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Transcription factor recognition surface on the RNA polymerase α subunit is involved in contact with the DNA enhancer element

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The carboxy-terminal one-third of *Escherichia coli* RNA polymerase α subunit plays a key role in transcription regulation by a group of protein transcription factors and DNA enhancer (UP) elements. The roles of individual amino acid residues within this regulatory domain of the α subunit were examined after systematic mutagenesis of the putative contact regions (residues 258–275 and 297–298) for the cAMP receptor protein (CRP). The reconstituted RNA polymerases containing the mutant α subunits were examined for their response to transcription activation by cAMP–CRP and the *rrnB*P1 UP element. Mutations affecting CRP responsiveness were located on the surface of the putative CRP contact helix and most of these mutations also influenced the response to the *rrnB* UP element. These observations raise the possibility that the CRP contact surface is also involved in contact with the DNA UP element, although some amino acid residues within this region play different roles in molecular communication with CRP and the UP element. Among the amino acid residues constituting the contact surface, Arg265 was found to play a major role in response to both CRP and the DNA UP element. Judged by DNase I footprinting analysis, α mutants defective in transcription from the CRP-dependent *lac*P1 promoter showed decreased activity in the cooperative binding of CRP. Likewise, mutants defective in *rrnB*P1 transcription showed decreased binding to the UP element. The amino acid residues important for contact with both CRP and DNA are conserved in the α subunits of not only bacterial, but also chloroplast RNA polymerases.

Keywords: point mutation/protein–DNA contact/protein–protein contact/RNA polymerase/transcription activation

Introduction

Escherichia coli RNA polymerase holoenzyme is composed of four different subunits ($\alpha_2\beta\beta'\sigma$). Core enzyme with the subunit structure $\alpha_2\beta\beta'$ is fully active in RNA polymerization, whereas the binding of one of the various species of σ subunit is required for specific initiation from promoters. The α subunit, consisting of 329 amino acid residues, plays a key role in RNA polymerase assembly (reviewed in Ishihama, 1981). Both *in vivo* (Hayward *et al.*, 1991) and *in vitro* (Igarashi and Ishihama, 1991; Igarashi *et al.*, 1991a; Kimura *et al.*, 1994) analyses of a set of deletion derivatives of *rpoA* have indicated that as many as 94 C-terminal amino acid residues can be removed without preventing the assembly of the RNA polymerase complex. The C-terminal proximal region is, however, needed for transcription regulation not only by transcription activators such as cyclic AMP receptor protein (CRP) (Igarashi and Ishihama, 1991), OxyR (Tao *et al.*, 1993) and OmpR (Igarashi *et al.*, 1991b), but also by transcription repressor GalR (Choy *et al.*, 1995). Transcription factors which require the C-terminal domain of RNA polymerase α subunit, directly or indirectly, for action are designated as class I factors (Ishihama, 1993). Most, if not all, class I factors bind to *cis*-acting DNA signals located upstream of the promoter –35 signal (Igarashi *et al.*, 1991b; reviewed by Ishihama, 1992, 1993). Later, the C-terminal proximal region was found to play a role in the recognition of promoter upstream (UP) elements, which consist of AT-rich sequences, that enhance transcription initiation (Ross *et al.*, 1993). Mutational mapping revealed that the amino acid residues required for CRP-dependent transcription on the α subunit are located within a narrow window between amino acid residues 258 and 270 (Zou *et al.*, 1992; Tang *et al.*, 1994). This region is also required for the response to OxyR (Tao *et al.*, 1995).

In order to investigate the role of each amino acid in this region (residues 258–275) and in the secondary OxyR-response region (residues 297–298), we carried out systematic mutagenesis to create a series of α derivatives carrying a single amino substitution, either Trp or Ala, from positions 258 to 275 and from 297 to 298. Each mutant α subunit was overexpressed, purified and reconstituted into mutant RNA polymerases. The ability of the mutant RNA polymerases to respond to CRP and the UP element was examined in transcription reactions *in vitro* using the cAMP/CRP-dependent *lac*P1 and UP element-dependent *rrnB*P1 promoters. Since Arg265 was found to play a major role in response to both CRP and the UP element, we also constructed another set of mutant RNA polymerases by replacing Arg265 with amino acids other than Trp and Ala, and examined their responses to CRP and UP. From the location of mutations affecting CRP and UP responses on the three-dimensional structure of the α subunit C-terminal domain recently resolved by Jeon *et al.* (1995), we propose that the same protein surface on α is involved in contact with both CRP and the UP element.

Results

Reconstitution of mutant RNA polymerases containing single-Trp α subunits

In order to determine the role of each amino acid residue within the putative CRP contact region on α, we con-

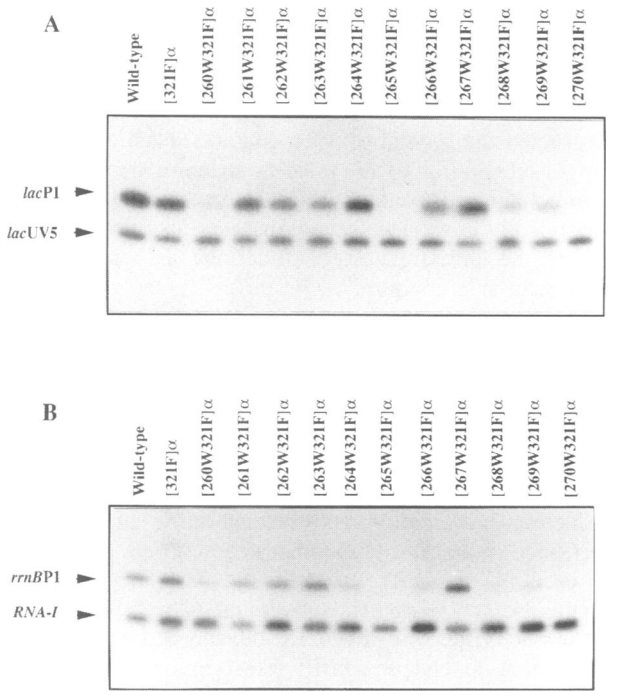

Fig. 1. CRP- and UP-dependent transcription by mutant RNA polymerases containing Trp-substituted α subunits. (**A**) CRP-dependent *lac*P1 promoter-directed transcription. A mixture of *lac* (0.1 pmol) and *lac*UV5 (0.04 pmol) promoter DNA fragments, CRP (2.5 pmol) and cAMP (10 μM) was pre-incubated at 37°C for 5 min. The reconstituted RNA polymerases (1 pmol each) containing wild-type or mutant α subunits were added as indicated (final volume, 35 μl), and incubation at 37°C was continued for another 30 min to allow the open complex formation. After addition of a substrate–heparin mixture, RNA synthesis was allowed for 5 min at 37°C. Transcription products were analyzed by electrophoresis on 8% polyacrylamide gel containing 8 M urea. The RNA polymerases used are shown above each lane. Specific transcripts are indicated by arrows (*lac*P1, transcript from the *lac*P1 promoter; *lac*UV5, transcript from the *lac*UV5 promoter). (**B**) UP-dependent *rrnB*P1-directed transcription. A 35 μl mixture containing one of the reconstituted RNA polymerases (0.6 pmol each) and a supercoiled plasmid DNA template (0.2 pmol), which carried both the *rrnB*P1 and the *RNA-I* promoters, was pre-incubated at 22°C for 30 min to allow open complex formation. After addition of a substrate–heparin mixture, RNA synthesis was allowed for 5 min at 22°C. Transcripts were separated by 5% polyacrylamide gel containing 8 M urea. The RNA polymerases used are indicated above each lane. Specific transcripts are indicated by arrows (*rrnB*P1, transcript from the *rrnB*P1 promoter; *RNA-I*, transcript from the *RNA-I* promoter).

