Mapping the Promoter DNA Sites Proximal to Conserved Regions of σ^{70} in an *Escherichia coli* RNA Polymerase−*lac*UV5 Open Promoter Complex†

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ABSTRACT: Base-specific interactions between promoter DNA and *Escherichia coli* RNA polymerase are regulated by a sigma (σ) protein during transcription initiation. To map spatial relations between evolutionarily conserved regions of the primary sigma (σ^{70}) and each DNA strand along the *lac*UV5 promoter in the transcriptionally active "open" complex, we have used a cysteine-tethered cutting reagent to cleave DNA strands. The chemical nuclease FeBABE [iron (*S*)-1-(*p*-bromoacetamidobenzyl)-ethylenediaminetetraacetate] was conjugated to single-cysteine mutants of σ^{70} at sites 132C, 376C, 396C, 422C, 496C, 517C, or 581C. After formation of open promoter complexes between *lac*UV5 DNA and RNA polymerase holoenzymes carrying conjugated σ^{70} subunits, we observed promoter DNA cleavage spanning at least 60 bases, between positions −48 and +12. The results show that σ^{70} region 2.1, otherwise implicated in core enzyme binding, is proximal to the nontemplate strand of *lac*UV5 DNA between the −10 promoter element and positions as far downstream of the transcription start site as +12. Conserved region 3.2 of σ^{70} is proximal to the template strand near the +1 transcription start site, and region 3.1 is positioned between the *lac*UV5−10 and −35 promoter elements. We propose a model for the orientation of σ^{70} and DNA in the open complex.

The binding of σ to the RNA polymerase core enzyme (subunit composition $\alpha_2\beta\beta'$) to form the holoenzyme is the first step in bacterial gene transcription (*1*−*4*). The $\alpha_2\beta\beta'$ core enzyme can catalyze RNA elongation, but a σ subunit is required for DNA promoter recognition and transcription initiation. In *Escherichia coli*, seven different species of σ subunits are presently known, each governing transcription from different sets of promoters (*4*). The primary sigma subunit (σ^{70}) of *E. coli* RNA polymerase is responsible for the transcription of most genes expressed during exponential cell growth (*2, 4, 5*). The $\alpha_2\beta\beta'\sigma^{70}$ holoenzyme recognizes two oligonucleotide sequences in *E. coli* promoters, a −10 region (consensus hexamer TATAAT) located approximately 10 bp upstream from the start point of transcription, and a −35 region (consensus hexamer TTGACA) (*6*−*8*). The −10 and −35 promoter elements are separated by approximately 17 bp, a distance that is sensed by the σ^{70} subunit (*9*).

After RNA polymerase binding, the DNA strands are separated to form an "open" promoter complex, capable of binding and polymerizing ribonucleotides for RNA synthesis. Although the mechanism of open complex formation is not resolved, important steps include binding of σ^{70} conserved region 4.2 to the −35 DNA promoter element and region 2.4 to the −10 promoter element, as well as base-specific interactions between the nontemplate strand of DNA and σ conserved region 2.3 to stabilize the open promoter complex (*10*−*14*). The untwisting and wrapping of the spacer DNA (between the −10 and −35 elements) around RNA polymerase may serve to destabilize the DNA duplex near the −10 element, where strand separation begins (*13*).

To explore spatial relations between the conserved regions of σ^{70} and each DNA strand along the *lac*UV5 promoter, we employed DNA cleavage by the cysteine-tethered chemical nuclease FeBABE (*15, 16*).[1] This approach allows for the identification of DNA sites proximal to cutting probes tethered in or near conserved regions of σ^{70}, within the open promoter complex. We introduced single cysteine residues into σ^{70} after first replacing native Cys residues at positions 132, 291, and 295 with serine.

The RNA polymerase-*lac*UV5 open complex was chosen for this study because it is well characterized and is stable at 37 °C in the absence of ribonucleoside triphosphates (*17*−

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[1] Abbreviations: FeBABE, iron (*S*)-1-(*p*-bromoacetamidobenzyl)-ethylenediaminetetraacetate. FeBABE also denotes the chelate moiety conjugated to a cysteine residue.

