The EMBO Journal (2007) 26, 1579–1590 | © 2007 European Molecular Biology Organization | All Rights Reserved 0261-4189/07
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THE EMBO JOURNAL

A σ-core interaction of the RNA polymerase holoenzyme that enhances promoter escape

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The σ subunit of bacterial RNA polymerase (RNAP) is required for promoter-specific transcription initiation and can also participate in downstream events. Several functionally important intersubunit interactions between *Escherichia coli* σ^{70} and the core enzyme ($\alpha_2\beta\beta'\omega$) have been defined. These include an interaction between conserved region 2 of σ^{70} (σ_2) and the coiled-coil domain of β′ (β′ coiled-coil) that is required for sequence-specific interaction between σ_2 and the DNA during both promoter open complex formation and σ^{70}-dependent early elongation pausing. Here, we describe a previously uncharacterized interaction between a region of σ^{70} adjacent to σ_2 called the nonconserved region (σ^{70} NCR) and a region in the N-terminal portion of β′ that appears to functionally antagonize the σ_2/β′ coiled-coil interaction. Specifically, we show that the σ^{70} NCR/β′ interaction facilitates promoter escape and hinders early elongation pausing, in contrast to the σ_2/β′ coiled-coil interaction, which has opposite effects. We also demonstrate that removal of the σ^{70} NCR results in a severe growth defect; we suggest that its importance for growth may reflect its role in promoter escape.

The EMBO Journal (2007) **26,** 1579–1590. doi:10.1038/sj.emboj.7601612; Published online 1 March 2007
Subject Categories: chromatin & transcription
Keywords: promoter escape; RNA polymerase; transcription pausing; λQ; σ^{70}

Introduction

Multisubunit DNA-dependent RNA polymerases are structurally conserved throughout all domains of life (Ebright, 2000). The bacterial RNA polymerase (RNAP) core enzyme (subunit composition $\alpha_2\beta\beta'\omega$) is catalytically active for RNA chain elongation during transcription. However, to recognize promoter sequences and initiate transcription, the core enzyme must associate with a σ factor, forming the RNAP holoenzyme (Gross *et al*, 1998). The primary σ factor of *Escherichia coli*, σ^{70}, directs transcription from promoters typically defined by two conserved sequence elements, the −10 and −35 elements that are separated by ∼17 base pairs (bp) (Gross *et al*, 1998). All primary σ factors share four regions of conserved sequence (regions 1–4) that are connected by intervening sequences of variable size (Lonetto *et al*, 1992), and regions 2 and 4 contain DNA-binding domains that recognize the −10 and −35 elements, respectively (Murakami and Darst, 2003). At least two other regions of σ (regions 1.2 and 3.0) can make sequence-dependent contact with auxiliary promoter elements (Bown *et al*, 1997; Feklistov *et al*, 2006; Haugen *et al*, 2006).

The transcription process can be divided into a number of distinct steps (deHaseth *et al*, 1998). First, the RNAP holoenzyme binds to duplex promoter DNA to form the closed RNAP–promoter complex. Next, a series of conformational changes leads to the formation of the initiation-competent open complex in which the DNA is locally melted (between −11 and +1) to expose the transcription start site. RNAP can then initiate transcription, typically synthesizing short abortive RNA products that are repetitively released and resynthesized before RNAP breaks its contacts with the promoter and escapes into productive elongation (Hsu, 2002). During the course of elongation, the transcription complex may encounter pause sites and potential arrest sites at which the nascent RNA remains stably bound to the enzyme (Artsimovitch and Landick, 2000). Finally, upon reaching a termination site, RNAP releases the RNA transcript and dissociates from the DNA.

In the context of the RNAP holoenzyme, σ^{70} forms extensive contacts with the core enzyme that involve each of the four conserved regions of σ^{70} (Sharp *et al*, 1999; Murakami *et al*, 2002; Vassylyev *et al*, 2002), including regions 2 and 4, which contact the coiled-coil domain of the β′ subunit (Arthur and Burgess, 1998; Young *et al*, 2001) and the flexible flap domain of the β subunit (Kuznedelov *et al*, 2002; Geszvain *et al*, 2004; Nickels *et al*, 2005), respectively. Both of these interactions have important implications for the functional properties of the holoenzyme at multiple stages of the transcription cycle. The interaction between σ^{70} region 2 (σ_2) and the β′ coiled-coil, in particular, which is essential for holoenzyme formation, is also required for functional interaction between σ^{70} and the promoter −10 element, allowing σ_2 to make sequence-specific contacts with bases of the nontemplate strand in the context of the open promoter complex (Marr and Roberts, 1997; Young *et al*, 2001).

σ^{70} can also play functional roles during the elongation phase of transcription (Mooney *et al*, 2005). The most well-characterized example involves the regulation of late gene transcription from the bacteriophage λ promoter $P_{R'}$, where σ^{70} mediates an early elongation pause that is essential for the function of the Q antiterminator protein (λQ), a regulator of late gene expression that enables RNAP to read through specific transcription terminators located within the late gene operon (Roberts *et al*, 1998) (Figure 1A). Although λQ induces terminator readthrough as a stable component of the transcription elongation complex (Deighan and Hochschild, 2007), it must first engage the RNAP holoenzyme

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Received: 2 October 2006; accepted: 24 January 2007; published online: 1 March 2007

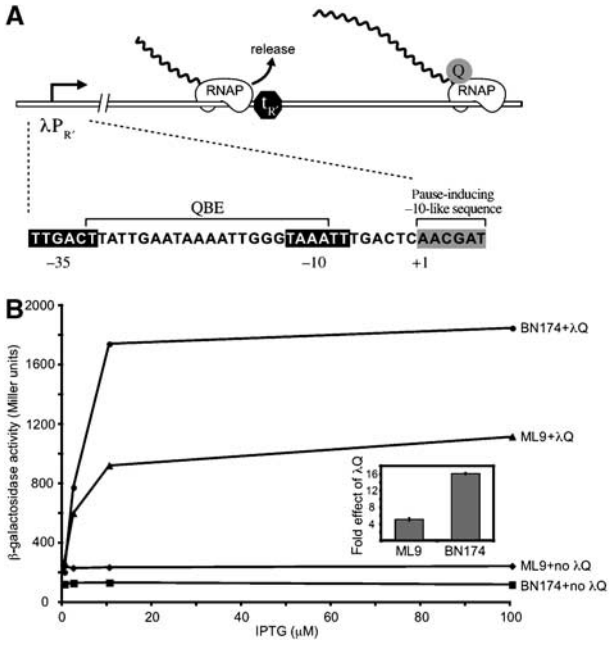

Figure 1 The σ^{70} L402F substitution impairs λQ-mediated antitermination *in vivo*. (**A**) Presence of λQ allows RNAP that has initiated from $P_{R'}$ to read through transcription terminator $t_{R'}$. Blow-up depicts functionally important elements at $P_{R'}$, including the promoter −10 and −35 elements, the QBE, and the pause-inducing −10-like element. (**B**) Effect of substitution L402F in chromosomally encoded σ^{70} on λQ-dependent *lacZ* expression from a $P_{R'}$-*lacZ* reporter *in vivo*. β-Galactosidase assays were performed to quantify *lacZ* expression levels in BN174 (WT) or ML9 (L402F) cells containing either a plasmid that directed the synthesis of λQ under the control of an IPTG-inducible promoter or a control plasmid that did not encode λQ. Note that the basal level of *lacZ* expression is ~2-fold higher in ML9 cells. Inset shows 'fold effect of λQ' values for cells grown in the presence of 100 μM IPTG; these values were determined by dividing the β-galactosidase activity in the presence of λQ by the β-galactosidase activity in the absence of λQ. Shown are the averages of three independent sets of measurements (and standard deviations).

during the early elongation pause to gain access to the elongation complex (Roberts *et al*, 1998). This engagement process depends on two DNA sequence elements, a Q binding element (QBE) that is located between the $P_{R'}$ −10 and −35 elements (Yarnell and Roberts, 1992) and a pause-inducing element that is located in the initial transcribed region (Ring *et al*, 1996) (Figure 1A). The requirements of the engagement process—that DNA-bound λQ interacts with the paused elongation complex—ensure that λQ specifically targets late gene transcription complexes.

The $P_{R'}$ pause-inducing element resembles a promoter −10 element and pausing, which manifests itself in complexes containing nascent RNAs of 16 or 17 nucleotides (nt), is mediated by a protein–DNA interaction between σ_2 and the nontemplate strand of the −10-like element (Ring *et al*, 1996). Moreover, as during open complex formation, establishment of sequence-specific contacts between σ_2 and the −10-like pause element depends on the interaction between σ_2 and the β′ coiled-coil. Thus, σ^{70} substitutions that weaken the σ_2/β' coiled-coil interaction (including σ^{70} substitution L402F) reduce pausing at $P_{R'}$, resulting in impaired λQ antitermination function both *in vitro* and *in vivo* (Ko *et al*, 1998; Sharp *et al*, 1999). These σ^{70} substitutions also destabilize open complexes (Ko *et al*, 1998), increasing promoter escape (Chan and Gross, 2001).

