

A hyper-mutant of the unusual σ^{70} -Pr promoter bypasses synergistic ppGpp/DksA co-stimulation

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ABSTRACT

The activities of promoters can be temporally and conditionally regulated by mechanisms other than classical DNA-binding repressors and activators. One example is the inherently weak σ^{70} -dependent Pr promoter that ultimately controls catabolism of phenolic compounds. The activity of Pr is up-regulated through the joint action of ppGpp and DksA that enhance the performance of RNA polymerase at this promoter. Here, we report a mutagenesis analysis that revealed substantial differences between Pr and other ppGpp/DksA co-stimulated promoters. *In vitro* transcription and RNA polymerase binding assays show that it is the T at the –11 position of the extremely suboptimal –10 element of Pr that underlies both poor binding of σ^{70} -RNAP and a slow rate of open complex formation—the process that is accelerated by ppGpp and DksA. Our findings support the idea that collaborative action of ppGpp and DksA lowers the rate-limiting transition energy required for conversion between intermediates on the road to open complex formation.

INTRODUCTION

The control of transcriptional initiation by RNA polymerase (RNAP) is a key step in differential gene expression required for adaptive and developmental responses. In bacteria, promoter-specific transcriptional initiation requires binding of a σ -factor to the catalytic core RNAP (subunit composition $\alpha_2\beta\beta'\omega$). All bacteria have a house-keeping σ -factor (σ^{70} in *Escherichia coli* and *Pseudomonas putida*) responsible for the bulk of transcription, but most also employ additional alternative σ s for recognition of different sets of promoters (1). The σ -factors direct the holoenzymes (σ -RNAP) by providing sequence-specific recognition determinants for promoter binding. For σ^{70} -promoters, the most prominent of these are the –35

(consensus TTGACA) and –10 (consensus TATAAT) elements that are contacted by the σ_4 and σ_2 subregions of σ^{70} , respectively (2). At extended –10 promoters, σ_3 contacts additional bases just upstream of the –10 element (consensus TGn), while the $\sigma_{1,2}$ subregion can provide additional contacts with DNA downstream of the –10 element (reviewed in 3). Natural promoters, however, do not necessarily possess all these DNA elements and the relative contribution of each for σ -RNAP binding to form the initial closed promoter complex differs from promoter to promoter. The σ -factor also plays a crucial role in the subsequent steps of DNA melting (isomerization) to form the transcriptionally competent open complex (2 and references therein).

The multiple steps of the transcriptional initiation pathway involve conformational changes in both the DNA and RNAP that present potential targets for regulation by DNA-binding transcriptional regulators and other factors, such as ppGpp (guanosine tetraphosphate) and DksA that directly target the active site of RNAP to alter its performance at specific promoters (reviewed in 3,4). The nucleotide alarmone ppGpp is the mediator of the ‘stringent’ response to amino acid starvation, where the translational capacity is matched to reduced demand through down-regulation of transcription from tRNA and rRNA operon promoters (stringent σ^{70} -promoters). However, other nutrient limitations and stresses also elicit the production of ppGpp to control an extensive network that re-directs the global transcriptional capacity of the cell from genes for growth and reproduction towards those for survival (5,6).

Targeting of the RNAP holoenzyme by ppGpp can alter its performance at susceptible σ^{70} -, σ^E - and σ^{FliA} -dependent promoters to result in either inhibition or stimulation of promoter activity (7–11). Binding of ppGpp is thought to lower the energy required for rate-limiting transition of an intermediate(s) in the pathway to open complex formation, with the negative or positive outcomes being dependent on the stabilities of intermediate complexes at the promoter (reviewed in 3). DksA

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accesses the active site of RNAP through the secondary channel, where it mediates long-range structural changes within RNAP that alter its interactions with promoter DNA (12–14). Although DksA and ppGpp can have mutually independent and sometimes counteractive effects (9,15 and references therein), DksA sensitizes RNAP to the cellular levels of ppGpp to account for their more frequent collaborative action (7,8).

