A surface of *Escherichia coli* σ^{70} required for promoter function and antitermination by phage λ Q protein

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The σ initiation factor σ^{70} of *Escherichia coli* acts not only in promoter recognition and DNA strand opening, but also to mediate the transformation of RNA polymerase (RNAP) to an antiterminating form by the phage λ gene Q protein. Q is able to bind and modify RNAP when α^{70}, still present in the initially elongating enzyme, recognizes a repeat of the –10 promoter element and induces a transcription pause. We have isolated mutations in the *rpoD* gene for σ^{70} that impair Q function because they reduce the ability of σ^{70} to recognize the downstream pause site. These mutations identify a locus of σ^{70} that is important for the formation and stability of open promoter complex, likely because it mediates protein interactions with RNAP core.

[*Key Words*: σ factor; promoter; antitermination]

Received July 2, 1998; revised version accepted August 21, 1998.

σ factors of bacterial RNA polymerase (RNAP) mediate promoter recognition and opening in transcription initiation (for review, see Gross et al. 1992). They act in the form of a holoenzyme, a complex with the universally conserved RNAP core enzyme E (composed of subunits $\alpha_2\beta\beta'$); thus $E\sigma^{70}$ is the holoenzyme of the primary σ factor of *Escherichia coli*, σ^{70}. Through conformational changes of an initial closed complex, $E\sigma^{70}$ mediates DNA strand separation to form an open complex that can template RNA chain synthesis (McClure 1985). During in vitro synthesis, σ^{70} becomes dispensable and can dissociate after transcripts reach 5–10 nucleotides in length (Hansen and McClure 1980), leaving the core to elongate RNA chains; thus, σ^{70} (and other σs) functions primarily in initiation.

In a variant of this simple initiation sequence, σ^{70} also acts during an early step of elongation to induce a paused transcription complex that is necessary for engagement of the bacteriophage λ gene *Q* antiterminator, the positive regulator that ensures lytic development in phage growth (Roberts 1992; Ring et al. 1996). Pausing is induced at RNA nucleotides +16 and +17 of the transcript of phage late gene promoter p_R' when σ^{70} recognizes a repeat of critical bases of the –10 promoter sequence that is displaced 11 bp downstream (Ring et al. 1996) (Fig. 1A). At both the promoter –10 sequence and the downstream pause-inducing sequence, $E\sigma^{70}$ recognizes primarily the nontemplate strand of DNA (Ring and Roberts 1994; Ring et al. 1996; Roberts and Roberts 1996; Marr and Roberts 1997); both the open and paused complexes are stabilized by this single-strand DNA-$E\sigma^{70}$ interaction. Q binds the paused complex by contacting DNA of the *qut* site, located between the –35 and –10 promoter elements, as well as unknown portions of RNAP (Fig. 1A). This interaction chases RNAP from the pause, allows incorporation of Q as a component of the elongation complex [at least for the related Q protein of phage 82 (W.S. Yarnell and J.W. Roberts, unpubl.)], and fundamentally alters the elongation property of RNAP so that it reads through terminators. σ^{70} is released at an unknown stage, but presumably early after Q engagement.

It is not known how σ^{70} acts to allow Q engagement. The presence of σ^{70} bound to the pause-inducing sequence is important per se and not simply to induce a pause: RNAP stopped at +16/+17 on a template carrying a mutant pause-inducing sequence, so that σ^{70} has dissociated or can be easily displaced, is not receptive to Q modification (Yarnell and Roberts 1992). Two nonexclusive possibilities are that σ^{70} alters the conformation of the rest of the transcription complex to make it receptive to Q modification, and that σ^{70} recruits Q through direct protein–protein interactions.

This activity provides an opportunity to find elements of σ^{70} important for Q activity, and, more important, for development of base-specific nontemplate strand DNA contacts in pausing and promoter recognition. For this purpose, we used a genetic screen to identify amino acid substitutions in σ^{70} that impair Q-mediated antitermination. We find that a major class of these mutations occurs on a surface in region 2.2 of σ^{70}, the most highly conserved region of the polypeptide (Gross et al. 1992).

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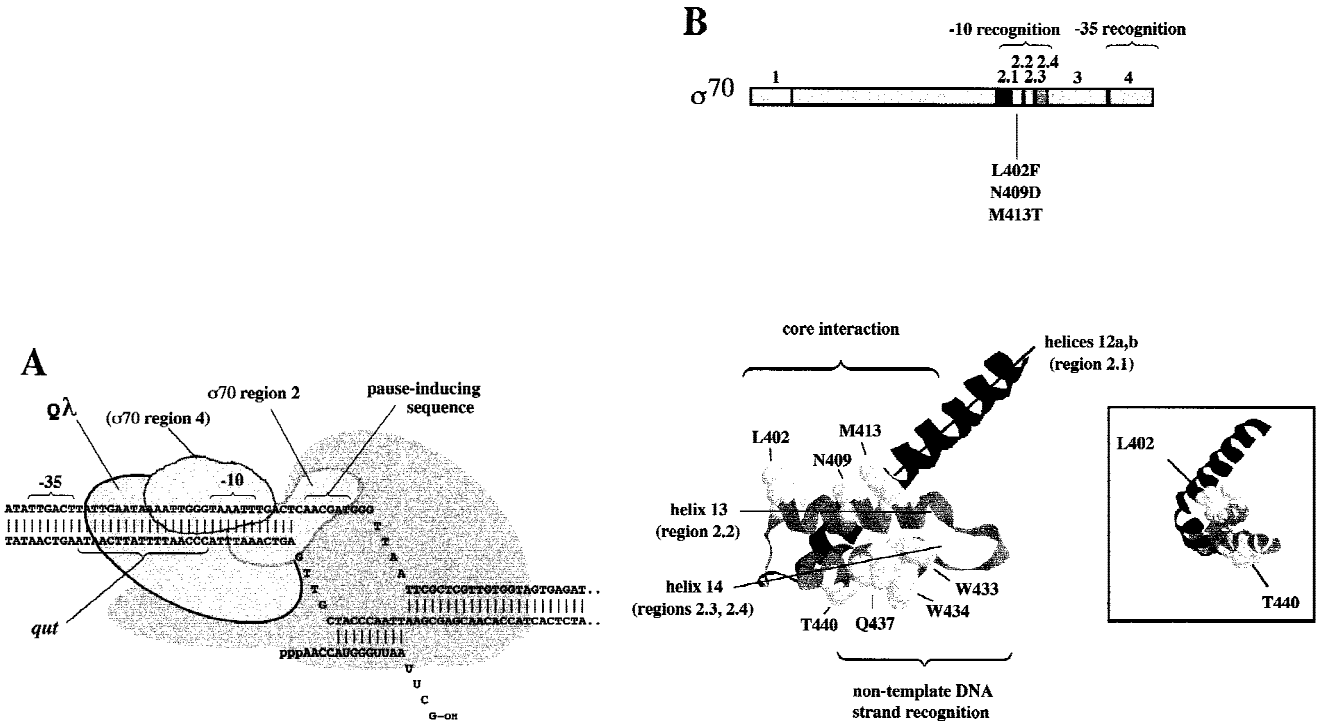

