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# Mapping of the OxyR Protein Contact Site in the C-Terminal Region of RNA Polymerase α Subunit

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**The *Escherichia coli* OxyR protein requires the C-terminal contact site I region of the RNA polymerase α subunit for cooperative interaction with and transcription activation at OxyR-dependent promoters, suggesting direct protein-protein contact between OxyR and the C-terminal region of the α subunit. To determine the precise location of the OxyR protein contact site(s) in this region, we carried out mutational analysis of the 3′ half of *E. coli rpoA*, the gene encoding the α subunit of RNA polymerase. We isolated a number of *rpoA* mutants defective in *oxyR*-dependent transcription activation at the *E. coli katG* promoter. Nucleotide sequence analysis of the *rpoA* gene from these mutants revealed that the mutations showing clear phenotypes are all clustered at two narrow regions (amino acid residues 265 to 269 and 293 to 300) within the C terminus of the α subunit. Reconstituted RNA polymerases containing the mutant α subunits were unable to respond to transcription activation in vitro at the *katG*, *ahpC*, and *oxyX* promoters by OxyR. These results suggest that these two regions comprise the contact surfaces on the α subunit for OxyR.**

Several lines of evidence indicate that transcription activation by DNA-binding transcription factors involves a direct protein-protein contact between those factors and RNA polymerase (1, 13). With RNA polymerases containing mutant α subunits with C-terminal truncation, we have demonstrated that the C-terminal region of α is involved in direct molecular communication with a group of transcription activator proteins (reviewed in reference 6). Together with other genetic, immunochemical, and biochemical evidence (15), it is proposed that the C-terminal one-third of the RNA polymerase α subunit (contact site I) makes direct contact with a group of transcription activator proteins (class I activator proteins), most of which bind upstream of basic promoter elements (5, 6). Besides the role in class I activator protein-dependent transcription, recent studies indicate that the C-terminal region of the α subunit is also involved in protein activator-independent transcription stimulation at several strong promoters, each containing an AT-rich sequence upstream of the −35 sequence (UP element) (14).

Recently, we showed that OxyR protein, an activator protein for hydrogen peroxide-inducible genes, activates transcription initiation in vitro from the *katG*, *ahpC*, and *oxyX* promoters by wild-type RNA polymerase but not by mutant RNA polymerases containing a C-terminal-truncated α subunit (19). Furthermore, we demonstrated that OxyR exerts cooperative binding to promoter DNA with wild-type RNA polymerase but not with the mutant RNA polymerases. These observations suggest that direct protein-protein contact between the OxyR protein and the C-terminal region of the α subunit plays an essential role in transcription activation at OxyR-dependent promoters.

To carry out detailed mapping of the contact site with the OxyR protein in the C-terminal contact site I region of the α subunit, we isolated a number of *rpoA* mutants defective in response to transcriptional activation by OxyR protein and then located the mutations by DNA sequencing. Our results suggest that OxyR makes contact with two short segments of the C-terminal region of the α subunit, both overlapping the contact sites for cyclic AMP (cAMP) receptor protein (CRP) in the case of *lac* P1 transcription activation (23).

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## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** *Escherichia coli* DH5 was used for measurement of β-galactosidase activity. KT1000 and KT1001 were constructed as follows. The *katG′-′lacZ* fusion gene on a plasmid vector (20) was transferred to lambda phage RS45 by homologous recombination, and this recombinant phage was lysogenized into MC4100 and TA4112 as described previously (17). RLG957, RLG2263, and RLG1336 are λ lysogenic derivatives of NK5031, each lysogenized with λ*rrnB* P1(−61 to +50)-*lacZ*, λ*rrnB* P1(−41 to +50)-*lacZ*, and λ*rrnB* P1(polylinker from M13)-*lacZ*, respectively (12). These strains were used for assessment of the effect of *rpoA* mutations on UP element-dependent transcription. *E. coli* BL21 (λDE3) was used for overproduction of subunits of RNA polymerase.

