σ^{38}-dependent promoter-proximal pausing by bacterial RNA polymerase

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ABSTRACT

Transcription initiation by bacterial RNA polymerase (RNAP) requires a variable σ subunit that directs it to promoters for site-specific priming of RNA synthesis. The principal σ subunit responsible for expression of house-keeping genes can bind the transcription elongation complex after initiation and induce RNAP pausing through specific interactions with promoter-like motifs in transcribed DNA. We show that the stationary phase and stress response σ^{38} subunit can also induce pausing by *Escherichia coli* RNAP on DNA templates containing promoter-like motifs in the transcribed regions. The pausing depends on σ^{38} contacts with the DNA template and RNAP core enzyme and results in formation of backtracked transcription elongation complexes, which can be reactivated by Gre factors that induce RNA cleavage by RNAP. Our data suggest that σ^{38} can bind the transcription elongation complex *in trans* but likely acts *in cis* during transcription initiation, by staying bound to RNAP and recognizing promoter-proximal pause signals. Analysis of σ^{38}-dependent promoters reveals that a substantial fraction of them contain potential pause-inducing motifs, suggesting that σ^{38}-depended pausing may be a common phenomenon in bacterial transcription.

INTRODUCTION

The σ subunit of RNA polymerase (RNAP) is the main transcription initiation factor in bacteria, which is involved in promoter recognition, DNA melting and RNA priming by the RNAP holoenzyme (1). The principal σ subunit (σ^{70} in *Escherichia coli*) recognizes two primary promoter elements, the −10 (TATAAT, or extended TGnTATAAT) and −35 (TTGACA) motifs, which are bound by σ conserved regions 2 and 4, respectively. During transition to transcription elongation, the σ-DNA contacts must be broken to allow RNAP escape from the promoter. Furthermore, regions 3 and 4 of the σ subunit, which in the open promoter complex occupy the RNA exit path in RNAP, must be displaced by nascent RNA to allow its extension (2–6). Nevertheless, the principal σ^{70} subunit of *E. coli* was shown to remain bound to at least a fraction of transcription elongation complexes (TECs) both *in vitro* and *in vivo* (7–13) and to recognize promoter-like motifs in transcribed DNA, leading to transcriptional pausing (reviewed in (14)). The primary contacts responsible for pause formation occur between σ region 2 and −10-like motifs in the nontemplate DNA strand (15), although the extended −10 (TG) (16) and −35-like motifs (17) were also shown to contribute to σ-dependent pausing.

The σ^{70}-dependent pauses were initially discovered in promoter-proximal regions of lambdoid phage late genes, where they serve to recruit an antitermination protein Q responsible for efficient transcription of distal operon genes (15,17–19). Later, σ^{70}-dependent pauses were detected in initially transcribed regions of many cellular genes (20–22), but their functional role remains unknown, except the notion that they increase σ^{70} retention in TECs during transcription of downstream genes (8,23), which may in turn affect the binding of other elongation factors that interact with the same RNAP site, such as NusG and RfaH (10,24). Although early studies suggested that σ^{70} primarily induces pausing *in cis*, by staying bound to RNAP after transcription initiation (19), later reports demonstrated that it can also access the TEC *in trans* both *in vitro* and *in vivo* (16,22,24–26), even at genes transcribed from promoters recognized by alternative σ subunits (26).

Analysis of the mechanisms of pause formation revealed formation of stressed TECs, in which several nucleotides of downstream DNA are 'scrunched' within RNAP due to ongoing transcription while the upstream part of the TEC remains fixed on the DNA template through specific σ-DNA interactions (16,27–29). This stress may be relieved either through breaking the σ-DNA contacts and pause escape or TEC backtracking, which is manifested by sensitivity of paused TECs to Gre-factors that induce RNA cleavage by RNAP in backtracked complexes (16,21,22,27,29,30). Following RNA cleavage, the reactivated TEC can enter the

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next cycle of RNA elongation and pausing, similarly to abortive cycling of RNAP in promoter complexes during transcription initiation (16,27,29).

In contrast to σ^{70}, the ability of most alternative σ subunits to induce pausing remained unexplored. The only σ that was studied, the main stationary phase and stress response σ^{38} factor in *E. coli* did not induce pausing on natural lambdoid phage (19) or semi-synthetic promoter templates containing downstream consensus pause-inducing motifs (24), even though its promoter specificity is highly similar to the σ^{70} subunit (31–33). Furthermore, activator-dependent σ^{54} factor was shown to be rapidly released from RNAP during transcription initiation (34), in contrast to stochastic release of σ^{70} during transcription elongation (8,10,12). Measurements of relative affinities of alternative σ subunits to RNAP revealed that they bind the core enzyme with 2- to 15-fold higher apparent K_ds in comparison with σ^{70}, with σ^{38} having the lowest affinity (35,36). Despite being most related to σ^{70} among other *E. coli* σ factors, σ^{38} contains amino acid substitutions at the core binding interface in σ region 2.2, and substitutions of neighboring residues in σ^{70} were shown to reduce σ-dependent pausing (Figure 1) (24,37). At the same time, σ^{38} and σ^{70} were shown to recognize almost identical promoter sequences and recent structural analysis of a σ^{38}-RNAP open promoter complex revealed that σ contacts with DNA and core RNAP are strikingly similar to the contacts of the principal σ subunit (Figure 1B) (38).

In this study, we demonstrate that σ^{38} can induce efficient pausing by *E. coli* RNAP on both synthetic and natural DNA templates *in vitro*, define the conditions for σ^{38}-dependent pausing and hypothesize that it might play a role in transcription regulation *in vivo*.

MATERIALS AND METHODS

Proteins and DNA templates

Escherichia coli core RNAP containing a His$_6$-tag in the N-terminus of the β subunit was expressed in *E. coli* cells from the pIA679 plasmid and purified as described (39). The *rpoS* gene encoding the *E. coli* σ^{38} subunit was cloned into the pET29 vector (between the NdeI and XhoI sites, without His-tag). The L117F mutation and region 3.2 deletion (Δ228-234) in σ^{38} were obtained by site-directed mutagenesis. All subunit variants were expressed in *E. coli* BL21(DE3) and purified by MonoQ chromatography of renatured inclusion bodies (40). The *E. coli* σ^{70} subunit was obtained as described previously (41). GreA and GreB factors were expressed from corresponding pET28 plasmids and purified as described in (42).

Nucleic acid scaffolds used for pause analysis were assembled from synthetic DNA and RNA oligonucleotides (DNA Synthesis and Eurogene, Moscow). DNA fragments containing wild-type and mutant variants of the *adhE* P1 (positions −90 to +48 relative to the start of transcription) and *ecnB* P promoters (positions −90 to +40) were obtained by PCR using genomic or synthetic DNA templates and primer oligonucleotides.