The σ^{70} -Pr promoter controls transcription of the *dmpR* gene that encodes the master regulator of the *dmp*-operon required for catabolism of dimethylphenol by *P. putida* CF600. The *dmp*-operon and the *dmpR* gene are divergently transcribed from a common intergenic region (Figure 1A). Transcription from the σ^{54} -dependent Po promoter strictly requires activation by DmpR. DmpR in turn only takes up its active transcriptional promoting form upon binding pathway substrates (or structural analogues, reviewed in 16). Physical interaction of DmpR bound to its upstream activation sites (UASs) and σ^{54} -RNAP bound to Po is aided by the DNA bending protein integration host factor (IHF). Substantial previous work has shown that ppGpp and DksA have separate inputs in the *dmp*-system to allow expression of the specialized Dmp-enzymes only under energy-limited conditions when other preferred carbon sources are absent (17–22). The effects of ppGpp and DksA ultimately converge to stimulate the activity of the intrinsically weak Pr promoter: (i) directly, via co-stimulatory effects on the performance of σ^{70} -RNAP at Pr and (ii) indirectly, through enhancing activity from the divergent Po *dmp*-operon promoter, which results in the stimulation of Pr output (21). In contrast to these ppGpp and DksA mediated stimulatory effects, transcription from Pr is also negatively affected by the initially transcribed region that encodes a 5'-untranslated region (5'-UTR) of the *dmpR* mRNA (21). These three regulatory processes act independently of each other but their molecular mechanisms are not yet well understood. Here, we report a mutational study to identify the critical components of the Pr promoter and potential rate-limiting steps that control its activity. The results show that the Pr promoter differs substantially from other ppGpp and DksA co-stimulated promoters and that it is the T at the –11 position of its extremely sub-optimal –10 element that underlies poor binding of σ^{70} -RNAP and susceptibility to synergistic co-stimulation by ppGpp and DksA.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Escherichia coli strains DH5 (23), MG1655 and its otherwise isogenic ppGpp-deficient [CF1693; (24)] or DksA null counterparts [RK201; (25)], were cultured in Luria-Bertani / Lennox (LB) medium (AppliChem GmbH). Cultures were supplemented with carbenicillin (Cb, 100 μ g/ml), kanamycin (Km, 50 μ g/ml) or tetracycline (Tc, 5 μ g/ml) when appropriate for strain or resident plasmid selection.

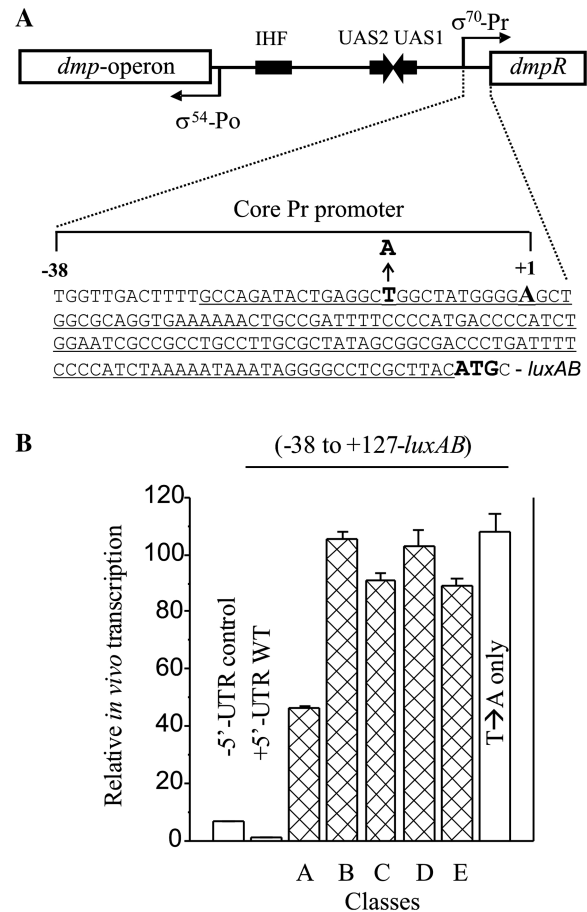


Figure 1. Identification of hyper-promoter mutants by random mutagenesis. (A) Schematic illustration of the *dmp*-system with the coding regions of *dmpR* and the *dmp*-operon shown as open boxes (not to scale). The relative locations of the binding sites for IHF (black box) and DmpR (inverted black arrows labeled UAS2 and UAS1) within the intergenic region are indicated. The DNA sequence (–38 to +127 relative to the +1 of *dmpR*) that encompasses the core Pr promoter (–38 to +1) is shown with the region targeted for random mutagenesis (–26 to +123) underlined. The ATG initiation codon, the +1 transcriptional start and the T-11A substitution found in all the hyperpromoter mutants are shown in bold. (B) Stationary phase luciferase activities of LB cultured *E. coli* MG1655 harbouring different luciferase transcriptional reporter plasmids in which Pr drives transcription of promoter-less *luxAB* genes: pVI931 (–38 to +8 Pr WT region, –5'-UTR control), pVI1017 (–38 to +127 Pr WT region, +5'-UTR WT), class A to E mutant derivatives (–38 to +127) obtained from the random mutagenesis as detailed in the text, and pVI1018 (–38 to +127, Pr T-11A). Values for relative transcription are from two to six independent experiments, normalized by setting the value of Pr WT (–38 to +127) as 1. Error bars are \pm SE.