Details about construction of the mutant *rpoA* library (pLAMC library) were provided previously (23). Briefly, the *rpoA* coding region was PCR amplified by *Taq* DNA polymerase in the presence of manganese ion. The *Eco*RI-*Bam*HI fragments of the PCR products, encoding the C-terminal half of the α subunit, were used to replace the corresponding region of the wild-type *rpoA* gene in pLAW2. pGEMC plasmids carrying the mutant *rpoA* genes under the control of the T7 promoter were constructed as described previously (23).

Lactose-tetrazolium agar plates (10) containing isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM) were used for selection of *rpoA* mutants defective in OxyR-dependent transcription activation at the *katG* promoter. LB medium (10) was used for the β-galactosidase assay.

**Proteins.** Subunits β, β′, $\sigma^{70}$, and wild-type and mutant α of *E. coli* RNA polymerase were overproduced, purified, and reconstituted in vitro into holoenzyme as described previously (4, 23). OxyR protein was purified as described previously (20).

**In vitro transcription.** In vitro transcription was carried out under standard single-round reaction conditions as described previously (8, 19). The following DNA fragments were used for templates: *katG*, the 194-bp *Eco*RI-*Hin*dIII fragment of pKT161 (20); *ahpC*, the 300-bp *Pst*I-*Eco*RI fragment of pDSA24 (a gift from D. Smille); *oxyX*, the 310-bp *Eco*RI-*Hin*dIII fragment of pKT908 (20); and *lacUV5*, the 205-bp *Eco*RI fragment of pKB252 (8).

**β-Galactosidase assay.** CRP-dependent *lacZ* expression was quantitated as described previously (23). UP element-dependent transcription was measured with the strains RLG957, RLG2263, and RLG1336 as described previously (14). For measurement of *katG′-′lacZ* fusion gene expression, overnight cultures of KT1000 and KT1001 cells carrying pLAW2 or one of the pLAMC plasmids were diluted with fresh LB medium containing IPTG (0.5 mM) and were grown to mid-log phase. Hydrogen peroxide (100 μM) was then added to one portion of each culture. After 10 min, the cultures were chilled on ice, and β-galactosidase activity was measured as described previously (10).

**DNA sequencing.** The *Eco*RI-*Bam*HI fragments carrying the downstream half

TABLE 1. Sequence alterations of *rpoA* mutants and effects on *katG'-'lacZ* fusion expression

| *rpoA* allele | Amino acid substitution | Base substitution | No. of isolates | Expression of *katG'-'lacZ* | | | | Activation (%)[b] | Induction ratio (%)[c] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Δ*oxyR*[a] | | *oxyR*$^+$[a] | | | |
| | | | | $-H_2O_2$ | $+H_2O_2$ | $-H_2O_2$ | $+H_2O_2$ | | |
| *rpoA*$^+$ | | | | 125 | 110 | 231 | 631 | 100 | 100 |
| *rpoA129* | R-265→C | CGC→TGC | | 86 | 81 | 107 | 186 | 29 | 59 |
| *rpoA138* | N-268→D | AAC→GAC | 3 | 93 | 88 | 106 | 191 | 30 | 61 |
| *rpoA140* | C-269→R | TGC→CGC | 1 | 96 | 93 | 139 | 328 | 52 | 79 |
| *rpoA141* | C-269→Y | TGC→TAC | 1 | 95 | 91 | 121 | 269 | 43 | 75 |
| *rpoA145* | P-293→S | CCT→TCT | 1 | 102 | 100 | 186 | 571 | 90 | 100 |
| *rpoA147* | P-293→L | CCT→CTT | 1 | 101 | 97 | 159 | 460 | 73 | 97 |
| *rpoA146* | N-294→D | AAC→GAC | 1 | 83 | 83 | 136 | 337 | 53 | 80 |
| *rpoA135* | K-298→E | AAA→GAA | 2 | 78 | 70 | 103 | 208 | 33 | 72 |
| *rpoA134* | S-299→P | TCT→CCT | 1 | 98 | 94 | 125 | 280 | 44 | 75 |
| *rpoA131* | L-300→P | CTT→CCT | 2 | 96 | 91 | 141 | 420 | 67 | 100 |
| *rpoA136* | L-307→P | CTG→CCG | 1 | 81 | 80 | 113 | 316 | 50 | 95 |

[a] β-Galactosidase activity of KT1001 (Δ*oxyR*) and KT1000 (*oxyR*$^+$) transformed by a plasmid carrying one of the *rpoA* alleles. Activity of β-galactosidase is the average of three independent assays and is given in Miller units.

