Architecture of a Complex between the $\sigma^{70}$ Subunit of Escherichia coli RNA Polymerase and the Nontemplate Strand Oligonucleotide

LUMINESCENCE RESONANCE ENERGY TRANSFER STUDY*

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We used luminescence energy transfer measurements to determine the localization of 5'- and 3'-ends of a 12-nucleotide nontemplate strand oligonucleotide bound to $\sigma^{70}$ holoenzyme. Five single reactive cysteine mutants of $\sigma^{70}$ (cysteine residues at positions 1, 59, 366, 442, and 596) were labeled with a europium chelate fluorochrome (donor). The oligonucleotide was modified at the 5'-end with Cy5 fluorochrome (acceptor). The energy transfer was observed upon complex formation between the donor-labeled $\sigma^{70}$ holoenzyme and the acceptor-labeled nontemplate strand oligonucleotide, whereas no interaction was observed with the template strand oligonucleotide. The oligonucleotide was bound in one preferred orientation. This observation together with the sequence specificity of single-stranded oligonucleotide interaction suggests that two mechanisms of discrimination between the template and nontemplate strand are used by $\sigma^{70}$: sequence specificity and strand polarity specificity. The bound oligonucleotide was found to be close to residue 442, confirming that the single-stranded DNA binding site of $\sigma^{70}$ is located in an $\alpha$-helix containing residue 442. The 5'-end of the oligonucleotide was oriented toward the COOH terminus of the helix.

Transcription initiation in Escherichia coli involves two essential steps: (i) initial promoter recognition by RNA polymerase (RNAP) and (ii) melting of DNA duplex in the vicinity of transcription start site (1–5). The simplest two-step model describing transcription initiation (Eq. 1) involves a rapid formation of a labile "closed" complex that in a second step isomerizes to a stable "open" complex.

$$\text{RNAP} + \text{DNA} \rightarrow \text{RNAP-DNA}_{\text{closed}} \rightarrow \text{RNAP-DNA}_{\text{open}} \quad (\text{Eq. 1})$$

In the open complex 10–15 base pairs of DNA duplex become single-stranded. This DNA duplex melting in the case of E. coli RNAP occurs spontaneously, therefore the energetic cost of duplex melting must be offset by some favorable RNAP-promoter interactions.

The E. coli RNA polymerase holoenzyme is a multisubunit enzyme (subunit composition $\alpha_2\beta\beta'\sigma$) (1). $\sigma^{70}$ (the primary $\sigma$ subunit) is the RNAP subunit thought to be responsible for the initial recognition of promoter DNA (1–6). Sequence homology between the conserved region 2.3 of $\sigma^{70}$ and eukaryotic single-stranded DNA (ssDNA)-binding proteins was used as a basis of the proposal that $\sigma^{70}$ subunit could also be actively involved in the promoter melting reaction through binding of ssDNA of the open complex (7). This favorable ssDNA-$\sigma^{70}$ interaction could reduce the energetic cost of DNA melting and facilitate open complex formation. The data from several laboratories provided experimental evidence in support of this proposal. The $\sigma^{70}$ subunit was shown to bind ssDNA (8–16) or single-stranded bubbles within the DNA duplex (17, 18). The binding was specific for the nontemplate DNA strand (9–16). The ssDNA binding site of $\sigma^{70}$ is most likely located in the conserved region 2.3 of the $\sigma^{70}$ subunit (13, 14, 16, 19–21). The ssDNA binding activity of $\sigma^{70}$ is regulated allosterically through binding to the core RNAP (16). Free $\sigma^{70}$ binds ssDNA weakly and does not discriminate between the template and nontemplate sequences. Binding of $\sigma^{70}$ to the core reduces the affinity of $\sigma^{70}$ to template ssDNA and increases the affinity to nontemplate ssDNA resulting in a ~200-fold difference in the affinity between nontemplate and template ssDNA (16). All of the above properties of $\sigma^{70}$ are consistent with its active role in the promoter melting reaction.

Region 2.3 of $\sigma^{70}$ is thought to be a location of the ssDNA binding site of $\sigma^{70}$. However, the architecture of the ssDNA-$\sigma^{70}$ complex and the structural determinants of the high selectivity for nontemplate ssDNA binding are not known. Therefore, in this work we used luminescence energy transfer (LRET) (22–25, 31, 32) distance measurements to determine localization of nontemplate strand oligonucleotide bound to $\sigma^{70}$ with respect to several functional domains of the protein.

