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Mapping the σ^{70} subunit contact sites on *Escherichia coli* RNA polymerase with a σ^{70}-conjugated chemical protease

(protein cleavage/single cysteine mutants/tethered FeEDTA/FeBABE)

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ABSTRACT **The core enzyme of *Escherichia coli* RNA polymerase acquires essential promoter recognition and transcription initiation activities by binding one of several σ subunits. To characterize the proximity between σ^{70}, the major σ for transcription of the growth-related genes, and the core enzyme subunits ($\alpha_2\beta\beta'$), we analyzed the protein-cutting patterns produced by a set of covalently tethered FeEDTA probes [FeBABE: Fe (S)-1-(*p*-bromoacetamidobenzyl)EDTA]. The probes were positioned in or near conserved regions of σ^{70} by using seven mutants, each carrying a single cysteine residue at position 132, 376, 396, 422, 496, 517, or 581. Each FeBABE-conjugated σ^{70} was bound to the core enzyme, which led to cleavage of nearby sites on the β and β' subunits (but not α). Unlike the results of random cleavage [Greiner, D. P., Hughes, K. A., Gunasekera, A. H. & Meares, C. F. (1996) *Proc. Natl. Acad. Sci. USA* 93, 71–75], the cut sites from different probe-modified σ^{70} proteins are clustered in distinct regions of the subunits. On the β subunit, cleavage is observed in two regions, one between residues 383 and 554, including the conserved C and Rif regions; and the other between 854 and 1022, including conserved region G, regions of ppGpp sensitivity, and one of the segments forming the catalytic center of RNA polymerase. On the β' subunit, the cleavage was identified within the sequence 228–461, including β' conserved regions C and D (which comprise part of the catalytic center).**

DNA-dependent RNA polymerase [EC 2.7.7.6] of *Escherichia coli* is composed of a four-protein core enzyme (subunit composition $\alpha_2\beta\beta'$) and one of several sigma subunits. The major sigma factor, σ^{70} (product of the *rpoD* gene), initiates the transcription cycle by specifying promoter recognition of most genes that are expressed during exponential cell growth and by promoting DNA strand separation (1–6). σ^{70} binds reversibly to the $\alpha_2\beta\beta'$ core enzyme (7–9), but the binding site has not yet been identified. Three-dimensional structures of the 380-kDa core enzyme (≈23 Å resolution) and the 450-kDa $\alpha_2\beta\beta'\sigma^{70}$ holoenzyme (≈30 Å resolution) provide graphic information about the overall conformations of these two macromolecular complexes (10). However, the resolution is not adequate to distinguish individual subunits, boundaries between subunits, or domains within subunits.

At the level of the primary sequence, highly conserved regions have been identified previously for each of the core subunits of RNA polymerase (11, 12), but it is not known whether one or more of these regions are responsible for binding σ^{70}. The results of chemical cross-linking (13–15), genetic studies (16–19), complex formation between subunits or subunit fragments (20, 21), and protein footprinting (22) have provided partial information concerning where σ^{70} binds to the surface of the core enzyme. However, when interpreting the results of genetic and footprinting studies it is difficult to discriminate between the effects of conformational changes and the effects of subunit proximity.

A way forward is provided by designing unique sites in σ^{70} at which a small reagent with cleavage activity can be tethered. This approach employs molecular cloning to prepare a set of mutant proteins, each with a unique cysteine residue at a chosen location, for conjugation to the cutting reagent. Cutting of the other subunits occurs only at sites located near this reagent in the holoenzyme structure.

As shown in Fig. 1, the 613-aa sequence of σ^{70} has regions that are associated with core enzyme binding, recognition of hexanucleotide promoter sequences at positions −10 and −35, DNA melting, and intramolecular contacts for regulation (5, 23, 24). Genetic analysis has implicated two conserved regions of σ^{70} in core binding (19, 25–28). Using this information, we selected seven sites of potential interest for tethering the cutting reagent FeBABE, which can cleave peptide backbones within ≈12 Å of its attachment site (29). With this set of mutants, sites on the core subunits that bind near the chosen σ^{70} sites have been identified.