Figure 1. (*A*) Paused complex of RNAP transcribing the λ late gene promoter. Region 2 of σ^{70} is placed to interact with the nontemplate strand of the pause-inducing sequence (AACGAT, in which underlining indicates match to the –10 promoter sequence consensus). Q^{λ} is shown bound to the *qut* site. The location of σ^{70} region 4 is unknown, but it is placed to suggest interaction with Q^{λ} (H. Sun and J.W. Roberts, unpubl.). The complex is shown in a backtracked form (Reeder and Hawley 1996; Komissarova and Kashlev 1997; Nudler et al. 1997) to account for its sensitivity to cleavage mediated by GreB (W. Yarnell and J.W. Roberts, unpubl.). (*B*) Map of the σ^{70} polypeptide, and structure of the region 2.1, 2.2, and 2.4 α-helix bundle of σ^{70} (Malhotra et al. 1996). Residues T440 and Q437 are believed to make base-specific contacts with promoter DNA of the nontemplate strand, and W433 and W434 to mediate DNA melting; residues L402, N409, and M413 are discussed in the text. (*Inset*) The structure rotated to view the helices from the left side, nearly end on.

We show that these mutations reduce the promoter proximal pause, an effect that can explain their interference with Q function. Furthermore, as might be expected from the similarity of the paused and open complexes, these mutations also reduce the stability of the open promoter complex. Our results, therefore, identify a surface of σ^{70} that is important to the structure of the open promoter complex, likely through contacts σ^{70} makes with core RNAP during the closed- to open-complex transition; this surface also may be part of the σ^{70}-core contacts that form holoenzyme (Malhotra et al. 1996). In addition, the identification of σ^{70} mutations provides genetic confirmation of the role of σ^{70} in antitermination and in steps of transcription after initiation of RNA chains.

Results

A genetic screen to identify amino-acid substitutions in σ^{70} that result in reduced Q antitermination activity

To measure antitermination by Q protein in vivo, and to provide a screen for mutations that alter Q function, we constructed a reporter strain that contains a fusion of p_R', two intrinsic terminators, and *lacZ* integrated into the *E. coli* chromosome on a prophage. This strain was transformed with an IPTG-inducible Q expression plasmid, so that IPTG-dependent *lacZ* expression reports Q function. To search for σ^{70} mutants that impair Q function, we mutagenized the *rpoD* gene (encoding σ70) in two halves on the multicopy plasmid pHTσ by PCR random mutagenesis (Zhou et al. 1991), introduced mutagenized plasmids into the reporter strain, and screened by colony color for defective Q function in conditions of *lacZ* induction. The *rpoD* gene of pHTσ is also controlled by the *lac* operator, producing 4–8 times the natural level of σ^{70} on IPTG induction (D. Ko and J.W. Roberts, unpubl.) The presence of this increased level of wild-type σ^{70} did not affect Q-dependent expression of *lacZ*. Note that the mutants recovered could not be dominant lethals for cell growth, and had to compete effectively with the endogenous σ^{70} for core enzyme to express a phenotype.

We isolated 10 mutations in the carboxy-terminal half of *rpoD* that support <70% wild-type Q function. We consider here one class of mutations that includes the only two which retain viability without an additional

wild-type σ^{70} gene (L402F and L409D), and thus affect Q function without destroying essential σ^{70} activity; we include a third (M413T) that is nearby, but has only slight effect in the original reporter assay. Mutations L402F, N409D, and M413T occur in a single stretch of region 2.2 in the atomic structure of residues 114–448 of σ^{70} (Malhotra et al. 1996), located on one side of a strongly bound three α-helix bundle of σ^{70} (Fig. 1B). The bundle includes part of helix 12a and helix 12b, which contains region 2.1, believed to be the major core enzyme-binding region (Lesley and Burgess 1989); helix 13, which contains most of region 2.2; and helix 14, which contains parts of regions 2.3 and 2.4. Region 2.4 includes amino acids thought to be involved in base-specific recognition of the –10 element nontemplate DNA strand (Gardella et al. 1989; Kenney et al. 1989; Siegele et al. 1989; Zuber et al. 1989; Roberts and Roberts 1996; Marr and Roberts 1997) and in melting or stabilizing single-stranded DNA (Helmann and Chamberlin 1988; Juang and Helmann 1994). L402, N409, and M413 are on a hydrophobic face of the α-helix 13 (Malhotra et al. 1996) that includes region 2.2 of σ^{70}.

To investigate the influence of the amino acid side chains, we also changed residues 402, 409, and 413 to alanine, to remove important side chain contacts but not introduce new ones (Niu et al. 1994). We also changed to alanine residue Q406, which is the remaining amino acid on the solvent exposed face of helix 13 of region 2.2, but which was not found in the screen. Mutation of the corresponding amino acid (Q80) in σ^{32} causes a defect in binding to core RNAP (Joo et al. 1997). Like the original mutations, the alanine substitutions support growth in the absence of wild-type σ^{70} (Fig. 2D and data not shown).

The effect of these mutations on Q-dependent β-galactosidase expression in two reporter strains growing uniformly in liquid culture is shown in Figure 2(A,B); expression with wild-type σ^{70} in the absence of Q is <10 units (Fig. 2C). Figure 2A shows expression levels in the reporter strain used for the selection, in which mutant σ^{70} competes with wild-type σ^{70} made from the chromosome; in Figure 2B, the mutants are assayed at 42°C in a strain carrying a temperature-sensitive σ^{70} (*rpoD285*), so that only the mutant σ^{70} is present. First, note that L402F, Q406A, both 409 mutants, and M413A show pronounced defects in Q-dependent *lacZ* expression in both strains and are more defective in the absence of wild-type σ^{70} (Fig. 2B). M413T shows slight or no defect in these liquid assays; however, it was visibly mutant in the different physiology of the colony screen, and we show evidence below that it shares defects with L402F and N409D (although more weakly). Note that the L402F, N409D, and M413T mutations show a decreasing severity of defect in this order, and that this relationship is true in other behavior described below.