EXPERIMENTAL PROCEDURES

Materials—Cy5, monosuccinimidyl ester, was purchased from Amersham Pharmacia Biotech. Succinimidyl ester of 7-amino-4-methylcoumarin-3-acetic acid (AMCA-NHS) was from Boehringer Mannheim. Oligonucleotides were obtained from Midland Certified Reagent Co. (Midland, TX). All other chemicals were of the highest purity commercially available. Core RNAP was purified from E. coli K12 cells (obtained from the University of Alabama fermentation facility) using the method of Burgess and Jendrisak (29).

Preparation of DTPA-AMCA(5)-Maleimide—5 mg of AMCA-NHS was dissolved in 250 µl of DMF, and 50 µl of 1 M ethylenediamine HCl (pH 7.0) was added. The mixture was incubated for 1 h at room temperature, and another 50 µl of 1 M ethylenediamine HCl was added followed by incubation for 1 h at room temperature. The mixture was diluted to ~4 ml with buffer A (25 mM triethylammonium acetate buffer (pH 7.0) containing 2% acetonitrile) + 300 µl of buffer B (buffer B is buffer A with 95% acetonitrile). A small amount of a white precipitate was removed by a centrifugation, and the sample was loaded on a fast
protein liquid chromatography reverse phase column (HR10/10 column (Amersham Pharmacia Biotech) packed with Resource 15RPC (Amersham Pharmacia Biotech). The column was eluted at 3 ml/min with 100 mM of 0–50% buffer B gradient. Fractions containing the adduct of 7-amino-4-methyl coumarin-3-acetic acid and ethylenediaene eluting at about 36% and 45% buffer B were pooled (7.5 ml) and lyophilized. Dried fractions were dissolved in 500 μl of DMF, and 5 mg of succinimidy ester of maleimide-propionic acid dissolved in 100 μl of DMF was added. The mixture was incubated for 1 h at room temperature, diluted to ~5 μl with 5% buffer B, and loaded onto a Resource 15RPC column. The column was eluted at 3 ml/min with 100 ml of 0–50% buffer B gradient. Fractions containing the AMCA-maleimide eluting at about 24% buffer B were pooled (7.5 ml) and lyophilized. Dried fractions were dissolved in 500 μl of DMF, and 20 mg of DTPA anhydride was added. The mixture was incubated for 1 h at room temperature, diluted to 5 ml with buffer A, and run on a Resource 15RPC column as described above. Fractions containing DTPA-AMCA-maleimide eluting at ~15% buffer B were pooled, dispensed to Eppendorf tubes such that each tube contained 0.1 μmol of the chelate, and dried. The yield of a purified product was 10–20%.

Single-cysteine Mutants of α70—The preparation of single-cysteine mutants of α70 is described elsewhere (27). Briefly, three endogenous cysteine residues of α70 (cysteines 132, 291, and 295) were replaced with Ser residues using site-directed mutagenesis. Single Cys residues were then replaced by the desired location using single residue replacements, with the exception of [1Cys]α70, in which the cysteine residue was inserted between the initiating Met and the second residue of the protein. The following single-cysteine mutants of α70 were used in LRET experiments: [1C]α70, [A59C]α70, [S366C]α70, [S442C]α70, and [R596C]α70. Single-cysteine mutants of α70 were expressed and purified as described above (27). Transcriptional activity of single-cysteine mutants of α70 compared with the wild type α70 was: 109% ([1C]α70), 83% ([A59C]α70), 64% ([S366C]α70), 95% ([S442C]α70), and 100% ([R596C]α70) (27).

Fluorochrome-labeled Oligonucleotides—In all experiments a 12-nt oligonucleotide (TCGTATAATGTG) corresponding to positions 15–~4 of the lacUV5 promoter nontemplate strand was used. The Cy5 fluorochrome was attached to the 5′ end by first adding a 5′-phosphatase amino group through a posttranslational modification of the oligonucleotide with ethylenediamine (28), which results in a two-carbon linker between the 5′-phosphate and the reactive amine. The 5′-amino-containing oligonucleotide was then reacted with ~1 mm succinimidy ester of Cy5 for 2–4 h at room temperature in 0.1 M sodium bicarbonate buffer (pH 8.3). The excess of Cy5 was removed on a G-25 spin column (Amersham Pharmacia Biotech), and the labeled oligonucleotide was purified from unlabeled DNA using a reverse phase high performance liquid chromatography column as described previously (26). To attach Cy5 fluorochrome to the 3′-end of the oligonucleotide a 3′-amino group was introduced during oligonucleotide synthesis using a three-atom linker. The reaction of the 3′-amino-containing oligonucleotide with Cy5 and the purification of labeled DNA were performed as described for 5′-labeled oligonucleotides. The concentration of the oligonucleotides was determined spectrophotometrically using absorbance at 260 nm corrected for the contribution due to Cy5 dye (26).

