Mutations in the CRE pocket of bacterial RNA polymerase affect multiple steps of transcription

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ABSTRACT

During transcription, the catalytic core of RNA polymerase (RNAP) must interact with the DNA template with low-sequence specificity to ensure efficient enzyme translocation and RNA extension. Unexpectedly, recent structural studies of bacterial promoter complexes revealed specific interactions between the nontemplate DNA strand at the downstream edge of the transcription bubble (CRE, core recognition element) and a protein pocket formed by core RNAP (CRE pocket). We investigated the roles of these interactions in transcription by analyzing point amino acid substitutions and deletions in Escherichia coli RNAP. The mutations affected multiple steps of transcription, including promoter recognition, RNA elongation and termination. In particular, we showed that interactions of the CRE pocket with a nontemplate guanine immediately downstream of the active center stimulate RNA-hairpin-dependent transcription pausing but not other types of pausing. Thus, conformational changes of the elongation complex induced by nascent RNA can modulate CRE effects on transcription. The results highlight the roles of specific core RNAP–DNA interactions at different steps of RNA synthesis and suggest their importance for transcription regulation in various organisms.

INTRODUCTION

To perform gene transcription, RNA polymerase (RNAP) should recognize promoters sequence-specifically and the DNA template with low specificity to guarantee smooth and efficient RNA elongation. In bacteria, this dilemma is resolved by using a dedicated promoter specificity factor, the σ subunit of RNAP that binds the catalytic core enzyme of RNAP and brings it to a promoter. The classical view of the transcription cycle suggested that after transcription initiation, the σ subunit dissociates and core RNAP continues RNA synthesis until it encounters a transcription termination signal (reviewed in (1,2)).

Subsequent studies demonstrated that transcription elongation is not a monotonous process and can be interrupted by transcription pauses of various nature. The σ subunit does not necessarily dissociate from core RNAP during transcription initiation and can cause pauses through specific interactions with promoter-like motifs in the transcribed DNA (3). Factor-independent transcription pauses may result from backtracking of the elongation complex (EC) promoted by unfavorable DNA sequences (4). In addition, core RNAP can respond to several types of specific pause-inducing signals that include: (i) consensus (or ‘ubiquitous’) pauses that are defined by several nucleotides in the transcription bubble and downstream DNA and result from stabilization of the pre-translocated state of the EC; this is the most widespread type of the pauses in E. coli that were recently revealed by whole-genome RNA sequencing analyses (5,6); (ii) hairpin-dependent pauses (such as hisP) that depend on the formation of a short RNA hairpin in the RNA exit channel of RNAP and participate in coordination of transcription and translation (7,8); (iii) opisP pauses that are recognized by RNAP in the nontemplate DNA strand in the transcription bubble and participate in the recruitment of transcription factor RfaH (7,9); and (iv) U-tract-induced pauses that are proposed to play an important role in transcription termination (reviewed in (10)). The molecular details of specific recognition of these signals are not fully understood.

Recent structural studies of transcription initiation complexes of Thermus thermophilus RNAP confirmed the principal role of the σ subunit in promoter recognition but unexpectedly revealed that core RNAP also participates in specific recognition of the nontemplate DNA strand around the starting point of transcription (positions −4/+2); this DNA segment was designated CRE (core recognition element) (11–13). In particular, a nontemplate guanine at promoter position +2 (+2G) is inserted into a pocket (‘CRE pocket’) formed by two segments of the β subunit (ca. 440–455 and 535–555 amino acid residues, the latter segment also known as ‘fork-loop 2’; E. coli numbering is used un-
MATERIALS AND METHODS

Proteins and DNA templates

Mutant variants of the E. coli rpoB gene were obtained by site-directed polymerase chain reaction (PCR)-mutagenesis in the pIA545 plasmid and recloned into the pLA679 vector encoding all four core RNAP subunits (with a His6-tag in the N-terminus). DNA fragments containing wild-type and mutant variants of T7A1 and rrnB P1 promoters were obtained by PCR using synthetic template and primer oligonucleotides; the rrnB P1* promoter contained a -7G substitution that stabilizes promoter complexes (19). T7A1 promoter followed by λ T2 R2 terminator was obtained as in (20). DNA template containing a fragment of the E. coli rpoB gene fused to the λ Pr promoter for measurements of elongation rates was described previously (21). DNA templates for analysis of hisP pausing were obtained by PCR from plasmid pIA226 containing λ Pr promoter followed by the hisP site. DNA template for analysis of opsP pausing was amplified with synthetic oligonucleotides, cloned into the pGEM-T vector, and the required DNA fragment was obtained by PCR. All promoter sequences are shown in Supplementary Figure S3.