Because the alanine substitutions at residues 406, 409, and 413 have mutant phenotypes, side-chain atoms beyond the β-carbon are important at these sites. For N409 and M413, the alanine mutations are more severe than the original screened mutations, which presumably retain some favorable interactions. N409D would disrupt a possible hydrogen bond, but might retain other side-chain interactions not possible for alanine; M413T might retain some polar and hydrophobic interactions that would be lost by the change to alanine. Of the four alanine substitutions, only L402A does not decrease Q-activated transcription. In fact, several alternative σ factors, including σ^{32}, have alanine at this amino acid position (Gross et al. 1992). The original mutation obtained in the screen, a change to phenylalanine, introduces a larger side chain that might sterically prevent the normal interaction of this entire surface of σ^{70}.

These mutations could preferentially affect initiation at promoter p_R', or another property of this transcription unit, rather than Q function. However, assay of p_R' in the absence of Q (Fig. 2C) shows that, in contrast to a deficiency, the basal transcription is increased. Furthermore, the increase is related to the severity of the defect in Q function. As discussed below, we ascribe this increased expression to lack of pausing at the promoter proximal site and consequent relief of this partially rate-limiting step in transcription.

The mutations modestly affect the cell growth rate (Fig 2D), thereby increasing the generation time ~50%. It is most evident for the alanine substitutions that severity of growth defect correlates with deficiency in Q function.

σ^{70} *mutations inhibit phage growth*

If the σ^{70} mutations interfere with Q function, they should impair growth of phage λ. In fact, plaques of phage λc17 grown at 42°C on CG19284 containing pHTσ(L402F) or pHTσ(N409D) are smaller than plaques on CG19284 containing pHTσ^+—one-quarter to one-half the diameter.

σ^{70} *mutants reduce Q-activated transcription in vitro*

To determine their effect on antitermination in vitro, and to probe the basis of their defects, we purified the mutant σs L402F, N409D, and M413T as His-tagged proteins, reconstituted them into holoenzyme RNAP, and measured terminated versus readthrough transcript from p_R' in the presence and absence of Q protein. In the absence of Q, holoenzyme reconstituted with mutant σ^{70} gives background terminator readthrough similar to that with wild-type σ^{70} (2%–3%) (Fig 3). Addition of 100 nM Q to reactions with wild-type Eσ^{70} produces the expected 50% readthrough, whereas Eσ^{70} containing mutants L402F, N409D, and M413T gives 8%, 12%, and 17%, respectively (Fig. 3). The relative ability of the three mutant RNAPs to be modified by Q in vitro corresponds to the ability of mutant strains to support Q function in vivo: L402F<N409D<M413T<wild type (Fig. 2).

The antitermination defect of the mutant σs was not overcome by increasing the Q concentration. At Q concentrations between 0.4 nM and 100 nM (saturating for wild type), RNAP reconstituted with mutant σ^{70} was defective in its ability to be modified by Q (Fig. 3); both

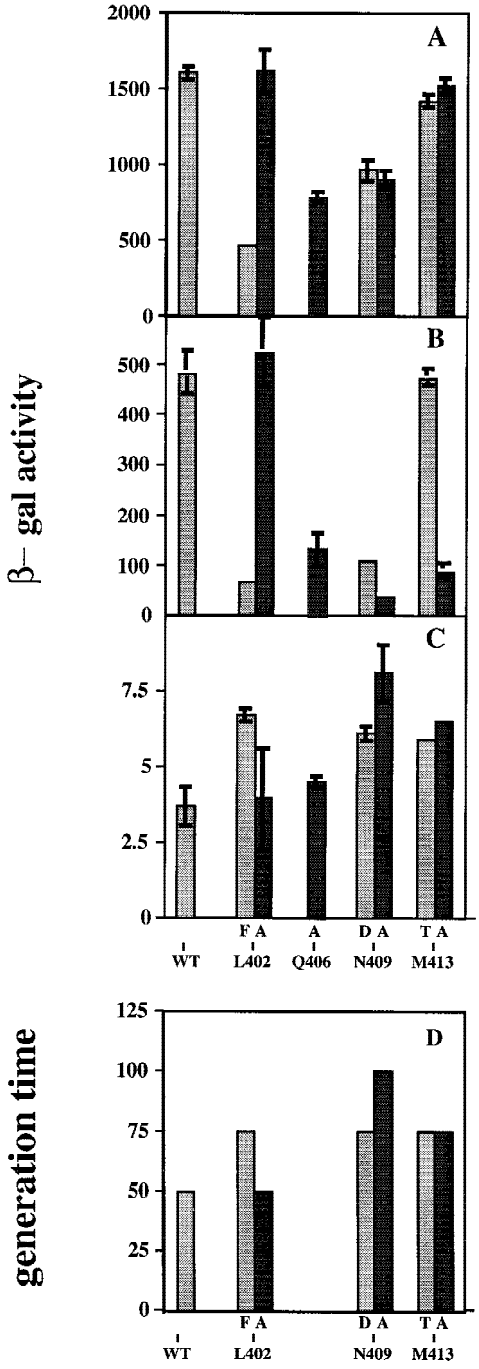

Figure 2. (*A*, *B*) Activity of σ^{70} region 2.2 mutants in Q function; (*C*) basal expression of p_R'; (*D*) cell growth rate. Each cell contains pHTσ expressing either wild-type or mutant σ^{70}. Q function or basal promoter activity is expressed as β-galactosidase activity from the reporter λ+49 as described. Q is expressed from plasmid pJG100 where it is present. (*A*) SG20250(pJG100), wild-type σ^{70} strain background. (*B*) CAG19284(pJG100), temperature-sensitive lethal σ^{70} strain background, grown at the restrictive temperature (42°C). (*C*) CAG19284, at 42°C (no Q present). (*D*) Growth rate of MTM9973 (recA⁻ version of CAG20176) carrying σ mutant plasmids, in the presence of tryptophan to repress the chromosomal σ^{70} gene.