MATERIALS AND METHODS

Materials. N- or C-terminal subunit peptides were purchased from Phoenix Pharmaceuticals (Mountain View, CA) and used for immunization of New Zealand White rabbits. Rabbit antibodies were affinity-purified on columns containing the immobilized terminal peptide antigens. Broad-range molecular weight marker proteins were from New England Biolabs. CPM (*N*-(4-(7-diethylamino-4-methylcoumarin-3-yl)phenylmaleimide) was purchased from Molecular Probes. QIAprep plasmid DNA purification kits were from Qiagen. Preparative electrophoresis grade agarose (SeaKem GTG) was used throughout. Ascorbic acid (vitamin C, microselect grade) was purchased from Fluka, and hydrogen peroxide (ultrex grade) was purchased from J.T. Baker. Pure water (18 MΩ cm^{-1}) was used throughout.

Site-Directed Mutagenesis. Seven single-cysteine mutants (132C, 376C, 396C, 422C, 496C, 517C, and 581C) of σ^{70} were prepared by PCR mutagenesis (30). The pGEMD expression vector (31) containing the σ^{70} gene (*rpoD*) under control of the T7 promoter was used as a backbone for all cloning experi-

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Abbreviations: BABE, (S)-1-(*p*-bromoacetamidobenzyl)ETDA; FeBABE, the iron chelate of BABE (also denotes the chelate moiety conjugated to cysteine).

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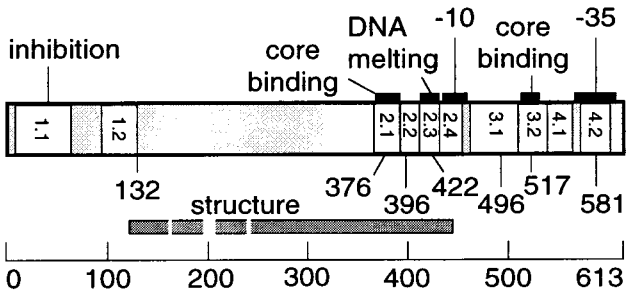

FIG. 1. Diagram of the primary structure of σ^{70} (613 aa), showing previously identified functions of the conserved σ^{70} regions and the positions of seven single-Cys mutants used for FeBABE conjugation. Residues included in the σ^{70} fragment crystal structure (23) are indicated by the dark bars below the diagram.

ments. Before single-cysteine residues could be introduced at desired positions along conserved regions of σ^{70}, the three native Cys residues C132, C291, and C295 of wild-type σ^{70} were first substituted with serine to produce a Cys-less *rpoD* (referred to as Cys(−)*rpoD* or Cys(−)σ^{70}). To accomplish this, an *Mlu*I-*Xho*I fragment of the double-mutant C291S, C295S *rpoD*, was prepared and ligated into a plasmid construct having the C132S substitution. This strategy allowed for the production of Cys(−)σ^{70} as well as the preparation of single-Cys mutant 132C, which contains a native cysteine residue on the C-terminal edge of subregion 1.2.

For the introduction of new cysteine residues at selected positions in the σ^{70} sequence, the Cys(−)*rpoD* was used as starting material plasmid. To prepare K376C σ^{70}, N396C σ^{70}, and R422C σ^{70}, PCR-generated fragments having these substitutions were ligated into the *Sac*II-*Bam*HI site of Cys(−)*rpoD*. K496C σ^{70} and S517C σ^{70} were produced by ligating PCR fragments into the *Sac*II-*Xho*I sites of Cys(−)*rpoD*. Mutant D581C σ^{70} was prepared by ligating a PCR fragment into the *Sac*II-*Ban*II site of Cys(−)*rpoD*.

The DNA sequence of each Cys-mutant gene was confirmed by using Shimadzu DSQ1000L and DSQ500L fluorescence sequencers. PCR primers used for site-directed mutagenesis were prepared on an Applied Biosystems 394 DNA/RNA synthesizer, PAGE-purified, and checked by UV absorbance.