In vitro transcription

Analysis of σ-dependent pausing in synthetic TECs assembled on oligonucleotide scaffolds was performed as described in (16). RNA oligonucleotide was 5′-labeled with γ-[^{32}P]-ATP and T4 polynucleotide kinase and mixed with the template DNA strand in buffer containing 40 mM Tris–HCl, pH 7.9 and 40 mM KCl at final concentrations 250 nM and 2.5 μM, respectively. The samples were incubated for 3 min at 65 °C, cooled down to 25 °C at about 1°C/min, diluted 3-fold in the same buffer, supplemented with core RNAP (190 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 5-fold with the same buffer. The σ subunit was added to 2.5 μM, unless otherwise indicated, and the samples were incubated for another 5 min. MgCl$_2$ (10 mM final concentration) and NTP substrates (100 μM each for the consensus scaffold; 200 μM ATP, GTP, CTP, 20 μM UTP for the *adhE* scaffold) were added, and the reaction was stopped after increasing time intervals by the addition of stop-solution (8 M urea, 20 mM EDTA). Gre factors were added to 1 μM prior to MgCl$_2$ and NTP substrates when indicated. RNA products were analyzed by 15% denaturing PAGE and quantified by phosphorimaging using Typhoon 9500 scanner (GE Healthcare). At each time point, the pause efficiency was calculated as the ratio of the paused RNA product to the sum of the paused and full-length products. Apparent σ affinities were calculated from the σ titration curves using the following equation: $P = P_{max} \times C/(C + K_{d,app}) + A$, where P is the pausing efficiency at a given σ concentration, P_{max} is maximal pausing at saturation, C is σ concentration, $K_{d,app}$ is the apparent dissociation constant for σ binding to the TEC, and A is the background level of pausing in the absence of the σ subunit. All measurements presented in the paper were independently repeated at least three times, and mean values and standard deviations were determined; the P-values were calculated by the Mann–Whitney U-test using the Statistica 6.0 software (StatSoft).

For analysis of σ-dependent pausing on natural promoter templates, holoenzyme RNAP (50 nM core RNAP and 250 nM σ subunit, or various σ concentrations in titration experiments) was incubated with promoter DNA (25 nM) in the transcription buffer containing 40 mM Tris–HCl, pH 7.9, 40 mM KCl and 10 mM MgCl$_2$ for 10 min at 37 °C. Nucleotide substrates were added to the following concentrations: 200 μM ATP, GTP, CTP, 20 μM UTP (with the addition of α-[^{32}P]-UTP) and 10 μg/ml heparin for *adhE* P1 and 100 μM GTP, CTP, 2 μM ATP and 1 μM UTP for *ecnB* P. In experiments with the Δ3.2 σ^{38} subunit (Figure 6C), the ApA primer was added (25 μM) to both WT and Δ3.2 RNAPs to compensate for possible defects of the mutant σ subunit in initiating nucleotide binding (2,41). The reactions were stopped after various time intervals by the addition of the stop-solution, RNA products were separated by 15% PAGE and analyzed by phosphorimaging.

Electrophoretic mobility shift assay

Synthetic TECs containing 5′-labeled RNA were assembled as described above and diluted with the transcription buffer
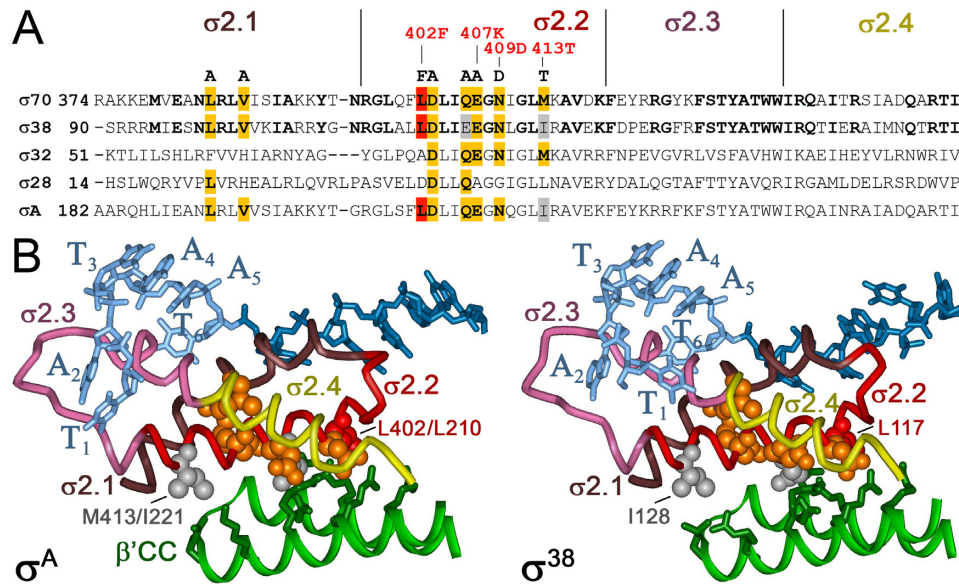
Figure 1. Interactions of σ region 2 with the -10 promoter element and core RNAP. (**A**) Alignment of region 2 from *E. coli* σ[70], σ[38], σ[32], σ[28] and *T. thermophilus* σ[A]. Subregions of region 2 are indicated. Amino acid residues identical in σ[70] and σ[38] are shown in bold. Residues in σ subregions 2.1 and 2.2 whose substitutions (shown in black above the alignment) impair σ-core interactions (57) are highlighted in orange, with the two residues that differ in σ[70] and σ[38] shown in gray; one of these residues is also substituted in *T. thermophilus* σ[A] (M413/I221 in σ[70]/σ[A]). Residue L402/L117 in the core-binding region of σ[70]/σ[38] is highlighted in red. Mutations in subregion 2.2 that decrease σ[70]-dependent pausing (24,37) are printed in red. (**B**) Open promoter complex structures of the *T. thermophilus* σ[A] (6) and *E. coli* σ[38] (38) RNAP holoenzymes; nucleotides of the −10 element are numbered. The colour scheme for σ subregions and amino acid substitutions corresponds to panel (A). The β′ coiled-coil element (β′CC) is shown in green, residues involved in direct interactions with σ are shown as stick models.

to 10 nM final concentration. BSA (Fermentas) was added to 1 mg/ml, followed by the addition of the σ subunit to indicated concentrations. The samples were incubated at 37°C for 15 min, mixed with loading buffer (50% glycerol, 2.5^x TBE; 2 μl per 10 μl sample) and immediately loaded onto pre-running native 5% gel (acrylamide:bisacrylamid = 37.5:1, 0.5^x TBE; 20 V/cm). The σ affinities were determined from σ titration curves using the following equation: $B = B_{max} \times C/(C + K_{d,app})$, where B is the binding efficiency at a given σ concentration (calculated as the ratio of σ-bound TEC to the sum of σ-free and σ-bound TECs), B_{max} is maximal binding at saturation, C is σ concentration, and $K_{d,app}$ is the apparent dissociation constant for σ binding to the TEC.

