Binding of the σ^{70} protein to the core subunits of *Escherichia coli* RNA polymerase, studied by iron–EDTA protein footprinting

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ABSTRACT We have used a nonspecific protein cleaving reagent to map the interactions between subunits of the multisubunit enzyme RNA polymerase (*Escherichia coli*). We developed suitable conditions for using an untethered Fe–EDTA reagent, which does not bind significantly to proteins. Comparison of the cleaved fragments of the subunits from the core enzyme ($\alpha_2\beta\beta'$) and the holoenzyme (core + σ^{70}) shows that absence of the σ^{70} subunit is associated with the appearance of several cleavage sites on the subunits β (within 10 residues of sequence positions 745, 764, 795, and 812) and β' (within 10 residues of sequence positions 581, 613, and 728). A cleavage site near β residue 604 is present in the holoenzyme but absent in the core, demonstrating that a conformational change occurs when σ^{70} binds. No differences are observed for the α subunit.

Gene transcription in living cells is a complex process, with large numbers of protein factors involved in selecting the correct initiation site on DNA and initiating and carrying out RNA synthesis to the appropriate end. The proteins responsible for transcription in both prokaryotic and eukaryotic cells are actively being identified and their functions explored (1–8). Bacterial RNA polymerase is a well-studied example. It is composed of five protein subunits ($\alpha_2\beta\beta'\sigma$), of which one (σ) is a member of a group of related proteins that convey different DNA-binding specificities. The most common σ subunit in *Escherichia coli* has a mass of 70 kDa and is designated σ^{70} .

It has long been known that σ^{70} binds reversibly to the $\alpha_2\beta\beta'$ core enzyme (9–11), but the actual binding site has been difficult to identify. This problem has been addressed by chemical cross-linking (12–14) and by molecular genetics (15), but identification of the particular amino acid residues on the core subunits involved in σ^{70} binding remains a challenge.

Recently, we and others (16–18) have developed reagents based on small metal chelates that cleave polypeptide chains at sites determined by proximity to the chelate, apparently independent of the amino acid residues involved. Taking advantage of the recently discovered peptide hydrolysis reaction, the chelate may be tethered to a single site (e.g., a cysteine side chain) and used to map its proximity to individual peptide bonds (16). This methodology has rapidly developed to the point where it can be applied to complex biological systems such as membrane proteins (19). In addition, untethered iron–EDTA has been used to study the accessibility of sites on the surface of a small DNA-binding protein (20). While the latter experiments do not have sufficient resolution to permit the precise identification of cut sites, they can supply a global picture of the regions protected when one protein binds to another. This could guide further studies with a tethered cutting reagent, which can reveal the proximity of a particular residue on one protein to a particular residue on another.

We have used iron–EDTA to map the σ^{70} binding sites on the core RNA polymerase subunits α , β , and β' . To accomplish

this, we have examined and modified the cutting technology as described below.

EXPERIMENTAL PROCEDURES

All lab ware was either purchased as metal-free or acid washed (21). Pure water ($18\text{ M}\Omega\cdot\text{cm}^{-1}$) was used throughout. All other reagents and solvents were the purest available.

***E. coli* RNA Polymerase Isolation and Purification.** RNA polymerase was prepared according to ref. 22. The purified enzyme was stored in 10 mM Tris·HCl, pH 7.9/15% glycerol/0.1 mM EDTA/0.1 mM dithiothreitol at -20°C . The activities of core and holoenzyme were assayed by a modification of the procedure of ref. 23.

Preparation of C- and N-Terminal Antibodies. The multiple antigenic peptide (MAP) method (24) was used to produce rabbit polyclonal antibodies. The C-terminal 14-mer peptides Leu-Ala-Glu-Leu-Leu-Asn-Ala-Gly-Leu-Gly-Gly-Ser-Asp-Asn and Leu-Ala-Ser-Arg-Gly-Leu-Ser-Leu-Gly-Met-Arg-Leu-Glu-Asn, corresponding to residues 1393–1406 of the 1407-residue β' subunit (25) and to residues 307–320 of the 329-residue α subunit (26), were synthesized on an 8-branch Fmoc-MAP resin with a model 430A peptide synthesizer (Applied Biosystems). After problems were encountered with antisera to the β subunit C terminus, the N-terminal 14-mer peptide Met-Val-Tyr-Ser-Tyr-Thr-Glu-Lys-Lys-Arg-Ile-Arg-Lys-Asp of the 1342-residue β subunit (27) was synthesized by Phoenix Pharmaceuticals (Mountain View, CA). Immunizations, boosts, and serum collection with New Zealand White rabbits were performed according to the protocols of Harlow and Lane (28). The serum was stored in 1-ml aliquots at -70°C until needed.