Overexpression and Purification of Mutant σ^{70} Proteins. Expression plasmids containing the mutant *rpoD* genes under control of the T7 promoter were transformed into *E. coli* BL21(DE3)pLysS cells and grown in Luria–Bertani medium containing ampicillin (200 μg/ml final) at 37°C. Upon reaching OD_{600} = 0.4, protein overexpression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 0.5 mM final), and cells were allowed to grow at 37°C for an additional 3 h before collecting. Purification of each mutant protein was carried out as described for wild-type σ^{70} (32).

Purification of *E. coli* RNA Polymerase and Transcriptional Activity. Core RNA polymerase was purified from *E. coli* W3350 cells and assayed for transcriptional activity (33, 34).

FeBABE Conjugation. Thiol-containing reducing agents such as 2-mercaptoethanol or DTT were removed before, and excluded after, conjugation with FeBABE (35). Each purified mutant σ^{70} in storage buffer [10 mM Tris, pH 7.6/10 mM $MgCl_2$/0.1 mM EDTA/1 mM DTT/50% (vol/vol) glycerol/0.2 M KCl] was dialyzed overnight at 4°C against conjugation buffer [10 mM Mops, pH 8/0.2 M NaCl/5% (vol/vol) glycerol/2 mM EDTA]. Conjugation reactions were initiated by the addition of excess FeBABE (0.3 mM final) to each purified σ^{70} mutant (.015 mM final) at pH 8. After 4 h at room temperature, excess FeBABE was removed by overnight dialysis against storage buffer (without DTT) at 4°C.

Thiol Assay for FeBABE Conjugation Yield. The presence of free Cys sulfhydryl groups on each single-Cys mutant σ^{70} was determined fluorimetrically by the CPM test (35). Assays were performed before and after FeBABE modification and were used to determine the yield of conjugation for each mutant protein.

Transcription Assay of Holoenzymes Carrying FeBABE-Modified σ^{70}. Before and after FeBABE conjugation, each purified mutant σ^{70} protein was mixed with the core enzyme and assayed for the ability to perform single-round runoff transcription from the *lac*UV5 promoter as described previously (31, 36, 37).

Affinity Cleavage of RNA Polymerase Core Subunits by FeBABE-σ^{70}. To avoid artifacts because of multiple cleavage, conditions were developed wherein only a small percentage of the holoenzyme complexes were cleaved. RNA polymerase holoenzyme was prepared by incubation of native core with FeBABE-modified σ^{70} proteins (1:1 molar ratio) at 30°C for 20 min in cleavage buffer [10 mM Mops, pH 8/10 mM $MgCl_2$/10% (vol/vol) glycerol/0.2 M NaCl/2 mM EDTA]. Stock solutions of sodium ascorbate (adjusted to pH ≈7, with dilute NaOH) and hydrogen peroxide were prepared just before use. Cleavage reactions were initiated by the rapid sequential addition of ascorbate (5 mM final) and peroxide (5 mM final) to RNA polymerase and allowed to proceed for 2 min at 30°C. Control reactions using nonconjugated σ^{70} mutants were treated identically. Reaction mixtures were quenched by the addition of 0.25 vol of 5× sample buffer [62.5 mM Tris·HCl, pH 8.2/2% (wt/vol) SDS/5% (vol/vol) 2-mercaptoethanol/10% glycerol/25 mM EDTA/0.02% (wt/vol) bromophenol blue], immediately frozen in liquid nitrogen, and stored at −70°C until needed for SDS/PAGE. Cleaved fragments were separated, blotted, and visualized by immunostaining with affinity-purified subunit terminal-specific antibodies essentially as described by Greiner *et al.* (22). Representative results are shown in Fig. 3.

Assignment of Cleavage Sites on β and β' Subunits. To compensate for sequence-dependent effects on the migration rate of proteins on SDS/PAGE, cleavage sites on β and β' were assigned by comparison with sequence markers generated from the same protein. As reference markers, β and β' were cleaved at either Cys or Met residues by chemical digestions of $\alpha_2\beta\beta'$ core to produce known marker fragments (38, 39). The cleavage sites for each fragment were assigned by using a third-order polynomial fit of log molecular weight versus migration distance (Rm) on SDS/PAGE for a set of known marker fragments (Fig. 2). The errors of these assignments were estimated (40) by refitting after removal of each point in turn and reanalyzing the data; β errors ranged from ±2 to ±9 residues (average ±6); β' errors ranged from ±7 to ±16 residues (average ±11).