[b] β-Galactosidase activity of $H_2O_2$-treated KT1000 (*oxyR*$^+$) transformed by a plasmid carrying one of the mutant *rpoA* alleles relative to that of KT1000 transformed by the *rpoA*$^+$ plasmid.

[c] Ratio of induction level in KT1000 (*oxyR*$^+$) and KT1001 (Δ*oxyR*) transformed by a plasmid carrying one of the mutant *rpoA* alleles relative to that of KT1000 and KT1001 transformed by the *rpoA*$^+$ plasmid.

of the mutant *rpoA* genes were subcloned into the *Eco*RI-*Bam*HI site of M13mp18 and then sequenced by the dideoxy chain termination method (16).

## RESULTS

**Isolation of *rpoA* mutants defective in *oxyR*-dependent *katG* transcription.** To isolate mutant α subunits defective in specific interaction with OxyR, we used a screening procedure similar to that used for isolation of *rpoA* mutants defective in CRP-dependent *lac* P1 transcription (23). For this purpose, we used a library of *rpoA* mutants (pLAMC library), each carrying a single point mutation on average in the downstream half of the *rpoA* gene (23). The mutant library was introduced into *E. coli* KT1000, a strain lysogenized with a lambda phage carrying a *katG'-'lacZ* fusion gene, and plated on lactose-tetrazolium agar plates containing IPTG. Each transformant carried one of the mutant *rpoA* alleles from the library. Mutant *rpoA* alleles, placed downstream of the *lpp* and *lac* promoter, were highly expressed upon addition of IPTG, resulting in replacement of the chromosomal *rpoA*$^+$-encoded wild-type α subunit in RNA polymerase by the plasmid-encoded mutant α subunits. After overnight incubation, approximately 1% of the colonies exhibited the color reaction, which is an indication of decreased expression of the *katG'-'lacZ* fusion gene. Plasmid DNA was extracted from each clone and transformed into *oxyR*$^+$ and *oxyR* deletion strains. Transformants were examined in detail for *katG'-'lacZ* fusion gene expression by measurement of β-galactosidase activity in cells grown in liquid media.

In an *oxyR* deletion background, the level of *katG* expression of the strains carrying the mutant *rpoA* plasmids was slightly lower than that of the strains carrying the wild-type *rpoA* plasmid regardless of the presence or absence of $H_2O_2$ (Table 1). As expected, the induction of *katG* expression by $H_2O_2$ was not observed for these strains. In the *oxyR*$^+$ strain transformed by the wild-type *rpoA*$^+$ plasmid, the level of expression of *katG* without $H_2O_2$ treatment was two times higher than that of the *oxyR* deletion strain, suggesting that $H_2O_2$ produced during aerobic growth acts as an intracellular inducer. The level of *katG* expression in this strain further increased 2.73-fold upon addition of $H_2O_2$. In contrast to the results with *oxyR* deletion strains, the *rpoA* alleles affected *katG* transcription in an *oxyR*$^+$ background to various degrees. The level of β-galactosidase activity in the mutant strains under $H_2O_2$-induced conditions ranged from 29 to 90% of the level of activity in the wild-type strain. These results indicate that the defect in *katG* expression caused by the mutant *rpoA* plasmids is *oxyR* dependent and that these plasmids encode a mutant α subunit with decreased interaction with OxyR. The induction ratios (the ratios of induction levels in the presence versus the absence of OxyR) were 59% for *rpoA129*, 61% for *rpoA138*, 72% for *rpoA135*, and 75% for *rpoA141* and *rpoA134*.

