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Interactions Between the *Rhodobacter sphaeroides* ECF Sigma Factor, σ^E, and its Anti-sigma Factor, ChrR

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Rhodobacter sphaeroides σ^E is a member of the extra cytoplasmic function sigma factor (ECF) family, whose members have been shown to regulate gene expression in response to a variety of signals. The functions of ECF family members are commonly regulated by a specific, reversible interaction with a cognate anti-sigma factor. In *R. sphaeroides*, σ^E activity is inhibited by ChrR, a member of a newly discovered family of zinc containing anti-sigma factors. We used gel filtration chromatography to gain insight into the mechanism by which ChrR inhibits σ^E activity. We found that formation of the σ^E:ChrR complex inhibits the ability of σ^E to form a stable complex with core RNA polymerase. Since the σ^E:ChrR complex inhibits the ability of the sigma factor to bind RNA polymerase, we sought to identify amino acid substitutions in σ^E that altered the sensitivity of this sigma factor to inhibition by ChrR. This analysis identified single amino acid changes in conserved region 2.1 of σ^E that either increased or decreased the sensitivity of σ^E for inhibition by ChrR. Many of the amino acid residues that alter the sensitivity of σ^E to ChrR are located within regions known to be important for interacting with core RNA polymerase in other members of the σ^{70} superfamily. Our results suggest a model where solvent-exposed residues with region 2.1 of σ^E interact with ChrR to sterically occlude this sigma factor from binding core RNA polymerase and to inhibit target gene expression.

Keywords: sigma factor; anti-sigma factor; transcription; regulation; *Rhodobacter sphaeroides*

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Introduction

Transcription is a key step in the regulation of prokaryotic gene expression, and is catalyzed by RNA polymerase. The sigma factor (σ) of this multi-subunit enzyme plays a key regulatory role in gene expression by recognizing specific promoter sequences.[1,2] Almost all bacteria currently studied possess multiple sigma factors, including a primary or "housekeeping" sigma (σ^{70}-type) and several classes of alternative sigma factors. These alternative σ factors allow cells to regulate transcription of specific genes in response to stress or changing environmental conditions.[3–5] Most of these alternative sigma factors are classified as members of the σ^{70} superfamily due to the conservation of amino acid sequence in regions that either interact with core RNA polymerase, promoter sequences, or facilitate the process of transcription initiation.[6–8]

Extra-cytoplasmic function (ECF) sigma factors are a group of alternative sigma factors whose target gene products often function outside the cytoplasm (in the membrane, periplasm of Gram-negative bacteria, or beyond).[9] ECF sigma factors control cellular responses to diverse environmental demands including: periplasmic stress,[10,11] resistance to cobalt and nickel,[12,13] high levels of light,[14] and oxidative stress.[15–17] Analyses of bacterial genome sequences suggest that ECF sigma factors could play a major role in gene regulation, since a large number of the σ^{70} superfamily currently present in the NCBI database are predicted to be ECF sigma factors.

A common feature of ECF sigma factor family members is the means by which their activity is regulated. The ECF sigma factor is often co-transcribed with a gene coding for a negative regulator, or "anti-sigma factor."[18–20] The regulation of transcription by an anti-sigma factor occurs either by

Abbreviations used: ECF, extra-cytoplasmic function.

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blocking sigma factor binding to core RNA polymerase (SpoIIAB,[21] RseA,[22] and RsrA[23]), by facilitating the dissociation of the sigma factor from RNA polymerase holoenzyme (FlgM),[24] or by preventing promoter recognition by RNA polymerase holoenzyme (AsiA).[25] To date, four ECF sigma factors have been shown to interact directly with their cognate anti-sigma factors: *Rhodobacter sphaeroides* σ^E:ChrR,[26] *Escherichia coli* σ^E:RseA,[22,27,28] *Pseudomonas aeruginosa* AlgU:MucA,[29] and *S. coelicolor* σ^R:RsrA.[16,30] While the molecular interactions between *E. coli* σ^E:RseA[22] and *S. coelicolor* σ^R:RsrA[23] have been analyzed, the lack of significant amino acid sequence similarity among anti-sigma factors makes it difficult to predict how each inhibitor will interact with its cognate ECF sigma factor.

We have been analyzing the interactions between the *R. sphaeroides* ECF sigma factor σ^E, and its inhibitor, ChrR. ChrR is predicted to be a soluble zinc-dependent anti-sigma factor that lacks significant amino acid sequence similarity to either *E. coli* RseA or other characterized membrane-bound inhibitors of ECF sigma factors.[16,22] Previous work has determined that ChrR forms a heterodimeric complex with *R. sphaeroides* σ^E, but the process by which this anti-sigma factor blocks σ^E function is unknown.[26] We show that ChrR can prevent σ^E from forming a stable complex with core RNAP. In addition, we characterize the effects of amino acid substitutions within region 2.1 of σ^E which alter the sensitivity of the sigma factor to inhibition by ChrR *in vivo* and *in vitro*. We propose that region 2.1 of σ^E defines a potential site of interaction between σ^E and ChrR by which the anti-sigma factor could prevent σ^E activity.

Results

ChrR prevents σ^E from binding *R. sphaeroides* core RNA polymerase

Previous work indicated that σ^E and ChrR form a heterodimeric complex,[26] but little was known about the mechanism by which ChrR blocks σ^E function. To address how ChrR inhibits σ^E activity, gel filtration chromatography was used to monitor the interactions of σ^E or the σ^E:ChrR complex with core RNA polymerase. *R. sphaeroides* core RNA polymerase and the σ^E:ChrR complex (predicted molecular mass of 43 kDa) were resolved on a Superdex 200 column (Amersham Pharmacia, Piscataway, NJ), with core RNA polymerase eluting in the void volume and complex eluting with an apparent molecular mass of ~32 kDa (Figure 1a). In addition, when σ^E (predicted molecular mass of 19.2 kDa) was analyzed on this column, it eluted as a species of an apparent molecular mass of ~17 kDa (Figure 1a). The ability to resolve core RNA polymerase, the σ^E:ChrR complex, and σ^E suggested that gel filtration experiments would provide insight into how ChrR blocks σ^E function.

When σ^E was incubated with core RNA polymerase and passed over this column, a decrease was seen in the amount of UV-absorbing material eluting at ~17 kDa (Figure 1b). SDS-PAGE of TCA precipitated column fractions showed that σ^E was present in the void volume along with RNA polymerase subunits (Figure 1b). This shift in the σ^E elution profile indicated that σ^E was able to bind to RNA polymerase and form a stable complex under these conditions. The presence of σ^E in the ~17 kDa fraction could be the result of excess σ^E over core RNA polymerase in the experiment, or some of the sigma factor was inactive due to the purification process and unable to bind core RNA polymerase.

To test if ChrR prevents σ^E from binding core RNA polymerase, we observed what happened when pure σ^E:ChrR complex was incubated with core RNA polymerase. When a mixture of core RNA polymerase and the σ^E:ChrR complex was passed over the Superdex 200 column, there was no detectable change in the area under the σ^E:ChrR complex peak, nor was there an appearance of a species with an apparent molecular mass predicted for ChrR (~21 kDa, Figure 1c). In addition, SDS-PAGE of TCA precipitated column fractions showed no detectable σ^E or ChrR in the void volume fractions that contained core RNA polymerase subunits (Figure 1c). This suggests that the σ^E:ChrR complex does not interact with core RNA polymerase to form a stable complex under conditions where σ^E can bind to this enzyme. These results also suggest that core RNA polymerase does not remove σ^E from ChrR under these conditions.

Screen for σ^E mutants having increased activity in the presence of ChrR

Since formation of a σ^E:ChrR complex appears to play a critical role in inhibiting σ^E activity, we sought to identify amino acid residue substitutions in σ^E that altered the sigma factor's sensitivity to inhibition by ChrR. To do this, we capitalized on the observation that *R. sphaeroides* σ^E and ChrR function in an *E. coli* tester strain that contains a chromosomal *rpoE* P1 :: *lacZ* reporter gene (ΦλJDN1).[26] This strain is white on MacConkey's lactose medium in the absence of *R. sphaeroides rpoE*, red when it contains *rpoE* on a plasmid under the control of its own promoter (*rpoE* P1), and pink when it contains the *rpoEchrR* operon on the same plasmid (data not shown). Thus, this tester strain provides a screen for *rpoE* mutations that alter the sensitivity of σ^E to ChrR.