Here, we describe the results of a genetic screen for σ^{70} mutations that suppress the $P_{R'}$ pause defect caused by σ^{70} substitution L402F. Designed to further probe the σ_2/β' coiled-coil interface, this screen unexpectedly uncovered amino-acid substitutions in the nonconserved region (NCR) of σ^{70}, a region located between conserved regions 1.2 and 2.1 that we show to be important for cell growth. The analysis of these substitutions led to the identification of a previously uncharacterized interaction between the σ^{70} NCR and a region of β′ located near its N-terminus. We show that this interaction inhibits σ^{70}-dependent pausing during early elongation and also facilitates promoter escape. In contrast, the σ_2/β' coiled-coil interaction is required for σ^{70}-dependent pausing (Ko *et al*, 1998) and impedes promoter escape (Chan and Gross, 2001). Our results suggest that the interaction between the σ^{70} NCR and the β′ N-terminal region may functionally antagonize the σ_2/β' coiled-coil interaction during specific stages of the transcription cycle. Furthermore, our finding that the σ^{70} NCR/β′ interaction facilitates promoter escape suggests a possible explanation for the important role of the σ^{70} NCR *in vivo*.

Results

Substitutions in the σ^{70} NCR that promote λQ-mediated antitermination

As a strategy to functionally probe the σ^{70}–core interface, we performed a genetic screen to identify second-site substitutions in σ^{70} that restore the ability of RNAP containing σ^{70} L402F ($E\sigma^{70}$ L402F) to engage the promoter-proximal pause site at $\lambda P_{R'}$. We designed this screen by taking advantage of the fact that the pause defect of $E\sigma^{70}$ L402F manifests itself as a defect in λQ-mediated antitermination both *in vitro* and *in vivo* (Ko *et al*, 1998). To assay λQ antitermination function at $P_{R'}$ *in vivo*, we used a $P_{R'}$-*lacZ* fusion, encompassing $P_{R'}$ sequence from −109 to +232, which includes terminator $t_{R'}$ (Figure 1A) (Nickels *et al*, 2002). The level of *lacZ* expression from this construct reports on the ability of plasmid-encoded λQ to function as an antiterminator for transcripts initiating from $P_{R'}$. We introduced the $P_{R'}$-*lacZ* reporter in single copy on an F′ episome into a strain containing the L402F mutation in the *rpoD* gene (encoding σ^{70}) or an otherwise isogenic strain containing wild-type (WT) *rpoD*. Plasmids directing the synthesis of λQ or no λQ under the control of an isopropyl-β-D-thiogalactoside (IPTG)-inducible promoter were introduced into these reporter strains. At maximal induction, λQ increased expression from the test promoter ~16-fold in cells containing σ^{70} WT (strain BN174) and only ~5 fold in cells containing σ^{70} L402F (strain ML9) (Figure 1B). Western blotting revealed similar levels of λQ in the two strains (data not shown).

To identify second-site substitutions in σ^{70} that suppress the effect of the L402F substitution on early elongation pausing at $P_{R'}$, we used error-prone PCR to introduce random mutations into the complete σ^{70} coding sequence present on plasmid pBRσ^{70} L402F, which directs the synthesis of σ^{70} L402F. The resulting expression libraries encoding σ^{70} L402F with other random substitutions were introduced into reporter strain cells bearing the L402F mutation in *rpoD* and already containing λQ (see Materials and methods). Clones

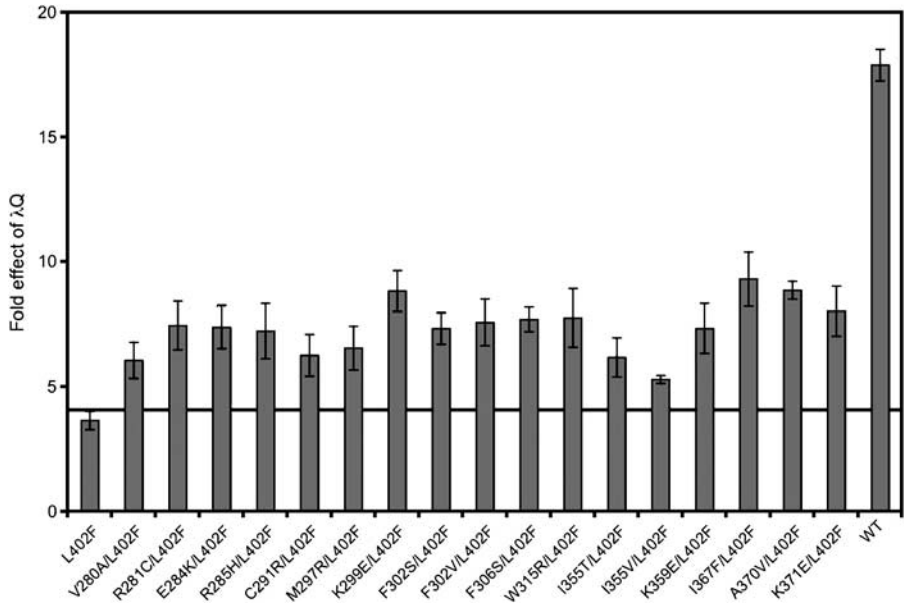

Figure 2 Substitutions in the σ^{70} NCR suppress the defect in λQ-mediated antitermination caused by the L402F substitution in σ^{70}. ML9 cells encoding σ^{70} L402F at the chromosomal *rpoD* locus and harboring the $P_{R'}$-*lacZ* fusion on an F′ episome were cotransformed with compatible plasmids directing the synthesis of the indicated σ^{70} protein and directing the synthesis of either λQ or no λQ. Cells were grown in the presence of 100 μM IPTG and assayed for β-galactosidase activity. 'Fold effect of λQ' values were calculated as described for Figure 1. Shown are the averages of three independent sets of measurements (and standard deviations).

expressing *lacZ* at increased levels were identified on indicator medium and the σ^{70}-encoding plasmids were isolated and their effects on *lacZ* transcription reconfirmed. A total of 17 suppressor substitutions at 15 amino-acid positions were uncovered that increased *lacZ* transcription specifically in the presence of λQ (Figure 2). Western blotting indicated that in general these second-site substitutions did not enhance *lacZ* expression by causing increases in λQ protein levels; the only exception was substitution A370V, which caused the λQ protein level to increase by ~50–100% (data not shown). Surprisingly, the screen did not yield any new substitutions in σ_2, although true revertants and other substitutions were frequently observed at residue 402. Rather, all of the second-site substitutions isolated in the screen mapped to the σ^{70} NCR, a domain located between conserved regions 1.2 and 2.1 that varies in both length and sequence among primary σ factors (Lonetto *et al*, 1992). We located the amino-acid residues affected by the suppressor mutations in a crystal structure of a σ^{70} fragment extending from region 1.2 to 2.4 (Malhotra *et al*, 1996). The substitutions affecting surface-exposed residues (R281C, E284K, R285H, K299E, F306S, K359E, I367F, A370V, and K371E), which define a semicontinuous surface (Figure 3), were selected for further study; we note that five of these nine residues are positively charged.

To confirm that the effects of the σ^{70} substitutions on transcription from $P_{R'}$ are specific to the antitermination function of λQ, we examined $P_{R'}$ transcription *in vitro* in the presence of λQ. After purifying the WT and mutant σ^{70} proteins, we assayed λQ function in the presence of the corresponding reconstituted holoenzymes by performing single-round transcription assays using a $P_{R'}$ template containing terminator $t_{R'}$. When λQ is present, it modifies RNAP so that it can read through the terminator, producing a full-length run-off transcript. Therefore, the percentage of full-length transcripts (% readthrough) is a measure of λQ function. Under our conditions, 33 and 5.5% readthrough

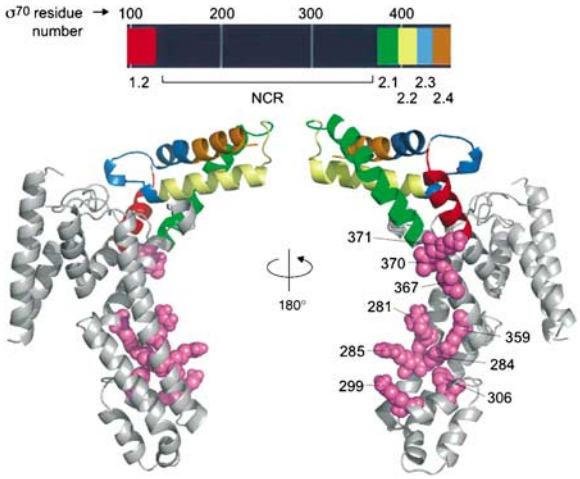

Figure 3 Substitutions in the σ^{70} NCR form a semicontinuous surface. Top: linear representation of the crystallized σ^{70} fragment, with conserved regions color-coded. Bottom: σ^{70} fragment structure, with conserved regions colored as above (adapted from Malhotra *et al*, 1996). The NCR is shown in gray and residues identified by suppressor substitutions are colored violet and labeled.

values were observed for $E\sigma^{70}$ WT and $E\sigma^{70}$ L402F, respectively, in the presence of λQ. We found that eight out of nine of the suppressor substitutions partially restored readthrough (to between 9 and 19%; data not shown) when tested in the context of $E\sigma^{70}$ L402F. The only exception was substitution A370V, which did not increase terminator readthrough above the experimental error; nevertheless, this substitution had effects on other functional properties of RNAP that were similar to those of the other substitutions (see below). None of the substitutions significantly increased the intrinsic ability of RNAP to read through $t_{R'}$ in the absence of λQ (data not shown).