structed a set of mutant α subunits, each carrying a single Trp substitution (single-Trp α) between amino acid residues 260 and 270. So that this set of α derivatives could also be used for fluorescence studies of α–CRP and α–UP interactions, we also replaced the pre-existing Trp at residue 321 with Phe prior to mutagenesis of the α subunit. This Trp321 to Phe substitution had no significant effect on either factor (CRP or UP)-dependent or independent transcription (see Figure 1). Mutant α subunits were overproduced using a gene expression system based on T7 promoter/polymerase, purified and reconstituted into mutant holoenzymes as described previously (Igarashi and Ishihama, 1991; Kimura *et al.*, 1994). Since the expression level of mutant *rpoA* on the plasmid was ~100-fold higher than that of wild-type *rpoA* on the chromosome (Kimura and Ishihama, 1995a), the contamination by wild-type α in the α derivatives used was <1%, if any. All α

Table I. Effect of amino acid substitutions in the α subunit on CRP- and UP element-dependent transcription

Amino acid residue	CRP-dependent *lac*P1 transcription[a]		UP element-dependent *rrnB*P1 transcription[b]	
(X)	X→Ala	X→Trp[c]	X→Ala	X→Trp[c]
258 Asp	++++[d]		++++[d]	
259 Asp	++++		++++	
260 Leu	+	–	+	+
261 Glu	++++	++++	++++	++++
262 Leu	–	++	+	++
263 Thr	++++	++	++++	++++
264 Val	++++	+++	++++	+
265 Arg	–	–	–	–
266 Ser	++++	+++	+++	–
267 Ala	++++	++++	++++	++++
268 Asn	++	+	–	–
269 Cys	++++	+	++	+
270 Leu	–	–	++++	–
271 Lys	++++		++++	
272 Ala	++++		++++	
273 Glu	++++		++++	
274 Ala	++++		++++	
275 Ile	++		++++	
297 Lys	+		–	
298 Lys	++		++++	

[a]The response of each reconstituted RNA polymerase to CRP was estimated comparing the *lac*P1 transcription level (normalized by *lac*UV5 transcript).
[b]The response of each reconstituted RNA polymerase to the UP element was estimated by measuring the transcription level from *rrnB*P1 (normalized by *RNA-I* transcript).
[c]The 'single-Trp α' mutants contained one additional amino acid substitution of Phe for Trp321, but this amino acid replacement did not influence transcription activities of the reconstituted RNA polymerases, at least from the promoters used.
[d]Activity ratio of the α-mutant RNA polymerase to wild-type RNA polymerase. ++++, >80%; +++, 80–60%; ++, 60–40%; +, 40–20%; –, <20%.

derivatives were assembled into the core enzymes as efficiently as the wild-type α subunit (data not shown). The amounts of enzymes used were adjusted based on the core enzyme activity as measured by poly(dAT)-dependent poly(AU) synthesis.

Transcription in vitro from lac promoters

To measure the response of these mutant RNA polymerases to CRP, we carried out *in vitro* mixed transcription experiments using two DNA fragments, one containing the *lac*UV5 promoter (a CRP-independent mutant version of *lac*P1) as a reference promoter, and the other containing the cAMP/CRP-dependent *lac*P1 promoter. A typical autoradiogram of the run-off transcripts is shown in Figure 1A, and the quantitative results are summarized in Table I. The reconstituted RNA polymerase containing α with the Trp321Phe substitution (hereafter designated as [321F]) showed essentially the same levels of transcription from the *lac*UV5 and *lac*P1 as the wild-type reconstituted enzyme. The result indicates that the replacement of Trp at 321 with Phe does not significantly influence the transcription activities of reconstituted RNA polymerase, at least from these two promoters.

All the mutant RNA polymerases containing α derivatives, each carrying a single Trp substitution plus the 321F mutation, exhibited essentially the same levels of transcription from the *lac*UV5 promoter as the wild-type

enzyme, indicating that single Trp substitutions in this region do not affect the factor-independent transcription activity. In contrast, significant differences were observed in transcription from the cAMP/CRP-dependent *lac*P1 promoter. Mutant enzymes containing [260W321F]α, [265W321F]α and [270W321F]α were virtually inactive (<20%), while those containing [268W321F]α and [269W321F]α were only partially active (<40%). The results agree with our *in vivo* mapping data of α mutations affecting its response to CRP (Zou *et al.*, 1992).

Transcription in vitro from rrnBP1 promoters

An UP element with an AT-rich DNA sequence located upstream of the core promoter region (–35 and –10 promoter sequences) of rRNA promoter *rrnB*P1 activates transcription *in vivo* and *in vitro* in the absence of protein factors. Ross *et al.* (1993) demonstrated that the α C-terminal region is essential for RNA polymerase binding to the *rrnB*P1 UP element and transcription activation by the UP element. One of the *rpoA* mutations, *rpoA*129 (R265C substitution), which renders α defective in CRP-dependent *lac*P1 transcription (Zou *et al.*, 1992), also influences UP element-dependent transcription (Ross *et al.*, 1993). Some of the new Trp-substituted mutants may also lack the ability to recognize the UP element. To test this hypothesis, we analyzed transcription of DNA containing the *rrnB*P1 promoter with or without its UP element. Figure 1B shows the results of a run-off transcription assay and the quantitative results are summarized in Table I.

Wild-type and all mutant RNA polymerases showed essentially the same low level of basal transcription from the mutant *rrnB*P1 promoter lacking the UP element sequences (data not shown). The *RNA-I* promoter, used as a reference promoter on the same plasmid, was transcribed by the mutant RNA polymerases as efficiently as by the wild-type RNA polymerase. However, significant differences were observed for the UP element-dependent *rrnB*P1 transcription. Transcription by the wild-type and [321F]α RNA polymerases was increased ~30-fold by the UP element. The amino acid substitutions at Arg265, Ser266, Asn268 and Leu270 completely inactivated the UP element-dependent transcription (or to <20% of the level of the wild-type enzyme), whereas the substitutions at Leu260, Val264 and Cys269 reduced the transcription to <40%. Interestingly, all the amino acid substitutions causing a defect in CRP-dependent transcription (substitutions at Leu260, Arg265, Asn268, Cys269 and Leu270) also resulted in a defect in UP element-dependent transcription. These results indicated that the amino acid residues required for CRP-dependent transcription are also involved in its response to the UP element. Since the critical residues required for the response to both CRP and the UP element are located on the surface of an amphipathic α-helix (helix 1) in the three-dimensional structure of α C-terminal domain (Jeon *et al.*, 1995; see also Figure 6A), the possibility arises that the same protein surface is involved in the molecular contact with both CRP and the DNA UP element (for details, see Discussion).

