# Conformation and DNA Binding Properties of a Single-Stranded DNA Binding Region of $\sigma^{70}$ Subunit from *Escherichia coli* RNA Polymerase Are Modulated by an Interaction with the Core Enzyme†

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ABSTRACT: A derivative of the $\sigma^{70}$ subunit from *Escherichia coli* RNA polymerase with specific fluorescence probes in conserved region 2.3 (DNA "melting motif") was prepared by replacing tryptophan residues at positions 314 and 326 of the wild-type $\sigma^{70}$ with alanine. The remaining two tryptophan residues (Trp 433 and 434) of [Ala 314, 326]$\sigma^{70}$ were biosynthetically replaced with 5-hydroxy-tryptophan (5OHTrp), a fluorescent tryptophan analogue with unique emission that can be selectively observed both in free 5OHTrp[Ala 314, 326]$\sigma^{70}$ as well as in 5OHTrp[Ala 314, 326]$\sigma^{70}$ bound to the core RNA polymerase. Fluorescence quenching experiments revealed that positions 433 and 434 were solvent exposed in free 5OHTrp[Ala314, 326]$\sigma^{70}$. The binding of $\sigma^{70}$ to core polymerase reduced the solvent exposure of these residues. In the presence of single-stranded oligonucleotides, fluorescence of 5OHTrp at position 433 and 434 was quenched ∼65% and these residues became inaccessible to the solvent. Using fluorescence of 5OHTrp at positions 433 and 434 as a specific signal of DNA binding, we show that free $\sigma^{70}$ bound single-stranded DNA weakly and did not discriminate between nontemplate and template strand of promoter DNA. Binding of $\sigma^{70}$ to the core increased the affinity for binding nontemplate DNA, whereas the affinity to template or "nonspecific" DNA was reduced, resulting in a holoenzyme which could bind nontemplate strand ∼200-fold better then the template strand. We concluded that Trp 433 and 434 of $\sigma^{70}$ are located within a single-stranded DNA binding region of $\sigma^{70}$ and that binding of $\sigma^{70}$ to the core enzyme induced conformational changes in a single-stranded DNA binding region of the protein. As a consequence of these conformational changes, $\sigma^{70}$ subunit gains the specificity for the nontemplate strand of the melted region in the "open" complex.

Transcription initiation in *Escherichia coli* is catalyzed by RNA polymerase holoenzyme (RNAP),[1] a multisubunit enzyme (subunit composition $\alpha_2\beta\beta'\sigma$) (for review, see ref *1*). Transcription initiation involves two major steps (eq 1):

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

In the first step, RNAP and promoter DNA rapidly form a labile "closed" complex which in a second step isomerizes to a stable "open" complex. This isomerization involves melting of 1−1.5 turns of DNA around the transcription start point and most likely involves more intermediate steps (recent reviews, refs *2* and *3*). Melting of DNA is an energetically costly step and, since in the case of *E. coli* RNAP does occur spontaneously, the energy spent on DNA melting must come from some favorable interactions between RNAP and promoter DNA. It was suggested that one of such favorable RNAP−promoter interactions could be the binding of a nontemplate strand of the melted DNA region by one of RNAP subunits (*3*−*5*). Such single-stranded DNA−RNAP interaction could reduce the energetic cost of DNA melting and facilitate "closed" to "open" complex isomerization. The binding of RNAP to nonspecific ss DNA has been observed (*6*) as well as the affinity of RNAP for artificially introduced single-stranded bubbles in the duplex DNA (*7, 8*). RNAP was shown primarily to recognize bases in a nontemplate strand of the −10 promoter region and these base-specific and strand-specific contacts were shown to persist even after melting of the DNA duplex (*9*). Sequence-specific binding of RNAP to nontemplate strand oligonucleotides corresponding to a −10 region sequences was also demonstrated (*10*−*15*). Such preference for the nontemplate strand was also observed by chemical probing of open complexes (*16*−*22*).

The involvement of the $\sigma^{70}$ subunit in nontemplate ss DNA binding was most clearly demonstrated by a preferential cross-linking of this subunit to ss oligonucleotides in holoenzyme-ss oligonucleotide complexes (*14, 15, 23*). On the basis of sequence homology to eukaryotic single-stranded DNA binding proteins region 2.3 was proposed to be

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[1] Abbreviations: RNAP, RNA polymerase, 5OHTrp, 5-hydroxy-tryptophan; NATA, *N*-acetyltryptophan amide; CD, circular dichroism.

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involved in promoter melting and ss DNA binding (*5*). Such role of region 2.3 is consistent with alanine scanning mutagenesis of regions 2.3 and 2.4 of *B. subtilis* σA. Substitutions of Tyr 425, Tyr 430, Trp 433, and Trp 434 (amino acid numbering of $\sigma^{70}$) produced $\sigma$ with impaired DNA melting properties (*24*). The DNA-melting defects of these mutants could be surmounted with an increase of temperature or with the use of supercoiled templates (*25, 26*). Marr and Roberts (*14*) showed that a mutation in region 2 (Q437H) known to affect promoter DNA recognition by the holoenzyme (*27*) resulted in a similar effect in binding of ss nontemplate oligonucleotide. This further pointed to regions 2.3/2.4 as the site for ss DNA−$\sigma$ interaction and showed that, indeed, specificity of RNAP interaction with −10 region was mostly dictated by $\sigma$ interactions with the nontemplate strand. Subsequently, Huang et al. (*15*) showed that replacing some of the aromatic residues of region 2.3 also affected binding of ss oligonucleotides. The hydrophobic residues of region 2.3 appear to be solvent exposed in a recently published three-dimensional structure of a fragment of $\sigma^{70}$ and are located on an extended α-helix which includes residues from region 2.4 found previously to be important for −10 duplex promoter region recognition (*28*). Such location is consistent with a "melting motif" role proposed for these hydrophobic residues (*3*).

In this work, we use a specific signal from fluorescence probes incorporated into conserved region 2.3 of $\sigma^{70}$ to study the conformation of this region and its DNA binding properties. Our results confirm that this region is involved in single-stranded DNA binding and show that DNA binding and conformational properties of this region are modulated by the $\sigma^{70}$−core RNAP interaction. The core RNAP acts as an allosteric regulator of $\sigma^{70}$ increasing its affinity for the nontemplate ss DNA, decreasing its affinity for the template strand, and thus, resulting in a core-bound $\sigma^{70}$ able to bind nontemplate strand ∼200-fold better then the template strand.

## EXPERIMENTAL PROCEDURES

*Materials.* Acrylamide, thallium(I) acetate, and 5OHTrp were purchased from Sigma Chemical Co. (St. Louis, MO), and potassium iodide was from Fisher Scientific (Chicago, IL). All other chemicals were of highest purity commercially available. Restriction enzymes were purchased from New England Biolabs, Inc. (Beverly, MA). Oligonucleotides were obtained from Midland Certified Reagent Co. (Midland, TX) or were synthesized in our laboratory on a model 392 automated oligonucleotide synthesizer (Applied Biosystems, Inc., Foster City, CA). The reagents for oligonucleotide synthesis were purchased from Glen Research (Sterling, VA). The oligonucleotides were purified by ion-exchange chromatography on a 1 mL monoQ FPLC column (Pharmacia), or by a reverse-phase HPLC of trityl-on oligonucleotides on a 4.1 × 150 mm PRP-1 column (Hamilton, Reno, NV).

