Mutational analysis of $\beta''_{260-309}$, a $\sigma^{70}$ binding site located on Escherichia coli core RNA polymerase.

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SUMMARY

In eubacteria, the sigma subunit binds to the core RNA polymerase and directs transcription initiation from any of its cognate set of promoters. Previously, our lab defined a region of the β' subunit that interacts with σ^{70} in vitro. This region of β' contained heptad repeat motifs indicative of coiled coils. In this work, we used ten, single point mutations of the predicted coiled coils, located within residues 260-309 of β', to look at disruption of the σ^{70}-core interaction. Several of the mutants were defective for binding σ^{70} in vitro. Of these mutants, three (R275Q, E295K, and A302D) caused cells to be inviable in an in vivo assay where the mutant β' is the cell’s sole source of β' subunit. All of the mutants were able to assemble into the core enzyme, however, R275Q, E295K, A302D were defective for Eσ^{70}-holoenzyme formation. Several of the mutants were also defective for holoenzyme assembly with various minor sigma factors. In the recently published crystal structure of *Thermus aquaticus* core RNA polymerase, the region homologous to β'_{260-309} of *Escherichia coli* forms a coiled coil. Modeling of our mutations onto that coiled coil places the most defective mutations on one face of the coiled coil.
INTRODUCTION

The DNA-dependent RNA polymerase (RNAP)$^1$ is a multisubunit enzyme that plays a central role in eubacterial gene regulation and expression. This enzyme has two functional forms: core and holoenzyme. Transcription elongation and termination are performed by the core enzyme with $\alpha:2,\beta:1,\beta':1$ stoichiometry (1). Core RNAP binds one of a variety of sigma subunit species at a given time to form a specific holoenzyme (2). The holoenzyme performs the tasks of promoter recognition and transcription initiation. Each sigma subunit directs its cognate holoenzyme to start transcription from only those promoters containing DNA sequences specifically recognized by that sigma factor (2-4).

Understanding the molecular basis of $\sigma$ binding to core RNAP will aid in the analysis of several questions involving how multiple $\sigma$ species come to sequester core and turn on subsequent genes. It has been hypothesized that all of the $\sigma$ species bind to the same site/sites on the core enzyme (1,6,7). Studies to identify the core binding site on $\sigma$ have resulted in the positive identification of a single binding site located on $\sigma^{70}$ overlapping conserved region 2.1 (5,8). A single point mutation in the homologous region of *Bacillus subtilis* $\sigma^E$ prevented binding to core (9). More recent genetic and biochemical studies suggest that region 2.1 may be only one of multiple contact sites that $\sigma$ uses in binding to the core enzyme (6,10,11).

Information about sites on core which bind $\sigma$ has come in most part from biochemical assays. Protein footprinting studies of the core enzymes susceptibility to hydroxy radical cleavage upon binding a modified sigma factor containing the cleavage catalyst have revealed that there are 3 regions on core, one located on $\beta'$ and two on $\beta$, that are in close proximity to $\sigma$ (12). One of the $\beta$ regions was identified earlier as being near the $\sigma^{70}$ binding region. It was noticed that this site, in the core enzyme complex, was cleaved by trypsin, while formation of E$\sigma^{70}$ prevented trypsin cleavage at
this site (13). Previous in vitro work from our lab identified a strong binding site for \( \sigma^{70} \) on the \( \beta' \) subunit (14). This site was mapped to within residues 260-309 of \( \beta' \).

The predicted secondary structure (15) of \( \beta'_{260-309} \) has two \( \alpha \) helices joined by a random coil. Another structural analysis program indicates that these two helices are amphipathic and have the potential for coiled coil formation (16). The coiled coil motif is based on a heptad repeat of residues designated a-g (17,18) (Fig. 1b). The a and d positions are hydrophobic while the other positions are usually charged or polar. Burial of the a and d hydrophobic residues during coiled coil formation provides a large amount of the binding energy. Specificity in binding comes from the e and g positions which can form ionic interactions or salt bridges.