To look for amino acid substitutions in σ^E that alter its sensitivity to ChrR, we screened a library of PCR-mutagenized *rpoE* genes for σ^E activity in this tester strain. After screening ~5500 colonies from eight independent mutagenesis experiments on MacConkey's lactose media, ~81% were pink, indicating "wild-type" σ^E activity, ~16% were white, indicating a decrease in σ^E activity, and

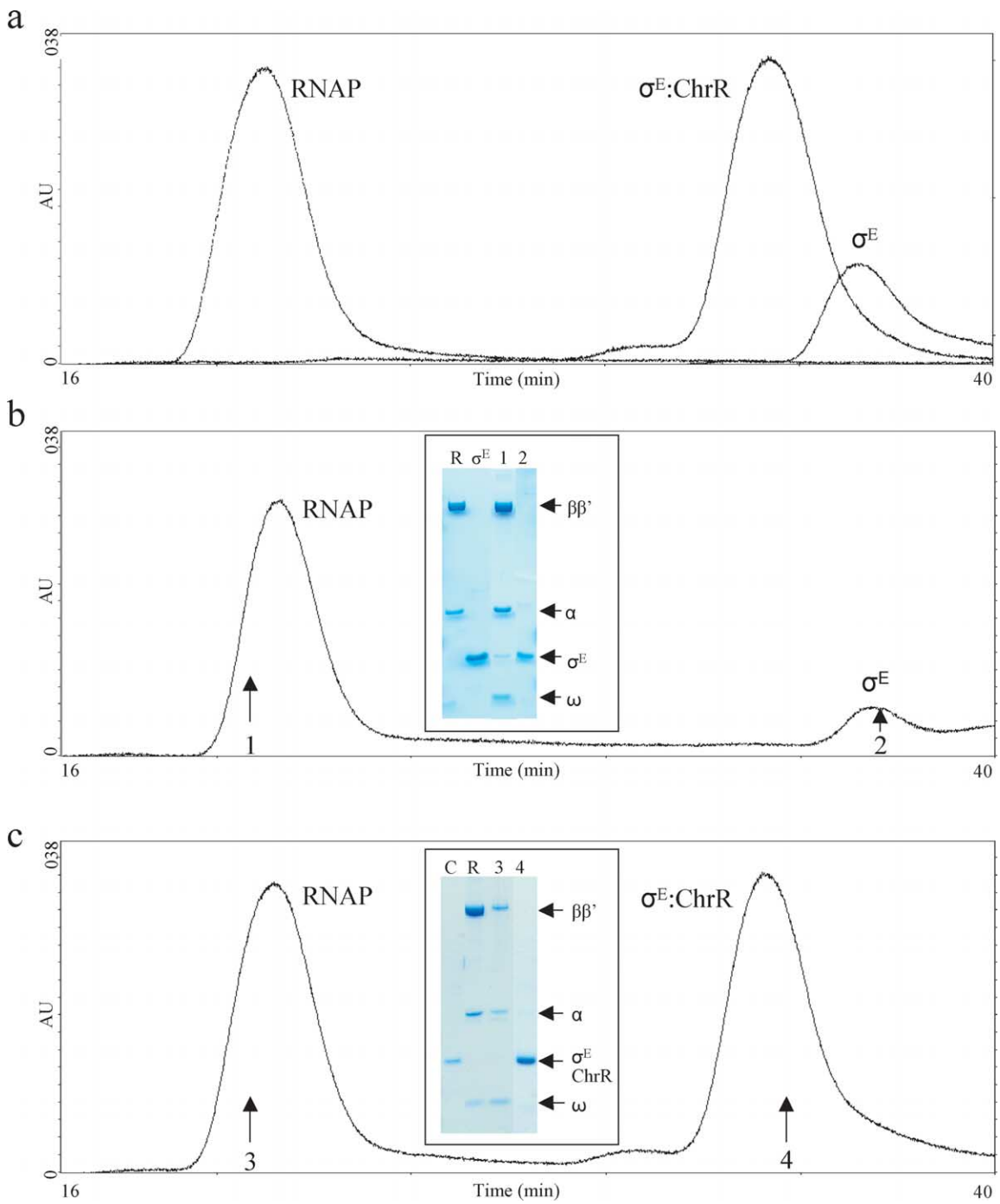

Figure 1. σ^E binding to ChrR prevents its ability to interact with RNA polymerase. (a) The elution profile of purified *R. sphaeroides* core RNA polymerase (0.25 μM), σ^E (4 μM), and σ^E:ChrR complex (4 μM) when passed separately over a Superdex 200 column. (b) The elution profile obtained when σ^E is incubated with *R. sphaeroides* core RNA polymerase, or (c) when the σ^E:ChrR complex is incubated with *R. sphaeroides* core RNA polymerase prior to passage over the same column. The inserts in b and c include SDS-PAGE analysis of core RNA polymerase (R), σ^E, σ^E:ChrR complex (C), and samples obtained from the indicated factions in each panel. The 19 kDa σ^E and 21 kDa ChrR proteins appear as a single band due to the low resolution of the SDS-PAGE gel.

~3% were red, indicating an increase in σ^E activity in the presence of ChrR. When the *R. sphaeroides rpoE* gene from 65 of the 151 red colonies was sequenced, four different single amino acid substitutions in σ^E were identified. Three of the amino acid substitutions (K38E, K38R, and F40S) were located in σ^E region 2.1, and one (F81I) was located in σ^E region 2.3 (Figure 2). The single amino acid substitutions in σ^E, K38E and K38R, were identified twice from independent mutagenesis screens. The *rpoE* genes in the remaining red colonies that were sequenced contained multiple mutations, including ~30 isolates which had K38E, K38R, F40S, or F81I as one of the multiple amino acid substitutions.

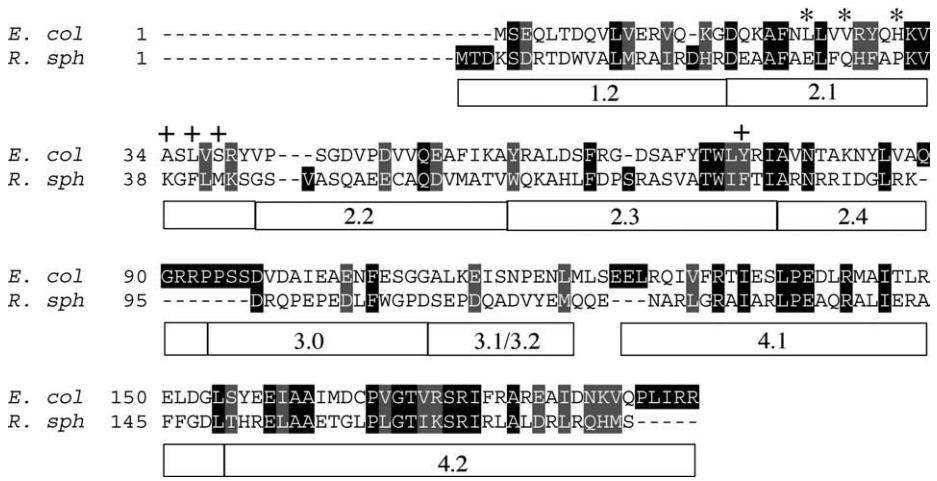

Figure 2. Alignment of *E. coli* and *R. sphaeroides* σ^E proteins. The amino acid sequence alignment was generated using ClustalW and the indicated sequences: *E. coli* σ^E *E. coli* (GenBank accession no. P334086) and *R. sph*, σ^E *R. sphaeroides* (GenBank accession no. AAB17906). Identical and similar amino acid residues are shaded in black and grey, respectively. Biochemically defined regions of eubacterial sigma factors are denoted with boxes.[8,56] Positions where amino acid substitutions in *R. sphaeroides* σ^E decrease activity in the presence of ChrR are indicated by *; positions where amino acid substitutions increase σ^E activity in the presence of ChrR are indicated by a +.

These results suggest that amino acid residues in regions 2.1–2.3 of σ^E may be important for its interaction with ChrR.

The effects of *rpoE* mutations *in vivo*

To assess the effects of each amino acid substitution in σ^E on its function, we measured levels of β-galactosidase produced from our tester strain, *E. coli* VH1000 (ΦλJDN1), which contained either wild-type, K38E, K38R, F40S, or F81I σ^E proteins. Cells containing either the K38E, K38R, or F81I mutant σ^E proteins had between ~three and five-fold more β-galactosidase activity than wild-type σ^E in the presence of ChrR (Figure 3a). Cells containing the F40S σ^E protein showed a slight, but reproducible, increase in β-galactosidase activity in the presence of ChrR as compared to those cells containing wild-type σ^E (Figure 3a).

To test if the amino acid substitutions in σ^E affected sigma factor function, we monitored β-galactosidase activity from strains that lacked ChrR but contained either wild-type, K38E, K38R, F40S, or F81I σ^E. If the amino acid substitutions in σ^E did not affect sigma factor function, we expected to find *rpoE* P1 :: *lacZ* reporter activity comparable to that seen in cells containing only wild-type σ^E. Cells containing the K38E, K38R, or F81I mutant σ^E proteins in the absence of ChrR had levels of β-galactosidase activity comparable to a strain containing wild-type σ^E in the absence of ChrR (Figure 3a), suggesting that these amino acid substitutions did not negatively affect sigma factor function. However, the F40S substitution in σ^E caused a slight (~33%) decrease in β-galactosidase activity in the absence of ChrR, suggesting that this amino acid substitution affects σ^E function. In addition, this analysis showed that the K38E, K38R, and F81I mutant σ^E proteins had a decrease in activity in the absence of ChrR when compared to activity in the presence of the anti-sigma factor. Possible explanations for the decreased σ^E activity in cells lacking the inhibitor will be presented in the Discussion.