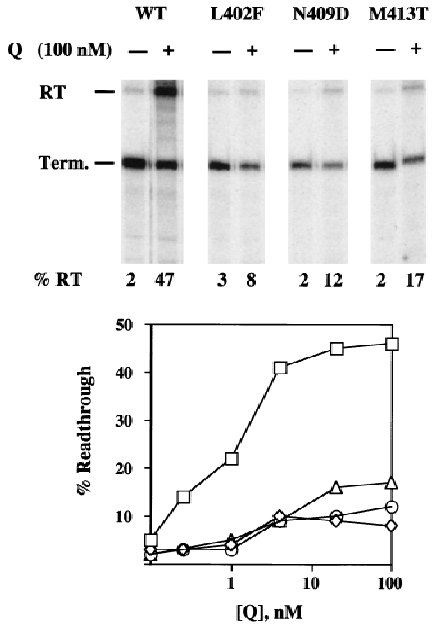

Figure 3. Activity of mutant σ^{70} proteins in Q-mediated antitermination in 5 min transcription reactions, as described. (□) Wild type; (◇) L402F; (○) N409D; (△) M413T.

wild-type and σ^{70}–mutant RNAP showed about half-maximal Q effect between 1 and 4 nM, despite their different saturation levels. This result suggests that the σ^{70} mutations do not act by weakening the binding of Q to the paused complex, which should be overcome by higher Q concentration, but instead change the capacity of the transcription complex to be modified.

σ^{70} mutants reduce promoter-proximal pausing

This reduced capacity for modification by Q might reflect the availability of substrate, namely the paused RNAP. To test this possibility, we analyzed products of single round in vitro transcription of promoter p_R' during a time course of 10 min (Fig 4A). Approximately 73% of wild-type enzyme pauses at the positions +16 and +17, with a half-life of ~5.9 min. The σ^{70} mutations reduce both the half-life of the pause—to ~2 min—and the percentage of RNAP that initially pauses (Fig. 4A). Significantly, the percent capture (percent paused extrapolated to zero time) corresponds to the ability of each mutant protein to support modification by Q. L402F supports the lowest Q activity (17% of wild type) and also has the lowest initial capture rate (30% of wild type), whereas M413T supports the highest Q activity (36% of wild type) and has the highest initial capture rate (75% of wild type) of the three mutants. Thus, these σ^{70} mutants are deficient in forming and/or maintaining the nontemplate strand binding that stabilizes the pause. The mutant defects follow the same order of severity as before.

The same pausing defect is detectable in vivo. Treatment of a growing culture with KMnO4 marks bases in the single-strand transcription bubble, thus revealing the

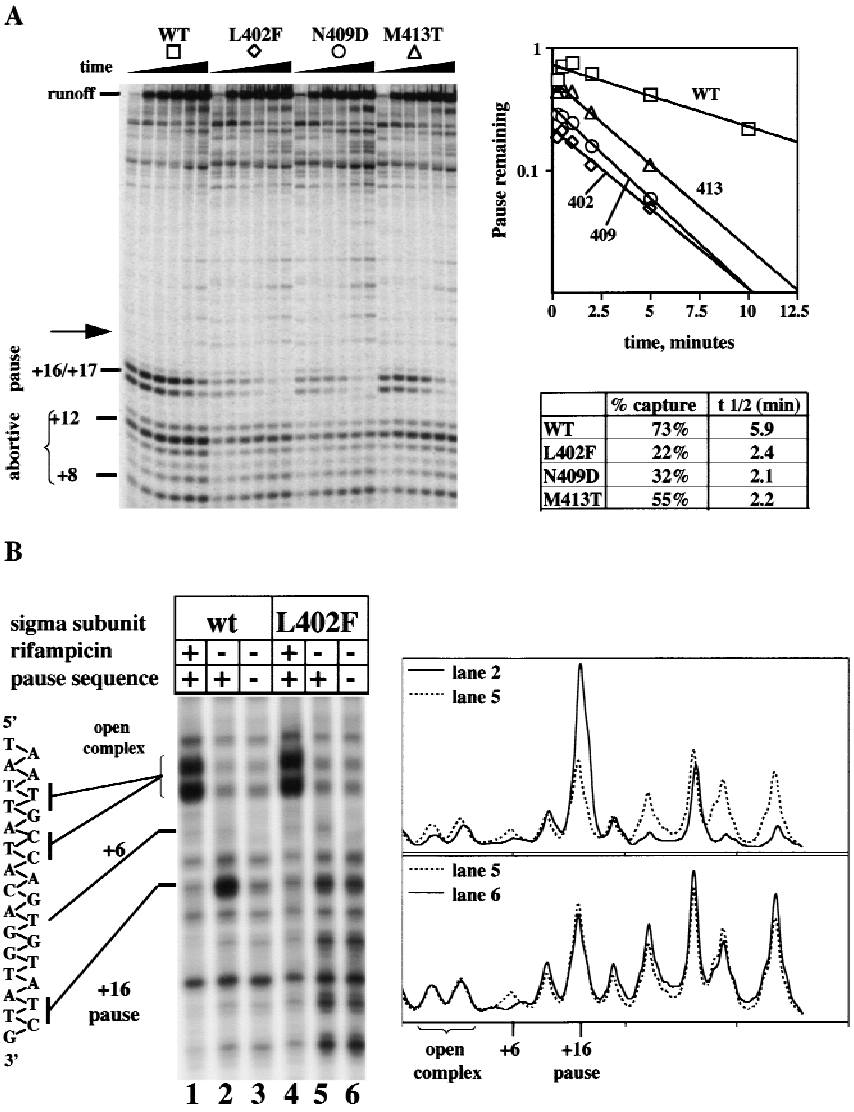

Figure 4. Deficiency of region 2.2 mutants in promoter-proximal pausing. (*A*) Pausing measured in vitro, in the absence of Q. Transcription reactions constructed as described were sampled at 0.25. 0.5, 1,2,5, and 10 min. (*B*) Pause detected in vivo by $KMnO_4$ treatment of strain MTM9973 (a *recA*$^-$ derivative of CAG20176) carrying plasmid pBAD33*spc rpoD* expressing either *rpoD*$^+$ or *rpoD*^{L402F}, and a plasmid with p_R' or the nonpausing derivative p_R'(+6C). Rifampicin was added 5 min before $KMnO_4$ treatment in lanes *1* and *4*.