We have undertaken a mutational analysis of this region to confirm that our in vitro binding results were relevant to in vivo binding and function. This work presents the analysis of 10 point mutations, most of which are change-of-charge mutations at the e and g residues, in the \( \beta'_{260-309} \) predicted coiled coil. Three of the mutations (R275Q, E295K, and A302D) were nonfunctional in binding \( \sigma^{70} \) in all of the assays in which they were tested, while still able to assemble into the core enzyme. We also report on mutations that were nonfunctional in some of our assays but functional in others, indicating that binding of other sites may compensate for loss of binding at the \( \beta'_{260-309} \) site. We use this analysis to demonstrate that the binding site identified previously by in vitro methods is important in vivo and that mutations in this region can greatly diminish core binding of \( \sigma^{70} \) and other minor sigmas. In the recently solved crystal structure for the core RNAP of \textit{Thermus aquaticus}, the region homologous to \textit{E. coli} \( \beta'_{260-309} \) was determined to form a “coiled coil-like” structure (19) consistent with our predictions. Modeling of our mutations onto the \textit{T. aquaticus} structure places all of the nonfunctional mutations on the same face of the \( \beta' \) coiled coil.

**EXPERIMENTAL PROCEDURES**
Construction of plasmids

Plasmid characteristics are described in Table I. Plasmids pTA577, 600-620 were made from the base plasmid pRL663 (20). Single HindIII and BamHI restriction sites, at bases 674 and 952 of rpoC respectively, were inserted into the rpoC gene of pRL663 via silent mutagenesis to create pTA577. pTA561 was created in the same manner as pTA577 except pRL308 (22) was the starting plasmid. The HindIII and BamHI restriction sites were used to insert PCR-generated DNA fragments containing the various mutations to generate pTA600-609. For pTA620, containing a truncated rpoC fragment coding for β’ residues 1-319, pRL663 was cut with Xba-HindIII for insertion of a PCR generated rpoC truncation. The σ70 binding site was previously mapped to residues 260-309 of β’, however we engineered some of the constructs for this work to extend to residue 319. This was done to incorporate the BamHI site mentioned previously. Therefore, the various mutations could be moved into the new plasmid to create pTA610-619. We have not seen any difference in behavior of the fragments ending at residue 309 as opposed to those ending at residue 319. All sequences generated via PCR were sequenced to ensure spurious mutations had not been incorporated.

Expression and purification of σ70

The cells were grown to an A600 of 0.6-0.8 in 1 L cultures at 37 °C in LB medium with 100 µg/ml ampicillin. Isopropyl β-D-thiogalactoside (IPTG) was then added to a concentration of 1 mM. Three hours after induction, the cells were harvested by centrifugation at 8,000 x g for 15 min and frozen at −20 °C.

The cell pellet from a 1 L culture was thawed and resuspended in 10 ml of lysis buffer (40 mM Tris, pH 7.9, 0.3 M KCl, 10 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride) and lysozyme was added to 0.1 mg/ml. The cells were incubated on ice for 15 min then sonicated in 3 x
60 s bursts. The recombinant protein in the form of inclusion bodies was separated from the soluble lysate by centrifugation at 27,000 x g for 15 min. The inclusion body pellet was resuspended by sonication in 10 ml of lysis buffer + 2 % (w/v) sodium deoxycholate (DOC). The mixture was centrifuged at 27,000 x g for 15 min and the supernatant was discarded. The DOC-washed inclusion bodies were resuspended in 10 ml deionized water and centrifuged at 27,000 x 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 (10 mg) were solubilized, refolded and purified according to a variation of the procedure of Gribskov and Burgess (21). The inclusion bodies were solubilized by resuspension in 10 ml of 6 M guanidine-HCl. The proteins were allowed to refold by diluting the denaturant 64-fold with buffer A (50 mM Tris, pH 7.9, 0.5 mM EDTA, and 5 % (v/v) glycerol) in 2-fold steps over 2 h. One gram of resin (DEAE-cellulose, 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 1.0 M NaCl in buffer A. The σ70 fractions were pooled and dialyzed overnight against 1 L of storage buffer (50 mM Tris, pH 7.9, 0.5 mM EDTA, 0.1 M NaCl, 0.1 mM DTT and 50 % (v/v) glycerol) and stored at –20 °C.

Quantitative Western blotting

Protein samples to be quantitated were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). The proteins were electrophoretically transferred out of the gel onto 0.05 μm nitrocellulose. The blot was blocked in Blotto (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1 % (v/v) Tween 20, and 3 % (w/v) nonfat dry milk) and probed with monoclonal antibodies. The signal was generated using the ECL Plus system (Amersham) and detected on a Storm PhosphorImager.
(Molecular Dynamics). The signal was quantitated using ImageQuant software (Molecular Dynamics).

