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Characterization of the *Escherichia coli* Transcription Factor σ^{70}: Localization of a Region Involved in the Interaction with Core RNA Polymerase†

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ABSTRACT: A set of internal deletions and frame-shift mutations was made in the structural gene for the major σ factor of *Escherichia coli* RNA polymerase (σ^{70}). The truncated proteins from these various mutants were examined to determine if they retained the ability to bind core RNA polymerase. Two assays were used to determine core-binding activity. Gel filtration was used to separate free σ^{70} from σ^{70} bound to core polymerase. Immunoprecipitation of polymerase using an anti-α-subunit monoclonal antibody was also used to determine if the various truncated proteins were bound to core. Results from these experiments indicate core-binding activity is retained when large portions of the σ^{70} protein are deleted. Deletion of a region in the central portion of the protein caused a large decrease in core-binding activity. The results suggest that the region spanning amino acids 361-390 is important for efficient core-binding activity. Sequence comparison of various σ factors shows highly conserved amino acids in this region. A synthetic peptide having the sequence of amino acids 361-390 was synthesized and examined for the ability to bind core RNA polymerase.

Specific initiation of transcription in *Escherichia coli* is dependent upon the σ subunit of RNA polymerase. The σ subunit binds to the core RNA polymerse ($\alpha_2\beta\beta'\omega$) to form the holoenzyme ($\alpha_2\beta\beta'\omega\sigma$). The holoenzyme recognizes and binds to a promoter permitting transcription initiation from a specific site (Burgess et al., 1969; Reznikoff et al., 1985). The core polymerase is unable to recognize promoter sequences, so it is the addition of the σ factor which specifies the location of transcription initiation. There are multiple σ factors in bacteria which recognize different sequences as

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promoters (Helmann & Chamberlin, 1988).

Every σ factor performs at least two functions. It binds to core polymerase and allows polymerase to recognize a particular DNA sequence as a promoter. Genetic evidence has indicated regions of σ factors which are involved in recognition of the promoter (Hu & Gross, 1988; Siegele et al., 1988). Less is known about what is required for σ to bind to core polymerase. We have generated deletions of various portions of the σ^{70} protein and tested directly whether they still retain the ability to bind to core polymerase. The results that we obtained have permitted us to identify a region of σ^{70} which is important for efficient core binding. We found that a synthetic peptide corresponding to this region was found to bind to core polymerase.

Materials and Methods

σ^{70} Deletions. Frame-shift mutations in the σ^{70} gene (*rpoD*) were generated by insertion of a *Bam*HI–*Sma*I adaptor into the first *Bgl*II site and the *Bam*HI site of pMRG7 (Gribskov, 1985) which contains *rpoD* under control of the *lacUV5* promoter. These frame-shift mutations delete amino acids 173–613 and amino acids 435–613, respectively, in the mutant protein. A mutant which deletes amino acids 35–360 from the protein was generated by deletion of an EcoRV fragment which is internal in *rpoD*.

A nested set of unidirectional deletions was made by exonuclease III deletion using the Erase-a-Base system (Promega) from the *Bam*HI site fused to the *Xho*I site of *rpoD*. This procedure generated deletions of amino acids 391–528, 378–528, and 288–529. Deletion of amino acids 435–527 was done by digesting pMRG7 with *Bam*HI followed by treatment with nuclease S1 to remove the single-stranded overhang. The S1 nuclease was inactivated by extraction with phenol, and the *Bam*HI digest was incubated with *Xho*I. The *Xho*I–*Bam*HI digest was then incubated with the Klenow fragment of DNA polymerase and nucleotides to provide blunt ends for ligation.

After transformation of the ligation mixtures for the various deletions, colonies were screened by immunoblotting of whole cell lysates to confirm the presence of in-frame deletions. The exact junction site for the exonuclease III deletions was determined by dideoxy sequencing (U.S. Biochemical Corp.) as described by the manufacturer.

Bacterial Strains and Media. All the plasmids coding for the σ deletions were in SK2267 (*gal*, *thi*, *Tl*^{r}, *endA*, *sbc15*, *hsdR4*, *recA*). JV554 [Δ(*lac*)74, *galK phoA20*, *phoR*, *trp*^{am}, *strA*, *relA*, *nadA*::Tn*10*] was used to make the S-30 extract (Jovanovich et al., 1989). Lysates were made from cells grown at 30 °C on LB medium containing 100 μg/mL ampicillin.