To test the behavior of these mutant σ^E proteins in its native host, we expressed ChrR and either wild-type, K38E, K38R, F40S, or F81I σ^E (under the control of its own promoter, *rpoE* P1) from a stable low copy plasmid in a *R. sphaeroides* strain that contains a chromosomal deletion of *rpoEchrR* (TF18). To determine σ^E function, we placed a *rpoE* P1:*lacZ* reporter fusion on a compatible low copy plasmid (pJDN30) in this strain. β-Galactosidase activity from this reporter fusion in TF18 cells containing a plasmid with the intact *rpoEchrR* operon is low (Figure 3b), because we know ChrR inhibits σ^E under these growth conditions.[26] Cells containing K38E σ^E had an ~100-fold increase in β-galactosidase activity, cells containing F81I σ^E exhibited an ~12-fold increase in β-galactosidase activity, and cells containing K38R σ^E had ~eightfold more β-galactosidase activity than the control strain containing wild-type σ^E (Figure 3b). In contrast, cells containing F40S σ^E only had ~1.4-fold more β-galactosidase activity, which is similar to the behavior of this mutant sigma factor in *E. coli*. When taken together, the properties of these mutant σ^E proteins in *E. coli* and *R. sphaeroides* suggest that the K38E, K38R, and F81I substitutions alter the sensitivity of σ^E to inhibition by ChrR without reducing their ability to function as sigma factors.

Region 2.1 of *R. sphaeroides* σ^E contains additional amino acid residues that are important for inhibition by ChrR

We sought to determine if the amino acid residues identified in our screen mapped to a specific region of sigma factors. Aligning the amino acid

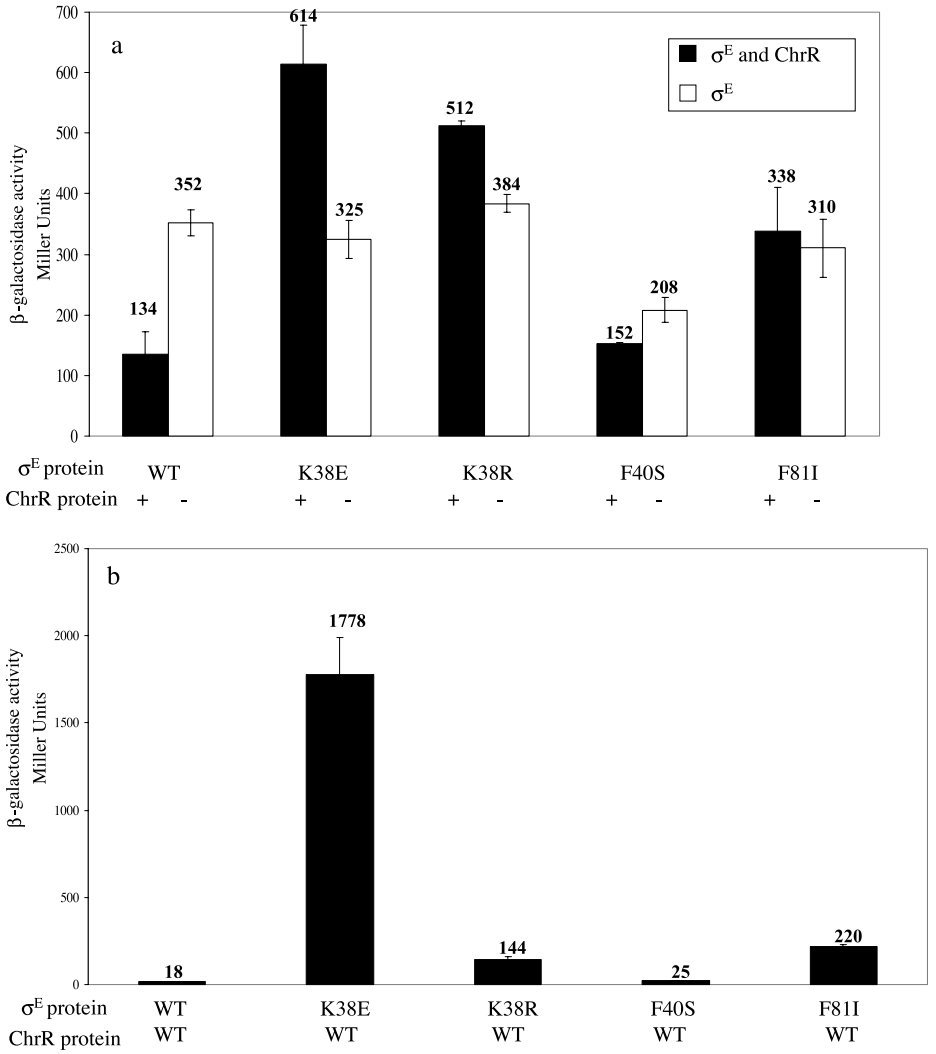

Figure 3. *R. sphaeroides* σ^E mutants have altered activity *in vivo* in the presence of the anti-sigma factor, ChrR. (a) β-Galactosidase activity from an *E. coli* tester strain containing a chromosomal *rpoE* P1 :: *lacZ* transcriptional fusion and the indicated *rpoE* gene (□), or *rpoE* and *chrR* genes (■). (b) β-Galactosidase activity of indicated σ^E mutants in a *R. sphaeroides* tester strain containing the indicated mutant σ^E and wild-type ChrR. All assays were performed in triplicate, with vertical bars denoting the average standard deviation from the mean.

sequences of *R. sphaeroides* σ^E and *E. coli* σ^E suggests that residues K38, F40, and F81 of *R. sphaeroides* σ^E correspond to amino acid residues A34, L36, and Y75 of *E. coli* σ^E (Figure 2). Mapping these residues of *R. sphaeroides* σ^E on the structure of *E. coli* σ^E,[22] predicts that residue K38 of this protein is surface exposed within the α-helical domain of region 2.1; residues F40 and F81 of *R. sphaeroides* σ^E appear to be involved in stabilizing interactions between the α-helices of regions 2.1 and 2.3 (Figure 4). In addition, this model predicts that residues K38 and F40 are within a part of σ^E region 2.1 that makes protein–protein contacts with other parts of the sigma factor,[31] core RNA polymerase,[32,33] or anti-sigma factors.[22,23] To determine if other amino acid residues within region 2.1 of *R. sphaeroides* σ^E are important for inhibition by ChrR, we individually substituted 21 amino acid residues within a 25 residue region (^{22}DEAAFAELFQHFAPKVKGFLMKSGS^{46}) with alanine (residues A24, A25, A27, and A34 are alanine in wild-type protein). These mutant σ^E proteins were expressed in the *E. coli* tester strain to determine if any of these alanine substitutions altered the sensitivity of σ^E to inhibition by ChrR or disrupted their function as sigma factors. β-Galactosidase levels from the strains tested suggest that the single amino acid substitutions in region 2.1 of σ^E can be classified into several categories.

Cells containing the D22A, E23A, H32A, K36A, K38A, G39A, F40A, L41A, K43A, S44A, G45A, or S46A mutant σ^E proteins had β-galactosidase activity similar to that found in cells containing either wild-type σ^E or those containing both wild-type σ^E and ChrR (Figure 5a). This suggests that alanine substitutions at these positions of *R. sphaeroides* σ^E do not have measurable effects on either sigma factor function (in cells lacking ChrR), or on their sensitivity to inhibition by

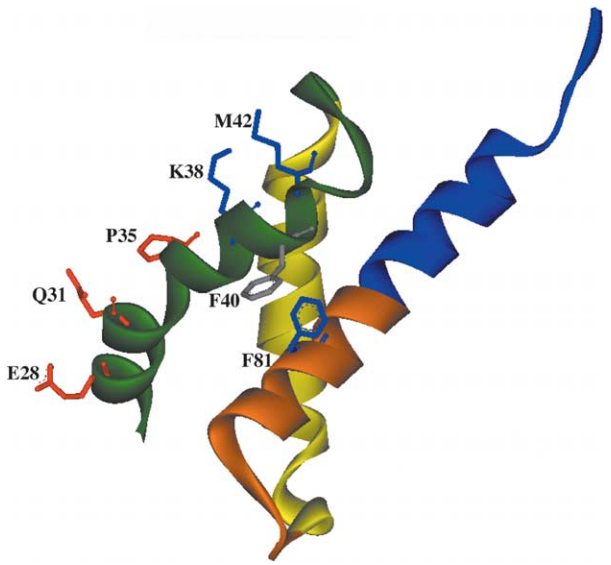

Figure 4. Amino acid substitutions in *R. sphaeroides* σ^E which alter activity in the presence of ChrR. Crystal structure of *E. coli* σ^E region 2,[22] highlighting the side-chains of residues in *R. sphaeroides* σ^E that alter sensitivity to ChrR. The σ^E structure is colored according to the biochemically defined regions of σ factors: region 2.1, green; region 2.2, yellow; region 2.3, orange; and region 2.4, blue. The side-chains of amino acid residues in *R. sphaeroides* σ^E whose substitutions resulted in an increase in σ^E activity in the presence of ChrR are indicated in blue (K38, M42, and F81), and those residues which resulted in a decrease in σ^E activity in the presence of ChrR *in vivo* are indicated in red (E28, Q31, and P35). *R. sphaeroides* σ^E residue F40 appears to affect σ^E activity in the absence of ChrR, and is indicated in grey.

ChrR. The K38A σ^E protein falls into this category, which suggests that removing the lysine side-chain has no affect on σ^E activity, but replacing the lysine with an arginine or a glutamate (K38R or K38E) reduces the sensitivity of this mutant sigma factor to inhibition by ChrR (Figure 3a).

