Transcription Activation Mediated by the Carboxyl-terminal Domain of the RNA Polymerase α-Subunit

OLGA N. OZOLINA,†‡ NOBUYUKI FUJITA,† and AKIRA ISHIHAMA‡¶

From the †Department of Molecular Genetics, National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan and the ¶Institute of Cell Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region 142292, Russian Federation.

Conformational changes within the carboxyl-terminal domain of the Escherichia coli RNA polymerase α-subunit (α-CTD) upon interaction with the DNA UP element or the transcription factor cAMP receptor protein (CRP) were studied by monitoring the spectral parameters of a fluorescent dye, fluorescein mercuric acetate, conjugated to various positions of α-CTD. When fluorescein mercuric acetate was conjugated to Cys located on helix I and the loop between helices III and IV, the spectral changes typical for DNA interaction were observed for the RNA polymerase-promoter binary complex with UP element-dependent rrnBP1 and the ternary complex with the CRP-dependent uxuAB promoter in the presence of cAMP/CRP. Likewise, the chemical nuclease iron-(p-bromoacetamidobenzyl)-EDTA conjugated to Cys-269 or Cys-272 introduced CRP-dependent cleavage of the uxuAB promoter, as in the case of rrnBP1 (Murakami, K., Owens, J. T., Belyaeva, T. A., Meares, C. F., Busby, S. J. W., and Ishihama, A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11274–11278), indicating that CRP rearranges the topology of the DNA contact surface in α-CTD. Conformational changes in α-CTD were also observed upon formation of a binary complex with the uxuAB (in the absence of CRP) and factor-independent T7D promoters. The spectral changes suggested that helix IV of α-CTD approaches the negatively charged phosphate moiety of DNA. In agreement with this prediction, iron-(p-bromoacetamidobenzyl)-EDTA conjugated to Cys-309 induced extensive DNA cleavage upstream from the uxuAB promoter −35 element. We propose that helix IV of α-CTD is involved in direct interaction with some promoters.

In transcription initiation by the prokaryotic RNA polymerase, exchangeable α-subunits are responsible for recognition of the core promoter DNA (1–3), whereas the carboxyl-terminal domain of the α-subunit (α-CTD) makes additional contacts with more distal regions (4). By both biochemical (4, 5) and physical (6, 7) data, the upstream sequence of the rrnBP1 promoter, generally designated as the UP element, was proven to directly contact α-CTD. α-CTD is also responsible for interaction with a set of transcription factors, designated as class I (or α-contact) factors, regulating transcription efficiency (3, 8). The most studied factor is cAMP receptor protein (CRP) (9–11), which regulates transcription of >80 genes (12, 13). The molecular mechanism of transcription regulation by CRP depends on the position of its binding site on the promoter sequence. Promoters that have the CRP-binding site centered between positions −60 and −100 usually require α-CTD for activation (3, 8). Random and site-directed mutagenesis within α-CTD revealed that the amino acid residues responsible for interaction with the UP element in rrnBP1 include Leu-260, Leu-262, Arg-265, Asn-268, Cys-269, and Lys-297, whereas Leu-260, Leu-262, Arg-265, Asn-268, Leu-270, Ile-275, Lys-297, and Lys-298 are involved in transcription activation at the lacP1 promoter by CRP (14–16). The amino acid residues mostly important for UP element-dependent transcription (Leu-262, Arg-265, Asn-268, and Lys-297) are also crucial for CRP-dependent activation. Two possibilities are considered to explain this overlapping: (i) the same surface of α-CTD participates in the specific interaction with both the DNA UP element and CRP, or (ii) CRP ensures the contact between the specific surface of α-CTD and promoter DNA. These two possibilities are not necessarily contradictory if one α-subunit is involved in interaction with CRP while another forms a specific contact with DNA, or if α-CTD participates in an initial and transient interaction with CRP prior to the final and stable interaction with DNA.

To get insight into the detailed mechanism of α-CTD interactions with the DNA UP element and CRP, we tried in this study to monitor CRP-induced conformational alterations in α-CTD upon transition from RNA polymerase-promoter binary complexes to ternary complexes. For this purpose, a set of single Cys mutant α-subunits at residues 263, 271, 272, 283, 285, 292, and 309 was constructed in addition to the Cys-269 mutant that was used in our previous studies (17, 18), and a fluorescent probe, fluorescein mercuric acetate (FMA), was conjugated to each of the single Cys mutant α-subunits. Using the reconstituted RNA polymerase holoenzymes containing the FMA-tethered mutant α-subunits, the fluorescent spectral changes were monitored after complex formation with the CRP-dependent promoter uxuAB, the factor-independent promoter T7D, and the UP element-dependent promoter rrnBP1. The topology of DNA contact surfaces was further studied by the recently developed approach employing a cutting reagent, FeBABE (19–21), tethered at the critical Cys-269, Cys-272, and Cys-309.