For cleavage at Cys with 2-nitro-5-thiocyanobenzoate (NTCB) (41), $\alpha_2\beta\beta'$ core was buffer-exchanged into unfolding buffer (0.1 M Mops, pH 8.5/8 M urea) at 37°C. Cleavage was initiated by the addition of 5× excess NTCB (in 0.1 M Mops

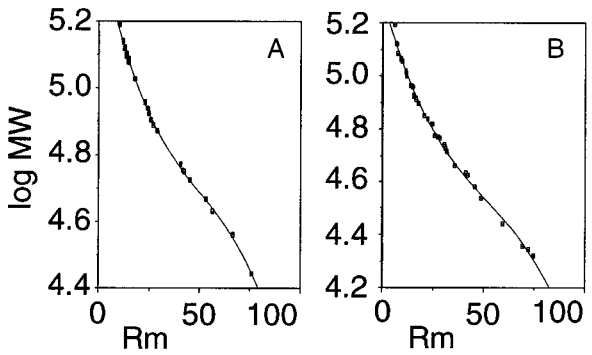

FIG. 2. Curve fits of β and β' fragments used as sequence markers to assign cleavage sites. The log molecular weight is plotted vs. relative migration distance (Rm) of markers on SDS/PAGE. (*A*) β sequence markers. (*B*) β' sequence markers.

