Localizatio

 other conserved and nonconserved regions of σ^2^ have led to the idea of multiple binding sites for the σ subunit on the core enzyme (14–18).

To date, little is known about the location of the σ binding sites on the core subunits. There have been two observations that have identified deletions in the β or β^′^ subunits that produce subunits still capable of forming core enzyme structures but not holo. First, a β subunit truncation, missing approximately 200 amino acids (aa)^1^ of the C terminus, was shown by glycerol gradient centrifugation to migrate with the other core subunits but was never seen in the σ-containing fractions (17). Second, when immunoprecipitation assays were performed using recombinant RNA polymerase containing β^′^ deletion mutants missing aa 201–477, the core subunits were recovered in the same fraction but lacked σ (18). The idea that σ binding is affected by perturbations of the C terminus of β and the N terminus of β^′^ is consistent with experiments showing that these two subunit termini are physically close together and can be fused through a flexible linker and still form a functional enzyme (19). Recent protein-protein footprinting data have identified a similar region on β^′^ and two new sites on β for possible interactions with the σ^70^ subunit (20).

The β and β^′^ subunits each contain regions that have high sequence homology with the two largest subunits of eukaryal polymerases (21–23). Some of these conserved regions may act as interaction domains. We define an interaction domain as the minimal region of a protein that can independently fold to form the secondary and tertiary structure required to interact with another protein, DNA, RNA, or ligand. Interaction domains will always be larger than the actual binding site, the amino acids in direct contact with the binding partner. Therefore, additional work will be needed to identify the critical residues involved in making binding contacts. Severinov et al. (24–26) demonstrated the domain-like properties of β and β^′^ by reconstitution of functional RNA polymerase from fragmented β and β^′^ subunits. This indicates that the properties of the protein do not require the entire intact length of the subunit but rather can be generated with smaller domain modules.

In this study, we set out to map the protein-protein interaction domains on both β and β^′^ required for the binding of σ^70^. Chemical and enzymatic cleavage as well as PCR methods were used to generate fragments of β and β^′^ for use in mapping the interaction domains. Using far-Western blotting and nickel nitrilotriacetic acid (Ni^2^+-NTA) co-immobilization assays (27–29), we were able to map a strong specific binding site for σ^70^ to the N terminus of β^′^. This report shows that this binding site is

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1 The abbreviations used are: aa, amino acids; BSA, bovine serum albumin; His6, hexahistidine; PAGE, polyacrylamide gel electrophoresis; N6^2^+-NTA, nickel nitrilotriacetic acid; HMK, heart muscle kinase; NT2CB, 2-nitro-5-thiocyanobenzoic acid; PCR, polymerase chain reaction; DTT, dithiothreitol; CHES, 2-cyclohexylaminoethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
characteristics of subunits and subunit fragments used in binding assays

| Plasmid | Subunit | Residues | Modifications | Ref. |
|---------|---------|----------|---------------|------|
| pTA499 | β'     | 1–1407   | N-terminal His₆ | This work |
| pTA500 | β'     | 1–1407   | C-terminal His₆ | This work |
| pTA501 | β'     | 1–1342   | N-terminal His₆ | This work |
| pTA502 | β'     | 1–1342   | C-terminal His₆ | This work |
| pTA515 | β'     | 1–260    | None           | This work |
| pTA516 | β'     | 1–280    | None           | This work |
| pTA517 | β'     | 1–300    | None           | This work |
| pTA518 | β'     | 1–309    | None           | This work |
| pTA519 | β'     | 150–309  | None           | This work |
| pTA522 | β'     | 1–260    | N-terminal His₆-HMK | This work |
| pTA523 | β'     | 1–280    | N-terminal His₆-HMK | This work |
| pTA524 | β'     | 1–300    | N-terminal His₆-HMK | This work |
| pTA525 | β'     | 1–309    | N-terminal His₆-HMK | This work |
| pTA528 | β'     | 60–309   | None           | This work |
| pTA530 | β'     | 100–309  | None           | This work |
| pTA531 | β'     | 33–309   | N-terminal His₆-HMK | This work |
| pTA532 | β'     | 60–309   | N-terminal His₆-HMK | This work |
| pTA533 | β'     | 100–309  | N-terminal His₆-HMK | This work |
| pTA534 | β'     | 150–309  | N-terminal His₆-HMK | This work |
| pTA535 | β'     | 178–309  | None           | This work |
| pTA536 | β'     | 200–309  | None           | This work |
| pTA537 | β'     | 178–309  | N-terminal His₆-HMK | This work |
| pTA538 | β'     | 200–309  | N-terminal His₆-HMK | This work |
| pTA540 | β'     | 260–1407 | C-terminal His₆ | This work |
| pTA546 | β'     | 260–1407 | C-terminal His₆ | This work |
| pTA547 | β'     | 270–1407 | C-terminal His₆ | This work |
| pTA548 | β'     | 280–1407 | C-terminal His₆ | This work |
| pTA549 | β'     | 290–1407 | C-terminal His₆ | This work |
| pRL663 | β'     | 1–1407   | C-terminal His₆ | This work |
| pRL706 | β      | 1–1342   | C-terminal His₆ | 30 |
| pHMK-His₆-σ⁷⁰ | a₇⁰ | 1–618    | N-terminal His₆-HMK | This work |
| pLN12  | a₇⁰    | 1–618    | None           | 35 |

