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NOTES

Amino Acid Residues in the α-Subunit C-Terminal Domain of *Escherichia coli* RNA Polymerase Involved in Activation of Transcription from the *mtr* Promoter

JI YANG,[1] KATSUHIKO MURAKAMI,[2] H. CAMAKARIS,[1] NOBUYUKI FUJITA,[2] AKIRA ISHIHAMA,[2] AND A. J. PITTARD[1]*

Department of Microbiology, University of Melbourne, Parkville, Victoria 3052, Australia,[1] and Department of Molecular Genetics, National Institute of Genetics, Mishima, Shizuoka 411, Japan[2]

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To examine the role of the amino acid residues (between positions 258 and 275 and positions 297 and 298) of the α-subunit of RNA polymerase in TyrR-mediated activation of the *mtr* promoter, we have carried out in vitro transcription experiments using a set of mutant RNA polymerases with a supercoiled *mtr* template. Decreases in factor-independent transcription in vitro by mutant RNA polymerases L262A, R265A, and K297A suggested the presence of a possible UP element associated with the *mtr* promoter. Mutational studies have revealed that an AT-rich sequence centered at −41 of the *mtr* promoter (SeqA) functions like an UP element. In vivo and in vitro analyses using a mutant *mtr* promoter carrying a disrupted putative UP element showed that this AT-rich sequence is responsible for interactions with the α-subunit which influence transcription in the absence of TyrR protein. However, the putative UP element is not needed for activator-dependent activation of the *mtr* promoter by TyrR and phenylalanine. The results from in vitro studies indicated that the α-subunit residues leucine-262, arginine-265, and lysine-297 are critical for interaction with the putative UP element of the *mtr* promoter and play major roles in TyrR-dependent transcription activation. The residues at positions 258, 260, 261, 268, and 270 also play important roles in TyrR-dependent activation. Other residues, at positions 259, 263, 264, 266, 269, 271, 273, 275, and 298, appear to play less significant roles or no role in activation of *mtr* transcription.

Activation of transcription initiation plays an important role in modulating gene expression. Recent studies with *Escherichia coli* have indicated that activation of transcription generally involves direct interactions between RNA polymerase bound at a promoter and a transcriptional activator bound at a site located upstream of the promoter (2, 11, 18). Based on the results of genetic, structural, and biophysical studies, it has been proposed that the α-subunit of RNA polymerase of *E. coli* is involved in such protein-protein interactions for a number of transcription activators (2, 10–12, 21). Work by Ross et al. (19) showed that the α-subunit of RNA polymerase can also interact with an AT-rich sequence (UP element) which is located upstream of the −35 region of the $rrnBp_1$ promoter of *E. coli* and is responsible for enhancement of transcription initiation. Such UP elements appear to be a feature of many promoters (8, 19, 22).

The TyrR protein of *E. coli* is both a repressor and an activator and controls the expression of a group of eight transcriptional units (TyrR regulon) whose translational products are involved in the biosynthesis or uptake of the three aromatic amino acids (17). The TyrR protein contains three structural domains (5), and genetic analysis has mapped the activation function of TyrR to the N-terminal domain (4, 24, 25). Alanine scanning mutagenesis has identified a number of amino acid residues whose side chains are specifically involved in activation (25).

Transcriptional expression of the *mtr* gene, which codes for the tryptophan-specific transport protein, is activated by the TyrR protein with tyrosine or phenylalanine as a cofactor. In *trpR* strains, which lack tryptophan repression, tryptophan can also mediate transcription activation of *mtr* but to a lesser extent than tyrosine or phenylalanine (9, 20). In vitro transcriptional analysis using a supercoiled template has shown that transcription from the *mtr* promoter (σ^{70}) is strongly inhibited by histone-like proteins HU and IHF or by $BaCl_2$ (26). In the presence of any of these reagents, which are known to alter DNA structure (6, 13), it is possible to demonstrate activation of transcription by purified TyrR protein and either of the cofactors, phenylalanine and tryptophan (26). The transcription activation of *mtr* does not occur if the activation-negative TyrR protein RQ10 is substituted for the wild-type protein or if RNA polymerase with a truncated α C-terminal domain is used (26). These results suggest that the activation of *mtr* transcription by TyrR is probably a direct result of interaction between TyrR protein and the C-terminal domain of the α-subunit of RNA polymerase (αCTD).

To study possible protein-DNA interaction between the α-subunit of RNA polymerase and the *mtr* promoter and to gain further insight into the protein-protein communication between TyrR and RNA polymerase, we screened a series of mutant RNA polymerases with alanine substitutions in the αCTD (15) using the previously described in vitro transcription system (26). In this report, we identify a number of amino acid residues in the αCTD which are involved in TyrR protein-dependent transcription activation of the *mtr* promoter, some

* Corresponding author. Phone: 61 3 9344 5679. Fax: 61 3 9347 1540.