Substitutions in the σ^{70} NCR enhance promoter-proximal pausing in vitro

To determine whether the substitutions in the σ^{70} NCR, like the L402F substitution, affect λQ antitermination function indirectly by affecting promoter-proximal pausing, we performed *in vitro* transcription time courses under single-round conditions using a λ $P_{R'}$ template, monitoring the RNA content of each reaction at various time points after the initiation of transcription. As observed previously (Grayhack *et al*, 1985; Yarnell and Roberts, 1992; Ko *et al*, 1998), the 16- and 17-nt RNA species appeared early in the time course and decayed over time, whereas the full-length transcript accumulated throughout the time course (Figure 4A, left panel). Furthermore, as expected, the L402F substitution in σ^{70} caused a marked reduction in the 16- and 17-nt pause species (Figure 4A, left panel) (Ko *et al*, 1998).

Compared to $E\sigma^{70}$ L402F, RNAP holoenzymes reconstituted with each of the double mutant σ^{70} proteins produced a relative increase in the amount of 16- and 17-nt RNA species (Figure 4A, middle panel, and Supplementary Figure S1). To quantify the effects of the suppressor substitutions on pausing, we calculated pause capture (i.e. the percentage of transcription complexes that functionally engage the pause element) and pause half-life (Ko *et al*, 1998) (see legend of Figure 4). Under our experimental conditions, we calculated pause capture values of 71 and 5.4% for $E\sigma^{70}$ WT and $E\sigma^{70}$ L402F, respectively. The pause capture values for RNAPs reconstituted with the double mutant σ^{70} proteins ranged from 9% for $E\sigma^{70}$ A370V/L402F to 54% for $E\sigma^{70}$ K299E/L402F (Figure 4B and Supplementary Figure S1). In contrast to the effects on pause capture, we did not detect significant effects of the suppressor substitutions on pause half-life (Figure 4B and Supplementary Figure S1). We conclude, therefore, that the σ^{70} NCR suppressor substitutions enhance λQ antitermination function by increasing the percentage of RNAP holoenzymes that functionally engage the pause element during early elongation at $\lambda P_{R'}$.

The suppressor substitutions also enhanced pausing at $\lambda P_{R'}$ in the context of otherwise WT σ^{70}, but these effects were more subtle (data not shown), presumably because the

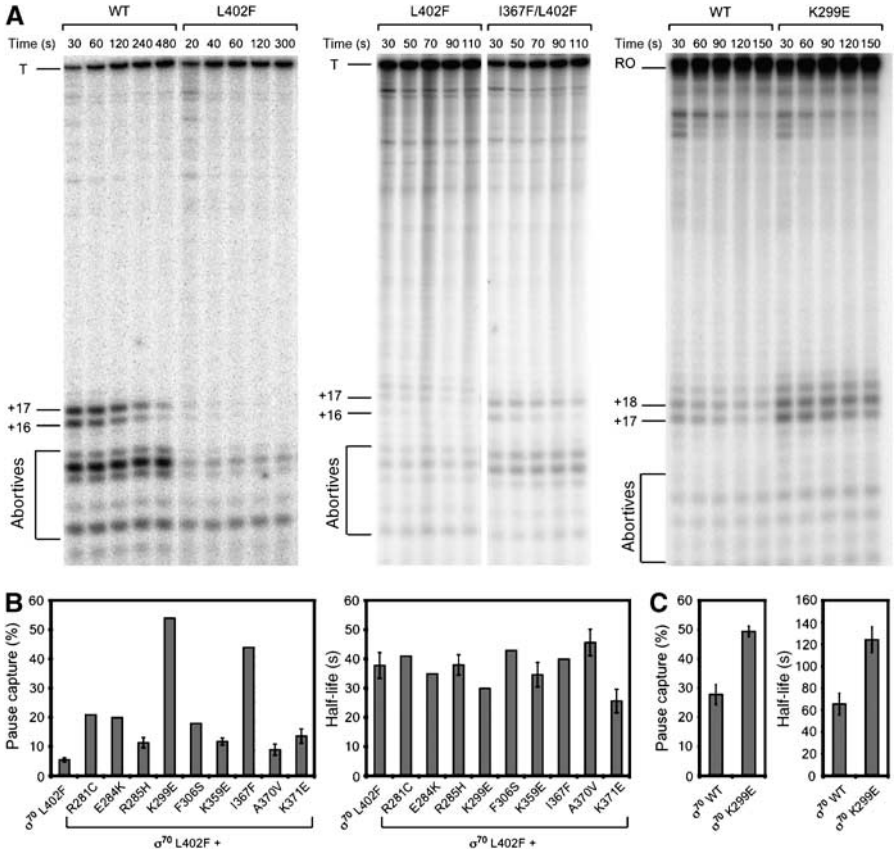

Figure 4 Substitutions in the σ^{70} NCR enhance pausing at $\lambda P_{R'}$ and at p*lac*UV5. (**A**) Single-round *in vitro* transcription time-course assays using a $P_{R'}$ template (left and middle panels) or a p*lac*UV5 template (right panel) and RNAP reconstituted with the indicated σ^{70} proteins. Aliquots of single reactions were removed and stopped at the indicated time points after transcription was initiated. The RNA was labeled internally with [α-^{32}P]UTP (middle and right panels) or end-labeled with [γ-^{32}P]ATP (left panel). +16 and +17, 16- and 17-nt RNA species, respectively, produced from the $\lambda P_{R'}$ template; T, 194-nt terminated transcript produced from the $\lambda P_{R'}$ template; +17 and +18, 17- and 18-nt RNA species, respectively, produced from the p*lac*UV5 template; RO, 96-nt runoff transcript produced from the p*lac*UV5 template. A faint 18-nt RNA species was also observed during time courses with the $\lambda P_{R'}$ template when the RNA was internally labeled. This is likely the result of nucleotide deprivation at U19 under conditions where the UTP concentration was reduced to improve incorporation of [α-^{32}P]UTP, and has been observed previously under similar reaction conditions (Ko *et al*, 1998). (**B**, **C**) Effects of substitutions on pause capture and pause half-life. The percentage of elongation complexes paused (100 (16-nt + 17-nt)/(16-nt + 17-nt + T) or 100 (17-nt + 18-nt)/(17-nt + 18-nt + T)) was approximated at each time point, plotted, and fit to the exponential equation $Y = Y_0 e^{-kt}$ (Supplementary Figure S1). Exponential equations were solved to obtain pause capture (left panels) and half-life (right panels) values for each holoenzyme. Pause capture was approximated by extrapolating the equations to $t = 0$. Error bars represent standard deviations from at least three separate experiments. In cases where error bars are not shown, the mutants were assayed twice, with similar results.

pause capture for $E\sigma^{70}$ WT is already quite high. To determine whether the suppressor substitutions could produce significant effects on σ^{70}-dependent promoter-proximal pausing even in the context of WT σ^{70}, we took advantage of a promoter (p*lac*UV5) that contains a promoter-proximal pause element that functions less efficiently than that of $P_{R'}$ (Nickels *et al*, 2004). We assayed transcription *in vitro* from a p*lac*UV5 template using $E\sigma^{70}$ WT and $E\sigma^{70}$ K299E (Figure 4A, right panel) and found that substitution K299E increased both pause capture (from 28 to 50%) and pause half-life (~2-fold) (Figure 4C and Supplementary Figure S1). In addition, we found that substitution K299E increased utilization of the p*lac* pause element *in vivo* (Supplementary Figure S1). We conclude that the σ^{70} NCR suppressor substitutions enhance σ^{70}-dependent early elongation pausing both in the presence and absence of substitution L402F.