Aminolink Plus immobilization kits (Pierce) were used to prepare affinity columns to purify the antibodies. Because of the insolubility of each peptide, 2 mg of each MAP was solubilized in coupling buffer (pH 10.5) containing 8 M urea and applied to 2 ml of resin. After overnight incubation at room temperature, each column was washed with 20 ml of the coupling buffer and then treated according to the manufacturer's instructions. One milliliter of antiserum was applied to each column and purified by a standard protocol (28). The effluent was adjusted to pH 8 with 0.02% sodium azide, and the affinity-purified solution was stored at 4°C until needed (up to 2 months).

Footprinting Reactions. All reactions were performed at 37°C in a cleavage buffer containing 10 mM Mops (pH 7.9), 120 mM NaCl, 10 mM MgCl_2 , and 1.0 mM EDTA. RNA polymerase was transferred from storage buffer to cleavage buffer via a spin column containing equilibrated Sephadex G-50 (fine) (29). Each reagent, Fe(III)–EDTA (Aldrich), ascorbic acid (vitamin C; Fluka Microselect grade), and H_2O_2 (J. T. Baker Ultrex grade), was prepared fresh before use as a $10\times$ stock solution. The ascorbic acid stock solution was titrated with 3 M NaOH until the pH was ≈ 7 . EDTA (1 mM

final concentration) was added to all reagents and buffers to ensure that any contaminating transition metals were chelated.

Conditions for the cleavage reaction were selected after determining the response of core RNA polymerase to a range of Fe-EDTA, ascorbate, and peroxide concentrations, with the individual reaction mixtures arrayed in the wells of a microtiter plate. For cleaved samples, 22 pmol of core or holo RNA polymerase was treated sequentially with Fe(III)-EDTA, ascorbate, and H_2O_2 to final concentrations of 20, 4, and 4 mM (20- μl final volume). Under these conditions, we found that each multisubunit enzyme assembly was unlikely to be cleaved more than once. After addition of each reagent, the reaction was gently Vortex mixed before the addition of the next reagent. After addition of the last reagent, the sample was mixed and the reaction was allowed to proceed for 1 min. For control samples, RNA polymerase was incubated in buffer only. All samples were quenched by adding 0.2 vol of 6 $\times$ sample application buffer [62.5 mM Tris-HCl, pH 6.8/2% (wt/vol) SDS/5% (vol/vol) 2-mercaptoethanol/10% glycerol/25 mM EDTA/0.05% (wt/vol) bromophenol blue]. After quenching, the samples were quickly frozen in liquid N_2 and stored at -70°C .

Reaction mixtures containing either free Fe(II) or Fe-EDTA formed *in situ* were performed as described above except EDTA was absent from all reagents and buffers. The 10 $\times$ Fe(II) stock solution was prepared by gently dissolving ferrous sulfate (Fisher) in pure water. Due to the rapid air oxidation of aqueous Fe(II), the stock solution was carefully agitated to avoid splashing and was used immediately after preparation. Free Fe(II) footprinting reagent concentrations were 0.2 mM Fe(II), 5 mM ascorbate, and 1 mM H_2O_2 . For footprinting with Fe-EDTA formed *in situ*, 22 pmol of core or holo RNA polymerase was cleaved by the sequential addition of Fe(II), ascorbate, EDTA (standardized solution; Aldrich), and H_2O_2 to final concentrations of 0.2, 5, 2, and 1 mM (20- μl final reaction volume) according to the protocol of ref. 20.