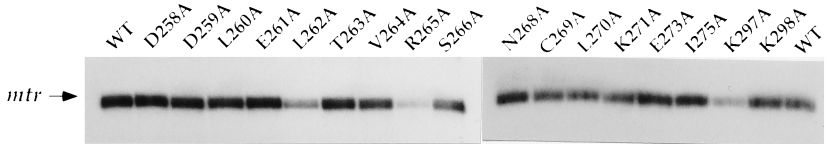

FIG. 1. Factor-independent in vitro transcription from the *mtr* promoter. Single-round in vitro transcription was carried out as described previously by Yang et al. (26). The activities of various RNA polymerases have been normalized based on the level of *lacUV5* transcription as described previously (15). The reaction mixture contained 0.5 pmol of wild-type (WT) or various mutant RNA polymerases and 0.015 pmol of DNA template (pDD3-mtr).

of which are also involved in a TyrR-independent transcription activation.

Factor-independent transcription from the *mtr* promoter. Murakami et al. (15) have used site-directed mutagenesis to construct a series of mutants of the α-subunit containing single alanine substitutions between positions 258 and 275 and positions 297 and 298. These mutant α-subunits have each been purified and reconstituted into RNA polymerase holoenzymes and have been used successfully in vitro to study interactions between the α-subunit of RNA polymerase and cyclic AMP receptor protein (CRP) and between the α-subunit and the DNA sequence referred to as the UP element of the $rrnBp_1$ promoter (15). To study the possible interaction of the α-subunit of RNA polymerase with the *mtr* promoter, we carried out single-round in vitro transcription experiments using the reconstituted wild type and each of the reconstituted mutant RNA polymerase holoenzymes. The transcription reactions were performed with a supercoiled DNA template (pDD3-mtr) (26) in the absence of HU and TyrR protein. As shown in Fig. 1, the transcription activities of mutant RNA polymerases containing mutant α, L262A, R265A, and K297A were about one-third of that seen with wild-type RNA polymerase, whereas the transcription activities of all the other mutant RNA polymerases were comparable to wild-type activities. As these three residues (leucine-262, arginine-265, and lysine-297) have been shown to be involved in interactions with the UP element of the $rrnBp_1$ promoter (7, 15), it seemed possible that the decreased transcription activities were due to the inability of these three α mutants to interact with an UP element of the *mtr* promoter.

Involvement of two upstream AT-rich sequences in basal level transcription or activation of the *mtr* promoter. We inspected the *mtr* upstream region to see if UP-like sequences were present. Two regions of AT-rich sequence were found, one located immediately upstream of the −35 region of the *mtr* promoter (centered at −41) and the other located further upstream (centered at −58) (Fig. 2). These two potential UP elements, each of which contains 7 bp, were designated SeqA and SeqB, respectively (Fig. 2). To determine whether either SeqA or SeqB contributes to expression and/or regulation of *mtr*, we carried out site-directed mutagenesis to change each of these two sequences to include GC bases (Fig. 2). SeqA was changed from TTTTTTT to TTCCGTT, and SeqB was changed from ATTTTTA to ATCCTTA. Each of the 180-bp DNA fragments (as shown in Fig. 2) carrying the *mtr* regulatory region containing the mutated putative UP element was cloned into the low-copy-number plasmid pMU575 (28) to construct an *mtr-lac* transcriptional fusion. The two resulting plasmids, along with the wild-type *mtr-lac* fusion plasmid pMU3190 (20), were each transformed into a *tyrR*$^+$ Δ*lac* strain (JP7740) and a *tyrR366* Δ*lac* strain (JP8042) (27). Following growth in minimal medium supplemented with tyrosine or phenylalanine, these strains were assayed for β-galactosidase activity. The results are shown in Table 1. In *tyrR366* cells, modification of SeqA led to about a fourfold reduction in basal level transcription from the *mtr* promoter, whereas modification of SeqB did not cause any change in basal level transcription. In the *tyrR*$^+$ background, the mutation in SeqA caused no significant changes in the activated level of *mtr* transcription. The mutation in SeqB resulted in reductions of the phenylalanine-activated level to 67% and the tyrosine-activated level to 54%. These results suggest that SeqA, but not SeqB, functions like an UP element, enhancing TyrR-independent transcription from the *mtr* promoter.

