type residue is present on α^{II}.

The equivalent in vivo experiment to address the effect of oriented α RNAP on *metH* expression is technically not possible. The *rpoA112* allele requires a temperature of at least 42°C for the orienting phenotype; unfortunately, the *metH* promoter is itself temperature sensitive and shows virtually no expression at 42°C (data not shown).

Orientation requirements of a wild-type αCTD within RNAP for MetR-dependent activation of *metE* and *metH*. Since the results above suggest that a point mutation in the CTD of α^I does not interfere with MetR-dependent activation of *metE*, we wanted to test whether an RNAP with a mutation in the CTD of α^{II} (and a wild-type α^I) would also activate *metE*, i.e., whether a single wild-type αCTD is sufficient for activation at *metE*. We also wanted to know what the requirements for the orientation of α were for activation at the *metH* promoter, since we were not able to test the effect of the L270Hα^I–R45Cα^{II} RNAP on *metH* in vivo due to the temperature sensitivity of the *metH* promoter. Although β-orienting substitutions (L48A, K86A, and V173A) in α analogous to the β′-orienting R45A substitution have been described (21), simple in vivo assays with such mutants are not possible because conditional, chromosomal versions of the β-orienting mutants are presently not available. We therefore designed an in vitro reconstitution-purification protocol to purify RNAP containing oriented α subunits. This protocol is an extension of the scheme developed by Tang et al. (40), which involves purification of RNAP from an in vitro reconstitution mixture by Ni^{2+} ion affinity chromatography based on a His_6-tagged α. We included in the reconstitution mixture a second source of α that carries an alternate affinity tag, allowing sequential purification of RNAP based on both tags, ensuring that the final RNAP incorporates an α dimer composed of two differently tagged monomers. Furthermore, by specifically including the R45A substitution in one of the two differently tagged α monomers, we can direct this monomer to the α^{II} position, and, by default, direct the other monomer to the α^I position. Thus, αCTD mutations can be incorporated into either the α^I- or α^{II}-specific monomers to test the orientation-dependent effects of the αCTD mutations in vitro. We constructed a plasmid, pREII-Strepα, that expresses an α containing the *Strep*-tag II sequence (Strep tag) between codons 1 and 2 of *rpoA*, the same location as the N-terminal His_6 tag in pHTT7f1-NHα (see Materials and Methods). It has been previously shown that incorporation of a His_6 tag into the N terminus of α does not impair enzyme function (41). To determine whether the Strep-tagged α is also functional, we tested for the ability of Strep-tagged α to complement a known *rpoA* mutant in vivo. The mutant strain GS1040 carries the chromosomal E261K *rpoA* allele, which prevents growth on glucose minimal medium (16). Transformants of GS1040 carrying the pREII-Strepα plasmid exhibit growth on glucose minimal medium indistinguishable from that of the wild-type strain (GS162) or from that of transformants of GS1040 carrying pREIIα, suggesting that Strep-tagged α is incorporated into RNAP and that the Strep tag does not interfere with function.

Since neither an N-terminal His_6 tag nor a Strep tag interferes with RNAP assembly or function, purified His_6- and Strep-tagged α were added simultaneously to the reconstitution mixture. Incorporation of the R45A substitution into the Strep tag α construct ensures that this monomer could be incorporated into RNAP only at the α^{II} position. Following renaturation-reconstitution, the RNAP was purified in two steps, first based on Ni^{2+} ion affinity of the His_6-tagged α subunit, then based on StrepTactin affinity of the Strep-tagged α subunit, generating a single population of RNAP containing oriented α subunits. The C-terminal domains of these *rpoA* plasmids can be altered to generate a set of αCTD mutant RNAPs with oriented α subunits. For this analysis, we chose to use a version of α in which the final 73 amino acids were deleted ($_{\Delta CTD}\alpha$) instead of the L270H mutant α, because the effect of substitutions at L270 may be indirect (see Discussion).