Donor Fluorochrome-labeled α70—Samples of each single-cysteine mutant of α70 (0.5–1.0 mg) were precipitated with 60% ammonium sulfate by the addition of the appropriate volume of saturated ammonium sulfate solution. The protein pellet was collected by centrifugation and dissolved in 75 μl of 50 mM Tris (pH 8.0), 1 mM EDTA, 5% glycerol, and 6 μl of GdHCl buffer. Dithiothreitol was added to a final concentration of 0.5 mM, and mixtures were incubated for 1 h at room temperature. Dithiothreitol was removed by a Microspin G-50 column (Amersham Pharmacia Biotech) equilibrated with the above buffer. DTPA-AMCA-maleimide was added to a final concentration of 0.5–1.0 mM, and the reaction was allowed to proceed for 1 h at room temperature. The excess of unreacted DTPA-AMCA-maleimide was removed by Microspin G-50 column equilibrated with 50 mM Tris (pH 8.0), 1 mM EDTA, 5% glycerol, and 6 μl of GdHCl buffer. The eluate from the G-50 column was diluted to ~0.75 ml with the above buffer, dialyzed first against the same buffer for few hours and next to 50 mM Tris (pH 8.0), 5% glycerol buffer overnight with three changes of 100 ml of buffer. Refolded modified α70, after a 1-h min incubation with 10 μM EuCl₃ and 10 μM EuCl₃, was mixed with purified core RNAP in a 1:1 molar ratio and incubated for 30 min at 4 °C. The reconstituted holozyme was purified from unbound labeled α70 on a fast protein liquid chromatography Superdex-200 size exclusion column (Amersham Pharmacia Biotech). For experiments with free donor-labeled α70, the protein after refolding through dialysis was purified further on Superdex-200 column.

LRET Measurements—All LRET experiments were performed in a 120-μl cuvette in 50 mM Tris- HCl (pH 8.0), 250 mM NaCl, 5% glycerol buffer at 25 °C. The concentration of the holozyme used was 25–50 nm. Luminescence decays of donor fluorochrome-labeled holozyme were recorded in the presence and absence of acceptor-labeled oligonucleotides (0–250 nm). Luminescence lifetime measurements were performed on a laboratory-built two-channel spectrofluorimeter with a pulsed nitrogen laser (LN300, Laser Photonics, Orlando, FL) as an excitation source (26). Donor emission was observed at 617 nm and acceptor emission at 670 nm. Decays of donors in the presence of acceptor and decays of sensitized acceptor emission were fitted to a three-exponential equation (see "Results"). Donor and sensitized acceptor decay curves were fitted simultaneously using global nonlinear regression with Scientist (Micromath Scientific Software, Salt Lake City, UT) to the following set of equations:

\[ I(t) = \alpha_0 e^{-\lambda t} \]

\[ I(t) = \sum \alpha_i e^{-\lambda_i t} \]

where \( \alpha \) is the amplitude and \( \tau \) is the luminescence lifetime, respectively. Decays of donors in the presence of acceptor and decays of sensitized acceptor emission were fitted to a three-exponential equation (see "Results"). Donor and sensitized acceptor decay curves were fitted simultaneously using global nonlinear regression with Scientist (Micromath Scientific Software, Salt Lake City, UT) to the following set of equations:

\[ I(t) = \alpha_0 e^{-\lambda t} \]

\[ I(t) = \sum \alpha_i e^{-\lambda_i t} \]

where \( I(t) \) and \( I(t) \) are luminescence intensity of donor and sensitized acceptor, respectively; \( \alpha_i \) and \( \alpha_i \) are amplitudes of the ith component in donor and sensitized acceptor decay, respectively; and \( \tau_i \) is the lifetime of the ith component. Such global fitting is possible because of unique properties of the europium chelate Cy5 donor-acceptor pair (long microsecond lifetime of the donor and nanosecond lifetime of the acceptor). The decay of sensitized acceptor in microsecond time scale under these conditions occurs with lifetimes of the donor engaged in energy transfer with the acceptor (26, 31–33). Thus, decays of the donor and sensitized acceptor are described by the same lifetimes and different amplitudes (Equations 3 and 4).