Fragment Separation and Visualization. To separate fragments, electrophoresis was performed using a mini Protean II gel apparatus (Bio-Rad) with 0.75-mm spacers. For separating the fragments of β and β' , typically 8% SDS resolving gels were overlaid with 4% stacking gels (30). The fragments of subunit α were typically separated with a 12% resolving gel and a 4% stacking gel. Several other acrylamide concentrations were used in control experiments to ensure that all the cleaved fragments on all the subunits had been identified. To avoid cleavage of the β and β' subunits in the sample application buffer, the RNA polymerase samples were not heated and gel electrophoresis was started within 10 min of thawing. Either Bio-Rad or New England Biolabs broad-range molecular weight markers were used as standards. The markers were combined with 0.20 vol of sample application buffer and heated at 95°C for 3 min. Electrophoresis was performed at 200 V until the bromophenol blue dye front reached the bottom edge of the gel.

After electrophoresis, the gel was equilibrated for 15 min in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, pH 11/10% methanol electroblotting buffer. Electroblotting was performed with a mini Trans-Blot cell (Bio-Rad) using a Problott membrane (Applied Biosystems) for 3 h at room temperature. Afterward, the membrane was cut to separate the molecular weight makers from the remainder of the blot. The marker lane was stained with Faststain (Zion) for 20 min and destained with 10% acetic acid/45% methanol. The remainder of the blot was incubated overnight at room temperature in 5% (wt/vol) nonfat milk in TBS (50 mM Tris-HCl, pH 7.4/150 mM NaCl). After incubation the membrane was washed three times with TBS containing 0.05% Tween 20 (TTBS). Typically, 15 ml of affinity-purified antibody solution was combined with 5 ml of 10 $\times$ TTBS buffer and 30 ml of H_2O . The blocked membrane was incubated for 2 h with this primary antibody solution,

washed three times with TTBS buffer, and incubated with 50 ml of a 1:3000 dilution of goat anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad) in TTBS. After 2 h at room temperature, the blot was washed three times with TTBS and once with TBS. The bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium using an Immun-Blot assay kit (Bio-Rad).

Fe(III)-EDTA and Fe(II) Binding to Core RNA polymerase. To make a radiolabeled solution of Fe(III)-EDTA, 20 μl of $^{59}\text{Fe(II)}$ (1 $\mu\text{Ci}/\mu\text{l}$; 1 Ci = 37 GBq) in 50 mM H_2SO_4 (DuPont/NEN) was mixed with 4 μl of 0.1 M EDTA. The pH was adjusted to 6 with 1- μl aliquots of diisopropylethylamine, and the mixture was allowed to stand at room temperature for 1 h to ensure complete oxidation to Fe(III). To confirm that all the ^{59}Fe was complexed, thin-layer chromatography (31) was performed with 10% NaOAc/ CH_3OH (1:1) as the developing solvent. In this system, uncomplexed iron remains at the origin while the Fe-EDTA complex migrates with $R_f = 0.15$. All the ^{59}Fe was confirmed to migrate as Fe-EDTA. A solution of Fe(III)-EDTA was then radiolabeled with the $^{59}\text{Fe-EDTA}$ solution to give a stock solution containing 100 mM $^{59}\text{Fe-EDTA}$ and 1 mM EDTA. To make a labeled solution of FeSO_4 containing $^{59}\text{Fe(II)}$, 90 μl of 22.2 mM FeSO_4 was combined with 10 μl of $^{59}\text{FeSO}_4$ in H_2O . This solution was diluted to 2.0 mM $^{59}\text{FeSO}_4$ with H_2O .

For iron-binding experiments, core RNA polymerase was exchanged from storage to standard cleavage buffer without EDTA, using a Sephadex G-50 spin column. To determine whether the core enzyme was capable of binding Fe(III)-EDTA, a 150 μl control reaction mixture containing 165 pmol of core RNA polymerase and 20 mM labeled Fe-EDTA in cleavage buffer was allowed to incubate at room temperature for 1 min. After incubation, 100 μl of solution was applied to a spin column with cleavage buffer containing no EDTA. After centrifugation, the collected effluent (100 μl) and column were γ -counted using a LKB model 1282 counter. The cpm in the column and the effluent were compared, and protein concentrations of the applied and the collected solutions were determined with a micro BCA protein assay kit (Pierce). Fe(II) binding by core RNA polymerase was determined using the same method as the Fe-EDTA, except the final concentration of the $^{59}\text{FeSO}_4$ was 0.2 mM.