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23). The midpoint of the transition from closed to open complex for the *lac*UV5 promoter is 22.5 °C, and the complex is completely open above 30 °C (*18*). In the RNA polymerase−*lac*UV5 open complex containing Mg^{2+}, the DNA strands are separated, approximately from positions −12 to +3 (*18, 24*−*28*). Well-developed transcriptional assays for this promoter are also available (*29*−*31*), which allow comparisons of the activity of mutant and conjugated proteins on the same template.

EXPERIMENTAL PROCEDURES

Materials. *Bam*HI and *Hin*dIII restriction enzymes were purchased from Promega. Ascorbic acid (vitamin C, microselect grade) was purchased from Fluka, and hydrogen peroxide (ultrex grade) from J. T. Baker.

Buffers. *Protein storage buffer*: 10 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 100 μM EDTA, 1 mM DTT, 50% (v/v) glycerol, and 0.2 M KCl. *Conjugation buffer*: 10 mM MOPS (3-morpholinopropanesulfonate), pH 8, 0.2 M NaCl, 5% (v/v) glycerol, and 2 mM EDTA. *DNA cleavage buffer*: 40 mM HEPES (*N*-2-hydroxypiperazine-*N*-2-ethanesulfonate), pH 8, 10 mM $MgCl_2$, 5% (v/v) glycerol, 0.1 M KCl, and 100 μM EDTA. *Quenching buffer*: 0.1 M thiourea and 100 μg/mL sonicated salmon sperm DNA. *0.5× TBE*: 45 mM Tris-borate and 1 mM EDTA. *TE*: 10 mM Tris-HCl (pH 8) and 1 mM EDTA (pH 8).

Preparation of End-Labeled lacUV5 Promoter DNA. The *Bam*HI (−127)-*Hin*dIII (+58) fragment of pLAC12 carrying the *lac*UV5 promoter (*28*) was either 3′ or 5′ end-labeled with ^{32}P at its *Hin*dIII end as described (*32*). Labeled promoter fragments were purified by electrophoresis on 5% nondenaturing polyacrylamide gels at 4 °C using 0.5× TBE at 200 V and isolated by the crush and soak method.

Site-Directed Mutagenesis. Single-cysteine mutants (132C, 376C, 396C, 422C, 496C, 517C, and 581C) of σ^{70} were prepared and expressed as described previously (*33*). Each purified single-Cys mutant σ^{70} protein was characterized for transcriptional activity, studied by circular dichroism, conjugated to FeBABE, and assayed for conjugation yield (*33*).

Transcriptional Activity of Mutant and Conjugated σ^{70} Proteins. Transcription factor independent single round runoff transcription assays from the *lac*UV5 promoter were performed as described (*34*−*36*) both before and after FeBABE conjugation. Briefly, holoenzymes were formed by incubation of native core RNA polymerase (1 pmol, 100 nM) with σ^{70} (2 pmol, 200 nM) at 30 °C for 15 min. Open complexes were formed by the addition of *lac*UV5 template (0.3 pmol, 8 nM) and incubating at 37 °C for 15 min. Transcription was initiated by the addition of heparin (200 μg/mL) and 160 μM each of ATP, CTP, GTP, and 50 μM UTP containing 2 μCi [α-^{32}P]UTP (400 Ci/mmol) and allowed to proceed for 5 min at 37 °C. Reactions were quenched, precipitated, electrophoresed on an 8% sequencing gel, and the 63 nt RNA transcript was visualized by film autoradiography. Transcriptional activities relative to wild-type σ^{70} were measured by densitometry and averaged for triplicate experiments, which agreed within ∼20%. Since the conjugation yields were not 100%, the activity a_{tot} of the mixture after conjugation is the weighted sum of the activities contributed by both species:

$$a_{tot} = (1 - f_{unc})a_{con} + f_{unc}a_{unc} \quad (1)$$

where f_{unc} is the fraction unconjugated (*33*), a_{unc} is the activity of the unconjugated mutant, and a_{con} is the activity of the conjugate.