The ability to analyze donor- and sensitized-acceptor decay data by global fitting to Equations 3 and 4 is an important advantage of using europium chelate in LRET experiments. It improves very significantly the precision and the confidence of lifetime determination in the presence of energy transfer.

The energy transfer (E) between europium chelate-labeled α70 and Cy5-labeled oligonucleotide was calculated from measurements of luminescence lifetime of a donor in the absence \( (\tau_d) \) and in the presence of acceptor \( (\tau_{da}) \),

\[ E = 1 - \tau_d/\tau_{da} \]

The distances between the donor and the acceptor were calculated according to Förster theory (22)

\[ R_0^2 = R^2 (1 - E/E) \]

where \( R \) is a distance between a donor and an acceptor, and \( R_0 \) is a distance at which the energy transfer is 0.5. The \( R_0 (55 \AA) \) was calculated as described previously using assumptions described by Selvin and Hearst (31).

One of these assumptions is that the orientation factor \( (x^2) \) value of 2/3 (completely random orientation of donors and acceptors (22-25)) could be used in calculating \( R_0 \). This assumption is justified by two factors: long lifetime of the donor and multiple transition dipole moments of the lanthanide (26, 31–35). The long donor lifetime increases the probability that a donor and an acceptor will rotate to all possible orientations during the donor excited state lifetime. Multiple transition dipole moments result in depolarization of donor emission even if the donor is completely immobile.

RESULTS

Acceptor-labeled 12-nt Oligonucleotide and Donor-labeled α70—Fig. 1, A and B, shows the structure of the two acceptor fluorochrome-labeled oligonucleotides used in this work. The 12-nt oligonucleotide sequence used corresponds to position ~15 to ~4 of the lacUV5 nontemplate strand sequence. We
have shown previously that σ^70 in a RNA polymerase holoenzyme is capable of binding this oligonucleotide ~200-fold better than the template or random sequence 12-nt oligonucleotide (16). The acceptor fluorochrome (Cy5) was attached to the 5'-end (panel A) or to the 3'-end (panel B) of the oligonucleotide. Panel C, absorption spectrum of a purified labeled Cy5-5'-NT oligonucleotide. The spectrum for NT-3'-Cy5 oligonucleotide was essentially the same.

Fig. 1. Structure of acceptor-labeled 12-nt nontemplate oligonucleotides. The acceptor (Cy5) was attached to the 5'-end (panel A) or to the 3'-end (panel B) of the oligonucleotide. Panel C, absorption spectrum of a purified labeled Cy5-5'-NT oligonucleotide. The spectrum for NT-3'-Cy5 oligonucleotide was essentially the same.

Fig. 2. Panel A, structure of europium chelate used as a donor in FRET measurements. Panel B, localization of single-reactive cysteine residues in the primary structure of σ^70 protein. Conserved regions of the protein (43) are indicated by boxes. Panel C, absorption spectrum of the purified donor-labeled [S366C] σ^70 protein. Spectra for other single-cysteine mutants of σ^70 were very similar.

Architecture of ssDNA-σ^70 Complex

Fig. 2A shows the structure of the fluorescence donor molecule used in our studies. The europium ion coordinated by the DTPA moiety of the probe is a luminescent component of the donor probe (26). We used a europium chelate for LRET measurements because, as shown recently, these probes offer several important advantages when used as donors in LRET measurements compared with classical organic dye fluorescence probes (26, 31–33). The fluorescence donors were incorporated into the specific sites of σ^70 protein through chemical modification of unique cysteine residues placed in different structural domains of the protein (Fig. 2B). Cys-1 and Cys-59 are in conserved region 1, which was shown to be involved in the
autoinhibition of promoter DNA binding in the free $\sigma^{70}$ (34, 35). Cys-366 is located in a nonconserved region of the protein near sequences thought to be important for core RNA polymerase (RNAP) binding (36, 37). Cys-442 is in region 2.4, responsible for −10 promoter DNA sequence recognition, and is adjacent to region 2.3, thought to be involved in nontemplate ssDNA binding (37–39). Cys-596 is in region 4.2, which was shown to be involved in promoter DNA sequence recognition (40). Fig. 2C shows an example of the absorption spectrum of the purified donor-labeled $\sigma^{70}$ (([S366C]$\sigma^{70}$). Characteristic peaks caused by protein (at −280 nm) and DTPA-AMCA (at −328 nm (26)) were observed. Using this spectrum we estimated that the labeled protein contained about 1 mol of the AMCA-DTPA/mol of the protein. The degree of labeling for other donor-labeled $\sigma^{70}$ proteins was 0.5–1.0 mol of AMCA-DTPA/mol of protein.