A 114-bp *lacUV5* promoter used in some of the experiments was prepared from synthetic 67-nt DNA strands corresponding to −89 to −23 of the top and −42 to +25 of the bottom strand of the *lacUV5* promoter. The strands (5 $\mu$M) were hybridized through their 3′-end 20-nt complementary regions and the single-stranded extensions were filled-in with the Klenow fragment of DNA polymerase. The 114-bp DNA duplex was purified using ion-exchange chromatography using a 1 mL ResourceQ column (Pharmacia).

*Cloning and Mutagenesis.* The plasmid containing the wild-type (wt) $\sigma^{70}$ gene under T7 polymerase promoter (pGEMD) was a kind gift of Dr. A. Ishihama (National Institute of Genetics, Mishima, Japan). The [Ala 314, 326]$\sigma^{70}$ mutant was prepared in pGEMD plasmid using the Clontech Transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA) according to manufacturer's instructions. Initial experiments showed that using this plasmid the expression of $\sigma^{70}$ in a media containing 5OHTrp was very poor. Therefore, $\sigma^{70}$ coding region was introduced to pQE60 plasmid (Qiagen, Chatsworth, CA). This was accomplished by abolishing (using site-directed mutagenesis) an endogenous *Nco*I restriction site in $\sigma^{70}$ DNA by changing the codon for Ser 506 (TCC to TCT). An *Nco*I site was then introduced at the ATG start codon using site-directed mutagenesis. The entire translated region of rpoD gene was excised using *Nco*I and *Hin*dIII restriction enzymes. Restriction digest product was purified using agarose gel electrophoresis and ligated into gel-purified pQE60 vector, also digested with *Nco*I and *Hin*dIII. The mutations were confirmed by dideoxy DNA sequencing (*29*).

*Expression and Purification of 5OHTrp[Ala 314, 326]$\sigma^{70}$.* The pQE60[Ala 314, 326]$\sigma^{70}$ construct was introduced into a Trp auxotrophic strain W3110 (a kind gift from Dr. J. B. Ross, Department of Biochemistry, Mount Sinai School of Medicine, NY) which was cotransformed with pREP4 plasmid (Qiagen). The cells were grown at 37 °C, shaking at 240 rpm, in 1 L of M9 minimal media supplemented with 2 mM magnesium sulfate, 100 $\mu$M calcium chloride, 0.4% glucose, 0.1% thiamine, 1% casamino acids, 250 $\mu$M L-Trp and 100 $\mu$g/mL ampicillin, until $OD_{600} = 0.5$. The cells were collected by centrifugation in a Sorvall GS-3 rotor at 5000 rpm for 20 min. The cell pellets were gently resuspended into another 1 L of the same media containing no tryptophan. After 30 min of shaking at 37 °C, 0.5 mM 5OHTrp was added to the media and the cells were shaken an additional 30 min. Protein overexpression was induced with 1 mM IPTG, and cells were shaken for an additional 3 h. The cells were collected and were stored at −80 °C until needed.

The purification protocol for 5OHTrp[Ala 314, 326]$\sigma^{70}$ was based on the procedure outlined by Igarashi and Ishihama (*30*). The cells were lysed and the $\sigma^{70}$ protein was extracted with guanidine-HCl from the inclusion bodies as described in ref *30*. The extracted denatured protein was then precipitated with 50% ammonium sulfate by the addition of saturated ammonium sulfate solution. The protein pellet was collected by centrifugation at 10000g for 20 min, and the pellet was dissolved in TGED buffer [10 mM Tris (pH 8), 5% glycerol, 0.1 mM EDTA, and 0.1 mM DTT) containing 6 M guanidine-HCl. Protein was renatured by dialyzing the sample against 500 mL TGED buffer containing no guanidine-HCl at 4 °C with two additional changes of the buffer. The sample was then purified on DEAE-cellulose column (Sigma, St. Louis, MO). After loading the protein, the column was washed with TGED buffer containing 0.1 M NaCl. The protein was eluted from the column using a linear gradient of 0.1 M to 0.5 M NaCl (in TGED). $\sigma$ eluted at ∼0.3 M NaCl. Peak fractions were pooled and diluted to the conductivity of TGED buffer containing 0.25 M NaCl. The sample was then applied to a 6 mL Resource

Q column (Pharmacia Biotech, Uppsala, Sweden) and eluted using a linear gradient of 0.25 to 0.65 M NaCl in TGED buffer. Purified protein was dialyzed against 50% glycerol, 10 mM Tris (pH 8), and 0.1 M KCl, and stored at −20 °C until needed. The yield from 1 L of culture was ∼10 mg. Concentrations of 5OHTrp[Ala 314, 326]$\sigma^{70}$ were determined by measuring the absorbance at 280 nm using an extinction coefficient of 25 970 $M^{-1}$ $cm^{-1}$ calculated according to Gill and von Hippel (*31*).

Core RNAP was purified from *E. coli* K12 cells (obtained from University of Alabama Fermentation Facility) using the method of Burgess and Jendrisak (*32*). Purified core RNAP was dialyzed against 50% glycerol, 50 mM Tris (pH 8), and 0.25 M NaCl and stored as aliquots at −20 °C until needed. Concentrations of core RNAP were estimated spectroscopically by measuring the absorbance at 280 nm and using an extinction coefficient of 0.55 mg/mL and a molecular mass of 378 kDa. For holoenzyme, an extinction coefficient of 0.62 mg/mL and molecular mass of 449 kDa were used (*33*).

For all experiments stored 5OHTrp[Ala 314, 326]$\sigma^{70}$ was dialyzed against 250 mL of 50 mM Tris/acetate (pH 8) and 0.25 M potassium acetate at 4 °C overnight. Aliquots of 100 μL of 5OHTrp[Ala 314, 326]$\sigma^{70}$ were then chromatographed on an FPLC sizing column (40 mL Superdex 200 column; Pharmacia) equilibrated with the same buffer to remove aggregates formed upon storage at high concentrations (>20 μM). The peak of monomeric 5OHTrp[Ala 314, 326]$\sigma^{70}$ was used in all measurements and was identified in Superdex 200 column profile by comparison with molecular mass markers.

Samples of holoenzyme were prepared by dialyzing aliquots of 5OHTrp[Ala 314, 326]$\sigma^{70}$ and core RNAP separately against 250 mL of the 50 mM Tris/acetate (pH 8), 0.25 M potassium acetate buffer. After dialysis, 5OHTrp-[Ala 314, 326]$\sigma^{70}$ and core RNAP were incubated together with 1.5 molar excess of $\sigma^{70}$ to the core, at 30 °C for 30 min (*1*). Samples of 100 μL of the mixture were chromatographed on 40 mL Superdex 200 column to separate holoenzyme from free $\sigma^{70}$. The holoenzyme peak was concentrated using either Microcon 10 or Microcon 30 microconcentrators (Amicon Inc., Beverly, MA) at 4 °C.