Gel Filtration Core-Binding Assay. Strains containing plasmids coding for the various σ deletions were inoculated 1:100 from overnight cultures into fresh media at 30 °C. The diluted cultures were grown to an A_{550} of 0.1–0.2, and isopropyl β-D-thiogalactopyranoside (IPTG) was added to 1 mM final concentration. After 45 min, 40 of mL of cells was harvested by centrifugation and resuspended in 0.4 mL of buffer 1 [20 mM Tris-HCl, pH7.9 at 25 °C, 0.2 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA),[1] and 0.1 mM DTT]. The cell suspension was frozen on dry ice and quickly thawed at 30 °C. The freeze–thaw procedure was repeated once more, and 115 units of DNase I and 10 μL of heat-treated RNase A (10 mg/mL) were added to the resulting lysate. The lysate was incubated for 15 min at 30 °C, and the remaining cell debris was removed by centrifugation in a microfuge. Excess core RNA polymerase (Burgess & Jendrisak, 1975) (2.3 μg) was added to the lysate and incubated 30 min at 30 °C. One hundred microliters of the lysate was then loaded onto a Waters Protein Pak 300SW sizing column (0.75 × 30 cm) previously equilibrated with buffer 1. The sample was eluted at 0.5 mL/min, and 0.25-mL fractions were collected.

Fractions were examined by SDS–PAGE using 12% gels. Gels were either stained with Coomassie blue to determine the presence of the core polymerase subunits in the various fractions or blotted onto nitrocellulose (Towbin et al., 1979). Immunoblots were probed with monoclonal antibodies to σ^{70} (Strickland et al., 1988) to determine the presence of full-length and truncated proteins in the various fractions. The antibody 2D1 was used to detect the Δ173–613 and Δ435–613 proteins since this antibody binds near the amino terminus of σ^{70}. All other blots were probed with the antibody 2D4 which binds near the carboxy terminus of σ^{70}. After protein transfer, immunoblots were blocked with 1% Blotto for 1 h (Johnson et al., 1984). Primary antibody was incubated at a 1:2000 dilution in PBS containing 1% BSA for 1 h and rinsed twice in TBST (10 mM Tris-HCl, pH 7.9 at 25 °C, 150 mM NaCl, and 0.1% Tween 20). A similar dilution of the secondary antibody (a goat anti-mouse IgG–alkaline phosphatase conjugate) was incubated for 30 min followed by two 5-min rinses in TBST. The immunoreactive bands were visualized by using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium as an alkaline phosphatase substrate (Blake et al., 1984).

S-30 Core-Binding Assay. Coupled transcription–translation extracts (S-30) were prepared essentially as described by Zubay (1973) with minor modifications (Jovanovich et al., 1989) from the *E. coli* strain JV554. One to ten micrograms of plasmid DNA coding for the various intact and truncated proteins was incubated for 70 min at 37 °C in 50 μL of the S-30 reaction containing [^{35}S]methionine (1.1 μCi/μmol) and 4.6 μg of added core polymerase. Precipitates in the reactions after incubation were removed by centrifugation in a microfuge.

Duplicate samples of 15 μL were removed from the reaction supernatant to determine the extent of binding of the ^{35}S-labeled proteins to core polymerase. To these samples was added 100 μL of buffer 2 (100 mM Tris-HCl, pH 8.0 at 4 °C, 100 mM NaCl, and 2 mM EDTA). Samples were treated with either 20 μL of a *Staphylococcus aureus* formalin-treated cell suspension or 20 μL of a *S. aureus* cell suspension (binds 1.3 mg of IgG/mL) which had been pretreated with an excess of an anti-α monoclonal antibody (4RA2). This antibody has been shown to bind the RNA polymerase holoenzyme (S. Lesley and N. Thompson, unpublished results). The cell suspensions were incubated for 60 min on ice to allow the antibody to bind the core polymerase. Cell suspensions were pelleted by centrifugation and washed twice with buffer 2 to remove unbound protein. The final pellet was resuspended in SDS sample buffer and heated for 2 min at 100 °C prior to SDS–PAGE on 12% gels. Five microliters of the original S-30 reaction was also examined by SDS–PAGE to determine the total amount of ^{35}S-labeled protein in the S-30 reaction.

After electrophoresis, the gels were soaked in Enhance (New England Nuclear) as directed by the manufacturer and dried under vacuum. After autoradiography, bands corresponding to the ^{35}S-labeled proteins were cut out and treated with 10 N HCl at 100 °C for 2 h. The HCl was neutralized with NaOH, and the ^{35}S-containing liquid was counted by scintillation to determine the relative amounts of protein in the

[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, 10 mM phosphate (pH 7.4) buffered saline (150 mM NaCl); DTT, dithiothreitol; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid.