Using this reconstitution and purification protocol, we generated four different RNAP species to test the positional requirements of the αCTD for MetR-dependent activation in vitro. The first RNAP contained α subunits with both αCTDs intact (wt α^I–wt α^{II}); in the second RNAP, the final 73 amino acids of α were deleted from both α subunits ($_{\Delta CTD}\alpha^I$–$_{\Delta CTD}\alpha^{II}$); in the third and fourth RNAP species, the αCTD was deleted from either the β-associated α^I ($_{\Delta CTD}\alpha^I$–wt α^{II}) or the β′-associated α^{II} (wt α^I–$_{\Delta CTD}\alpha^{II}$). The activity of each RNAP was normalized to the wild-type enzyme using αCTD-independent promoters (Fig. 4A), and the effect of deleting each αCTD on *metE* and *metH* expression was examined. As expected from previous studies (15a), MetR-dependent activation of *metE* transcription seen with the wild-type RNAP was almost completely lost when both αCTDs were deleted (Fig. 4B; compare lanes 6 and 12). However, both single-αCTD RNAP derivatives, $_{\Delta CTD}\alpha^I$–wt α^{II} and wt α^I–$_{\Delta CTD}\alpha^{II}$, responded to MetR-mediated activation nearly as well (50% ± 2% and 70% ± 2%, respectively) as the wild-type RNAP (Fig. 4B, lanes 6, 8, and 10). These results indicate that a single wild-type αCTD in either the α^I or α^{II} position of RNAP is sufficient for MetR-dependent activation of *metE*.

The oriented α RNAPs were also tested for their effects on

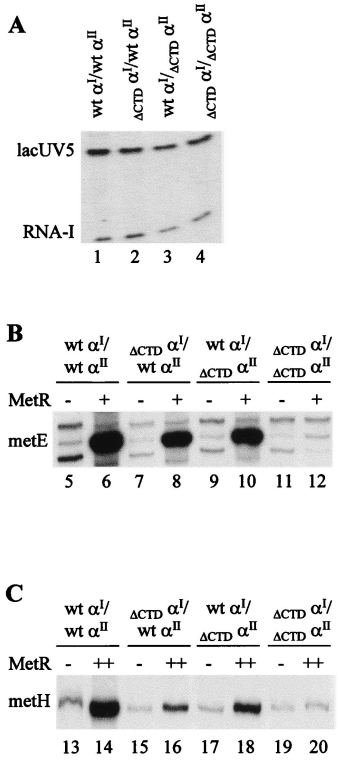

FIG. 4. In vitro transcriptions using RNAP with oriented α subunits. The RNAP is designated by the α subunits in the holoenzyme. (A) Transcripts from single-round transcription experiments with the supercoiled plasmid pRLG593 as a template to equalize the activity of each RNAP. Indicated are the positions of the αCTD-independent transcripts initiated at the *lacUV5* promoter and the plasmid-derived RNA-I transcript. The RNAPs were used at the following concentrations in these experiments: 10 nM wt α^I–wt α^{II} and $_{\Delta CTD}\alpha^I$–wt α^{II} RNAPs, and 13 nM wt α^I–$_{\Delta CTD}\alpha^{II}$ and $_{\Delta CTD}\alpha^I$–$_{\Delta CTD}\alpha^{II}$ RNAPs. (B) Transcripts from single-round transcription experiments using a linear template carrying the *metE-metR* control region in the absence (−) and presence (+) of 135 nM MetR. The transcript initiated at the *metE* promoter is indicated. (C) Transcripts from single-round transcription experiments using a linear template carrying the *metH* promoter in the absence (−) and presence (++) of 270 nM MetR. The transcript initiated at the *metH* promoter is indicated.