The results herein described show that within the RNA polymerase-promoter binary complexes with uxuAB and T7D promoters, the α-subunit of RNA polymerase makes contacts
Fluorescent Monitoring of RNA Polymerase-DNA Interaction

with the upstream DNA, and this interaction involves Cys-309 on α-CTD helix IV rather than helix I, which is crucial for UP element-dependent transcription (15, 16). Upon ternary complex formation in the presence of CAP/CRP, both Cys-269 and Cys-272 approach the promoter DNA as in the case of rnnBP1 UP element interaction (15, 16), indicating a conformational rearrangement in the topology of the DNA contact surface in α-CTD.

**EXPERIMENTAL PROCEDURES**

**Construction and Purification of Mutant α-Subunits**—Eight mutant derivatives of the rpoA gene were used in this study. One mutant, bearing the single Cys residue at its native position, 269, was constructed previously (17, 18), whereas seven were prepared in this study by the overlapping polymerase chain reaction mutagenesis method (22). Two synthetic oligonucleotides (CGGAGAAGATGGAGGCC/CCAGGG and CCCTCGTGTGTTGACAGCTGTCAGCC) were used as primers, and seven species of oligonucleotides (CTTGGCAAGAACCGGAGTGGACAGAC/CCAGG and CGGACTCGTTCTGAGGAGCTCAACCT (T292C), and GAGACGA (Q283C), GGAGCTCAACCTC(GCA)ACGCTGTACCAGA (T285C), ACGCGGGCCCAGTTCG, GCGTTAGCAGAGCGGAC(GCA)CAATTC-...}

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emission intensities registered at λm, using different orientations of polarizers.

FeBABE-mediated Cleavage of DNA—The DNA cleavage reaction induced by protein-bound FeBABE was carried out as described in our previous studies (19, 20). In brief, the reconstituted core enzymes containing FeBABE-modified α-subunits were dialyzed against DNA cleavage buffer (40 mM HEPES (pH 8.0), 10 mM MgCl2, 0.1 mM KCl, 0.1 mM EDTA, and 5% glycerol) without DTT and stored at ~80 °C until use. For DNA cleavage, mixtures of 32P-end-labeled DNA fragments (6 nt) and FeBABE-conjugated RNA polymerase (20 nM) were incubated at 37 °C for 10 min in 90 μl of DNA cleavage buffer in the absence and presence of CRP (100 nM) and cAMP (10 μM). To remove nonspecifically bound RNA polymerase, salmon sperm DNA (40 μg/ml final concentration) was added 0.5 min before the start of the DNA cleavage reaction. The cleavage reaction was initiated by the addition of sodium ascorbate (pH 7.0; 2 mM final concentration) and hydrogen peroxide (1 mM final concentration) and then allowed to proceed at 37 °C for 20 min before quenching with 0.1 M thiourea and 100 mM final ascorbic acid. After dilution with Tris/EDTA buffer up to 200 μl, DNA was extracted with phenol/chloroform, precipitated with ethanol, and analyzed by electrophoresis on 8% polyacrylamide gel containing 8M urea. Bands were visualized with the BAS2000 BioImage analyzer. The temperature mutant RNA polymerases, each containing a different single Cys mutant α-subunit at residue 269, reconstituted wild-type RNA polymerase thus calculated was set as 100%.

The enzymes analyzed were the same as described for A, C, the activities of the mutant RNA polymerases were measured as described for A using a mixture of 4 nM T7D and 0.4 nM lacUV5 promoters, and the relative activities were calculated as described for B. The enzymes analyzed were the same as described for A, D, the activities of the mutant RNA polymerase containing a mixture of 12 nM uxuAB and 2 nM lacUV5 in the presence of 100 nM CRP and 10 μM cAMP, and the relative activities were calculated as described for B. The enzymes analyzed were the same as described for A, Error bars indicate the deviations in results from a minimum of three repetitions.

RESULTS
Construction of Single Cys Mutant α-Subunits
Previously, we constructed the single Cys mutant α-subunit derivative bearing Cys only at its natural position, 269 (17). In addition, we constructed in this study a set of mutant α-subunit derivatives carrying a single Cys residue after replacement by Cys of Thr-263, Lys-271, Ala-272, Gln-283, Thr-285, Thr-292, or Ser-309, starting with the Cys-null α-subunit mutant possessing all four Cys residues substituted with Ala (C54A, C131A, C176A, and C269A) (17). The site of Cys substitution was chosen so as to preserve the activities of the α-subunit by avoiding mutagenesis of amino acid residues generally crucial for biological activities and by replacing amino acid residues that are similar in chemical nature to Cys (with the exception of Lys-271). We tried to replace Lys-271 because it is located on the surface of helix I, which is essential for transcription activation by the UP element and CRP.