In vitro transcription

For analysis of promoter complex stabilities, holoenzyme RNAPs (50 nM wild-type or mutant core RNAP plus 250 nM σ70 subunit) were incubated with promoter DNA (25 nM) in transcription buffers containing 40 mM Tris-HCl, pH 7.9 and 10 mM MgCl2 with addition of 40 mM or 10 mM KCl for T7A1 and rrnB P1* promoters, respectively, for 10 min at 37°C. Heparin was added to 100 μg/ml and the samples were incubated for different time intervals, followed by the addition of nucleotide substrates: Cpa (25 μM) and uridine triphosphate (UTP) (1 μM, with the addition of α-[32P]-UTP) for wild-type T7A1; Cpa (25 μM) and guanosine triphosphate (GTP) (1 μM, with the addition of α-[32P]-GTP) for T7A1+2G; Cpa, cytidine triphosphate (CTP) (25 μM) and UTP (1 μM, α-[32P]-UTP) for rrnB P1*; Cpa, GTP (25 μM) and UTP (1 μM, α-[32P]-UTP) for rrnB P1*+2G. The reactions were stopped after 1 min by the addition of stop-solution (8M urea, 20 mM EDTA), RNA products were separated by 23% Polyacrylamide gel electrophoresis (PAGE) and quantified by phosphorimaging. Pause efficiencies were calculated as ratios of paused RNAs to the sum of paused and run-off RNA.

DNA fragments containing wild-type and mutant variants of T7A1 and rrnB P1 promoters were obtained by PCR using synthetic template and primer oligonucleotides; the rrnB P1* promoter contained a -7G substitution that stabilizes promoter complexes (19). T7A1 promoter followed by λ T2 R2 terminator was obtained as in (20). DNA template containing a fragment of the E. coli rpoB gene fused to the λ Pr promoter for measurements of elongation rates was described previously (21). DNA templates for analysis of hisP pausing were obtained by PCR from plasmid pIA226 containing λ Pr promoter followed by the hisP site. DNA template for analysis of opsP pausing was amplified with synthetic oligonucleotides, cloned into the pGEM-T vector, and the required DNA fragment was obtained by PCR. All promoter sequences are shown in Supplementary Figure S3.
Figure 1. RNAP–CRE interactions in the open promoter complex. (A) Structure of *T. thermophilus* RNAP with a promoter DNA fragment (4Q4Z, (13)). The β flap and β′ clamp domains are indicated. The σ subunit is not shown. (B) RNAP–DNA interactions at the downstream edge of the transcription bubble. The active site Mg$^{2+}$ ions are shown as purple spheres; the +1 and +2 initiating nucleotides are shown in orange and yellow, respectively. The template and nontemplate DNA strands are black and blue, respectively; DNA nucleotides at promoter position +2 are shown in red, nontemplate thymine at position +1 is dark blue. β subunit segments forming the CRE pocket are shown in green (168–176; 443–451; 533–546; *E. coli* numbering); residues W183, D446 and E546 are shown as stick models.

Analysis of *hisP* pausing on synthetic oligonucleotide scaffolds was performed as described in (22) with modifications. RNA oligonucleotide was 5′-labeled with γ-[32P]-ATP and T4 polynucleotide kinase and mixed with the template DNA strand in transcription buffer (40 mM Tris-HCl, pH 7.9, 10 mM MgCl$_2$, 40 mM KCl) at final concentrations of 500 and 600 nM, respectively. The samples were incubated for 2 min at 95°C and cooled down to 25°C at about 1°C/min. The samples were supplemented with core RNAP (800 nM) and incubated for 15 min at 37°C. Nontemplate DNA oligonucleotide was added (1 μM), the incubation was continued for another 15 min and the samples were diluted 10-fold with the transcription buffer. Antisense RNA (asRNA) oligonucleotide was added to 2 μM when required and incubated for 10 min at 37°C. CTP, UTP and GTP were added to 2 μM and the reaction was stopped after increasing time intervals. A control chase reaction was performed with 500 μM of CTP, UTP and GTP to reveal a background fraction of the complexes that were irreversibly stalled at the pause site. RNA products were analyzed by 19% denaturing PAGE. At each time point, the pause efficiency was calculated as the ratio of the paused RNA product to the sum of the paused and read-through products (minus background values measured in the chase reaction). Observed rate constants for the pause decay and corresponding pause half-life times were calculated by fitting the data to the single-exponential equation.

Analyses of elongation rates and transcription termination were performed on the λP$_R$-rpoB and T7A1-λr2 templates, respectively, as previously described (21). ECs stalled at positions +26 and +20 of the λ P$_R$ and T7A1 promoters were obtained by the addition of corresponding limited substrate sets to preformed promoter complexes at 37°C. For analysis of elongation rates, the samples were transferred to 20°C, transcription was restarted by the addition of all four NTPs (200 μM each) and heparin (15 μg/ml), and the kinetics of full-length RNA synthesis was monitored at 20°C. For analysis of transcription termination, NTPs were added to 10 μM together with heparin (15 μg/ml) and transcription was performed for 5 min at 37°C, resulting in synthesis of 100 nt terminated and 150 nt run-off RNAs. Reactions were terminated by the addition of stop-solution and RNA products were analyzed by 10% denaturing PAGE. Termination efficiencies are shown in percent of RNA transcripts released at the point of termination relative to the sum of terminated and run-off RNAs.