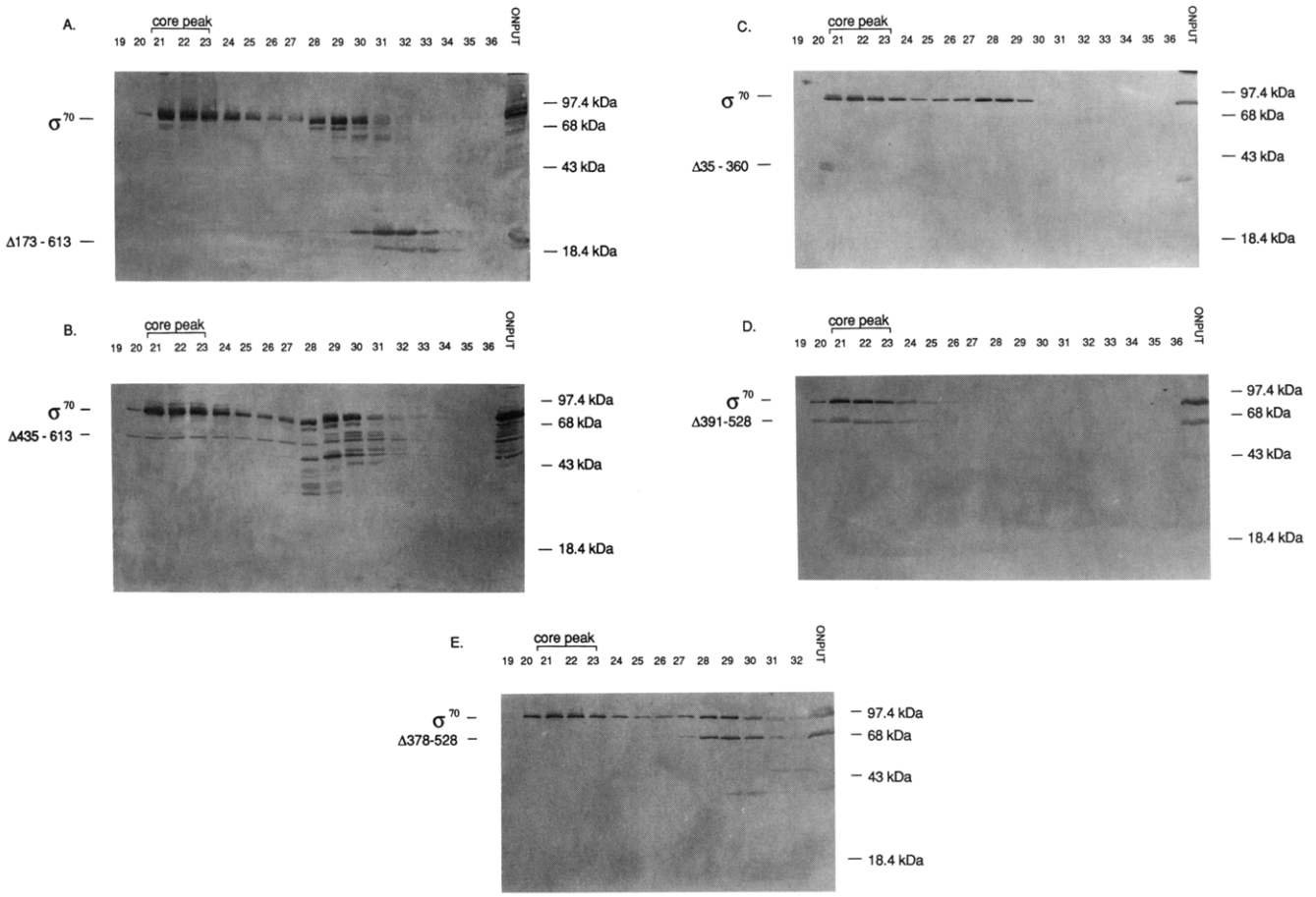

FIGURE 1: Gel filtration core-binding assay results. Lysates were made from *E. coli* strains carrying plasmids coding for various σ^{70} deletions. Proteins from lysates were separated by gel filtration as described under Materials and Methods. Fractions were screened by immunoblotting, and deletions were detected by using the anti-σ^{70} monoclonal antibodies 2D1 (A and B), which reacts with the amino terminus of σ^{70}, or 2D4 (C–E), which reacts with the carboxy terminus. Intact and truncated proteins are indicated in each blot [(A) Δ173–613; (B) Δ435–613; (C) Δ35–360; (D) Δ391–528; (E) Δ378–528]. Fractions containing the core polymerase subunits are indicated in each blot.

various bands. The binding of the ^{35}S-labeled proteins to core polymerase was estimated by determining the percent of the total ^{35}S-labeled σ or mutant protein that was contained in the immunoprecipitate. This binding value was compared to a value for σ^{70} to obtain the relative binding ratio.

Synthetic Peptide and Binding Assay. A synthetic peptide having the sequence INRRMSIGEAKARRAKKEMVEANLRLVISI corresponding to amino acids 361–390 of σ^{70} was synthesized (University of Wisconsin Biotechnology Center). The peptide was purified by chromatography on a C18 reverse-phase column. Peptide was conjugated to CNBr-activated Sepharose 4B as described by the manufacturer (Pharmacia) to give a final peptide concentration of approximately 4 mg/mL of resin.