In vitro transcription with a mutant *mtr* template. To study the effect of SeqA on transcription in vitro, we carried out in vitro transcription experiments using a supercoiled *mtr* template carrying the mutation which disrupts SeqA (Fig. 2) and using either wild-type RNA polymerase or each of the three RNA polymerases containing the substitutions L262A, R265A, and K297A. As can be seen in Fig. 3 (lanes 1 to 4), in the absence of HU, the differences in the levels of transcription between the mutant RNA polymerases and the wild type which had been observed previously with the wild-type template were not seen when the *mtr* template with the disrupted SeqA was used. This suggests that the TyrR-independent enhancement

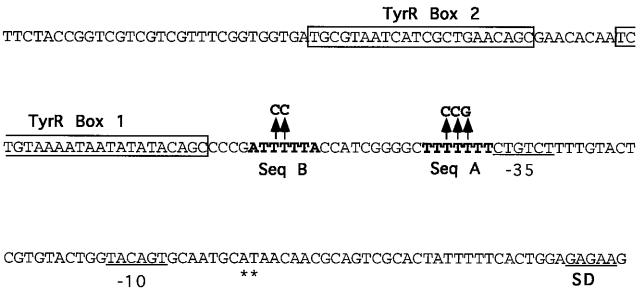

FIG. 2. Nucleotide sequence of a 180-bp fragment carrying the regulatory region of the *mtr* gene. The two TyrR boxes are boxed in. The −35 and −10 regions of the *mtr* promoter and the Shine-Dalgarno (SD) sequence are underlined. The transcription initiation site is marked by a double asterisk. SeqA and SeqB sequences are shown in boldface. Mutations of SeqA and SeqB are indicated above the sequence.

TABLE 1. Effect of mutations in the putative UP elements on transcription or activation of the *mtr* promoter

mtr promoter	β-Galactosidase sp act (U)[a]			
	tyrR366		*tyrR*$^+$	
	MM + Tyr	MM + Phe	MM + Tyr	MM + Phe
WT[b]	407	568	3,426	6,492
SeqA	126	102	2,983	7,331
SeqB	405	597	1,849	4,330

[a] Units of β-galactosidase specific activity are those defined by Miller (14). MM + Tyr, minimal medium containing 1 mM tyrosine; MM + Phe, minimal medium containing 1 mM phenylalanine.

[b] WT, wild type.

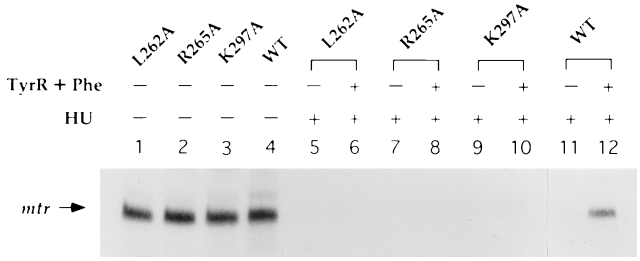

FIG. 3. In vitro transcription from the mutant *mtr* promoter with a disrupted SeqA. Single-round in vitro transcription was carried out as described in the legend to Fig. 1. The reaction mixture contained 0.5 pmol of wild-type (WT) or various mutant RNA polymerases and 0.015 pmol of *mtr* DNA template containing a disrupted SeqA. When added, the final concentrations of HU, TyrR, and phenylalanine were 400, 200, and 1 mM, respectively.

of transcription caused by SeqA involves the three residues of the αCTD (leucine-262, arginine-265, and lysine-297). The in vitro transcription experiments using the mutant template were also performed in the presence of HU with and without purified TyrR protein and phenylalanine. The results show that in the presence of HU alone, transcription of *mtr* was severely inhibited in every case. Furthermore, TyrR-phenylalanine-mediated activation occurred only with the wild-type RNA polymerase and not with the mutant RNA polymerases (Fig. 3, lanes 5 to 12), indicating that these mutations also affect TyrR-dependent transcription of *mtr*. The results also confirm that the interaction of wild-type RNA polymerase and SeqA is not necessary for TyrR-mediated activation of *mtr* transcription.