MetR-dependent expression of *metH* in vitro. Consistent with previous results (15a), the $_{\Delta CTD}\alpha^I$–$_{\Delta CTD}\alpha^{II}$ RNAP derivative did not show any appreciable increase in *metH* transcription in the presence of MetR (Fig. 4C; compare lanes 13 and 14 to lanes 19 and 20). Consistent with its activity at the *metE* promoter, the RNAP derivative having a deletion of the αCTD only at α^{II} responded to MetR activation of *metH* 47% ± 3% as well as the wild-type RNAP (Fig. 4C, lanes 14 and 18). In contrast, the RNAP derivative having a deletion of the αCTD only at α^I responded poorly to MetR activation of *metH*, showing only 21% ± 3% of the levels seen with the wild-type RNAP (Fig. 4C, lanes 14 and 16). These results indicate that MetR exhibits a more stringent requirement for a functional CTD on the β-associated α^I subunit than for the CTD of α^{II} for activation of *metH*.

DISCUSSION

Amino acids in the αCTD important for MetR activation of *metE*. Through random PCR mutagenesis of *rpoA* we have identified two residues, N268 and L270, that are important for MetR-dependent activation of *metE*. Residue N268 has previously been characterized as a DNA binding residue of α (10, 17, 28). The N268D substitution has also been shown to affect CRP- and OxyR-dependent activation of the *lacP1* and *katG* promoters, respectively (44, 57). An alanine substitution at position 268 not only disrupts activation at promoters containing an UP element (10, 28), where the αCTD contacts the DNA directly, but also has been found to decrease activation at nearly every native promoter tested that requires the αCTD for activation (for examples, see reference 30).

Amino acid L270 is not an α-DNA interaction residue; however, an L270A substitution has been shown to disrupt CRP-dependent activation at *lacP1* and TyrR-mediated activation of *mtr* to nearly the same extent as substitutions in α-DNA binding residues (e.g., R265A) (28, 55). In addition, an L270P substitution in α disrupts CRP activation of *lacP1* and CysB activation of *adi* (39, 57). As previously suggested by Murakami et al. (28), the effect of a mutation at 270 may not define a point of contact between α and activator proteins but instead may be indirect. Amino acid 270 is located in helix 1 of the αCTD, which also contains three of the DNA binding residues (R265, N268, and C269) (10, 17); therefore, substitutions at 270 may disrupt the structure of helix 1, thereby altering a portion of the α-DNA binding surface.

The N268D and L270H substitutions are interesting in that the *metE* phenotypes of these mutations can be partially to completely suppressed by high levels of the activator in vivo. The phenomenon of activator overexpression suppressing an *rpoA* phenotype is not novel. In *Salmonella* serovar Typhimurium, if the FNR homologue OxrA is overexpressed, the phenotype of the *rpoA8* mutation (G311Rα) is partially suppressed (24). However, suppression of the N268D and L270H phenotypes is not observed in the in vitro transcriptions where purified RNAP containing N268Dα or L270Hα is used (Fig. 3B), suggesting that when MetR levels are high in vivo, wild-type α RNAP will be preferentially recruited to *metE*.

Screening of an αCTD alanine substitution library identified additional residues that are important for *metE* activation by MetR. Most of the surface-exposed residues identified in this screen cluster to a complex face of α that includes residues important for activation of both *metE* and *metH* (Fig. 5). Residues L262, R265, N268, C269, G296, K298, and S299, which have previously been identified as DNA binding residues of α (10, 17, 28), localize to this complex face. It is possible that these residues define an interaction surface on α for contact with MetR because they affect MetR-dependent activation of *metE* in vivo; however, we favor the alternate hypothesis previously proposed for these residues in CRP-, Mor-, and Ogr-dependent activation (1, 3, 10, 36, 53): L262, R265, N268, C269, G296, K298, and S299 are involved in nonspecific protein-DNA interactions that stabilize the activator-α interaction.