Binary Open Complex Formation. RNA polymerase core enzyme was purified from *E. coli* W3350 cells and assayed for transcriptional activity (*37, 38*). Holoenzymes were prepared by incubation of mutant σ^{70} proteins (100 nM final) with $\alpha_2\beta\beta'$ core enzyme (25 nM final) at 30 °C for 10 min in DNA cleavage buffer as described (*39*). Binary open promoter complexes of RNA polymerase and *lac*UV5 DNA were prepared essentially as described (*21, 22, 26*), by the addition of ^{32}P-end-labeled *lac*UV5 promoter (5 nM final) followed by incubation at 37 °C for 15 min. To remove nonspecifically bound RNA polymerase, heparin (50 μg/mL final) was added 3 min before DNA cleavage reactions were initiated.

DNA Cleavage of the lacUV5 Promoter. DNA cleavage by RNA polymerase holoenzymes carrying FeBABE-modified σ^{70} proteins was initiated by the rapid sequential addition of sodium ascorbate (pH ∼7, 2 mM final), and hydrogen peroxide (1 mM final) to the heparin-containing open binary complexes described above. Reactions (90 μL total) were allowed to proceed at 37 °C for 10 min before quenching with 30 μL of quenching buffer and 80 μL of TE buffer. Quenched reaction mixtures were extracted with phenol: chloroform, then chloroform, precipitated with ethanol, and electrophoresed on DNA sequencing gels as described (*32*). Gels were dried and visualized by film autoradiography at room temperature.

Molecular Modeling. Atomic coordinates for the σ^{70} tryptic fragment (1SIG) were obtained from the Protein Data Bank. The FeBABE structure was generated in InsightII (Biosym/Molecular Simulations) and fit to the known crystal structure of an analogous iron chelate contained in structure 1INE (*40*). Side chains at positions 132, 376, 396, and 422 were converted to Cys residues, and FeBABE moieties were docked onto these sites within the σ^{70} fragment structure. A composite image depicting these conjugates having minimal steric clashes between probes and σ^{70} protein was then rendered. Positions 496, 517, and 581 are not included in the σ^{70} tryptic fragment and are not modeled here. The *lac*UV5 promoter DNA sequence from −25 to +12 was composed in InsightII, and strand separated from −12 to +3 to depict the single-stranded (open) region (*18, 24*−*28*). The DNA was manually docked onto the σ^{70} fragment in an orientation consistent with our observed DNA cleavage results. Previous studies which report interactions between the −12 A/T base pair and residues Gln-437 and Thr-440 (*41, 42*) and point mutations which result in impaired DNA melting (*43, 44*) were also considered.

RESULTS

As measured in *lac*UV5-directed transcription, Cys(−) σ^{70} retains ∼95% activity relative to wild-type (Figure 1). Single Cys residues were introduced along conserved regions of σ^{70}; no significant difference was observed in the circular dichroism of any of the σ^{70} mutants discussed here (data not shown). The seven single Cys mutants used for DNA cleavage reactions were found to retain 70-95% of wild-

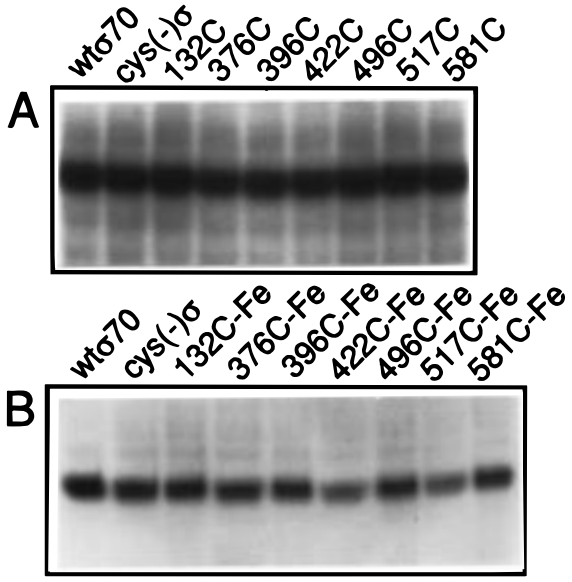

FIGURE 1: *lac*UV5 directed transcriptional activity of mutant σ^{70} proteins. (A) Before conjugation. (B) After FeBABE conjugation.