DNA footprinting

ExoIII. In the ExoIII footprinting experiments, synthetic TECs were obtained as described above, but the template DNA oligonucleotide was 5′-end labeled with γ-[32P]-ATP. The TECs were immobilized on Ni-NTA agarose (Qiagen) and washed two times with 1 ml of buffer containing 40 mM Tris-HCl, pH 7.9 and 40 mM KCl). MgCl$_2$ was added to 10 mM, followed by the addition of 10 units of ExoIII (New England BioLabs, Inc.) per 10 μl reaction point at 37 °C in the transcription buffer (40 mM Tris–HCl, pH 7.9, 40 mM KCl and 10 mM MgCl$_2$). For footprinting of 23 and 24-mer TECs, ExoIII was added 30 seconds after the addition of corresponding NTPs sets (100 μM of each CTP, GTP or CTP, GTP, ATP, respectively). The reaction was stopped by the addition of 10 μl of stop-solution containing EDTA (50 mM) and chicken erythrocyte DNA (1 μg/μl). RNAP-

DNA complexes were eluted with 100 mM imidazole (pH 7.9) for 5 min at 65°C and 15 min at 25 °C with shaking and treated with chlorophorm. DNA was ethanol-precipitated, washed with 80% ethanol and dissolved in formamide loading buffer. The samples were analyzed by 15% denaturing PAGE. The A+G cleavage marker was obtained by treatment of the labeled DNA oligonucleotide with formic acid and piperedine (16).

KMnO$_4$. For the KMnO4 footprinting experiments, the *adhE* P1 promoter was labeled at the template strand with 5′-[32P]-labeled primer during PCR. Holoenzyme RNAP (100 nM core plus 500 nM σ[38]) was incubated with the labeled promoter fragment (10 nM) in the transcription buffer for 15 min at 37 °C. KMnO$_4$ was added to 2 mM and the reaction was stopped after 30 s by the addition of an equal volume of solution containing 1 M β-mercaptoethanol and 1 M sodium acetate (pH 4.8). DNA was ethanol precipitated and dissolved in 100 μl of 10% piperedine. The samples were heated for 15 min at 95 °C and treated with equal volume of water-free chloroform. DNA from the water phase was ethanol-precipitated, dissolved in urea-containing loading buffer and analyzed by 17% PAGE.

RESULTS

σ[38]-dependent pausing in synthetic TECs

To reveal possible effects of the σ[38] subunit on transcriptional pausing, we first tested whether σ[38] could cause pausing *in trans*, when added to σ-free TECs. We assembled TECs from core RNAP and synthetic oligonu-
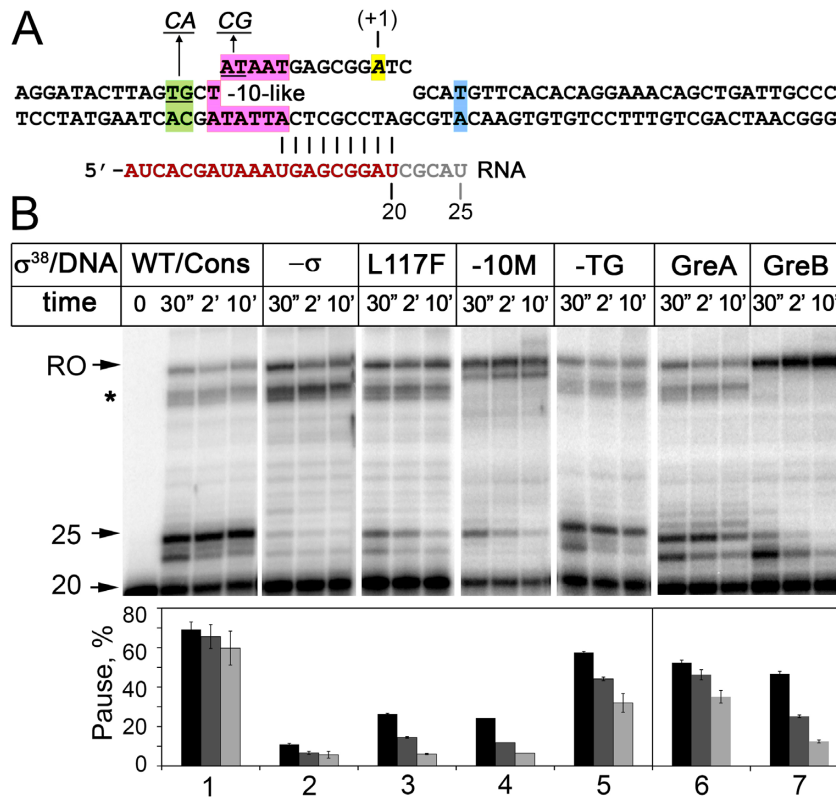
Figure 2. Analysis of σ^{38}-dependent pausing in synthetic TECs. (**A**) Structure of the consensus nucleic acid scaffold used for pause analysis. The −10 and TG motifs are light violet and green, respectively, analyzed nucleotide substitutions in these motifs are shown above the sequence. Nucleotide corresponding to the transcription start point in promoters is yellow, the major pause site is blue. The starting 20 nt RNA is red, nucleotides added during transcription until the pause site are gray. (**B**) RNA products formed on the nucleic scaffold templates (Cons, −10 M and –TG). Transcription was performed with wild-type (WT) or mutant L117F σ^{38} subunits (2.5 μM); GreA and GreB (1 μM) were added where indicated. Positions of the starting 20 nt, paused 25 nt and full-length (RO) RNAs are shown; asterisk indicates an additional pause that is observed near the end of the template. Quantitation of the pause efficiencies are shown below the gels (in percent of the paused 23–25 nt RNAs to the sum of RNAs ≥23 nt; means and standard deviations from three independent experiments are shown).

cleotides containing the consensus extended −10 element (TGcTATAAT) recognized by σ^{38}, previously revealed by *in vitro* selection experiments (31). The RNA 3′-end in reconstituted TECs was positioned eight nucleotides downstream of the -10-like element, corresponding to the optimal distance between the −10 element and the starting point of transcription in promoters (Figure 2A). Because σ^{70} and σ^{38} recognize essentially the same -10 consensus motifs (31), the same nucleic acid scaffold was previously used to analyze fine details of σ^{70}-dependent pausing (16); this allowed us to make direct comparison of the effects of both subunits on pausing. The reconstituted TECs were supplemented with purified σ^{38} subunit, followed by the addition of NTP substrates and analysis of RNA extension. We observed strong transcriptional pausing on this template (with ∼60–70% efficiency and half-life time exceeding 10 min) that occurred five nucleotides downstream of the starting 20 nt RNA transcript (Figure 2B, panel 1), at the same position as σ^{70}-dependent pausing (16). Only minor RNAP stalling was observed at this position in the absence of the σ^{38} subunit (panel 2).