For the experiments with wt $\sigma^{70}$, the samples of free $\sigma^{70}$ and the holoenzyme were prepared as described above for the 5OHTrp[Ala 314, 326]$\sigma^{70}$.

*Transcription Activity Assay*. Transcriptional activity of 5OHTrp[Ala 314,326]$\sigma^{70}$ was measured using a runoff transcription assay described in ref *34* with T7 DNA as a template or using abortive initiation assay (*35*) with 114 bp *lacUV5* DNA fragment as a template. In place of the radioactive UTP used originally, we used a fluorescent derivative of UTP (UTP-γ-ANS) prepared as described previously (*36*). To prepare the holoenzyme for the activity measurement, a 4 molar excess of 5OHTrp[Ala 314, 326]$\sigma^{70}$ was incubated with the core RNAP at 30 °C for 30 min. The activity of the wt $\sigma^{70}$ reconstituted with the core RNAP as described above was also measured for comparison.

*CD Measurements*. CD spectra of wt $\sigma^{70}$ and 5OHTrp-[Ala 314, 326]$\sigma^{70}$ were recorded using JASCO model 720 in 0.1 cm cuvette. Spectra between 200 and 260 nm were recorded taking measurements every 0.5 nm using 8 μM protein solutions in 20 mM sodium phosphate (pH 7.5) containing 100 mM NaCl.

*Fluorescence Measurements*. Fluorescence measurements were performed using an Aminco-Bowman Series 2 spectrofluorometer (Spectronic Instruments, Inc., Rochester, NY). All fluorescence quenching experiments were performed in 50 mM Tris/acetate (pH 8) and 0.25 M potassium acetate containing 5% spectroscopic grade glycerol (Aldrich). Samples of 200 μL of free 5OHTrp[Ala 314, 326]$\sigma^{70}$ (250 nM) or 5OHTrp[Ala 314, 326]$\sigma^{70}$ holoenzyme (250 nM) were titrated with 4 μL aliquots of 0.54 M quencher stock solution. Fluorescence intensity at 334 nm (excitation at 313 nm) was measured after each addition of the quencher. The intensities for free 5OHTrp[Ala 314, 326]$\sigma^{70}$ were corrected for the background signals measured with blank samples in which the protein was omitted. The intensities for the holoenzyme were corrected for background using blank samples containing 250 nM core RNAP. All quenching experiments were performed at 25 °C.

After correction for dilution, data were plotted according to the Stern−Volmer equation:

$$F_o/F = 1 + K_{SV}[Q] \quad (2)$$

$F_o$ and $F$ are the fluorescence intensities in the absence and presence of quencher, respectively, $K_{SV}$ is the Stern−Volmer dynamic quenching constant, and [Q] is the concentration of quencher. The Stern−Volmer quenching constants were calculated from the slopes of corresponding plots. The bimolecular collisional quenching constant $k_q$ was calculated by:

$$k_q = K_{SV}/\langle\tau\rangle \quad (3)$$

where $\langle\tau\rangle$ is the mean (intensity weighted) fluorescence lifetime of 5OHTrp[Ala 314, 326]$\sigma^{70}$. The fluorescence lifetimes of 5OHTrp[Ala 314, 326]$\sigma^{70}$ were measured using cross-correlation phase and modulation technique (*37*) at the Laboratory of Fluorescence Dynamics, University of Illinois, Urbana, IL. The relative quenching constants ($K_{rel}$) were calculated using

$$K_{rel} = (K_{SV}/K_{SV,acryl})/(K_{SV}^{NATA}/K_{SV,acryl}^{NATA}) \quad (4)$$

$K_{SV}$ and $K_{SV,acryl}$ are Stern−Volmer quenching constants for a given quencher and acrylamide**,** respectively, determined for a 5OHTrp[Ala 314, 326]$\sigma^{70}$. $K_{SV}^{NATA}$ and $K_{SV,acryl}^{NATA}$ are Stern−Volmer quenching constants for a given quencher and acrylamide**,** respectively, determined for a model compound (N-acetyl-tryptophan amide; NATA).

*DNA Binding Experiments*. The following DNA's were used in DNA binding experiments: (i) a 12-nt ss DNA fragment (TCGTATAATGTG) corresponding to positions −4 to −15 of the nontemplate strand of the *lacUV5* promoter, (ii) a 12-nt ss DNA fragment (CACATTATACGA) corresponding to position −15 to −4 of the *lacUV5* template strand, (iii) a "nonspecific" 12-nt ss DNA fragment (TCGCGCGGCGTG) in which all conserved As and Ts in −4 to −15 were changed to C's and G's, (iv) a 12-nt nontemplate randomized ss DNA (TTGATATCGTAG) having the same base composition as the nontemplate strand but randomized sequence, and (v) a 20-bp DNA duplex (CGGCTCGTATAATGTGTGGA, nontemplate strand sequence) corresponding to positions +1 to −19 of the *lacUV5* promoter DNA. DNA binding was monitored by a decrease