Residues L260, T263, H276, P293, E302, V306, S309, N320, and P322 also localize to this complex face of α, with H276, N320, and P322 forming an outlying extension of the face and the others situated near the DNA binding residues (Fig. 5). Since the phenotype caused by alanine substitutions at these positions is MetR dependent and because many of these residues are situated near the α-DNA binding residues, these residues could be stabilizing the interaction of α with DNA after being positioned by MetR. Alternatively, these residues could form a protein-protein interaction surface for contacting MetR. It has been postulated that if indeed the αCTD is involved in both nonspecific DNA interactions and specific protein-protein interactions, then mutations in the residues involved in the protein-protein interactions should result in a stronger phenotype than mutations in the DNA binding residues (1). Despite the fact that the alanine substitution exper-

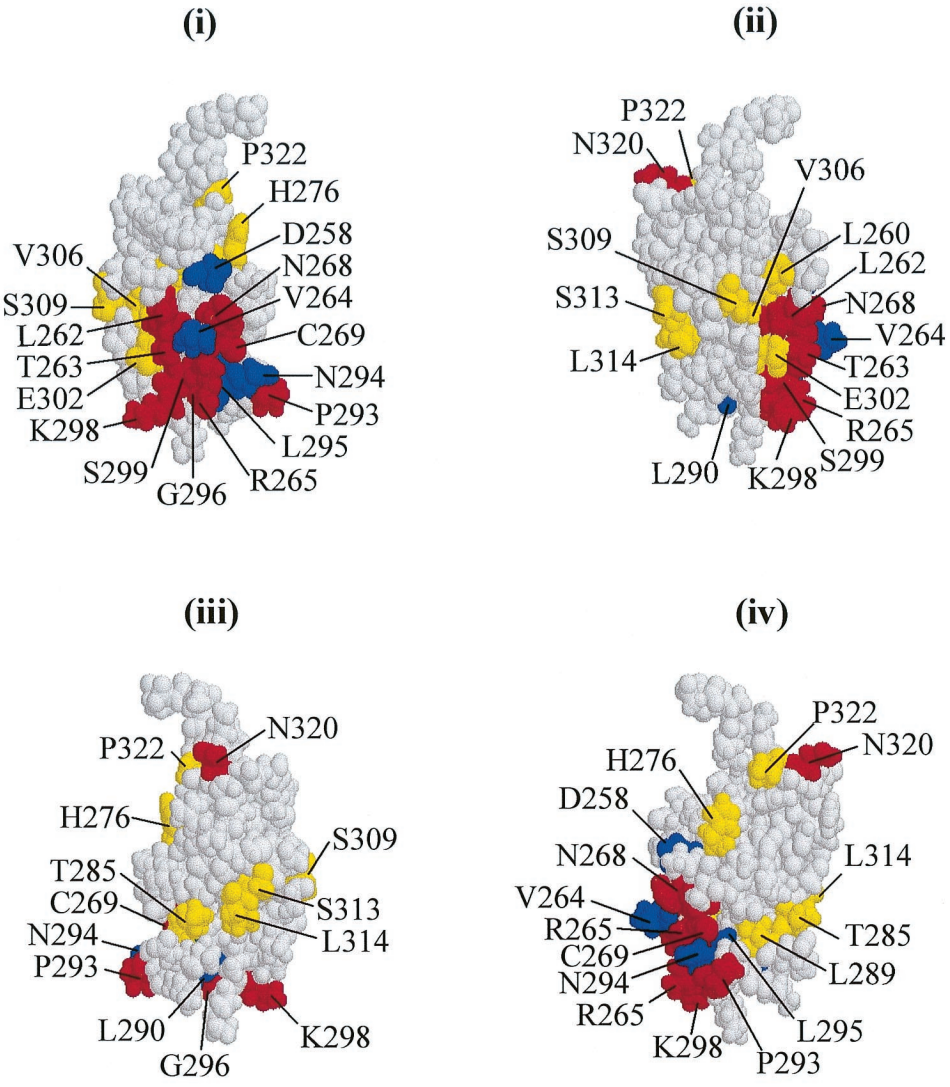

FIG. 5. The solution structure of the αCTD (17), showing the positions of residues identified as important for MetR-dependent activation at *metE* and *metH* in vivo and, in some cases, verified in vitro. Red, residues important for both *metE* and *metH* expression; yellow, residues found to be important for MetR-dependent expression of *metE* only; blue, residues found to be important for MetR-dependent expression of *metH* only. Views (i) to (iv) are related by 90° rotations about the vertical axis.