Cell viability assays

For analysis of in vivo effects of the rpoB mutations, *E. coli* cells of strain RL585 bearing a temperature-sensitive chromosomal rpoB allele (23) were transformed with an empty pBAD vector or with pBAD-based plasmids expressing wild-type and mutant rpoB alleles under the control of inducible arabinose promoter. The cells were plated on Petri dishes containing LB agar with addition of 2% glucose and 200 μg/ml ampicillin and incubated for 2–4 days at 30°C. Single colonies were grown in 1 ml of liquid LB for 20 h, diluted to OD$_{600} = 0.4$ followed by serial dilutions, put on LB plates containing 0.2% arabinose and 200 μg/ml ampicillin, and incubated for 2–3 days at 30 or 42°C.

Analysis of the effects of the mutations on rifampicin (Rif) sensitivity was performed in *E. coli* strain DH 5α. The cells were transformed with the same plasmids (but without the addition of glucose), single colonies were picked-up and grown overnight, the cultures were diluted to OD$_{600} = 0.4$ and put on LB plates containing 0.2% arabinose, 200 μg/ml ampicillin and 0, 20 or 50 μg/ml Rif. The plates were incubated for 2 days at 37°C.
RESULTS

We obtained the following mutations in the CRE pocket of *E. coli* RNAP (Figure 1B and Supplementary Figure S1): (i) alanine substitutions of residues D446 and E546; residue D446 is directly involved in specific interactions with non-template +2G in promoter complexes; residue E546 is adjacent to D446 and can potentially participate in such interactions on its different positioning in various RNAP structures (11,13,24,25); (ii) alanine substitution of residue W183 that stacks with the +1 non-template promoter nucleotide; and (iii) deletions of the two β subunit segments that form the CRE pocket, Δ444–451 (substituted with five glycine residues) and Δ533–546 (substituted with three glycine residues). All mutant variants of the rpoB gene were cloned into expression plasmids encoding all core RNAP subunits, and corresponding RNAPs were expressed and purified from *E. coli* cells (see the ‘Materials and Methods’ section for details).

Effects of CRE pocket mutations on promoter complex stability

We first tested the effects of the RNAP mutations on promoter binding to confirm the proposed role of the CRE pocket in specific DNA recognition. Previously, the +2G residue in the non-template DNA strand was shown to increase stability of RNAP complexes with synthetic DNA scaffolds corresponding to the downstream part of the promoter bubble, in comparison with other three nucleotide variants (11). Furthermore, several mutations in the CRE pocket impaired transcription from a strong β promoter, which forms very stable complexes with RNAP. However, other substitutions, including D446A, had only minor effects on RNAP activity (11). Thus, to highlight the role of the RNAP-CRE interactions in promoter complex formation, we analyzed the effects of the CRE-pocket mutations on the open complex stability using promoters that form less stable complexes with RNAP and their mutant variants with substitutions at position +2.

We found that the +2T→G substitution significantly increased the half-life time of the open promoter complex (RP₀) formed by wild-type RNAP on the T7A1 promoter (t₁/₂ = 260 and 660 s, respectively; Figure 2 and Table 1). Similarly, a +2G variant of the rrnB P₁* promoter was more stably bound by RNAP than the wild-type +2C promoter (t₁/₂ = 14 and 25 s; Table 1). Therefore, guanine at position +2 increases the stability of complexes formed by RNAP on natural promoters.

We further measured RP₀ stabilities for mutant RNAPs with amino acid substitutions and deletions in the CRE pocket. The E546A substitution did not affect promoter complex stability. D446A and W183A substitutions significantly decreased the RP₀ half-lives (t₁/₂ = 100 and 49 s for wild-type T7A1 promoter, respectively) and both deletions dramatically destabilized the complex (t₁/₂ < 10 s; Table 1). Similarly, several other mutations in the same regions of the β subunit were previously shown to decrease promoter complex stabilities on various promoters (Supplementary Figure S1, see Discussion) (26–28).

![Figure 2](https://academic.oup.com/nar/article-abstract/43/12/5798/2902728)

Figure 2. Effects of mutations in the CRE pocket on promoter complex stability. (A) Outline of experiment. Heparin was added to preformed promoter complexes, followed by nucleotide addition after increasing time intervals. The reactions were performed with CpA+(UTP/GTP) in the case of T7A1 and CpA+(GTP/CTP)+UTP in the case of rrnB P₁* promoter variants (with addition of α[32P]UTP), resulting in the synthesis of 3 and 4 nt RNA products, respectively. (B) Kinetics of promoter complex dissociation for the wild-type and +2G T7A1 promoter variants, measured for wild-type and D446A RNAPs. Averages and standard deviations from two to three independent experiments are shown; the lines correspond to single-exponential fits of the data.