**LRET Measurements**—Figs. 3 and 4 show representative examples of LRET data for the donor-labeled $\sigma^{70}$ reconstituted with the core RNAP, in the presence and absence of acceptor-labeled oligonucleotides. The data shown in Figs. 3 and 4 are for the donor-labeled ([S366C]$\sigma^{70}$ and 12-nt oligonucleotide labeled with Cy5 at the 5′-end (Cy5-5′-NT). The results for other donor-labeled $\sigma^{70}$ and Cy5-5′-NT or NT-3′-Cy5 oligonucleotides were qualitatively similar. Only the extent of LRET observed was different with different combinations of labeled $\sigma^{70}$ and labeled oligonucleotides. LRET data for all of these combinations are summarized in Tables II and III.

In the absence of acceptor-labeled oligonucleotide, luminescence decays of donor-labeled $\sigma^{70}$ reconstituted with the core RNAP were monoexponential (Fig. 3A). In the presence of acceptor-labeled oligonucleotide, decays of donor-labeled $\sigma^{70}$ were no longer single exponential, and the presence of faster decaying component(s) was observed (Fig. 3A). The decay of the donor-labeled holoenzyme in the presence of acceptor-labeled oligonucleotide could be fitted to a three-exponential decay function. At any given concentration of acceptor-labeled oligonucleotide, we expected in solution an equilibrium mixture of RNAP-oligonucleotide complex (capable of LRET) and free RNAP (incapable of LRET). In accordance with this expectation, the slowest decay time ($\tau_3$) observed in the presence of acceptor-labeled oligonucleotide was very similar to the decay time observed in the absence of acceptor, and its amplitude decreased with the increase of oligonucleotide concentration (Table I). The faster decaying component(s) were thus interpreted to be caused by LRET between donor-labeled $\sigma^{70}$ and acceptor-labeled oligonucleotide. Three observations support this interpretation. First, in the presence of unlabeled 12-nt nontemplate oligonucleotide, decay of the donor was monoexponential with the lifetime very similar to the one in the...
The data in the table are for Cy5–5’-NT 12-nucleotide oligonucleotide and the 50 nM holoenzyme reconstituted with σ70 labeled with fluorescence donor at Cys-366.

| DNA | Amplitude (slow) t_{slow} | Amplitude (fast) t_{fast} | DNA | Amplitude (slow) t_{slow} | Amplitude (fast) t_{fast} |
|-----|--------------------------|--------------------------|-----|--------------------------|--------------------------|
| 0   | 100 596 54 72            |                          | 100 | 36 610 64 78            |                          |
| 250 | 26 562 74 63            |                          |     |                         |                          |

a Amplitude (fast) is a sum of amplitudes of two fast components in the three-exponential decay observed in the presence of acceptor-labeled oligonucleotide. (see “Results”).

b t_{fast} is the weighted average of the lifetimes of two fast components (τ_{1i} and τ_{2i}) in the three-exponential decay observed in the presence of acceptor-labeled oligonucleotide.

Two lifetimes (τ_{1i} and τ_{2i}) were necessary to describe the fast decaying portion of the decay curve adequately. There are several possible interpretations for the two fast decaying components observed in the presence of acceptor. They could be a result of two populations of species capable of LRET; for example, the oligonucleotide could bind to the primary binding site and, to a lesser degree, to a secondary site. The appearance of two fast decaying components in LRET with lanthanide chelates was observed previously, and the very fast component was interpreted to be an instrumental artifact (30–32). In cases where the donor-acceptor distance was large, the two fast lifetimes could be resolved. For example, in the case of donor-labeled [R596C]σ70 and NT-3’-Cy5 DNA these lifetimes were: τ_{1} = 66 μs (9% of total fast component amplitude) and τ_{2} = 412 μs (91% of total fast component amplitude). However, when the distance between the donor and acceptor was smaller, the values of these two lifetimes became correlated and could not be well resolved. Thus, for all LRET calculations presented here we used the weighted average of two fast decaying components, avoiding the arbitrary decision of which lifetime component to use. The impact of the mode of LRET calculations on the results was small because the distances obtained using the average of τ_{1} and τ_{2} were very similar to distances calculated using only τ_{2}(the average difference in distances between these two modes of calculation was only 4 ± 2 Å).