All these mutant α-subunits were overexpressed, purified, and reconstituted into the mutant holoenzymes. The efficiency of core enzyme assembly by the mutant α-subunits was essentially the same as that by the wild-type α-subunit, in agreement with the finding that the contact surfaces on the α-subunit for RNA polymerase assembly are all located within the N-terminal domain (8, 9).

Transcription Activity of the Mutant Enzymes
The functional activity of wild-type and mutant RNA polymerases was analyzed using a single-round transcription assay directed by the UP element-dependent promoter rrnB1 (Fig. 1, A and B), the CRP-dependent promoter uxuAB (Fig. 1D), and the control promoter T7D (Fig. 1C). The lacL8UV5 promoter was always added to the transcription mixture as an internal reference. Fig. 1A shows one example of the gel electrophoresis pattern of rrnB1 transcription by eight different samples of the mutant RNA polymerases, each containing a different single Cys mutant α-subunit (lanes 4–11), together with transcripts by the enzymes containing the wild-type α-subunit (lane 1), the Cys-null mutant α-subunit (lane 2), and the C-terminal deletion mutant α235 (lane 3). The level of lacL8UV5 transcription stayed almost constant for all 11 RNA polymerases because transcription of lacL8UV5 without the UP element was virtually unaffected by the presence or absence of α-CTD (9).

After correction for the experimental fluctuations based on the intensity of the lacL8UV5 transcripts in the same reactions. The activity of the reconstituted wild-type RNA polymerase thus calculated was set as 100%.

The enzymes analyzed were the same as described for A. The activities of the mutant RNA polymerases were measured as described for A using a mixture of 4 nM T7D and 0.4 nM lacUV5 promoters, and the relative activities were calculated as described for B. The enzymes analyzed were the same as described for B, A.

The activities of the mutant RNA polymerase containing α235, devoid of the C-terminal 84 residues, in transcription directed by UP element-dependent rrnB1 (Fig. 1, A, lane 3; and B, bar 3) and by cAMP/CRP-dependent uxuAB (Fig. 1D, bar 3) were <20 and ~25%, respectively, of the level of wild-type RNA polymerase, in confirmation of the concept that α-CTD is required for both UP element- and class I factor-dependent transcription (4, 9). The T7D-dependent transcription by the α235 mutant RNA polymerase also decreased to ~50% of the level of the wild-type enzyme (Fig. 1C, bar 3), implying that the T7D promoter may have a weak UP element that is recognized by α-CTD (see below).

All the single Cys mutant RNA polymerases, except for the enzyme containing a single Cys α-subunit at residue 269, retained the rrnB1 transcription activity at ~40–60% (Fig. 1B, bars 2, 4, and 6–11) and the CRP-dependent uxuAB transcription activity at ~50–70% (Fig. 1D, bars 2, 4, and 6–11) of the level of the wild-type enzyme. The enzyme with a single Cys mutant α-subunit at position 269 (note that Cys is the original
amino acid residue in the wild-type α-subunit, but this enzyme was reconstituted in parallel with other mutant enzymes) retained, as expected, almost all the full activities of UP element-dependent rrrnP1 transcription (Fig. 1B, bar 5) and CRP-dependent uuxuAB transcription (Fig. 1D, bar 5). After replacement of Cys-269 by Ala, however, the UP element- and CRP-dependent transcription activities were markedly reduced because Cys-269 is located on helix I, which is critical for transcription activation (15). All the mutant enzymes containing single Cys-α-subunits, including the Cys-269 mutant α-subunit enzyme, showed T7D transcription activities as high as the wild-type enzyme (Fig. 1C, bars 2 and 4–11).

### Efficiency of FMA Conjugation to Mutant α-Subunits

All these mutant α-subunits were modified with the fluorescent probe FMA. The level of conjugation by FMA was estimated as an average value by two different measurements: (i) the absorbance change in a-associated FMA at \( \lambda_{\text{max}} \) (505–510 nm) and (ii) the emission intensity change in FMA after removal from the RNA polymerase by treatment with DTT (Table I). The modification level of the α-subunit varied significantly depending on the positions of FMA conjugation, from −30% (Cys-285 and Cys-309) to 96% (Cys-269) (note that if the efficiency of single α-subunit modification is 30%, for instance, the populations of RNA polymerase containing one FMA-modified (and one unmodified) and two modified α-subunits are 42 and 9%, respectively, assuming that the modified α-subunit has equal affinity to the β- and β′-subunits compared with the unmodified α-subunit). Cys residues located at positions 269, 271, and 283 were modified at high levels (60–100%), indicating that these residues are exposed on the surface of α-CTD. On the other hand, modification at positions 272, 285, and 309 was at lower levels (30–40%). All weakly modified Cys residues appear to locate in highly polar surroundings sandwiched between positively and negatively charged residues (Lys-271 and Asp-273 for Cys-272, Arg-284 and Glu-286 for Cys-285, and Asp-305 and Arg-310 for Cys-309).