Substitutions in the σ^{70} NCR disrupt an interaction with the β′ subunit

Because the L402F substitution specifically weakens the interaction between σ_2 and the β′ coiled-coil, we sought to determine whether the substitutions in the σ^{70} NCR restore early elongation pausing by strengthening the σ_2/β′ coiled-coil interaction. To do this, we employed a transcription-based bacterial two-hybrid assay (Dove *et al*, 1997; Dove and Hochschild, 2004). In this assay, interaction between a protein domain X fused to a subunit of RNAP and a partner domain Y fused to a DNA-binding protein activates transcription from a test promoter bearing a recognition site for the DNA-binding protein in the upstream region. This two-hybrid assay enabled us to detect an interaction between σ^{70} residues 94–448 (specifying the structurally characterized σ^{70} fragment comprising region 1.2, the NCR, and region 2) (Malhotra *et al*, 1996) and β′ residues 262–309 (specifying the coiled coil) (Young *et al*, 2001). Specifically, the σ^{70} fragment was fused to the CI protein of bacteriophage λ (λCI) and the β′ coiled-coil fragment was fused to the N-terminal domain of the RNAP α subunit in place of its C-terminal domain (Figure 5A). Introduction of plasmids encoding these two fusion proteins into strain FW102 $F'O_L2$–62, which bears test promoter p*lac*O_L2–62 linked to a *lacZ* reporter gene, results in the activation of *lacZ* transcription (up to ~13-fold) (Figure 5B). As expected, introduction of the L402F substitution into the σ^{70} moiety of the λCI-σ^{70} fusion protein weakened this interaction substantially (Figure 5C). However, the interaction was neither weakened nor significantly strengthened by any of the suppressor substitutions (which were individually introduced into the otherwise WT σ^{70} moiety) (Figure 5C).

Using the two-hybrid assay, we also detected an interaction between the same σ^{70} (94–448) fragment and a more N-terminal region of β′, present within a fragment extending from residue 55 to 261 (Figure 5D). In contrast to the σ^{70}/β′ coiled-coil interaction, this second interaction was unaffected by the σ^{70} L402F substitution, but was disrupted by all but one (A370V) of the suppressor substitutions (Figure 5E). That the substitutions in the σ^{70} NCR did not affect the σ^{70}/β′ coiled-coil interaction suggests that their disruptive effects on the σ^{70}/β′ 55–261 interaction are specific and not the result of defects in the synthesis, stability, or folding of the mutant λCI-σ^{70} fusion proteins.

We conclude from the results of these two-hybrid assays that the substitutions in the σ^{70} NCR isolated in our genetic screen specifically weaken an interaction with a region of β′ contained within the 55–261 fragment that we call the sigma NCR interaction domain (S_{NCR}ID). Furthermore, our results suggest that, in contrast to the interaction between σ_2 and the β′ coiled-coil, the interaction between the σ^{70} NCR and the β′ S_{NCR}ID inhibits early elongation pausing (because substitutions that disrupt the interaction enhance pausing). Consistent with this idea, we found that strengthening the σ^{70} NCR/β′ S_{NCR}ID interaction decreased early elongation pausing (Supplementary Figure S2).

The results of a previous study revealed that the σ^{70} NCR is not required for transcription *in vitro*, at least from some promoters, but raised the possibility that it is essential for viability (Kumar *et al*, 1995). We sought to revisit this issue by replacing the σ^{70} NCR with a seven-residue linker that connects conserved regions 1.2 and 2.1 in the stationary phase σ factor σ^{38} (see Supplementary Results). We found that although this chimeric σ factor was stably produced, cells containing the chimera in the absence of WT σ^{70} were severely compromised for growth (Supplementary Figure S3).

Substitutions in the β′ subunit that enhance early elongation pausing

The hypothesis that an interaction between the σ^{70} NCR and the β′ S_{NCR}ID inhibits σ^{70}-dependent early elongation pausing suggests that it should be possible to isolate substitutions on the interacting surface of β′ that disrupt its interaction with the σ^{70} NCR and enhance early elongation pausing. To identify such substitutions, we screened for β′ mutants that enhanced λQ-mediated antitermination in cells containing the L402F mutation in *rpoD*. After random PCR mutagenesis of a fragment encoding β′ residues 1–258 (present on plasmid pBRβ′), we identified eight substitutions at five amino-acid positions (E148, E162, E170, E171, and E175—all negatively charged residues) that suppressed the defect in λQ-mediated antitermination caused by σ^{70} substitution L402F. At maximal induction, λQ stimulated *lacZ* transcription from the λ$P_{R'}$-*lacZ* test promoter 8.2- to 9.7-fold in ML9 (*rpoD* L402F) cells directing the synthesis of the mutant β′ proteins from pBRβ′, compared with only 4.8-fold in ML9 cells directing the synthesis of WT β′ from pBRβ′ (Figure 6A).

To determine whether these β′ substitutions affect the interaction between the β′ S_{NCR}ID and the σ^{70} NCR, we again used the two-hybrid assay. After introducing mutations encoding substitutions E148K, E162K, E170K, E171K, and E175K into plasmid pBRα-β′ 55–261, we transformed FW102 $F'O_L2$–62 cells with pACλCI-σ^{70} 94–448 together with pBRα-β′ 55–261 and the mutant variants. Each of the β′ substitutions reduced or nearly abolished the stimulatory effect of the λCI-σ^{70} fusion protein on *lacZ* transcription (Figure 6B). Western blotting confirmed that these substitutions did not affect α–β′ 55–261 protein levels (data not shown). Thus, these β′ substitutions, like those on the putative partner surface of the σ^{70} NCR, weaken the σ^{70} NCR/β′ S_{NCR}ID interaction and enhance λQ function *in vivo*.

To confirm that the effects of the β′ substitutions on transcription from $P_{R'}$, like those of the σ^{70} NCR substitutions, are specific to the antitermination function of λQ, we examined transcription from $P_{R'}$ *in vitro* in the presence of λQ. RNAP core enzymes containing β′-His_6(WT),