Far Western blotting

Cells, containing truncated β' expression plasmids pT3A610-620, were grown to A<sub>600</sub> = 0.6-0.8 and induced with 1 mM IPTG. The cells were grown for an additional 30 min. A 200 µl sample was removed and sonicated 3 x 30 s. 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. The lysates were separated by SDS-PAGE. The proteins were electrophoretically transferred onto 0.05 µm nitrocellulose. The nitrocellulose was blocked by incubating in HYB buffer (20 mM Hapes, pH 7.2, 200 mM KCl, 2 mM MgCl₂, 0.1 µM ZnCl₂, 1 mM DTT, 0.5% (v/v) Tween 20, 1% (w/v) non-fat dry milk) for 16 h at 4 °C.

A chimeric σ⁷₀ was created by fusing a heart muscle kinase (HMK) recognition sequence to the N terminus of σ⁷₀ to facilitate radio labeling of σ⁷₀. Labeling of σ⁷₀ was done in a 100 µl reaction volume. 50 µl of 2x kinase buffer (40 mM Tris, pH 7.4, 200 mM NaCl, 24 mM MgCl₂, 2 mM DTT) was added to 50 µg of HMK-σ⁷₀ protein. 240 U of cAMP-dependent kinase catalytic subunit (Promega) was added and the total volume was brought to 99 µl with deionized water. One microliter of γ⁻³²P-ATP (0.15 mCi/µl) was added. The mixture was incubated at room temperature for 30 min. The reaction mixture was then loaded onto a Biospin-P6 column (BioRad) pre-equilibrated with 1x kinase buffer and centrifuged at 1100 x g for 4 min. The flow-through was collected and stored at –20 °C.

The blocked nitrocellulose was incubated in 10 ml of HYB buffer with 4 x 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 dried and the signal was visualized with a PhosphorImager and quantitated with IMAGEQUANT software (Molecular Dynamics).

**Growth assessment**

Plasmids pTA577, 600-609 (0.1 µg) were transformed into strain RL602 (22,23). After heat shock and incubation on ice, 300 µl of LB was added to the 50 µl cell mixture. 10 µl of the transformation reaction was spotted onto LB plates plus ampicillin (100 µg/ml) and incubated at 30 °C. Another 10 µl was spotted onto identical plates and incubated at 42 °C. The plates were incubated between 24-48 h and assessed for growth.

**Purification of core/ho101 complexes**

1 L flasks containing 200 ml of LB with ampicillin (100 µg/ml) and IPTG (0.15 mM) were inoculated with 200 µl from overnight cultures of cells containing plasmids pTA561, 577, 600-609. The cultures were grown at 37 °C with shaking until the $A_{600} = 0.4$ for log phase assays and 2 h longer ($A_{600} ~ 2.0$) for the early stationary phase assays. The cells were harvested by centrifugation at 6,000 x g for 10 min and stored at −20 °C until use. The cell pellets were resuspended in 5 ml TE (10 mM Tris, pH 7.9 and 0.1 mM EDTA) plus 0.15 M NaCl and lysozyme (0.1 mg/ml), then incubated on ice for 15 min. The cells were sonicated 3 x 30 s and centrifuged for 25 min at 27,000 x g to remove the insoluble material. The supernatant was loaded onto a 1.5 ml immunoaffinity column containing the polyl-responsive, anti-β’ monoclonal antibody (MAb), NT73 (24). The column was washed with 15 ml TE plus 0.15 M NaCl followed by a second wash with 10 ml TE plus 0.5 M NaCl. The protein was eluted from the column with 4 ml TE plus 0.7 M NaCl and 30 % propylene glycol. The eluted sample (4ml) was diluted with 6 ml buffer B (20 mM Tris, pH 7.9, 500 mM NaCl, 5 mM imidazole, 0.1 % (v/v) Tween 20, and 10 % (v/v) glycerol) and loaded (2x) onto 500 µl of Ni²⁺-NTA resin. The resin was washed twice with 5 ml of buffer B and eluted with 0.5 ml
buffer B plus 0.25 M imidazole. Samples from the elution fractions were assayed by Western blot as described above, using MAbs to each subunit or \( \sigma \) factor. The secondary antibodies were horseradish peroxidase-labelled goat anti-mouse IgG antibodies and the signal was generated using the ECL Plus substrate system (Amersham) and detected using the STORM PhosphorImager and quantitated with IMAGEQUANT software (Molecular Dynamics).

