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A σ^{32} mutant with a single amino acid change in the highly conserved region 2.2 exhibits reduced core RNA polymerase affinity

(protein–protein interaction/sigma factor/transcription initiation)

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ABSTRACT **σ^{32}, the product of the *rpoH* gene in *Escherichia coli*, provides promoter specificity by interacting with core RNAP. Amino acid sequence alignment of σ^{32} with other sigma factors in the σ^{70} family has revealed regions of sequence homology. We have investigated the function of the most highly conserved region, 2.2, using purified products of various *rpoH* alleles. Core RNAP binding analysis by glycerol gradient sedimentation has revealed reduced core RNAP affinity for one of the mutant σ^{32} proteins, Q80R. This reduced core interaction is exacerbated in the presence of σ^{70}, which competes with σ^{32} for binding of core RNAP. When a different but more conserved amino acid was introduced at this position by site-directed mutagenesis (Q80N), this mutant sigma factor still displayed a significant reduction in its core RNAP affinity. Based on these results, we conclude that at least one specific amino acid in region 2.2 is involved in core RNAP interaction.**

In eubacteria there exist a number of different sigma factors that are involved in the expression of specific sets of genes. The primary sigma factor controls the expression of primary housekeeping genes, and a number of alternative sigma factors regulate gene expression at specific stages of growth, or as a response to outside stimuli. In spite of the diversity of sigma factors, they all have similar activities during transcription initiation (reviewed in refs. 1 and 2). They direct transcription initiation by interacting with core subunits of RNA polymerase to form a holoenzyme, by recognizing the −10 and −35 regions of DNA promoters, and by stabilizing the separation of DNA strands. Ultimately, the sigma factor, at least in the case of σ^{70}, dissociates from the rest of the RNAP, and the core RNAP subunit enters the elongation phase of transcription.

As expected of a group of proteins with similar functions, a sequence alignment of various sigma factors has revealed four regions of homology (2, 3). Region 2 is further divided into four subregions, and these regions have been implicated in various functions during transcription initiation. Genetic studies by a number of groups have indicated that region 2.4 is involved in the recognition of the −10 region of the promoter (4–7). Based on DNA footprinting studies, conserved aromatic amino acids in region 2.3 have been shown to promote the separation of DNA strands (8, 9). Mutation and deletion analyses have implicated region 2.1 in core RNAP binding (10, 11). However, the role of the most highly conserved region, 2.2, during transcription remains unknown.

Several groups have speculated that the most conserved region on sigma factors may be the core RNAP binding region (2, 12, 13). Their assumption was based on the premise that all sigma factors bind on the same region of core RNAP. If this idea were true, then region 2.2 should form the primary core RNAP binding region. Contrary to this assumption, a deletion analysis of σ^{70}, the primary sigma factor in *Escherichia coli*, has shown that a deletion of parts of regions 1.2 and 2.1 reduces binding to core RNAP (10). However, deletions can profoundly affect the structure of a protein and do not indicate the specific contact site on an intact protein.

In an attempt to elucidate the function of region 2.2 during transcription, we have analyzed four mutants of σ^{32}, an alternative sigma factor that confers heat shock promoter specificity (14). All four mutants contain a single amino acid change in region 2.2. Because the mutations occurred in the highly conserved region and because most of the affected amino acids themselves were conserved, we explored the possibility that one or more of these σ^{32} mutants might be defective in their ability to interact with core RNAP. Using glycerol gradient sedimentation to examine protein–protein interaction, we have shown that a conserved amino acid in region 2.2 is critical for efficient core RNAP interaction.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Bacterial strains and plasmids are listed in Table 1. Isolation of *rpoH* alleles has been described (15).

Cloning of *rpoH* Alleles. Cloning of *rpoH173*, *rpoH181*, *sidB2*, and *rpoH28–2*(ts) was essentially the same as previously described (20). After the site of mutation in each allele was determined by DNA sequencing, these alleles were subcloned into an *rpoH* histidine-tagged vector, pUHE211–1, by a fragment exchange of *Mlu*I and *Pst*I segment of the *rpoH* gene. The plasmid-encoding σ^{32} mutant, *rpoH185*, was generated by amplifying pUHE211–1 with a primer that changed nucleotides 238–240 from CAG to ACC in the *rpoH* gene. The PCR product was then sequenced to check for any nucleotide misincorporations. None were found.