iments were performed in the presence of a wild-type, chromosomal copy of *rpoA*, several of these substitutions do indeed exhibit stronger *metE* phenotypes than do substitutions in the α-DNA binding residues (Fig. 2A). In addition, a change from isoleucine to alanine at residue 303, which is buried within α but behind residues E302, V306, and S309, causes the most drastic reduction in MetR-dependent activation of *metE* (Fig. 2A), presumably by disrupting a MetR interaction surface of α. In contrast, a change from leucine to alanine at 270, which we propose disrupts the α-DNA interaction by altering helix 1 of the αCTD, has a more modest effect on MetR-dependent *metE* expression (Fig. 2A). Although we favor a model where these residues interact with MetR for activation, we have not ruled out the possibility that substitutions at these residues may cause a *metE*-down phenotype due to some indirect effect, e.g., altering levels of MetR or expression of homocysteine biosynthetic enzymes in vivo.

The other surface-exposed residues of the αCTD that caused decreases in *metE* activation when changed to alanine, L289, S313, and L314, do not localize to the complex face of α. However, these residues, along with T285 (which causes nearly a twofold decrease in *metE* activation in vivo when changed to alanine [Fig. 2A]), form a discrete patch on the face of the αCTD opposite the complex face (Fig. 5). These residues lie within the 20- by 10-Å surface of the αCTD that has been shown to be important for CRP activation of the synthetic CC(−41.5) promoter and is proposed to be the surface used for an αCTD-CRP interaction (36). The CC(−41.5) synthetic promoter has an activator binding site in essentially the same location (−41.5) as the activation site for MetR on the *metE* promoter (−42). Therefore, these residues could define a third MetR contact patch on the αCTD. The analogy between *metE* and CC(−41.5) is not quite straightforward because of the second, upstream MetR binding site (site 1) at *metE* that is located in the position thought to be contacted by the αCTD at CC(−41.5); however, since the rotational geometry of the proteins at these promoters is not known, this does not necessarily present a steric problem.

At the well-studied promoter CC(−41.5), αCTD-CRP and αCTD-DNA interactions increase the binding of RNAP to the promoter but have no effect on the isomerization to open complex. CRP uses additional interactions with residues in the αNTD to facilitate the closed-to-open-complex isomerization (reviewed in reference 4a). Using CC(−41.5) as a model for *metE*, we propose that RNAP is recruited to *metE* through specific interactions between MetR and residues T285, L289, S313, and L314 of the αCTD. These residues lie within the same 20- by 10-Å surface of α that contains the residues important for the recruitment of CRP to CC(−41.5) (4a, 36). The MetR-RNAP interaction may then be stabilized by αCTD-DNA interactions involving residues L262, R265, N268, C269, G296, K298, and S299, which are properly positioned to contact DNA upon interaction between the αCTD and MetR. The αCTD-DNA contacts made at other promoters where the activator binds overlapping the −35 sequence can be observed as an extension of the footprint upstream of the promoter in the presence of wild-type RNAP but not with RNAP carrying α subunits with CTD deletions (for an example, see reference 2), suggesting that the αCTD reaches over the activator to contact the DNA. At *metE*, however, an upstream extension of the MetR footprint is not observed with wild-type RNAP (15a); therefore, we favor a model where the αCTD interacts with DNA within the MetR footprint, probably by binding to a face of the helix different from that where MetR binds. This second, upstream MetR binding site at *metE* may be the reason why we identified additional residues within the complex face of α that are important for MetR-dependent activation. Residues L260, T263, H276, P293, E302, V306, S309, N320, and P322 may also be used to stabilize the MetR-RNAP interaction. We did not identify any residues in the αNTD that were important for MetR-dependent activation; however, αNTD substitutions did not, in general, cause strong decreases in CRP-dependent activation at CC(−41.5) (31), so our initial selection for PCR-mutagenized *rpoA* genes that decreased *metE* expression may not have been sensitive enough to detect αNTD mutants. Alternatively, residues L260, T263, H276, P293, E302, V306, S309, N320, and P322 of the αCTD may replace the αNTD-activator interaction used at CC(−41.5) to facilitate the closed-to-open-complex isomerization.