Binding reactions and washes were performed in 100-μL volumes containing 20 mM Tris, pH 7.9 at 25 °C, and 0.5 M NaCl. Samples containing 4 μg of core polymerase, 4 μg of core and 50 μg of peptide, 2.4 μg of σ^{70}, 4.3 μg of holoenzyme, or 180 μg of S-30 extract supplemented with 1 μg of core were incubated in the reaction buffer for 15 min at 37 °C. To these samples was added 2.5 μL of peptide–Sepharose (10 μg of peptide) or control Sepharose and mixed frequently for 15 min at 37 °C. The Sepharose was then pelleted by brief centrifugation and the supernatant removed. Three washes of 100 μL binding reaction buffer were performed to remove unbound protein. Washes of pellets from the extract-containing samples were substituted with 0.3 M NaSCN and 0.2 M NaCl for 0.5 M NaCl to reduce nonspecific binding. After the final wash, supernatants were removed, and pellets were resuspended in 20 μL of SDS sample buffer prior to SDS–PAGE on 4–20% gels.

Results

σ^{70} Deletions. Frame-shift mutations and internal deletions were made in the σ^{70} structural gene (*rpoD*) which was under control of the *lacUV5* promoter. Truncated σ^{70} proteins were produced in cells harboring the mutant σ^{70} plasmids by treating cultures with IPTG to induce transcription from the *lacUV5* promoter. Whole cell lysates from these induced strains were immunoblotted and the mutant proteins visualized using monoclonal antibodies to σ^{70} which have been described previously (Strickland et al., 1988). The epitopes for these monoclonal antibodies have been mapped and were used to confirm the identity of the truncated proteins in the lysate.

Gel Filtration Core-Binding Assay. To determine whether the mutant proteins retained the ability to bind to core RNA polymerase, an assay was used which was based on the size difference between σ which is bound to core RNA polymerase (450 kDa) versus σ which is not bound (70 kDa). This difference in size allows the two forms to be separated on a gel filtration column.

Lysates from cells containing the mutant protein were loaded onto an HPLC sizing column and the fractions assayed by immunoblot analysis. Results are shown in Figure 1. Since the column onput is basically a whole cell lysate, it contains both wild-type and mutant σ^{70}. The column fractions contain two peaks of σ^{70}. The first peak also contains the core polymerase subunits (data not shown), indicating that these

Table I: Core-Binding Results from Immunoprecipitation of Labeled Proteins Using an Anti-α Monoclonal Antibody[a]

plasmid	protein produced	binding relative to σ^{70}
pMRG7	σ^{70}	1.0
pSAL120	$\sigma^{70}\Delta 435$–613	0.2
pSAL121	$\sigma^{70}\Delta 173$–613	0.01
pSAL119	$\sigma^{70}\Delta 35$–360	0.3
pMRG7ΔB9	$\sigma^{70}\Delta 391$–528	2.8
pMRG7ΔB10	$\sigma^{70}\Delta 378$–528	0.1
pAG37	σ^{32}	2.1
pKM11	galactokinase	0.01

[a] Plasmids coding for intact or truncated σ protein were incubated in a coupled transcription–translation system (S-30) with [^{35}S]methionine to generate radiolabeled proteins. Relative binding ratios were calculated by determining the percentage of the intact or truncated proteins found in immunoprecipitates of the polymerase.

fractions contain σ which is bound to core polymerase. The second peak contains σ which is not bound to core.

Any free σ should be able to bind since excess core polymerase was added to the extract. Significant levels of free σ^{70} are seen in most of the lysates. This suggests that a portion of the σ in the cell is unable to bind to core polymerase. When excess purified σ^{70} is added to the extract, the majority of the added σ is found in the "bound" peak (data not shown), indicating that the extract has the ability to bind all the free σ^{70} present in the extract. Whether the population of σ^{70} which cannot bind core has any physiological relevance or whether it is a consequence of the assay procedure is unclear.

The mutant σ's $\Delta 435$–613, $\Delta 35$–360, $\Delta 391$–528, and $\Delta 435$–527 (Figure 1B, 1C, 1D, and data not shown) are found in the fractions containing the core polymerase subunits, indicating that these mutants retain the ability to bind core polymerase. The $\Delta 35$–360 and $\Delta 391$–528 deletions are not found in the fractions where the free protein would be expected to run. Presumably, this is due to the degradation of the protein which is not bound to core polymerase although the $\Delta 391$–528 protein may bind to core with greater affinity (see Table I). Mutants $\Delta 173$–613, $\Delta 378$–528, and $\Delta 288$–529 (Figure 1A, 1E, and data not shown) are only found in significant amounts in the fractions where the free protein would be expected to run, indicating that these mutants lack the ability to bind to core polymerase.

The number and level of degradation products detected from mutant and wild-type σ^{70} in Figure 1 vary between mutants and are dependent upon the monoclonal antibody used. The antibody 2D4, which recognizes the carboxy terminus of σ^{70}, shows the least degradation products while extracts probed with the antibody 2D1, which reacts with the amino terminus, show numerous bands. The monoclonal antibody 1H6 (Strickland et al., 1988), which binds near the amino terminus, shows similar bands (data not shown), indicating that the bands are due to degradation rather than cross-reactivity. These results indicate that the carboxyl terminus is the first portion of σ which is degraded. This is interesting since it is the carboxy terminus which has been implicated in the recognition of the −35 portion of the promoter (Hu & Gross, 1988; Siegele et al., 1988). Some of these degradation products should have the ability to bind to core polymerase but not the ability to recognize a promoter.