pause (Kainz and Roberts 1992). Figure 4B shows KMnO4 analysis of the nontemplate strand around p-$_R'$ in growing cultures containing wild-type or L402F Eσ^{70}. Lanes 1 and 4 illustrate cultures treated with rifampicin to collect RNAP on the promoter, revealing open complex reactivity at –2 through –8. Lanes 2 and 5 show cultures without rifampicin, revealing pause bubble reactivity at +14 to +16. Comparison of lanes 2 and 3 shows that a +6C mutation destroying the pausing consensus eliminates the +16 pause signal in wild type, as expected. Signal in the pause position is weaker by two- to threefold for σ^{70} L402F (Fig. 4B, cf. lanes 2 and 5). Unexpectedly, there is more signal downstream of the pause site in σ^{70} L402F than in wild type; this reactivity represents active transcription complexes, because it is dispersed on incubation with rifampicin (Fig 4B, lane 4). Apparently, σ^{70} L402F not only fails to engage the pausing signal efficiently, but also induces elongation anomalies farther downstream. Presumably the signal that does appear in L402F at the pause position is part of the generalized downstream reactivity and not pause signal recognition, because it is not reduced by the +6C mutation (Fig. 4B, cf. lanes 5 and 6). Traces of this anomalous pausing may appear in in vitro transcription as well (Fig. 4A, cf. bands at arrow in lane 1 of each set).

L402F and N409D affect the rates of dissociation and association of the open complex

Because pausing at +16/+17 reflects the same binding between nontemplate strand DNA sequence and Eσ^{70} that also stabilizes the open complex, helix 13 mutations should affect the stability of the open complex. To show this, we used the method of Zhang et al. (1996), who showed that native gel electrophoresis traps open complex and freezes the distribution of free and bound promoter in a reaction mixture. We formed open complex by incubating labeled DNA with excess Eσ^{70} for 10 min at 37°C and measured dissociation during incubation at 17°C in the presence of excess competitor DNA (Fig. 5);

under these conditions, the half-life for dissociation of open complex of wild-type RNAP is ~30 min (Roberts and Roberts 1996). Open complex made with either L402F or N409D is severalfold less stable than wild-type complex (Fig. 5A,B); L402F is the least stable, consistent with its stronger phenotype for pausing and Q function. M413T is indistinguishable from wild type, consistent with its milder phenotype and with its similar yield of abortive products (see below).

We also measured the rate of association of Eσ^{70} and p_R' promoter DNA to form open complex (Fig. 5C). Wild-type and M413T holoenzyme show similar association kinetics, reasonably consistent with the expected first order capture of free DNA into complex at RNAP excess, and saturating by 5 min. In contrast, L402F and N409D give apparently biphasic curves, with a rapid rise in the first minute followed by a much slower rise. Possibly part of the initial closed complex diverts into a nonproductive pathway that is only slowly reversible; whatever the precise explanation, it is clear that L402F and N409D are defective in open complex formation. Similar kinetic anomalies have been found previously for weak RNAP–promoter interactions (Zhang et al. 1996).

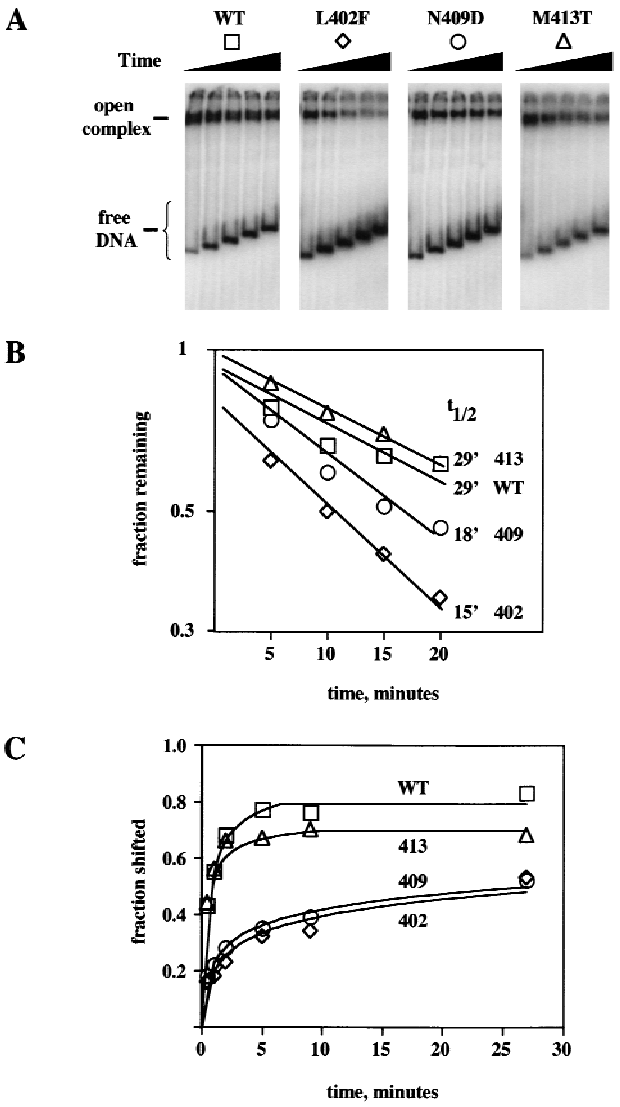

Figure 5. Effect of region 2.2 mutations on rate of open promoter complex dissociation and formation by Eσ^{70}. (*A*) Gel analysis of dissociation kinetics of preformed complexes on promoter p_R'. The DNA fragment was as described, except that it contained a –12 T–C mutation of promoter p_R' to weaken the complex and accentuate the rate of decay. (*B*) Graphical analysis of *A*. (*C*) Association of Eσ^{70} with DNA containing (wild type) promoter p_R'. (□) Wild type; (◇) L402F; (○) N409D; (△) M413T.