Effect of various α-subunit mutants on TyrR-mediated transcription activation of the *mtr* wild-type promoter. Previous studies involving in vitro experiments with RNA polymerase with a truncated α-subunit indicated that in the presence of HU, TyrR protein, and phenylalanine, the level of transcription from the *mtr* promoter is dependent on an intact α-subunit of RNA polymerase (26). To identify the amino acid residues in the α-subunit essential for TyrR-activated transcription, we carried out single-round in vitro transcription experiments using the reconstituted wild-type and each of the reconstituted mutant RNA polymerase holoenzymes. These experiments involved a supercoiled DNA template (pDD3-mtr) (26), in the presence and absence of HU, with and without purified TyrR protein and the cofactor phenylalanine. As shown in Fig. 4, addition of HU to the transcription mixtures caused severe inhibition of transcription by each of the RNA polymerases used. In the reactions carried out by the wild-type RNA polymerase, as previously reported (26), transcription was partially restored with the addition of purified TyrR protein and phenylalanine. With the mutant RNA polymerases, the extent of the TyrR-mediated reversal of transcription inhibition caused by HU varied considerably. The degree of TyrR-mediated transcription activation (fold activation) was measured for each of the RNA polymerases by comparing the intensity of the *mtr* band produced in the presence of HU, TyrR, and phenylalanine with that produced in the presence of HU alone.

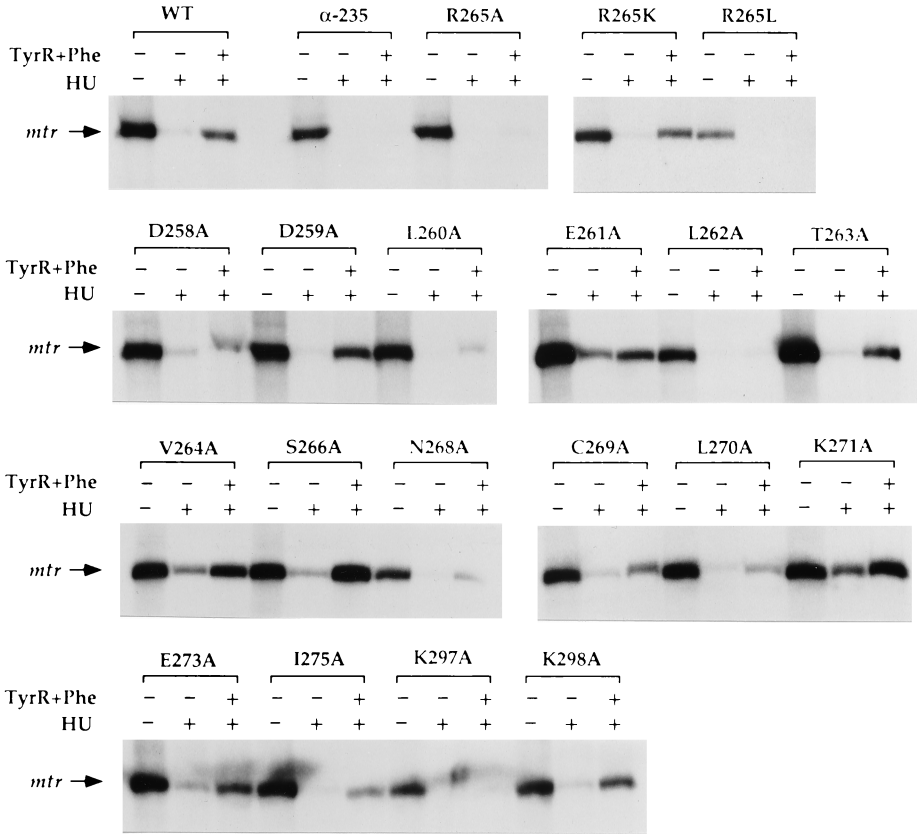

FIG. 4. In vitro transcription from the *mtr* promoter by the wild-type (WT) and various mutant forms of RNA polymerase. Single-round in vitro transcription was carried out as described in the legend to Fig. 1. The reaction mixture contained 0.5 pmol of WT or various mutant RNA polymerases and 0.015 pmol of DNA template (pDD-mtr). When added, the final concentrations of HU, TyrR, and phenylalanine were 400, 200, and 1 mM, respectively.

TABLE 2. Effect of amino acid substitutions in the α-subunit on TyrR-mediated activation of the *mtr* promoter

α-Subunit	Activation (fold)[a]
WT[b]	4.9
α-235	1.0
D258A	2.0
D259A	6.6
L260A	1.7
E261A	1.4
L262A	1.0
T263A	5.2
V264A	2.8
R265A	1.0
S266A	5.0
N268A	1.9
C269A	2.4
L270A	1.7
K271A	2.4
E273A	3.3
I275A	2.8
K297A	1.0
K298A	3.7
R265L	1.0
R265K	4.5

[a] Intensity of the *mtr* band produced in the presence of HU, TyrR, and phenylalanine divided by that produced in the presence of HU alone.
[b] WT, wild type.