LRET between the donor-labeled σ70 and acceptor-labeled 12-nt nontemplate strand oligonucleotide was specific only for σ70 reconstituted with the core RNAP. Free σ70 showed no evidence of significant energy transfer in the presence of 100 nM oligonucleotide (Fig. 3, C and D). Donor decays in the presence or absence of acceptor-labeled DNA were monoeponential (Fig. 3C), and essentially no sensitized emission of the acceptor was observed (Fig. 3D). At the same concentration of labeled oligonucleotide a very efficient LRET was observed in the case of holoenzyme (Fig. 3, A and B). These observations are consistent with and further confirm our previous report that the specificity for binding the nontemplate single-stranded oligonucleotide is induced in σ70 by an interaction with the core RNAP (16).

Competition experiments were used to determine the specificity of LRET in the nontemplate strand oligonucleotide-holoenzyme complex (Fig. 4). In the presence of 50 nM acceptor-labeled nontemplate strand oligonucleotide an efficient LRET was observed as indicated by a much faster decay of the donor compared with the decay in the absence of DNA (Fig. 4A). This efficient LRET could be eliminated by the addition of excess unlabeled 12-nt nontemplate strand oligonucleotide as indicated by almost overlapping decay curves observed in the absence of any DNA and in the presence of 50 nM acceptor-labeled and 1 μM unlabeled nontemplate oligonucleotide (Fig. 4B). In contrast, excess unlabeled nontemplate randomized sequence oligonucleotide had essentially no effect, and the efficient LRET was still observed, as indicated by a much faster decay observed in the presence of 50 nM acceptor-labeled nontemplate oligonucleotide and 1 μM unlabeled nontemplate randomized sequence oligonucleotide (Fig. 4C).}

**Table I**

| Donor-labeled σ70 | τ_{1} | τ_{2} | E | R |
|-------------------|------|------|---|---|
| [1C]σ70          | 648  | 114  | 0.82 | 43 |
| [A59C]σ70        | 653  | 71   | 0.89 | 39 |
| [S366C]σ70       | 596  | 205  | 0.66 | 49 |
| [S442C]σ70       | 632  | 42   | 0.93 | 35 |
| [R596C]σ70       | 605  | 380  | 0.37 | 60 |

* Lifetimes of donor-only samples are for donor-labeled σ70 reconstituted with the core RNAP in the presence of 1 μM unlabeled nontemplate strand oligonucleotide.

**Table II**

| Donor-labeled σ70 | τ_{1} | τ_{2} | E | R |
|-------------------|------|------|---|---|
| [1C]σ70          | 648  | 114  | 0.82 | 43 |
| [A59C]σ70        | 653  | 71   | 0.89 | 39 |
| [S366C]σ70       | 596  | 205  | 0.66 | 49 |
| [S442C]σ70       | 632  | 42   | 0.93 | 35 |
| [R596C]σ70       | 605  | 380  | 0.37 | 60 |

* Lifetimes of donor-only samples are for donor-labeled σ70 reconstituted with the core RNAP in the presence of 1 μM unlabeled nontemplate strand oligonucleotide.

**Table III**

| Donor-labeled σ70 | τ_{1} | τ_{2} | E | R |
|-------------------|------|------|---|---|
| [1C]σ70          | 648  | 114  | 0.82 | 43 |
| [A59C]σ70        | 653  | 71   | 0.89 | 39 |
| [S366C]σ70       | 596  | 205  | 0.66 | 49 |
| [S442C]σ70       | 632  | 42   | 0.93 | 35 |
| [R596C]σ70       | 605  | 380  | 0.37 | 60 |

* Lifetimes of donor-only samples are for donor-labeled σ70 reconstituted with the core RNAP in the presence of 1 μM unlabeled nontemplate strand oligonucleotide.