Plasmid constructions

Plasmids (Table 1) were constructed by standard DNA techniques. The fidelity of the DNA regions generated by PCR amplification or by insertion of synthetic double stranded linkers was confirmed by DNA sequencing. Luciferase transcriptional reporter plasmids are all based on pVI928 (21) that carries the promoter-less *luxAB* genes of *Vibrio harveyi* downstream of a polycloning site. Plasmids of the random mutagenesis library carry XhoI to BglIII fragments encompassing the –38 to +127 region

relative to the +1 of *dmpR* (Figure 1). These custom-synthesized fragments (GeneScript) carry 0 to 9 mutations within the -26 to +123 region that was targeted for mutagenesis. Transcriptional reporter plasmids that carry the -38 to +8 region were constructed by inserting linkers with SmaI and BglII/BamHI compatible ends between the SmaI and BglII sites of pVI928. All linkers are as linker-WT (Supplementary Table S1) except for the desired substitution(s). Analogous *in vitro* transcription templates were generated by inserting the same linkers between SmaI and BamHI sites of pTE103 (26).

Luciferase assays

For rapid screening for Pr hypermutations, individual colonies of *E. coli* MG1655 harbouring the library of mutant transcriptional reporter plasmids were streaked on solid medium and incubated overnight at 30°C. After addition of 100 µl of 1:1 diluted decanal (luciferase substrate) to the lid of inverted plates, light emission was documented using X-ray film. Quantitative luciferase assays were performed on cultures grown and assayed at 30°C essentially as described by ref. (17). To ensure balanced growth, overnight cultures were diluted 1:50 and grown into exponential phase before a second dilution to an OD₆₀₀ of 0.05–0.08 and initiation of the experiment. Light emission from 100 µl of whole cells using a 1:2000 dilution of decanal was measured using an Infinite M200 (Tecan) luminometer. Specific activity is expressed as relative luciferase units per OD₆₀₀ of 1.0.

Purified proteins and ppGpp for *in vitro* assays

The nucleotide ppGpp (27), native *P. putida* σ^{70} -RNAP holoenzyme (28) and *P. putida* DksA with an N-terminal His tag (22) were purified as previously described.

Electromobility shift assays

Mixtures (15 µl final volume) contained 2 nM radio-labelled DNA probe and the indicated amounts of purified *P. putida* σ^{70} -RNAP and DksA. Binding reactions were incubated for 30 min at 30°C, or for 60 min at 4°C, in buffer (35 mM Tris-Ac, pH 7.9, 70 mM KAc, 5 mM MgAc₂, 20 mM NH₄Ac, 1 mM DTT) that also contained 100 µg/ml non-specific competitor (salmon sperm) DNA, where stated. The resulting complexes were resolved using 4.5% native polyacrylamide gels buffered with 45 mM Tris-borate / 1 mM ethylenediaminetetraacetic acid (EDTA).

DNA probes of 329 bp spanning the wild-type or mutant variants of the -38 to +8 region relative to the +1 of *dmpR* (and a corresponding promoter-less control fragment of 283 bp) were amplified from pTE103-based plasmids using primers 2587 and 2588 (Supplementary Table S1). The 5'-overhangs of XhoI-digested probes were radio-labeled using Klenow fragment DNA polymerase (Roche), [α^{32} -P]dATP (250 µCi at 3000 Ci/mmol, Perkin Elmer) and cold dGTP, dCTP and dTTP. Unincorporated radio-label was removed using Micro Bio-Spin P30 Tris Chromatography Columns (Bio-Rad). DNA probes spanning the -100 to +123 region relative to the +1 of *dmpR* were generated by PCR amplification

using primers 2614 and 2183 (Supplementary Table S1) with pVI1027 (wild-type Pr) or pVI1038 (Pr T-11 A) as templates (Table 1). The amplified fragments were subsequently digested with EcoRI and labeled as described above.