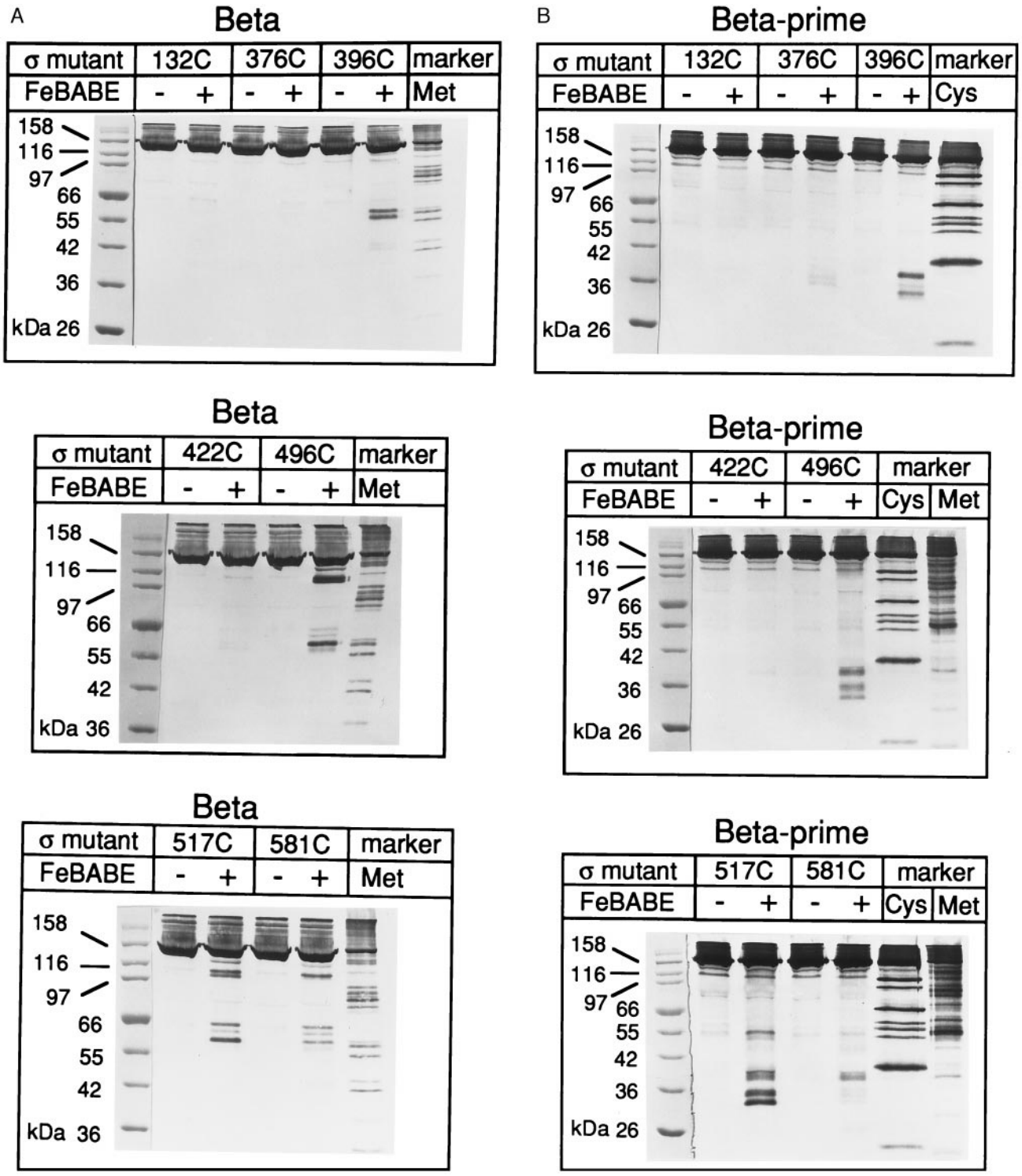

FIG. 3. Holoenzyme cutting by σ^{70} single-Cys FeBABE conjugates. Immunostained blots of SDS/PAGE detecting either β or β' subunit fragments by using affinity-purified N-terminal antibodies. (*Left*) Cleavage of the β subunit. (*Right*) Cleavage of the β' subunit. Holoenzyme cleavage reactions that contained FeBABE-modified σ^{70} proteins are indicated by a plus sign, and those that did not carry the FeBABE probe are indicated by a minus sign. All samples were treated with ascorbate and hydrogen peroxide (whether conjugated or not). Immunostained sequence markers prepared by cleavage at methionine and/or cysteine are shown along the right side of each gel. Broad range molecular weight markers, stained with Coomassie blue, are shown along the left edge of each gel.

buffer) over total sulfhydryl groups. If necessary, the pH of the solution was readjusted to 8.5. After overnight incubation at 37°C, the cleavage reactions were quenched with 1% SDS/1% 2-mercaptoethanol.

Methionine-specific cleavage reactions using cyanogen bromide were performed as described by Grachev *et al.* (38).

RESULTS AND DISCUSSION

Mutant σ^{70} Proteins. The crystal structure (23) of a fragment of σ^{70} has provided a framework for the design of experiments that focus on the interaction of σ^{70} with other components in the transcription cycle. However, this structure does not include the evolutionarily conserved regions 1.1, 3.1,

3.2, 4.1, and 4.2. Positions for the cysteine mutants discussed here were chosen within or flanking the evolutionarily conserved regions of the σ^{70} family. Invariant residues were preserved to maintain the overall structure and function of σ^{70}. After constructing the Cys(−)*rpoD* gene, the purified Cys(−)σ^{70} mutant protein was found to maintain ≈95% of its transcriptional activity. Seven single-Cys mutants (Fig. 1) were prepared, overexpressed, purified, and reconstituted into holoenzymes by mixing with the $\alpha_2\beta\beta'$ core enzyme as described by Fujita and Ishihama (32). All single-Cys mutant proteins were found to retain 70–95% of wild-type transcriptional activity.

It is important that the amino acid substitutions do not disrupt the overall structure of σ^{70}. CD has been used previously to detect mutations in region 1.1 that disrupt sigma structure (42). The far-UV CD spectrum of each mutant protein was found to be nearly identical to that of wild-type σ^{70} (data not shown), suggesting the preservation of overall structure for all the σ mutants constructed.

FeBABE conjugation yields were determined to be: 132C, 31%; 376C, 40%; 396C, 48%; 422C, 49%; 496C, 69%; 517C, 72%; and 581C, 67%. To determine the effect of conjugation on the transcriptional activity of each mutant, single-round transcription assays also were performed after conjugation with the FeBABE probe. After correction for the presence of unconjugated σ^{70}, the conjugates showed the following transcriptional activity 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% (43).