**Mapping of the mutations in the *rpoA* gene.** To locate the mutations, we determined the DNA sequence of the mutagenized region of each *rpoA* mutant allele, between the *Eco*RI site and the termination codon. The results, summarized in Table 1, indicated that most of the mutant clones carried a single-base substitution. The mutations strongly affecting *oxyR*-dependent transcription were all mapped at two distinct dipeptide regions: one at amino acid residues 268 to 269 (*rpoA138*, *rpoA140*, and *rpoA141*) and the other at residues 298 to 299 (*rpoA134* and *rpoA135*). Amino acid substitution mutations at residues 293, 294, 300, and 307 had weaker effects. One of the three N-268→D (*rpoA138*) mutant clones and one of the two K-298→E (*rpoA135*) mutant clones had an additional amino acid substitution (E-181→G and V-242→M, respectively. Because the phenotypes of these double mutants are almost the same as those of the mutants with the single N-268→D and K-298→E substitutions (data not shown), we concluded that the substitutions at amino acid residues 268 and 298 are responsible for the mutant phenotypes. Although we have previously shown the involvement of Arg-265 of the α subunit in interaction with OxyR (19), mutations affecting this residue were not obtained in this study, suggesting that our genetic screening has not yet saturated all of the sites leading to the mutant phenotype.

**Effect of mutations on CRP-dependent *lac* transcription.** In our previous study, we found that one of the *rpoA* mutations, *rpoA129* (R-265→C substitution), which is defective in CRP-dependent *lac* P1 transcription (23), also affects the response to activation by OxyR (19). To investigate whether the *rpoA* mutations isolated in this study also affect CRP-dependent *lac* transcription conversely, we quantitated the level of *lacZ* expression by measuring β-galactosidase activity in *E. coli* DH5

TABLE 2. Effects of *rpoA* mutations on *lacZ* and *rrnB* expression

| *rpoA* allele | Promoter activity | |
|---|---|---|
| | *lacZ* (%)[a] | *rrnB* P1 (+UP/−UP element ratio)[b] |
| *rpoA*$^+$ | 100 | 37.2 |
| *rpoA129* | 43 | 3.5 |
| *rpoA138* | 84 | 5.7 |
| *rpoA140* | 75 | 5.7 |
| *rpoA141* | 54 | 4.5 |
| *rpoA145* | 77 | 9.5 |
| *rpoA147* | 68 | 6.6 |
| *rpoA146* | 83 | 5.7 |
| *rpoA135* | 52 | 4.9 |
| *rpoA134* | 65 | 7.4 |
| *rpoA131* | 65 | 13.2 |
| *rpoA136* | 74 | 13.0 |

[a] β-Galactosidase activity of DH5 transformed by a plasmid carrying one of the mutant *rpoA* alleles relative to the activity of DH5 carrying the wild-type *rpoA*$^+$ plasmid.

[b] β-Galactosidase activities of RLG957 (lysogenic for λ*rrnB* P1 [−61 to +50]-*lacZ*) and RLG2263 (lysogenic for λ*rrnB* P1 [−41 to +50]-*lacZ*), each transformed by a plasmid carrying one of the *rpoA* alleles, were corrected by subtraction of the background β-galactosidase activities of RLG1336 (lysogenic for λ [promoterless *lacZ*]) carrying the same plasmid. The ratio of corrected β-galactosidase activity of RLG957 and RLG2263 is used to express *rrnB* P1 promoter activity.

cells transformed by each of the plasmids carrying the mutant *rpoA* gene. All of the test strains showed lower levels of β-galactosidase activity than that transformed by the wild-type plasmid, pLAW2 (Table 2). In general, the decrease in the level of OxyR-dependent transcription paralleled the decrease in the level of *lacZ* expression, although two mutants (N-268→D and N-294→D) had a more severe effect on activation by OxyR. These results suggest that the region of the α subunit involved in OxyR interaction overlaps, if it is not identical to, the region that is essential for transcription activation of the *lac* promoter by CRP.

**Effect of the mutations on UP element-dependent transcription.** The AT-rich UP element upstream of the −35 sequence of the *rrnB* P1 promoter is known to enhance transcription in the absence of protein activators (12). The purified α subunit of RNA polymerase alone binds to the *rrnB* P1 UP element in vitro, and its C-terminal region is essential for both binding to and transcription activation by the UP element (14). To examine the effect of the *rpoA* point mutations on the responsibility of the α subunit to the *rrnB* P1 UP element, we compared β-galactosidase activities in vivo of two *rrnB* P1-*lacZ* fusion genes, one carrying and the other lacking the UP element, for each mutant α subunit (Table 2).