Alanine scanning mutagenesis

Trp substitutions for specific residues in the C-terminal domain rendered α inactive in the responses to both CRP and UP. However, the Trp substitutions may disrupt the local conformation of α subunit, leading to inactivation indirectly in its molecular contacts with CRP or the UP element. As an attempt to exclude this possibility, we constructed the second set of α mutants, each containing a single substitution of Ala (alanine scanning mutagenesis; Cunningham and Wells, 1989). Alanine scanning makes a chemically consistent set of mutants and, in addition, permits the investigation of functions associated with amino acid side chains.

Alanine scanning was performed not only for the region including amino acid residues 258–275, which forms helix 1 (Jeon *et al.*, 1995), but also for a region from residues 297 and 298 on helix 4, which includes the secondary site for the response to OxyR (Tao *et al.*, 1995). Helices 1 and 4 are located close together on the three-dimensional structure (Jeon *et al.*, 1995; see also Figure 6A). Overproduction, purification and reconstitution of the Ala-substituted mutant α subunits were carried out as in the case of single-Trp α mutants. The mutant RNA polymerases carrying Ala-substituted α were examined for their activities of cAMP/CRP-dependent *lac*P1 and the UP element-dependent *rrnB*P1 transcription.

Figure 2A, shows the autoradiograms of *in vitro* transcription from the CRP-dependent *lac*P1 promoter and Table I summarizes the quantitative results. Mutant RNA polymerases containing [260A]α, [262A]α, [265A]α, [268A]α and [270A]α were defective in the response to CRP, as in the case of Trp-substituted mutants. However, the mutant enzymes containing [263A]α, [264A]α, [266A]α and [269A]α showed essentially the same levels of transcription as the wild-type holoenzyme, suggesting that the slight decrease observed for the mutants carrying the Trp substitution at the same sites was due to conformational disturbance of the α subunit structure. Among the residues tested only in the alanine scanning experiment, substitutions at Ile275, Lys297 and Lys298 showed decreased activity in CRP-dependent *lac*P1 transcription.

The same set of Ala-substituted mutants was also tested for UP-dependent *rrnB*P1 transcription. The results, shown in Figure 2B and Table I, indicate that Ala substitutions at Leu260, Leu262, Arg265, Asn268, Cys269 and Lys297 reduced the UP element-dependent transcription, whereas the mutants with Ala substitutions at Val264, Ser266 and Leu270 showed the same response to the UP element as the wild-type enzyme. Taking the results using the two sets of mutant RNA polymerases together, we concluded that five residues (Leu260, Leu262, Arg265, Asn268 and Lys297) are essential for transcription activation by both CRP and the UP element, and that three residues (Leu270, Ile275 and Lys298) are involved only in the response to CRP, while one residue (Cys269) is involved only in the response to the UP element. Among all the mutants examined in this study, the substitution at Arg265 for both Trp and Ala gave the most severe reduction in the responses to both CRP and the UP element. The reduction level was as high as that in C-terminal truncated α mutants.

Role of Arg265: amino acid substitution analysis

The above experiments using Trp- or Ala-substituted α derivatives indicated that, for both CRP-dependent and UP element-dependent transcription, Arg265 is the most critical amino acid within the α subunit C-terminal domain.

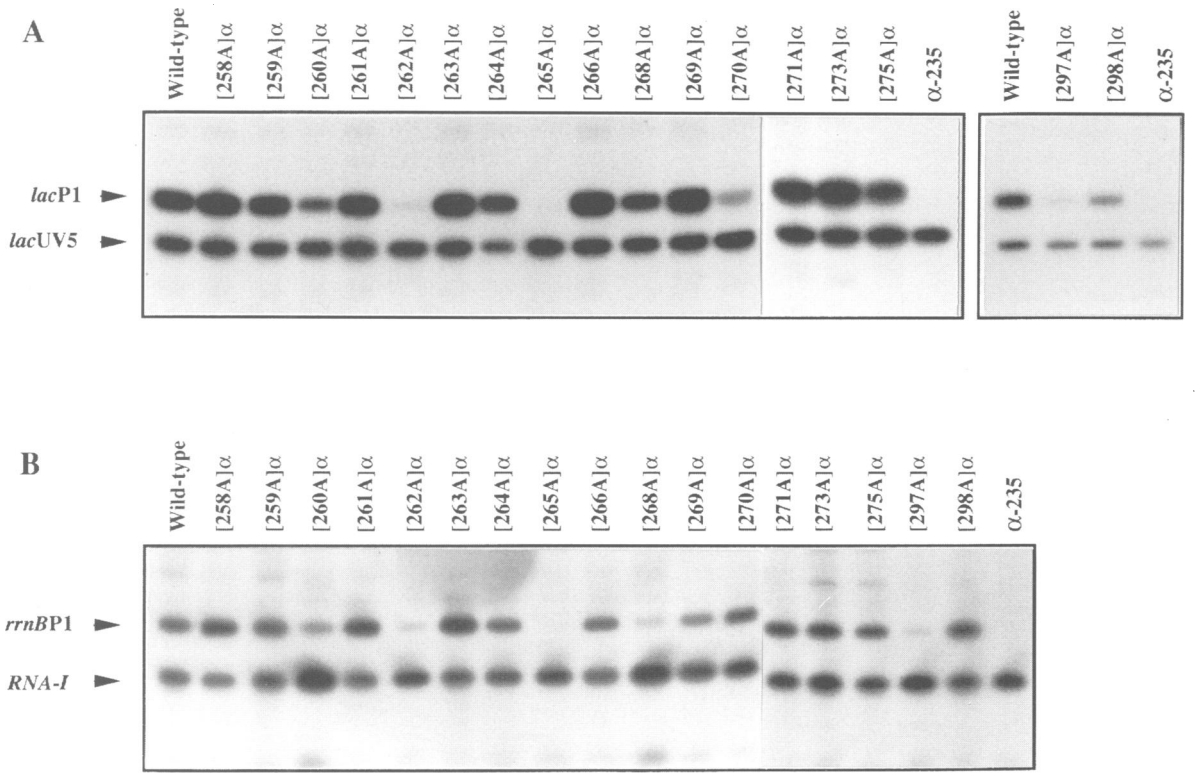

Fig. 2. CRP- and UP-dependent transcription by mutant RNA polymerases containing Ala-substituted α subunits. (**A**) CRP-dependent *lac*P1 promoter-directed transcription. The reaction conditions for *in vitro* transcription and the gel electrophoresis conditions were as in Figure 1A. The RNA polymerases used are indicated above each lane. Specific transcripts are indicated by arrows. (**B**) UP-dependent *rrnB*P1-directed transcription. The reaction conditions for *in vitro* transcription and the gel electrophoresis conditions were as in Figure 1B. The RNA polymerases used are indicated above each lane. Specific transcripts are indicated by arrows.