**Architecture of σ70-Nontemplate ssDNA Complex**—Results of LRET measurements with all five single-cysteine mutants of σ70 and nontemplate 12-nt oligonucleotides with acceptors at the 3'- or 5'-end are summarized in Tables II and III. A wide range of energy transfer efficiencies (from 0.37 to >0.99) was observed. The range of distances corresponding to these energy transfer efficiencies was from 60 Å to <25 Å. For several
residues of σ70 very significant differences between the distance to 5′ and 3′ of the oligonucleotide were found. Region 2.4 (Cys-442) was found to be the closest to the oligonucleotide bound to σ70. It appears also that the 5′-end of the bound oligonucleotide was much closer (<25 Å) to residue 442 than the 3′-end (35 Å). Residue 596 was the farthest from the bound oligonucleotide, and this residue seems to be located almost at the same distance from the 5′- and 3′-ends of the oligonucleotide. Also, the N-terminal cysteine was found at an approximately equal distance from the 5′- and 3′-ends of the oligonucleotide. Residue 59 found closer to the 5′-end of the oligonucleotide, and residue 366 found closer to the 3′-end.

Using distances determined by LRET, three-dimensional models of relative localization of different domains of σ70 could be built. An example of such a model superimposed on the crystal structure of σ70 fragment is shown in Fig. 5. Building these models allowed an indirect determination of several additional distances between the sites in the complex (Table IV). Because there were not enough distance constraints to determine all possible distances uniquely, an analysis of a relative precision of these indirect distance determinations was performed. A set of 25 independent models fulfilling distance constraints from LRET experiments was built, starting each from randomly “scrambled” initial distances between the sites in the complex. In each of these models all possible distances were then measured, and the mean and standard deviation were calculated (Table IV). The standard deviation can thus be used as a convenient measure of a relative precision of these indirect distance estimations. Inspection of the data presented in Table IV shows that two sets of distances can be identified easily: those very poorly defined (standard deviation > 20 Å) and those whose precision is good enough (standard deviation < 10 Å) for use in discussing the architecture of the complex. Distances with relatively high precision of estimation were 5′ of the oligonucleotide to 3′ of the oligonucleotide, residues 1–442, 9–442, 366–442, and 442–596. We have recently measured several interdomain distances in the holoenzyme,3 and these distances appear to be in general agreement with the distances calculated by model building (Table IV). Additionally, using these directly determined interdomain distances we attempted to obtain a better estimate of 5′ ⇔ 442 distance by building models that included this distance as a variable. This distance could not be determined by LRET measurements because the donor and acceptor were too close (Table II). A distance of ~14 Å was obtained from model building, consistent with LRET results (Table II).

**DISCUSSION**

We have determined the distances between several sites in the σ70 and the 5′- or the 3′-end of 12-nt nontemplate strand oligonucleotide in complex with RNAP holoenzyme. The measured distances allowed us to build a model describing a three-dimensional architecture of the oligonucleotide-RNAP com-

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**Table IV**

Calculated distances between sites in holoenzyme-oligonucleotide complex using distance constraints from LRET measurements

| Positions in holoenzyme-oligonucleotide complex | Distance$^a$ (Å) |
|-----------------------------------------------|-----------------|
| 5′ of oligo ⇔ 3′ of oligo                     | 32 ± 7          |
| 1 ⇔ 59                                        | 41 ± 24         |
| 1 ⇔ 366                                       | 48 ± 21         |
| 1 ⇔ 442                                       | 45 ± 6          |
| 1 ⇔ 596                                       | 51 ± 29         |
| 59 ⇔ 366                                      | 47 ± 21 (54)$^b$|
| 59 ⇔ 442                                      | 51 ± 6 (46)$^b$|
| 59 ⇔ 596                                      | 66 ± 26 (55)$^b$|
| 366 ⇔ 442                                     | 38 ± 6 (39)$^b$|
| 366 ⇔ 596                                     | 58 ± 24 (51)$^b$|
| 442 ⇔ 596                                     | 58 ± 6 (50)$^b$|

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$^a$ Distances were calculated by building 25 independent models of three-dimensional architecture of oligonucleotide-RNAP complex fulfilling distance constraints from LRET measurements. Each model was built using distance constraints routine of ChemSite (Pyramid Learning, Stanford, CA) starting from random and different initial distances between the sites in σ70.

$^b$ The numbers in parentheses correspond to distances between the sites determined directly by LRET in the free holoenzyme (see Footnote 3).

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3 S. Callaci, E. Heyduk, and T. Heyduk, unpublished data.
plex. Several conclusions regarding the architecture of the complex can be made.