In contrast to wild-type RNAP, the +2T→G substitution in the T7A1 promoter did not increase promoter complex stability in the case of D446A RNAP and only slightly increased it in the case of E546A RNAP (Figure 2B and Table 1). At the same time, promoter complex stability was still increased in the case of the W183A mutant. Therefore, in agreement with structure-based predictions (11), specific interactions of residues D446 and E546 with +2G appear to be involved in stabilization of promoter complexes.

General effects of CRE-pocket mutations on transcription elongation and termination

Our next goal was to reveal the effects of the CRE-pocket mutations on the elongation step of transcription. We first measured average elongation rates for mutant RNAPs using a ~500 bp *E. coli* rpoB template fused to the MPK promoter. ECs stalled at the +26 promoter position were chased with all four NTPs and the kinetics of the run-off RNA synthesis was analyzed (Figure 3 and Supplementary Figure S4). The D446A and W183A substitutions did not significantly change the average rate of RNA synthesis, the E546A substitution increased it ∼2-fold, while the Δ444–451 deletion slightly decreased the rate and the Δ533–546 deletion dramatically impaired RNA elongation.

Previously, the D446A substitution was shown to increase consensus (ubiquitous) pausing at most genome positions (6). However, careful inspection of the RNA products synthesized during transcription of the rpoB template revealed that this substitution stimulated pausing at some sites (probably, corresponding to consensus pause motifs)
Table 1. Promoter complex stabilities of wild-type and mutant E. coli RNAP variants

| RNAP       | Promoter       | $t_{1/2}$ (s) | Rel. |
|------------|----------------|--------------|------|
| WT         | T7A1           | 257 ± 5      | 1    |
|            | T7A1+2G        | 662 ± 45     | 2.6  |
|            | $rrnB$ P1*     | 14 ± 3       | 1    |
|            | $rrnB$ P1*+2G | 25 ± 1       | 1.8  |
| D446A      | T7A1           | 104 ± 9      | 0.40 |
|            | T7A1+2G        | 107 ± 15     | 0.42 |
|            | 245 ± 30       | 0.96         |
| E546A      | T7A1           | 356 ± 13     | 1.4  |
|            | T7A1+2G        | 94 ± 22      | 0.37 |
| W183A      | T7A1           | < 10         | < 0.04|
|            | T7A1+2G        | < 10         | < 0.04|
| $\Delta 443-451$ | T7A1     | < 10         | < 0.04|
| $\Delta 533-546$ | T7A1     | < 10         | < 0.04|

Promoter complex half-life times were determined from single exponential fits of the heparin challenge experiment shown in Figure 2. Wild-type (WT) T7A1 and $rrnB$ P1 promoters contain T and C at position +2, respectively. A -7G variant of the $rrnB$ P1 promoter ($rrnB$ P1*) was used to increase promoter complex stability (19). See Supplementary Figure S3 for all promoter sequences. The last column shows $t_{1/2}$ values relative to WT RNAP.

Table 2. Efficiencies of transcription termination at the $\lambda$ tR2 terminator by wild-type and mutant RNAPs

| RNAP       | tR2 (%) | Rel. |
|------------|---------|------|
| WT         | 50 ± 1  | 1    |
| D446A      | 34 ± 1  | 0.68 |
| E546A      | 41 ± 3  | 0.82 |
| W183A      | 53 ± 6  | 1.1  |
| $\Delta 443-451$ | 52 ± 5 | 1.0  |
| $\Delta 533-546$ | 69 ± 3 | 1.4  |

The last column shows termination efficiencies relative to wild-type RNAP.

Role of the CRE pocket in hairpin-dependent pausing

The observed effects of the CRE-pocket mutations on transcription elongation and termination prompted us to more thoroughly investigate their effects on transcription pausing which plays essential roles in both processes. We focused on the analysis of the D446A and E546A substitutions that could affect specific recognition of the nontemplate guanine residue in the CRE pocket.

To reveal mechanistic differences in the effects of the CRE-pocket mutations on the recognition of various types of pause signals, we analyzed pausing at two specific pause sites, $hisP$ (stabilized by RNA hairpin formation) and $opsP$ (stabilized by RNAP interactions with the nontemplate DNA strand) (7). Importantly, both sites share some sequence features with the consensus pause site; in particular, both contain guanine nucleotides at positions immediately downstream of the pause sites (+1G, corresponds to +2G in promoter complexes in respect to the position of the RNAP active site; Figure 4A and Supplementary Figure S7A, see below).
We first compared pause-inducing properties of wild-type hisP site and its variants with substitutions of +1G and +2G with C (cytosine substitutions were chosen to preserve the stability of DNA duplex at corresponding positions). The +1G→C substitution significantly reduced the pause half-life time for wild-type RNAP (from 64.0 to 11.7 s; Figure 4 and Table 3). This agrees with previous reports showing that downstream DNA sequences contribute to hairpin-dependent pausing (29–31). The effect was specific for the +1 position since the +2G→C substitution did not decrease but slightly increased it (∼1.5 fold; Table 3).