### Transcription Activity of FMA-modified Mutant RNA Polymerases

For quantitative measurement of the effect of chemical modification on the enzyme activity, we carried out the in vitro transcription assay with all the reconstituted RNA polymerases containing the FMA-modified mutant α-subunits before and after removal of the fluorescent probe by treatment with 1 mM DTT. For all the templates (uuxuAB, T7D, and rrrnP1) used, the sizes of transcripts were the same for FMA-modified and unmodified RNA polymerases, but the levels of transcription increased to various extents for some mutant RNA polymerases after removal of FMA depending on the templates used. Fig. 2A shows an example of the transcription pattern for the enzyme carrying the Cys-292 mutant α-subunit with or without FMA. Fig. 2 (B–D) shows the activity level of each FMA-modified RNA polymerase (before DTT treatment) relative to that of the respective unmodified enzyme (after DTT treatment). The yields of RNA transcripts derived from the uuxuAB and T7D promoters were normalized by the yield of product derived from lacL8UV5, whereas the level of rrrnP1 was normalized by the product of RNA-I. Since the conjugation yields were not 100% in many cases, the level of FMA-modified RNA polymerase was treated, prior to the transcription assay, with 1 mM DTT for 15 min at 30 °C to remove FMA. The concentrations of RNA polymerase and templates used were as follows: lanes 1 and 4, 12 nM uuxuAB, 2 nM lacL8UV5, and 20 nM holoenzyme (in the presence of 100 nM CRP and 10 μM cAMP); lanes 2 and 5, 2 nM T7D, 8 nM lacUV5, and 10 nM RNA polymerase; lanes 3 and 6, 6 nM pSLUP and 12 nM RNA polymerase. RNA products were analyzed by polyacrylamide gel electrophoresis. B–D, the relative activities of RNA polymerases, each containing a mutant α-subunit with a single Cys in different positions, were measured using the templates uuxuAB, T7D, and rrrnP1, respectively. For quantitation, polyacrylamide gels were exposed to imaging plates, and the plates were analyzed with a BAS2000 BioImage analyzer. The yields of uuxuAB and T7D transcripts were corrected based on the yield of the lacL8UV5 transcript measured as an internal control, whereas the yield of the rrrnP1 transcript was normalized using the RNA-I transcript as an internal control. The relative level of transcripts produced by the modified enzyme was estimated as described under “Experimental Procedures.” The data represent the averages of two or three assays using the different enzyme preparations obtained from independent reconstitution experiments under standard conditions.

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**TABLE I**

Level of FMA conjugation to single Cys residues within α-CTD

The level of FMA conjugation to single Cys mutant α-subunits was calculated by measuring the absorbance change at 505 nm or the intensity change after DTT treatment.

| Cys substitution | Level of FMA conjugation (FMA/α-subunit) | Absorbance change at 505 nm | Intensity change after DTT treatment | Average |
|-----------------|------------------------------------------|-----------------------------|--------------------------------------|---------|
| T263C           | 44 ± 3                                    | 40 ± 2                      | 42 ± 2                               |         |
| C269C           | 95 ± 3                                    | 97 ± 3                      | 96 ± 2                               |         |
| A272C           | 68 ± 3                                    | 65 ± 13                     | 67 ± 8                               |         |
| A271C           | 39 ± 7                                    | 36 ± 8                      | 38 ± 3                               |         |
| Q283C           | 60 ± 6                                    | 77 ± 3                      | 68 ± 8                               |         |
| T285C           | 35 ± 5                                    | 28 ± 3                      | 32 ± 4                               |         |
| T292C           | 43 ± 10                                   | 41 ± 4                      | 42 ± 4                               |         |
| S309C           | 22 ± 6                                    | 35 ± 4                      | 34 ± 4                               |         |