Amino acids in the αCTD important for MetR activation of *metH*. In the initial selection for α mutations that alter MetR-dependent activation, we were able to identify only one substitution, N268D, which had a significant effect on *metH* expression. Interestingly, we were able to detect a *metH* phenotype only for the *rpoA* alleles tested in GS972, whereas *rpoA* mutations that affected *metE* expression were best detected in GS162 (Table 2). We speculate that a *metH*-down phenotype is observed only in GS972 because GS162 does not produce enough MetR to fully activate *metH*, and a *metH*-down phenotype is apparent only when there is sufficient MetR available.

In the screen of the αCTD alanine substitution library, only α-DNA interaction residues (R265, N268, K298, and S299) were found to significantly disrupt *metH* activation if changed to alanine (Fig. 2B). We confirmed the *metH* phenotypes of R265 and N268 substitutions in vitro as well as identifying another α-DNA interaction residue, G296, as important for *metH* activation (Fig. 3D). Therefore, five of the residues previously identified as important for α-DNA interactions are also important for *metH* activation: R265, N268, G296, K298, and S299 (Fig. 5). We were also able to show that mutations with a slight phenotype in vivo, e.g., L270H, could indeed cause a significant phenotype in vitro (Fig. 3D). Therefore, we consider mutations that cause less than a twofold down phenotype for *metH* expression in vivo to be potentially necessary for MetR activation of *metH*. By these less stringent criteria, a number of other surface-exposed αCTD residues, including D258, L262, T263, V264, C269, L290, P293, N294, L295, G296, and N320, also disrupt *metH* activation if changed to alanine. Several of these residues have also previously been shown to be important for activator-dependent transcription at other promoters: D258 is important for CRP, TyrR, and bacteriophage Mu Mor protein activation of *lac*, *mtr*, and P_m, respectively (1, 42, 55); V264 mutations can suppress a positive control mutant of OmpR to partially restore activation at *ompF* (19); L290 is necessary for P2 Ogr activation at P4 late promoters (53); N294 is required for activation at *katG* by OxyR and UP element activation of $rrnBp_1$ (10, 44); and L295 mutations disrupt UP element activation of $rrnBp_1$ (10).

All of the residues identified as crucial for *metH* activation are located within the complex face of α (Fig. 5). While some of the residues in this complex face are important for activation of both *metE* and *metH*, others are important for *metH* activation only (Fig. 5). Substitutions in residues D258, V264, L290, N294, and L295 all affect *metH* but not *metE* expression. Since a number of the *metH*-specific residues have been shown to be important for activator-dependent expression in other systems, we propose that residues D258, T263, V264, L290, P293, N294, L295, and N320 contact MetR for activation at *metH*. Alternatively, D258 may interact with the σ^{70} subunit, a role previously proposed for this residue in CRP-dependent activation at *lac* (4a). Furthermore, we propose that the MetR-αCTD interaction is stabilized by interactions between residues L262, R265, N268, C269, G296, K298, and S299 and *metH* promoter DNA.