S-30 Core-Binding Assay. A second type of assay was used to confirm whether the mutant proteins retained an ability to bind core polymerase. The assay is based on immunoprecipitation of holoenzyme using a monoclonal antibody to the subunit of RNA polymerase which is known to bind the holoenzyme. The mutant binding measurement was compared to one for σ^{70}, and a ratio of the binding relative to σ^{70} was determined. Since this number is also a function of the relative stability of the mutant proteins in the reaction, it is not necessarily a measurement of the relative affinities of the mutant proteins for core polymerase. There is no competition between wild-type and mutant σ factors since excess core polymerase is available in the binding reaction. These experiments provide a qualitative measurement of the affinity of the mutant σ factors for the core polymerase. Because the S-30 assay is based on immunoprecipitation rather than size differential, artifacts such as aggregation of the mutant proteins would not affect the results. Results for various deletions are shown in Table I.

The mutant σ's $\Delta 435$–613, $\Delta 35$–360, and $\Delta 391$–528 as well as σ^{32} and the wild-type σ^{70} control are all found in significant levels in the immunoprecipitate, indicating that these proteins are capable of binding core RNA polymerase. The $\Delta 173$–613 protein and galactokinase control are not found in significant amounts in the immunoprecipitates which indicates that these proteins do not have the ability to bind to core polymerase. The $\Delta 378$–528 protein is found at a low level in the precipitate which may indicate that this protein has some affinity for core. This result conflicts with the gel filtration result for $\Delta 378$–528. If is clear from Table I, however, that there is a large loss in core-binding activity when amino acids 378–391 are deleted.

Figure 2 summarizes the core-binding data for the mutants. Large portions can be removed from either end of the σ^{70} protein without destroying its ability to bind core polymerase. Deletions in the central portion of σ^{70} result in a large decrease in the ability to bind core polymerase, implicating amino acids 361–390 as being important for core-binding activity.

Peptide-Binding Assay. A peptide was synthesized containing amino acids 361–390 to determine whether these amino acids are sufficient to bind the core polymerase. Purified peptide was conjugated to Sepharose and the conjugate incubated with polymerase to allow binding to occur. After the Sepharose–peptide conjugate was washed to remove unbound protein, the samples were run on SDS–PAGE to determine the extent of binding to the conjugate. The results shown in Figure 3 demonstrate that core polymerase binds well to the peptide–Sepharose but not to the control Sepharose which contains no peptide. Addition of free peptide to the binding reaction prevents core polymerase from binding to the conjugate. This indicates that the binding of the peptide is to a specific site on the polymerase rather than a general affinity of the peptide for polymerase.

Surprisingly, the peptide was also found to bind to σ^{70} and the holoenzyme. This is contrary to what was expected. Data from the deletions indicated that this region was involved in binding to core, so it was expected that the binding site would be inaccessible in the holoenzyme. The results could be explained if the region of σ^{70} contained in the peptide (361–390) interacts with another complementary site on σ^{70}. Proteolysis experiments show that residues 361–390 are in a protease-resistant fragment of σ^{70} (Burgess et al., 1987), which indicates that these amino acids may be involved in forming a tight internal structure. σ^{70} could undergo a conformational change when binding to core which allows 361–390 to interact with core. This change in conformation could expose the complementary site on σ^{70} so that the holoenzyme could bind to the peptide conjugate. A biphasic model of σ^{70} binding to core to form the holoenzyme has been proposed previously (Wu et al., 1976).

The specificity of binding of the peptide to polymerase was checked by incubating the conjugate with an extract. Polymerase was almost completely bound from the extract while