type transcriptional activity before conjugation with FeBABE. FeBABE conjugation yields of each single Cys protein were 132C, 31%; 376C, 40%; 396C, 48%; 422C, 49%; 496C, 69%; 517C, 72%; and 581C, 67%. After FeBABE conjugation, all conjugates apparently retained transcriptional activity, with the possible exception of 422C−Fe. Using eq 1, the conjugates showed the following activity a_{con} relative to wild-type: 132C−Fe, 50%; 376C−Fe, 30%; 396C−Fe, 30%; 422C−Fe, 10%; 496C−Fe, 50%; 517C−Fe, 30%; and 581C−Fe, 30%. Because of experimental variations, these measurements are only accurate to ±20%.

Nontemplate Strand DNA Cleavage. Proximity cleavage of the *lac*UV5 nontemplate strand by holoenzymes carrying FeBABE-modified σ^{70} was found to vary both in position and intensity along the entire promoter (Figure 2). At the C-terminal edge of conserved region 1.2 of σ^{70}, FeBABE positioned on 132C produced very faint but reproducible DNA cleavage in two areas near the −10 promoter element (easily visible upon longer film exposure). This is consistent with genetic evidence (*45*) which made use of amino-terminal σ^{70} deletions to determine that region 1.2 is required for DNA strand separation during open complex formation.

At the N-terminal edge of σ^{70} conserved region 2.1 (previously implicated in σ-core interactions), FeBABE positioned on 376C cuts the nontemplate strand of DNA in the −10 promoter region (between −10 and −14) with moderate yield. At the C-terminal edge of conserved region 2.1, FeBABE positioned on 396C cuts DNA from −3 to +4 within the open (strand-separated) region and also (faintly) as far downstream as +12. This is the farthest downstream cutting observed.

Within conserved region 2.3, FeBABE positioned on 422C (previously implicated in DNA strand separation) cuts between −13 and −8. FeBABE positioned on 496C of σ^{70} (conserved region 3.1) cuts the nontemplate strand of DNA with high yield along the spacer region between the −10 and −35 promoter elements, from −12 to −28. FeBABE conjugated to 517C, in the polymerase core binding region 3.2, was found to cut DNA within the strand-separated region from −3 to +4 with low yield. FeBABE positioned on