**RESULTS**

*Mutational design*

Structural prediction, using the Coils program(16), scored both of the predicted \( \alpha \)-helices of \( \beta'_{260-309} \) as having a high probability of forming coiled coils (Fig. 1a). To test this prediction we constructed two \( \beta' \) mutants with proline residues inserted into either helix. These \( \beta' \) mutants were no longer predicted to form helices or coiled coils. When assayed for function in both the far Western and in vivo growth assays, both mutants were found to be nonfunctional (data not shown). We took this to indicate that the helical/coiled coil structure in this region was important for function. The solubility of these mutant proteins was not 100\%, so we ceased using them since their loss of function could simply be due to gross folding defects. We decided to concentrate in most part on the “e” and “g” positions of the \( \alpha \)-helices for the next phase of our analysis. The e and g residues of coiled coils often engage in interhelical interactions such as the formation of ionic interactions or salt bridges (17,18). Such interactions in this case could be intramolecular (between the two helices of \( \beta'_{260-309} \)) forming a coiled coil structure necessary for binding by the sigma subunit (Fig. 1b). Alternatively, the e and g residues of \( \beta'_{260-309} \) could be making intermolecular contacts with sigma upon binding. Our efforts were directed toward making change-of-charge mutations at these residues of \( \beta' \) (Fig. 1b) and assaying their effects on binding. Two of the mutations described in this work do not involve e or g residues and were chosen for other reasons.
Based on the findings that tyrosine and arginine residues are often located in “hot spots” of protein-protein interactions (26), we changed the tyrosine residue at position 269 to an alanine and arginine 297 to a serine. Previous studies in our lab found that insertion of a leucine at position 297 generated a β’ subunit that was nonfunctional for binding σ70 (unpublished results). Therefore, we were interested to determine if a less drastic mutation at this position would also affect σ binding.

Several of the mutations in the β’260-319 region disrupt interaction with σ70 in a far Western assay.

Previously, we had used far Western blotting to map a σ70 binding site to the N-terminal region of the β’ subunit (14). We again applied this procedure as an initial assay for functionality of our β’ mutants. The mutations were cloned into a gene fragment coding for amino acids 1-319 of the β’ subunit. Cells containing these genes were induced for a short period to give moderate levels of the β’ fragment, comparable to other proteins in the extract. Samples were analyzed for binding σ70 by far Western analysis as described in the Experimental Procedures. The amount of σ70 probe bound by each β’1-319 mutant fragment was compared to the amount bound by wt β’1-319 fragment. Each signal was normalized to the amount of β’1-319 contained in the supernatant as determined by Western blotting.

Five of the mutations (R275Q, R293Q, E295K, R297S, and A302D) were greatly reduced in their ability to bind σ70 (Fig. 2). The Q300E and N309D mutations had the opposite effect, binding more σ70 than wild type β’1-319. Q300E exhibited an increase in relative binding of greater than 7-fold. There were no effects on binding seen with the N266D, Y269A, or K280E mutations.

Complementation with mutant β’ subunits

To assess the importance of the σ70 binding site in vivo, we assayed the ability of mutant β’ subunits to function as the cell’s sole source of β’.

Plasmids containing mutant or wild type, full
length β’ were transformed into strain RL602 (22,23). The chromosomal rpoC gene of RL602 has an amber mutation that prevents functional β’ from being produced in the absence of a suppressor tRNA. RL602 also has a chromosomal, temperature-sensitive, amber suppressor. At the permissive temperature (30 °C) the amber suppressor is active and allows chromosomal β’ to be produced and the cell can grow. The amber suppressor is not active at the non-permissive temperature (42 °C). Therefore, at 42 °C, chromosomal β’ is not made and the cell cannot grow without another source of β’. If the plasmid-derived β’ can complement the loss of β’, then the cells will grow and form colonies on plates at the non-permissive temperature. If the mutant β’ cannot complement, there will be no growth on the plates at this temperature.

Three of the β’ mutants that were defective for sigma binding in the far Western assay (R275Q, E295K, and A302D) could not support growth at the non-permissive temperature, indicating that these mutations were also caused defects in binding sigma in vivo (Fig. 3). N266D, a mutation that had no detectable effect in the far Western assay, allowed some growth at the non-permissive temperature but not enough to be considered wild type. In contrast, the R293Q and R297S mutations that did not bind σ70 in the far Western assay could support growth in vivo. Mutations Y269A, K280E, Q300E, and N309D had no detectable effects on growth. Expression levels for nonfunctional β’ mutants were determined to be equivalent to that of plasmid derived, wild type β’ when grown at 37 °C (data not shown).