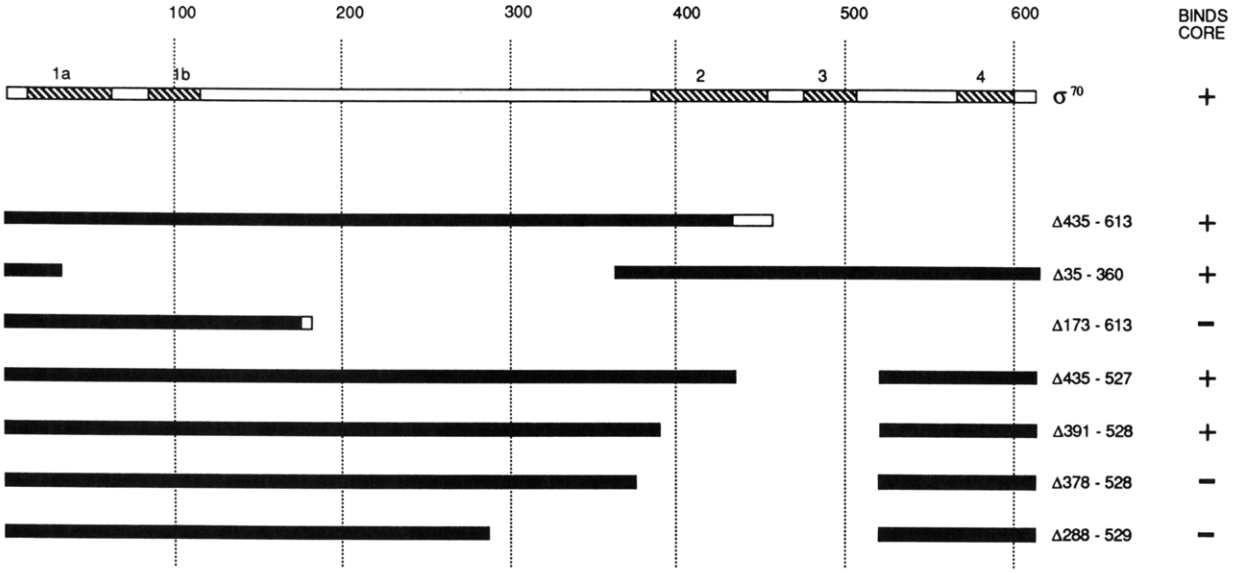

FIGURE 2: Deletion core-binding summary. The core-binding ability of the various deletions is summarized. Regions 1–4 which are conserved among σ factors are indicated by the hatched regions in σ^{70}. Amino acids from σ^{70} which are retained in the various deletions are indicated by the shaded lines. The unshaded areas in the Δ435–613 and Δ173–613 deletions indicate nonsense amino acids generated by the frame-shift. Numbering indicates amino acid position in intact σ^{70}.

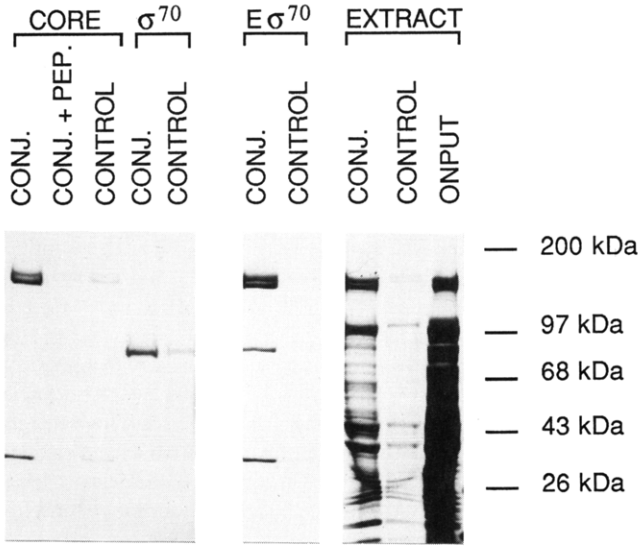

FIGURE 3: Peptide core-binding assay results. Peptide-conjugated or control Sepharose was incubated with core polymerase, σ^{70}, holoenzyme, or a cell extract. Competing free peptide was added to one sample containing the core polymerase as indicated. Approximate molecular masses are indicated in kilodaltons.

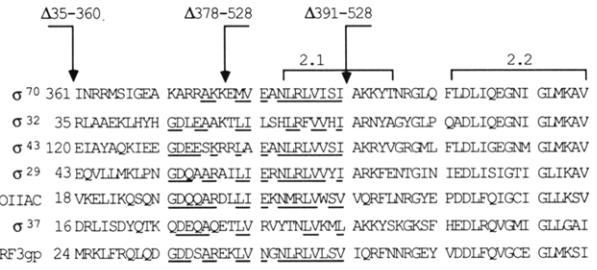

FIGURE 4: Sequence alignment of several σ factors in the region implicated in core binding. Sequences were aligned as described previously (Gribskov & Burgess, 1986; Helmann & Chamberlin, 1988; Masuda et al., 1988). Subregions 2.2 and 2.4 are indicated. *E. coli* σ^{70} and σ^{32} and *B. subtilis* σ^{43}, σ^{29}, spoIIAC, σ^{37}, and ORF3gp sequences are shown. End points of σ^{70} deletions are indicated by arrows. Conserved amino acids in the region implicated in core binding (361–390) are underlined. Conserved amino acids are grouped as follows: (I, L, M, V), (H, K, R), (D, E, N, Q), (A, G), (F, Y, W), (S, T).

the majority of the other proteins were not. Several other proteins were retained by the conjugate, however, indicating that the binding under these conditions is not completely specific. Wash conditions of up to 1 M NaSCN were used to reduce nonspecific binding to the conjugate (data not shown). In this high-salt wash, polymerase binding was reduced but above control levels, and nonspecific binding was also reduced.