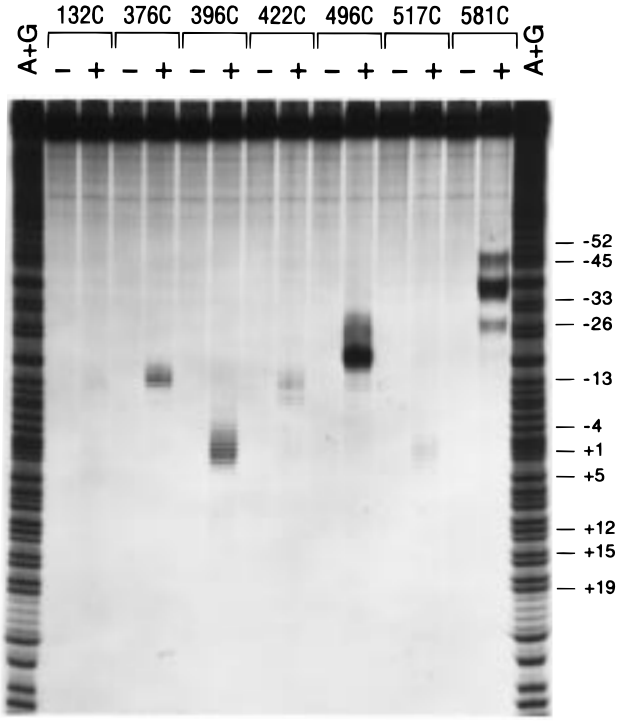

FIGURE 2: Nontemplate strand *lac*UV5 DNA cleavage within the open promoter complexes by holoenzymes carrying FeBABE-modified σ^{70} proteins. Positions of the single Cys residues are indicated across the top of the figure. Cleavage reactions containing FeBABE modified σ^{70} proteins are marked +, and those without the probe are marked −. All samples (whether conjugated with FeBABE or not) were treated identically with ascorbate and hydrogen peroxide. Maxam−Gilbert A+G lanes are shown along each side of the gels. DNA sequencing gels were visualized by film autoradiography.

581C, located in conserved region 4.2 (−35 DNA binding) was found to cut promoter regions near −16 (faint), −25 to −27 (medium), −34 to −40 (strong), and −45 to −48 (medium). This is the farthest upstream cutting observed.

Template Strand DNA Cleavage. Cleavage of the *lac*UV5 template strand (Figure 3) by holoenzymes carrying FeBABE-modified σ^{70} proteins differed in both intensity and position from cleavage of the nontemplate strand. In particular, the relative yield of template strand cleavage by FeBABE positioned on 376C and 396C was much lower than that observed for the nontemplate strand. In contrast, we find *stronger* cutting of the template strand in the strand-separated region by FeBABE attached to 517C (region 3.2).

FeBABE positioned on 132C very faintly yet reproducibly cuts the template strand near position −18. Faint cleavage was observed between positions −16 to −20 for FeBABE-modified 376C. Two areas of the template strand were also faintly cut by FeBABE positioned on 396C: between −5 and −12 and between +6 and +9. FeBABE tethered to 422C cut the template DNA strand between positions −14 and −19 with low yield.

Intense DNA cleavage was observed for FeBABE-modified 496C along the spacer between the −10 and −35 promoter elements, from positions −14 to −31. Within the strand-separated region of the promoter near the +1 start site, FeBABE positioned on 517C produced strong DNA cleavage of the template strand from −7 to +2. Finally, FeBABE-modified 581C cuts the template strand from −27

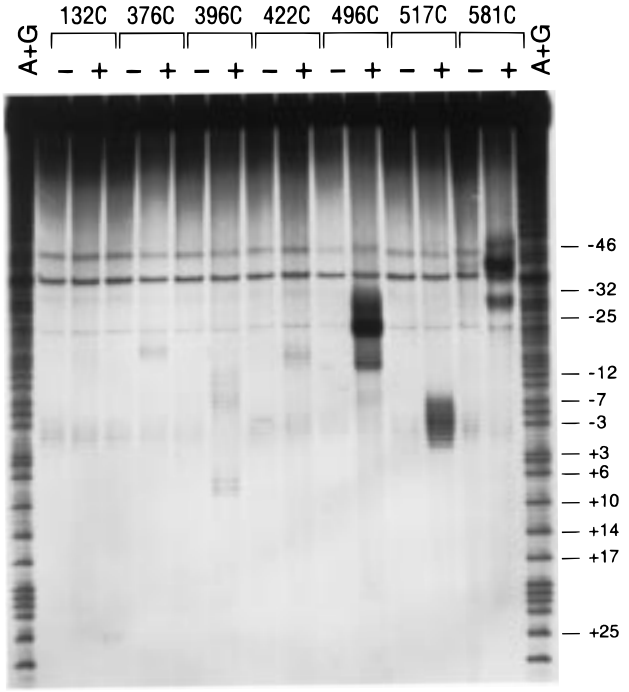

FIGURE 3: Cleavage of the template strand of *lac*UV5 promoter DNA (conditions and format as in Figure 2).