The results agreed well with our previous observations that *rpoA*129 (R265C substitution) RNA polymerase is defective in activation by cAMP/CRP (Zou *et al.*, 1992), *rrnB* UP element (Ross *et al.*, 1993) and OxyR (Tao *et al.*, 1993, 1995). Arg265 is also the target of ADP-ribosylation by bacteriophage T4. To characterize the function(s) of this amino acid in detail, we constructed the third set of mutant RNA polymerases by replacing Arg265 with some other amino acids, and characterized their responses to CRP and the UP element. We selected six amino acids which differ in the chemical properties of their side chains: Leu and Met are neutral-non-polar amino acids; Ser and Gln are neutral-polar amino acids; Glu is an acidic amino acid; and Lys is a basic amino acid. All the mutant α subunits were overexpressed, purified and reconstituted into the respective mutant holoenzymes. Figure 3 shows the results of *in vitro* transcription experiments from the *lac*P1 and *rrnB*P1 promoters.

All the mutant RNA polymerases except [265K]α were virtually inactive in their responses to CRP and the UP element. RNA polymerase carrying [265K]α retained about one-fourth the CRP-dependent activity of the wild-type enzyme (Figure 3A), but it was virtually inactive in UP-dependent transcription (Figure 3B). We thus conclude that Arg265 plays the most essential role in transcription activation by both CRP and the DNA UP element.

DNase I footprinting: mutant α–CRP interaction

In *E.coli*, CRP regulates >50 promoters (Botsford and Harman, 1992; Kolb *et al.*, 1993a) which can be classified

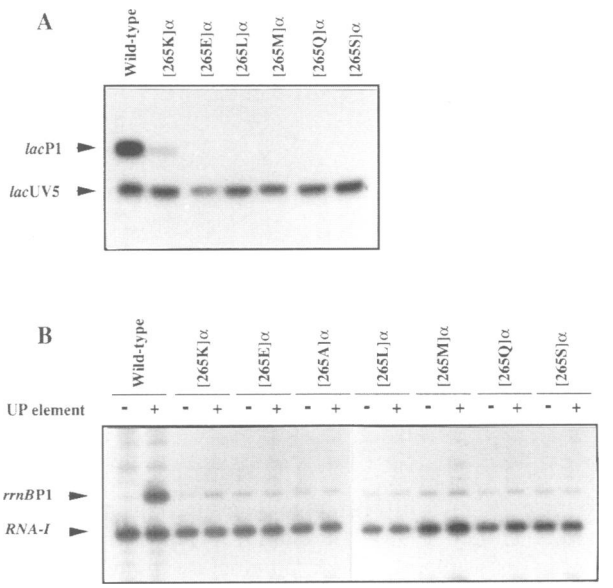

Fig. 3. Effect of substitutions of Arg265 in the α subunit. (**A**) *In vitro* transcription of the *lac* promoter fragment. The reaction conditions for *in vitro* transcription and the gel electrophoresis conditions were as described in Figure 1A. The RNA polymerses used were as shown above each lane. Specific transcripts are shown by arrows. (**B**) *In vitro* transcription of the *rrnB*P1 promoter plasmids. The reaction conditions for *in vitro* transcription and the gel electrophoresis conditions were as described in Figure 1B. The RNA polymerases and the promoters used are shown above each lane. The promoters were: +, *rrnB*P1 with the UP element (pRLG862): –, *rrnB*P1 without the UP element (pLR14). Specific transcripts are indicated by arrows.