σ^{32} Purification. Overproducers of his-tagged σ^{32} were grown at 30°C in 1 liter of 2 × YT medium with 100 μg/ml ampicillin and 50 μg/ml kanamycin. At A_{600} = 1, isopropyl B-D-thiogalactoside was added to 0.5 mM. Cells were grown for 20 min and poured into tubes with ice. All subsequent steps were performed at 4°C. After centrifugation at 5,000 rpm for 10 min in a Sorvall SLA-3000 rotor, the cell pellet was resuspended in 18 ml of ice-cold buffer X (50 mM KH_2PO_4, pH 7.9 at 4°C/300 mM KCl/50 mM Ile/50 mM Phe) and subjected to 10,000 lb/in^2 in an ice-cold French press. The cell lysate was centrifuged for 30 min at 15,000 rpm in a Sorvall SS-34 rotor. The supernatant was loaded onto a 3-ml nickel-nitrilotriacetic acid agarose column at a rate of 0.4 ml/min. The column was subsequently washed with 40 ml of buffer X and then with 10

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Abbreviation: RNAP, RNA polymerase.

Table 1. Bacterial strains and plasmids used in this study

Strain	Plasmid	Relevant genotype	Characteristics	Source
285c		*rpoD285*		(15)
BB1554		Δ*dnaK52 sidB2*		(16)
TC28-2		*rpoH28-2*(ts)		(17)
CG410		*dnaK756*		(18)
RL721		*rpoC3531*(His6) *zja*::*kan*		Gift of R. Landick (University of Wisconsin, Madison)
NUT21		*dnaK756*/pUHE211-1 pDMI,1		This lab
NUT25		*dnaK756*/phis173 pDMI,1		This lab; pUHE211-1 derivative
NUT81		*dnaK756*/phisB2 pDMI,1		This lab; pUHE211-1 derivative
NUT84		*dnaK756*/phis28-2 pDMI,1		This lab; pUHE211-1 derivative
NUT95		*dnaK756*/phis185 pDMI,1		This lab; pUHE211-1 derivative
	pUHE211-1		σ^{32}-c-his, Ap^r	(19)
	pDMI,1		lacIq, Km^r	(19)
	pJet40		*dnaK*-P1 promoter	Gift of J. Erickson (Columbia University, New York)

ts, temperature sensitive.

ml of buffer X + 15 mM imidazole. Nickel-bound proteins were eluted with 30 ml of 15–150 mM linear gradient of imidazole in buffer X. Purified σ^{32} proteins were dialyzed against two changes of 1 liter of 50 mM KH_2PO_4/300 mM KCl/50% glycerol. All mutant and wild-type σ^{32} protein concentrations were determined according to Pace *et al*. (21).

Core RNA Polymerase (RNAP) and σ^{70} Purification. Proteins were purified according to the protocol received from R. Landick's lab (personal communication) with the following modification. σ^{70} was stripped from RNAP using BioRex 70 (Bio-Rad) as described (22). Purified proteins were dialyzed with two changes of 1 liter of TGED (10 mM Tris·HCl, pH 7.9 at 20°C/5% glycerol/100 mM EDTA/100 mM DTT) + 50% glycerol. A molar extinction coefficient of 198,500 M^{-1} cm^{-1} and 41,745 M^{-1} cm^{-1} was used to calculate core RNAP and σ^{70} concentration, respectively (20, 23).