Molecular Weight Determination of Fragments. The molecular weight of each RNA polymerase subunit fragment, detected by an antibody that binds to one original terminus, was determined by comparison to a standard curve (either a third- or a fifth-order polynomial fit of $\log M_r$ vs. relative migration) of commercial molecular weight markers. The molecular weight of each fragment was compared to the subunit sequence to locate the cut site.

RESULTS

Some of the effects of varying cleavage conditions are illustrated in Fig. 1. In particular, lanes 5, 6, and 7 compare the cleavage of the β subunit by unchelated iron, sequentially added iron and EDTA, and preformed iron-EDTA. The slight fragmentation observed with incomplete reaction mixtures in lanes 3 and 4 is presumably due to the presence of small amounts of alternative oxidizing agents (O_2) and reducing agents (cysteine residues) in the mixtures. Cleavage under these conditions is sometimes more pronounced. Only when Fe-EDTA, H_2O_2 , and ascorbate are all present is reproducibly efficient cleavage of the protein observed (lane 7). Table 1 shows that most of the unchelated iron binds to the protein, while none of the iron-EDTA does.

Figs. 2-4 are typical gels that show the cleavage by preformed iron-EDTA of the β , β' , and α subunits in the core and holoenzyme. In Fig. 2, multiple cleavage sites 84-91 kDa from the N terminus of β are readily evident in the core, but not in

FIG. 1. Comparison of iron footprinting reaction conditions using core RNA polymerase (8% PAGE; immunoblot stained with anti- β antibody; N terminus). Lanes: 1, core only; 2, H_2O_2 and ascorbate; 3, Fe(III)-EDTA and ascorbate; 4, Fe(III)-EDTA and H_2O_2 ; 5, unchelated Fe(II), ascorbate, and H_2O_2 ; 6, sequentially added Fe(II), ascorbate, EDTA, and H_2O_2 ; 7, Fe(III)-EDTA, ascorbate, and H_2O_2 .

the holoenzyme. Also, a 69-kDa fragment is present in the holoenzyme (lane 2) but absent in the core (lane 3). Several other, more modest changes in band intensities were also observed. These are indicated by different line lengths in Fig. 5. In Fig. 3, cleavage sites approximately 89, 86, and 73 kDa from the C terminus of the β' subunit are evident in the core but not the holoenzyme. In Fig. 4, little difference is observed in cleavage of the α subunit. All the cleavage sites detected on all the core subunits in a collection of electrophoretic analyses using 5–12% polyacrylamide gels are summarized in Fig. 5.

DISCUSSION

To be most useful, protein footprinting methodology should cause minimal perturbation of the protein under study. It is well understood that conditions should be chosen so that the probability of more than one cleavage event occurring in any molecular complex is negligible. It is also important that fragment bands be as sharp as possible; we found that an excess of Fe-EDTA over ascorbate and peroxide gave the best results. In our experience, the most successful buffers are Mops $\approx$ Hepes $>$ Tris $>$ imidazole $>>$ phosphate. Thiols should be avoided and glycerol should be kept well below 5% (vol/vol). Moderate concentrations of NaCl, KCl, $MgCl_2$, and NaOAc do not seem to interfere, and the reaction does not appear to be sensitive to pH. Detection of cleavage products with high sensitivity using antibodies against the naturally occurring terminal peptide sequences of the RNA polymerase subunits allows the cutting experiments to be carried out on the native protein.

The use of a cutting reagent that does not bind to the protein is also preferred, since extensive binding by the reagent might interfere with binding by the protein ligand of interest. Table 1 shows that unchelated iron binds extensively to RNA polymerase (161 mol of Fe per mol of core enzyme), but Fe-EDTA does not. Lane 6 of Fig. 1 shows that subsequent addition of EDTA removes only a part of the bound iron (compare fragment bands in lane 5 due to bound iron and lane 7 due to Fe-EDTA with lane 6, which shows a mixture of the two). Since the side chains of amino acids such as Cys, His, Glu, Asp, etc. cause proteins to bind metals,

Table 1. $^{59}Fe(II)$ and $^{59}Fe(III)$ -EDTA binding to core RNA polymerase

	$^{59}Fe(II)$	$^{59}Fe(III)$ -EDTA
cpm column	6,427	269,492
cpm effluent	26,504	22
% bound to core	80.5	0
mol of Fe per mol of core	161	0

Measured by spin-column gel filtration.