located within a span of residues (260–309) that overlaps conserved region B of β' (23).

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids**

Plasmid characteristics are described in Table I. An overexpression vector for C-terminal hexahistidine (His₆)-tagged β' (pTA500) was constructed by removing the XbaI-HindIII fragment from pRL663 and placing it in pET28b (Novagen) (31). N-terminally His₆-tagged β' was expressed from pTA499 that was constructed using PCR to place the His₆ tag on the N terminus of a fragment that overlapped the NraI site of β'. This fragment was placed into the pET28b vector followed by the insertion of the C-terminal portion of the gene on a NruI-HindIII fragment from pRL663. The C-terminal His₆ tag from the pRL663 fragment was removed by replacement of the RsrII-HindIII fragment with a PCR product coding for the wild type C terminus. pTA501 was constructed by creating an N-terminal His₆ tag via PCR for a fragment with a PCR product coding for the wild type C terminus. pTA502, coding for the C-terminal His₆-tagged β subunit, was derived using PCR to insert a N-terminal NcoI site on a fragment overlapping KpnI. The C-terminal His₆-containing fragment was inserted on a KpnI-HindIII fragment from pRL706 (19). Vectors expressing unmodified fragments were obtained by PCR cloning of the desired fragment and placement of the fragment into either pET21a (Novagen) for pTA528, pTA530, pTA535, and pTA536 or pET24a (Novagen) for pTA519 using NdeI and XhoI restriction sites. pTA522–525, pTA531, and pTA533 were all created by amplifying the specified β' region via PCR and inserted into a pET21a derivative that had been modified to fuse a N-terminal His₆ and heart muscle kinase (HMK) recognition site to the expressed proteins. pTA532 and pTA534 were constructed in the same fashion with the exception that the His₆-HMK vector derivative was constructed from pET28b, pTA547–549 were created by insertion of the fragments, N-terminally truncated via PCR, that overlapped the SnaBI site of β' into pET24a. The C-terminal coding region of the gene was inserted on a SnaBI-HindIII fragment from pTA500. pTA546 was created by fusing a C-terminal His₆ tag directly after residue 309 via PCR. The fragment was placed into the pET24a vector using NdeI and XhoI sites. To use σ⁷₀ as a radioactive probe, the HMK site was fused to the N terminus of σ⁷₀ along with a His₆ purification tag. pHMK-His₆-σ⁷₀ was created by placing the σ⁷₀ gene into a derivative of pET28b vector that contained the N-terminal His₆ and HMK fusion and adds a total of 13 extra aa (MHHHHHHHARRASV) to the N terminus of σ⁷₀. All products created by PCR were sequenced to ensure that no mutations had been introduced.