Previously, amino acid substitutions in σ^{70} region 2.2 were shown to reduce σ^{70}-dependent pausing by destabilizing the σ-core interface (see Introduction). We introduced

one of this substitutions, L117F (corresponds to L402F in σ^{70}, Figure 1) in the σ^{38} subunit and analyzed its effects on pausing. The mutation dramatically reduced the ability of σ^{38} to induce pausing (Figure 2B, panel 3), suggesting that interactions of σ^{38} region 2 with core RNAP are essential for its efficient binding to the TEC.

To confirm that the binding of the σ^{38} subunit to the TEC depends on its specific interactions with the promoter-like element, we analyzed two variants of the DNA template with nucleotide substitutions in this element (shown in Figure 2A). Substitutions in the −10 motif (TCGAAT, '-10M') dramatically reduced pausing, while substitutions in the TG motif (CA, '-TG') moderately decreased the pausing efficiency (Figure 2B, panels 4 and 5). Thus, the σ^{38} subunit can likely recognize both motifs in the context of the TEC.

The σ^{70} subunit was shown to induce backtracking of paused TECs, resulting in their high sensitivity to Gre factors that stimulate RNA cleavage in the RNAP active site (see Introduction). We found that both *E. coli* GreA and GreB decreased σ^{38}-dependent pausing in reconstituted TECs when present in the transcription reactions (Figure 2B, panels 6 and 7). This effect was especially pronounced in the case of GreB, which decreased both the pause effi-
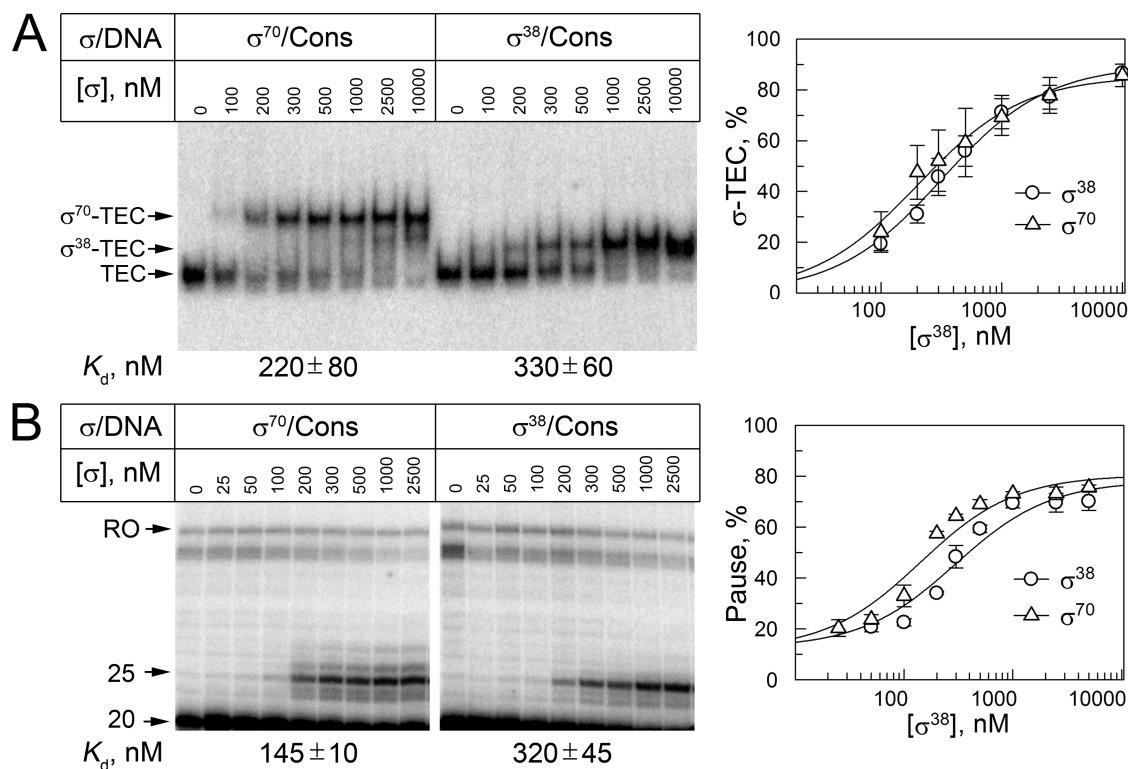
Figure 3. Comparison of σ^{70} and σ^{38} interactions with synthetic TECs. (**A**) Analysis of σ binding to the consensus TEC by EMSA. Positions of σ-free and σ-bound TECs are indicated. See Figure 2 for the consensus scaffold structure. (**B**) Analysis of σ-dependent pausing at different σ concentrations. Positions of the starting 20-mer RNA, paused 25-mer and full-length (RO) transcripts are indicated with arrows. Apparent K_ds for σ binding are shown below the gels (means and standard deviations from three-four independent experiments).

ciency and half-life (to about 30 s), suggesting that the σ^{38} subunit also induces TEC backtracking at the pause site.

Analysis of σ binding to the TEC

To confirm that σ^{38} directly interacts with the TEC during pausing, we analyzed σ binding by the electrophoretic mobility shift assay (EMSA). We reconstituted consensus TEC containing radiolabeled RNA as described above, supplemented it with either σ^{38} or σ^{70} and analyzed complex formation by gel electrophoresis under native conditions. We found that both σ subunits can indeed bind the TEC, resulting in different changes in its mobility (Figure 3A). Titration curves revealed that σ^{38} and σ^{70} bind the TEC with comparable affinities (apparent dissociation constants $K_{d,app}$ of 330 ± 60 nM and 220 ± 80, respectively, $P > 0.05$).

We further compared affinities of σ^{70} and σ^{38} to the consensus TEC by measuring pausing at increasing σ concentrations in the transcription assay (Figure 3B). Both σ subunits induced pausing with similar efficiencies, and the apparent affinity of σ^{38} to the TEC was only 2-fold lower than the affinity of σ^{70} ($K_{d,app} = 320 \pm 45$ and 145 ± 10 nM, respectively, $P < 0.05$). These values were in a good agreement with the apparent dissociation constants measured in the EMSA experiments. Thus, σ^{38} does not differ greatly from σ^{70} in its ability to interact with TECs containing consensus pause-inducing motifs.