Discussion

Many different changes in amino acids could destroy σ binding without being directly involved in the binding process. However, when core-binding activity is retained after the amino acid sequence is altered, this indicates that the deleted sequence is not necessary for binding. We have identified regions, based on the deletion boundaries, which are dispensible for core binding using retention of binding activity as a criterion. The deletions examined here identify a region of σ^{70} from amino acids 361 to 390 which is important for binding to the core RNA polymerase. Amino acids 330–343 have been shown previously to not be required for function (Hu & Gross, 1983). That result is in agreement with the results presented here in that the region which can be deleted and still retain function is in the area that can be deleted and still retain core-binding activity. Previously, a proteolytic fragment of σ^{70} containing amino acids from approximately positions 106–449 was observed to have core-binding activity (Burgess et al., 1987). This observation is also in agreement with the results presented in this study.

It has not yet been determined whether any of the deletions described here retain the ability to initiate transcription when bound to core polymerase. The Δ35–360 mutant is most likely to function since it still contains sequences implicated in recognition of the −10 and −35 portions of the promoter in addition to being able to bind core polymerase. Attempts to complement the *rpoD*800 mutation with this mutant were unsuccessful (data not shown); however, this mutant protein may also be unstable at the higher temperature required to inactivate *rpoD*800. Preliminary results indicate that the Δ35–360 protein copurifies with the holoenzyme. We are presently attempting to purify the Δ35–360 protein to homogeneity to determine whether it can initiate transcription

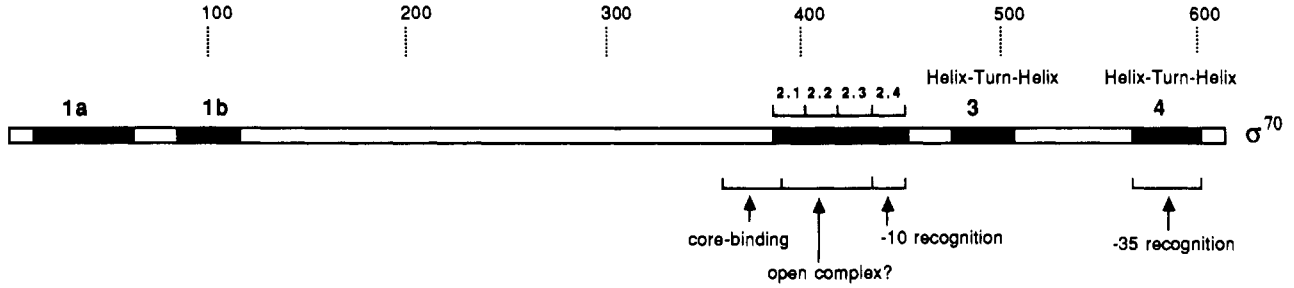

FIGURE 5: Summary of proposed σ^{70} functional domains. Conserved regions 1–4 are shown in the shaded areas. Genetic studies have indicated regions involved in recognition of promoter sequences at −35 (Hu & Gross, 1988; Siegele et al., 1988) and −10 (Siegele et al., 1989). Helix–turn–helix structures have been predicted by primary sequence analysis (Gribskov & Burgess, 1986). Results presented here indicate a region required for core binding. A region involved in open complex formation has been proposed (Helmann & Chamberlin, 1988), and results presented here indicate that subregion 2.2 could be included.

when added to core polymerase.

Several groups have noted regions indicating homology between various σ factors (Gribskov & Burgess, 1986; Stragier et al., 1985; Hellman & Chamberlin, 1988). Gribskov and Burgess have identified four regions of similarity. Region 1 is found only in *E. coli* σ^{70} and *Bacillus subtilis* σ^{43}. Region 4 has been implicated by genetic studies to be involved in recognition of the −35 portion of the promoter (Hu & Gross, 1988; Siegele et al., 1988) and has been postulated to have the helix–turn–helix structure common to many DNA-binding proteins (Gribskov & Burgess, 1986). Region 3 is also predicted to form a helix–turn–helix, but so far, no function has been assigned to this region. Gribskov and Burgess have shown that region 2 is the most highly conserved of the regions identified. The high degree of conservation of amino acid sequence indicates that this region contains a structure which is necessary for σ function. Helmann and Chamberlin have further divided region 2 into four subregions which are conserved to varying degrees among σ factors.