**FIG. 2.** Transcription activities of mutant RNA polymerases containing FMA-conjugated α-subunits. A, *in vitro* transcription was carried out using the RNA polymerase (RNAP) containing the single Cys-292 mutant α-subunit previously conjugated with FMA (lanes 1–3). In lanes 4 and 5, the FMA-modified RNA polymerase was treated, prior to the transcription assay, with 1 mM DTT for 15 min at 30 °C to remove FMA. The concentrations of RNA polymerase and templates used were as follows: lanes 1 and 4, 12 nM uuxuAB, 2 nM lacL8UV5, and 20 nM holoenzyme (in the presence of 100 nM CRP and 10 μM cAMP); lanes 2 and 5, 2 nM T7D, 8 nM lacUV5, and 10 nM RNA polymerase; lanes 3 and 6, 6 nM pSLUP and 12 nM RNA polymerase. RNA products were analyzed by polyacrylamide gel electrophoresis. B–D, the relative activities of RNA polymerases, each containing a mutant α-subunit with a single Cys in different positions, were measured using the templates uuxuAB, T7D, and rrrnP1, respectively. For quantitation, polyacrylamide gels were exposed to imaging plates, and the plates were analyzed with a BAS2000 BioImage analyzer. The yields of uuxuAB and T7D transcripts were corrected based on the yield of the lacL8UV5 transcript measured as an internal control, whereas the yield of the rrrnP1 transcript was normalized using the RNA-I transcript as an internal control. The relative level of transcripts produced by the modified enzyme was estimated as described under “Experimental Procedures.” The data represent the averages of two or three assays using the different enzyme preparations obtained from independent reconstitution experiments under standard conditions.
60–70% inhibition of transcription by the mutant RNA polymerases carrying FMA at position 269 or 292, whereas the inhibition was <30%, if any, for the enzymes with FMA conjugation at position 263, 271, 272, 285, or 309 (Fig. 2D). The direct involvement of Cys-269 in UP element recognition has been established by genetic and physical studies (7, 11). CRP-dependent RNA synthesis directed by the uxuAB promoter was affected by the presence of the label at Cys-269, Cys-271, Cys-285, and Cys-292 (Fig. 2B). FMA tethered at these positions interfered with either CRP-α-CTD interaction or CRP-induced α-CTD-DNA interaction. We conclude that all the FMA-modified enzymes retain the basic activity of RNA synthesis, but the modification of some positions affects transcription by some factor-dependent promoters.

Spectral Parameters Monitored for α-Subunit-conjugated FMA

Previously, we reported that the spectral parameters of FMA conjugated to Cys-269 underwent specific changes when the modified enzyme interacted with the UP element-dependent rrnB P1 promoter (18). The typical spectral changes investigated in this study include the following parameters: (i) the shift in the emission spectrum (Δλ), (ii) the change in emission intensity (ΔI), and (iii) the change in polarization anisotropy (ΔA).

To identify the interacting surfaces on α-CTD with the DNA UP element and the transcription factor CRP, we performed a systematic spectral characterization for the set of eight mutant RNA polymerases bearing FMA at various positions on α-CTD upon interaction with the factor-independent T7D UP element-dependent rrnBP1, and CRP-dependent uxuAB promoters. Typical spectra for some mutant RNA polymerases are shown in Fig. 3, which represents all type of changes registered in the spectral parameters of FMA conjugated to the single Cys residues. The summary of the spectral measurements for all eight mutant RNA polymerases is shown in Fig. 4.

The emission spectra of fluorescent dyes are usually sensitive to the polarity of their local surroundings. Interaction of the dipole moment of a fluorophore with that typical for the local surroundings as well as chemical interaction between a fluorophore and water molecules may result in the spectral shift. In many cases, we observed a reproducible blue shift in the spectra (for example, see Fig. 3C), but a red shift in the spectra was never observed. The spectral blue shift may represent the interaction of protein-conjugated FMA with the negatively charged phosphate moiety of DNA (see below for detailed discussion). When the change in λmax was >2.5 nm, the molecular interaction giving rise to the spectral change was assumed to be specific (Fig. 4). The polarization anisotropy (A) was calculated from the emission spectra recorded in the presence of polarizers set in different orientations (see the equation under “Experimental Procedures”). This parameter characterizes an average angular displacement of a fluorophore, which occurs between absorption and subsequent emission of the photon, and depends on the rate of rotational diffusion and the lifetime of the excited state. One example of the depolarization of emission is shown in Fig. 3D. Reproducible change in A if >20% were taken as significant in Fig. 4.

Spectral Characterization of α-CTD-tethered FMA

Interactions with uxuAB and T7D Promoters—Fig. 4B presents a spatial topology of the spectral changes that took place in FMA tethered to the mutant enzymes upon binary complex formation with the promoter uxuAB (in the absence of CRP). A reproducible blue shift in the emission spectra (shown in blue) was observed when FMA was placed in positions 283, 292 (see also Fig. 3D), and 309, while a significant anisotropy (A) change (or depolarization) (shown in green) was observed in three cases, FMA at positions 283, 285, and 292. In particular, the change at position 285 was the strongest. A slight anisotropy decrease was observed for FMA at position 309. A low level of quenching of fluorescence (shown in orange) was observed only at position 269 (see also Fig. 3B). The spectral changes in FMA observed with the T7D promoter (Fig. 4C) were essentially the same as those with uxuAB (Fig. 4B), except that the emission intensity decrease (shown in orange) at positions 271 and 272 on helix I was significant for the T7D promoter complex. Thus, we conclude that α-CTD undergoes topologically similar changes when the RNA polymerase forms binary complexes with uxuAB and T7D.