**Expression and Purification of Proteins**

Plasmids were transformed into BL21(DE3) (Novagen) for expression (31). The cells were grown in 1-liter cultures at 37 °C in LB medium with either 100 μg/ml ampicillin or 50 μg/ml kanamycin. The cultures were grown to an A₆₀₀ between 0.6 and 0.8 and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside. Three hours after induction, the cells were harvested by centrifugation at 8,000 × g for 15 min and frozen at −20 °C until use.

The cells were thawed and resuspended in 10 ml of lysis buffer (40 mM Tris-HCl, pH 7.9, 0.3 M KCl, 10 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride), and lysozyme was added to 100 μg/ml. The cells were incubated on ice for 15 min then sonicated three times in 60-s bursts. The recombinant protein in the form of inclusion bodies was separated from the soluble lysate by centrifugation at 27,000 × g for 15 min. The inclusion body pellet was resuspended, by sonication, in 10 ml of lysis buffer plus 2% (w/v) sodium deoxycholate. The mixture was centrifuged at 27,000 × g for 15 min and the supernatant discarded. The deoxycholate-washed inclusion bodies were resuspended in 10 ml deionized water and centrifuged at 27,000 × g for 15 min. The water wash was repeated, and the inclusion bodies were aliquoted into 1-mg pellets and frozen at −20 °C until use.

σ⁷₀ inclusion bodies were solubilized, refolded, and purified according to a variation of the procedure of Gribkov and Burgess (32). The inclusion bodies were solubilized by resuspension in 6 M guanidine HCl. The proteins were allowed to refold by diluting the denatured 64-fold with buffer A (50 mM Tris-HCl, 0.5 mM EDTA, and 5% (v/v) glycerol) in 2-fold steps over 2 h. One gram of DE52 resin (Whatman) was added and mixed with slow stirring for 24 h at 4 °C. The resin was then collected in a 10-ml column, washed, and the protein eluted with a gradient from 0.1 to 1 M NaCl in buffer A. The σ⁷₀ fractions were pooled and dialyzed overnight against 1 liter of storage buffer (50 mM Tris-HCl, 0.5 mM EDTA, 0.1 mM NaCl, 0.1 mM DTT, and 50% (v/v) glycerol) and stored at −20 °C.
Whole cell lysates were prepared as follows. Cells containing truncated β' expression plasmids were grown to an A$_{600}$ of 0.6–0.8 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside. The cells were grown for an additional 30 min. A 200-μl sample was removed and sonicated 5 × 30 s at 20 μl of glycerol and 20 μl of SDS-sample buffer were added and heated for 2 min at 95 °C then stored at −20 °C until use.

**Protein Cleavage**

β and β' inclusion bodies were subjected to chemical or enzymatic cleavage (see below) and then purified by nickel affinity chromatography as follows. The cleavage reaction was loaded onto 300 μl of Ni²⁺-NTA resin (Qiagen) pre-equilibrated with a Bio-Rad mini-column. The mixture was diluted to 4M urea by adding an equal volume of buffer B pre-equilibrated with buffer B (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole, 0.1% (v/v) Tween 20, and 10% (v/v) glycerol) + 8 μm urea. The protein bound resin was washed with 10 column volumes of buffer B + 8 μm urea followed by 10 column volumes of buffer B to allow refolding. The resin was then washed with 500 μl of buffer B + 40 mM imidazole. The protein was eluted with 500 μl of buffer B + 200 mM imidazole. The eluted fractions were stored at −20 °C.

**NTCB Cleavage** (33)—1 mg of inclusion body protein was resuspended in 1 ml of buffer B + 8 μm urea. DTT was added to 5-fold molar excess over the thiols in the protein. The mixture was incubated for 15 min at 37 °C to reduce any disulfide bonds. NTCB was added to be in 5-fold molar excess over total sulfhydryl groups. The pH was adjusted to 9.5 with NaOH. The reaction mixture was incubated for 2 h at room temperature. The cleavage mixture was diluted 1:10 in buffer B + 8 μm urea and loaded onto a Ni²⁺-NTA column as described above.