Analysis of the '-10M' and '-TG' TEC variants showed that substitutions in the -10 element abolished σ^{38} binding

in EMSA experiments ($K_d > 10$ μM), while substitutions in the TG element significantly decreased σ affinity measured by both EMSA and the transcription assay ($K_d = 900 \pm 140$ and 525 ± 100 nM, respectively; 1.6–3-fold differences with the consensus TEC, $P < 0.05$) (Supplementary Figure S1A and S1B) thus explaining the negative effect of these substitutions on σ-dependent pausing.

Previously, binding of the σ^{70} subunit to the TEC was shown to result in extension of upstream RNAP–DNA contacts, manifested in protection of upstream DNA from exonuclease III (ExoIII) cleavage (16). To reveal similar changes in the upstream RNAP-DNA contacts during σ^{38}-dependent pausing, we performed ExoIII footprinting of the rear TEC border in reconstituted TECs during RNA extension (Supplementary Figure S2A). In the absence of the σ^{38} subunit, the TEC border gradually moved downstream when the starting 20 nt RNA was extended by three or four nucleotides (Supplementary Figure S2C, lanes 8–13). In the presence of the σ^{38} subunit, the rear TEC border was shifted 10–11 nucleotides upstream in the majority of TECs, indicative of σ^{38} binding (Supplementary Figure S2C, compare lanes 2 and 8). A minor fraction of TECs in σ-containing reactions that revealed the same ExoIII stops as in σ-less reactions likely corresponded to complexes that did not bind the σ subunit.

Previous analysis of σ^{70}-dependent pausing demonstrated that during RNA extension at the pause site downstream DNA is 'scrunched' within the TEC while the upstream part of DNA is hold in the same register through
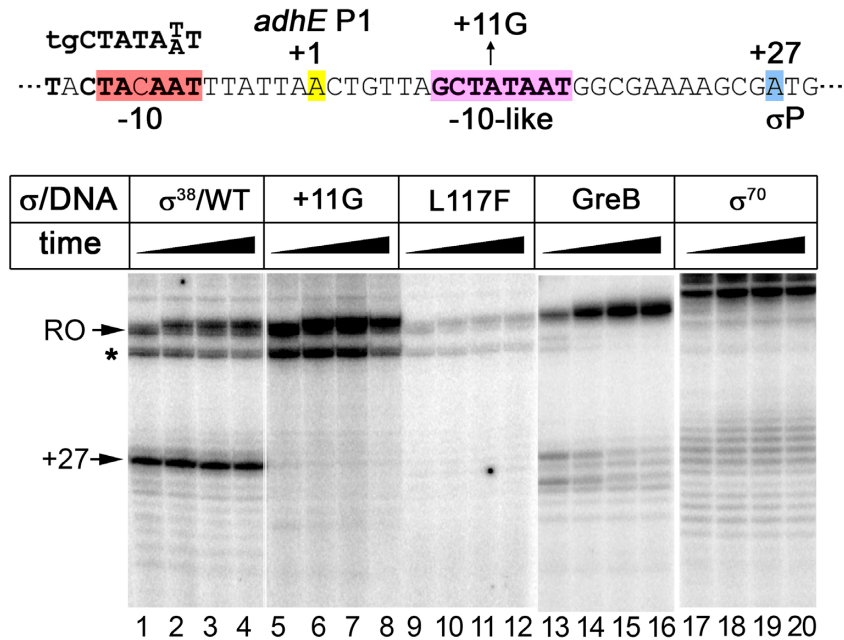
Figure 4. σ^{38}-dependent pause in the initially transcribed region of the *adhE* P1 promoter. The nontemplate promoter strand is shown on the top; the −10 and −10-like elements are shown in pink and violet, respectively; the −10 consensus (31) is shown above the sequence; the transcription start point is yellow, the pause site (+27, σP) is light blue. Transcription was performed on the wild type (WT) or +11G templates with WT and L117F σ^{38} subunits or the σ^{70} subunit. GreB was added to σ^{38}-containing reactions where indicated. The reactions were performed for 0.5, 1.5, 3 and 5 min. Positions of the pause site and full-length (RO) RNAs are shown. An uncharacterized Gre-sensitive pause observed near the end of the template is indicated with an asterisk.

specific contacts of the σ subunit with the promoter-like element (see Introduction). We observed that the upstream RNAP-DNA contacts remained static in 20-, 23- and 24-mer TECs (Supplementary Figure S2C, lanes 2–7). Control experiments demonstrated that a major fraction of reconstituted TECs (≥80%) are active under these conditions and extend RNA to the expected position (Supplementary Figure S2B). This suggests that in σ^{38}-bound complexes the transcribed DNA part must be scrunched within RNAP to allow RNA extension.

σ^{38}-dependent pausing on natural promoter templates

Experiments presented above demonstrated that σ^{38} can efficiently induce transcriptional pausing at consensus pause sites in synthetic TECs. To reveal whether σ^{38}-dependent pauses could potentially occur during transcription of natural templates, we analyzed initially transcribed sequences of σ^{38}-dependent promoters present in the Regulon database (43). We found that a considerable fraction of all σ^{38}-dependent promoters contained -10-like motifs between the +1 and +20 nucleotides in the initially transcribed regions. In particular, 42 out of 143 available promoters (almost 30%) revealed potential pause-inducing sequences with at least five matches to the consensus extended -10 motif TGcTATA(A/C)T (the −10 element is underlined) (Supplementary Table S1).

To analyze σ^{38}-dependent pausing on natural promoter templates, we amplified four promoters, *adhE* P1, *ecnB* P, *glgS* P1 and *talA* P2, together with their initially transcribed regions, from genomic DNA and performed *in vitro* transcription assays with the σ^{38} RNAP holoenzyme.

With two of these templates, *adhE* P1 and *ecnB* P, we observed pauses at the expected positions relative to the pause-inducing sequences (10-12 nucleotides downstream of the -10-like element in each template; promoter positions +27 and +17/+18, respectively, deduced from comparison with marker RNA transcripts of known lengths) (Figure 4 and Supplementary Figure S3). The pausing was especially prominent in the case of the *adhE* P1 template (∼45% efficiency, with the pause half-life time >> 5 min) (Figure 4). For the *ecnB* P template, the pausing was weaker and additional strong pauses of unknown nature were observed downstream of the predicted σ-dependent pause site (Supplementary Figure S3B).

Substitutions of the second adenine in the −10-like motifs in the *adhE* P1 (+11G) and *ecnB* P (+5G) templates dramatically decreased pausing at the expected positions (+27 and +17/+18), thus confirming that the pausing depends on these motifs (Figure 4, lanes 5–8, and Supplementary Figure S3B, lanes 6–10). Previously, cytosine residue immediately upstream of the −10 element was shown to be important for promoter recognition by the σ^{38} subunit (33). Substitution of this residue in the pause-inducing motif on the *adhE* P1 template ('+9T') decreased pausing (down to ∼35%), while substitution of the upstream G ('+8A') did not change the pausing efficiency (Supplementary Figure S4A).