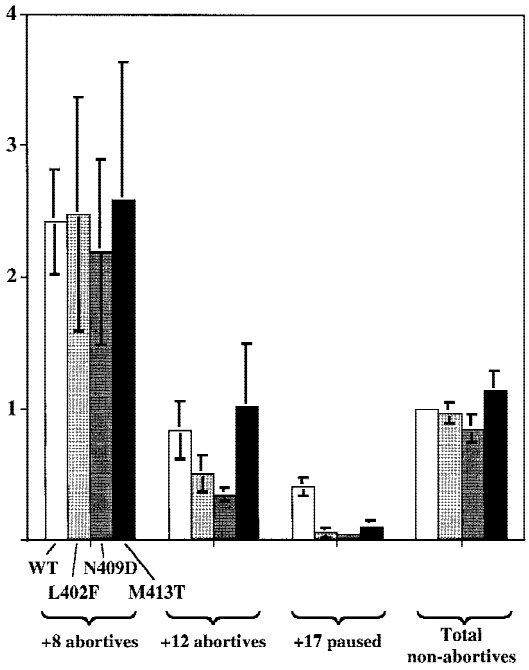

Figure 6. Deficiency of region 2.2 mutant σ^{70} in abortive initiation. Products of three separate 5′ in vitro synthesis experiments were analyzed by gel, quantified as described, and averaged. (Open bars) WT; (light gray bars) L402F; (dark gray bars) N409D; (solid bars) M413T.

L402F and N409D reduce abortive transcription

The yield of abortive transcripts, which are produced by repeated synthesis of oligonucleotides in the open complex (Johnston and McClure 1976), reflects open complex stability, because a less stable complex can escape into productive elongation after fewer cycles of abortive synthesis. This is true, for example, for nontemplate strand promoter nucleotide changes that affect the Eσ^{70}-DNA interaction (Roberts and Roberts 1996). Inspection of Figure 4A shows a deficiency in abortive synthesis by open complexes with mutant σs, particularly evident as the yield of +12 relative to +8. For a different experiment, we measured the yield of 8-mer and 12-mer abortive RNAs for wild-type and σ^{70} mutant holoenzyme (Fig. 6). Quantitation of abortive and total elongated transcripts (+16/+17 pause and longer) reveals that +12 abortive transcripts are two- to threefold deficient relative to wild type for L402 and N409D, although normal for M413T

(Fig. 6). Thus, the two most severe σ^{70} mutants show a distinct defect in abortive synthesis. The defect in longer (+12) but not shorter (+8) abortive RNAs might reflect failure of the mutant Eσ^{70}–DNA bond to sustain greater strain associated with greater extension of the catalytic center.

Discussion

We have identified a surface in subregion 2.2 of *E.coli* σ^{70} that stabilizes the open promoter complex, and, by its ability to mediate pausing near the start site, is required for the antitermination activity of phage λ Q protein. The region 2.2 mutations we identified appear to act both by impairing formation of the paused complex, allowing more RNAP to transcribe through without being caught, and destabilizing the complex once it is formed. They might more severely affect pausing than promoter function in general if pausing requires relatively fast engagement of Eσ^{70} into the DNA bound state as it passes the site.

We considered that the pause-reducing phenotype might result from weaker core-σ binding after initiation, allowing release of σ^{70} before RNAP reaches the pause-inducing sequence. However, if the pause defect were solely the result of early release of σ^{70}, the percent capture would be reduced, but the pause half-life would be unchanged; because all three mutations reduce the pause half-life as well as percentage capture, they must reduce stability of the paused complex, as is also true for the open complex at the promoter.

If the pause limits transcription, which it does slightly for pR′ and much more for a stronger promoter (Kainz and Roberts 1995), then the reduced-pausing σ^{70} mutations should have higher levels of transcription than wild-type σ^{70} in transcription not affected by Q. This would account for the higher basal transcription rate of p_R' by holoenzyme containing region 2.2 mutant σ^{70} relative to wild-type σ^{70}.

Of the four regions of extensive similarity among σ factors, the most conserved is region 2 (Lonetto et al. 1992), encompassing amino acids 371–456 in σ^{70}, and believed to mediate core RNAP binding, DNA melting, and sequence-specific promoter contacts. Until recently, no function had been assigned to subregion 2.2, the most conserved segment among σ factors. Because the exposed surface of region 2.2 helix 13 is hydrophobic and is near the core-binding segment—residues 361–390 of region 2.1—Malhotra et al. (1996) suggest that the helix of region 2.2 also binds core RNAP, or possibly another protein surface. In agreement, point mutation analysis of alternative σ factors (Shuler et al. 1995; Tintut and Gralla 1995) implicates helix 13 in core contacts, and the mutation Q80R in σ^{32} (corresponding to amino acid 406 in σ^{70}) decreases core binding, according to a sedimentation assay (Joo et al. 1997).

We show that mutations in region 2.2 decrease Q-activated transcription in vivo and in vitro, stimulate basal transcription from p_R' in vivo, decrease promoter-proximal pausing, and impair open complex formation and stability. Our mutations lie on the solvent-exposed surface of α-helix 13 of region 2.2, which contains amino acids 401–418. We suggest the mutations do not affect promoter binding through direct effect on DNA-σ contacts, but instead act indirectly: The DNA-binding surface of region 2.4 and the region 2.2 residues are on opposite sides of a helix bundle and, thus, are unlikely to both contact promoter DNA. Furthermore, the strongest mutation of our set occurs at the solvent-exposed residue L402, which is nonpolar and, thus, is likely to contact another hydrophobic surface.

We suggest that region 2.2 of σ^{70} makes contacts with the subunits of core RNAP that form and strengthen during the transition from closed to open promoter complex. Conformational changes could be transmitted allosterically to the DNA-binding helix, which is packed against helix 13, thereby explaining the effect of helix 13 mutations on open complex formation and stability (Fig. 7). Region 2.2 may function differently from region 2.1 in core interaction; deletion analysis showed that amino acids downstream of 390, which includes all of region 2.2, are dispensable for core binding, whereas deletions in region 2.1 impair core binding (Lesley and Burgess 1989). Furthermore, the activity of region 2.2 mutants in our screen requires that they still bind core, and initiate efficiently at promoter p_R', to mediate expression that is insensitive to Q function; in addition, mutant σ had to compete effectively against wild-type σ^{70} expressed from the chromosomal gene in the initial mutant screen.

We can estimate roughly how defective the mutant σ^{70} might be in competing with wild-type σ^{70} to form holoenzyme. By Western analysis, we found that the plasmid expresses mutant σs at four- to eightfold excess over the wild-type σ^{70} in SG20250 (λ+49), the strain used for the screen. On the basis of the measurements of

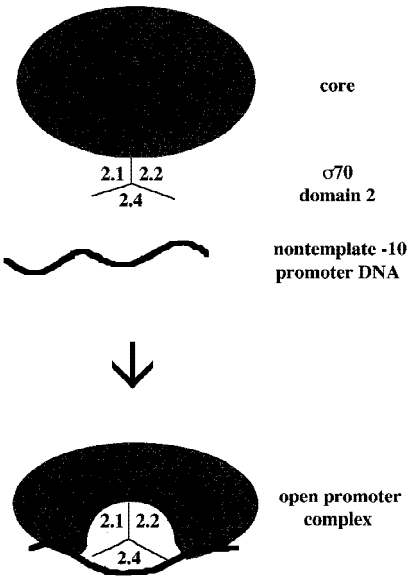

Figure 7. Model of nontemplate –10 region promoter DNA interaction with holoenzyme to form the open promoter complex.