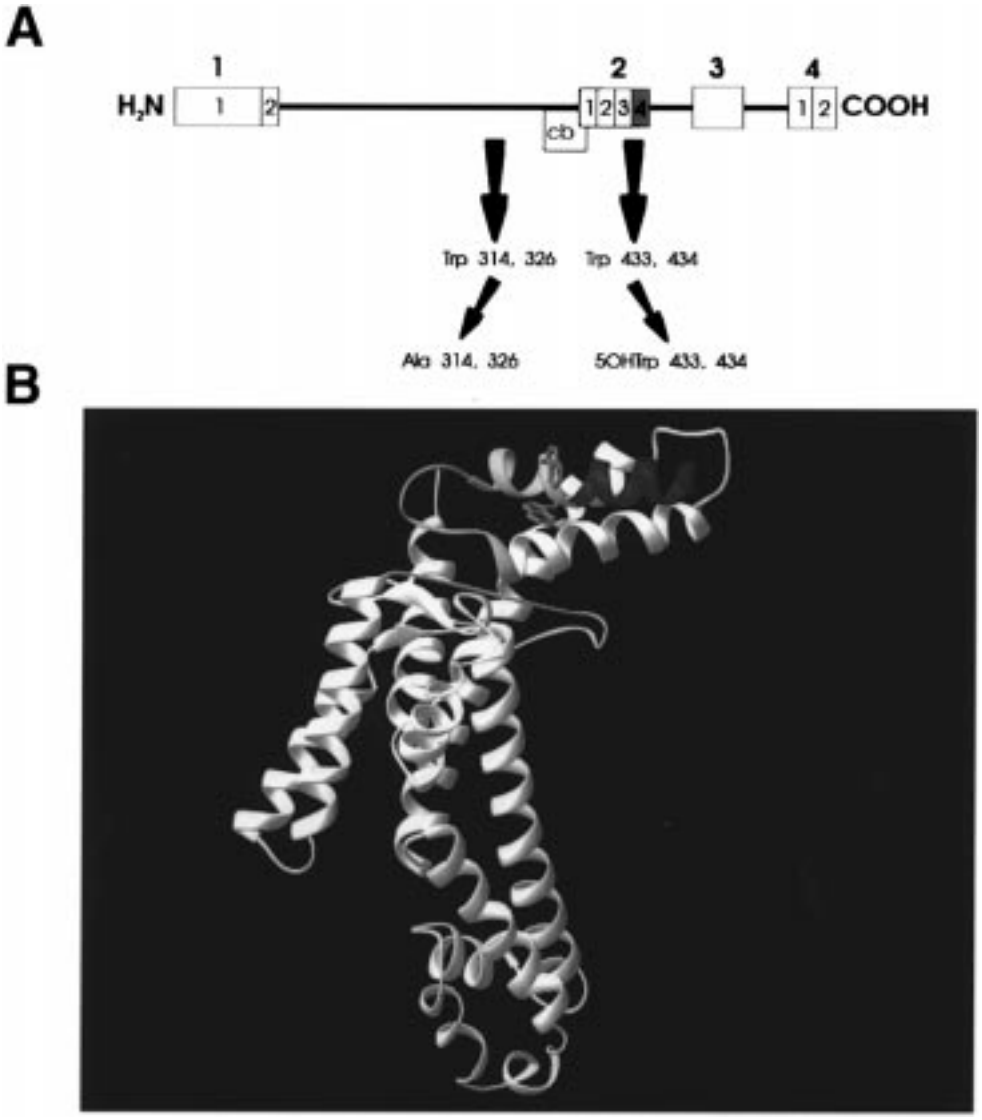


FIGURE 1: (A) Location of the Trp residues of $\sigma^{70}$ in the primary structure of the protein. The conserved regions of the protein (*56*) are marked by boxes with corresponding numbers. Conserved regions 2.3 and 2.4 are colored yellow and blue, respectively. The arrows illustrate the steps which were used to obtain a $\sigma^{70}$ derivative with specific fluorescence probes in the 2.3 region. These steps were (i) replacement of Trp 314 and 326 with Ala through site-directed mutagenesis and (ii) replacement of Trp 433 and 434 with 5OHTrp residues through a biosynthetic incorporation (see Materials and Methods). (B) Location of Trp 433 and 434 (red) in the three-dimensional structure of 114−448 $\sigma^{70}$ fragment. The figure was drawn with RIBBONS (*54*) using coordinates for 114−448 fragment of $\sigma^{70}$ (*28*). The conserved regions 2.3 and 2.4 are colored yellow and blue, respectively.

in fluorescence of 5OHTrp[Ala 314, 326]$\sigma^{70}$ (excitation at 313 nm, emission at 334 nm). All DNA binding experiments were performed in 50 mM Tris/acetate (pH 8) and 0.1 M potassium acetate containing 5% spectroscopic grade glycerol (Aldrich) at 25 °C. Aliquots of 0.2−0.4 $\mu$L of DNA were added to 200 $\mu$L protein sample (250 nM), and 60 s after mixing, the fluorescence intensity was measured. The data for free 5OHTrp[Ala 314, 326]$\sigma^{70}$ were corrected for a background of the buffer containing the respective DNA concentration, and the holoenzyme data were corrected for a background of the core RNAP containing respective DNA concentration. The correction for the inner filter effect was not necessary because at the excitation wavelength used (313 nm), even at the highest concentration (25 $\mu$M), these oligonucleotides had an absorbance <0.022.

*Detection of Nontemplate Strand−5OHTrp[Ala 314, 326]$\sigma^{70}$ Contact Formation in the Open Complex*. The fluorescence of 200 $\mu$L of 5OHTrp[Ala 314, 326]$\sigma^{70}$ holoenzyme (190 nM) was monitored as a function of time for 45 s. The 114 bp *lacUV5* promoter fragment was added to a final concentration of 290 nM and after a 30 s delay during which the sample was mixed, the recording of fluorescence intensity as a function of time was resumed and continued for 2 min. The experiment was performed in 50 mM Tris/acetate (pH 8), 0.1 M potassium acetate, 10 mM $MgCl_2$ 5% glycerol at 10 and 20 °C.

## RESULTS

*$\sigma^{70}$ Derivative with Fluorescence Probes in Region 2.3*. Figure 1A shows the strategy used to prepare a derivative of $\sigma^{70}$ with fluorescence probes in region 2.3. This strategy relies on the use of 5OHTrp, an analogue of tryptophan which can be biosynthetically incorporated into proteins (*38, 39*). Unique spectroscopic properties of 5OHTrp (absorbance extending to approximately 325 nm) allow observation of the fluorescence signal of 5OHTrp in the presence of even a large excess of Trp. Native $\sigma^{70}$ contains four Trp residues: Trp 314, 326, 433, and 434. Residues 314 and

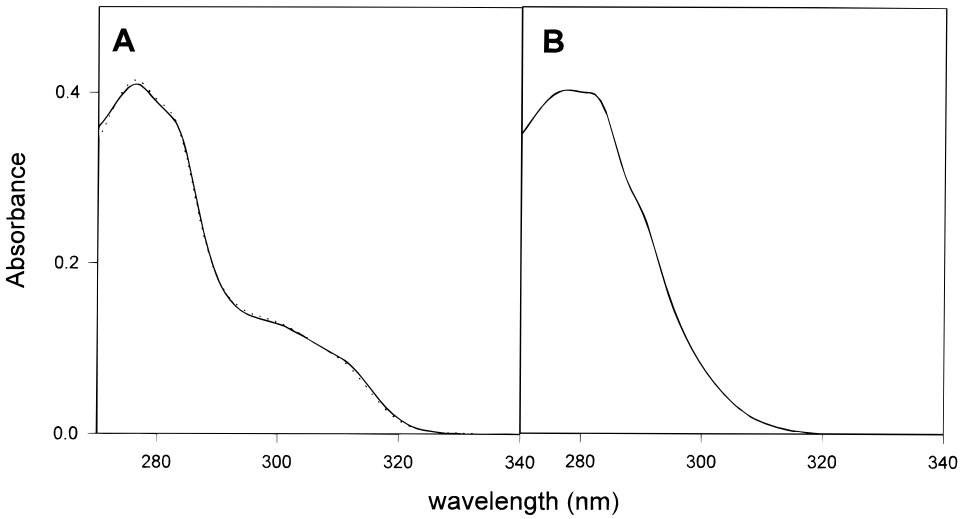


FIGURE 2: Absorption spectra of 5OHTrp[Ala 314, 326]$\sigma^{70}$ (A) and wt $\sigma^{70}$ (B). The dotted line in panel A represents the fit using LINCS program (*39, 40*) to the protein absorbance spectrum using the t-boc-5OHTrp, *N*-acetyltryptophan amide, and *N*-acetyltyrosine amide basis spectra. The fit revealed that ~91% of Trp residues were replaced with 5OHTrp in 5OHTrp[Ala 314,326]$\sigma^{70}$. The ratio of Tyr to total Trp from the fit was 5.9 which was in a good agreement with the value of 6.5 expected from the primary sequence data for $\sigma^{70}$ (*55*).