Effects of β’ mutations on core/holoenzyme assembly

An alternate explanation for the inviability caused by some of the β’ mutations would be that they are no longer able to be assembled into the core enzyme. To evaluate the potential assembly defects caused by the various mutations, we expressed His6-tagged, mutant β’ subunits in cells that were also expressing wild type, chromosomal β’ proteins. We used a Ni²⁺-NTA mediated pull-out
assay to purify the mutant β', subunits together with associated cell proteins. An immunoaffinity column was used to clean up the samples in order to reduce any non-specific binding to the Ni\(^{2+}\)-NTA column.

All of the mutant β’ subunits tested retained the ability to assemble into the core enzyme demonstrated by the association of the α and β subunits throughout the purification (Fig. 4a,b). Again, mutations R275Q, E295K, and A302D caused defects in binding σ\(^{70}\) in both log and stationary phase samples (Fig. 4c). Also reduced in Eσ\(^{70}\) formation were N266D in both log and stationary phase samples and R297S in log phase samples. Q300E again showed properties of binding σ\(^{70}\) better than wild type. Y269A, K280E, R293Q, and N309D had no detectable effect on Eσ\(^{70}\) assembly. When a non-His\(_6\) tagged β’ was expressed from the plasmid, there was no detectable nonspecific binding to the Ni\(^{2+}\)-NTA column.

All of the sample eluates were also assayed for the presence of any minor sigma species. The only minor σ’s whose concentrations were sufficient for detection were σ\(^{32}\) in log phase and σ\(^{32}\) and σ\(^{F}\) in stationary phase samples. The results for these σ’s were essentially the same as for σ\(^{70}\) with the exception of mutants R297S and Q300E. In stationary phase samples from the Q300E mutant the σ\(^{32}\) and σ\(^{F}\) levels are greatly reduced while the σ\(^{70}\) levels are above wt. The log phase samples for this mutant also contained a decreased amount of σ\(^{32}\) indicating a defect in Eσ\(^{32}\) formation but not as severe as in stationary phase.

*Molecular modeling of β’ mutations*

Recently, Zhang et al. published the crystal structure of *T. aquaticus* core RNAP (19). The β’\(_{260-309}\) region of *E. coli* RNAP has a high degree of sequence conservation with its *T. aquaticus* homolog\(^2\) (Fig. 5a). This region of the *T. aquaticus* β’ subunit forms a “coiled coil-like” structure. When the mutations studied here are modeled onto the *T. aquaticus* structure using the Rasmol
software program (25), those that are most defective in $\sigma$ binding are grouped on one face of the coiled coil. Those that had defective phenotypes in some assays but not others are on the outer edges of this face. Mutations that had no detectable effects are clustered on the opposite face of the coiled coil, with the exception of N309D which is located at the very C terminus of the coiled coil immediately next to the “rudder” (19)(Fig. 5b,c).

**DISCUSSION**

Binding of various sigma factors to the core polymerase is a major step in the process of global gene expression and regulation. It is not known if this step is part of the regulation, via a competition for binding to a limited core population, or merely a straight-forward binding of free $\sigma$’s to an excess of core (27-30). If there is competition among populations of sigma species for core binding, that competition may be influenced by binding specificity of the $\sigma$’s. In light of the high sequence conservation of most sigma species, it has been thought that all sigma factors bind to the same location/s on the core enzyme (1). We had previously identified a binding site for $\sigma^{70}$ in vitro (14). In this report, we verify the importance of this site for in vivo binding and function, identify important residues for $\sigma$ binding, define a potential binding interface for the sigma-core interaction, and show that this binding site is involved in binding at least some of the minor sigma factors.

Our mutational analysis was designed to look for loss of $\sigma^{70}$ binding by targeting residues of the 260-309 region of the $\beta'$ subunit that were identified as occupying e or g positions in the predicted coiled coil structure. Based on our results, we have divided the mutations into 3 groups: nonfunctional for sigma binding in all assays tested, nonfunctional in some assays but not others and those that were functional in all assays tested. The first group contains mutations R275Q, E295K, A302D. These three mutations were nonfunctional for $\sigma^{70}$ binding in vitro and in vivo indicating that they play a very important role in binding $\sigma^{70}$. Arginine 275 is located near the C terminus of
the first of the two putative helices while glutamate 295 and alanine 302 are in the middle and near the C terminus of the second helix, respectively. This confirms what we had found with our earlier mapping work that both predicted helices of $\beta'$$_{260-309}$ were involved in binding $\sigma$$^{70}$. The mutations made at these residues were the only ones tested that could not support any detectable growth when the expression of the chromosomal $\beta'$ subunit was turned off. This is significant in light of the fact that these mutant $\beta'$ subunits, along with all those tested in this study, had no detectable defect in interactions with the alpha and beta subunits necessary to form the core enzyme. We conclude from these results that there are no gross folding defects that are responsible for the lack of $\sigma$$^{70}$ binding.