The L117F mutation in the σ^{38} subunit strongly decreased holoenzyme RNAP activity on both promoters, likely because of impaired σ-core interactions. At the same time, it had even stronger effects on σ-dependent pausing, since almost no paused RNA products were observed at the expected positions with this mutant (Figure 4, lanes 9–

12; Supplementary Figure S4A; Supplementary Figure S3, lanes 11–15).

Finally, the pausing was reduced in the presence of the GreB factor, whose addition resulted in the appearance of shortened RNA products at the pause site (Figure 4, lanes 13–16). Moreover, when the paused 27-mer complexes were immobilized on an affinity sorbent and washed with the transcription buffer to remove NTPs, intrinsic RNA cleavage was observed even in the absence of Gre factors, resulting in shortening of the transcript by 3–4 nucleotides (Supplementary Figure S4B, lane 2). Thus, pause formation is likely accompanied by relaxation of the stressed TEC through RNAP backtracking. Addition of NTPs to the TEC after RNA cleavage resulted in its reactivation and RNA extension through the pause site, again resulting in the pause formation (Supplementary Figure S4B, lane 9).

Overall, these assays revealed typical characteristics of σ-dependent pausing previously described for the σ^{70} subunit. Surprisingly, we observed that the σ^{70} subunit could not induce as efficient pausing on the *adhE* P1 template as the σ^{38} subunit (Figure 4, lanes 17–20, Supplementary Figure S4A). However, the size of full-length RNAs synthesized by the σ^{70}-RNAP on this template was higher than in the case of σ^{38}-RNAP (Supplementary Figure S4C), suggesting that σ^{70}-RNAP likely initiates transcription from another starting point located upstream of the σ^{38}-dependent start, thus precluding direct comparisons of the pausing process for the two σ subunits on this template. σ^{70}-containing RNAP also poorly recognized the *ecnB* promoter, so that only faint synthesis of paused or full-length RNAs could be detected (Supplementary Figure S3, lanes 21–25).

Analysis of promoter-proximal pausing by permanganate footprinting

To detect transition from the promoter to the paused complex during transcription initiation on the *adhE* P1 template, we performed footprinting of transcription complexes with potassium permanganate, which allows to specifically modify thymine residues in single-stranded DNA regions (Figure 5A). We expected that in the process of promoter escape the transcription bubble should migrate from the promoter to the downstream pause site. Indeed, we observed that the *adhE* P1 template was melted between positions −11 and +1 in the open promoter complex (Figure 5B, compare lanes 2 and 3). This melting disappeared upon addition of NTP substrates, and a new melted region was detected at the proposed pause site (lane 4). The positions of modified thymines in the paused complex corresponded to the backtracked TEC conformation, in agreement with its sensitivity to the GreB cleavage factor (Figure 5A).

RNAP containing the mutant L117F σ^{38} subunit could fully open the promoter, but no DNA melting was detected at the pause site in the presence of NTP substrates, in accordance with the inability of the mutant σ subunit to induce pausing (Figure 5B, lanes 5 and 6). Furthermore, the +11G template with substitution in the pause-inducing sequence was normally melted by the wild-type σ^{38} RNAP holoenzyme but no pausing was detected upon NTP addition (lanes 8 and 9). Overall, these experiments revealed TEC stalling at the proposed pause site after transcrip-
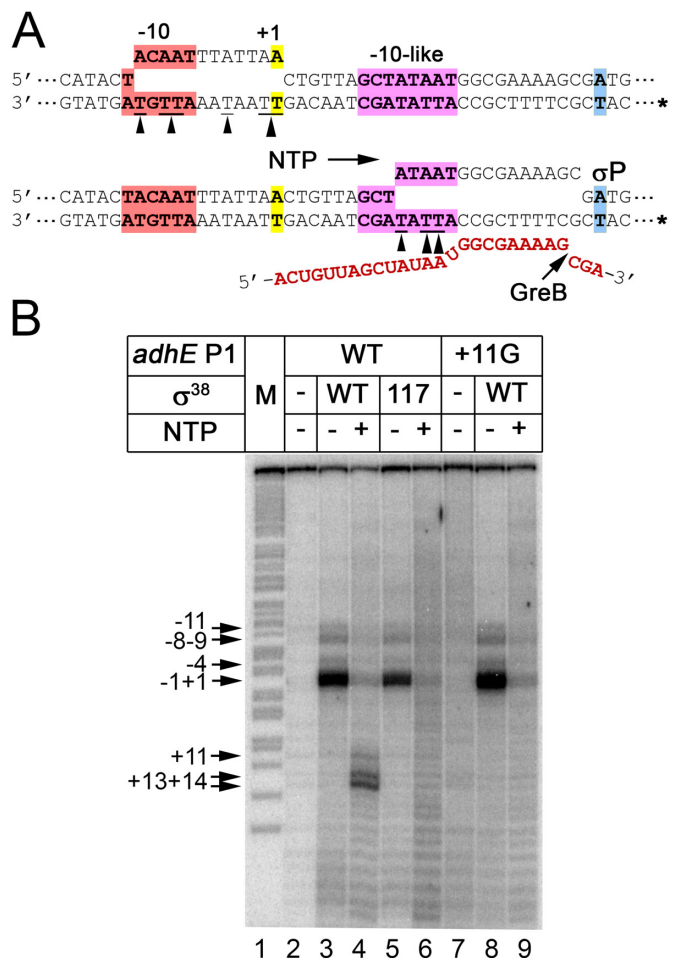

Figure 5. Analysis of open complex formation and σ^{38}-dependent pausing by KMnO₄ footprinting. (**A**) Schematics of DNA melting in the open promoter complex (top) and paused TEC (bottom); only central part of the promoter DNA fragment is shown, the RNA transcript is shown in red. Positions of modified thymines in the template strand are indicated with arrowheads below the sequences. Position of GreB-induced RNA cleavage is shown with an arrow. (**B**) Analysis of permanganate DNA cleavage. Transcription complexes were formed with either wild-type or L117F σ^{38} subunits on the wild-type or +11G templates. 'M' is the A+G cleavage marker. Positions of modified thymine residues are indicated.

tion initiation, which depended on the σ^{38} subunit and the pause-inducing sequence.