326 are in a nonconserved region of the protein, and Trp 433 and 434 are located in the conserved region 2.3. In the crystal structure of 116−448 fragment of $\sigma^{70}$ (*28*), Trp 433 and 434 are located in an α-helix containing residues from regions 2.3 and 2.4 and appear to be solvent exposed (Figure 1B). To prepare a derivative of $\sigma^{70}$ with specific fluorescence probes in region 2.3, Trp 314 and 326 were replaced with alanine. The resulting [Ala 314, 326]$\sigma^{70}$ mutant was expressed in a tryptophan auxotroph strain in media containing 5OHTrp resulting in a replacement of Trp 433 and 434 with 5OHTrp.

Comparison of the absorption spectra of 5OHTrp[Ala 314, 326]$\sigma^{70}$ (Figure 2A) and the wt $\sigma^{70}$ (Figure 2B) showed efficient incorporation of 5OHTrp. Additional absorbance in the 300−320 nm range characteristic for 5OHTrp residues is apparent in the 5OHTrp[Ala 314,326]$\sigma^{70}$ spectrum. Using a linear combination of spectra analysis (LINCS) (*39, 40*) we estimated that the degree of 5OHTrp incorporation was 91%. The transcriptional activity of 5OHTrp[Ala 314, 326]$\sigma^{70}$ holoenzyme was 80−90% of the wt $\sigma^{70}$ holoenzyme. Replacement of Trp 314 and 326 with alanine and Trp 433 and 434 with 5OHTrp did not affect significantly the overall conformation of the protein as shown by CD measurements (Figure 3A) and sensitivity to proteolytic digestion (Figure 3B). The far-UV CD spectra of 5OHTrp[Ala314, 326]$\sigma^{70}$ and wt $\sigma^{70}$ were identical (Figure 3A). The spectra obtained were characteristic for a protein with a high α-helical content and were very similar to a CD spectrum of a wt $\sigma^{70}$ purified from *E. coli* cells under nondenaturing conditions (*41*). Digestion of both 5OHTrp[Ala 314, 326]$\sigma^{70}$ and wt $\sigma^{70}$ with trypsin resulted in a generation of protease-resistant 40 kDa fragment (Figure 3B). The same characteristic 40 kDa protease-resistant product of trypsin digestion was observed previously for native $\sigma^{70}$ (*42*). Thus, we concluded that 5OHTrp[Ala 314, 326]$\sigma^{70}$ had the biological activity and the conformation very similar to wt $\sigma^{70}$.

*DNA Binding Properties of Region 2.3 of 5OHTrp[Ala 314, 326]$\sigma^{70}$.* The presence of a tryptophan residue in the vicinity of a DNA binding site in the protein often results in quenching of Trp fluorescence upon formation of protein−DNA complex as shown by nucleic acid-binding experiments with model Trp-containing peptides (*43, 44*). We therefore

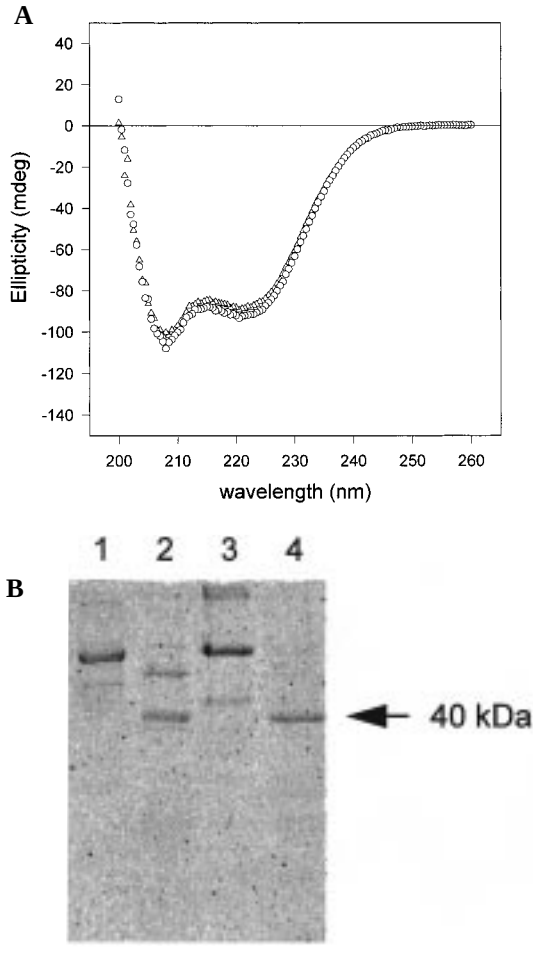


FIGURE 3: (A) Far-UV CD spectra of wt $\sigma^{70}$ (circles) and 5OHTrp-[Ala 314, 326]$\sigma^{70}$ (triangles). The spectra shown are averages of three scans. (B) SDS−PAGE of products of limted proteolysis with trypsin of wt $\sigma^{70}$ (lanes 1 and 2) and 5OHTrp[Ala 314, 326]$\sigma^{70}$ (lanes 3 and 4). Lanes 1 and 3 show untreated proteins, lanes 2 and 4 show protein digested with 10 ng of trypsin for 30 min at room temperature. Positions of 40 kDa protease-resistant band is indicated with an arrow.