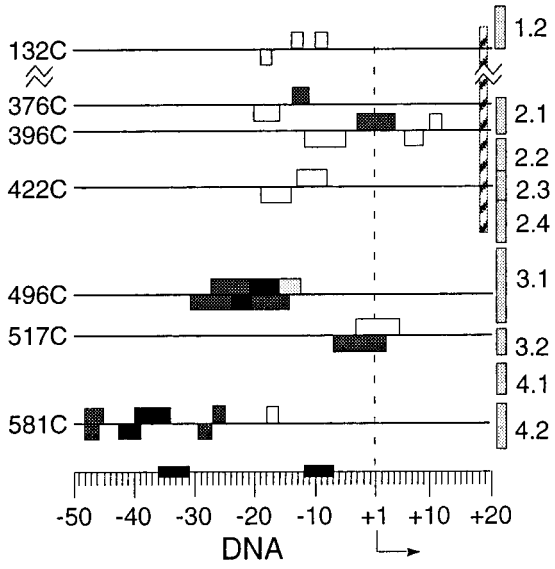

FIGURE 4: Summary of *lac*UV5 DNA cleavage results. Boxes above horizontal lines indicate nontemplate (top strand) cleavage. Boxes below the horizontal lines indicate template (bottom strand) cleavage. Darkness of the boxes indicates cleavage efficiency observed by film autoradiography. Numbered vertical bars along the right indicate conserved regions of σ^{70}. The crystallized fragment of σ^{70} is indicated by a striped vertical bar along the right. FeBABE probes were positioned at sites listed vertically along the left. Positions along the DNA template, including the +1 start site of transcription and the −10 and −35 promoter elements, are indicated along the bottom of the figure.

to −30 (medium), −39 to −43 (strong), and −46 to −48 (medium).

DISCUSSION

The retention of transcriptional activity after replacement of native Cys residues with serine, as well as the preservation of the overall structure of σ^{70} as indicated by circular dichroism, suggests that these residues are not essential to the structure and function of the σ^{70} subunit. Circular dichroism has previously been used to detect mutations in region 1.1 that disrupt σ structure (*46*); however, none of the mutants in this study show such alteration. Of the σ^{70} mutants constructed, mutagenesis and FeBABE conjugation at residue 422 had the greatest impact on transcriptional activity. Since σ^{70} position 422 is located close to the promoter −10 recognition helix 14 (Figure 5), it is credible that this mutant is the least active in the set.

As summarized in Figure 4, both DNA strands in the *lac*UV5 promoter are within cutting range [∼12 Å from the Cys sulfur, plus the 3−4 Å diffusion distance of the actual cutting species (*47*)] of conserved regions 1.2, 2.1, 2.3, 3.1, 3.2, and 4.2 of σ^{70}. The DNA cleavage data in Figure 2 and Figure 3 indicate that conserved regions of σ^{70} are proximal to specific regions along the entire promoter, spanning 60 bases between positions −48 and +12 (Figure 4).

The results demonstrate that σ^{70} conserved region 2.1, previously implicated in σ-core interactions, preferentially interacts with the *nontemplate* strand of the *lac*UV5 promoter between the −10 element and positions downstream of the transcription start site near +12 (Figures 2−4).

Conserved region 3.2 of σ^{70} is near each DNA strand within the strand-separated region near the +1 transcription start site (with a strong preference for the template strand). And σ^{70} region 3.1 is positioned between the *lac*UV5−10 and −35 promoter elements (with similar cleavage of each strand). Region 4.2 of σ^{70} is near the −35 promoter region, which agrees with previous genetic and biochemical studies (*3*, *8*, *14*, *40*, *46*−*48*).