The intensity of each of the *mtr* bands on polyacrylamide gels was quantified by using a phosphorimager (FUJIX BASS-2000). It can be seen in Table 2 that, as was the case with mutant *mtr* template, the α-subunits with alanine substitutions at positions 262, 265, and 297 were completely defective in TyrR-mediated activation of the wild-type *mtr* promoter. The alanine substitutions at positions 258, 260, 261, 268, and 270 showed greater than 60% reduction in activation. In contrast, the alanine substitutions at positions 263, 264, 266, 269, 271, 273, 275, and 298 had lesser effects and in some cases no effect on activation. One α mutant, alanine-259, showed a 40% increase in activation.

The arginine residue at position 265 of the αCTD has been shown to be critical for protein-protein interactions with CRP at the *lac* promoter (15). Replacing this arginine residue by other amino acids (leucine, methionine, glutamine, glutamate, serine, and lysine) resulted in a complete loss of CRP-mediated activation. To test in more detail the role of arginine-265 in transcription activation of the *mtr* promoter by TyrR, we carried out in vitro transcription assays using RNA polymerases with either a leucine or lysine substitution at position 265 of the α-subunit (15). It is shown in Table 2 that changing arginine-265 to leucine completely inactivated TyrR-mediated activation of the *mtr* promoter, whereas replacing arginine-265 by a lysine, another basic residue, had virtually no effect on TyrR-mediated activation. These results suggest that it is the positive charge and not the hydrophobic side chain of arginine-265 that is required for the in vitro transcription of *mtr* in the presence of HU and TyrR protein.

Discussion. As previously reported, in vitro activation of transcription of *mtr* by TyrR protein and phenylalanine is observed on supercoiled templates in the presence of HU, IHF, or $BaCl_2$ (26). The fact that HU can be replaced by $BaCl_2$ indicates that the action of both of these reagents is more likely to involve a direct effect on DNA topology than specific interactions with RNA polymerase. The results reported in this paper confirm the involvement of the carboxyl domain of the α-subunit of RNA polymerase in TyrR-dependent activation of transcription of *mtr*. The α-subunit residues leucine-262, arginine-265, and lysine-297 all play major roles in this process and are also implicated in a TyrR-independent enhancement of transcription involving an UP-like sequence (SeqA) adjacent to the *mtr* promoter. These three residues have previously been implicated in the interaction of RNA polymerase with the UP element of the $rrnBp_1$ promoter (7, 15). The side chains of other residues, at positions 258, 260, 261, 268, and 270, were also shown to be important in TyrR-mediated transcription and may be involved in protein-protein interactions between TyrR protein and the α-subunit. Each of these residues has been implicated in transcription activation of one or more of the class 1 activation systems (1, 3, 15, 23). This may reflect a common overlapping recognition region on the α-subunit available for interactions with different activator proteins. The possibility that they may be important for nonspecific interactions with DNA cannot be ruled out, but such reactions are perhaps more likely to involve residues such as arginine-265, whose substitution affects all systems examined so far.

Alanine-scanning mutagenesis of the *tyrR* gene has shown that the side chains of arginine-2, aspartate-9, and arginine-10 are most critical for transcription activation of *mtr* (4, 25). It is interesting to note that these three critical amino acids in the TyrR activating region are all charged residues, and out of the eight amino acid residues in αCTD whose side chains are involved in TyrR-mediated activation, two (aspartate-258 and glutamate-261) are negatively charged and two (arginine-265 and lysine-297) are positively charged. Protein-protein interactions involving opposing negatively and positively charged residues have frequently been reported and have recently been described for CRP and RNA polymerase (16).

With respect to arginine-265, it has been shown that both the hydrophobic chain and the positively charged amino group are required for CRP-mediated activation (15). This is in contrast to our observation that only the positively charged amino group of arginine-265 is necessary for TyrR-mediated activation of the *mtr* promoter. Apparently, the role played by arginine-265 in TyrR-mediated activation of the *mtr* promoter is less specific than that in CRP-mediated activation of the *lac* promoter. Of all the αCTD mutants screened, only mutant D259A had an increased ability to respond to TyrR at the *mtr* promoter. The close proximity of aspartate-259 to aspartate-258, which is critical for TyrR-mediated activation, may suggest that the TyrR protein is also able to interact with aspartate-259. However, this interaction may unfavorably affect the interactions of TyrR with other critical residues of αCTD, resulting in a submaximal level of activation at the *mtr* promoter.

One interesting observation from the in vivo results is that when transcription of *mtr* is activated by TyrR protein and phenylalanine, the same high levels of activity are observed regardless of the presence or absence of the SeqA element. In other words, the proposed interaction between the α-subunit and SeqA, which enhances transcription in the absence of TyrR, either does not occur or, if it does, it has no effect on transcription in the presence of TyrR protein and phenylalanine.

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