Jishage and Ishihama (1995), core should be present in limiting concentration relative to σ^{70}. Taking the activity of each mutant σ^{70} in supporting Q function in the σ^{70} temperature-sensitive reporter CG19284 (λ+49) as its intrinsic activity in the absence of competitor, we calculate the theoretical Q function when plasmid-expressed mutant competes with the single copy amount of wild-type σ^{70} in SG20250 (λ+49), assuming the mutant has no defect in core binding; and we then calculate the severity of the core-binding defect if the predicted and observed numbers do not match (Table 1). For the most severe mutation, L402F, calculation indicates no core-binding defect. Others show small defects, except for the ~70-fold defect of M413A. We note that the calculated severity of the core-binding defect increases inward along helix 13. Thus, helix 13 appears to have some role in binding free core RNAP in addition to its postulated function in the closed- to open-complex transition.

The notion that binding of the exposed face of helix 13 of region 2.2 into core repositions region 2.4 (and possibly regions of core) to bind single-stranded DNA in the open promoter complex implies significant conformational changes in promoter binding; such changes were predicted by kinetic studies of initiation at the promoters *lacUV5* and pR. Buc and McClure (1985) and Roe et al. (1985) presented evidence for two distinct closed intermediates (I_1 and I_2) of λp_R: $R + P \leftrightarrow I_1 \leftrightarrow I_2 \leftrightarrow RP_o$. Roe et al. (1985) proposed that the first isomerization ($I_1 \leftrightarrow I_2$), which presumably establishes the major contacts that stabilize the open complex, involves a conformational change in RNAP that nucleates DNA melting. Furthermore, they proposed that energetics of this step are dominated by burial of a hydrophobic surface, on the basis of a large and negative heat capacity of the reaction, its lack of salt dependence, and its relative insensitivity to DNA supercoiling. We suggest that L402 is a major component of this hydrophobic surface, and that because of their similar phenotypes, the other mutated amino acids, Q406, N409, and M413, are also buried during the transition from I_1 to I_2. It is consistent with this assignment that the $I_1 \leftrightarrow I_2$ transition is rate limiting for both the association and dissociation of open complex (Roe et al. 1985), and that the L402F and N409D mutations affect both steps. It is further consistent that formation of open complex by L402F and N409D mutant $E\sigma^{70}$ is stimulated to wild-type levels (although not to wild-type rates) by high glycerol concentration (D. Ko and J.W. Roberts, unpubl.), which, Roe et al. (1985) suggest, acts to stabilize the conformational change in RNAP.

Materials and methods

Strains and plasmids

The reporter strains were constructed as single-copy lysogens of λ+49, a derivative of λRS45 that contains an operon fusion of $\lambda p_R'$ and two transcription terminators preceding *lacZ*, as described by Simons and Kleckner (1987) (J. Guo and J.W. Roberts, unpubl.). Bacterial strains were SG20250 (MC4100, *F⁻araD139* Δ (*lacIPOZY*)*U169 strA thi*), from S. Gottesman (NCI, NIH, Bethesda, MD); CAG19284 [*rpoD285 arg lac thi recA argG75 lacZ49(F'laci^Q)*], carrying a temperature-sensitive lethal mutation in *rpoD*, from A. Dombroski; CAG20176 [*araD139Δara–leu)7696 galE15 galK16 ΔlacX74 rpsL hsdR2(r⁻m⁻) mcrA mcrB::Δcm → trp rpoD:Tn(tet)*], a strain in which the endogenous copy of *rpoD* can be repressed by tryptophan, from C. Gross (UCSF School of Medicine); and MTM9973, a *recA⁻*-derivative of CAG20176. The Q-source plasmid pJG100 contains λ gene *Q* controlled by the lactose operator, and one copy of *lacI^q* (J. Guo and J.W. Roberts, unpubl.). Plasmid pHTσ contains *rpoD* transcribed from the *lac* promoter, and was obtained from M. Kainz (University of Wisconsin, Madison). Plasmid pBAD33*spc rpoD* is a derivative of pBAD33 (Guzman et al. 1995) carrying spectinomycin resistance and *rpoD* (M.T. Marr and J.W. Roberts, unpubl.). Plasmid M650, the template for PCR reaction to make DNA for in vitro synthesis, contains promoter p_R' fused to terminators t_c and t_o (McDowell et al. 1994) (M. Marr and J.W. Roberts, unpubl.). λc17 phage was from laboratory stock.

PCR mutagenesis and screening

rpoD was mutagenized in two segments, according to Zhou et al. (1991). The segment from –19 to +847 was amplified in six reactions, which were pooled and cloned into the *Xba*I and *Sph*I sites of pHTσ. The segment from +720 to +1908 was amplified in five reactions, which were pooled and cloned into the *Sph*I and *Hin*dIII sites of pHTσ. Ligation mixes were electroporated into the reporter strain SG20250 (λ+49) (pJG100), and grown on

Table 1. *Estimation of RNAP core-binding defect of σ^{70} mutants*

	Q activity				
	mutant alone (%)	mutant plus wild type σ^{70} (%)	theoretical, no binding defect (%)	Calculated core binding (% of WT)	Calculated binding defect
L402F	14	30	28	88	1.1
L402A	108	101	107	107	—
Q406A	28	49	40	49	2.1
N409D	23	60	36	22	4.6
N409A	8	56	23	18	5.5
M413T	98	89	98	—	—
M413A	19	95	32	1.3	76

Theoretical Q activity, assuming no core binding defect, was calculated by assuming that 1/6 of Q activity was provided by wild-type (WT) σ^{70} and 5/6 by mutant σ^{70}, as implied by the measured excess of mutant over wild type (four- to eightfold) due to expression of mutant σ^{70} from the plasmid. The core-binding ability of each mutant then was calculated as proportional to the fraction of mutant σ^{70} present that would have to be functional to account for the measured Q activity.