Interactions with CRP and the CRP-uxuAB Promoter Complex—When the FMA-conjugated RNA polymerases were mixed with cAMP/CRP in the absence of DNA, we found a

![FIG. 3. Typical changes in the emission spectra of α-subunit-conjugated FMA. A, titration of the RNA polymerase (RNAP; 25 nm) conjugated with FMA (FMMA) at Cys-283 (spectrum 1) with increasing concentrations of cAMP/CRP up to final concentrations of 300 nM CRP and 10 μM cAMP (spectra 2–5). ΔI represents the average increase (and deviation) in the emission intensity (I) of three independent measurements carried out using three different preparations of the reconstituted RNA polymerase. B, titration of the RNA polymerase (25 nm) conjugated with FMA at Cys-269 (spectrum 1) with increasing concentrations of promoter uxuAB up to a final concentration of 40 nM (spectra 2–5). ΔI represents the average decrease (and deviation) in the emission intensity (I) of two independent measurements carried out with two different preparations of the reconstituted RNA polymerase. C, emission spectra of the RNA polymerase (25 nm) conjugated with FMA at Cys-269 (spectrum 1) and a ternary complex formed in the presence of 30 nM uxuAB, 200 nM CRP, and 10 μM cAMP (spectrum 5). The emission spectra of samples 2–4 containing intermediate concentrations of DNA and cAMP/CRP were omitted for clarity. Δλmax represents the spectral shift (and reliability interval) observed in three independent measurements using three different RNA polymerase preparations. D, emission spectra of the RNA polymerase (50 nm) conjugated with FMA at Cys-292 (spectrum 1) and a binary complex formed in the presence of 60 nm uxuAB (spectrum 5), and 50 μM cAMP (spectrum 5). The emission spectra of samples 2–4 containing intermediate DNA concentrations were omitted for clarity. Δλmax represents the average decrease in the polarization anisotropy (and deviation) that was calculated on the basis of three independent measurements using three different RNA polymerase preparations. All spectra were corrected for the background fluorescence derived from the DNA-RNA polymerase complexes in the absence of FMA.](http://www.jbc.org/content/1123/2/1123/F2.large.jpg)
5–14% increase in the emission intensity for FMA conjugated to Cys at positions 263, 283, 285, and 309 (Fig. 4D, shown in red). In the case of positions 263 and 283, the intensity increase was accompanied by depolarization (24–27%) (shown in green), but for FMA conjugated to positions 285 and 309, the depolarization was not as significant (9–14%). A reliable spectral blue shift (~3 nm) was registered at positions 271 and 309 (shown in blue). Since, in control experiments, neither CRP nor cAMP alone was able to induce similar changes in the spectral parameters, we conclude that the changes observed are a reflection of the specific conformational changes induced by direct protein-protein interaction between the RNA polymerase and cAMP/CRP, supporting the previous predictions (29–31).

When the FMA-conjugated RNA polymerases bound to the preformed cAMP-CRP-DNA complex, we observed spectral changes (Fig. 4E) that were significantly different from those obtained with the DNA-RNA polymerase (Fig. 4B) and CRP-RNA polymerase (Fig. 4D) binary complexes. The final conformation of the transcription machinery that was formed on the promoter in the presence of cAMP/CRP was not simply a superposition of the topological changes realized in two forms of the binary protein-protein and protein-DNA complexes. The most significant changes were observed at positions 269 and 272, which were not detected for any binary RNA polymerase complex with uxuAB or cAMP/CRP. For both positions, the depolarization was ~23% (shown in green), and the blue spectral shift (shown in blue) ranged from 3 to 6 nm in different experiments. The changes at positions 269 and 272 were similar to those observed when the same FMA-conjugated RNA polymerases bound to the rrnBP1 promoter (for details, see below), suggesting that these residues within helix I of α-CTD interact with DNA only in the cAMP/CRP-activated state. The local environments in the ternary complex of FMA conjugated to positions 292 and 283 were similar to those in the binary complex with uxuAB (Fig. 4B), whereas both the depolarization and emission intensity increase at positions 285 and 309 in theuxuAB complex (Fig. 4B) almost disappeared after formation of the ternary complex (Fig. 4E). The cAMP-CRP-induced increase in emission intensity (shown in purple) with FMA attached at positions 283 and 285 (Fig. 4D) also disappeared in the ternary complex (Fig. 4E). From these results and those regarding the spectral changes in the rrnBP1 complex (see below), we conclude that the presence of CRP induces specific conformational transitions in the binary complex between RNA polymerase and promoter uxuAB that take place in the region of helix I.

Interaction with the UP Element-dependent rrnBP1 Promoter—Spectral measurements were also performed after formation of the complex of the FMA-conjugated RNA polymerases with the rrnBP1 promoter. As shown in Fig. 4F, the changes observed in the region of helix I appeared to be very similar to those observed with the uxuAB-CRP-RNA polymerase ternary complex (Fig. 4E). In all cases, however, the spectral alterations with the rrnBP1-RNA polymerase binary complex were more significant (25–29% depolarization, 3–7-nm spectral shift, and 7–9% quenching) than those with the uxuAB-CRP-RNA polymerase ternary complex. On the other hand, some difference was observed between the spectral properties of the rrnBP1 binary and uxuAB ternary complexes. For instance, the changes observed at positions 263 and 283 in the ternary complex were not observed in the rrnBP1 promoter complex, except for the emission increase at position 283. The change at position 263 was specific for the CRP-RNA polymerase binary complex (Fig. 4D), and that at position 283 was observed for the uxuAB-RNA polymerase binary complex (Fig. 4B).