***In Vitro* Transcription Assay.** Holoenzyme containing different concentrations of σ^{32} proteins was reconstituted under the following conditions: buffer A (50 mM Hepes, pH 7.9 at 4°C/0.1 mM EDTA/1 mM DTT/100 mM NaCl/10 mM $MgCl_2$), 0.5 mg/ml BSA, 150 μM ATP, 150 μM CTP, 1.5 μM UTP, 1.1 nM core RNAP, and 3.5 μg of DNA template containing the *dnaK*-P1 promoter. The size of the DNA template is 3.0 Kbp, and the molar ratio of promoter to core RNAP is 16:1. After incubating the mixture for 10 min at 30°C, 150 μM GTP and 33 nM [α-^{32}P]UTP were added. The final reaction volume was 100 μl. Then, 5 μl of 2 mg/ml rifampicin was mixed into the reaction. After 10 min, the reaction was stopped by adding 100 mM EDTA, 2 M NH_4OAc, and 0.4 μg/μl tRNA. Samples were precipitated in isopropanol and washed with 70% ethanol. RNA transcripts were then dissolved in 16 μl of loading dye (80% formamide/10 mM EDTA/1 mg/ml xylene cyanol FF/1 mg/ml bromophenol blue). Twenty-five percent of the transcripts were loaded on a 6% polyacrylamide gel. RNA transcripts were visualized using a PhosphorImager and quantitated under IMAGEQUANT software (Molecular Dynamics).

Glycerol Gradient Sedimentation. Holoenzyme was reconstituted in buffer A at 30°C for 15 min in a 200-μl volume. The concentration of sigma factors, σ^{70} and σ^{32}, and core RNAP in each experiment was 100 nM. Samples were loaded on top of a 5-ml linear 15–35% (vol/vol) glycerol gradient and centrifuged at 4°C in a Beckman SW50.1 rotor for 24 hr at 48,000 rpm. Eighteen fractions were collected from the bottom of the tube and subjected to Western blot analysis with σ^{32} antiserum to detect the sedimentation pattern of σ^{32}.

RESULTS

Mutants of σ^{32} in Region 2.2. We have isolated and cloned 15 *rpoH* alleles by suppressing the temperature sensitivity of *rpoD285* (15), which contains a 42-bp deletion mutation in σ^{70}, the primary sigma factor in *E. coli*. DNA sequence analysis has revealed two alleles with a single amino acid change in region 2.2. These are *rpoH173* (Q80R) and *rpoH181* (P74R). Another allele *sidB2* (E81G) was isolated by suppressing the growth deficiency of Δ*dnaK52* (16). We have cloned and sequenced the fourth *rpoH* allele, *rpoH28–2* (G82S), isolated by Waghorne and Fuerst (17). G82S fails to permit growth of lambda phage at high temperatures. Amino acid alignment of sigma factors has revealed that G82 is an absolutely conserved residue among all sigma factors, whereas Q80 and E81 are highly conserved residues (3). P74, on the other hand, may be conserved only among heat shock sigma factors (24).

Purified Proteins Exhibit Different Levels of Transcriptional Activity. We were able to purify σ^{32} proteins to greater than 95% purity with the exception of G82S, which was unstable. The remaining purified proteins were assayed for their activity using an *in vitro* transcription assay. The sigma factors were preincubated with core RNAP to facilitate the reconstitution of Eσ^{32} holoenzyme. The reconstituted RNAP then recognized the DNA template, which contained the *dnaK*-P1 promoter and a terminator from an *E. coli* rRNA transcription unit, producing a transcript of 290 nucleotides. When only core RNAP or σ^{32} was present in the reaction, no transcripts were seen (data not shown). However, when both proteins were present, significant levels of transcripts were detected (Fig. 1).

Our analysis revealed a relatively high activity for wild-type σ^{32}. When the ratio of σ^{32} to core RNAP was one to one, approximately 60 fmol of transcripts were produced from a possible maximum of 110 fmol. A Lineweaver–Burk plot was drawn to obtain a better estimate of the maximal level of transcripts and the dissociation equilibrium constant, K_d, which would be equivalent to the concentration of the sigma factors at the half-maximal level of transcription (graph not shown). Based on the points plotted on the graph, the following linear equation was obtained:

$$1/Y = (1.10 \times 10^{-2}) + (1.05 \times 10^{-2})(1/X)$$

where Y is the concentration of the product and X is the concentration of σ^{32}. Calculating the Y and the X intercepts provided the values for the maximal level of transcripts (91 fmol) and K_d (1 nM).