In vitro transcription assays

Single-round *in vitro* transcription assays (final volume 20 µl) were performed at 30°C with 10 nM supercoiled plasmids (transcript size 282 nt) or linear DNA (transcript size 143 nt) in a buffer containing 35 mM Tris-Ac, pH 7.9, 70 mM KAc, 5 mM MgAc₂, 20 mM NH₄Ac, 1 mM DTT and 0.275 mg/ml bovine serum albumin, as previously described (21). When added, ppGpp and/or DksA (or a mock solution of the DksA storage buffer) were pre-incubated with *P. putida* σ^{70} -RNAP for 5 min prior to addition of template DNA. Unless otherwise stated, reactions were subsequently incubated for 10 min to allow open complex formation, followed by initiation of transcription by addition of nucleotides (final concentrations: 500 µM ATP, 200 µM GTP, 200 µM CTP, 80 µM UTP and [α^{32} P]UTP (5 µCi at >3000 Ci/mmol, Perkin Elmer)). Simultaneous addition of heparin (0.125 mg/ml) was used to prevent re-initiation. Reactions were terminated after a further 10-min incubation by addition of 5 µl stop/loading buffer (150 mM EDTA, 1.05 M NaCl, 14 M urea, 10% glycerol, 0.037% xylene cyanol, 0.037% bromophenol blue). Transcripts were resolved on a 5 or 6% polyacrylamide gel containing 7 M urea, and quantified using phosphorimaging.

DNase I footprinting assays

Binary complexes were formed by incubating 40 ng of radio-labelled DNA fragments with different concentrations of *P. putida* σ^{70} -RNAP for 30 min at 30°C or for 60 min at 4°C. Binding reactions (final volume 20 µl in buffer containing 35 mM Tris-Ac, pH 7.9, 70 mM KAc, 5 mM MgAc₂, 20 mM NH₄Ac, 1 mM DTT and 1 mg/ml bovine serum albumin) were additionally supplemented with ppGpp and DksA (or DksA storage buffer) when required. After complex formation, heparin sensitivity was determined by exposure to heparin (final concentration 0.15 mg/ml) for 10 min. Subsequent DNase I digestion (upon addition of 3 µl of a solution containing 0.3 U of DNase I from Roche prepared in 40 mM Tris-HCl, pH 7.9, 10 mM NaCl, 6 mM MgCl₂, 2 mM CaCl₂) was at 37°C for 40–60 s or at 4°C for 10–14 min. Reactions were terminated by the addition of 40 µl of phenol and 180 µl of a solution containing 2 M sodium acetate, 12.5 mM EDTA, 250 µg/ml salmon sperm DNA and 0.3 µl/ml glycogen. After centrifugation at 14000 rpm for 10 min, DNA was ethanol precipitated from the aqueous phase at -80°C. DNA pellets were recovered by centrifugation at 14000 rpm for 30 min at 4°C, and subsequently washed with 70% ethanol, dried and resuspended in 5 µl formamide loading buffer (90% [v/v] formamide, 10 mM Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, 0.05% [w/v] bromophenol blue, 0.05% [w/v] xylene cyanol). Samples were denatured at 95°C for 5 min and analyzed on a 6% denaturing polyacrylamide-8.3 M urea gel. A+G Maxam