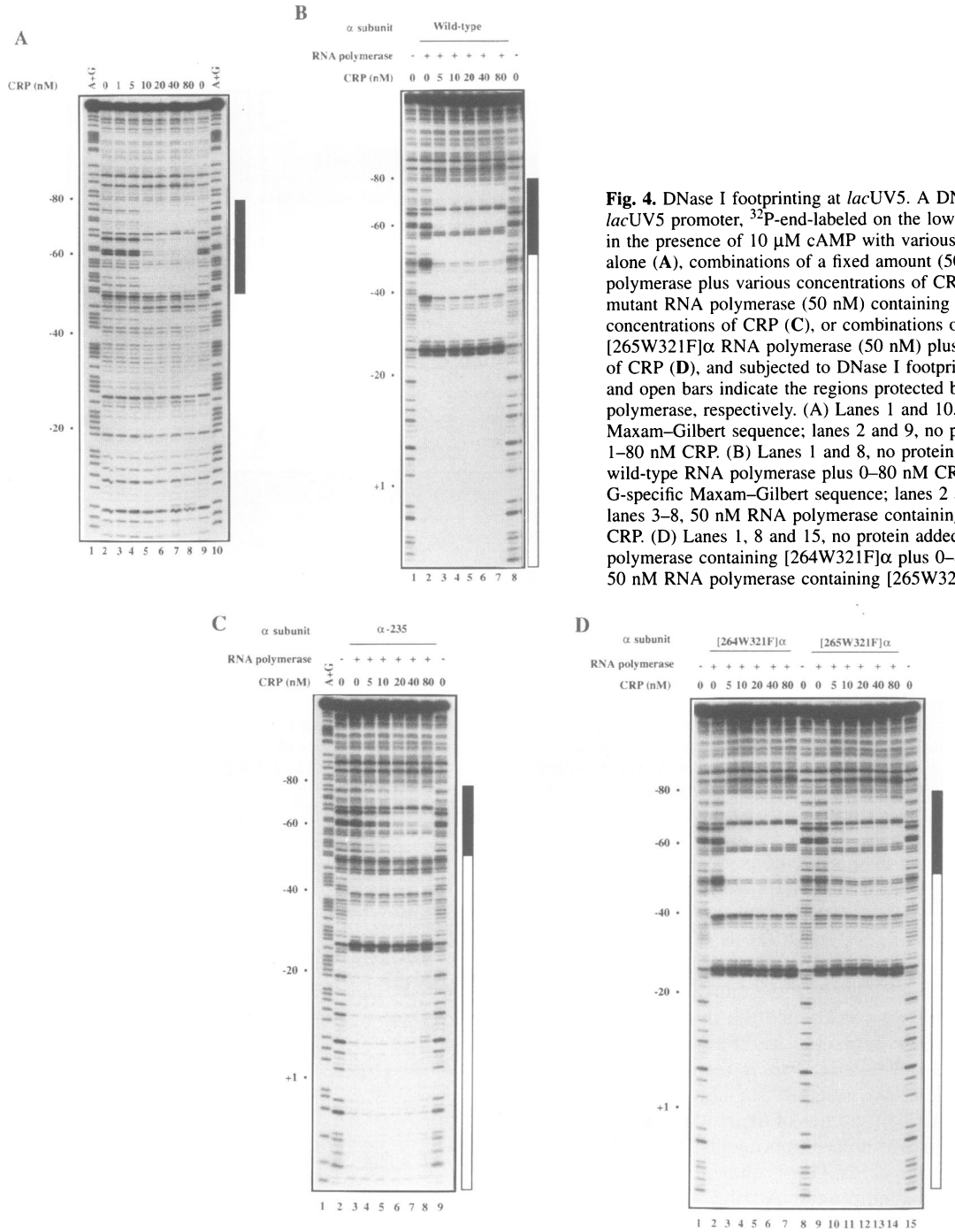

Fig. 4. DNase I footprinting at *lac*UV5. A DNA fragment carrying *lac*UV5 promoter, ^{32}P-end-labeled on the lower strand, was incubated in the presence of 10 μM cAMP with various concentrations of CRP alone (**A**), combinations of a fixed amount (50 nM) of wild-type RNA polymerase plus various concentrations of CRP (**B**), combinations of mutant RNA polymerase (50 nM) containing α-235 plus various concentrations of CRP (**C**), or combinations of either [264W321F]α or [265W321F]α RNA polymerase (50 nM) plus various concentrations of CRP (**D**), and subjected to DNase I footprinting analysis. The filled and open bars indicate the regions protected by CRP and RNA polymerase, respectively. (A) Lanes 1 and 10, A + G-specific Maxam–Gilbert sequence; lanes 2 and 9, no protein added; lanes 3–8, 1–80 nM CRP. (B) Lanes 1 and 8, no protein added; lanes 2–7, 50 nM wild-type RNA polymerase plus 0–80 nM CRP. (C) Lane 1, A + G-specific Maxam–Gilbert sequence; lanes 2 and 9, no protein added; lanes 3–8, 50 nM RNA polymerase containing α-235 plus 0–80 nM CRP. (D) Lanes 1, 8 and 15, no protein added; lanes 2–7, 50 nM RNA polymerase containing [264W321F]α plus 0–80 nM CRP; lanes 9–14, 50 nM RNA polymerase containing [265W321F]α plus 0–80 nM CRP.

into three groups with respect to their mechanism of action (Merkel *et al.*, 1995). At class I promoters including *lac*P1, CRP supports RNA polymerase binding to the promoters by making direct protein–protein contact with the α subunit (Malan *et al.*, 1984; Kolb *et al.*, 1993b). To measure the CRP-binding activities of mutant RNA polymerases directly, we performed DNase I footprinting analysis using the *lac*UV5 promoter as a template. RNA polymerase is able to form a stable open complex with this CRP-independent mutant promoter in the absence of CRP (Kolb *et al.*, 1993b). Since the CRP binding site is retained on *lac*UV5 in the same sequence as on the original *lac*P1, CRP alone is able to bind to the target DNA site, but in the presence of RNA polymerase, CRP binding to DNA is enhanced. Thus, the level of CRP binding to *lac*UV5 reflects its affinity for a given RNA polymerase.

Mixtures of a fixed amount of ^{32}P-labeled *lac*UV5 fragment with RNA polymerase, CRP or different combinations of the two proteins were incubated in the transcription buffer without substrates and then subjected to DNase I digestion. As shown in Figure 4B–D, RNA polymerases containing α-235, [264W321F]α and [265W321F]α protected the same promoter region from –43 to +20 on the lower strand as efficiently as wild-type α. This protection was observed almost at the same

protein concentrations for all the enzymes used, indicating that the C-terminal region of α is not essential for the RNA polymerase binding to the promoter.

On the other hand, CRP protein alone protected the CRP-binding site, from –76 to –50 relative to the transcription start point, from DNase I digestion (Figure 4A), only when a 40- to 80-fold molar excess of CRP over DNA (1 nM) was added. When both CRP and wild-type RNA polymerase were incubated together with the same DNA fragment, clear protection of the CRP-binding site was observed at lower CRP concentrations than that in the presence of CRP alone. The increased DNA-binding affinity of CRP resulted from the cooperative binding with RNA polymerase. When CRP was incubated with the DNA fragment in the presence of α-235 RNA polymerase, however, the cooperative binding of CRP was not observed (Figure 4C), indicating that the C-terminal domain of α is required for the contact with CRP.

The substitution mutation at Val264 strongly affected the response of α to the UP element (Figure 1B), but not to CRP (Figure 1A). In agreement with these observations, no difference was observed in the CRP–RNA polymerase interaction between α-WT and [264W321F]α enzymes. In contrast, a >20-fold molar excess of CRP over DNA was needed to achieve the same extent of CRP binding in the presence of [265W321F]α RNA polymerase, indicating that the binding activity of [265W321F]α with CRP is lower than that of α-WT. These results demonstrate that the mutant RNA polymerases inactive in CRP-dependent *lac*P1 transcription have decreased affinity for CRP, even though the R265W mutant α still retained a low level affinity for CRP, but α-235 lost the affinity completely.

DNase I footprinting: mutant α–DNA UP element interaction

Using the footprinting assay, we next examined the ability of the mutant RNA polymerases to interact with the DNA UP element. As shown in Figure 5, wild-type RNA polymerase strongly protected both the promoter and the UP element regions even though the cleavage of AT-rich UP element DNA by DNase I was relatively inefficient (Figure 5, lanes 3–5). In contrast, the two mutant RNA polymerases containing [265A]α and α-235 protected the core promoter regions (from –35 to +20) strongly, but the UP element region was not at all protected (Figure 5, lanes 6–11). These results showed that the α-mutant holoenzymes which are defective in activated transcription at *rrnB*P1 are unable to interact with the UP element, and that the residue Arg265 is important in this interaction.

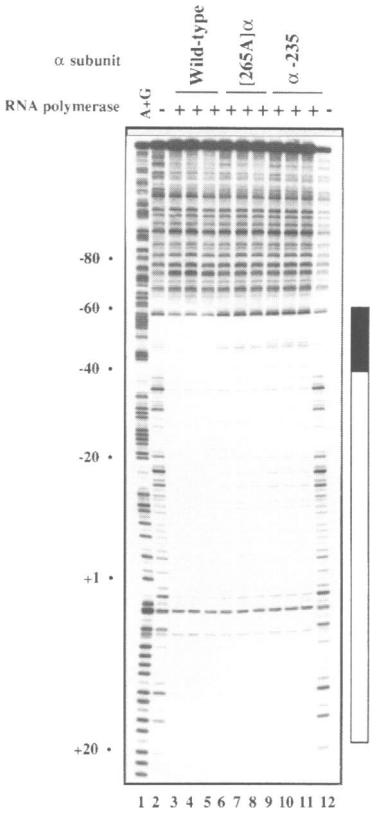

Fig. 5. DNase I footprinting at *rrnB*P1. A ^{32}P-end-labeled DNA fragment was incubated at 37°C for 30 min with the reconstituted RNA polymerases containing either wild-type or mutant α subunits in 25 μl of transcription buffer containing 5 mM $CaCl_2$ in the presence of initiating nucleotides, ATP (500 μM) and CTP (50 μM). The experiments were: lane 1, A + G-specific Maxam–Gilbert sequence; lanes 2 and 12, no protein added; lanes 3–5, wild-type RNA polymerase; lanes 6–8, RNA polymerase containing [265A]α; lanes 9–11, RNA polymerase containing α-235. The final concentrations of RNA polymerases added were: lanes 3, 6 and 9, 30 nM; lanes 4, 7 and 10, 50 nM; lanes 5, 8 and 11, 70 nM. The filled and open bars indicate the regions protected by the C-terminal region of α subunit and the rest of the RNA polymerase, respectively.