Cleavage by FeBABE-σ^{70} Conjugates. Single-Cys mutant 132C, located at the C-terminal end of conserved σ^{70} region 1.2, contains the only naturally occurring Cys residue included in this panel of mutants. In the σ^{70} fragment crystal structure (23), residue 132 is adjacent to conserved region 2.1, which is implicated in core binding. No cutting of the core subunits was observed by FeBABE attached to 132C (Figs. 3 and 4). This result serves as an essential control experiment, demonstrating that under these conditions there is no artifactual cutting of the subunits. Of the seven single-Cys mutants discussed in this study, the N-terminal four (132C, 376C, 396C, and 422C) can be found on the σ^{70} fragment crystal structure. A summary of the β and β' cleavage sites produced by the FeBABE conjugates of these four mutants is shown in Fig. 4. Because residues beyond 448 of σ^{70} were not included in the crystal structure, cleavage of the β and β' subunits by 496C-Fe, 517C-Fe, and 581C-Fe are discussed below.

Near the putative core-binding surface in σ^{70} region 3.1, cutting was observed by FeBABE attached to 496C near residues 471, 489, 515, and 537 of β (between β regions C and D, including Rif cluster I) and also near β residues 854 and 900 (β region G, and at the N terminus of dispensable region 2), as well as strong cuts near residues 278, 298, and 330 of β' (β' region C). Conserved region 3 of σ^{70} and region G of β have been found to mediate ppGpp-dependent functions *in vivo* (44, 45).

Near the putative active center 5′ face (46), σ^{70} region 3.2, cutting was observed by FeBABE attached to 517C near residues 388, 403, 470, 493, 523, and 550 of β (a pattern similar to 496C, plus two faint cuts before the N-terminal end of β region C), and also near β residues 858, 913, and 1020 (including conserved region G and bracketing dispensable region 2), as well as strong cuts near residues 276, 294, 328, 434, and 461 of β' (β' regions C and D).

Near the putative –35 DNA promoter binding site, σ^{70} region 4.2, cutting was observed by FeBABE attached to 581C near residues 384 (faint), 395 (faint), 477, 501, 527, and 554 of β (Rif I and region D), and also near β residues 875, 913, and 1022 (a pattern similar to 517C, though with different relative yields), as well as cuts near residues 228, 278, 289, 301, 328, and 401 of β' (shifted toward the N terminus of β' and with lower yields relative to the pattern of 517C).

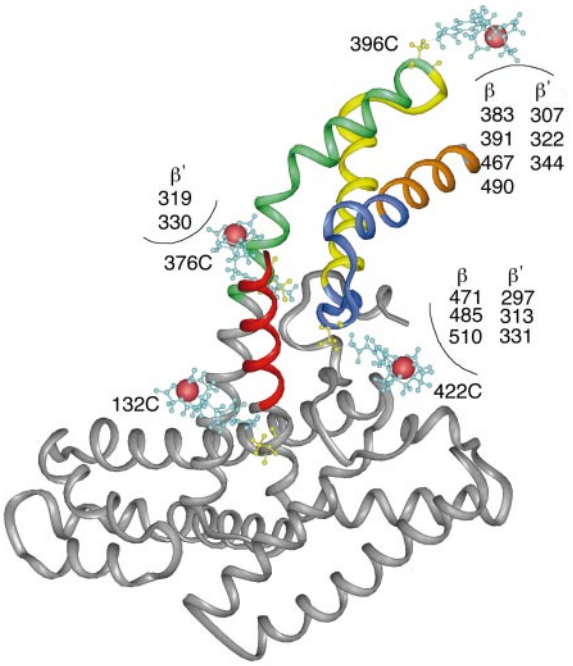

FIG. 4. Representation of four of the conjugated single-Cys mutants and their cut sites on the core subunits, by using the crystal structure of a σ^{70} fragment (23). Conserved regions of σ^{70} are indicated. Regions: 1.2, red; 2.1, green; 2.2, yellow; 2.3, cyan; 2.4, orange.