The results for the mutants were significantly different from those of the wild type. The level of transcripts for P74R at the equimolar concentration of core RNAP was only 20% of the wild type. E81G, a mutant that altered a conserved residue, was slightly lower in activity than P74R. Attempts to increase the concentration of mutant sigma factors to raise the maximal

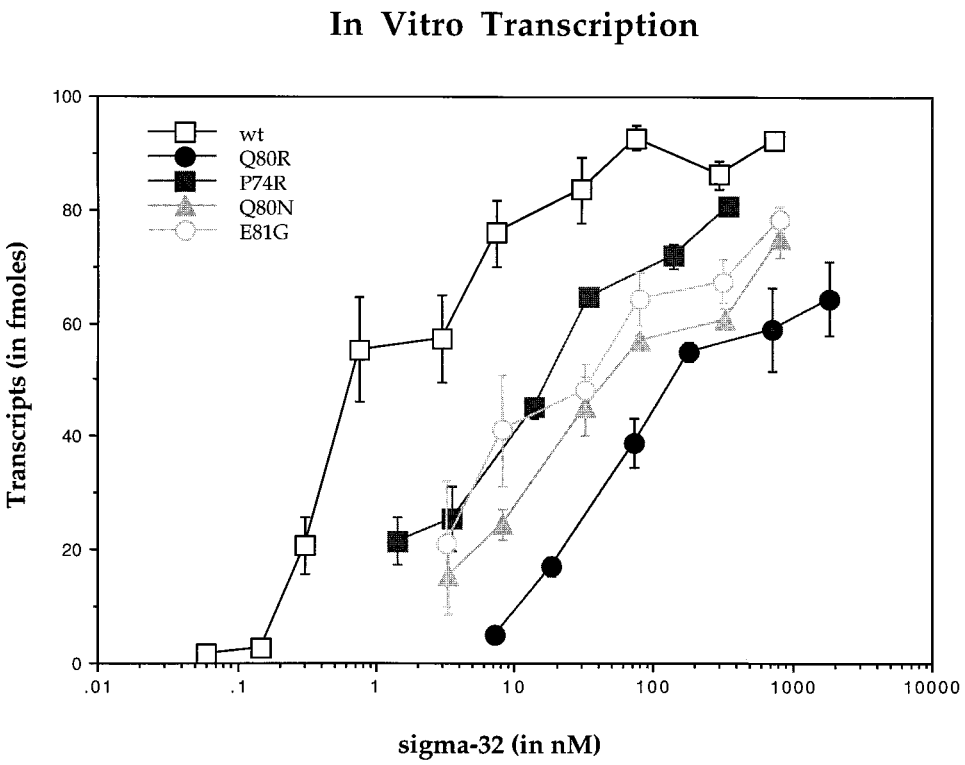

FIG. 1. Graphical representation of transcripts from a *dnaK*-P1 containing promoter using holoenzyme reconstituted with wild-type or four σ^{32} mutants and core RNAP. Core RNAP (1.1 nM) was used in each reaction with increasing concentration of sigma factors. Data are expressed as means ± SD of at least four experiments in each curve.

activity resulted in the precipitation of proteins. Q80R displayed the lowest level of activity. It was difficult to detect any transcriptional activity with equimolar amounts of sigma and core RNAP for this mutant sigma factor. Although it was not possible to delineate the maximal level of transcripts, it was clear from the titration curve that the K_d of Q80R–core RNAP complex would be considerably less than that of the wild type. Therefore, our findings indicate that although wild-type σ^{32} exhibits high activity, the three purified mutants are defective at some stage of transcription.

Binding to Core RNAP. To substantiate further whether any of the mutants might be defective in core RNAP interaction, we used the glycerol gradient sedimentation technique. In the absence of core RNAP, σ^{32} sedimented near the top of the gradient (Fig. 2*B*). When an equimolar concentration of core RNAP was added into the reaction, σ^{32} sedimented to the bottom of the gradient, indicating that the sigma factor was binding to core RNAP (Fig. 2*C*). The sedimentation pattern of core RNAP with (Fig. 2*A*) or without (data not shown) σ^{32} was indistinguishable. The concentration of both σ^{32} and core RNAP used in this experiment was 100 nM. Even though this technique does not measure protein–protein interaction at equilibrium because of the changing conditions during sedimentation, a binding constant for a number of protein complexes has been estimated while taking into consideration the dilution effect during sedimentation (25–27). In our assay, an efficient interaction of the proteins suggests that the K_d of the complex is less than 100 nM. If the 2- to 3-fold dilution effect during glycerol gradient sedimentation is accounted for, then the K_d can be less than 33 nM. The above *in vitro* transcription analysis indicated that the K_d of σ^{32}–core RNAP complex may be 1 nM.