Comparison of the *in cis* and *in trans* modes of σ-dependent pausing

Previous studies demonstrated that the σ^{70} subunit preferably induces promoter-proximal pausing *in cis* but can also access the TEC *in trans* during transcription elongation, if present at elevated concentrations (see Introduction). Experiments with the consensus nucleic acid scaffold demonstrated that σ^{38} can bind the TEC *in trans* similarly to the σ^{70} subunit (the first two sections of Results). To reveal the mode of σ^{38}-TEC interactions in the case of promoter-proximal pausing, we analyzed pausing at various σ^{38} concentrations. As expected, the efficiency of transcription initiation (the amount of full-length RNA synthesized) on
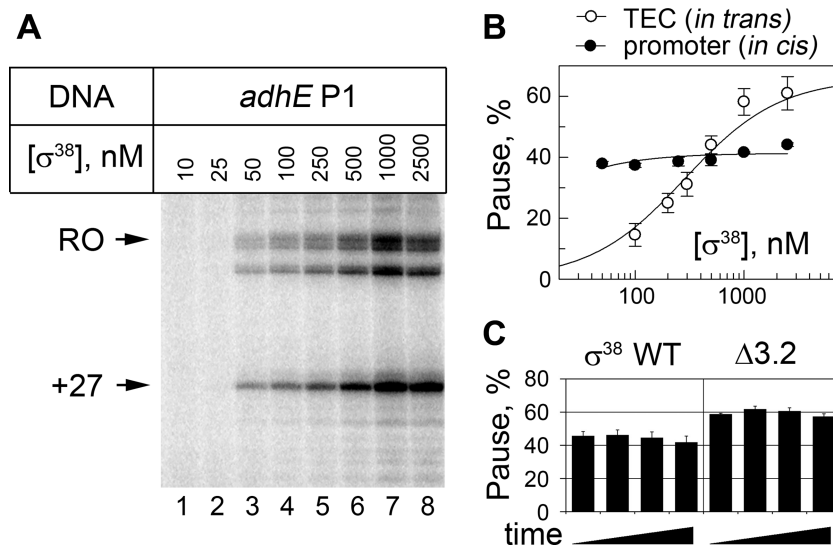
Figure 6. The σ^{38} subunit acts *in cis* during transcription initiation to induce pausing. (**A**) Analysis of promoter-proximal pausing on the *adhE* P1 template at different σ^{38} concentrations. Positions of the paused and run-off (RO) RNAs are indicated. (**B**) Plot of the pausing efficiencies (shown in percent of the paused RNA to the sum of paused and RO transcripts) at different σ concentrations (black circles). The data for σ^{38} titration in the synthetic *adhE* TEC, where σ^{38} was added *in trans*, are shown for comparison (open circles) (see Supplementary Figure S5C). (**C**) Comparison of the pausing efficiencies on the *adhE* P1 template for WT and $\Delta3.2$ σ^{38} subunits. The reactions were performed in the same way as in Figure 4. Means and standard deviations from three-four independent experiments are shown.

the *adhE* P1 template greatly depended on σ concentration (Figure 6A). However, in contrast to the experiment with synthetic TECs (Figure 3), the pausing efficiency remained constant at all σ^{38} concentrations (corresponding to an apparent K_d value much below the concentration range used in this experiment) (Figure 6B, closed circles). This suggested that after transcription initiation, the σ^{38} subunit remains bound to the TEC and recognizes the pause signal *in cis* to induce pausing.

To directly compare the *in cis* and *in trans* modes of σ^{38} action on the same template, we assembled synthetic TECs on a nucleic acid scaffold corresponding to the *adhE* pause site, with RNA 3′-end positioned four nucleotides upstream of the pause site (Supplementary Figure S5A), and analyzed pausing after *in trans* σ^{38} addition. We observed pause formation exactly at the same position as in the case of the natural *adhE* P1 template (Supplementary Figure S5B, compare σ-less and σ^{38}-containing reactions, panels 1 and 3). In contrast to the promoter template, however, the pause efficiency strongly depended on σ^{38} concentration, with $K_{d,app}$ for σ binding of 490 ± 75 nM (Figure 6B and Supplementary Figure S5C). This value was comparable to the $K_{d,app}$ of σ^{38} measured for the consensus TEC (see above, second section of Results), suggesting that the *adhE* pause-inducing sequence *per se* does not promote stronger binding of σ to the transcription complex.

In contrast to the *adhE* P1 promoter template, the σ^{70} subunit induced pausing on the *adhE* scaffold template with the same efficiency and specificity as σ^{38} (Supplementary Figure S5B, panel 2) and had about 2-fold higher affinity to the synthetic TEC than σ^{38} ($K_{d,app} = 270 \pm 10$ nM, $P < 0.05$; Supplementary Figure S5C), similarly to the relative affinities of these σ subunits to the consensus TEC. This further suggested that the differences in promoter-proximal

pausing between σ^{38} and σ^{70} observed on the *adhE* P1 promoter template result from their different fates during transcription initiation, and not differences in the pause site recognition (see Discussion).

Since the σ^{38} subunit must remain bound to core RNAP during transcription initiation to ensure the *in cis* action, any changes in the efficiency of σ dissociation should result in changes in the pausing efficiency at promoter-proximal sites. To test this proposal, we obtained a mutant variant of the σ^{38} subunit with a deletion in its region 3.2 ($\Delta228$–234). Region 3.2 occupies the RNA exit channel in the RNAP holoenzyme and should be displaced by the nascent RNA to allow efficient promoter escape (2,3,6,38). Previously, we demonstrated that deletion of corresponding residues in region 3.2 in the σ^{70} subunit impaired its dissociation during transcription initiation and enhanced σ^{70}-dependent promoter-proximal pausing (41). The region 3.2 deletion also increased σ^{38}-dependent pausing on the *adhE* P1 template by 10–15% ($P < 0.05$ for all time points; Figure 6C). Similarly, the pausing was stimulated on the *ecnB* P template (Supplementary Figure S4B, lanes 16–20). At the same time, the region 3.2 deletion did not increase the efficiency of pausing and σ^{38} affinity in synthetic *adhE* TEC ($K_{d,app} = 410 \pm 77$ nM; Supplementary Figure S5B and S5C). Thus, stimulation of pausing by the region 3.2 deletion observed on the natural promoter template cannot be explained by changes in the affinity of σ^{38} to the TEC or its increased propensy to induce pausing.

In summary, these results suggest that σ^{38} does not completely dissociate during transcription initiation but remains bound in a fraction of TECs and acts *in cis* to induce transcriptional pausing.

DISCUSSION

For years, σ^{70}-dependent pausing by *E. coli* RNAP has served as a paradigm in studies of transcriptional pausing induced by transcription initiation factors in bacteria. Extensive studies of σ^{70}-dependent pausing revealed its dependence on two DNA elements: a promoter-like σ-binding motif and a downstream sequence responsible for pause formation and TEC backtracking (15,16,21,22,28–30). The pausing is induced by initial σ^{70} interactions with the −10-like element followed by formation of a stressed 'scrunched' TEC, which can be stabilized in the paused state by an elemental pause sequence (also found in other common pause types, including consensus and hairpin-dependent pausing, (44–46)). Further RNA extension results in either pause escape or TEC backtracking, followed by Gre-dependent RNA cleavage after which the TEC can enter the next pausing cycle.