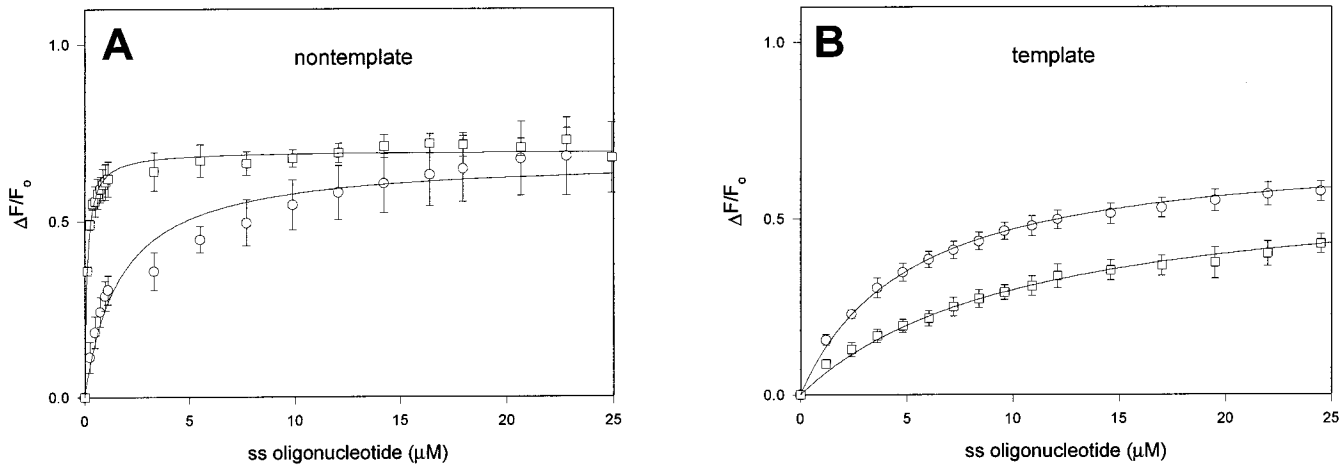


FIGURE 4: DNA binding to the 2.3 region of 5OHTrp[Ala 314,326]$\sigma^{70}$ measured by quenching of 5OHTrp fluorescence. The binding was measured with free (open circles) and core-bound (squares) 5OHTrp[Ala 314,326]$\sigma^{70}$. (A) Binding of a 12-nt ss DNA fragment (TCGTATAATGTG) corresponding to positions −4 to −15 of the nontemplate strand of the *lacUV5* promoter. (B) Binding of a 12-nt ss DNA fragment (CACATTATACGA) corresponding to positions −15 to −4 of the template strand of the *lacUV5* promoter. Solid lines correspond to a nonlinear regression fit of the data to a simple binding equation describing formation of a 1:1 5OHTrp[Ala 314,326]$\sigma^{70}$−DNA complex.

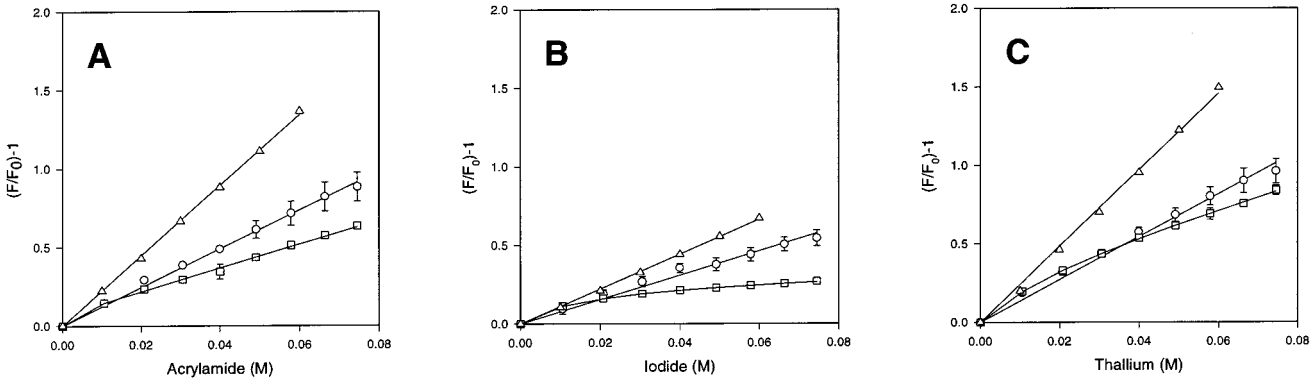


FIGURE 5: Fluorescence quenching of 5OHTrp[Ala 314,326]$\sigma^{70}$ free (circles) and in complex with the core RNAP (squares). Fluorescence quenching of a model compound (NATA, *N*-acetyl-tryptophanamide) which structurally mimics a freely accessible Trp residue involved in a peptide bond is also shown (triangles). The quenching experiments were performed with acrylamide (A), iodide (B), and thallium (C). The data shown are the average of three experiments and the error bars shown correspond to standard deviations. The solid lines are linear regressions to a simple Stern−Volmer equation (Materials and Methods) in the case of free 5OHTrp[Ala 314,326]$\sigma^{70}$. In the case of the holoenzyme, the lines are shown to guide the eye only.

expected that if region 2.3 is involved in ss DNA binding we should be able to detect the ss DNA binding to region 2.3 by quenching of fluorescence of 5OHTrp at positions 433 and 434 in the presence of ss DNA. Figure 4A shows a result of a titration of free 5OHTrp[Ala 314, 326]$\sigma^{70}$ and 5OHTrp[Ala 314, 326]$\sigma^{70}$ holoenzyme with a 12-nt ss oligonucleotide corresponding to −4 to −15 sequence of the nontemplate strand of *lacUV5* promoter. With an increase of ss DNA concentration, the fluorescence was quenched in a manner characteristic for complex formation between DNA and 5OHTrp[Ala 314, 326]$\sigma^{70}$. The maximal quenching was ∼65% both for free and the core-bound protein. Figure 4B shows results of similar titration but now with a 12-nt ss oligonucleotide corresponding to −15 to −4 sequence of the template strand of *lacUV5* promoter. As in the case of the nontemplate strand, 60−70% of fluorescence quenching was observed in the presence of ss DNA. However, a striking difference between the data in Figure 4 is that, in the case of the nontemplate strand, the binding was much tighter for the holoenzyme, whereas in the case of template strand, the binding was weak for free $\sigma^{70}$ and became even slightly weaker for the holoenzyme. The binding was specific for ss DNA since in the presence of ds DNA only small quenching of fluorescence was observed (not shown). The experimental data in Figure 4 could be fitted to an equation describing a simple 1:1 complex formation between $\sigma^{70}$ and ss DNA. Table 1 shows values of apparent dissociation constants for complexes of $\sigma^{70}$ with nontemplate and template strands, with nonspecific oligonucleotide in which all conserved A's and T's of the nontemplate strand were replaced with C's and G's and with randomized nontemplate strand (the same base composition as the nontemplate oligonucleotide but random sequence). All four oligonucle-

Table 1: Apparent Dissociation Constants for Binding of ss Oligonucleotides to Free $\sigma^{70}$ ($K_{free}$) and Core-Bound $\sigma^{70}$ ($K_{holo}$)

| ss oligonucleotide | $K_{free}$ ($\mu$M) | $K_{holo}$ ($\mu$M) |
|---|---|---|
| 12-nt nontemplate[b] | 1.7 (1.1; 3.2)[a] | 0.05 (0.03; 0.13) |
| 12-nt template[c] | 5.0 (4.6; 5.6) | 10.5 (9.1; 12.5) |
| 12-nt "nonspecific"[d] | 2.0 (1.5; 2.7) | 11.0 (9.3; 14.0) |
| 12-nt nontemplate randomized[e] | 10.3 (7.8; 13.9) | 17.6 (11.1; 30.0) |

[a] The numbers in paranthesis correspond to 95% confidence limits of the equilibrium constant. [b] TCGTATAATGTG. [c] CACATTATACGA. [d] TCGCGCGGCGTG. [e] TTGATATCGTAG.