Cells containing M42A σ^E had a small, but reproducible (1.2-fold) increase in σ^E activity in the presence of ChrR, and an ~fourfold decrease in activity when compared to wild-type σ^E in the absence of ChrR (Figure 5b). Amino acid sequence alignments indicate that residue M42 of *R. sphaeroides* σ^E corresponds to residue S38 of *E. coli* σ^E (Figure 2). By mapping *R. sphaeroides* σ^E residue M42 on the *E. coli* σ^E structure,[22] the side-chain of this amino acid appears to lie on the same face as that of residue K38. From the mutational studies of *R. sphaeroides* σ^E residues K38 and M42, it appears that amino acid changes in this region can reduce the sensitivity of mutant sigma factors for inhibition by ChrR (Figure 4).

Cells containing alanine substitutions at position E28, Q31, and P35 of σ^E had between ~three and sevenfold less activity in the presence of ChrR than cells containing wild-type σ^E (Figure 5b). In the absence of ChrR, the E28A, Q31A, and P35A mutant σ^E proteins have activity similar to or slightly lower than cells containing wild-type σ^E (Figure 5b). These results suggest that alanine substitutions at positions E28, Q31, and P35 increase σ^E sensitivity to inhibition by ChrR without affecting function. When the *R. sphaeroides* σ^E E28, Q31, and P35 side-chains were mapped onto the *E. coli* σ^E structure,[22] these residues are predicted to lie on the same surface exposed face of region 2.1 as residues K38 and M42 (Figure 4). Therefore, it appears that single amino acid substitutions in a potential surface exposed face of *R. sphaeroides* σ^E region 2.1 can either decrease or increase the sensitivity of this sigma factor to inhibition by ChrR.

Finally, cells containing mutant σ^E proteins with amino acid substitutions at residues F26, L29, F30, F33, and V37 show a ~two to fourfold decrease in σ^E activity in the presence of ChrR, and a ~three to ninefold decrease in σ^E activity in the absence of ChrR when compared to cells containing wild-type σ^E (Figure 5c). It has been impossible to determine if the abundance of any mutant σ^E proteins in *E. coli* is significantly different from their wild-type counterparts (data not shown). Thus the behavior of this class of mutant proteins suggests that alanine substitutions at these positions affect the activity or stability of σ^E. If the *R. sphaeroides* σ^E F26, L29, F30, F33, and V37 side-chains are mapped on the structure of *E. coli* σ^E,[22] they are predicted to stabilize helix–helix interactions between region 2.1 and the other parts of region 2 (data not shown). The negative effects of alanine substitutions in residues F26, L29, F30, F33, and V37 on *R. sphaeroides* σ^E activity *in vivo* also suggests that these residues are involved in stabilizing potential helix–helix interactions between region 2.1 and other regions of σ^E.

Sensitivity of mutant σ^E proteins to inhibition by ChrR *in vitro*

The behavior of these mutant σ^E proteins *in vivo* predicts that these amino acid substitutions should alter the sensitivity of the sigma factor to inhibition by ChrR *in vitro*. To test the sensitivity of mutant σ^E proteins to inhibition by ChrR *in vitro*, a His_6-tagged version of each protein was purified and used for *in vitro* transcription reactions with the *rpoE* P1 template. We focused on several mutant σ^E proteins because they showed either altered sensitivity for ChrR (E28A, Q31A, P35A, K38E, K38R, and M42A), or because they provided controls which had essentially wild-type σ^E activity *in vivo* (G39A).

Before testing the sensitivity of each mutant σ^E protein to inhibition by ChrR, the amount of sigma factor required to produce maximal transcription from the *rpoE* P1 reporter was determined. By measuring the amount of *rpoE* P1 transcript produced as the concentration of wild-type or mutant σ^E was increased (0–100 nM), the relative activity of each mutant sigma factor was measured. Maximal transcript levels were seen with 50–100 nM concentrations of each protein

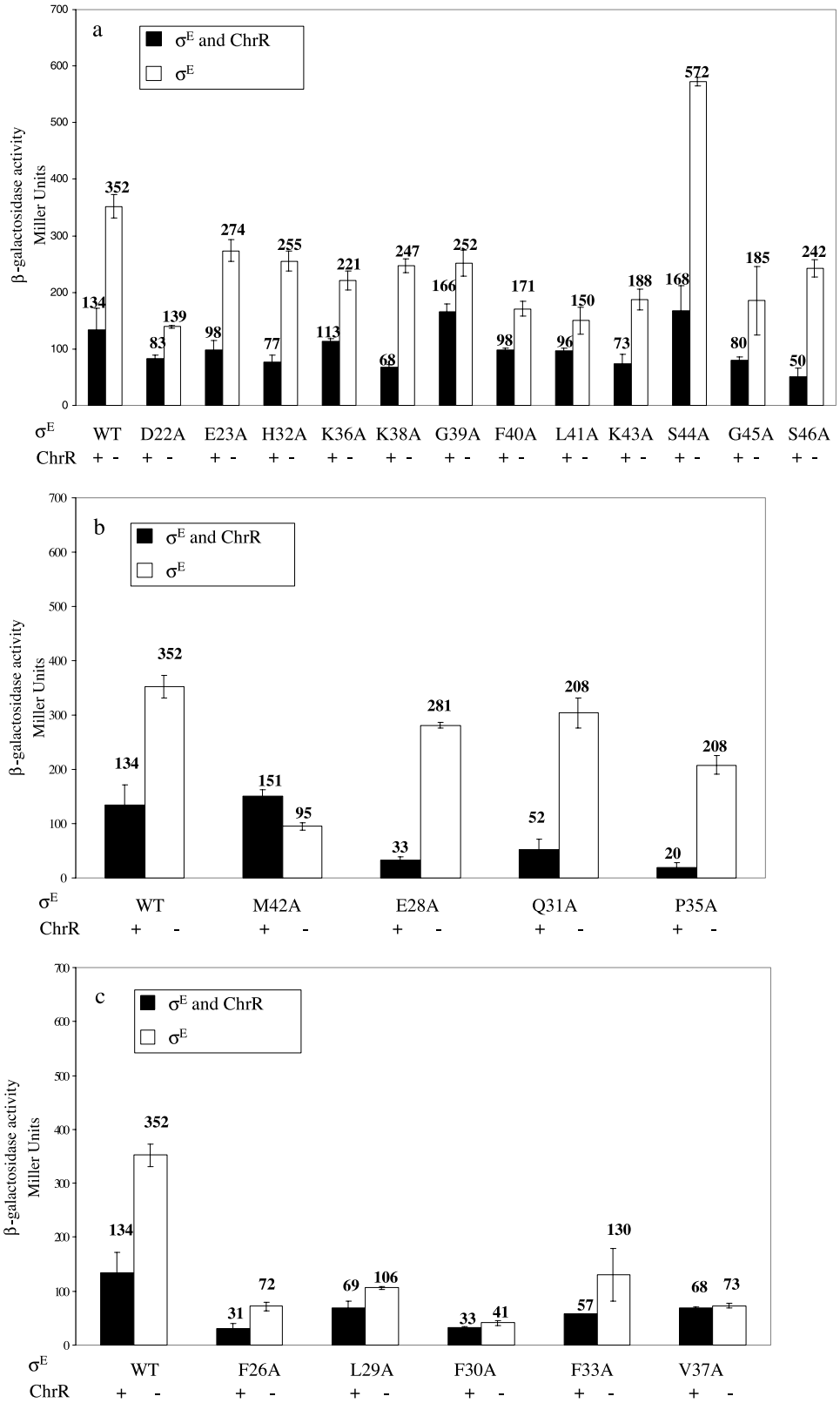

Figure 5. Effects of alanine substitutions on σ^E activity *in vivo*. β-Galactosidase activity from *E. coli* tester strains containing either *rpoE* (□), or *rpoE* and *chrR* (■). All assays were performed in triplicate, with vertical bars denoting the average standard deviation from the mean. (a) Amino acid substitutions in σ^E that result in wild-type activity in the presence and absence of ChrR. (b) Amino acid substitutions in σ^E that result in either an increased (M42A) or decreased (E28A, Q31A, and P35A) activity in the presence of ChrR. (c) Amino acid substitutions in σ^E that result in a decrease in activity both in the presence and absence of ChrR.