It is possible that the local structure of these mutant proteins is disturbed. Sequence analysis of all 10 mutant subunits predicted no change in secondary structures as compared to the wild type protein (15,31). The A302D change would be the most likely, though, of the three group 1 mutations to be disturbing the local structure. This introduces a bulky charged side chain in place of a single methyl group. Also, based on the crystal structure of the $T. aquaticus$ core RNAP (19), the A302 alpha carbon is directed more toward the opposite helix of the coiled coil than are the side chains of R275 or E295 which are solvent exposed. If R275Q and E295K are not affecting the local $\beta'$ structure then most likely the negative $\sigma$$^{70}$ binding properties are coming from steric hindrance, charge repulsion, or loss of a specific interaction with the sigma subunit.

The group 2 mutants, N266D, R293Q, and R297S, are of particular interest since they seem to have some function depending on the assay in which they are analyzed. R293Q and R297S were not functional for in vitro $\sigma$$^{70}$ binding in far Western assays but could support growth and were able to form core enzymes that were capable of binding $\sigma$$^{70}$, although R297S does cause a decrease in the binding efficiency of the mutant core enzyme in log phase. The differences in the in vivo and in vitro assay results for these mutants can be explained in multiple ways. First, a positive result from
the far Western assay requires that a β’ fragment (1-319 a.a) refold the secondary structure needed to bind σ^{70} while part of the protein is immobilized on a membrane. Therefore, mutations found to cause defects may be introducing in vitro folding deficiencies. Secondly, the in vivo assays are analyzing σ binding to the multisubunit core enzyme and not just an individual subunit or fragment. A great deal of evidence has been reported suggesting multiple binding sites on core RNAP for the sigma factor (6,10-12). Thus, loss of one of those sites may be compensated for by the remaining binding interactions. We believe that while R293Q and R297S mutations are disrupting σ binding to β’_{260-309}, they are not obstructing σ^{70} from making its other contacts on core polymerase.

In contrast to the previous group 2 mutations, N266D had no effect on σ^{70} binding to β’, but caused reductions in Εσ^{70} formation comparable to group 1 mutations and had a weak growth deficiency. N266 is located at the base of the coiled coil and when mutated could change the local structure. This change may be causing a shift in the orientation of the coiled coil with respect to the rest of the core enzyme. This would not affect the binding of σ^{70} to the coiled coil but may disrupt other contacts normally made by σ^{70} with core.

The group 3 mutants, Y269A, K280E, Q300E, and N309D, were all fully functional indicating that these residues are not making critical contacts with σ^{70}. The Q300E change was rather interesting. This mutation seems to cause an increase in binding of σ^{70} to β’. The large increase in relative binding seen in the far Western may not have been derived strictly from an increase in affinity of the mutant β’ fragment for σ^{70}. The β’ fragment containing this mutation could be better suited than the wild type fragment to refold its native conformation while attached to the nitrocellulose. Therefore, a larger population of properly folded protein may exist to bind σ^{70}.

The increase in relative σ^{70} binding by the Q300E β’ mutant in the far Western was not as dramatic in vivo possibly due to the σ^{70}-core interaction having a larger K_{eq} than the σ^{70}-β’ interaction.
However, the assembly of Eσ70 for this mutant was still almost twice that of wild type. Inhibitors based on coiled coil interactions have proven to be useful in disrupting such processes as viral entry into cells and topoisomerase activity (32-34). Our lab has begun work to design inhibitors of the σ-core interaction for potential use as antibacterial therapeutics. The Q300E mutation may provide useful information on increasing the binding constant of such an inhibitor.