The *metE* and *metH* promoters differ not only in the number of MetR binding sites but also in the locations of the activation sites. However, a simple comparison of the locations of the active sites relative to the transcriptional start sites is problematic because both S1 nuclease mapping (47) and primer extension (data not shown) show that *metH* has two transcription start sites separated by 3 intervening bp that appear to be used with equal efficiency in the absence and in the presence of MetR; thus, it is likely that both transcripts depend on the same Pribnow box. Because of the dual start sites at *metH*, we use the 3′-most T base of the Pribnow box, which is highly conserved in most promoters (13), as a reference point to determine the relative locations of the MetR sites at the *metE* and *metH* promoters. The center of the MetR activation site (site 2) at *metE* is 36 bp upstream of this reference point, while the center of the single MetR site at *metH* is 52 bp upstream of this point (Fig. 1); therefore, the MetR site at *metH* is 16 bp, or one and one-half helical turns, further upstream than the *metE* promoter. This means that MetR binds to opposite helical faces of DNA at these promoters. We propose that the differences in the αCTD residues that are important for MetR activation result from the differences in the locations of the MetR activation sites at *metE* and *metH*.

Differential orientation requirements for wild-type αCTD within RNAP for activation at *metE* and *metH*. Our experiments with RNAP containing oriented α subunits indicate that the CTD of either α subunit is capable of making the interactions necessary for MetR-dependent activation at *metE*. This interchangeability of the αCTD was also observed for UP element subsite recognition at $rrnBp_1$ by Estrem et al. (9). It has also been reported that the αCTD functions interchangeably for CRP activation at *lac* and CC(−41.5) (4a). In contrast, MetR activation at *metH* has a more stringent requirement for an intact αCTD on the β-associated α^I; the CTD of the β′-associated α^{II} substitutes very poorly for the α^I CTD. The

metH promoter is similar to the *lacP1* promoter in that the activators bind well upstream of the −35 sequence in both cases; however, using the 3′ T base of the Pribnow box as a reference, CRP binds to a site centered 54.5 bp upstream (6), while MetR binding at *metH* is centered 52 bp upstream (5). This means that CRP binds one-quarter of a helical turn further upstream at *lac* than MetR binds at *metH*. We speculate that the restriction on which of the two αCTDs is capable of activation at *metH* is due to limits on the rotational flexibility of the αCTD with respect to the rest of RNAP. It has previously been shown that the α linker confers considerable two-dimensional flexibility on the αCTD, allowing it to reach long distances from the core promoter to interact with activator proteins, as long as the helical phasing of the activator is maintained (11, 50, 52). Phasing experiments with FNR have shown that changes of as little as 1 or 2 bp from a position favorable for FNR activation can destroy FNR-dependent activation (52). From these results, we speculate that the αCTD has considerable flexibility such that it can stretch to reach distant activators but lacks a rotational flexibility that would allow it to both reach for activators and wrap around the DNA to contact activators that bind to a helical face of the DNA different from that where RNAP binds. This restricted rotational flexibility could also limit the mobility of each αCTD such that an activator that binds "off to the side" of the DNA (relative to the plane set by RNAP) would be able to contact one αCTD but the other could not substitute if the critical αCTD was mutated or deleted. This model would predict that other αCTD-dependent activators that bind "off to the side" may display an α-specificity, as was seen for MetR at *metH*. One example might be the OxyR-dependent promoter *katG*, which has previously been shown to require the αCTD (43). OxyR at *katG* binds 47 bp upstream of the 3′ T of the Pribnow box (45), meaning that it also binds "off to the side" of the DNA but it binds to the side opposite MetR at *metH*; therefore, we would predict that if OxyR at *katG* does show an αCTD specificity, it may require that the αCTD on the β′-associated α^{II} be intact. Furthermore, the limited rotational flexibility of the αCTD proposed in this model would predict that αCTD-dependent activators that bind to the opposite helical face of the DNA relative to RNAP would need to bind in such a way that the activating region of the activator protein would be wrapped around the DNA and would thus be accessible to the αCTD. Such a mechanism has the potential to lead to an α-specificity which could be examined in vitro by using oriented α RNAPs.

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