These results indicate that not only the previously identified DNA-binding regions 2.3, 2.4, and 4.2 but also conserved regions 1.2, 2.1, 3.1, and 3.2 of σ^{70} are located close to specific regions along the *lac*UV5 promoter. Previous work (*14*, *50* and references therein) has suggested that the primary interactions between σ^{70} and DNA occur in the *nontemplate* strand near the −10 promoter element. Our results corroborate this, and also provide a picture relating the positions of conserved σ^{70} regions within the transcription complex to each DNA strand along the *lac*UV5 promoter. This leads to a partial model depicting certain σ^{70}-DNA spatial orientations within the *lac*UV5 open promoter complex (Figure 5) based on our DNA cleavage data, the existing σ^{70} fragment crystal structure (*51*), and information from the σ literature.

In general, this study supports previous studies (*2*, *3*, *8*, *14*, *50*, *52*) which indicate that region 2 is located near the −10 DNA promoter element, region 3 is near +1, and region 4 is located near the −35 promoter element. Of the four FeBABE conjugates which could be positioned on the crystallized σ^{70} fragment, we have placed the +1 DNA site near the 396C−FeBABE conjugate (C-terminal edge of region 2.1) due to the strong DNA cleavage of the nontemplate strand in this region (Figure 2). The upstream edge of the strand-separated region was placed near the 132C−FeBABE probe, which agrees with a recent genetic study indicating that region 1.2 is required for open complex formation (*45*). Position −12 of the promoter was oriented near the 376C−FeBABE probe (N-terminal edge of region 2.1), in general agreement with previous models of the open-