Mutations in the CRE pocket had distinct effects on hisP pausing. Both the D446A and E546A substitutions significantly decreased the pause half-life times (about 2.5-fold; Table 3). This contrasts previously reported stimulatory effect of the D446A substitution on consensus pausing (6). At the same time, the W183A substitution did not significantly affect pausing (t1/2 = 69.5 s), suggesting that this residue does not play an important role in the hisP recognition.

We then tested whether RNAPs with mutations of D446 and E546 are responsive to nucleotide substitutions in the hisP pause signal. Remarkably, the +1G→C substitution had significantly weaker effects on pausing by mutant RNAPs in comparison with the wild-type enzyme. The pause half-life times were reduced ∼1.8- and 3-fold for the D446 and E546 mutants, in comparison with the 5.5-fold change observed for wild-type RNAP (Figure 4C and Table 3). Again, this effect was specific for the +1 position since the +2G→C substitution did not decrease but stimulated hisP pausing by mutant RNAPs to the same extent as in the case of wild-type RNAP (∼1.5-fold; Table 3).

Furthermore, when analyzed on the +1C template, the D446A substitution did not decrease the pause half-life but even increased it in comparison with the wild-type RNAP (13.9 versus 11.7 s; Figure 4C and Table 3). Thus, the deleterious effect of this mutation on pausing at the wild-type hisP site can be fully attributed to the loss of interactions of the D446 residue with +1G. Similarly, the E546A substitution had a weaker effect on pausing at the +1C hisP site relative to wild-type RNAP (∼1.4-fold versus 2.6-fold differences in the pause half-life times on the wild-type template).

Importantly, neither the +1C substitution nor the D446A mutation significantly affected the pausing efficiency since the maximal pausing, measured at the first time point (15 s,
which roughly corresponds to the time required for RNAP to reach the hisP site on this DNA template, was comparable for all reactions (80–85%; Figure 4C). Thus, interactions with +1 guanine are likely important for stabilization of the paused state but not for initial recognition of the pause-inducing signal. Overall, the role of CRE-pocket–DNA interactions in the recognition of the hisP signal is apparently different from that in consensus pausing, which is suppressed by these interactions (6).

### Role of the RNA hairpin in CRE-dependent pausing

One important difference between consensus and hisP pauses is the presence of a folded RNA hairpin in the RNA exit channel of RNAP in the latter case. The hairpin was shown to induce long-range conformational changes in RNAP, resulting in clamp opening and preventing EC translocation ((8,32); see the ‘Discussion’ section). To understand a possible role of the hairpin in the modulation of the RNAP–CRE response, we employed a recently described oligonucleotide-based pausing assay (8,22). In this assay, EC is assembled on a scaffold based on the hisP sequence but lacking the RNA hairpin, followed by addition of NTP substrates, either in the absence or presence of complementary asRNA (Figure 5A and B).

In agreement with published studies (8,22), we found that RNA duplex greatly increases both the pause half-life time \( t_{1/2} \) and the pause efficiency \( P_{max} = 58.3 \) and \( 85.9\% \) for wild-type RNAP. Similarly to the wild-type hisP template, the D446A mutation decreased the pause half-life in the presence of the RNA duplex (although the effect was less dramatic; \( t_{1/2} = 34.0 \) s). In contrast, the same mutation did not decrease but even slightly increased the pause duration in the absence of asRNA \( t_{1/2} = 13.3 \) s; Figure 5C and D). We therefore conclude that the difference in the RNAP response to +1G in the two types of the pause-inducing signals is mediated by the RNA hairpin-induced changes in the EC structure (see the ‘Discussion’ section).

Since the D446A substitution suppressed hisP pausing we proposed that it may promote forward translocation of the paused complex, which was shown to predominantly adopt the pre-translocated state (8,33). To test the effects of the D446A substitution on translocation, we compared the rates of RNA pyrophosphorylation by wild-type and mutant RNAPs in ECs assembled on the synthetic scaffold and stalled at the pause site (Supplementary Figure S6). Surprisingly, we observed that the D446A substitution increased the reaction rate (Supplementary Figure S6C and D). At the same time, wild-type and D446A RNAPs revealed similar dependencies of the reaction rates on the pyrophosphate concentration (Supplementary Figure S6E) suggesting that the mutation did not significantly affect pyrophosphate binding. Therefore, the mutation might either favor the pre-translocated EC conformation or facilitate the pyrophosphorolysis reaction in the RNAP active center (see the ‘Discussion’ section).