***β* Subunit Cleavage Sites.** Five FeBABE-conjugated σ^{70} mutants (396C-Fe, 422C-Fe, 496C-Fe, 517C-Fe, and 581C-Fe) were found to cleave the β subunit of RNA polymerase (1,342 residues) between amino acids 383 and 554 (Figs. 3 *Left* and 5*A*). This segment of β contains the evolutionarily conserved region C, part of region D (11, 12, 39), sites for recessive lethal mutants (47), the Rifampicin-binding cluster I [amino acids 507–534 (48, 49)], and streptolydigin-resistance sites [amino acids 543–546 (50)]. In addition, ribonucleotide analogs (51) bound in the initiation site of the RNA polymerase open promoter complex have been cross-linked to β between residues 516 and 540. The C-terminal part of σ^{70} region 3, between residues 508 and 561, also has been cross-linked to ribonucleotide analogs (46, 52). Our data suggest that σ^{70}-conserved regions 2.1, 2.3, 3.1, 3.2, and 4.2 are all proximal to conserved β regions C and D and the Rif I cluster.

Three of these same FeBABE-modified Cys-mutant proteins (496C-Fe, 517C-Fe, and 581C-Fe) also were found to cut β within a region spanning amino acids 854-1022, encompassing conserved region G and dispensable region 2 (DR2) (53). The primary contact site for the α subunit has been mapped to a similar region, between residues 738 and 936 (including regions F and G), although the β region downstream from residue 1139 (including region H) is required for stabilization of α binding to β (54). β-conserved regions D and H are also targets of iron-mediated cleavage within the active center of RNA polymerase (39, 55). Evidently, σ^{70}-conserved regions 3.1, 3.2, and 4.2 are proximal to at least part of the RNA polymerase catalytic center.

***β′* Cleavage Sites.** The six conjugated σ^{70} mutants (376C-Fe, 396C-Fe, 422C-Fe, 496C-Fe, 517C-Fe, and 581C-Fe) having Cys located in conserved regions 2.1, 2.3, 3.1, 3.2, or 4.2 of σ^{70} were found to cleave the β' subunit (1,407 residues) between amino acids 228 and 461 (Figs. 3 *Right* and 5*B*). Cleavage of β' always includes conserved region C and sometimes includes B and D. Conserved region D of β' has been mapped near the