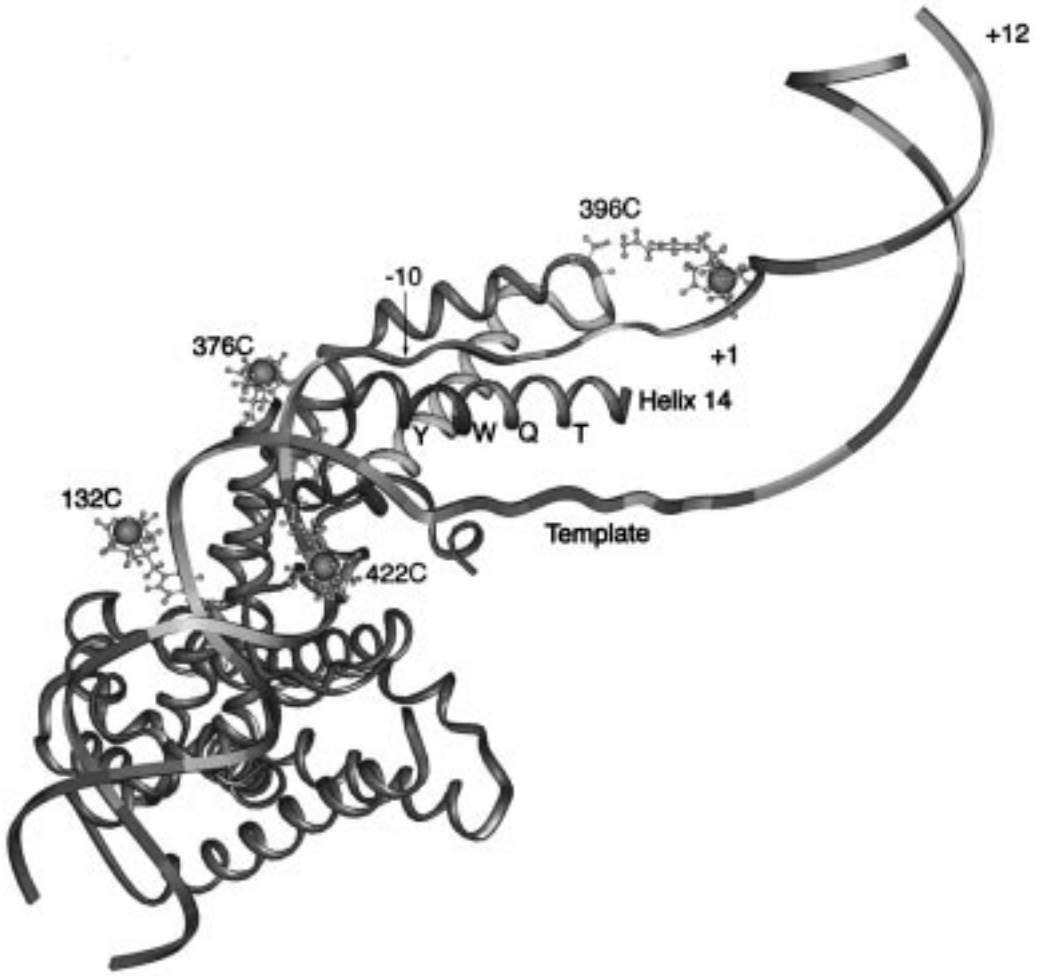

FIGURE 5: Proposed model of σ^{70}-DNA spatial orientations within the open promoter complex along the −25 to +12 region of the *lac*UV5 promoter. The σ^{70} fragment (*51*) is shown as a composite structure having FeBABE positioned at four sites: 132, 376, 396, and 422. Conserved regions of σ^{70} are indicated: region 1.2, red; 2.1, green; 2.2, yellow; 2.3, cyan; 2.4, orange. The DNA bases are colored red, adenine; blue, guanine; yellow, cytosine; green, thymidine. The DNA strands are separated from −12 to +3 and oriented to coincide with the cleavage data. Amino acids on helix 14 are identified by single letter code: Tyr-430, Y; Trp-433, W; Gln-437, Q; Thr-440, T.

promoter complex (*50, 52*). Our model also places −12 near the 422C−FeBABE probe, which locates residues Tyr-430 and Trp-433 near DNA residues at −11/−10. This is consistent with reports that identify point mutations at residues Tyr-430 and Trp-433 which render σ^{70} defective in nucleation of strand separation (*43, 44*).

When the *lac*UV5 promoter is juxtaposed to the σ^{70} fragment in a manner consistent with the cleavage data for each strand, the orientation of σ^{70} helix 14 (residues 427−444) relative to DNA differs significantly from that proposed earlier (*51*). With respect to the nontemplate DNA strand, σ^{70} residues Gln-437 and Thr-440 are located *downstream* relative to Tyr-430 and Trp-433. It may be that the interactions of Gln-437 and Thr-440 with DNA residue −12 are separated in time from the interactions of Tyr-430 and Trp-433 with DNA residues −11/−10.

The uncrystallized regions of σ^{70} (most notably residues 1−113 and 449−613) are not included in Figure 5, nor are the other RNA polymerase subunits $\alpha_2\beta\beta'$, which may exert steric hindrance to affect the DNA cleavage patterns. In addition, the actual extent of FeBABE rotational freedom for each single Cys mutant within the open transcription complex is not known. However, even if the FeBABE probes at either end of helix 12b on 376C and 396C are rotated as close to each other as possible, they do not overlap. The orientation of DNA relative to helix 12b depicted in Figure 5 is clearly indicated by our cleavage data; the orientation of helix 14 follows from the crystal structure.

The model presented in Figure 5 contains no modifications to the original σ^{70} fragment coordinates (1SIG). Minor rotations within the flexible loop connecting helices 13 and 14 would result in a roll of helix 14 to place the aromatic side chains of Tyr-430 and Trp-433 in a more favorable orientation for interaction with the single-stranded bases −10 and −11 of the nontemplate strand, as proposed earlier (*41, 42, 51*).

Internal mapping of RNA polymerase−DNA complexes provides new information about the relationship between σ^{70} and DNA in the open promoter complex. When compared with external footprinting by DNaseI or untethered FeEDTA, which reveals only the perimeter outlined by bound RNA polymerase, the strength of the Cys-tethered FeEDTA mapping strategy becomes clear. More importantly, the same set of Cys-tethered FeBABE probes can be used for mapping both protein−protein (*33*) and protein−nucleic acid spatial orientations within biological complexes. This provides a

route to the elucidation of molecular arrangements in systems that would be difficult to study by other means.

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