FIG. 2. Fe-EDTA cleavage sites on the β subunit of RNA polymerase holoenzyme compared to core (8% PAGE; immunoblot stained with anti- β antibody, N terminus). Lanes: 1, holoenzyme control; 2, cleaved holoenzyme; 3, cleaved core; 4, core control.

and since the binding of metal ions can change the conformation of the metal-binding groups and the net electric charge of the protein, metal binding introduces uncertainty into the results. In our hands, removing EDTA from buffer solutions in order to study protein cleavage by unchelated iron led to various amounts of cleavage of the large subunits of RNA polymerase without the addition of iron, ascorbate, or peroxide. This is almost certainly due to the traces of transition metals present as contaminants in even the purest biochemicals and the cleanest containers.

The extensive studies of energy transfer from lanthanide-EDTA chelates to chromophores in proteins (refs. 32 and 33 and references therein) led to the conclusion that EDTA chelates of trivalent terbium did not bind significantly to RNA polymerase or to a number of other proteins. These small chelates evidently diffuse freely through the solution, randomly encountering the macromolecules of interest. The EDTA chelate of iron has a structure related to that of terbium, except that Fe-EDTA normally binds one water molecule instead of the three bound by Tb-EDTA (34, 35). Thus, it is not unexpected that Fe-EDTA does not bind to RNA polymerase.

How untethered Fe-EDTA cuts the protein is a matter of some uncertainty, because of the rich chemistry involved (36). It is well known that iron can cause oxidative damage to proteins (18, 37, 38), which can lead to chain scission, and

FIG. 3. Fe-EDTA cleavage sites on the β' subunit of RNA polymerase holoenzyme compared to core (8% PAGE; immunoblot stained with anti- β' antibody, C terminus). Lanes: 1, cleaved holoenzyme; 2, cleaved core; 3, core control.

FIG. 4. Fe-EDTA cleavage sites on the α subunit of RNA polymerase holoenzyme compared to core (12% PAGE; immunoblot stained with anti- α antibody, C terminus). Lanes: 1, holoenzyme control; 2, cleaved holoenzyme; 3, cleaved core; 4, core control. The impurity causing the bands at 22 kDa in lanes 1 and 2 has not been identified.

it has recently been demonstrated that a tethered iron-EDTA chelate can hydrolyze—not oxidize—peptide bonds (17). However, the first process is thought to act at the α -carbon of an amino acid residue, while the second acts at the adjacent carbonyl carbon, so that the products will differ by less than one amino acid residue. This uncertainty is well within the experimental error of the technology available to analyze subpicomole quantities of protein fragments. An alternative approach to protein footprinting is to use a reversible lysine-modifying reagent and a proteolytic enzyme such as a lysine-specific endoprotease (39). While practically limited to lysine side chains, this technique might complement the broad sampling of the protein surfaces illustrated in Figs. 2–4. The more cut sites that can be studied, the higher will be the resolution of the answer.

The most obvious overall result in Fig. 5 is that binding σ^{70} to core does not affect the great majority of the cut sites. Thus, the technique is not hypersensitive to small changes in conditions. However, a number of specific, reproducible changes are observed. We estimate that the uncertainty in assigning the sequence positions of the cut sites is ± 10 residues.

For the core enzyme, 39 fragments are produced on the β subunit; for the holoenzyme, there are 36. A distinctive series of four bands near β sequence positions 745, 764, 795, and 812 is present in the core but absent in the holoenzyme (Fig. 5, box 2). The protection of this extensive region of the protein when σ^{70} is bound to core appears to be due to steric hindrance—i.e., it appears to be part of the footprint of σ^{70} on the β subunit of the enzyme. When one protein binds to another, an interfacial region typically encompassing 10^3 – 10^4 Å² becomes buried (40). Our literature search for mutants mapped to this region of β revealed a Glu-813 to Lys mutant with diminished catalytic activity (41).

While conformational changes in DNA upon ligand binding often are not considered in detail, this possibility cannot be ignored with proteins. In Fig. 5, box 1, a β cut site near residue 604 found in the holoenzyme is not seen in the core. This difference indicates unambiguously that a conformational change has occurred due to the binding of σ^{70} to the core enzyme and adds a note of caution to the interpretation of these experiments. However, such bands are rare; this is the only clear example we found.