Table 2: Solute Fluorescence Quenching Parameters for 5OHTrp[Ala 314, 326]$\sigma^{70}$

| quencher | Stern−Volmer quenching constant, $K_{SV}$ ($M^{-1}$)[a] | bimolecular quenching constant, $k_q$ ($M^{-1}$ $s^{-1}$)[b] | relative quenching constant, $K_{rel}$[c] |
|---|---|---|---|
| acrylamide | 12.3 | $4.2 \times 10^9$ | 1.00 |
| iodide | 7.7 | $2.7 \times 10^9$ | 1.30 |
| thallium | 13.6 | $4.7 \times 10^9$ | 0.99 |

[a] Stern−Volmer quenching constants were calculated using eq 2 (Materials and Methods). [b] Bimolecular quenching constants were calculated using eq 3 (Materials and Methods). The intensity averaged fluorescence lifetime (2.9 ns) for 5OHTrp[Ala 314, 326]$\sigma^{70}$, measured as described in Materials and Methods, was used in theses calculations. [c] Relative quenching constants were calculated using eq 4 (Materials and Methods).

otides bind free $\sigma^{70}$ with a similar micromolar affinity. The template strand, the nonspecific oligonucleotide, and the randomized nontemplate oligonucleotide bind the holoenzyme with a similar affinity and in each case slightly worse then the free $\sigma^{70}$. In contrast, nontemplate strand binds the holoenzyme very tightly, with an affinity ~200-fold higher compared to the binding of template, nonspecific, and randomized nontemplate oligonucleotides to the holoenzyme and with affinity ~40 higher compared to the binding of all oligonucleotides to the free $\sigma^{70}$. Thus, free $\sigma^{70}$ is unable to discriminate between different ss DNA sequences, whereas the holoenzyme shows high selectivity for binding the nontemplate strand.

*Solvent Exposure of Region 2.3 in Free and Core-Bound 5OHTrp[Ala 314, 326]$\sigma^{70}$.* It has been previously suggested that duplex DNA binding properties of regions 2.4 and 4.2 of $\sigma^{70}$ could be regulated by the core RNAP through a steric "unmasking" of these regions induced by $\sigma^{70}$−core RNAP interactions (*45*, *46*). Since region 2.3 is adjacent to region 2.4, a similar mechanism could be involved in the regulation of ss DNA binding properties of region 2.3 by the core RNAP. Thus, we have measured solvent accessibility of Trp 433 and 434 in free and core-bound $\sigma^{70}$ using fluorescence quenching experiments (*47*). These experiments were performed with neutral quencher (acrylamide; Figure 5A), negatively charged quencher (iodide; Figure 5B), and positively charged quencher (thallium; Figure 5C). The Stern−Volmer plots in the case of free $\sigma^{70}$ (Figure 5, circles) were linear. Stern−Volmer quenching constants and bimolecular quenching constants were calculated from these plots (Table 2). The bimolecular quenching constant of $4.2 \times 10^9$ $M^{-1}$ $s^{-1}$ for acrylamide quenching is characteristic for a fully solvent-accessible Trp residue (*48*) and thus shows that in free 5OHTrp[Ala 314, 326]$\sigma^{70}$ Trp 433 and 434 are exposed to the solvent. These residues show preference for quenching with a negatively charged quencher (iodide), as shown by comparing the relative quenching constants (*49*, *50*) for iodide and thallium (Table 1).

The Stern−Volmer plots for 5OHTrp[Ala 314, 326]$\sigma^{70}$ bound to the core RNAP were significantly different than for the free 5OHTrp[Ala 314, 326]$\sigma^{70}$ and were nonlinear. Since the quantum yield (and thus most likely the averaged fluorescence lifetimes) was not affected by 5OHTrp[Ala 314, 326]$\sigma^{70}$−core interaction, the interaction with the core RNAP had apparently changed the solvent exposure of at least one of the tryptophan residues of 5OHTrp[Ala 314, 326]$\sigma^{70}$. Due to the limited accuracy and the limited range of quencher

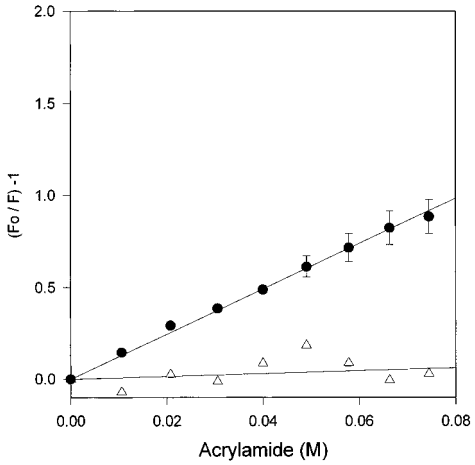


FIGURE 6: Comparison of fluorescence quenching of 5OHTrp residues with acrylamide in free 5OHTrp[Ala 314, 326]$\sigma^{70}$ (filled circles) and in 5OHTrp[Ala 314, 326]$\sigma^{70}$ in complex with a 12-nt nontemplate ss DNA fragment (open triangles). Solid lines correspond to a linear fit to a simple Stern−Volmer equation (eq 1). The Stern−Volmer constants were 12.3 and 0.8 $M^{-1}$ for free and DNA-bound 5OHTrp[Ala 314, 326]$\sigma^{70}$, respectively.

concentration used, the quantitative analysis of nonlinear Stern−Volmer plots for the holoenzyme was not possible, but it is apparent from the data in Figure 5 that Trp 433 and (or) 434 became less solvent exposed in the holoenzyme. We have previously observed in preliminary experiments a similar decrease in solvent exposure in wt $\sigma^{70}$ substituted with 5OHTrp upon binding of wt $\sigma^{70}$ to the core RNAP (*51*). Now, using 5OHTrp[Ala 314, 326]$\sigma^{70}$ this decrease in solvent accessibility can be specifically assigned to residues 433 and 434. Decreased solvent exposure in core-bound $\sigma^{70}$ is observed with all three quenchers. The nonlinearity of Stern−Volmer plots in the case of the holoenzyme suggests that the environments of Trp 433 and Trp 434 were affected to a different extent by core binding, consistent with protein−DNA cross-linking data of Huang et al. (*15*).

Solvent accessibility of Trp 433 and 434 was also determined for $\sigma^{70}$ in complex with ss DNA. The pronounced quenching of 5OHTrp fluorescence observed in the presence of ss DNA, although indicative of the close proximity of bound ss DNA to these residues, does not directly prove it. We expected that if these Trp residues are located in ss DNA binding site of $\sigma^{70}$, they should become inaccessible or less accessible to the solvent in the presence of saturating concentrations of ss DNA. Figure 6 shows that this indeed was a case.