Several lines of observations indicate the participation of α-CTD helix I and the loop between helices III and IV in the interaction with rrnBP1 DNA, including the following: (i) mutant studies indicated the participation of helix I and the loop region between helices III and IV in UP element-dependent transcription (15, 16); (ii) FeBABE, a chemical nuclease, conjugated to Cys-269 introduces cleavage in the UP element of rrnBP1 (11); and (iii) the NMR spectra indicate the direct contact of α-CTD with the DNA UP element in this region (7). We then interpret that the blue spectral shift observed for FMA conjugated to Cys-269, Cys-272, and Cys-292 in both the uxuAB ternary and rrnBP1 binary complexes reflects close contacts of α-CTD, including these residues, with negatively charged phosphate moieties of DNA. In addition, the blue spectral shift in FMA tethered at Cys-309 was reproducibly observed for the binary complexes with the uxuAB and T7D promoters. This may indicate that another surface including
the C-terminal proximal region of helix IV approaches DNA in binary complexes with a group of promoters.

**Conjugation of FeBABE to Single Cys Mutant α-Subunits**

To verify this possibility, we used the chemical nuclease FeBABE with the contact-dependent cleavage activities of nucleic acids and proteins. When promoter complexes of the RNA polymerase with FeBABE conjugated to specific positions are treated with sodium ascorbate and hydrogen peroxide, DNA is cleaved at specific positions near the protein-bound FeBABE due to the generation of hydroxy radicals from chelate-bound irons (32). Three single Cys mutant α-subunits at positions 269, 272, and 309 were modified with FeBABE. The levels of modification, estimated by measuring unmodified Cys using the CPM test (32), were found to be 58, 48, and 53% for Cys-269, Cys-272, and Cys-309, respectively. The FeBABE-conjugated mutant α-subunits were reconstituted into the respective mutant holoenzymes by adding purified intact β, β′, and σ70-subunits. The possible effect of the chemical modification by FeBABE on the enzyme activity was analyzed using single-round transcription assays directed by three promoters: CRP-dependent uxuAB, T7D, and rrrBP1. Before and after removal of the protein Cys-conjugated FeBABE by DTT, we observed little change (<20%) in the activity of all modified enzymes (data not shown), indicating that the critical functional sites for interaction with CRP and UP elements are preserved after FeBABE conjugation at least at these three sites.

**Identification of DNA Cleavage Sites by α-Subunit-conjugated FeBABE**—The contact sites of α-CTD on the uxuAB and T7D promoters were determined after analysis of the cleavage sites by FeBABE conjugated to positions 269 and 272 on helix I and position 309 on helix IV of α-CTD. In the absence of RNA polymerase addition, the uxuAB promoter was cleaved at restricted positions by low levels of hydroxy radicals generated after the addition of ascorbate and H$_2$O$_2$ (Fig. 5, *lanes*). These positions include −53, −24, −20, and −3 on the top strand and −58, −50, −25, −23, and −19 on the bottom strand (Fig. 5, *A* and *B*), whereas such nonspecific cleavage was hardly detected for T7D promoter (Fig. 5, *C* and *D*). The sites hypersensitive to cleavage by low levels of hydroxy radicals are located at or next to kissable dinucleotides such as TA, TG, and CA (for the sequence, see Fig. 6), implying a local distortion of the DNA helix in these regions. After RNA polymerase binding, slight changes were observed in the pattern of intrinsic hypersensitive sites on DNA, exposing new hypersensitive sites (for instance, −27, −48, and −49 on the uxuAB top strand; −32 on the uxuAB bottom strand; −19 and −37 on the T7D top strand; and −25 and −36 of the T7D bottom strand). Moreover, the hypersensitive site at position −53 of uxuAB became protected after the binding of both RNA polymerase and CRP (Fig. 5A). The cleavage at these intrinsic hypersensitive sites takes place independent of the site of FeBABE conjugation.

The FeBABE conjugation site-dependent specific DNA cleavage was observed for both of the test promoters. In the case of the uxuAB promoter, the weak cleavage by FeBABE conjugated to Cys-272 was observed in the absence of CRP around the −38/−39 region of the top strand (Fig. 5A; also see Fig. 6) and around the −28/−29 and −39/−40 regions of the bottom strand (Fig. 5B; also see Fig. 6). After the addition of CRP, the cleavage at these sites was significantly enhanced for FMA conjugated to both Cys-269 and Cys-272, suggesting that CRP induces the conformational change in α-CTD, probably of the downstream α-subunit (11), so as to make helix I in closer contact with the uxuAB promoter at two regions. In the case of the T7D promoter, FeBABE conjugated to Cys-272 introduced DNA cleavage around positions −44 and −32/−33 on the bottom strand (Fig. 5D; also see Fig. 6).