**Hydroxylamine Cleavage** (34)—1 mg of inclusion body protein was resuspended in 1 ml of buffer B + 8 μm urea. Five-hundred microliters of the solubilized protein were added to 500 μl of hydroxylamine cleavage solution (0.4 M CHES, pH 9.5, 4 μl hydroxylamine HCl) and incubated 2 h at 42 °C. β-Mercaptoethanol was added to 0.1 M and incubated 10 min at 37 °C. The mixture was diluted 1:10 in buffer B + 8 μm urea and loaded onto a Ni²⁺-NTA column as described above.

**Thermolysin Cleavage** (35)—1 mg of inclusion body protein was resuspended in 100 μl of buffer B + 8 μm urea and incubated for 15 min at 37 °C. Thermolysin was added at protein-to-protease ratios of 4,000:1, 8,000:1, and 16,000:1 (w/w). Reactions were carried out for 30 min at room temperature. The reactions were loaded onto a Ni²⁺-NTA column as described above.

**Tryptsin Cleavage** (35)—1 mg of inclusion body protein was resuspended in 1 ml of buffer B + 8 μm urea and incubated for 15 min at 37 °C. The mixture was diluted to 4 μl urea by adding an equal volume of buffer B. Tryptsin was added at protein-to-protease ratios of 4,000:1, 8,000:1, and 16,000:1 (w/w). Digestion was performed at room temperature for 30 min. The reactions were loaded onto a Ni²⁺-NTA column as described above.

**Far-Western Blotting**

**Dot Blot**—Inclusion body proteins that were resuspended in buffer B + 8 μm urea were spotted directly onto a nitrocellulose membrane (Schleicher & Schuell) using a Schleicher & Schuell “MINIFOLD” dot blot apparatus. The wells were washed three times with buffer B. The nitrocellulose was blocked by incubation in HYB buffer (20 mM Heps, pH 7.2, 200 mM KCl, 2 mM MgCl₂, 0.1 mM ZnCl₂, 1 mM DTT, 0.5% (v/v) Tween 20, 1% (v/v) non-fat dry milk) for 1 h at 4 °C.

**Gel Blot**—Protein cleavage fragments or whole cell lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE). The proteins were electrophoretically transferred onto 0.05% nitrocellulose. The nitrocellulose was blocked by incubating in HYB buffer for 1 h at 4 °C.

**Labeling**—Labeling of σ⁷⁰ was done in a 100-μl reaction volume, 50 μl of 2 × kinase buffer (40 mM Tris-HCl, pH 7.4, 200 mM NaCl, 24 mM MgCl₂, 2 mM DTT, and 50% (v/v) glycerol) was added to 50 μg of σ⁷⁰ protein. 240 units of cAMP-dependent kinase-catalytic subunit (Promega) was added, and the total volume was brought up to 99 μl with deionized water. One microliter of [γ-³²P]ATP (0.15 μCi/μl) was added. The mixture was incubated at room temperature for 30 min. The reaction mixture was then loaded onto a BioSpin-P6 column (Bio-Rad) pre-equilibrated with 1 × kinase buffer and spun at 1,100 × g for 4 min. The flow-through was collected and stored at −20 °C.