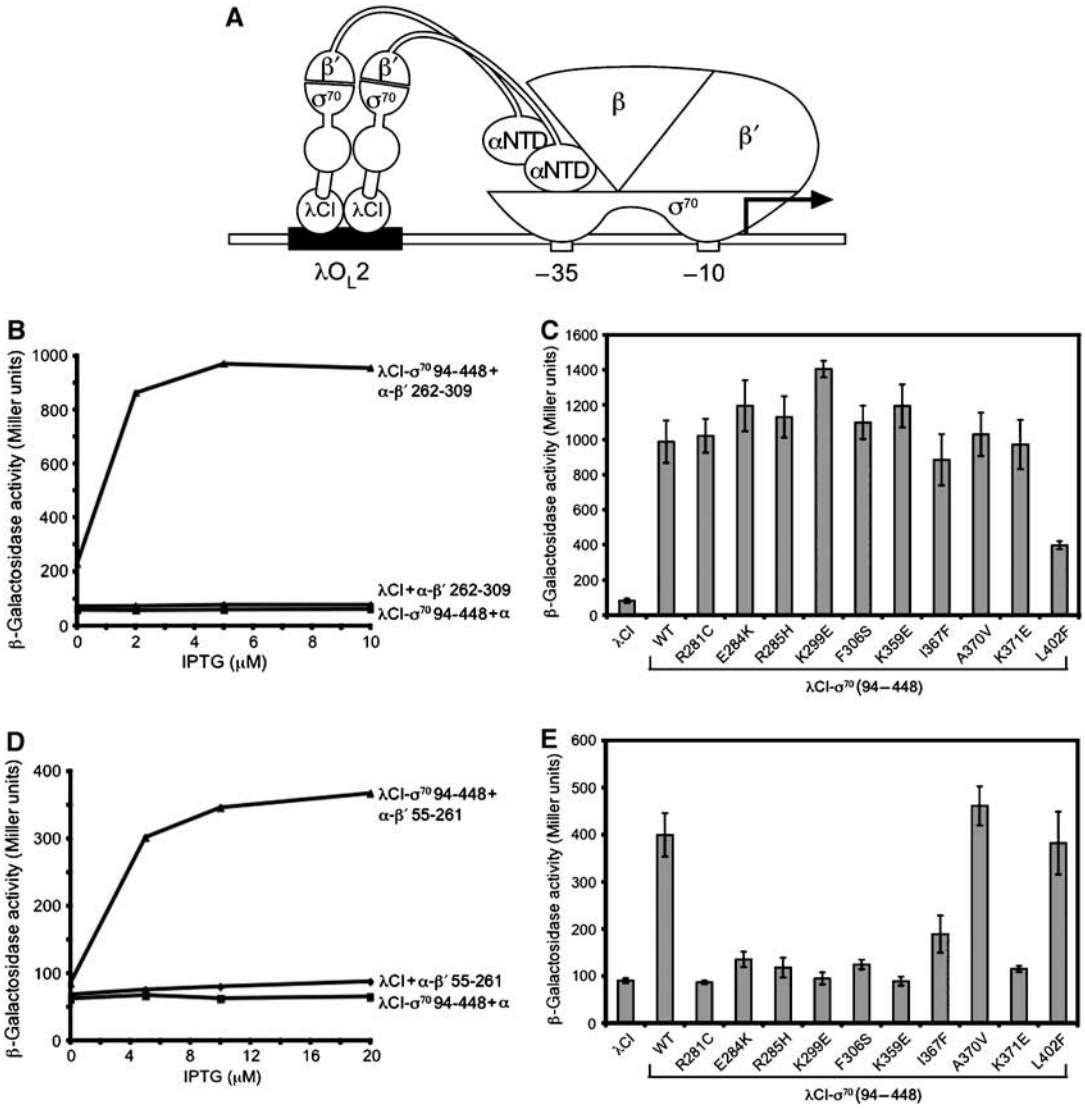

Figure 5 Substitutions in the σ^{70} NCR specifically disrupt an interaction with β′ 55–261. (**A**) Schematic of bacterial two-hybrid assay used to detect protein–protein interactions between fragments of σ^{70} and β′. (**B**) λCI-σ^{70} 94–448 activates transcription from the test promoter in cells containing the α–β′ 262–309 fusion protein. Strain FW102 F′O_L2–62 cells containing compatible plasmids directing the synthesis of the indicated proteins were grown in the presence of the indicated concentrations of IPTG and assayed for β-galactosidase activity. (**C**) Effects of substitutions in the σ^{70} NCR on the interaction between the σ moiety of the λCI-σ^{70} 94–448 fusion protein and the β′ moiety of the α–β′ 262–309 fusion protein. Cells were grown in the presence of 10 μM IPTG and assayed for β-galactosidase activity. (**D**) λCI-σ^{70} 94–448 activates transcription from the test promoter in cells containing the α–β′ 55–261 fusion protein. Strain FW102 F′O_L2–62 cells containing compatible plasmids directing the synthesis of the indicated proteins were grown in the presence of the indicated concentrations of IPTG and assayed for β-galactosidase activity. (**E**) Effects of substitutions in the σ^{70} NCR on the interaction between the σ moiety of the λCI-σ^{70} 94–448 fusion protein and the β′ moiety of the α–β′ 55–261 fusion protein. Cells were grown in the presence of 20 μM IPTG and assayed for β-galactosidase activity. (C, E) Assays were performed three times in duplicate on separate occasions; shown are the average values from all trials with standard deviations.

β′-His_6(E148K), β′-His_6(E171K), and β′-His_6(E175K) were purified (see Materials and methods) and used to reconstitute holoenzymes with σ^{70} L402F. The β′ substitutions E148K, E171K, and E175K increased terminator readthrough in the presence of λQ protein to 13, 10, and 24%, respectively, compared with a 5.5% readthrough for RNAP containing WT β′ (data not shown).

To determine whether the effects of the β′ substitutions on λQ function were caused by enhanced pausing, we performed single-round *in vitro* transcription time-course assays with Eσ^{70} L402F, E[β′ E148K]σ^{70} L402F, and E[β′ E175K]σ^{70} L402F (Figure 7A and Supplementary Figure S1). Both these β′ substitutions increased pause capture significantly, with values of 19 and 71% for E[β′ E148K]σ^{70} L402F and E[β′ E175K]σ^{70} L402F, respectively, compared with 5.4% for Eσ^{70} L402F (Figure 7B). Thus, substitutions in β′ that weaken the σ^{70} NCR/β′ S_{NCR}ID interaction, like those in the σ^{70} NCR, enhance early elongation pausing. These results provide strong support for the hypothesis that the σ^{70} NCR/β′ S_{NCR}ID interaction inhibits early elongation pausing at $\lambda P_{R'}$.

Weakening the interaction between the σ^{70} NCR and the β′ S_{NCR}ID increases abortive RNA synthesis

While examining the effects of the σ^{70} NCR and β′ S_{NCR}ID substitutions on early elongation pausing by means of *in vitro* transcription assays, we noticed that the RNAP holoenzymes

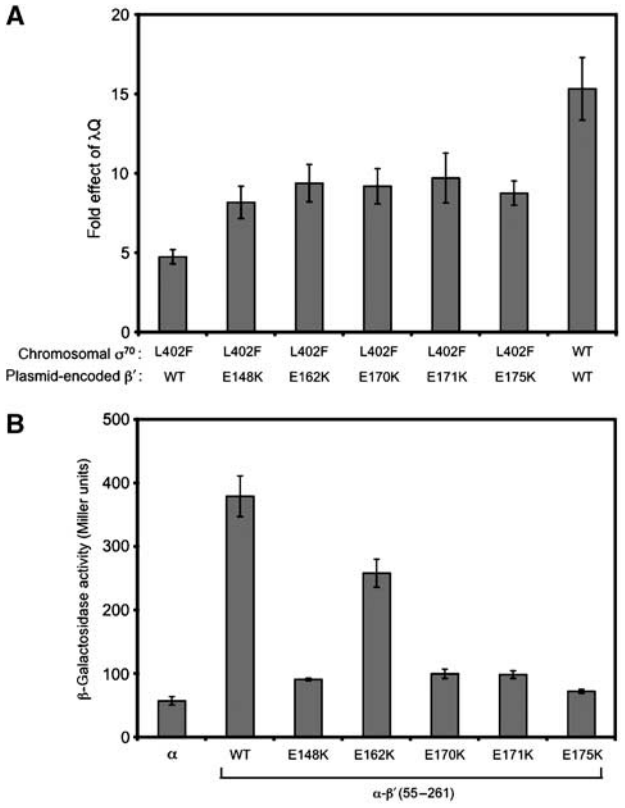

Figure 6 Substitutions in β′ suppress the defect in λQ-mediated antitermination caused by the σ^{70} L402F substitution and weaken the interaction between the β′ S_{NCR}ID and the σ^{70} NCR. (**A**) Cells encoding either σ^{70} L402F or WT σ^{70} at the chromosomal *rpoD* locus and harboring the $P_{R'}$-*lacZ* fusion on an F′ episome were cotransformed with compatible plasmids, one directing the synthesis of the indicated β′ protein (in addition to chromosomally encoded WT β′) and the other directing the synthesis of either λQ or no λQ. Cells were grown in the presence of 100 μM IPTG and assayed for β-galactosidase activity. 'Fold effect of λQ' values were calculated as described for Figure 1. Shown are the averages of three independent sets of measurements (and standard deviations). (**B**) Effects of substitutions in the β′S_{NCR}ID on the interaction between the β′ moiety of the α–β′55–261 fusion protein and the σ moiety of the λCI-σ^{70} 94–448 fusion protein. Strain FW102 F′O_L2–62 cells containing compatible plasmids directing the synthesis of the λCI-σ^{70} fusion protein and either α or the indicated α–β′ fusion protein were grown in the presence of 20 μM IPTG and assayed for β-galactosidase activity. Assays were performed three times in duplicate on separate occasions; shown are the average values from all trials with standard deviations.