We now demonstrate that the alternative σ^{38} subunit can also induce highly efficient RNAP pausing at both synthetic and natural DNA sites, with the properties of the paused complexes being highly similar to σ^{70}-paused TECs. In particular, formation of σ^{38}-paused TECs depends on σ^{38} contacts with the extended −10 motif at the pause site and the RNAP core enzyme, since both site-specific DNA substitutions and mutations in the β'-binding site of σ^{38} strongly reduce pausing. Interestingly, however, the σ^{38}-dependent pausing does not seem to require the presence of the elemental pause site, since no characteristic sequence motifs (such as pyrimidine and guanine nucleotides at pause positions −1 and +1, respectively) can be found in the analyzed promoter-proximal pause sites (Supplementary Figure S3A). Similarly to σ^{70}-dependent pauses (16,18), the rear TEC border in σ^{38}-containing complexes remains fixed on DNA during stepwise RNA extension, suggesting formation of stressed 'scrunched' intermediates, which are further converted to backtracked TECs sensitive to Gre-factors. Despite similar Gre-sensitivity, σ^{70}-dependent pauses can be detected in both ΔGre and wild-type *E. coli* cells *in vivo* and are important for the lytic phase of growth of lambdoid phages (20,27), suggesting that σ^{38} pausing might also have functional consequences for gene expression.

The discovery of strong σ^{38}-dependent pausing is surprising since no σ^{38} effects on RNAP pausing were observed on previously analyzed σ^{70}-dependent DNA templates (19,24). The σ^{38} subunit was also shown to have lower affinity to the core RNAP enzyme in comparison with σ^{70} (35,36) and this was proposed to explain its inability to induce transcriptional pausing (24). However, the affinities of σ^{38} and σ^{70} to the TEC have never been directly compared. At the same time, the possibility of σ^{38}-induced pausing was discussed previously (33), and this is what could be expected given the highly similar promoter specificities of the σ^{38} and σ^{70} RNAP holoenzymes. Although σ^{38} contains amino acid substitutions in region 2.2 interacting with the β' coiled-coil motif of core RNAP (Figure 1A), they do not significantly change the σ-core interface, as revealed in the recent σ^{38} open promoter complex structure (Figure 1B) (38). Furthermore, although the M413T substitution at one of the positions that differ between σ^{70} and σ^{38} (M413 and

I128, respectively; Figure 1A) was shown to reduce σ^{70}-dependent pausing, its effects were modest in comparison with other analyzed substitutions (37). In fact, the *Thermus aquaticus* σ^{A} subunit, which contains the same substitution as σ^{38}, was shown to efficiently induce pausing by its cognate RNAP (47).

Our experiments revealed comparable pausing for σ^{38} and σ^{70} in synthetic TECs containing either consensus or *adhE*-derived -10-like sequences and demonstrated that σ^{38} has about 2-fold higher K_d for TEC binding *in trans* in comparison with σ^{70}. Thus, σ^{38} and σ^{70} reveal smaller differences in the binding to the TEC than previously reported differences in their interactions with core RNAP, likely because the −10-like element in the DNA template contributes to σ-TEC interactions. Given the slightly lower affinity of σ^{38} to the −10-like pause-inducing sequences and its faster release during transcription elongation (12), we propose that efficient σ^{38}-dependent pausing may require the presence of nearly consensus pause-inducing motifs located close to the starting point of transcription, which were not analyzed in previous studies (19,24).

Analysis of the sequences of known σ^{38}-dependent promoters revealed that many of them indeed contain such motifs. In our experiments, we revealed σ^{38}-dependent pausing in two out of four analyzed transcription units, *adhE* and *ecnB*, suggesting that σ^{38}-induced pausing may be a widespread phenomenon in bacterial transcription. The σ^{38} pausing was especially strong in the case of the *adhE* P1 promoter, which contained a consensus pause-inducing signal resulting in permanent transcription arrest in the absence of Gre factors. Interestingly, both the *adhE* and *ecnB* templates are differently utilized by σ^{38} and σ^{70} RNAP holoenzymes, resulting in prominent differences in transcription patterns. In particular, the lower efficiency of σ^{70}-dependent pausing on the *adhE* template might be explained by suboptimal positioning of the pausing site relative to the start of transcription, which is shifted upstream in comparison with σ^{38}-dependent initiation, probably resulting in a different architecture of σ^{70}-containing complexes at the pause site.

We demonstrated that during promoter-dependent transcription initiation, σ^{38} can likely remain bound to the TEC and induce pausing *in cis*, thus avoiding competition with other σ subunits. In particular, the *in cis* action may allow σ^{38} to induce pausing on its native templates even in the presence of large excess of the σ^{70} subunit, which has similar DNA specificity. Indeed, DNA immunoprecipitation experiments revealed the presence of σ^{38} in promoter-proximal regions of several analyzed genes suggesting that it remains bound to at least a fraction of TECs *in vivo* after transcription initiation (12). Intriguingly, it was recently reported that σ^{70}-dependent pausing at a consensus promoter-proximal pause-inducing site is increased during the stationary phase (26). While it was proposed to reflect an increased half-life of the σ^{70}-dependent pause, an alternative explanation would be that σ^{38} contributes to such pausing in stationary cells.

Both the *adhE* P1 and *ecnB* P promoters were reported to be activated under specific conditions: during fermentative growth on sugars in anaerobic conditions (48–51) and in stationary phase under high osmolarity conditions (52),

respectively. We note that in both promoters the pause sites overlap with the binding sites of transcription repressors, Cra and OmpR for *adhE* P1 and *ecnB* P, respectively (Supplementary Figure S3A). These repressors were proposed to control the expression of their target genes, alcohol dehydrogenase and entericidin B (51–53). We therefore speculate that temporary RNAP stalling at the pause sites might be important for transcription regulation, for example, by affecting the binding of transcription repressors to promoter-proximal regions during ongoing transcription.

Our results suggest that not only the principal σ subunit but also alternative σ subunits and, probably, other transcription initiation factors may induce transcriptional pausing in bacteria. Hypothetically, similar mechanisms of promoter-proximal pausing might operate in other domains of life. Promoter-proximal pausing plays an essential role in transcription regulation in higher eukaryotes, where it serves to rapidly change transcription levels and coordinate RNA synthesis with RNA processing, and the paused complexes were shown to adopt backtracked conformation, similarly to σ^{70}- and σ^{38}-paused TECs (54,55). While no promoter recognition factors have yet been demonstrated to regulate transcriptional pausing in eukaryotes, the eukaryotic orthologue of NusG, elongation factor Spt4/5 was shown to prevent RNAP II pausing through interactions with the nontemplate DNA strand (56). Thus, interplay of various factors that compete for RNAP and DNA binding during transcription initiation and elongation likely contributes to regulation of pausing in eukaryotes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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