Discussion

The α subunit C-terminal domain interacts with both protein factors and DNA enhancer sequences using the same protein surface

The results of *in vitro* transcription experiments by the mutant RNA polymerases containing mutant α subunits with single amino acid substitutions indicated that most mutations affected the response of RNA polymerase to both CRP and the UP element simultaneously, although some mutations affected the response to only one of these two factors. The first group includes mutations at Leu260, Leu262, Arg265, Asn268 and Lys297, indicated by either red or yellow in Figure 6, which all gave a marked reduction in the response to both CRP and the UP element. The second group includes mutations at Leu270, Ile275 and Lys298, indicated by either green or yellow in Figure 6, which affected only CRP-dependent *lac*P1 transcription, while the third group includes mutations at Cys269, indicated by blue in Figure 6, which affected only UP element-dependent *rrnB*P1 transcription. The distribution of mutations affecting UP element-dependent transcription is generally in good agreement with that obtained by Gaal *et al.* (1996), except for two residues, Lys297 and Lys298. Our K297A mutant has a defect in the response to the UP element, but instead the K298A mutant in their mutant collection is defective. The discrepancy may be due to the difference in assay conditions. We employed a single-round transcription assay, while they carried out multiple-round transcription.

Judging from the three-dimensional structure of the α C-terminal domain (Jeon *et al.*, 1995; see Figure 6A), the side chains of Arg265 (red), Asn268 (red), Cys269 (blue), Lys297 (red) and Lys298 (green) are considered to be exposed on the protein surface.

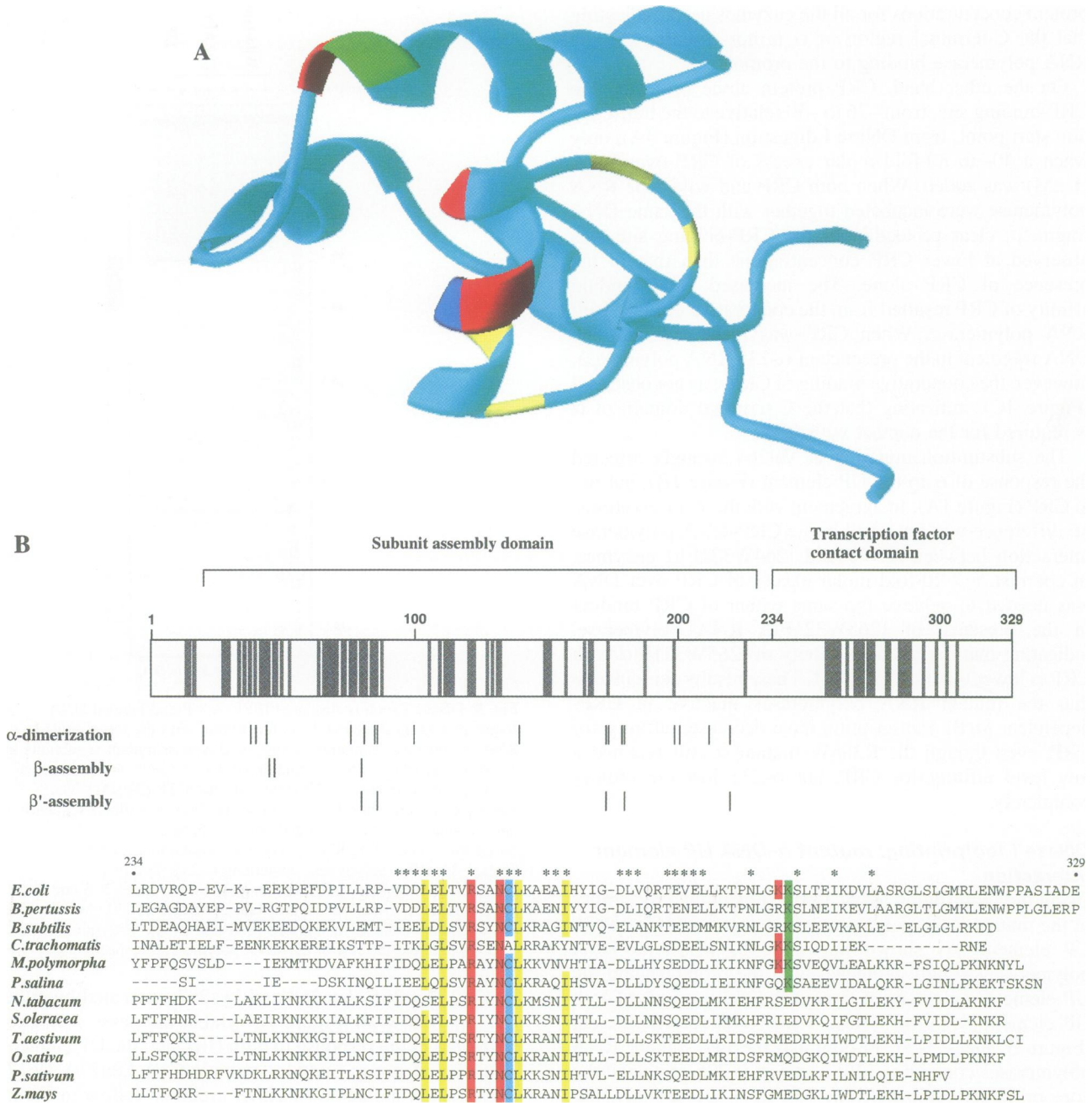

Fig. 6. Mapping of the contact sites for CRP and/or the UP element. (**A**) The amino acid residues involved in contact with CRP and/or the UP element are mapped on the three-dimensional structure of the α subunit C-terminal domain (Jeon *et al.*, 1995), constructed using the program Ribbons (Carson, 1991). The amino acid residues in contact with both CRP and UP, red; the residues in contact with only CRP, green; and the residues in contact with only the UP element, blue. The amino acids important for protein folding are shown in yellow. (**B**) Conservation of the contact site on α with transcription factors. The locations of the conserved amino acid residues and of the functional regions are indicated on the *E.coli* α subunit. The top line shows (to scale) the locations of the conserved sequences in the α subunit and their functional assignments are shown beneath. The mutations of α affecting the subunit–subunit contacts (Kimura *et al.*, 1994; Kimura and Ishihama 1995a,b) are indicated by bars, while the C-terminal conserved sequences include the RNA polymerase–transcription factor contact sites (Ishihama, 1993). The sequence between residues 234 and 329 of the *E.coli* α subunit was compared with the corresponding region of α subunits from both bacteria (*Bordetella pertussis*, *Bacillus subtilis* and *Chlamydia trachomatis*) and chloroplasts (*Marchantia polymorpha*, *Pyrenomoras salina*, *Nicotiana tabacum*, *Spinocia oleracea*, *Triticum aestivum*, *Oryza sativa*, *Pisum sativum* and *Zea mays*). The highly conserved residues are indicated by stars, while the amino acids important for interaction with CRP and/or the UP element are indicated using the same color scheme as in (A).