**Probing**—The blocked nitrocellulose was incubated in 10 ml of HYB buffer with 4 × 10⁵ cpm/ml ³²P-labeled σ⁷⁰ for 3 h at room temperature. The blot was washed three times with 10 ml of HYB buffer for 3 min each. The blot was then dried and exposed to film or PhosphorImager (Molecular Dynamics).
conditions using Ni\textsuperscript{2+}-NTA resin to isolate cleavage fragments containing a His\textsubscript{6} tag. These purified fragments were then identified based on their mobility in SDS-PAGE, and their exact size was determined based on the cleavage site which produced them (Fig. 3A). When the cleavage fragments were fractionated by SDS-PAGE, they produced a ladder of descending sized fragments with a common end (either N or C terminus depending on the placement of the His\textsubscript{6} tag). The use of both N- and C-terminally His\textsubscript{6}-tagged fragments allows the positive identification of both the N and C termini of the interaction domain. The \( \sigma^{70} \) probe will only bind the fragments that have an intact interaction domain. The N-terminally His\textsubscript{6}-tagged \( \beta' \) ladders produced by hydroxylamine and NTCB cleavage both produced the same ladder. However, the ladder produced by hydroxylamine had additional early fragments, which may correspond to sites of cleavage that are not cleaved by NTCB. Therefore, there are many more sites of cleavage available. To increase the number of proteolytic fragments with a common end (either N or C terminus), we made constructs that were truncated at either the N or C terminus. DNA coding for the truncated fragments was cloned into overexpression plasmids. When cells containing these plasmids had been grown to an \( A_{600} \) of 0.6, expression was induced. The cells were only allowed to grow for 30 min after induction. A whole cell lysate from each culture was made and used for far-Western blotting assays (Fig. 4). Short expression times kept the expression level of the induced protein comparable with the other proteins in the lysate. The use of the whole cell lysate in far-Western blotting assays was an internal control to ensure binding was specific for the protein of interest. This also meant that the various proteins would not have to be purified and could be expressed without purification tags. When constructs were made where the C terminus of \( \beta'_{1-309} \) was truncated beyond amino acid 300, the binding of \( \sigma^{70} \) was lost (Fig. 4A). However, the N terminus of the same fragment could be truncated up to 60 aa without diminishing the signal (Fig. 4B). \( \beta'_{100-309} \) still showed binding, but at a lower level, and \( \beta'_{150-309} \) did not bind \( \sigma^{70} \). These results narrowed the \( \sigma^{70} \) binding site to \( \beta'_{60-309} \). Western blot experiments using anti-\( \beta' \) monoclonal antibodies were done to ensure that the protein fragments were being transferred to the nitrocellulose and that they were fragments of \( \beta' \) (data not shown).

**Co-immobilization Assays Further Narrow Interaction Site to 260–309 aa of \( \beta' \)—Ni\textsuperscript{2+}-NTA co-immobilization assays were used to confirm and extend the results that had been produced using far-Western blotting. The proteins to be assayed for binding \( \sigma^{70} \) were fused to His\textsubscript{6} purification tags and overexpressed in the form of inclusion bodies. The inclusion body protein was solubilized with 8 M urea and loaded onto Ni\textsuperscript{2+}-NTA resin. The denaturant was washed away allowing the proteins to refold while still remaining bound to the resin. Native \( \sigma^{70} \) was then loaded onto the column. The column was washed, and the bound proteins were then eluted with imidazole. Any truncated protein that contained the interaction domain for \( \sigma^{70} \) would cause \( \sigma^{70} \) to be bound and to be in the eluted fraction. The results of the these binding experiments are consistent with the far-Western blotting experiments in respect to defining the C-terminal boundary of the domain, \( \beta'_{1-309} \) bound \( \sigma^{70} \), while \( \beta'_{1-300} \) and \( \beta'_{1-280} \) did not bind \( \sigma^{70} \) (Fig. 5A–C). Refolded \( \beta'_{1-309} \) without a His\textsubscript{6} tag was mixed with \( \sigma^{70} \) and passed over the Ni\textsuperscript{2+}-NTA to ensure the complex was not nonspecifically binding to the column. The complex passed through the column and was not seen in the eluted fraction (Fig. 5D). As a control, BSA was loaded onto a column containing \( \beta'_{1-309} \) (Fig. 5E). BSA was seen only in the flow-through and not in the eluted fraction, suggesting \( \beta'_{1-309} \) binds \( \sigma^{70} \) specifically.

For the N-terminal boundary, the results showed that more of the N terminus could be removed without affecting \( \sigma^{70} \) binding than was seen by the far-Western assay. Several N-terminally truncated fragments, all having aa 309 as the C-terminal boundary followed by a His\textsubscript{6} tag, were constructed and used in co-immobilization assays. Truncations to residues 33, 60, 100, 178, and 200 still produced fragments capable of binding \( \sigma^{70} \) (data not shown). \( \beta'_{260-309} \), the smallest fragment we could make and still manipulate efficiently in our assay, retained the ability to bind \( \sigma^{70} \) (Fig. 6A). To find the N terminus of the interaction domain, truncations greater than residue 240 were made from full-length \( \beta' \). A truncation of the first 260 residues of \( \beta' \) (\( \beta'_{260-C} \)) bound \( \sigma^{70} \), while \( \beta'_{270-C} \) showed diminished binding, and \( \beta'_{280-C} \) showed no detectable binding of \( \sigma^{70} \) (Fig. 6, B–D). Taken together these results indicate that a strong \( \sigma^{70} \) binding site on the core polymerase is located within the aa 260–309 region of \( \beta' \).