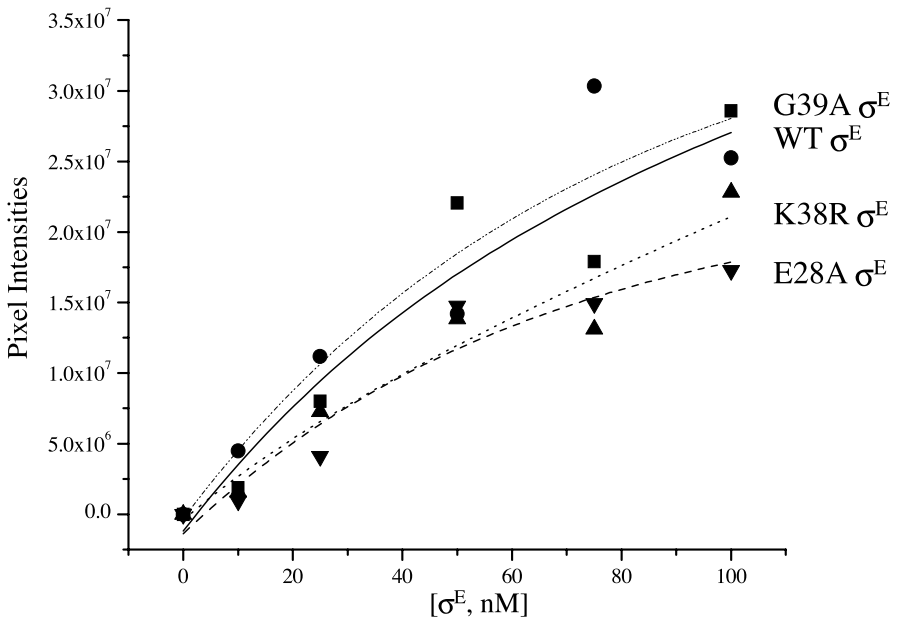

Figure 6. Activity of wild-type and mutant σ^E proteins *in vitro*. Multiple round *in vitro* transcription assays performed with increasing amounts of σ^E proteins. Shown is the amount of *rpoE* P1 transcript produced with a constant amount of *R. sphaeroides* core RNA polymerase and increasing amounts of wild-type σ^E (■), or mutant σ^E proteins that had either wild-type (G39A, ●), increased (K38R, ▲), or decreased (E28A, ▼) activity in the presence of ChrR *in vivo*.

(Figure 6), suggesting that the activity of each purified mutant σ^E protein was within twofold of wild-type σ^E. From this we conclude that none of the single amino acid substitutions dramatically reduce the ability of these mutant σ^E proteins to function in transcription.

To test the ability of ChrR to inhibit wild-type and mutant σ^E proteins *in vitro*, we measured the amount of *rpoE* P1 transcript remaining after the addition of increasing amounts of ChrR fused to maltose-binding protein (ranging from one to 20-fold molar excess compared to σ^E).[26] For these assays, a concentration of σ^E protein was used that produced 50% of the maximal *rpoE* P1 transcript. This concentration increased our ability to monitor any alterations in the sensitivity of individual sigma factors to inhibition by ChrR. In assays containing wild-type σ^E, we saw a concentration-dependent decrease in the *rpoE* P1 transcript when ChrR was added. When ChrR concentrations were at fivefold excess over σ^E, an ~80% decrease in σ^E-dependent transcription was seen (Figure 7a and inset). As a control, a mutant σ^E protein which appeared to have wild-type sensitivity to ChrR *in vivo* (G39A) was also inhibited in a concentration-dependent manner by ChrR, with a fivefold excess of ChrR causing ~70% inhibition of sigma factor activity (Figure 7a and inset).

When this assay was used to analyze the effects of ChrR on the other mutant σ^E proteins, transcription using the K38E, K38R, or M42A σ^E proteins was less sensitive to inhibition by ChrR. Only a 5–25% reduction in *rpoE* P1 transcript levels was seen when ChrR was present at fivefold excess over K38E, K38R, or M42A mutant σ^E proteins (Figure 7a). Therefore, the K38E, K38R, and M42A substitutions in σ^E result in mutant sigma factors which are less sensitive to ChrR *in vivo* and *in vitro*, reinforcing the conclusion that residues K38 and M42 could define a site of interaction with the anti-sigma factor.

In contrast, σ^E-dependent transcription from the E28A and Q31A mutant σ^E proteins reproducibly required only twofold excess ChrR to obtain maximal inhibition (80%), instead of the fivefold excess required for maximal inhibition of either wild-type or G39A σ^E proteins (Figure 7b). These results are consistent with the *in vivo* behavior of the E28A and Q31A mutant σ^E proteins, and they suggest that alanine substitutions at these positions increase the sensitivity of this sigma factor to ChrR.

The last mutant σ^E protein tested, P35A, also appeared to be more sensitive to inhibition by ChrR *in vivo* (Figure 5b). However, when P35A σ^E was tested *in vitro* for sensitivity to ChrR, its activity was only reduced ~25% in the presence of fivefold excess ChrR (Figure 7a). This level of inhibition is similar to that observed with other mutant σ^E proteins that appear less sensitive to ChrR, suggesting that the P35A substitution in σ^E reduces the sensitivity of this sigma factor to its inhibitor. Possible explanations for the different behaviors of the P35A σ^E mutant protein in the presence of ChrR *in vivo* and *in vitro* will be presented in the Discussion.

Discussion

In previous studies, *R. sphaeroides* σ^E was shown

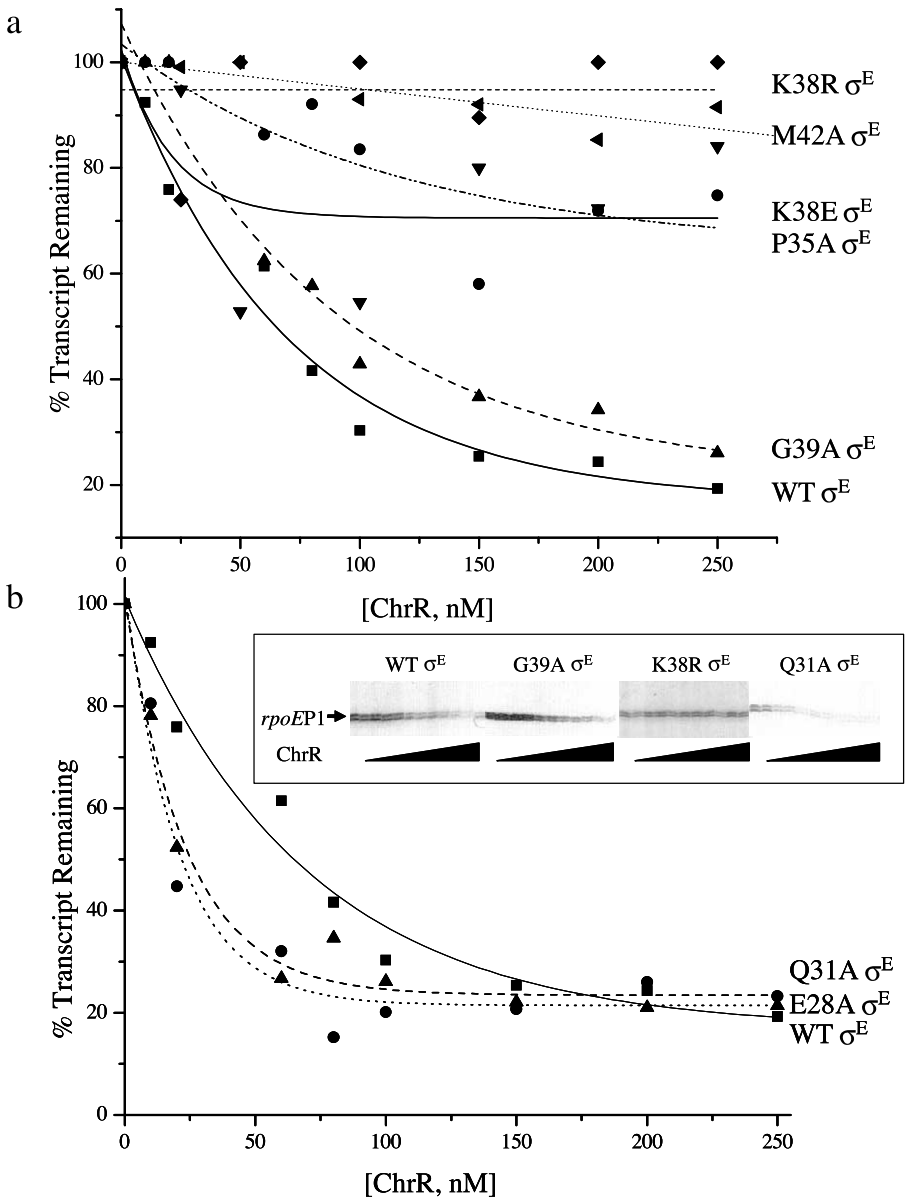

Figure 7. Amino acid substitutions in *R. sphaeroides* σ^E that affect the ability of ChrR to inhibit σ^E-dependent activity *in vitro*. The percent of *rpoE* P1 transcript produced in multiple round transcription assays with increasing amounts of ChrR. All data are normalized to the amount of transcript present from each Eσ^E enzyme in the absence of ChrR, which is defined as 100% activity. (a) Behavior of mutant σ^E proteins (P35A, ●, K38E, ▼, K38R, ◆, and M42A, ◄) compared to wild-type σ^E (■) and a mutant σ^E protein that has wild-type activity *in vivo* (G39A, ▲). (b) Activity of mutant σ^E proteins (E28A, ●, and Q31A, ▲) compared to wild-type σ^E (■). The inset in b shows the amount of *rpoE* P1 transcript produced by RNA polymerase holoenzyme containing the indicated sigma factor (wild-type (WT), G39A σ^E (G39A), Q31A σ^E (Q31A) and K38R σ^E (K38R)) in assays with increasing amounts of ChrR (0–500 nM). The *rpoE* P1 transcript appears as two species due to two termination sites within the SpoT 40 transcription terminator.

to form a 1 : 1 complex with ChrR, and it was suggested that binding of ChrR to σ^E was sufficient to inhibit σ^E-dependent transcription.[26] However, it was not known how ChrR binding inhibited σ^E activity and what regions of the σ factor were important for inhibition by ChrR. In this work we determined a possible mechanism of σ^E inhibition by ChrR and identified amino acid substitutions in σ^E which alter sensitivity of this sigma factor to inhibition by its anti-sigma factor, ChrR.