In the strain containing only the wild-type α subunit, the *rrnB* P1 promoter activity with the UP element was about 40-fold higher than that in the strain lacking the UP element, and the activity ratio agreed well with those from previous observations (12, 14). In the strains containing a mutant α subunit in addition to the wild-type α subunit expressed from the chromosomal *rpoA*$^+$ allele, the ratios of the two promoter activities were 3 to 10 times lower than that of the strain containing the wild-type α subunit alone. Amino acid substitutions N-268→D, C-269→Y, K-298→E, and S-299→P (and R-265→C) had strong effects on both *oxyR*-dependent and UP element-dependent transcription. Amino acid substitutions C-269→R, P-293→L, N-294→D, and K-298→E (and R-265→C) had stronger effects on UP element-dependent transcription than on *oxyR*-dependent transcription. The differential effect of some amino acid substitutions suggests that the defect in response to OxyR could not be directly related to the inability to utilize the UP element. Differential effects of *rpoA* mutations on CRP-dependent and UP element-dependent transcription have been observed (11).

**In vitro transcription by mutant RNA polymerases.** To directly examine the responsibility of mutant RNA polymerases containing the mutant α subunits for transcription activation by OxyR, we carried out in vitro transcription studies with purified proteins. For this purpose, we selected four typical *rpoA* mutants, *rpoA134*, *rpoA135*, *rpoA138*, and *rpoA141*, each carrying a single-amino-acid substitution, S-299→P, K-298→E, N-268→D, and C-269→Y, respectively. Mutant α subunits were overproduced with a gene expression system based on T7 promoter and polymerase, purified, and reconstituted into mutant holoenzymes as described previously (4, 23). All of the mutant α subunits as well as the wild-type α subunit were assembled into the holoenzymes (data not shown). With the reconstituted wild-type and mutant RNA polymerases, we carried out in vitro mixed-transcription experiments with the *katG* and *lacUV5* promoters (Fig. 1A). The *lacUV5* promoter, used as a reference promoter, was transcribed by the mutant RNA polymerases as efficiently as by the wild-type RNA polymerase. The wild-type RNA polymerase exhibited OxyR-dependent

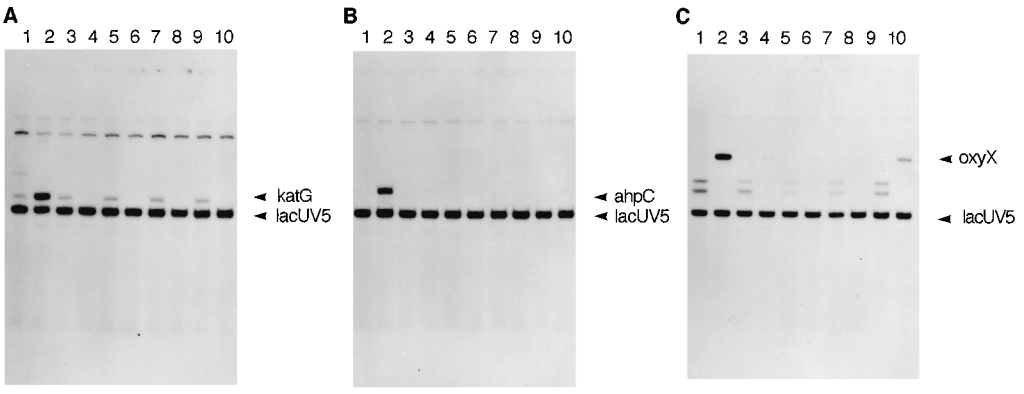


FIG. 1. In vitro transcription of the *katG* (A), *ahpC* (B), and *oxyX* (C) promoters by the reconstituted wild-type and mutant RNA polymerases. The following RNA polymerases were used (by lane): 1 and 2, wild type; 3 and 4, RpoA134 holoenzyme; 5 and 6, RpoA136 holoenzyme; 7 and 8, RpoA138 holoenzyme, and 9 and 10, RpoA141 holoenzyme. The amount of RNA polymerase used is 1 pmol for each reaction. The reactions were carried out in the presence (lanes 2, 4, 6, 8, and 10) or absence (lanes 1, 3, 5, 7, and 9) of OxyR protein. Specific transcripts are indicated by arrowheads. Slowly migrating bands in each panel are template-sized end-to-end products.