We examined the core RNAP binding affinities of E81G and P74R (Fig. 2 *D* and *E*, respectively). Although E81G appeared to trail the core RNAP by one-half, both mutants were almost identical to the sedimentation pattern of the wild type. These observations suggest that the cause of their decreased activity may lie elsewhere in the transcriptional process. However, another interpretation of these data is that the mutant proteins are indeed defective in core RNAP interaction, but the technique used in the experiment is not sensitive enough to detect the slight reduction in core RNAP affinity. Nevertheless, these two mutants did possess strong core RNAP affinity at the concentration used in the experiment. Q80R produced a strikingly different pattern of sedimentation (Fig. 2*F*). The majority of Q80R was unbound. The broad sedimentation behavior of Q80R in the presence of core RNAP also suggested that the complex is unstable, constantly associating and dissociating during sedimentation. To obtain an estimate of the binding constant of this complex, densitometry was used to calculate the percentage of the core RNAP-bound sigma. Our analysis indicated that only 20% was associated with core RNAP. Because one molecule of sigma interacts with one molecule of core RNAP, we estimated that the K_d for the mutant RNAP complex was approximately 100 nM, taking into consideration the dilution effect during sedimentation.

Competition for Core RNAP with σ^{70}. The K_d for the σ^{70}–core RNAP interaction has been measured to be approximately 2 nM (23). If the K_d for the Q80R–core RNAP complex were 100 nM, then the addition of the core RNAP competitor σ^{70} in equimolar quantity would completely displace Q80R from the polymerase. To test this idea, we added σ^{70} into the reaction. In the following experiments, core RNAP was added into a mixture that already contained both types of sigma factors, either σ^{70} and σ^{32}, or σ^{70} and Q80R. The results showed that even when σ^{70} is present, σ^{32} could effectively compete for core RNAP (Fig. 2*G*). Approximately 50% of σ^{32} was found in a complex with core RNAP. A similar percentage was observed in another report (28). However, this high affinity for core RNAP in σ^{32} was drastically reduced when a specific amino acid in region 2.2 was altered. As expected, Q80R was almost completely displaced in the presence of σ^{70} (Fig. 2*H*).