ChrR prevents σ^E from interacting with core RNA polymerase

Previous work has shown that the σ^E:ChrR complex is unable to transcribe σ^E target genes when added to core RNA polymerase.[26] One possible explanation for this observation is that ChrR binding to σ^E prevents this sigma factor from binding to core RNA polymerase. A similar situation occurs between *E. coli* σ^E and its anti-sigma factor

RseA, as well as between *S. coelicolor* σ^R and RsrA. For *E. coli* RseA, it is proposed that interactions with σ^E regions 2 and 4 sterically occlude binding sites for the RNA polymerase β' and β subunits.[22] In the case of *S. coelicolor* RsrA, an ~10 kDa fragment of the sigma factor that contains region 2 has been shown to interact with RNA polymerase.[23] In addition, the interaction between this fragment of σ^R and core RNA polymerase can be prevented by the presence of RsrA, suggesting that region 2 of this sigma factor is involved in forming a complex with RNA polymerase and with RsrA.[23] However, there are other examples of anti-sigma factors that allow the σ factor to interact with RNA polymerase but prevent the resulting holoenzyme from initiating transcription.[34–37] Given the lack of significant amino acid similarity between ChrR and other characterized anti-sigma factors, it was important to understand how this protein prevents σ^E activity.

We found that the σ^E:ChrR complex was unable to form a stable complex with core RNA polymerase under conditions where σ^E was able to bind to this enzyme to form Eσ^E. In addition, the σ^E:ChrR complex did not appear to dissociate when incubated with core RNA polymerase, suggesting that ChrR binding to σ^E is mutually exclusive with core RNA polymerase binding to σ^E. These observations, when considered with previous work, provide strong evidence that formation of the σ^E:ChrR complex is key to inhibiting *R. sphaeroides* σ^E-dependent transcription.

Region 2.1 of *R. sphaeroides* σ^E is important for sensitivity to ChrR

One possible explanation for the inability of σ^E to bind to core RNA polymerase when this sigma factor interacts with ChrR is that the anti-sigma factor masks RNA polymerase-binding determinants on σ^E. Analysis of members of the σ^{70} superfamily of sigma factors suggests that regions 2.1, 2.2 and 4.1 are principal sites for RNA polymerase binding.[33,38–40] If ChrR were to inhibit σ^E holoenzyme formation by blocking RNA polymerase-binding determinants, one might expect that residues within one or more of these regions of σ^E are also important for sensitivity to this anti-sigma factor.

Our results implicate region 2.1 of σ^E as being important for inhibition by the anti-sigma factor, ChrR. Specifically, we identified amino acid changes in region 2.1 of *R. sphaeroides* σ^E that either decrease (K38E, K38R, and M42A) or increase (E28A and Q31A) the sensitivity of σ^E to inhibition by ChrR. When these residues are modeled on the *E. coli* σ^E structure,[22] they appear to be located on a solvent-exposed face of region 2.1. Thus it is possible that one or more of these amino acid side-chains constitute a site on σ^E that is involved in making direct interactions with ChrR. If this hypothesis is correct, ChrR binding to σ^E could sterically occlude a major RNA polymerase-binding determinant on σ^E and thereby prevent formation of σ^E holoenzyme. Each of the mutant σ^E proteins studied (E28A, Q31A, P35A, K38E, K38R, and M42A) were able to form transcriptionally competent complexes with core RNA polymerase, suggesting that these amino acid residues are not essential for RNA polymerase holoenzyme formation. We propose that these determinants in region 2.1 of σ^E are part of a larger domain used by σ^{70} family members to interact with core RNA polymerase, and that ChrR interactions within this domain interfere sterically with RNA polymerase holoenzyme formation.[22,23]

The crystal structure of the *E. coli* σ^E:RseA complex shows that the N terminus of RseA makes both van der Waals and hydrogen bond contacts with residues of *E. coli* σ^E region 2.1, including Leu24 and Val27.[22] The corresponding *R. sphaeroides* σ^E region 2.1 residues, Glu28 and Gln31, were identified as important for sensitivity to ChrR since alanine substitutions at these two positions increased inhibition of σ^E by this anti-sigma factor *in vivo* and *in vitro*. It is interesting to note that the loss of a large charged side-chain (Glu and Gln) at each position in *R. sphaeroides* σ^E results in increased sensitivity to ChrR, perhaps by allowing additional main-chain interactions between the sigma factor and its anti-sigma factor. Experiments are in progress to probe the nature of the interactions between σ^E and ChrR, and to test if these and other changes in region 2.1 directly alter the interactions of these two proteins.

The organization of region 2.1 of *R. sphaeroides* σ^E

Our data suggest that the overall structure of region 2.1 in *R. sphaeroides* σ^E is α-helical as is the case of other members of the σ^{70} superfamily.[22,23,31,41] If we map the amino acid side-chains in region 2.1 of *R. sphaeroides* σ^E onto a helical wheel model, all of the alanine substitutions that reduce sigma factor activity map to one face of the predicted helix (Figure 8). This face is predicted to make helix–helix interactions with regions 2.2 and 2.3 based on the analysis of other σ^{70} family members (see below).[22,23,31,41] In addition, residues identified by our studies which alter the sensitivity of σ^E to inhibition by ChrR map to the opposite face of these helical wheel projections, and could be surface exposed (Figure 8, see below).

The position of region 2.1 in *E. coli* σ^{70} and σ^E appears to be stabilized by hydrophobic side-chain interactions with residues in regions 2.3 and 2.4.[22,31,41] The properties of *R. sphaeroides* σ^E mutant proteins, F40S and F81I, could be explained if these side-chains made Van der Waals interactions with amino acid side-chains in regions 2.3 and 2.1, respectively. If this is true, interrupting these interactions could both alter the sensitivity of σ^E proteins for ChrR, and reduce their ability to function as sigma factors.

In the case of *E. coli* σ^{70}, *E. coli* σ^E and *S. coelicolor*

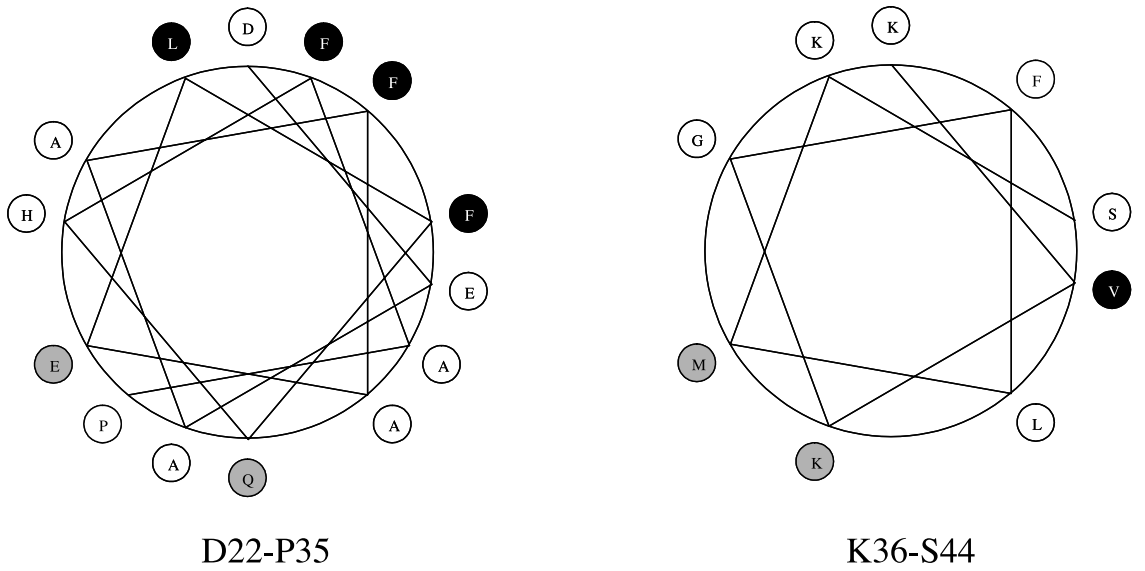

Figure 8. Helical wheel model of region 2.1 of *R. sphaeroides* σ^E. Due to the presence of a proline at position 35 in σ^E we modeled region 2.1 of *R. sphaeroides* σ^E as two helical domains. Residues in σ^E are highlighted as follows: grey, substitutions that increase (K38 and M42A) or decrease (E28 and Q31) σ^E activity in the presence of ChrR; and black, substitutions that decrease σ^E activity both in the presence and absence of ChrR (F26, L29, F30, F33, and V37).