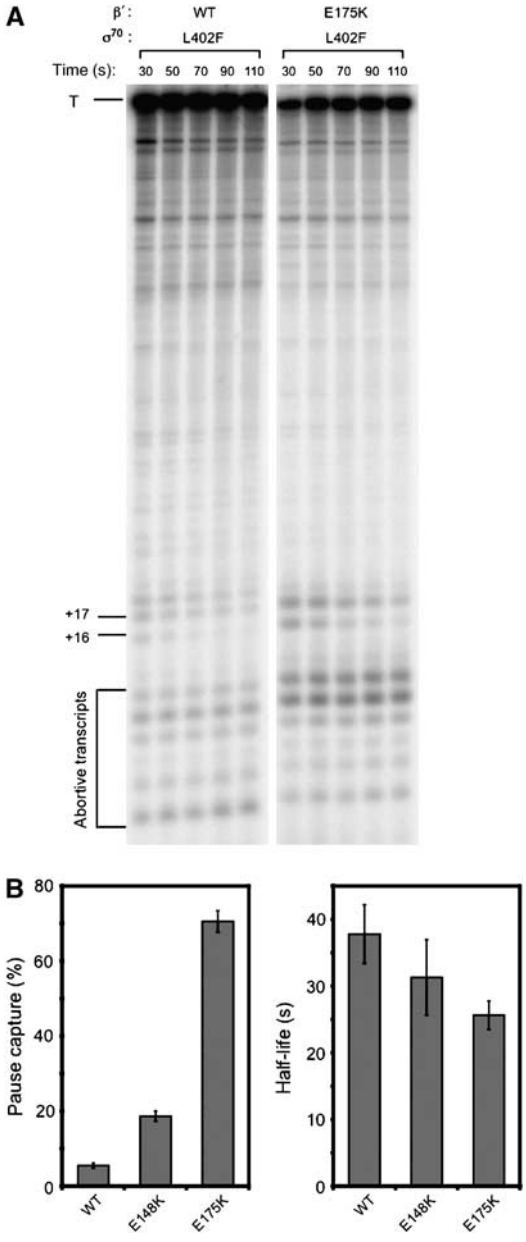

Figure 7 Substitutions in the β′ S_{NCR}ID enhance pausing at $\lambda P_{R'}$. (**A**) Single-round *in vitro* transcription time-course assays using a $P_{R'}$ template and RNAP containing the indicated β′ and σ^{70} proteins. +16 and +17, 16- and 17-nt RNA species, respectively, produced from the $\lambda P_{R'}$ template; T, 194-nt terminated transcript produced from the $\lambda P_{R'}$ template. (**B**) Effects of substitutions on pause capture and pause half-life (calculated from plots shown in Supplementary Figure S1). Error bars represent standard deviations from at least three separate experiments. All data were obtained and analyzed as described for Figure 4.

containing σ^{70} L402F with one of the suppressor substitutions in either σ^{70} or β′ synthesized significantly more 11-, 12-, and 13-nt abortive RNAs than Eσ^{70} L402F (Figures 4A and 7A). Note that the synthesis of these abortive transcripts was reduced by substitution L402F, consistent with previous observations with this and other σ^{70} substitutions that weaken the interaction between σ_2 and the β′ coiled-coil (Ko *et al*, 1998; Chan and Gross, 2001). To quantify the effects of the σ^{70} NCR and β′ S_{NCR}ID substitutions on abortive RNA synthesis, we performed single-round *in vitro* transcription reactions under conditions (see Materials and methods) that permitted us to calculate abortive probabilities (defined as the number of transcripts of a given size divided by the total number of transcripts equal or greater in size) (Hsu, 1996) for the detectable abortive RNA products (8, 9, 11, 12, and 13 nt in length). Figure 8A and B shows that the σ^{70} L402F substitution reduced the abortive probabilities at +11, +12, and +13 (but not at +8 and +9), consistent with the results of previous studies of abortive initiation on the N25 anti promoter (Chan and Gross, 2001). The σ^{70} suppressor substitutions (assayed in the context of σ^{70} L402F) significantly increased the abortive probabilities at +11, +12, and +13, but not at +8 and +9 (Figure 8A and B). Similarly, the β′ suppressor substitutions (β′ E148K and β′ E175K) increased the abortive probabilities at +11, +12, and +13 without affecting the abortive probabilities at +8 and +9 (Figure 8C and D). Therefore, we conclude that

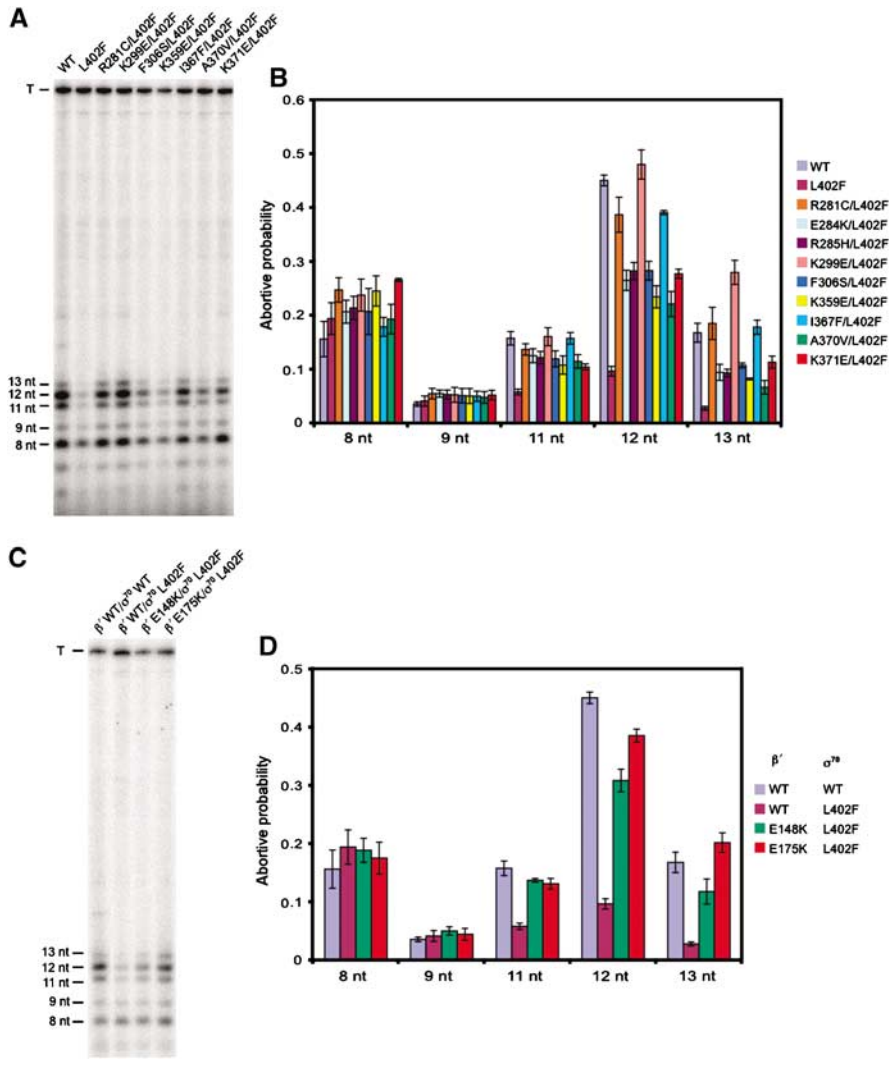

Figure 8 Substitutions that weaken the interaction between the σ^{70} NCR and β′ S_{NCR}ID increase abortive transcript synthesis. (**A, C**) Single-round *in vitro* transcription assays using a $P_{R'}$ template and RNAP holoenzymes containing the indicated σ^{70} and β′ ((C) only) proteins. All RNAP holoenzymes in (A) contain wild-type β′. Shown are the 8-, 9-, 11-, 12-, and 13-nt abortive transcripts and the 194-nt terminated transcript (T) produced from the $P_{R'}$ template. (**B, D**) Effects of σ^{70} and β′ substitutions on abortive probabilities at +8, +9, +11, +12, and +13. Abortive probability values, which describe the probability that RNAP will release an abortive RNA rather than extend at least one more nucleotide, were calculated for each position by dividing the number of moles of each RNA species by the total number of moles of all RNA species of equal or greater size (e.g. 11-nt/(11-nt + 12-nt + 13-nt + T)). Error bars represent standard deviations from at least three separate experiments.

weakening the interaction between the σ^{70} NCR and β′ S_{NCR}ID increases the likelihood that an initial transcribing complex will abort and release 11-, 12-, or 13-nt transcripts rather than escape into productive elongation. Conversely, we found that strengthening the σ^{70} NCR/β′ S_{NCR}ID interaction resulted in decreased abortive probabilities (Supplementary Figure S2D). The σ^{70} NCR/β′ S_{NCR}ID interaction thus functionally counteracts the σ_2/β′ coiled-coil interaction, disruption of which facilitates promoter escape (Chan and Gross, 2001).

Weakening the interaction between the σ^{70} NCR and the β′ S_{NCR}ID does not specifically affect open complex stability

As well as decreasing abortive transcript synthesis, the L402F substitution destabilizes open complexes (Ko *et al*, 1998), suggesting a relationship between open complex stability and abortive yield (Roberts and Roberts, 1996; Hsu, 2002). To address the possibility that disrupting the σ^{70} NCR/β′ S_{NCR}ID interaction inhibits promoter escape (increases abortive yields) by increasing open complex stability, we measured the kinetics of open complex dissociation for RNAP reconstituted with WT σ^{70}, σ^{70} L402F, and the doubly substituted σ^{70} proteins (see Supplementary Results). We observed no correlation (positive or negative) between the effects of our σ^{70} NCR substitutions on open complex stability, on the one hand, and abortive yields, on the other (Supplementary Figure S4). We therefore conclude that the interaction between the σ^{70} NCR and the β′ S_{NCR}ID affects abortive transcript synthesis through a mechanism that does not involve open complex stability.