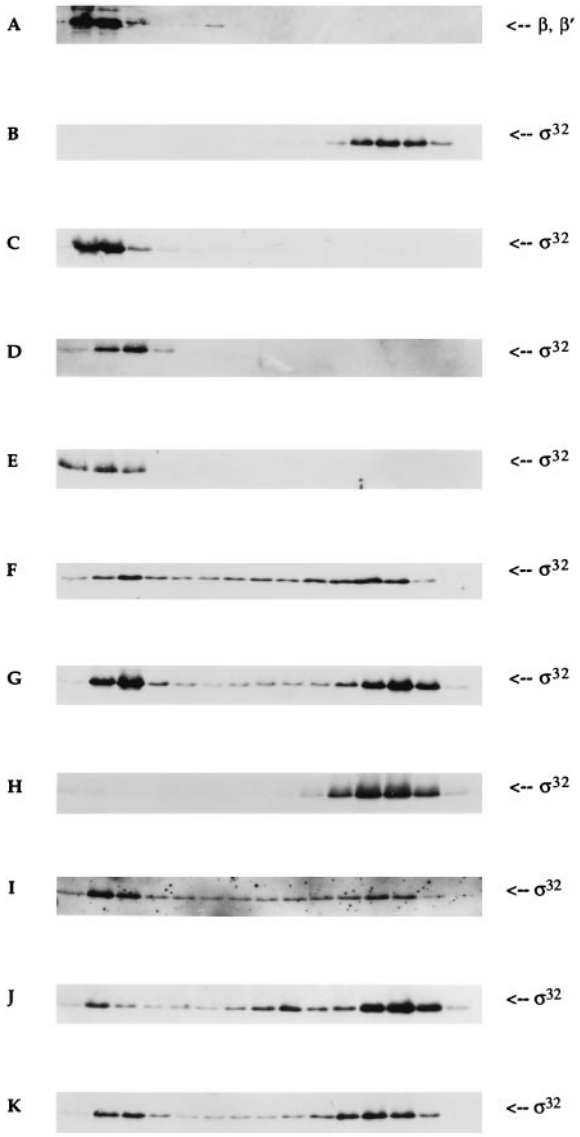

FIG. 2. Western blot analysis of glycerol gradient sedimentation of sigma factors and core RNAP. The concentration of each protein in all experiments was 100 nM. The sedimentation pattern of both σ^{32} and core RNAP was determined using polyclonal antibodies [gift of C. Gross (University of California, San Francisco) and Y. N. Zhou (National Institutes of Health, Bethesda, MD)]. The left-most region of each panel represents the bottom of the tube (35% glycerol). A typical sedimentation pattern of β and β' subunits of core RNAP with σ^{32} (*A*); σ^{32} only (*B*); σ^{32} with core RNAP (*C*); E81G with core RNAP (*D*); P74R with core RNAP (*E*); Q80R with core RNAP (*F*); σ^{32} + σ^{70} with core RNAP (*G*); Q80R + σ^{70} with core RNAP (*H*); P74R + σ^{70} with core RNAP (*I*); E81G + σ^{70} with core RNAP (*J*); and Q80N with core RNAP (*K*).

We then examined the effect of σ^{70} on the core RNAP binding affinity of E81G and P74R. In the absence of a core competitor, E81G and P74R were observed to interact with core RNAP quite efficiently (Fig. 2 *D* and *E*, respectively). When an equimolar amount of σ^{70} was introduced, P74R revealed a sedimentation pattern that was reminescent of the result obtained from σ^{32} (Fig. 2*I*). Slightly more than half of the sigma factor was found to be in complex with core RNAP. The other mutant, E81G, revealed a different result when σ^{70} was included in the reaction (Fig. 2*J*). Approximately 90% was found dissociated from core RNAP.

The Conserved Mutant Q80N Possesses Lower Transcriptional Activity. To examine residue 80 of σ^{32} in greater detail, we generated the mutant Q80N (see *Materials and Methods*). This is a conserved change, as both are polar amino acids with an amide. The difference between the two residues is the shortening of the side chain by one angstrom. Although the change from a polar to a basic amino acid may potentially alter the stability of the structure, this is much less likely to occur with a conserved change, such as in glutamine to an asparagine. On the other hand, such a change may have a significant effect on protein–protein interaction.

Q80N was purified, and its activity was also determined using an *in vitro* transcription assay (Fig. 1). Surprisingly, the activity of this mutant was similar to the transcriptional activity seen in other mutants. In spite of the conserved change of amino acid, Q80N displayed a significantly lower activity than that of the wild type. In addition, the dose-response curve of Q80N was quite comparable to that of Q80R. This result suggests that Q80 is an essential residue for the function of the subunit of RNAP.

Q80N also Exhibits Reduced Core RNAP Affinity. Encouraged by the above result, we determined the affinity of Q80N for core RNAP by glycerol gradient sedimentation and found that Q80N was as defective as Q80R in core RNAP binding (Fig. 2*K*). Approximately 25% of this mutant sigma factor was bound to core. Furthermore, the broad sedimentation behavior of Q80N indicated the unstable association with core RNAP. When the core RNAP competitor σ^{70} was included in the reaction, majority of Q80N failed to interact with core RNAP (data not shown). Therefore, we conclude that the length of the side chain is important for proper core RNAP association.

DISCUSSION

Mutations of Sigma that Affect Core RNAP Binding. A few studies have been performed to identify the core RNAP binding region of sigma factors (10, 11, 29). Initially, a deletion analysis on σ^{70} identified a short peptide fragment containing sequences from region 1.2 and 2.1 that might be essential for core RNAP interaction (10). In support of this finding, a single amino acid substitution in region 2.1 of σ^{E} in *Bacillus subtilis* was shown to destabilize core RNAP interaction (11). The same study also reported that a number of single amino acid substitutions in region 2.2 had no effect on the function of the sigma factor, except for one residue that was believed to have destabilized the structure of the polypeptide. Unfortunately, the analogous residue corresponding to Q80 of σ^{32} was not investigated in this report.