In good agreement with the mutant studies, DNA footprinting experiments indicated that the defect of mutant RNA polymerases defective in UP element-dependent transcription is attributable to direct effects on UP element binding (Figure 5; see also Gaal *et al.*, 1996). Moreover, evidence for the direct involvement of Arg265 and Asn268 in UP element binding was obtained by NMR studies. Chemical shift or signal broadening perturbations were obtained for the signals originating from these residues only in the presence of the DNA UP element (Jeon *et al.*, 1995). Thus, we propose that Arg265, Asn268 and Cys269 on helix 1, in conjunction with Lys297 on helix 4, form

the target site for physical contact with the UP element. Although amino acid substitutions at Leu260 and Leu262, indicated by yellow in Figure 6, resulted in considerable effects on UP element-dependent transcription, these effects might be indirect due to resultant conformational changes of the α C-terminal domain, because these two hydrophobic amino acids exist inside this domain forming the hydrophobic core. We suggest that these amino acids are important for protein folding in order to expose the DNA contact site for direct interaction with the UP element.

The present study indicated that most Ala- or Trp-substitution mutations on α affecting the response to the UP element also affected the response to CRP, as measured by CRP-dependent *lac*P1 transcription. Furthermore, DNase I footprinting data indicate that the mutant RNA polymerase containing [265W321F]α, which is inactive in the response to the UP element, has decreased affinity for CRP. The most straightforward interpretation would be that the UP element contact surface exposed on helix 1 in the α C-terminal domain is involved in recognition of not only the UP element, but also CRP. Two mutations at Leu270 and Ile275, indicated by yellow in Figure 6, which affected the response to CRP but not to the UP element, may be due to a defect in proper folding of this amphipathic helix because these residues form the hydrophobic surface of this helix and are buried inside the α C-terminal domain.

Previously, Ebright and colleagues proposed that the contact sites on α with CRP and the UP element are located adjacent to, but not overlapping, each other (Tang *et al.*, 1994; Gaal *et al.*, 1996), because the mutants that they found to be defective in the response to CRP isolated after *in vivo* random screening carried changes at residues 258, 259 and 261. Under our *in vivo* screening conditions, however, such mutations were mapped at residues 265, 268 and 269 (Zou *et al.*, 1992). The phenotypes of the mutants we isolated were confirmed in *in vitro* transcription assays using the reconstituted RNA polymerases containing the mutant α subunits. Furthermore, in this study, we carried out a systematic mutagenesis by replacing each amino acid residue of α in a wide region, including both positions 258–261 and 265–269, with either Ala or Trp. The results of *in vitro* transcription by the mutant RNA polymerases containing these mutant α subunits demonstrate that the most defective mutations on the α subunit for CRP-dependent *lac*P1 transcription and UP element-dependent *rrnB*P1 transcription were almost overlapping, including residues 265, 268 and 269. Under the *in vitro* transcription assay conditions employed in this study, the replacement of Asp258 and Asp259 with Ala and that of Glu261 with either Ala or Trp had little or no effect on CRP-dependent transcription. Taking our results altogether, we conclude that the contact sites on the α subunit with CRP and the UP element are located on the same protein surface, and that the same amino acid residues play important roles in both protein–protein and protein–DNA contacts. The isolation of the third group of mutants, such as V264W, S266W and C269A mutants, which are defective in response only to the UP element, indicates *per se* that the mechanism of CRP action is not simply attributable to the support of α binding to the UP element.

A number of DNA-binding transcription factors have been identified in both prokaryotes and eukaryotes, which interact with other protein factors. The contact sites on these factors with DNA and proteins are believed to be located on different protein surfaces. In this aspect, the α subunit is the first unique protein that interacts with both DNA and proteins using the same protein surface. If this is the case, the α subunit cannot interact with DNA and protein factors at the same time. Most of the essential genes constitutively expressed in exponentially growing *E.coli* carry UP elements, while inducible genes require regulatory proteins to be transcribed. Under most physiological growth conditions, the α subunit seems to make contact with only one of the two group factors. It has, however, not yet been excluded that two α subunits in RNA polymerase have different conformations, one facing with β subunit and the other with β′, and interact with two different factors at the same time.

The functional role of Arg265

In our collection of RNA polymerase mutants, the substitution at Arg265 gave the most dramatic reduction in the response to both CRP and the UP element. The response resembles that seen with the C-terminal truncated mutant α. From the random screening *in vivo* of α mutants defective in transcription activation by CRP, mutations at residue 265 gave the most severe effects on both CRP-dependent (Zou *et al.*, 1992) and OxyR-dependent transcription (Tao *et al.*, 1993, 1995). Mutants carrying mutations at Arg265 were also defective in interaction with GalR (H.E.Choy *et al.*, in preparation). Therefore, we conclude that Arg265 is the most important amino acid for molecular communications with both regulatory protein factors and DNA sequences. To confirm this prediction, we made a set of α mutants by replacing Arg265 with various amino acids, and found that Lys is the only amino acid that can substitute for Arg in the response to CRP, indicating that the positive charge at this position is involved in communication with CRP. In the three-dimensional structure of the α subunit C-terminal domain (see Figure 6A), Arg265, Lys297 and Lys298 are located close together, probably forming a basic region. In agreement with this prediction, substitutions at residues 297 and 298 produced α derivatives partially inactive in the CRP response.

On the other hand, all the mutant RNA polymerases containing α with substitutions for Arg265, including [265K]α RNA polymerase, are virtually inactive in their response to UP element. These results suggest that not only the positive charge, but also the side chain structure, play certain roles in the recognition of the DNA UP element.

The α C-terminal domain is a common target for transcription regulators

Amino acid sequence analysis of bacterial and chloroplast α subunits demonstrates that three conserved regions exist within the α homologues (Figure 6B), two highly conserved regions, residues 28–53 and 64–88 (on the *E.coli* sequence), in the N-terminal assembly domain and one conserved region at residues 257–290 in the C-terminal transcription factor contact domain. The N-terminal conserved regions contain the sites for α-dimerization and