The alternative sigma factors have been thought to bind to the same sites on core RNAP as σ70. Mutating conserved residues of different sigma species will disrupt core binding (6). Traviglia et al. used tethered Fe-EDTA cleavage to determine that several of the minor σ species of E. coli are in close proximity to the same regions of core RNAP as σ70 within the Eσ complex (7). We found that, at least for σ32 and σF, minor sigma factors do bind one of the same sites on core as σ70. Though they are binding to the same site, there is some difference in the manner of binding. The Q300E mutation that increased binding of σ70 had the opposite effect, especially in stationary phase, on σ32 and σF. R297S also had different binding properties for the sigma factors. This mutation caused an increased binding of the minor sigmas and reduced binding of σ70. It is interesting that these mutations both had opposing effects on σ70 and the minor sigmas, although only two minor sigmas were at detectable levels. This suggests that changes in the local environment could favor or hinder minor sigma binding as a whole as compared to σ70. The patterns of binding to the various β’ mutants need to be determined for more of the minor sigmas in order to substantiate this hypothesis.

Finally, the crystal structure of T. aquaticus core RNAP has been of great utility in trying to understand the results of the mutations. Based on the computer predictions and our mutational results, we could not have concluded that β’260-309 formed a coiled coil structure. However, combining this information with the T. aquaticus vs. E. coli β’ sequence alignment and the T. aquaticus crystal structure, it is clear that β’260-309 adopts a coiled coil conformation. Upon σ
binding though, it is not clear what structure this region takes on. Conserved region 2.1 of $\sigma^{70}$, implicated in core binding (8), forms a coiled coil with region 1.2 in the crystal structure of the $\sigma^{70}$ protease-resistant domain (35). Also, a predicted coiled coil in $\sigma^{54}$ of *E. coli* has been found to be important in the sigma-core interaction (36). These sigma factor structures may be interacting with $\beta^{'260-309}$ to form a four helix coiled coil. It is also known that the sigma factor undergoes a conformational change upon binding core (11,37,38). This may be caused by a rearrangement of the coiled coils to form new contacts (39,40).

From the clustering of the group 1 mutations on the same face of the coiled coil structure while having the group 2 mutations on the edges of the cluster and the group 3 members on the opposite side of the coiled coil, we conclude that we have defined the binding interface for $\sigma^{70}$ on $\beta^'$. Recent work in our lab has localized the region of $\sigma^{70}$ that is interacting with $\beta^{'260-309}$ to a peptide containing a portion of the nonconserved region and region 2.1 of the $\sigma^{70}$ subunit (41). $\beta^'$ region 198-237 was identified by Brodolin *et al.* as interacting with the nontemplate strand of the lacUV5 promoter which also is known to be contacted by region 2.4 of $\sigma^{70}$ (42-44). We are now in a position to model the $\sigma$-core interaction using these results as landmarks.

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FOOTNOTES

1 The abbreviations used are: BSA, bovine serum albumin, His_{6}, hexahistidine; DOC, deoxycholate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Ni^{2+}-NTA, nickel nitrilotriacetic acid; HMK, heart muscle kinase; IPTG, isopropyl-β-D-thiogalactopyranoside; RNAP, RNA polymerase; MAb, monoclonal antibody; PCR, polymerase chain reaction.

2 L. Minakhin and K. Severinov, personal communication

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FIGURES LEGENDS

Figure 1 The β′_{260-309} region. (a) Schematic diagram of β′_{260-309} interaction domain. The lettered boxes represent the conserved regions of eukaryal and prokaryal RNAP largest subunits (45). β′_{260-309} interaction domain overlaps part of the β′ subunit conserved region \( \beta \). Below the interaction domain are diagrams of the predicted α helices and coiled coils. (b) Hypothetical helical wheel drawing of predicted β′_{260-309} coiled coil. The two predicted helices are shown as interacting with one another to form an antiparallel coiled coil. Mutations are shown next to original residues along with the residue number. The N-terminus is at amino acid N266 on the right helix. This helix is drawn as coming out of the page, while the left helix is going into the page and terminates at N309.

Figure 2. Western and far Western blots of cell extracts containing wt or mutant β′_{1-319}. Cell extracts of indicated β′ mutants were separated by 8-16% Tris-glycine SDS-PAGE, blotted to nitrocellulose, and probed with (a) an anti-β′ antibody or (b) \(^{32}\)P-labeled σ\(^{70}\). (c) Relative binding of σ\(^{70}\) by wt and mutant β′ fragments. The values for relative σ\(^{70}\) binding by wt vs. mutant β′_{1-319} fragments determined from far Western blotting analysis were normalized to the amount of β′_{1-319} fragment loaded as determined by quantitative Western blot analysis (wt = 1.0). Error bars represent standard deviation. Results are average of three different experiments.