A high level of FeBABE-mediated DNA cleavage was observed when FeBABE was conjugated to Cys-309. In the case of the uxuAB promoter, the FeBABE-conjugated RNA polymerase alone introduced cleavage at positions −35/−36 on the top strand (Fig. 5A; also see Fig. 6) and positions −36/−37 and −46/−50 on the bottom strand (Fig. 5B; also see Fig. 6). In the presence of cAMP/CRP, the cleavages at positions −35/−36 on the top strand and at positions −36/−37 on the bottom strand were markedly enhanced, but the cleavage at the −46/−50 region on the bottom strand disappeared (Fig. 5B; also see Fig. 6).

**DISCUSSION**

Since the discovery of the involvement of α-CTD in transcription activation by transcription factors (8, 9, 33) and DNA UP elements (4), detailed mapping of the structural elements within α-CTD for molecular contacts with the protein factors and promoter DNA has been carried out mainly by the genetic approach (14–16). Based on the results of mutant studies alone, however, we cannot discriminate between effects arising from the direct molecular contacts and the indirect effects due to conformational changes in proteins. The approach we employed in this and previous studies (17, 18) allowed us to register conformational alterations that take place in α-CTD upon transcription complex formation. The method includes the measurement of fluorescent signals originating from a probe attached at specific positions on α-CTD and comparison of their topological distribution. By this method, we identified...
structural elements in α-CTD involved in transcription complex formation at different templates. A combination of this approach with the protein contact-dependent DNA cleavage by FeBABE tethered at specific sites on the protein (32) allowed us to localize the protein and DNA sites that are involved in the direct interaction.

The type of spectral changes registered in the case of the UP element-dependent promoter rnbBP1 is in good accordance with the genetic, biochemical, and physical data suggesting involvement of helix I and the loop region between helices III and IV in the interaction with the DNA UP element (7, 16). This observation indicates that the method employed in this study is useful for monitoring the functional interaction of different α-CTD surfaces with transcription factors and regulatory DNA sequences. One unique finding is the involvement of helix II and the C-terminal part of helix IV in the interaction with promoter DNA. The interaction of α-CTD with uuxuAB (in the absence of CRP) and T7D, both lacking the typical UP element, induced spectral changes when the fluorescence probe was conjugated to helix II and the C-terminal part of helix IV. One interpretation is that these parts of the α-subunit directly interact with a promoter element(s) other than the basic −35/−10 signals and the typical UP element. In good agreement with this prediction, the chemical nuclease FeBABE conjugated to the single Cys-309 induced specific DNA cleavage in the upstream region of both promoters (for the cleavage site sequences, see Fig. 6). The strong cleavage took place upstream from the promoter element (Fig. 5). Both promoters have a G/C-rich sequence in this region, and such a sequence is absent in the rnbBP1 promoter. The site of weaker cutting by FeBABE is located between positions −45 and −50, located −1.5 helix turns upstream from the promoter element. In the case of T7D, the upstream cleavage sites are located at the upstream edge of the sequence CTTTA, which has five matches with the frequently identified non-canonical sequence element (CTTTAC) in this region (34). The distal cleavage sites in uuxuAB are also located immediately upstream from a similar sequence, ATTTC. In addition, both promoters have easily deformable dinucleotides, TA and CA(TG), in the upstream interaction sites. Such sequences are often observed upstream from bacterial promoters (35). This structural peculiarity might be favorable for additional contacts made in the upstream region. These results indicate that the mode of newly identified interaction between α-CTD and upstream promoter DNA is sequence- or structure-dependent. The spectral changes observed for FMA conjugated to helix II and/or the C-terminal edge of helix IV might also be due to an indirect effect originating from the DNA contact with other surfaces of α-CTD. Thus, detailed genetic and biochemical studies are needed to confirm our prediction.

Some class I transcription factors have been proposed to interact in the C-terminal proximal regions of the α-subunit, including OmpR, Fnr, and MerR (reviewed in Refs. 3 and 8). Transcription activation by Fnr is reduced by mutations at positions 300, 302, 305, 308, 315, and 317 of α-CTD (36). Likewise, substitution at positions 311 and 323 renders α-CTD defective in transcription by the MerR-dependent merR promoter (37). Thus, the newly identified DNA contact surface including Ser-309 appears to overlap with the contact sites for Fnr and MerR. Both Fnr and MerR are known to induce the change in DNA conformation near the respective binding sites and thus may affect the DNA-binding activity of this C-terminal proximal DNA contact surface.

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Transcription Activation Mediated by the Carboxyl-terminal Domain of the RNA Polymerase α-Subunit: MULTIPOINT MONITORING USING A FLUORESCENT PROBE

Olga N. Ozoline, Nobuyuki Fujita and Akira Ishihama

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