*Fluorescence Detection of ss DNA−Region 2.3 Contact Formation in the Open Complex.* Preferential quenching of 5OHTrp fluorescence in 5OHTrp[Ala 314, 326]$\sigma^{70}$ holoenzyme by the nontemplate ss oligonucleotide suggested that a similar quenching should be also observed upon mixing of RNAP and promoter DNA under conditions allowing open complex formation. Figure 7 shows the results of a kinetic experiment in which fluorescence intensity of 5OHTrp at positions 433 and 434 was monitored as a function of time after the addition of 114 bp fragment of *lacUV5* promoter DNA. These experiments were performed at lower temperatures (20 and 10 °C) such that the time scale of open complex formation would be compatible with the manual mixing of the reagents. Fluorescence was quenched upon addition of the promoter DNA and the decrease in fluores-

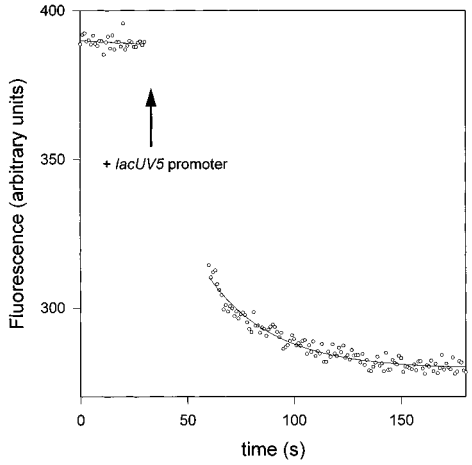


FIGURE 7: Detection of a ss DNA region 2.3 of 5OHTrp[Ala 314, 326]$\sigma^{70}$ contact formation during "closed" to "open" complex isomerization of the *lacUV5* promoter. Fluorescence of 190 nM 5OHTrp[Ala 314, 326]$\sigma^{70}$ holoenzyme (excitation at 313 nm, emission at 334 nm, 20 °C) was measured as a function of time. At a point indicated by an arrow, 290 nM 114-bp *lacUV5* promoter DNA fragment was added. The recording of fluorescence signal was resumed ~30 s after adding the DNA. The solid line for the data after DNA addition corresponds to a nonlinear regression fit of the data to an equation describing a kinetic process involving a single first-order rate constant [$F = F_0 + \Delta F^*(1 - \exp(-kt)]$, where $F$ is the fluorescence signal, $F_0$ is a signal at $t = 0$, $\Delta F$ is an amplitude of fluorescence signal change, and $k$ is the rate constant. The fitted rate constants was $4.4 \times 10^{-2}$ s$^{-1}$.

cence as a function of time could be fitted to a single-exponential decay equation. The rate constants obtained from this analysis were 0.032 and 0.044 s$^{-1}$ at 10 and 20 °C, respectively. For comparison, the value of the first-order rate constant of open complex formation previously determined at 20 °C was 0.01 s$^{-1}$ for wt $\sigma^{70}$ (*52*). The reasonably close correspondence of the rate constants at 20 °C suggests that indeed a ss DNA contact involving region 2.3 forms concomitant with the open complex formation. This contact can be detected by fluorescence quenching of 5OHTrp at positions 433 and 434, and thus, it is very likely that the ss oligonucleotide binding detected by fluorescence quenching corresponds to a biologically important ss DNA−$\sigma^{70}$ binding event.

## DISCUSSION

The results presented in this paper show that a ss DNA−$\sigma^{70}$ interaction can be detected using a fluorescence signal of specific fluorescence probes incorporated into region 2.3 of $\sigma^{70}$. Fluorescence of 5OHTrp at positions 433 and 434 of region 2.3 was quenched by ss DNA, and these residues became inaccessible to the solvent in $\sigma^{70}$−ss DNA complex. Thus, our data confirm earlier proposals that such a ss DNA binding site is present in $\sigma^{70}$ (*5*) and that it is located in region 2.3 of the protein (*3*, *5*, *13*−*15*, *24*, *26*, *28*). Our data also showed that, in the case of the holoenzyme, interaction with ss DNA was specific for the nontemplate strand of promoter DNA as previously observed (*9*−*15*). Additionally, our data allowed insights into how this nontemplate specificity in the case of the holoenzyme is accomplished. It appears that the nontemplate specificity is a result of an allosteric modification of $\sigma^{70}$ DNA binding properties by $\sigma^{70}$−core interaction. Core RNAP decreased $\sigma^{70}$ affinity for the template strand and increased $\sigma^{70}$ affinity for the nontemplate strand, resulting in ~200-fold discrimination between nontemplate and template strands. A core-induced conformation change in $\sigma^{70}$ is involved in regulation of ss DNA binding activity of $\sigma^{70}$. It has been shown previously that $\sigma^{70}$ ability to recognize ds −10 and ds −35 DNA sequences is also regulated by core RNAP (*45*, *46*). Using fragments of $\sigma^{70}$ protein, Dombroski et al. (*45*) showed that conserved region 1 is most likely responsible for this regulation. It was proposed that, in free $\sigma^{70}$ protein, region 1 is positioned such that it sterically blocks the DNA binding domains of $\sigma^{70}$ (regions 2.4 and 4.2), preventing free $\sigma^{70}$ from binding to promoter DNA. Interaction with the core RNAP was suggested to induce a conformational change in the $\sigma^{70}$ protein in which region 1 moved away and unmasked DNA binding regions. It was also proposed that Trp 434 may directly participate in interacting with region 1 (*53*). Our results show that most likely the ss DNA binding properties of region 2.3 are not regulated by a simple steric occlusion mechanism involving region 1. This conclusion is based on the observation that interaction with the core RNAP results in a *decrease* of solvent exposure at region 2.3, contrary to the expectation if a steric unmasking of this region would be induced by the core RNAP. Thus, some other conformational changes in this region of $\sigma^{70}$ induced by the core RNAP have to be involved in the regulation of ss DNA binding properties of the protein. This conclusion is consistent with the observation by Severinova et al. (*13*) that 114−448 fragment of $\sigma^{70}$ (lacking region 1) was able to bind ss DNA only when bound to the core RNAP. It was also proposed that the autoinhibition of $\sigma^{70}$ DNA binding could be due to a presence of a disordered acidic loop (residues 192−211) in a cleft containing residues from region 2.3 and 2.4 (*28*). The presence of this highly negatively charged loop near or at the DNA binding site of $\sigma^{70}$ would inhibit $\sigma^{70}$ interaction with negatively charged DNA. This mechanism is also unlikely since we observe that Trp 433 and 434 are solvent exposed in free $\sigma^{70}$ and are preferentially quenched by negatively charged quencher, iodide. Both observations are inconsistent with the presence of a highly negatively charged loop in the vicinity of Trp residues.

Thus, the exact nature of core-induced conformational change in region 2.3 remains to be established. A possible functional role for the observed decrease in solvent accessibility of region 2.3 induced by core RNAP could be the improved specificity of $\sigma^{70}$ DNA binding site for ss DNA. Single-stranded and double stranded DNA differ significantly in their diameters and one simple mechanism to facilitate discrimination between these two forms of DNA could be to restrict the accessibility of the binding site to molecules of the size compatible with ss DNA only.

In summary, the currently available experimental data clearly suggests that a favorable interaction between region 2.3 of $\sigma^{70}$ and the nontemplate strand of the transcription bubble is an important element of "closed" to "open" complex isomerization. We could directly detect the $\sigma^{70}$−ss nontemplate strand interaction during "closed" to "open" complex isomerization using the fluorescence signal of 5OHTrp at positions 433 and 434 (Figure 7). The function of this interaction would be to lower the energetic cost of promoter melting. Preparation of $\sigma^{70}$ fluorescent derivative which allows specific detection of a nontemplate ss DNA−$\sigma^{70}$ contact formation opens possibilities for in-depth studies

of the role of this contact in the "open" complex formation. The experiments in which this fluorescent derivative is used to study the thermodynamics of ss DNA−$\sigma^{70}$ interaction and the kinetics of ss DNA−$\sigma^{70}$ contact formation are currently being pursued in our laboratory.

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