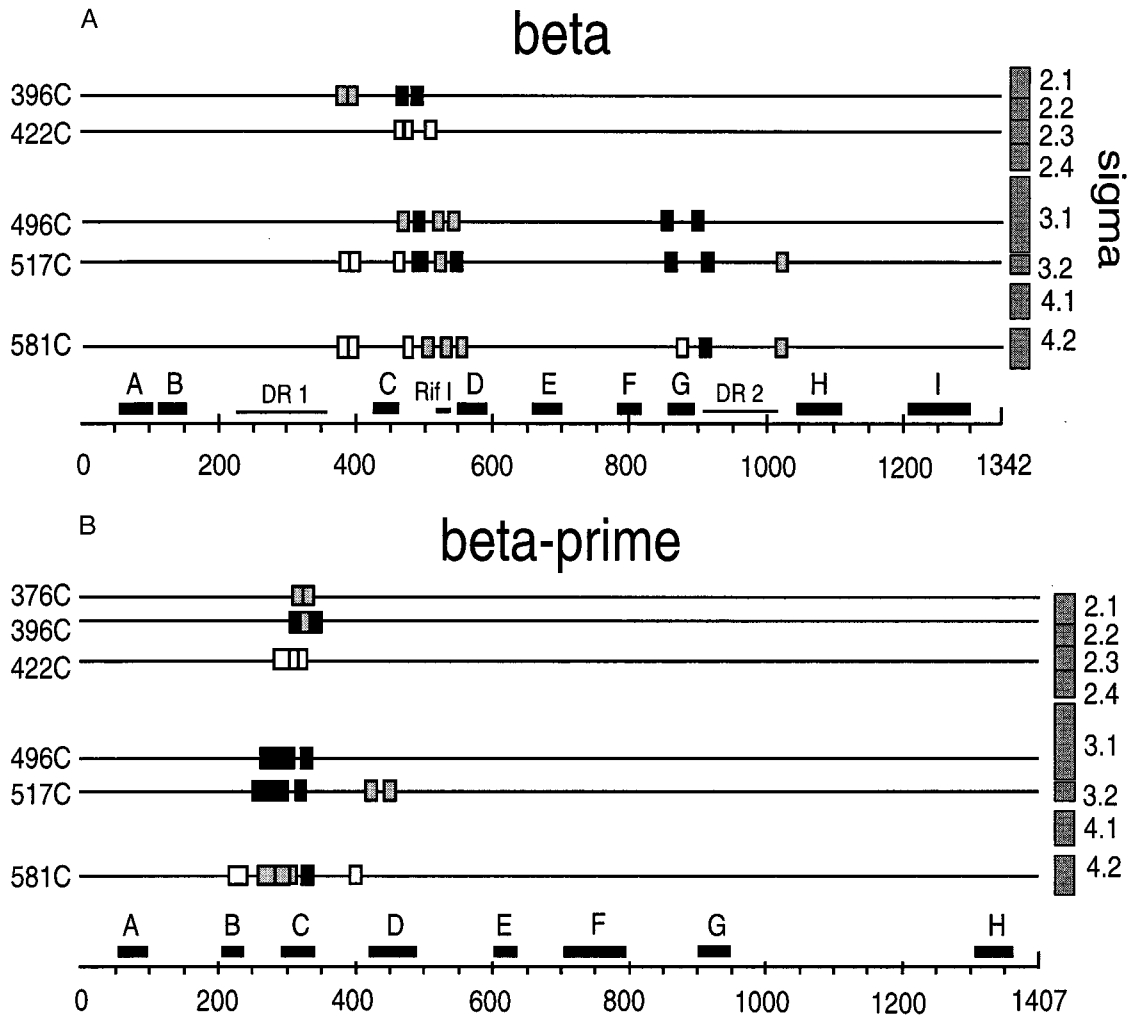

FIG. 5. Summary of cleavage data in Fig. 3, relating proximities of conserved regions of σ^{70} to sites on β subunit (*A*), and β' subunit (*B*). Darkness of the shaded boxes indicates cleavage efficiency observed on Western blots; box widths indicate estimated errors in assignment of cut sites. Conserved regions of β and β' are indicated along the horizontal axes.

catalytic center of RNA polymerase (39, 55). Termination-altering mutations (56) also are found in the region between amino acids 228–461.

Genetic analysis of β' deletion mutants suggests that the N-terminal region of β' is involved in the assembly of core enzyme and that the region between amino acids 201–477 on β' is involved in its interaction with the σ^{70} subunit (21). Mutant β' proteins lacking this region fail to form holoenzyme but retain the ability to form core enzyme.

Comparison with Protein Footprinting. Greiner *et al.* (22) used random cleavage with untethered FeEDTA to study the effects of σ^{70} binding on the accessibility of $\alpha_2\beta\beta'$ surfaces. From comparison of the randomly cleaved fragments of the subunits from the $\alpha_2\beta\beta'$ core and the holoenzyme, they found 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 a conformational change when σ^{70} binds.

Our results with tethered probes are quite different. Part of this may be attributed to the fact that cleavage by an untethered reagent cannot distinguish between the effects of conformational changes and steric hindrance, which could lead to finding sensitive cut sites that are not near a binding region. Also, because random cleavage is not focused on the region of interest, it produces many fragments. These may not be resolved fully on a protein gel, making it difficult to detect changes in the region of interest. On the other hand, cutting from 7 positions out of 613 probably does not reveal the entire surface of σ^{70} on core; there is still much to be learned by using tethered cutters.

CONCLUSION

Although footprinting revealed detectable changes at 8 sites on β and β', the present work has identified 53 sites that are unambiguously proximal to bound σ^{70}. We have found that probes placed not only in previously identified core-binding regions 2.1 and 3.2, but also in conserved regions 2.3, 3.1, and 4.2 are proximal to specific sites and conserved regions on β and β'. Strikingly, the cut sites are clustered in discrete segments of the primary structure of these two large subunits. Further development of this mapping strategy should increase substantially our understanding of the architecture of RNA polymerase and provide tools for the characterization of other macromolecular complexes.

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