σ^R, the α-helical nature of region 2.1 is disrupted at the position equivalent to the proline at residue 35 in *R. sphaeroides* σ^E.[22,23] For this reason, we have chosen to model region 2.1 of *R. sphaeroides* σ^E as two helices extending from residue D22 to P35 and K36 to S44 (Figure 8). A search of the protein database indicates that a proline at this position is present in several ECF sigma factors with a high degree of amino acid sequence identity to *R. sphaeroides* σ^E (unpublished data). In addition, the genes encoding for each of these σ^E homologues are linked to genes predicted to encode proteins related to ChrR. Therefore, it is possible that a structural feature created by a proline at this position in σ^E may be important for recognition of this particular group of ECF sigma factors by their cognate anti-sigma factors.

Properties of mutant σ^E proteins *in vivo* and *in vitro*

Somewhat surprisingly, the *in vivo* analysis of the K38E, K38R, and F81I mutant σ^E proteins revealed that the absence of ChrR led to a decrease in σ^E activity when compared to that found in the presence of the anti-sigma factor. Western blot analysis with antiserum to σ^E showed a significant decrease in the amount of each of these three mutant σ^E proteins in cells lacking ChrR (unpublished data), suggesting that σ^E turnover could influence the amount of target promoter activity measured *in vivo*. Protein turnover is a critical part of the regulatory circuit which controls σ^E activity in *E. coli*.[42,43] By analogy, proteolysis of free *R. sphaeroides* σ^E may influence the activity of wild-type and mutant sigma factors *in vivo*. If this were true, turnover of K38E, K38R, and F81I mutant σ^E proteins might account for the decreased target promoter activity observed in the absence of ChrR.

Proteolysis may also explain why the P35A mutant σ^E protein appeared to have increased sensitivity to ChrR *in vivo*, while *in vitro* studies showed this protein was less sensitive to inhibition by this anti-sigma factor (compare Figures 5b and 7a). We propose that P35 is important for the structure of *R. sphaeroides* σ^E region 2.1 and the ability of the ECF sigma factor to interact with its cognate anti-sigma factor. Experiments are underway to determine whether residue P35 of σ^E makes direct interactions with ChrR, or if the proline at this position is important for maintaining a structure that is necessary for recognition of σ^E by ChrR.

Information available from the analysis of several anti-sigma factors suggests that these proteins share little sequence similarity, and have different structures when bound to their cognate σ factors.[21,22,30] ChrR also shares little sequence similarity with any of the anti-sigma factors that have been structurally characterized to date, so we believe that its structure when bound to σ^E will be novel. Thus, it will be interesting to determine if *R. sphaeroides* σ^E makes additional contacts with ChrR that were not identified in our analysis. Indeed, ChrR belongs to a recently discovered class of zinc-binding anti-sigma factors common among various α and γ proteobacteria,[15,17,26,44,45] so understanding more about its structure, mechanism of inhibition, and the factors that control its regulation will shed light on a process that is likely to be conserved in the microbial world.

In summary, we have identified one mechanism by which ChrR inhibits *R. sphaeroides* σ^E activity,

Table 1. Bacterial strains and plasmids

	Description	Source or reference
Strains		
R. sphaeroides		
2.4.1	Wild-type	Lab strain
TF18	*rpoEchrR* mutation in 2.4.1	50
E. coli		
DH5-	*supE44 lacu169*(80 *lacZ M15*) *hsdR178 recA1 endA1 gyrA96 thi-1 relA-1*	BRL
VH1000	*pyrE*$^+$*lacI lacZ*	57
S17-1	C600 :: RP-4 2-(Tc :: Mu) (Kn :: Tn7) *thi pro hsdR Hsd* M$^+$ *recA*	58
M15	*lac*$^-$ *ara*$^-$ *gal*$^-$ *mtl*$^-$ *F*$^-$ *rec*A$^-$ *uvr*$^+$	59
Plasmids		
PREP4	*lacI*^{q}; Kn^r	Qiagen
pQE31	N-terminal His6-tag expression vector; Ap^r	Qiagen
pUC19	General cloning vector; Ap^r	60
pUC19rpoE	~550 bp fragment containing *rpoE* in pUC19	50
pBS16	~1.8 kb SalI–SacI fragment containing the *rpoEchrR* operon in pUC19; Ap^r	50
pRK415	Shuttle vector for expression in *R. sphaeroides* RK2 replicon, Mob$^+$; Tc^r	61
PRKK96	Vector for *in vitro* transcription; Ap^r, Sp^r	51
PRKK200	*lacZ*-fusion reporter vector; Kn^r, Sp^r	62
PJDN14	533 bp *rpoE* fragment ligated into pQE31 to create in frame $His_6 - {}^E$ fusion; Ap^r	51
PJDN18	~1.8-Kb *rpoEchrR*-His EcoRI–HindIII restriction fragment cloned into EcoRI–HindIII site of pRK415	51
PJDN30	−39 to +17 *rpoE* P1 promoter fragment cloned into KpnI–StuI sites of pRKK200	51
PJDN34	−39 to +17 *rpoE* P1 promoter fragment ligated into pRKK96; Ap^r, Sp^r	51
PJDN48	pBS16 with addition of *E. coli* ribosome-binding site prior to the ATG start of *chrR*; Ap^r	51
PJRW8	pBS16 containing *rpoE* F40S; Ap^r	This work
PJRW16	pJDN48 digested with EcoRI to remove *chrR*; Ap^r	This work
PJRW19	pJDN48 containing *rpoE* F40S digested with EcoRI to remove *chrR*; Ap^r	This work
PJRW21	pJDN48 containing *rpoR* F81I digested with EcoRI to remove *chrR*; Ap^r	This work
PJRW24	pBS16 containing *rpoE* F81I; Ap^r	This work
PJRW25	pBS16 containing *rpoE* K38E; Ap^r	This work
PJRW27	pJDN48 containing *rpoR* K38E digested with EcoRI to remove *chrR*; Ap^r	This work
PJRW31	pBS16 containing *rpoE* K38R; Ap^r	This work
PJRW33	pJDN48 containing *rpoR* K38R digested with EcoRI to remove *chrR*; Ap^r	This work
PJRW43	pJDN48 containing *rpoR* K38A digested with EcoRI to remove *chrR*; Ap^r	This work
PJRW49	pBS16 containing *rpoE* K38A; Ap^r	This work
PJRW61	pJDN14 containing *rpoE* F40S; Ap^r, Kn^r	This work
PJRW62	pJDN14 containing *rpoE* F81I; Ap^r, Kn^r	This work
PJRW63	pJDN14 containing *rpoE* K38E; Ap^r, Kn^r	This work
PJRW64	pJDN14 containing *rpoE* K38R; Ap^r, Kn^r	This work
PJRW65	pJDN14 containing *rpoE* K38A; Ap^r, Kn^r	This work
PJRW66	pJDN18 containing *rpoE* F40S; Tc^r	This work
PJRW67	pJDN18 containing *rpoE* F81I; Tc^r	This work
PJRW68	pJDN18 containing *rpoE* K38E; Tc^r	This work
PJRW69	pJDN18 containing *rpoE* K38R; Tc^r	This work
PJRW71	pBS16 containing *rpoE* E28A; Ap^r	This work
PJRW72	pBS16 containing *rpoE*Q31A; Ap^r	This work
PJRW73	pBS16 containing *rpoE* P35A; Ap^r	This work
PJRW74	pBS16 containing *rpoE*M42A; Ap^r	This work
PJRW97	pBS16 containing *rpoE* F26A; Ap^r	This work
PJRW98	pBS16 containing *rpoE* L29A; Ap^r	This work
PJRW99	pBS16 containing *rpoE* F30A; Ap^r	This work
PJRW101	pBS16 containing *rpoE* F33A; Ap^r	This work
PJRW103	pBS16 containing *rpoE*V37A; Ap^r	This work
PJRW104	pBS16 containing *rpoE*G39A; Ap^r	This work
PJRW126	pJDN48 containing *rpoR* F26A digested with EcoRI to remove *chrR*; Ap^r	This work
PJRW127	pJDN48 containing *rpoR* E28A digested with EcoRI to remove *chrR*; Ap^r	This work
PJRW128	pJDN48 containing *rpoR* L29A digested with EcoRI to remove *chrR*; Ap^r	This work
PJRW129	pJDN48 containing *rpoR* F30A digested with EcoRI to remove *chrR*; Ap^r	This work
PJRW130	pJDN48 containing *rpoR*Q31A digested with EcoRI to remove *chrR*; Ap^r	This work
PJRW132	pJDN48 containing *rpoR* F33A digested with EcoRI to remove *chrR*; Ap^r	This work
PJRW133	pJDN48 containing *rpoR* P35A digested with EcoRI to remove *chrR*; Ap^r	This work
PJRW135	pJDN48 containing *rpoR*V37A digested with EcoRI to remove *chrR*; Ap^r	This work
PJRW136	pJDN48 containing *rpoR*G39A digested with EcoRI to remove *chrR*; Ap^r	This work
PJRW139	pJDN48 containing *rpoR*M42A digested with EcoRI to remove *chrR*; Ap^r	This work
PJRW144	pJDN14 containing *rpoE* E28A; Ap^r, Kn^r	This work
PJRW145	pJDN14 containing *rpoE*Q31A; Ap^r, Kn^r	This work
PJRW146	pJDN14 containing *rpoE* P35A; Ap^r, Kn^r	This work
PJRW147	pJDN14 containing *rpoE*M42A; Ap^r, Kn^r	This work
PJRW149	pJDN14 containing *rpoE*G39A; Ap^r, Kn^r	This work

Ap^r, ampicillin resistance; Sp^r, spectinomycin resistance; Kn^r, kanamycin resistance; Tc^r, tetracycline resistance.

and have identified region 2.1 as a likely site of interaction on this ECF sigma factor for its cognate anti-sigma. Studies are under way to identify the region(s) of the anti-sigma factor that are required for inhibition of σ^E, to determine the structure of this complex, and to identify the signal which controls the interaction between ChrR and σ^E.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

E. coli strains (Table 1) were grown at 37 °C in Luria–Bertani medium.[46] *R. sphaeroides* strains (Table 1) were grown at 30 °C in Sistrom's succinate-based minimal medium A.[47] When necessary, media was supplemented with 100 μg/ml ampicillin, 25 μg/ml kanamycin, 25 μg/ml spectinomycin, or 1–10 μg/ml tetracycline to maintain plasmids. Sequences of primers used in this study are available upon request.