**DISCUSSION**

Since the discovery of the \( \sigma \) subunit in 1968, a great deal of effort has gone into characterizing the interactions between \( \sigma \) and the core enzyme (4). To date, several biochemical and
the major binding interaction for $\sigma^{70}$ in the holoenzyme while $\beta$ adds a secondary binding interaction. Multiple core binding sites on $\sigma$ have been suggested in light of $\sigma$ mutations apparently affecting core binding that map outside of conserved region 2.1 (14–16).

The primary finding of this work is that a strong binding site for $\sigma^{70}$ is located within residues 260–309 of $\beta'$. A deletion of residues 201–477 of $\beta'$ has been reported previously to produce a mutant protein that could still form core but not holoenzyme (18). The problem with such deletion studies is that one cannot conclude that the binding site is located in the region deleted, but merely that the region, when deleted, prevents correct formation of the interaction domain. Results obtained from protein-protein footprinting experiments indicated that a similar region of $\beta'$ (residues 228–461) was physically close to $\sigma^{70}$ (20). There is difficulty in interpreting these results, since the assay gives indications of physical proximity of the proteins that do not necessarily correspond to protein-protein binding. From our findings it can be concluded that a major $\sigma^{70}$ binding site is located within these regions.

The $\sigma^{70}$ interaction domain on $\beta'$ contains several residues located in conserved region B (23). This region does not have any known function. Secondary structural predictions derived from the PHD program (39) for residues 260–309 indicates one helix from residue 264 to residue 283 connected by a loop to the second helix from residue 292 to residue 309. These predicted helices are also predicted to form coiled coils (40). This is of particular interest, since similar predictions were made for residues 355–391 of $\sigma^{70}$. These residues overlap conserved region 2.1. The crystal structure of the protease-resistant fragment of $\sigma^{70}$ confirmed the prediction that the helix containing region 2.1 is forming a coiled coil with conserved region 1.2 (41). Since coiled coils have been shown to be involved in many protein-protein interactions (42–44), this would suggest that $\beta'_{260–309}$ may be interacting with region 2.1 of $\sigma^{70}$. Our laboratory is investigating where the $\beta'_{260–309}$ interaction domain is contacting $\sigma^{70}$.

Previously in our laboratory we have developed a method for quickly mapping epitopes for monoclonal antibodies (35). We describe here an application of that method to identify domains involved in other protein-protein interactions. Ordered fragment ladder far-Western blotting was used to map the $\sigma^{70}$ binding site on $\beta'$ to within $\beta'_{60–309}$. This method relies on the fact that after the removal of the denaturant some fraction of

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**Fig. 3.** Ordered fragment far-Western. $\beta$ and $\beta'$ subunits were cleaved with hydroxylamine and NTCB. The Ni²⁺-NTA purified fragments were separated on identically loaded 8–16% SDS-PAGE gels (Novex). One gel (A) was stained with Coomassie Blue, and the other (B) was transferred to nitrocellulose and probed with $^{32}$P-$\sigma^{70}$, Lanes: 1, markers; 2, E. coli lysate; 3, N-His$_6$$\beta'$-hydroxylamine; 4, C-His$_6$$\beta'$-hydroxylamine; 5, N-His$_6$$\beta$-hydroxylamine; 6, C-His$_6$$\beta$-hydroxylamine; 7, N-His$_6$$\beta'$-NTCB; 8, C-His$_6$$\beta'$-NTCB; 9, N-His$_6$$\beta$-NTCB; 10, C-His$_6$$\beta$-NTCB; 11, purified core polymerase.