Discussion

Through a genetic screen for σ^{70} mutations that affect σ^{70}-dependent promoter-proximal pausing, we have identified

a previously uncharacterized interaction between the σ^{70} NCR and an N-terminal domain of β′ that we call the β′ S_{NCR}ID. Our genetic data suggest that this interaction is mediated in part by positively charged residues in the σ^{70} NCR and negatively charged residues in the β′ S_{NCR}ID, and mutant-suppressor analysis identified oppositely charged residues that likely approach one another closely at the σ^{70} NCR/β′ S_{NCR}ID interface (Supplementary Figure S5). Interaction between the σ^{70} NCR and the β′ S_{NCR}ID is consistent with FRET-based structural models of the *E. coli* RNAP holoenzyme and open complex (Mekler *et al*, 2002). We found that the σ^{70} NCR/β′ S_{NCR}ID interaction functions to facilitate promoter escape and inhibit σ^{70}-dependent promoter-proximal pausing, as substitutions that weakened the interaction increased abortive transcript synthesis, and also increased pausing, whereas substitutions that strengthened the interaction had the opposite effects. The interaction between the σ^{70} NCR and the β′ S_{NCR}ID functionally counteracts the interaction between σ_2 and the β′ coiled-coil in that the latter inhibits promoter escape (Chan and Gross, 2001) and promotes pausing (Ko *et al*, 1998).

Mechanistic significance of σ^{70} NCR/β′ S_{NCR}ID interaction

The correlation between the promoter escape and pausing phenotypes caused by altering the strength of the σ^{70} NCR/β′ S_{NCR}ID interaction underscores the relationship between these two processes. During both transcription initiation and early elongation pausing, σ_2 is bound to the nontemplate strand of a −10 (or −10-like) element (Ring *et al*, 1996; Marr and Roberts, 1997; Young *et al*, 2001). According to current models, the abortive phase of transcription involves the synthesis and release of short RNA products while RNAP maintains its contacts with the promoter (with the −10 element, in particular) (Roberts and Roberts, 1996; Hsu, 2002). For this to occur—that is, for transcript elongation to occur without forward translocation of the enzyme with respect to the core promoter elements—it has been proposed that excess template DNA must transiently be accommodated in the main channel of the enzyme in a process referred to as DNA 'scrunching', a proposal that has recently received direct experimental support (Kapanidis *et al*, 2006; Revyakin *et al*, 2006). The strain that accumulates during this DNA scrunching process can be relieved either by the release of an abortive RNA product or by breakage of the promoter contacts (i.e. promoter escape).

Events similar to those that occur during abortive initiation are thought to occur during early elongation pausing at $P_{R'}$ (Marr and Roberts, 2000). Specifically, the location of the −10-like pause element (12 bp downstream from the $P_{R'}$ −10 element) suggests that σ_2 initially engages the −10-like element when the nascent transcript is ∼12 nt in length. The nascent RNA is then extended while σ_2 remains bound to the −10-like element (requiring DNA scrunching) to produce either a 16- or 17-nt pause product. At this point, the interaction between σ_2 and the pause element evidently hinders further extension and the accumulated strain can be relieved in one of two ways. Either the contacts between σ_2 and the −10-like element are broken and pause capture does not occur, or RNAP enters a backtracked state in which the catalytic center of the enzyme slides back relative to the 3′ end of the nascent RNA transcript (returning to the +12 position; Marr and Roberts, 2000) and pause capture occurs. For RNAP to escape the pause, transcript cleavage (or forward translocation) must occur to regenerate a 3′ end (or reposition the existing 3′ end) at the enzyme's catalytic center (Marr and Roberts, 2000). According to this model, the events that cause RNAP to release longer abortive RNA products during the early stages of transcription are analogous to events that cause RNAP to pause during early elongation.

The analogy between the events that occur during the abortive phase of transcription and the events that occur during early elongation pausing suggests a possible explanation for the effect of the σ^{70} NCR/β′ S_{NCR}ID interaction on the two processes. In particular, we propose that the σ^{70} NCR/β′ S_{NCR}ID interaction might destabilize the interaction of σ_2 with nontemplate strand DNA, specifically under conditions of DNA scrunching, thus facilitating promoter escape and hindering pause capture. In support of this proposal is our finding that disrupting the σ^{70} NCR/β′ S_{NCR}ID interaction did not have a specific effect on open complex half-life; an increase in open complex half-life would have been expected if the σ^{70} NCR/β′ S_{NCR}ID interaction functioned to destabilize the σ_2/nontemplate strand interaction under all conditions.

Function of the σ NCR in other bacteria

Although not strictly required for transcription *in vitro* (Kumar *et al*, 1995), we found that removal of the σ^{70} NCR severely compromised cell growth (see also Kumar *et al*, 1995), suggesting that it plays an important role in transcription from at least some promoters. Moreover, the σ^{70} NCR (despite its name) resembles the nonconserved regions of primary σ factors from other Gram-negative proteobacteria including *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Haemophilus influenzae*, and *Caulobacter crescentus*. Interestingly, the majority of substitutions that we isolated in our genetic screen affect residue positions that are among the most conserved within the family of σ^{70}-type NCRs. For example, while the NCRs of the primary σ factors in *P. aeruginosa* and *V. cholerae* are only 37 and 56% identical, respectively, to the *E. coli* σ^{70} NCR (residues 127–373), the residues at 13 of the 15 positions identified in our screen were conserved across all three species. We suggest, therefore, that the σ^{70} NCR/β′ S_{NCR}ID interaction is likely to be conserved as well.

Although high-resolution structures are available for RNAP holoenzymes from *Thermus aquaticus* (*Taq*) and *Thermus thermophilus* (Murakami *et al*, 2002; Vassylyev *et al*, 2002), they are not informative with regard to the *E. coli* σ^{70} NCR/β′ S_{NCR}ID interaction because the corresponding regions of *Thermus* σ^{A} and β′ are greatly diverged from their counterparts in *E. coli*. In particular, β′ from the *Thermus* species contains a large (293 residue) inserted domain (the β′ NCD) in the region corresponding to the β′ S_{NCR}ID and conversely, the *Thermus* σ^{A} NCR is significantly smaller than the σ^{70} NCR (72 versus 247 residues) (Iyer *et al*, 2004). Nevertheless, the σ^{A} NCR is positioned to interact with a portion of the β′ NCD in high-resolution structural models of the *Thermus* RNAP holoenzymes (Chlenov *et al*, 2005). Consistent with this structural information, the bacterial two-hybrid assay permits detection of an interaction between the *Taq* σ^{A} NCR and the *Taq* β′ NCD (M Leibman and A Hochschild, unpublished data). Experiments are underway to determine

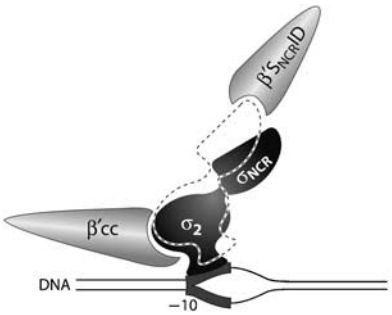

Figure 9 Model depicting opposing σ_2/β′ coiled-coil and σ^{70} NCR/β′ S_{NCR}ID interactions. According to this model, optimal interaction between σ_2 and the β′ coiled-coil (β′cc) promotes interaction between σ_2 and the nontemplate strand of the −10 element (σ domains shown in black). The functionally antagonistic interaction between the σ^{70} NCR and β′ S_{NCR}ID distorts the interaction between σ_2 and the β′cc, facilitating the release of σ_2 from the DNA (σ domains shown as dashed outline).

whether this interaction is functionally analogous to the σ^{70} NCR/β′ S_{NCR}ID interaction in *E. coli*.

Functional antagonism between the σ_2/β′ coiled-coil and the σ^{70} NCR/β′ S_{NCR}ID interactions

The σ_2/β′ coiled-coil interaction and the σ^{70} NCR/β′ S_{NCR}ID interaction have opposing effects on both promoter escape and early elongation pausing, raising the question of how this facilitates the transcription process. Previous work has demonstrated that the σ_2/β′ coiled-coil interaction is critical during promoter open complex formation, being required for promoter melting and contributing importantly to open complex stability (Young *et al*, 2001, 2004). However, by the same token, the σ_2/β′ coiled-coil interaction, which is required for σ_2 to establish sequence-specific contact with nontemplate strand DNA, limits promoter escape (and also escape from σ^{70}-dependent promoter-proximal pause sites) (Ko *et al*, 1998; Chan and Gross, 2001). Moreover, evidence suggests that the β′ coiled-coil interacts more strongly with region 2 of σ^{70} than with region 2 of σ^{38} (which lacks the NCR) (S Garrity, A Yuan, and A Hochschild, unpublished data). Thus, we speculate that the σ^{70} form of the RNAP holoenzyme may require an opposing interaction to facilitate its escape from the promoter into productive elongation (see Figure 9). Our suggestion that the σ^{70} NCR/β′ S_{NCR}ID interaction functions to destabilize (directly or indirectly) the interaction of σ_2 with nontemplate strand DNA specifically under conditions of DNA scrunching explains how the σ^{70} NCR/β′ S_{NCR}ID interaction might facilitate promoter escape in a manner that does not compromise open complex formation. We speculate further that the σ^{70} NCR/β′ S_{NCR}ID interaction may provide a target for regulatory factors that could modulate either promoter escape or escape from an early elongation pause.