Our study indicates that the most highly conserved region, 2.2, is involved in core RNAP binding. Using glycerol gradient sedimentation to observe holoenzyme formation with purified σ^{32} and core RNAP, we have shown that the mutation Q80R exhibits reduced core RNAP affinity. A similar result was obtained using a "small zone" gel filtration column (data not shown) (27). These results supported the initial observation of potential defects in core RNAP binding through *in vitro* transcription analysis. In addition, the use of purified proteins in our protein–protein interaction assay allowed us to estimate the K_d of the Q80R–core RNAP complex as approximately 100 nM. This is a 100-fold reduction in the core RNAP binding affinity, because the K_d of σ^{32}–core RNAP complex was estimated to be 1 nM by our *in vitro* transcription experiment. Therefore, the reduction in the mutant's K_d allowed us to predict that competition with equimolar σ^{70} would displace Q80R from core RNAP almost completely. Such displacement was observed.

The Structural Basis for the Effects of *rpoH* Mutations. This report's analysis of the σ^{32} mutants in region 2.2 is consistent with the recently determined crystal structure of a protease-resistant fragment of σ^{70}, which is composed entirely of alpha helices and connecting loops (30). Q80, which we believe is

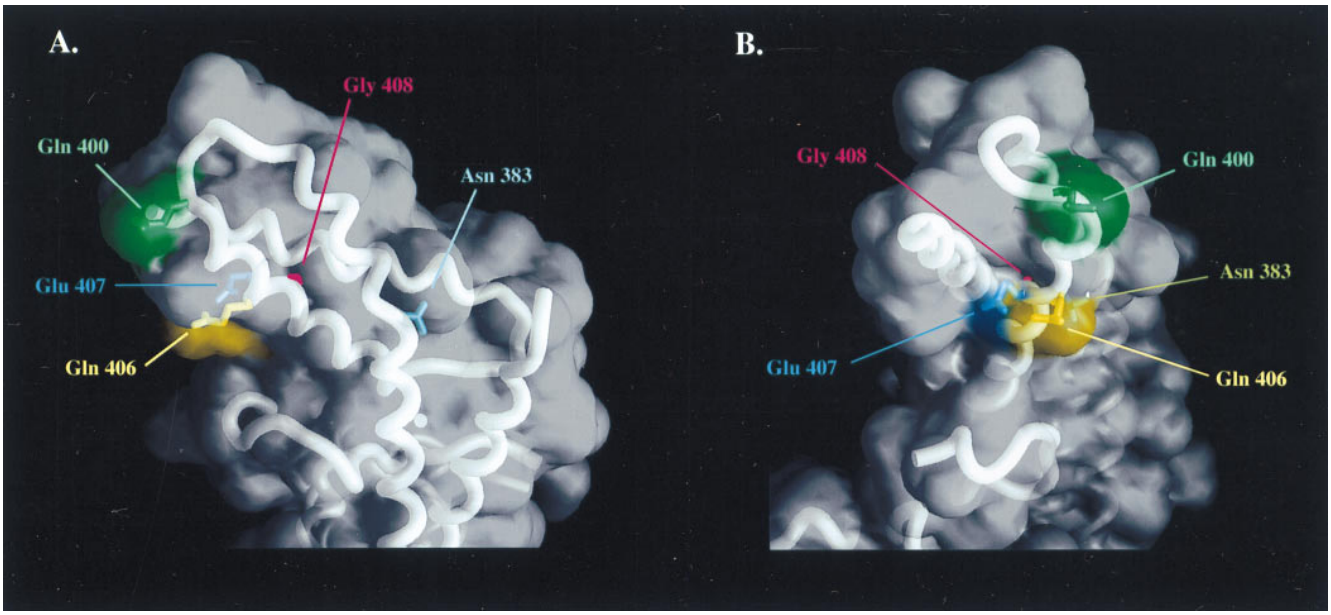

FIG. 3. Core RNAP binding region of the crystallized structure of σ^{70} (30). Residues His^{57}, Pro^{74}, Gln^{80}, Glu^{81}, and Gly^{82} of σ^{32} correspond to Asn^{383}, Gln^{400}, Gln^{406}, Glu^{407}, and Gly^{408} of σ^{70}, respectively. (*A*) The frontal view of the region presumed to be important for core RNAP interaction is illustrated, where the kink is centered about Asn^{383}. (*B*) The left-side view of *A*. [The figure was generated by using the program GRASP (31).]