### Effect of the D446A substitution on opsP pausing

We next analyzed RNAP pausing at the opsP site, a 12-nt sequence motif that also has similarities with the consensus pause motif and induces pausing at two positions, before the last nucleotide of the site and one nucleotide downstream of it (Supplementary Figure S7). Both pausing positions are followed by guanines in the non-template strand, suggesting that they might also be recognized by the CRE pocket of RNAP. However, in contrast to the hisP signal, no RNA hairpin is formed during pausing at the opsP site.

Comparison of the pausing kinetics for wild-type and D446A RNAPs revealed that the mutation significantly stimulated pausing at the upstream position (37P in Supplementary Figure S7C and D) and had essentially no effect on downstream pausing (39P). This contrasts hisP-pausing that is suppressed by the D446A substitution. The mechanism of the 37P pause may therefore be similar to consensus pausing (in accord with the sequence similarities between these two sites, Supplementary Figure S7A) that is also stimulated by the D446A substitution (6). At the same time, the mechanism of pausing at the 39P site may be different; indeed, a significant fraction of the downstream paused complex was shown to enter a backtrack state, susceptible to Gre-induced RNA cleavage (34).

### In vivo effects of the CRE-pocket mutations

Finally, we tested whether the mutant RNAP variants can support cell growth when introduced into a temperature-sensitive E. coli strain with defects in expression of chromosomal rpoB allele (RL585; (23)). Expression of wild-type plasmid-encoded rpoB restores the growth of this strain under restrictive conditions (at 42°C; Figure 6A). The D446A and E546A mutants are also viable suggesting that these mutations do not dramatically perturb cellular RNAP function. At the same time, deletions in the CRE pocket (Δ443–451 and Δ533–546) do not support cell growth under these conditions.
Figure 5. Role of RNA duplex in RNAP pausing at the hisP site. (A) Structure of the scaffold template used in the experiments. RNA is red, with the four 3′-terminal nucleotides added during transcription shown in lowercase letters. Positions of the 3′-end in the starting, paused and read-through (R) transcripts are indicated. Antisense RNA (asRNA) oligonucleotide is orange. The +1 G:C base pair is shown in yellow. (B) Outline of experiment. (C) Pausing kinetics measured in the absence or presence of asRNA for wild-type and D446A RNAPs. (D) Pause half-life times and pause efficiencies ($P_{\text{max}}$, predicted pausing at zero time point) determined from the pause decay curves.

conditions. Moreover, the cells containing these rpoB alleles revealed pronounced growth defects even at permissive temperature (30°C) (Figure 6B) suggesting that both deletions are toxic when expressed in vivo.

The CRE pocket is located close to the Rif pocket also formed by the β subunit, and some mutations of Rif resistance were mapped within fork-loop2 and adjacent regions (Supplementary Figure S1). We therefore tested the effects of the mutations in the CRE pocket on Rif resistance of a sensitive E. coli DH5α strain. Expression of the wild-type, D446A and E546β subunit variants did not change its Rif sensitivity (Figure 6B). Surprisingly, however, the Δ533–546 cells could grow at elevated Rif concentrations (50 μg/ml) demonstrating that RNAP with this deletion can support cell growth despite its toxicity in the RL585 strain (see above) (Figure 6A). The observed differences may be likely explained by the decreased viability of the temperature-sensitive RL585 strain (23). The Δ443–451 cells also revealed low level of Rif resistance (20 μg/ml). The resistance of these deletion alleles of rpoB is consistent with the localization of some known Rif-resistance mutations in the deleted regions (amino acid substitutions at positions 447, 448, 533, 534, 536, 537; deletions Δ531–533, Δ535–542; Supplementary Figure S1) (26,35–38) and the involvement of fork-loop2 in direct interactions with Rif (35,36).

DISCUSSION

Our results suggest that sequence-specific interactions of the core enzyme of bacterial RNAP with the downstream edge of the transcription bubble play important roles at dif-
different steps of transcription and, in particular, make different contributions to different types of transcription pausing. The roles of these interactions in transcription is illustrated in Figure 7. As is shown in the figure, the active center of RNAP contains two functional sites, $i$ and $i+1$ that accommodate the NTP and RNA substrates during transcription, and can adopt two principal conformations, pre- and post-translocated. In the RP$_o$, the $i$ and $i+1$ sites bind the two initiating NTPs corresponding to promoter positions +1 and +2, respectively. Similarly, in the post-translocated EC these sites accommodate the 3'-end of RNA and the incoming NTP (at position +1 relative to the RNA 3'-end). Following nucleotide addition, EC occurs in the pre-translocated conformation in which both sites are occupied by nascent RNA. Available data suggest that the interactions of the CRE pocket with the nontemplate DNA strand can affect the conformational state of the RNAP active center and EC translocation.