The oligonucleotide binds to RNAP apparently in a preferred orientation. In principle, the oligonucleotide could bind to its binding site either in 5′ → 3′ or 3′ → 5′ orientation. If the binding could occur equally well in either orientation, the apparent distances measured between sites in σ70 and the 5′ or 3′-end of the oligonucleotide should be the same. We observed, however, that for several sites in σ70 the distances between the 5′-end and the 3′-end were very significantly different (Tables II and III), showing that the oligonucleotide was bound in one preferred orientation. Such preference for a specific orientation may be an additional mechanism by which RNAP in the open complex could discriminate between template and non-template strands in the transcription bubble. Previous binding experiments with oligonucleotides corresponding to the 10 region of the non-template and the template strand showed that RNAP holoenzyme could bind non-template sequence oligonucleotides 200-fold better then the template sequence oligonucleotides (16). Oligonucleotides can freely assume any orientation when they bind to the ssDNA binding site of σ70. The situation will be different in the open complex when ssDNA strands in the 10 region have restricted mobility. Thus, if the non-template strand in the open complex is in a correct orientation for binding to the ssDNA binding site of σ70, the template strand will be forced to be in the opposite, unfavorable for the binding orientation. Thus, the two different mechanisms for discrimination between single-stranded non-template and single-stranded template strands in the 10 region of promoter DNA are apparently being used by σ70. One mechanism is the sequence specificity of the binding, the other mechanism is the strand polarity specificity. This dual mode of discrimination employed by σ70 seems to be well suited for the tasks that the ssDNA binding site of σ70 needs to perform: selective binding of the 10 sequences and selective binding of the non-template strand.

Region 2.4 (Cys-442) was found to be the closest to the oligonucleotide bound to σ70. This is consistent and confirms the proposals that the ssDNA binding site of σ70 is localized in region 2.3 (13, 14, 16, 19–21) because this region is adjacent to region 2.4 and is located in the same α-helix (helix 14 (37)). It appears also that the 5′-end of the bound oligonucleotide was much closer (<25 Å) to residue 442 than the 3′-end was (39 Å). Thus, the relative orientation of the bound oligonucleotide with respect to helix 14 appears to be 5′ → COOH terminus of the helix. Such an orientation is consistent with the proposed model of non-template ssDNA-σ70 interaction based on the crystal structure of the σ70 fragment (37). It is also consistent with data relating mutations in σ70 and mutations in the 10 region of promoter DNA. Based on these studies it was proposed that residues 437 and 440 are involved in recognition of position –12, whereas residue 441 is involved in recognition of position –13 (38, 39, 41, 42). Such an alignment of bases in the 10 region and amino acids in helix 14 is consistent with a 5′ → COOH terminus alignment of helix 14 and the non-template oligonucleotide in a holoenzyme-oligonucleotide complex. However, an opposite orientation of the non-template strand with respect to helix 14 was also proposed (44). The reasons for this discrepancy are not clear.

Cys-596 is in region 4.2 of σ70 which was suggested to bind the 35 region of promoter DNA (40). Assuming a simple linear arrangement of –10 and –35 DNA sequences and protein domains involved in binding of these sequences, it could be expected that the 5′-end of the bound oligonucleotide should be much closer to residue 596 than the 3′-end is. Data in Tables II and III show that this was not observed. In contrast, residue 596 seems to be located almost at the same distance from the 5′- and 3′-ends of the oligonucleotide. This suggests that the orientation of the bound oligonucleotide with respect to residue 596 is as illustrated in Fig. 5, i.e. it is more or less perpendicular, not parallel, to the line joining regions 2.4 and 4.2. This observation suggests that promoter DNA in the open complex is not straight, and thus formation of the open complex involves significant deformation of DNA.

Based on LRET distance measurements it was possible to estimate indirectly with reasonable precision several other distances in the oligonucleotide-RNAP complex. The predicted distance between the 5′- and 3′-ends of the oligonucleotide was 32 ± 7 Å, a distance somewhat shorter than expected for a linear 12-nt DNA, consistent with the deformation of DNA in the open complex. The predicted distance between residues 366 and 442 was found to be 38 ± 6 Å. This distance is comparable, within the error of estimation, to the distance between these two residues (35 Å) observed in the crystal structure of σ70 fragment (37). The agreement of this predicted distance with the crystal structure of the σ70 fragment provides an additional validation of distances measured by LRET. The predicted distance between residues 442 and 596 was 58 ± 6 Å. Residues 442 and 596 are located in σ70 domains involved in recognition of –10 and –35 DNA sequences, which are separated by ~17 base pairs. Thus, the predicted distance of 58 ± 6 Å is compatible with the distance expected from the ~17-base pair separation between binding sites for these two structural domains of the protein.

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