The β' cleavage pattern shows that three cut sites near sequence positions 581, 613, and 728 are present in the cleaved core (30 total cut sites) but absent in the cleaved holoenzyme (Fig. 5, box 3 and 4). The loss of these bands presumably is due to steric protection by bound σ^{70} . More modest changes in the intensities of other fragment bands are indicated by different length lines in Fig. 5. Our conclusions are limited to the most obvious changes in cleavage patterns; the outright disappearance or appearance of fragment bands.

Cleavage of the α dimer produces 14 fragments in both the core and holoenzyme. No obvious differences in cut sites or band intensities are observed for the α subunit under these

FIG. 5. Summary of Fe-EDTA cleavage sites on all core subunits from gels containing 5%, 8%, and 12% acrylamide. Each vertical line represents a cut site along the amino acid sequence of each subunit. Alternating shaded bars represent 100 amino acid residues. The height of each vertical line corresponds to the relative band intensity. Cut sites on the core are shown on top; those on the holoenzyme are shown below. Assignments of individual cut sites on both β and β' are estimated to be accurate to within ± 10 amino acid residues. Because of its anomalous migration, cut sites on α subunit are less certain. BETA, box 1 shows a cut site near residue 604 due to a conformational change in core upon binding of σ^{70} ; box 2 shows the region spanning residues 745–812 that is protected when σ^{70} is bound to core. BETA PRIME, boxes 3 and 4 show three fragments near residues 581, 613, and 728, which are protected when σ^{70} is bound. ALPHA, no differences were observed between core and holoenzyme.

reaction conditions. In contrast to β and β' , the α subunit was observed to migrate anomalously on SDS/PAGE; though its molecular mass is known to be 36 kDa, it moves as a 40-kDa protein. In Fig. 5, a constant molecular mass correction of 4 kDa was applied when assigning the molecular mass of each fragment. A nested set of markers comprised of specific truncations of the α subunit may provide a more accurate determination of their true masses.

Other studies of σ -core interactions have provided limited information on the sites involved. The use of chemical cross-linking has shown which subunits are adjacent to one another (12–14). For example, McMahan and Burgess, using an aryl azide cross-linker, found that it was possible to cross-link unspecified amino groups on σ^{70} to all three of the subunits of core RNA polymerase. They investigated the α subunit further and demonstrated that a σ^{70} - α cross-link occurred somewhere between α residues 209 and 329. The electrophoresis and blotting technology used here does not efficiently visualize fragments within ≈ 10 kDa of either the C or the N terminus, which may explain why no σ^{70} - α interaction is evident in Fig. 4.

Subunit interactions have also been studied genetically via deletion and site-directed mutations of the subunits. For example, Glass *et al.* (15) have found that removal of 20% of the β subunit from the C terminus prevents the binding of σ^{70} to core RNA polymerase, suggesting that this region is involved in σ^{70} binding. Our results indicate that σ^{70} binds near the center of β (Fig. 5, box 2) but cannot exclude sites very near the C terminus.

The core subunits ($\alpha_2\beta\beta'$) have a total of 3075 distinct peptide bonds (3078 α carbons), of which perhaps 500–600 ($\approx 20\%$) are too near the ends of the primary structures to be studied with full sensitivity. A total of 83 cuts can be detected in the core and 77 can be detected in the holoenzyme. Thus, about 1 bond in 40 is potentially subject to cleavage and detection by these techniques. The α subunit, with 328 peptide bonds (329 residues) is detectably cleavable at 14 sites (4.3%), while the much larger β' subunit (1406 peptide bonds) is detectably cleavable at 30 sites (2.1%) and β subunit (1341 bonds) is detectable at 39 (2.9%). Of these cut sites, 8 ($\approx 10\%$) are sensitive to the presence of σ^{70} . One cut site on β subunit is seen only in the presence of σ^{70} , while 7 on β and β' are seen only in its absence. The former is obviously due to a conformational change, but it stands to reason that most of the latter reflect the binding site of σ^{70} on the core. This degree of ambiguity is a consequence of any experiment that detects changes in the accessibility of sites. Nonetheless, global information of this type clarifies our understanding of where σ^{70} binds. Evidently a region near the middle of the β sequence (Fig. 5, box 2) and a region near the middle of the β' sequence (Fig. 5, boxes 3 and 4), neither of which was implicated in previous studies by other techniques, are components of this site.

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