During promoter recognition, the nontemplate +2G base, if present in the promoter sequence, can be accommodated in the CRE pocket and this increases the open complex stability (Figure 7A; (11) and this work). Residues D446 and E546 likely make direct contribution to these interactions since their substitutions abolish the stimulating effect of the +2G residue on promoter binding. The +2G position in promoter complexes corresponds to the $i+1$ site of the RNAP active center that accommodates the +2 NTP substrate. Thus, such interactions may also play a role in positioning of the template DNA strand in the correct register. In addition, the CRE pocket as a whole plays an important role in the RP$_o$ formation since many mutations in this region including those studied in this work destabilize RNAP-promoter complexes, and some of them mimic the effects of small alarmone ppGpp and its cofactor DksA on transcription (Supplementary Figure S1, (26–28,39)). This may be explained by the loss of favorable interactions of this pocket with the nontemplate DNA strand. Indeed, previously studied deletion $\Delta$436–445 in the $E. coli$ $\beta$ subunit, which overlaps with the $\Delta$443–451 deletion studied in this work, was shown to impair melting of the downstream part of the transcription bubble around the starting point of transcription, likely due to the loss of RNAP–DNA interactions in this region (28). Substitution of residue W183, which is conserved in most bacteria (Supplementary Figure S1), also destabilizes promoter complexes suggesting that its stacking interactions with the +1 nontemplate nucleotide observed in the X-ray structures are important for open complex formation. Interestingly, recent analysis of the effects of several W183 mutations on transcription initiation by $E. coli$ $\sigma^{54}$ holoenzyme RNAP revealed no defects in promoter complex stability but suggested that this residue is involved in initial steps of RNA synthesis and RNAP translocation (40). It remains to be established whether it might play similar roles during transcription initiation by the $\sigma^{70}$ holoenzyme.

During transcription elongation, the interactions of the CRE pocket with the DNA template likely modulate transcription pausing. Depending on the type of the pause, these interactions can either suppress or stimulate RNA elongation. In the case of the consensus pauses, which are stabilized in the pre-translocated state because of an unfavorable DNA sequence (5,6), the binding of the +1G in the CRE pocket was proposed to stimulate forward RNAP translocation by stabilizing the post-translocated EC conformation (Figure 7B and C). Interestingly, +1G by itself promotes pausing and prevents RNAP translocation, likely because the G:C base pair is more difficult to melt when present in the $i+2$ position in the pre-translocated complex, and the template C is poorly accommodated in the $i+1$ site after translocation (41). Similarly, $opsP$ pausing, which is dependent on the nontemplate DNA strand recognition, may be alleviated by the interactions of the CRE pocket with nontemplate guanine immediately downstream of one of the two major pausing positions (Supplementary Figure S7).

In contrast to the previously proposed antipausing role of the RNAP–CRE interactions (6), these interactions stimulate hairpin-induced pausing, which also occurs in the pre-translocated EC conformation (8,33). In particular, we demonstrated that substitutions of residues D446 and E546 in the CRE pocket significantly decrease the pause half-lives, depending on the presence of +1G in the pause se-
demonstrated that $+1_G$ in the hisP promoter preferentially adopts a frayed conformation in the $i+1$ site, thus preventing its further extension (8). Clamping may occur only transiently (8). For +1G to be melted and accommodated in the CRE pocket in the DNA strand in the melted region, thus allowing +1G to be bound in the CRE pocket in the pre-translocated conformation defined by several conserved nucleotides, including a G:C pair at the upstream boundary of the RNA:DNA hybrid, pyrimidine:purine pair (Y:R) in the $i+1$ site and a G:C pair downstream of the active site. Interactions of +1G with the CRE pocket promote forward EC translocation and suppress consensus pausing. RNA hairpins or duplexes formed in the RNA exit channel modulate the effects of +1G/CRE-pocket interactions, likely through changes in the clamp conformation and positions of the downstream duplex and the nontemplate DNA strand. This results in stabilization of the paused state, either by preventing EC translocation (upper) or by inhibiting nucleotide addition in the post-translocated state (lower).

First, clamp opening and associated changes during translocation alter the position of the downstream DNA duplex in the RNA exit channel of RNAP. Previously, the RNA hairpin, which folds under the flap domain of RNAP, was shown to increase the pause half-life by inducing clamp-opening and preventing the trigger loop folding in the active center and RNAP translocation (8,32). In addition, the RNA 3′-end in the paused complex was proposed to adopt a frayed conformation in the $i+1$ site, thus preventing its further extension (33). We speculate that these conformational changes might modulate the effects of +1G on pausing in two alternative ways.