Purification of *R. sphaeroides* core RNA polymerase, σ^E:ChrR complex, MBP-ChrR, and σ^E

R. sphaeroides core RNA polymerase was purified *via* a combination of affinity chromatography using the polyol-responsive 4RA2 monoclonal antibody (Dr Richard Burgess, Madison, WI) and anion exchange chromatography.[48] A σ^E:ChrR complex, containing either wild-type or mutant σ^E proteins, was purified by Ni^{2+} affinity chromatography from *E. coli* cells containing an intact *rpoEchrR* operon behind an inducible promoter.[48] MBP-ChrR was purified by affinity chromatography as described.[26]

To purify σ^E proteins, we constructed expression plasmids by digesting pBS16 derivatives containing either wild-type σ^E or the various σ^E mutants with BsmFI and BamHI. The resulting 430 bp fragment containing the appropriate *rpoE* was cloned into pJDN14, and introduced into M15pREP4 (QIAGEN, Valencia, CA). Wild-type and mutant σ^E proteins were overexpressed and purified as described.[48]

Gel filtration chromatography

σ^E (4 μM), *R. sphaeroides* core RNA polymerase (0.25 μM), σ^E:ChrR complex (4 μM), σ^E incubated with core RNA polymerase, or σ^E:ChrR complex incubated with core RNA polymerase was mixed in HPLC buffer (25 mM Tris–HCl, pH 7.9, 150 mM NaCl) at room temperature for 30 minutes. Samples were loaded onto a Superdex 200 column (Amersham Pharmacia, Piscataway, NJ) that was equilibrated with HPLC buffer at a flow rate of 0.5 ml/minute for 50 minutes using a System Gold 125NM solvent module connected to a model 168 diode array detector (Beckman Coulter, Fullerton, CA). Apparent molecular masses were estimated by comparison to elution of low and high molecular mass standards (Amersham Pharmacia, Piscataway, NJ). Column eluates were TCA precipitated, separated on a 4–12% Bis-Tris polyacrylamide gel (Invitrogen, Carlsbad, CA), and visualized using the Gelcode Blue system (Pierce, Rockford, IL).

Mutagenesis of *rpoE*

Error-prone PCR was carried out in EasyStart PCR tubes (Molecular BioProducts, San Diego, CA) with 1% (w/v) Triton X-100, using 10 pmol of primers 1212 and 1233 (New England BioLabs, Beverly, MA), 20 ng of pUC19rpoE plasmid (Table 1), 0.5 mM of each dNTP, 1 × Taq DNA polymerase buffer, and 2.5 units Taq DNA polymerase (Promega Corp., Madison, WI). Reactions were allowed to proceed for 35 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for two minutes. PCR products were cloned into pBS16 using restriction sites NdeI and StuI, which were within *rpoE*.

Alanine substitutions within σ^E region 2.1 (^{22}DEAAFA ELFQHFAPKVKGFLMKSGS^{46}) were generated *via* site-directed mutagenesis using the QuickChange Mutagenesis kit (Stratagene, La Jolla, CA) with pBS16 or pJDN48 templates. Genes encoding mutant σ^E proteins were placed in plasmids lacking ChrR by digesting pJDN48 derivatives with EcoRI. All plasmids encoding mutant *rpoE* were confirmed by DNA sequencing, and transformed into VH1000 (ΦλJDN1).

Screening of σ^E proteins for activity in the presence of ChrR

pBS16 plasmids containing wild-type or mutant *rpoE* genes were transformed into the VH1000 tester strain that contains an *rpoE* P1 :: *lacZ* fusion as a lambda lysogen (ΦλJDN1).[26] Strains were plated on MacConkey's media supplemented with 0.5% (w/v) lactose. Colonies that became red after overnight incubation at 37 °C, indicating lactose utilization (and increased σ^E-dependent transcriptional activity) had the *rpoE* gene from their plasmid sequenced using specific primers to determine the nature of the mutation(s).

Mutant *rpoE* genes encoding σ^E proteins with single amino acid changes were cloned into pJDN48 using BsmFI and BamHI restriction digests. This pBS16-derived plasmid increases the abundance of ChrR by placing a strong *E. coli* ribosome-binding site in front of *chrR*.[26] Additional plasmids lacking *chrR* were created by EcoRI digestion of the appropriate pJDN48 derivatives.

Placing σ^E mutant proteins in *R. sphaeroides*

Wild-type or mutant *rpoEchrR* operons were excised from pBS16 *via* an EcoRI and HindIII restriction digest. The resulting 1.8 kb fragments were cloned into pJDN18, a pRK415 derivative that is stable in *R. sphaeroides*. Plasmid constructs were confirmed by DNA sequencing, transformed into *E. coli* S17-1, and placed in *R. sphaeroides* TF18 (pJDN30) by conjugation.[49] *R. sphaeroides* TF18 has a deletion in the *rpoEchrR* operon that renders σ^E and ChrR inactive,[50] so σ^E activity is dependent on the *rpoE* gene within pJDN18. Plasmid pJDN30 contains a *rpoE* P1:*lacZ* transcriptional fusion on a compatible, low copy plasmid used to quantify σ^E activity in *R. sphaeroides*.[51]

β-Galactosidase assays

β-Galactosidase activity assays were performed in triplicate as described.[52] Results from β-galactosidase assays are presented in Miller units.[53]

Modeling of *R. sphaeroides* σ^E region 2.1

The structure of *E. coli* σ^E (PDB accession no. 1OR7) was used as a scaffold to model the position of residues of *R. sphaeroides* σ^E analyzed in this study.[22] The program Deep View—Swiss PDB Viewer[54]† was used to replace the appropriate *E. coli* residues with the corresponding *R. sphaeroides* residues. For reference, *R. sphaeroides* σ^E Glu28, Gln31, Pro35, Lys38, Phe40, Met42, and Phe81 correspond to *E. coli* σ^E Leu24, Val27, His31, Ala34, Leu36, Ser38 and Tyr75.

Figure 4 was generated using DS ViewerPro 5.0 from Accelrys‡. Figure 8 was constructed using helical wheel software§.

In vitro transcription assays

To determine the relative activity of wild-type and mutant σ^E proteins, *in vitro* transcription assays were performed. Increasing concentrations (0–100 nM) of wild-type and mutant His_6-σ^E proteins were added to 50 nM *R. sphaeroides* core RNA polymerase in transcription buffer (40 mM Tris–HCl, pH 7.9, 200 mM KCl, 10 mM Mg acetate, 1 mM DTT, 62.5 μg/ml acetylated BSA). Plasmid pJDN34, containing *rpoE* P1 cloned upstream of a known transcription terminator,[39] was added to a final concentration of 20 nM and the reactions were incubated at 30 °C for 30 minutes. Transcription was initiated with the addition of ribonucleotides at final concentrations of 250 μM GTP, CTP, ATP; 25 μM UTP; and 1 μCi [α-^{32}P]UTP (3000 Ci/mmol). Reactions were incubated at 30 °C for 30 minutes, and terminated with the addition of 95% (w/v) formamide loading buffer.[55] RNA products were analyzed for the amount of σ^E-dependent transcript using 6% (w/v) denaturing PAGE and the Molecular Dynamics phosphorimaging system (Sunnydale, CA).

To determine the effects of MBP-ChrR on the activity of wild-type and mutant σ^E proteins, *in vitro* transcription assays were performed. Increasing amounts of MBP-ChrR (0–500 nM) were added to an assay containing an amount of His_6-σ^E (~25–100 nM) that produced 50% of the maximal activity (see above) in transcription buffer. This mixture was allowed to incubate for 30 minutes at 30 °C. Template (pJDN34, 20 nM) and *R. sphaeroides* core RNA polymerase (50 nM) were added and the mixture was incubated at 30 °C for an additional 30 minutes. Transcription was initiated with the addition of ribonucleotides (see above) and allowed to proceed for 30 minutes at 30 °C. RNA products were analyzed as described above. The amount of σ^E-dependent transcript for each reaction was analyzed using ImageQuant software (Molecular Dynamics, Sunnydale, CA), and data were plotted using Origin 7.0 (OriginLab Corp., Northampton, MA).

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† http://us.expasy.org/spdbv
‡ http://www.accelrys.com/dstudio/ds_viewer
§ http://marqusee9.berkeley.edu/kael/helical.html

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