MacConkey plates supplemented with ampicillin (100 μg/ml) and spectinomycin (70 μg/ml). Expression of wild-type σ^{70} from pHTσ gives red color, indicating Q function; putative mutants were picked as white or pink, verified by retransformation, and characterized by β-gal assay. The plasmid was also transformed into CAG19284(λ+49) to measure the effect of the mutations without active endogenous wild-type σ^{70}. Basal expression in the absence of Q was measured in the same strain lacking pJG100. More than half of the original plasmids had multiple mutations, but fragments containing a single mutation to which the phenotype could be ascribed were isolated and recloned. In addition to the viable mutants described here, our screen also yielded σ^{70} mutants that are lethal as haploids, including sites in DNA recognition regions 2.4 and 4.2 (D. Ko and J.W. Roberts, unpubl.); analysis of these may reveal other functions of σ^{70} in Q activity.

Cell growth and β-galactosidase assay

Overnight cultures were diluted 1:50 into LB medium supplemented with the appropriate antibiotics and 1 mM IPTG (to induce Q from pJG100), or (for MTM9973) 100 μg/ml tryptophan to repress expression of the chromosomal *rpoD* and 2 mg/ml arabinose to induce *rpoD* from the plasmid. For growth of CAG19284 at restrictive temperature, overnight cultures were grown at 32°C and growth after dilution was at 42°C. β-Galactosidase activity was measured at an OD_{600} of 0.5 in duplicate samples from at least two different cultures; the mean and standard deviation are given.

Site-directed mutagenesis

Alanine substitutions were introduced by PCR overlap mutagenesis (Ring et al. 1996) and verified by sequencing.

Proteins and DNA

Mutations in *rpoD* were subcloned from pHTσ into a pET28-based σ^{70} expression vector. Wild-type and mutant 6-His-tagged σ^{70} were purified as described (Marr and Roberts 1997). RNAP holoenzyme was purified as described (Hager et al. 1990); Bio-Rex 70 was used to remove σ as described (Burgess and Jendrisak 1975). RNAP was reconstituted by incubating wild-type or mutant σ^{70} at a 25-fold molar excess over core for 30 min at room temperature (23°C). λ Q protein and NusA protein were purified as described (Yarnell and Roberts 1992). DNA template for in vitro transcription and band-shift analysis of open promoter complexes was synthesized by PCR as described (Ring et al. 1996). The 292-bp DNA used for band-shift contained λ promoter p_R' located such that the transcription start point was at 142 bp. The 459-bp DNA used for in vitro transcription to measure Q activity (Fig. 3) contained p_R' located such that the transcription start point was at 88 bp and the release site of terminator t_C at 271 bp, giving a terminated RNA of 184 nucleotides and a readthrough RNA of 372 nucleotides. The template DNA used to measure pausing (Fig. 4) gave a runoff RNA of 147 nucleotides and had no terminators.

In vitro transcription

Reactions containing 2 nM template, 10 nM reconstituted holoenzyme, and 100 nM NusA were preincubated at 37°C for 5 min in 20 mM Tris-Cl (pH 8.0), 0.1 mM EDTA, 1 mM DTT, 50 mM KCl, 200 μM ATP, GTP, and CTP, 50 μM UTP, 0.3 μC/μl [α-^{32}P]UTP, and 10%–25% glycerol; when antitermination was measured, λQ was then added at up to 100 nM. Synthesis reactions for experiments of Figures 6 and 7 contained high (23%–25%) glycerol, which equalizes total synthesis between mutant and wild-type enzymes. Synthesis was started by the addition of 2.5 μl of 40 mM MgCl and 100 μg/μl rifampicin to a final reaction volume of 25 μl. Synthesis was stopped by the addition of 125 μl of 0.6 MTris (pH 8.0), 12 mM EDTA, and 80 μg/ml tRNA. Transcription reactions were extracted with 150 μl of phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with 2.5 volumes of 100% EtOH. Samples were resuspended in 4 μl of 80% formamide, 1× TBE, and 0.05% bromophenol blue, and transcripts were resolved in 12% polyacrylamide gels containing 8 M urea. Bands were visualized and quantified by PhosphorImager (Storm 840) and ImageQuant software.

KMnO4 reactivity in vivo

Reactivity was measured in strain MTM9973 carrying pBAD33*spc rpoD*$^+$ or pBAD33*spc rpoD*^{L402F}, and pXY306 or pXY306(+6C), as described (Kainz and Roberts 1992), except that growth was in LB supplemented with 0.2% arabinose and 100 μg/ml tryptophan; $KMnO_4$ was used at 30 mM; reactions were quenched with 100 mM Tris (pH 8.0), 100 mM EDTA, 150 mM β-mercaptoethanol, and 100 mM NaCl; and modified pyrimidines were detected by 15 cycles of linear PCR with end-labeled oligonucleotide.

Assay of open complex by native gel electrophoresis

The formation and dissociation of open complex were measured essentially according to Zhang et al. (1996) by use of PCR-synthesized DNA end-labeled with T4 polynucleotide kinase. For measurement of dissociation, reaction mixtures containing 20 mM Tris HCl (pH 8.0), 0.1 mM EDTA, 1 mM DTT, 50 mM KCl, 4 mM $MgCl_2$, 100 μg/ml BSA, 0.1 nM ^{32}P-labeled DNA, and 20 nM reconstituted RNAP were incubated for 10 min at 37°C. Heparin was added to 100 μg/ml and reactions were shifted to 17°C; 5-μl aliquots were removed over time and loaded onto a 5% polyacrylamide/1× TBE gel running at 100 V at room temperature (23°C). After 2.5 hr, the gel was dried and bands visualized and quantified by PhosphorImager. To measure formation of open complex, reaction mixtures constructed as above were incubated at 37°C; 5-μl samples were taken at indicated times and added to 1-μl of heparin to give a final concentration of 100 μg/ml. After 45 sec at room temperature, the entire 6 μl was loaded onto a gel and analyzed as described above.

Acknowledgments

We thank C. Gross and members of the laboratory for criticizing the manuscript; C. Gross, A. Dombrowski, S. Gottesman, and M. Kainz for bacterial strains and plasmids; S. Darst for providing coordinates of the σ^{70} atomic structure; and the National Institutes of Health for support. D.K. thanks the Cornell Hughes Scholars Program for support.

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Genes Dev. 1998, **12:**
Access the most recent version at doi:10.1101/gad.12.20.3276