Second, +1G may be bound in the CRE pocket in the post-translocated EC conformation that may be transiently formed in the paused complex. This binding may help to prevent NTP addition in the $i+1$ site by affecting the conformation of the adjacent elements of the active center (Figure 7D, lower), including the trigger loop and the bridge helix, which directly contacts the flexible fork-loop 2 region forming a part of the CRE pocket (Figure 1B). The EC in the elemental paused state, which is proposed to precede other pausing events, was shown to adopt a post-translocated conformation in which the $i+1$ site is partially occluded by the kinked bridge helix (15). The interactions of +1G with the CRE pocket may contribute to such unproductive conformation thus favoring backward RNAP translocation over nucleotide addition. In support of this idea, our data on stimulation of pyrophosphorolysis at the pause site by the D446A substitution suggest that in wild-type RNAP the interactions of residue D446 with +1G actually promote forward RNAP translocation, similarly to consensus pausing. However, in contrast to consensus pausing, the hairpin formation likely stabilizes inactive active center conformation and shifts the equilibrium toward the pre-translocated state with frayed RNA (8,15).

The CRE pocket also likely plays a general role in transcription elongation since CRE-pocket mutations significantly affected the rate of RNA synthesis by bacterial and eukaryotic RNAPs (42,43) and this work). Furthermore, β subunit substitutions D444G and H447R in the CRE pocket in E. coli RNAP were proposed to destabilize transcription complexes during transcription-replication collisions (44). We observed that the D446A substitution decreased hisP pausing but stimulated other types of transcription pauses (see above). At the same time, the E546A substitution suppressed most transcription pauses suggesting that this residue may contribute to various types of pausing through direct contacts with DNA (as proposed for hisP pausing) and/or by affecting the fork-loop 2 conformation. The most dramatic changes in RNA elongation were observed in the case of the Δ533–546 deletion that significantly increased transcription pausing. The observed effects can be explained by either changes in RNAP interactions with the nontemplate DNA strand or indirect effects of the mutations on the active site conformation (see above).

The mutations in the CRE pocket also affected intrinsic transcription termination, probably as a result of al-

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**Figure 7.** CRE–RNAP interactions at different steps of transcription. RNAP is schematically shown as a yellow oval; the CRE pocket is green; the β flap is shown as a blue semi-oval; the downstream DNA binding channel is gray; the nontemplate guanine downstream of the active site is red. The $i, i+1$ and $i+2$ positions are indicated. (A) +2G bound in the CRE pocket stabilizes the open promoter complex. (B) Consensus pause complexes are stabilized in the pre-translocated conformation defined by several conserved nucleotides, including a G:C pair at the upstream boundary of the RNA:DNA hybrid, pyrimidine:purine pair (Y:R) in the $i+1$ site and a G:C pair downstream of the active site. (C) Interactions of +1G with the CRE pocket promote forward EC translocation and suppress consensus pausing. (D) RNA hairpins or duplexes formed in the RNA exit channel modulate the effects of +1G/CRE-pocket interactions, likely through changes in the clamp conformation and positions of the downstream duplex and the nontemplate DNA strand. This results in stabilization of the paused state, either by preventing EC translocation (upper) or by inhibiting nucleotide addition in the post-translocated state (lower).
tered pausing: indeed, the termination effects of the mutations correlated with their effects on hairpin-dependent pausing. In particular, suppression of termination by the Δ446A and E546A substitutions indicates that contacts of these residues with DNA may be important for termination-associated pausing and/or subsequent EC dissociation. It remains to be established whether the RNAP–CRE interactions may play a stimulatory or an inhibitory role in the case of other types of transcription pauses and related regulatory events, including factor-dependent pausing and termination.

Given considerable level of evolutionary conservation of the CRE pocket (Supplementary Figure S1), its regulatory functions in DNA recognition likely extend to eukaryotic homologs of bacterial RNAP. In particular, it was noted that S. cerevisiae RNAPII can also accommodate a non-template base in this pocket (Supplementary Figure S2C) (11,45). Mutations in the fork-loop 2 region in S. cerevisiae RNAPII were shown to affect RNAPII interactions with downstream DNA and EC translocation (42). Furthermore, substitution D370A in the second largest subunit of S. cerevisiae RNAPIII was shown to decrease termination (46), similarly to the equivalent Δ446A substitution in E. coli RNAP.

In accordance with their significant effects on various steps of transcription, many studied mutations in the CRE pocket have pronounced in vivo phenotypes including changes in stringent response and antibiotic sensitivity (this work, (26,27,37)). Even the Δ533–546 deletion that revealed dramatic changes in promoter binding, RNA elongation and termination, and was toxic under certain conditions in vivo could nevertheless confer Rif-resistance to the bacterial cells. Thus, the CRE pocket is an evolvable RNAP determinant that plays important roles in transcription regulation and antibiotic resistance and may be a target for rational design of RNAP variants with altered DNA specificities.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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