Materials and methods

Expression vectors

Plasmid pBRσ^{70} contains the complete coding sequence of the σ^{70} subunit of *E. coli* RNAP under the control of a weak constitutive synthetic promoter with the sequence TTTACAACATGAAG TAACTTCTCGCATTATGTCTCGA. Plasmid pBRβ′ contains the complete coding sequence of the β′ subunit of *E. coli* RNAP under the control of the same weak constitutive synthetic promoter. Additionally, pBRβ′ contains the following restriction sites introduced as silent mutations into the β′ coding sequence: *Not*I at codons 257–259, *Xho*I at codons 872–873, and *Bam*HI at codons 1182–1183. Plasmid pBRα (Dove *et al*, 1997) encodes the α subunit of *E. coli* RNAP under the control of tandem *lpp* and *lacUV5* promoters. Plasmids pBRα-β′ 55–261 and pBRα-β′ 262–309 are pBRα derivatives that encode residues 1–248 of the α subunit of *E. coli* RNAP fused to residues 55–261 and 262–309, respectively, of the β′ subunit of *E. coli* RNAP under the control of tandem *lpp* and *lacUV5* promoters. Plasmid pACλCI (Dove *et al*, 1997) encodes λCI under the control of the *lacUV5* promoter. Plasmid pACλCI-σ^{70} 94–448 is a pACλCI derivative that encodes λCI (residues 1–236) fused to residues 94–448 of the σ^{70} subunit of *E. coli* RNAP. Plasmid pACλQ (Nickels *et al*, 2002) encodes λQ under the control of the *lacUV5* promoter. Plasmid pACΔQ (Nickels *et al*, 2002) encodes no functional λQ.

Strains and test promoters

Reporter strain BN147 contains sequence extending from −109 to +232 of $\lambda P_{R'}$ fused to a *lacZ* reporter gene. This reporter fusion is present on an F′ episome and has been described (Nickels *et al*, 2002). Reporter strain ML35 is identical to BN147 except that it contains the $\lambda P_{R'}$ mutation A(+5)T (Ring and Roberts, 1994). We constructed a derivative of strain FW102 (Whipple, 1998) that has a mutation encoding the L402F substitution at the chromosomal *rpoD* locus linked to a kanamycin resistance gene. The resulting strain, ML6, was mated with donor strains BN147 and ML35 to create the *rpoD(L402F)* reporter strains ML9 ($\lambda P_{R'}$) and ML37 ($\lambda P_{R'}$ A[+5]T), respectively. Strain ML102, a derivative of strain BL21(DE3), carries the L402F mutation at the chromosomal *rpoD* locus linked to a kanamycin resistance gene.

Libraries and screening

Expression libraries were generated by error-prone PCR of the complete coding sequence of σ^{70} (in three segments) and of the sequence encoding residues 1–258 of β′ in pBRσ^{70} and pBRβ′, respectively. Libraries were cotransformed with pACλQ into reporter strain ML37 and plated on indicator medium containing X-gal (60 μg/ml), IPTG (10 μM), and tPEG (250 μM). The $\lambda P_{R'}$ A(+5)T mutation (Ring and Roberts, 1994) in strain ML37 enhanced the difference in λQ-dependent *lacZ* activity between cells containing plasmid-encoded and chromosomally encoded σ^{70} L402F, and cells containing plasmid-encoded σ^{70} WT in addition to chromosomally encoded σ^{70} L402F. Library clones were screened for those that resulted in increased *lacZ* activity relative to unmutagenized pBRσ^{70}(L402F) or pBRβ′. Identified mutations were recloned into pBRσ^{70} or pBRβ′ before cotransforming with either pACλQ or pACΔQ into reporter strain ML9 for β-galactosidase assays.

β-Galactosidase assays

Cells were grown in LB supplemented with the appropriate antibiotics at the following concentrations: carbenicillin (100 μg/ml), tetracycline (10 μg/ml), chloramphenicol (25 μg/ml), and kanamycin (50 μg/ml). IPTG was used at the indicated concentrations. SDS–$CHCl_3$-permeabilized cells were assayed as described (Dove and Hochschild, 2004).

Proteins

His_6-tagged σ^{70} proteins were purified as described after overproduction from plasmid pLNH12-His (Panaghie *et al*, 2000) in BL21DE3 cells. RNAP core enzymes were purified as described (Vrentas *et al*, 2005) from strain ML102 cells containing plasmid pVS10 (Artsimovitch *et al*, 2003), which directs the synthesis of high levels of α, β, β′-His_6, and ω. The presence of the L402F mutation at the chromosomal *rpoD* locus in ML102 cells ensured that the core enzyme preparations were not contaminated with WT σ^{70}. Holoenzyme was formed by incubating the core enzyme with a 5- to 10-fold excess of the appropriate σ^{70} protein. λQ and NusA proteins were gifts from J Roberts.

In vitro transcription

The transcription assays were performed with holoenzymes prepared as described above except for the assay shown in the left-hand panel of Figure 4A, which was performed using holoenzyme reconstituted from commercially obtained *E. coli* RNAP core (Epicentre) and purified σ^{70} proteins. For analysis of promoter-proximal pausing under single-round conditions, open

complexes were formed by incubating 40 nM RNAP with 5 nM $\lambda P_{R'}$ or p*lac*UV5 template for 10 min at 37°C in transcription buffer (20 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 10 mM DTT, 50 mM KCl, and 100 μg/ml BSA) plus either 200 μM each of ATP, GTP and CTP and 50 μM [α-^{32}P]UTP (4 mCi/ml) to generate internally labeled RNA or 200 μM each of GTP, CTP and UTP and 50 μM [γ-^{32}P]ATP (1 mCi/ml) to generate end-labeled RNA. Transcription was initiated by addition of 4 mM $MgCl_2$ and 5 μg/ml rifampicin, and the reactions were incubated at 37°C. At the indicated times after the addition of the $MgCl_2$–rifampicin mixture, aliquots of each reaction were removed and quenched in five volumes of stop buffer (600 mM Tris–HCl, pH 8.0, and 12 mM EDTA) supplemented with tRNA. Samples were then extracted with phenol/chloroform (1:1), precipitated with ethanol, resuspended in 4.5 μl loading buffer (95% (v/v) formamide, 20 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol), and electrophoresed on 15% (w/v) polyacrylamide sequencing gels. Bands were visualized by phosphorimager.

For analysis of abortive transcript synthesis under single-round conditions, open complexes were formed by incubating 40 nM RNAP with 5 nM $\lambda P_{R'}$ template for 10 min at 37°C in transcription buffer. Reactions were initiated by the simultaneous addition of 50 μg/ml heparin plus 100 μM each of UTP, GTP and CTP and 50 μM [γ-^{32}P]ATP at 1 mCi/ml. Reactions were incubated for 20 min at 37°C before they were quenched with five volumes of stop buffer. Samples were then prepared for electrophoresis and electrophoresed as described above.

For all *in vitro* transcription assays with $\lambda P_{R'}$, linear template DNA, which was generated by the PCR from plasmid pFW11-$P_{R'}$-*lacZ* (Nickels *et al*, 2002), contained $P_{R'}$ sequence extending from −109 to +232. For *in vitro* transcription assays with p*lac*UV5, linear template DNA was generated by the PCR from a pFW11-derived plasmid carrying p*lac*UV5 sequence extending from −60 to +36 (Nickels *et al*, 2004).

Supplementary data

Supplementary data are available at *The EMBO Journal* online (http://www.embojournal.org).

Acknowledgements

We thank S Dove and B Nickels for their comments on the manuscript, S Garrity for assistance in preparing Figure 3 and C Vrentas, T Gaal, W Ross, and R Saecker for helpful suggestions. We also thank J Roberts for purified NusA and λQ proteins, V Svetlov and I Artsimovitch for RNAP expression vectors, C Gross for σ^{70} shut-off strain CAG20153, and S Garrity for plasmids pBRα-β′ 55–261, pBRα-β′ 262–309, and pACλCI-σ^{70} 94–448. This work was supported by NIH grant GM44025 to AH.

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