Figure 3. Growth with plasmid-derived wt or mutant β′ as the sole source of β′ subunit. Strain RL602 was transformed with plasmids encoding either wt or mutant, full length β′. Transformed cells (10 µl) were then spotted onto duplicate plates, incubated at either 30 °C (permissive) or 42 °C (nonpermissive) for 24-48 h, and then assessed for growth.
Figure 4. Assembly of core and/or holoenzyme. Cells grown with wt or mutant \( \beta' \) expression plasmids were harvested and subjected to purification to isolate the plasmid-derived, His\(_6\)-tagged, \( \beta' \) and any of its assembled complexes. Proteins from Ni\(^{2+}\)-NTA purified samples were separated via SDS-PAGE and blotted to nitrocellulose. The blots were then probed with MAb’s against the indicated subunits. (a) Log phase samples. (b) Stationary phase samples. No His\(_6\): strain expressing plasmid-derived, wt \( \beta' \) without a hexahistidine tag. (c) Quantitation of relative \( \sigma^{70} \) binding for the mutants vs. wild type \( \beta' \), normalized to the amount of the \( \alpha \) subunit retained (wt = 1.0). Results are the average of three different experiments. Error bars represent standard deviation. (d and e) Log and stationary samples, respectively, probed for minor sigma factors.

Figure 5. Modeling of mutations. (a) Protein sequence alignment of *E. coli* \( \beta' \)\(_{260-309}\) and the homologous region from *T. aquaticus*. Shaded letters represent those not identical to *E. coli*. (b and c) Two views of mutations modeled onto the crystal structure of *T. aquaticus* core RNAP (19) using Rasmol software program (25). (b) Looking down center of coiled coil toward polymerase. (c) Side view of coiled coil. The mutations that were defective in all assays tested are colored green. Mutations that were defective in some assays, but not all, are colored cyan. Mutations that were always functional are colored purple. “Rudder”, colored maroon, is added to orient the structure (19).

Table 1 - Plasmid List and Characteristics

| Plasmid   | \( \beta' \) residues | Mutations | Modifications | Reference |
|-----------|------------------------|-----------|---------------|-----------|
| pRL308    | 1-1407                 | none      | none          | 22        |
| pRL663    | 1-1407                 | none      | C-terminal His\(_6\) | 20        |
| pTA561    | 1-1407                 | silent    | none          | This work |
| Code     | Start | End   | Mutation | Tag          | Source  |
|----------|-------|-------|----------|--------------|---------|
| pTA577   | 1-1407| silent| C-terminal His$_6$ | This work |
| pTA600   | 1-1407| N266D | C-terminal His$_6$ | This work |
| pTA601   | 1-1407| Y269A | C-terminal His$_6$ | This work |
| pTA602   | 1-1407| R275Q | C-terminal His$_6$ | This work |
| pTA603   | 1-1407| K280E | C-terminal His$_6$ | This work |
| pTA604   | 1-1407| R293Q | C-terminal His$_6$ | This work |
| pTA605   | 1-1407| E295K | C-terminal His$_6$ | This work |
| pTA606   | 1-1407| R297S | C-terminal His$_6$ | This work |
| pTA607   | 1-1407| Q300E | C-terminal His$_6$ | This work |
| pTA608   | 1-1407| A302D | C-terminal His$_6$ | This work |
| pTA609   | 1-1407| N309D | C-terminal His$_6$ | This work |
| pTA610   | 1-319 | N266D | none      | This work |
| pTA611   | 1-319 | Y269A | none      | This work |
| pTA612   | 1-319 | R275Q | none      | This work |
| pTA613   | 1-319 | K280E | none      | This work |
| pTA614   | 1-319 | R293Q | none      | This work |
| pTA615   | 1-319 | E295K | none      | This work |
| pTA616   | 1-319 | R297S | none      | This work |
| pTA617   | 1-319 | Q300E | none      | This work |
| pTA618   | 1-319 | A302D | none      | This work |
| pTA619   | 1-319 | N309D | none      | This work |
| pTA620   | 1-319 | silent| none      | This work |
Fig. 1b
Fig. 4a,b
Fig. 4c
Fig. 4d,e
Fig. 5a
Mutational analysis of b'260-309, a s70 binding site located on Escherichia coli core RNA polymerase
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