Subregion 2.2 (amino acids 403–416) is identical between σ^{70} and σ^{32}. σ^{43} is identical in 12 of 14 amino acids. The high degree of conservation is indicative of a structure required for proper function. This conservation of protein sequence has led to the proposal that subregion 2.2 is required for binding of the protein to core polymerase (Gribskov & Burgess, 1986). This possibility is attractive since this is a common function of all the σ factors. Evidence from the deletions examined here, however, suggests that subregion 2.2 is not required for binding to core polymerase since the Δ391–528 deletion binds core polymerase well. Another possible function of subregion 2.2 is to permit the formation of the promoter open complex. This is an attractive possibility due to the proximity of this subregion to subregion 2.4 which is implicated in −10 recognition (Gardella et al., 1989; Zuber et al., 1989; Siegele et al., 1989). Helmann and Chamberlin have proposed that subregions 2.1 and/or 2.3 are involved in open complex formation based on sequence comparison of these subregions with known single-stranded DNA-binding proteins. These subregions might initiate open complex formation directly or by interaction with core. Helmann and Chamberlin identify a group of conserved aromatic amino acids in subregion 2.3 which could be directly involved in the melting out of the −10 region. Subregions 2.1 and 2.2 are low in aromatic amino acids and may be involved in an interaction with core polymerase. Since neither core polymerase nor σ can form an open complex individually, some interaction at the −10 region seems likely.

The Δ391–528 deletion retains most of subregion 2.1 and still binds core polymerase. The Δ378–528 mutation deletes past subregion 2.1 and does not bind core polymerase well. This indicates that amino acids around subregion 2.1 are important for core binding. Figure 4 shows a sequence alignment of several σ factors in this region. The synthetic peptide corresponding to the region of σ^{70} which is implicated in core binding (amino acids 360–391) shows affinity for polymerase as well as for σ^{70}. We were unable to find conditions where the peptide could bind only these proteins from a crude lysate It is unclear how specific the binding of the peptide is in this assay. The fact that the polymerase binding to the peptide conjugate can be prevented by the presence of free peptide indicates that the peptide is interacting with a specific region of polymerase. Studies have shown that σ is in contact with the β and β' subunits (Coggins et al., 1977; Yura & Ishihama, 1979). It seems unlikely that only a small sequence (amino acids 361–390) is uniquely responsible for σ binding to core polymerase. A more probable model is that many amino acids throughout the protein contribute to the binding with amino acids 361–390 being critical contacts for proper orientation of the protein on the core polymerase.

A summary of the results shown here and by various other groups has been used to propose a model for σ^{70} functional domains and is shown in Figure 5. Although no experimental evidence has been shown for the proposed helix–turn–helix structure and open complex domain, they have also been included in the figure. The combination of genetic and biochemical techniques used by several groups is yielding a rough model of σ functional domains. As more information becomes available on the functional and structural domains, a clearer picture of the mechanism of transcription initiation will be formed.

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Independent Flexible Motion of Submolecular Domains of the Ca^{2+},Mg^{2+}-ATPase of Sarcoplasmic Reticulum Measured by Time-Resolved Fluorescence Depolarization of Site-Specifically Attached Probes

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ABSTRACT: The Ca^{2+}-transporting ATPase of rabbit skeletal muscle sarcoplasmic reticulum was site-specifically labeled with either *N*-(1-anilinonaphth-4-yl)maleimide (ANM) or 5-[[(iodoacetamido)-ethyl]amino]naphthalene-1-sulfonate (IAEDANS), and the segmental motion of submolecular domains of the ATPase molecule was examined by means of time-resolved and steady-state fluorescence anisotropy measurements. The ANM-binding domain showed wobbling with a rotational relaxation time ϕ = 69 ns in the absence of free Ca^{2+} without any independent wobbling of the ANM moiety. The IAEDANS-binding domain showed a significantly slower wobbling with ϕ = 190 ns in the absence of Ca^{2+}. The present results demonstrated for the first time that the ATPase molecule is composed of distinct domains whose mobilities are considerably different from each other. The binding of Ca^{2+} to the transport site increased the segmental motion of ANM-labeled domain, leading to a ϕ value of 65 ns. Solubilization of the ANM-labeled SR membranes by deoxycholate led to a further increase in the segmental flexibility (ϕ = 48 ns in the absence of free Ca^{2+}), indicating that the mobility of the ANM-binding domain was considerably restricted through interaction with the membrane. The mobility of the ANM-binding domain of solubilized ATPase was also increased to some extent upon binding of Ca^{2+}.

Active Ca^{2+} transport across the sarcoplasmic reticulum (SR)[1] membranes is carried out by Ca^{2+},Mg^{2+}-ATPase (SR-ATPase), an intrinsic membrane protein of M_r 110000 whose amino acid sequence has recently been deduced from the nucleotide sequence of a cDNA clone (Brandl et al., 1986). The mechanism of coupling between ATP hydrolysis and the uphill movement of Ca^{2+} ions against the concentration gradient has long been a subject of intensive studies [for a recent

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[1] Abbreviations: ANM, *N*-(1-anilinonaphth-4-yl)maleimide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; E-P intermediate, enzyme-phosphate complex intermediate; IAEDANS, 5-[[(iodoacetamido)ethyl]amino]naphthalene-1-sulfonate; SR, sarcoplasmic reticulum; SR-ATPase, Ca^{2+},Mg^{2+}-ATPase of the sarcoplasmic reticulum; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

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