Table II. Plasmids

Plasmid	Amino acid substitution		Protein	Source
pGEMAX185	None		α-WT	Igarashi *et al.* (1991)
pGEMA321F		W321F	[321F]α	this work
pGEMA260W321F	L260W	W321F	[260W321F]α	this work
pGEMA261W321F	E261W	W321F	[261W321F]α	this work
pGEMA262W321F	L262W	W321F	[262W321F]α	this work
pGEMA263W321F	T263W	W321F	[263W321F]α	this work
pGEMA264W321F	V264W	W321F	[264W321F]α	this work
pGEMA265W321F	R265W	W321F	[265W321F]α	this work
pGEMA266W321F	S266W	W321F	[266W321F]α	this work
pGEMA267W321F	A267W	W321F	[267W321F]α	this work
pGEMA268W321F	N268W	W321F	[268W321F]α	this work
pGEMA269W321F	C269W	W321F	[269W321F]α	this work
pGEMA270W321F	L270W	W321F	[270W321F]α	this work
pGEMA258A	D258A		[258A]α	this work
pGEMA259A	D259A		[259A]α	this work
pGEMA260A	L260A		[260A]α	this work
pGEMA261A	E261A		[261A]α	this work
pGEMA262A	L262A		[262A]α	this work
pGEMA263A	T263A		[263A]α	this work
pGEMA264A	V264A		[264A]α	this work
pGEMA265A	R265A		[265A]α	this work
pGEMA266A	S266A		[266A]α	this work
pGEMA268A	N268A		[268A]α	this work
pGEMA269A	C269A		[269A]α	this work
pGEMA270A	L270A		[270A]α	this work
pGEMA271A	K271A		[271A]α	this work
pGEMA273A	E273A		[273A]α	this work
pGEMA275A	I275A		[275A]α	this work
pGEMA297A	K297A		[297A]α	this work
pGEMA298A	K298A		[298A]α	this work
pGEMA265K	R265K		[265K]α	this work
pGEMA265E	R265E		[265E]α	this work
pGEMA265L	R265L		[265L]α	this work
pGEMA265M	R265M		[265M]α	this work
pGEMA265Q	R265Q		[265Q]α	this work
pGEMA265S	R265S		[265S]α	this work

assembly of β and β′ subunits (Kimura *et al.*, 1994; Kimura and Ishihama, 1995a,b). On the other hand, the C-terminal conserved domain includes the contact sites for CRP (and probably OxyR, CysB and GalR) (Ishihama, 1993) and the UP element. In particular, most of the amino acids involved in the response to CRP and/or the UP element (residues 260, 262, 265, 268–270, 275 and 297–298) are well conserved within not only bacterial but also chloroplast α subunits, suggesting that the protein surface on RNA polymerase formed by these residues provides a common and fundamental target site for transcription regulators. Along this line, it is important to identify the α-contact site on each protein factor and to compare its conformation between the regulatory proteins sharing the same contact site on the α subunit.

Materials and methods

Construction of plasmids for the expression of mutant α subunits

Plasmids used for the expression of mutant α subunits are summarized in Table II. Site-directed mutagenesis was performed as described by Kunkel *et al.* (1991) by using uracil-containing single-strand DNA of pGEMAX185, an expression plasmid for the wild-type α subunit (Igarashi and Ishihama, 1991). All the plasmid constructions were checked by DNA sequencing.

Expression and purification of RNA polymerase subunits

α-WT, β, β′ and σ^{70} subunits were expressed and purified according to Igarashi and Ishihama (1991). *Escherichia coli* strain BL21(λDE3) was used for the expression of α derivatives, except for [268W321F]α, which was expressed in strain BL21(λDE3) $pLys^S$. Transformed cells were cultured with shaking at 37°C in Luria broth (LB) medium containing 200 μg/ml of ampicillin. When the culture reached 50 Klett units, the expression of α derivatives was induced by adding IPTG at 0.5 mM. After incubation for an additional 3 h, cells were harvested and stored at –80°C until use. Cell lysates were prepared essentially according to Igarashi and Ishihama (1991).

Overexpressed α proteins were purified from insoluble fractions ([258A]α, [259A]α, [261A]α, [263A]α, [264A]α, [266A]α, [269A]α, [271A]α, [273A]α, [275A]α, [321F]α, [261W321F]α, [262W321F]α, [264W321F]α and [267W321F]α), soluble fractions ([260A]α, [262A]α, [265A]α, [268A]α, [270A]α, [297A]α, [298A]α, [260W321F]α, [263W321F]α, [265W321F]α, [266W321F]α, [268W321F]α, [269W321F]α, [265E]α, [265K]α, [265L]α, [265M]α, [265Q]α and [265S]α) or inclusion bodies ([270W321F]α) as described previously (Igarashi and Ishihama, 1991; Kimura *et al.*, 1994).

Reconstitution and purification of core enzyme and holoenzyme

Reconstitution of wild-type and mutant core enzymes, and separation of the assembled enzymes from unassembled subunits, were carried out as described (Igarashi and Ishihama, 1991). Holoenzymes were prepared by mixing the reconstituted core enzymes with a 4-fold molar excess of purified σ^{70}, and incubated for 25 min at 30°C immediately before use for transcription and DNase I footprinting assays.

Transcription in vitro

RNA polymerase core enzyme activity was assayed under the standard conditions as described previously (Igarashi and Ishihama, 1991). The standard conditions for promoter-dependent transcription were as described (Kajitani and Ishihama, 1983a,b). Transcription from the *lac* promoters was carried out using truncated DNA templates (Igarashi and Ishihama, 1991) (CRP was a kind gift from Hiroji Aiba, Nagoya

University). Transcription from the *rrnB*P1 promoter was carried out using as template a closed circular plasmid carrying the promoter (the *rrnB* promoter plasmids were gifts from Richard Gourse, University of Wisconsin). Transcripts were analyzed by gel electrophoresis. Gels were visualized by autoradiography on X-ray films or with a Bioimage Analyzer BAS2000 (Fujix). Radioactivity associated with each gel band of the transcripts was quantified using a Bioimage Analyzer BAS2000 (Fujix).

The templates used were: a 205 bp *Pvu*II–*Xba*I fragment of pUC19 carrying the wild-type *lac* promoter; a 205 bp *Eco*RI fragment of plasmid pKB252 carrying the *lac*UV5 promoter (Kajitani and Ishihama, 1983a); the plasmid pRLG862 carrying the *rrnB*P1 promoter region from –88 to +1; and the plasmid pLR14 carrying the *rrnB*P1 promoter region from –88 to +1 with the non-*rrnB*P1 sequence substituted for the UP element sequence from –59 to –41. pRLG862 and pLR14 carried the *rrnB*T1T2 terminators (Ross *et al.*, 1993). Closed circular plasmid DNAs were purified by standard CsCl methods.

DNase I footprinting

DNA footprinting by DNase I was carried out under the same reaction conditions as employed for *in vitro* transcription experiments. Mixtures of ^{32}P-end-labeled DNA fragment (1 nM) and proteins were incubated for 25 min at 37°C in 25 μl transcription buffer containing 5 mM $CaCl_2$, and then incubated at 25°C for 5 min. DNase I was added and the incubation was continued for 30 s at 25°C. Digestion was terminated by adding 100 μl of a stop solution (1% SDS, 0.2 M NaCl, 20 mM EDTA and 40 μg/ml of yeast tRNA). DNA was extracted with phenol, precipitated with ethanol, and analyzed by electrophoresis on an 8% polyacrylamide gel containing 8 M urea. Gels were visualized by autoradiograpy or with a Bioimage Analyzer BAS2000 (Fujix).

The templates used for DNase I footprinting were: the *Bam*HI(–127)–*Hin*dIII(+58) fragment of pLAC12 carrying the *lac*UV5 promoter (Tagami and Aiba, 1995), which was ^{32}P labeled on the bottom strand at its 5′ *Hin*dIII end; and the *Xho*I(–160)–*Hin*dIII(+50) fragment of pSL9 carrying the *rrnB*P1 promoter, which was ^{32}P labeled on the top strand at its 3′ *Hin*dIII end.

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