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**Fig. 4.** Probing cloned fragments. Whole cell lysates from cells expressing $\beta'$ fragments were fractionated by SDS-PAGE using 8–16% Tris-glycine gels. A, C-terminally truncated fragments. Lanes 1–4, Coomassie Blue-stained; lanes 5–8, far-Western probed with $^{32}$P-$\sigma^{70}$. Lanes: 1 and 5, $\beta'_{1–300}$; 2 and 6, $\beta'_{1–309}$; 3 and 7, $\beta'_{1–260}$; and lanes 4 and 8, $\beta'_{1–309}$ B, N-terminally truncated fragments. Lanes 1–7, Coomassie-stained; lanes 8–14, far-Western probed with $^{32}$P-$\sigma^{70}$. Lanes: 1 and 8, $\beta'_{1–309}$; 2 and 9, $\beta'_{31–309}$; 3 and 10, $\beta'_{60–309}$; 4 and 11, $\beta'_{100–309}$; 5 and 12, $\beta'_{150–309}$; 6 and 13, $\beta'_{258–309}$; and 7 and 14, $\beta'_{260–309}$.

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Genetic studies have contributed to what is known about the putative core binding domains on $\sigma$, however, much less is known about the sites on core that bind $\sigma$ (2). In the holoenzyme assembly pathway, $\beta'$ is added to the $\alpha_2\beta$ complex and then $\sigma$ is added to form the holoenzyme (38). This would suggest that either the major $\sigma$ binding site is located on $\beta'$ or is formed in cooperation with $\alpha$ and/or $\beta$ upon $\beta'$ assembly into the core enzyme. The isolation of $\sigma^{70}$-$\beta'$ complexes provides evidence for the former (18). In this report we have localized a strong binding site for $\sigma^{70}$ on $\beta'$, as well as identified low level binding affinity for $\sigma^{70}$ to $\beta$. Thus, we conclude that $\beta'$ provides...
the blotted protein will be able to refold and produce the proper conformation for binding of the probe. The specificity of the assay was demonstrated by probing whole cell lysates and identifying β' as the major binding interaction. The combination of specific chemical cleavage of proteins and far-Western blotting provided a very rapid and effective way to localize this protein-protein interaction. Cloning and screening individual, truncated fragments was necessary only after the interaction was identified.

To confirm and extend the results obtained with far-Western blotting, Ni²⁺-NTA co-immobilization assays were performed. These experiments also demonstrated that fragments from the N terminus to residue 309 could still bind σ⁷⁰, while removal of just 9 C-terminal residues to aa 300 would abolish binding. The results obtained from the N-terminally truncated fragments in these assays gave much better resolution of the binding site location than was obtained from far-Western assays. Up to 260 residues could be removed from the N terminus without affecting σ⁷⁰ binding. When 270 residues were removed, binding of σ⁷⁰ was diminished but not abolished, suggesting that either part of the binding site had been removed or the binding site was intact but hindered from refolding due to the loss of upstream residues. To ensure that the binding site was what we were actually mapping and not just a region required for proper folding of the actual binding site, protein fragments were made from 260–309 of β' and shown to be sufficient for binding. We believe that the difference in the identified interaction domain size between the far-Western assay (β'260–309) and the co-immobilization assay (β'260–309) is consistent with the properties of each assay. The far-Western assay requires the interaction domain to refold and properly present the binding site while some portion of the protein is attached to the nitrocellulose membrane. We believe that as such the proteins are more conformationally restricted than proteins bound only at one terminus as in the Ni²⁺-NTA co-immobilization assay. Therefore, more of the protein length is required to form a scaffold-like structure to keep the interaction domain away from the membrane surface. The combination of mapping methods provides a rapid, high resolution procedure for identification of protein interaction domains.

We have work in progress to map the binding sites on core for the other 6 known E. coli σ factors. It has been hypothesized that the core binding region on the σ factors must be highly conserved, since they all must bind core polymerase (1, 2, 7). This has led to region 2 as the primary candidate, since it is the most highly conserved region. This suggests that all of the σ factors in the cell may bind to the same site or sites on core polymerase. We are now in a position to test this hypothesis and identify if the other σ factors can also bind β'260–309.

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