*katG* promoter-directed transcription. For all of the mutant RNA polymerases examined, however, virtually no activation was observed in OxyR protein-dependent transcription, although the basal level of *katG* transcripts in the absence of OxyR was detected. These in vitro observations support the in vivo finding that these amino acid substitutions impair the interaction between OxyR and RNA polymerase.

To examine whether the mutations isolated on the basis of defective *katG* activation also affect transcription activation of other OxyR-regulated promoters, we carried out in vitro transcription of the *ahpC* and *oxyX* promoters. The binding sites of OxyR protein on the *ahpC* and *oxyX* promoters are located at similar positions, relative to the respective transcription initiation sites, as on the *katG* promoter (21), and the C-terminal region of α is known to be necessary for transcriptional activation of these genes by OxyR (19). As shown in Fig. 1B and C, all of the mutant RNA polymerases were unable to respond to OxyR in transcription activation at the *ahpC* and *oxyX* promoters. These results strongly suggest that these amino acid substitutions affect protein-protein contacts of the α subunit with OxyR but do not affect promoter recognition properties.

## DISCUSSION

A large number of amino acid substitution mutations, which impair transcription of positively regulated systems, have been mapped in the C-terminal contact site I region of the RNA polymerase α subunit (2, 9, 18, 22, 23). Taken together with the analyses of deletion mutations (3, 4), it is proposed that the C terminus of the α subunit makes direct contact with a group of transcription activator proteins (5, 6, 15). Most of the point mutations obtained on the basis of defective activation of a given system are clustered in narrow regions composed of several amino acid residues within the contact site I region. The sites of mutations are, however, different among the systems activated by different factors, and each mutation affects the expression of only a specific set(s) of systems. Thus, multiple subsites seem to exist in the contact site I region on the α subunit, each interacting with a different activator protein(s).

In our systematic mapping of the contact site I region on the α subunit, we isolated in this study a set of *rpoA* mutants defective in OxyR-dependent transcription by random mutagenesis of the C-terminal proximal half of the *rpoA* gene followed by in vivo screening for decreased expression of *lacZ* under the control of the OxyR-regulated *katG* promoter. The mutations showing the clear mutant phenotype are clustered at two regions, around amino acid residues 268 to 269 (and 265) and 298 to 299. Mutations with less profound phenotypes were also obtained in the vicinity of the downstream contact site, including residues 293, 294, 300, and 307. The results of in vitro transcription experiments with the reconstituted RNA polymerases containing these mutant α subunits are generally in good agreement with the in vivo data. Thus, we concluded that the contact site for OxyR is composed of the two short segments, one between residues 265 and 269 and the other between residues 293 and 300. These two segments forming the OxyR contact surface are both located on the hydrophilic side of helix-1 and helix-4, respectively, as analyzed by multidimensional heteronuclear magnetic resonance spectroscopy (7).

Interestingly, these two segments involved in interaction with OxyR are also involved in CRP-dependent activation at the *lac* P1 promoter (11, 23). These results suggest that CRP and OxyR share a common contact surface on α, although CRP and OxyR are structurally unrelated and the locations of their DNA-binding sites relative to the transcription start site are different. The role of each amino acid residue on the two contact surfaces, however, seems different in terms of molecular contact between OxyR and CRP, because the levels of activation defect for some mutations are different between OxyR and CRP. (For instance, the mutations in the C-terminal proximal contact surface on helix-4 affect the response to OxyR more than they affect the response to CRP.) The contact surface with CRP and OxyR was also found to be involved in interaction with the *rrnB* P1 UP element (11, 14). The effects of mutation of individual amino acids on CRP-, OxyR-, and UP element-dependent transcription are different. In addition, the observed defects in *katG* transcription by the α subunit mutant RNA polymerases are OxyR dependent both in vivo and in vitro. Thus, we concluded that the mechanism of activation by OxyR is due to direct protein-protein contact(s) with the RNA polymerase α subunit.

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