critical for core RNAP interaction, has a corresponding amino acid, Q406, in σ^{70} (Fig. 3). The crystal structure reveals several aspects of Q406 that may support our observation with mutants at this position. Q406 is exposed to the solvent, which may promote favorable protein–protein interaction by being readily exposed on the surface of the protein. Structurally, it is located within the solvent-exposed hydrophobic patch, composed of highly conserved residues, and believed to be a critical region for core RNAP interaction (30). Finally, Q406 lies very close to the kink, centered about N383, which is thought to be an important structural motif for core RNAP interaction (30).

Glutamine is a polar amino acid with an amide group and has the potential to form hydrogen bonds with a residue or residues in core RNAP. Because the strength of hydrogen bonds is heavily dependent on the distance and the colinearity between hydrogen bond donors and acceptors, a substitution of glutamine with any other amino acid may have deleterious consequences to the polypeptide's core RNAP affinity. When the substitution was a conserved amino acid, as it was the case with Q80N, a significant reduction in core RNAP interaction was noticed.

We were not able to purify G82S because of its extreme instability *in vivo*. This instability of G82S was first noticed by Yan Ning Zhou, using Western blot analysis to detect the level of proteins *in vivo* (personal communication). This residue is one of three residues in region 2.2 that are absolutely conserved among all sigma factors in the σ^{70} family (3). The other two are G85 and L86. G94C of σ^{E} in *B. subtilis*, which is analogous to G85 of σ^{32}, failed to accumulate this sigma factor *in vivo* (11). We believe that this residue has also destabilized the alpha helix. The crystal structure of this region of σ^{70} has provided confirmation of our speculation (Fig. 3). In σ^{70}, both G408 and G411, which correspond to G82 and G85 of σ^{32}, lie in a region of closest proximity with neighboring alpha helices (30). Therefore, the introduction of amino acids bulkier than a glycine in the space-restricted hydrophobic environment should perturb the stability of the structure.

P74 is one of few residues in region 2.2 that are not conserved among all sigma factors. We thus were not surprised to discover that P74R was able to interact with core RNAP efficiently. Even when σ^{70} was added into the reaction, P74R was able to compete efficiently for core RNAP. The crystal structure of σ^{70} may provide an explanation for this mutant's behavior (Fig. 3). The analogous residue on σ^{70} is Q400, an unconserved change. In addition, this residue is located somewhat removed from the hydrophobic patch consisting of solvent-exposed residues and is not part of an alpha helix but of a connecting loop. These features indicate that this residue may not be critical for core RNAP binding.

E81G appeared to associate with core RNAP almost as tightly as did wild type, even though its *in vitro* transcriptional activity was decreased. It is worth noting that E81 is a conserved residue, and the analogous residue of σ^{70}, E407, is partially exposed to the solvent (Fig. 3*B*). There was the possibility that the core RNAP binding defect was subtle and undetectable by our assay. By introducing the core RNAP competitor, σ^{70}, we were able to visualize the effect of the mutation on core RNAP affinity. Although there was a significant level of the sigma factor that interacted with core RNAP, most were found free of core RNAP. This evidence suggests that although Q80 has a profound effect, E81 has a subtle effect, at least in core RNAP interaction. However, we cannot rule out the possibility that there may be other defects for either of the mutants at this time.

Our work finally confirms the speculation that the most highly conserved region, 2.2, is involved in core RNAP binding. We conclude that mutations in Q80 lead to reduced core RNAP affinity, which directly contributes to its decreased activity during *in vitro* transcription. Because Q80 is a highly conserved residue, it would be interesting to determine whether mutations at this residue in other sigma factors can lead to the unstable Eσ complex. We are currently investigating this possibility.

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