Table 1. Plasmids used in this study

Plasmid	Relevant properties	Reference/source
<i>In vivo</i> luciferase transcriptional reporter plasmids based on pVI928		
pVI928	Cb ^R , RSF1010-based broad-host-range promoter-less <i>luxAB</i> promoter probe vector	(21)
pVI931	Pr- <i>luxAB</i> (−38 to +8 relative to Pr +1)	(21)
pVI934	As pVI931 with multiple substitutions (−38 to −30, mut 1)	(21)
pVII1017	Pr- <i>luxAB</i> (−38 to +127 relative to Pr +1)	This study
pVII1018	As pVII1017 with a T-11A substitution	This work
pVII1019	As pVI931 with a T-11A substitution	This study
pVII1020	As pVI931 with an A-15T substitution	This study
pVII1021	As pVI931 with a G-9A substitution	This study
pVII1022	As pVI931 with two substitutions (−38 and −37, mut 2)	This study
pVII1023	As pVI931 with a T-7A substitution	This study
pVII1024	As pVI931 with a T-7G substitution	This study
pVII1025	As pVI931 with a T-7C substitution	This study
pVII1026	As pVI931 with multiple substitutions: T-11A, A-15T, G-9A (triple mut) and those of mut 1	This study
pVII1027	Pr- <i>luxAB</i> (−265 to +127 relative to Pr +1)	This study
<i>In vitro</i> transcription plasmids based on pTE103		
pTE103	Cb ^R , carries a strong T7 transcriptional terminator downstream of the polycloning site.	(26)
pVI948	−265 to +8 Pr on pTE103	(21)
pVI950	−38 to +8 Pr on pTE103	(21)
pVI951	As pVI950 with multiple substitutions (−38 to −30, mut 1)	(21)
pVII1030	As pVI950 with a T-11A substitution	This study
pVII1031	As pVI950 with a A-15T substitution	This study
pVII1032	As pVI950 with a G-9A substitution	This study
pVII1033	As pVI951 (mut 1) but with a T-11A substitution	This study
pVII1034	As pVI951 (mut 1) but with a A-15T substitution	This study
pVII1035	As pVI951 (mut 1) but with a G-9A substitution	This study
pVII1036	As pVI948 but with a T-11A substitution	This study
Plasmids based on pBluescript II SK+		
pVII1037	Cb ^R , pBluescript II SK+ with a modified poly-cloning site: BglIII-BamHI-SmaI-HindIII-NotI-NdeI-NcoI-XhoI-KpnI	This study
pVII1038	Cb ^R , −265 to +127 relative to Pr +1 with a T-11A substitution	This study

and Gilbert sequencing reactions (29) with the same DNA fragment were loaded alongside the footprinting samples. Gels were dried on Whatman 3MM filter paper and analysed by exposure to X-ray film or phosphorimaging.

The DNA probes used were generated by PCR amplification using pVII1027 (for wild-type Pr) or pVII1038 (for Pr T-11 A) as DNA templates and either the primer pair 1204/2183 (which amplifies the −265 to +126 Pr region) or 133/2183 (which amplifies the −112 to +126 Pr region, Supplementary Table S1). Radio-label was incorporated via primer 2183 or 133, which were pre-labelled by using [γ -³²P]ATP (>3000 Ci/mmol; Perkin Elmer) and T4 kinase (Invitrogen). The resulting radioactive PCR products were purified using Micro Bio-Spin P30 Tris Chromatography Columns (Bio-Rad).

KMnO₄ footprinting assays

Assays were performed essentially as described in ref. (30) but with minor modifications of incubation times. Initial binary complexes were formed with DNA prepared and treated as described under DNase I footprinting. Ternary initiating and ternary elongating complexes were then generated by the addition of ATP, GTP and TTP or all the four nucleoside triphosphates (NTPs), respectively (final concentration 200 μ M of each) and further incubation at 30°C (20 s for Pr T-11 A or 1 min for Pr WT). Binary complexes (but not the ternary complexes) were subsequently disrupted by exposure to 350 mM NaCl for 20 s. Reactions were then supplemented with KMnO₄ (to a

final concentration of 5.5 mM) and incubated at 30°C for 2 min prior to termination of the reaction by addition of 20 μ l of a STOP solution (0.69 M sodium acetate, 1 M β -mercaptoethanol and 200 μ g/ml salmon sperm DNA). Samples were precipitated and subsequently treated as described under ‘DNase I footprinting assay’ section.

RESULTS

Random mutagenesis identifies an T-11 A hypermutation of Pr

The core Pr promoter region encompasses −38 to +1 relative to the transcriptional start of *dmpR*, as determined by deletion analysis and primer extension of *in vitro* and *in vivo* generated RNA (21). Substitutions of a potential extended −10 promoter element 29 bp upstream of the transcriptional start (in the −38 to −30 region; TGGTTGACT, extended −10 consensus TGnTATAAT) abolished promoter activity (21) (Figures 1B and 2). However, as detailed below, we show here that this region actually encompasses an essential −35 element of the Pr promoter.

As an initial step to identify additional determinants crucial for Pr output, we generated a library of luciferase transcriptional reporter plasmids in which the −38 to +127 region of *dmpR* controls the promoter-less *luxAB* genes. The DNA introduced into these constructs was synthesized to incorporate an average of two substitutions at random in positions −26 to +123, leaving the remaining

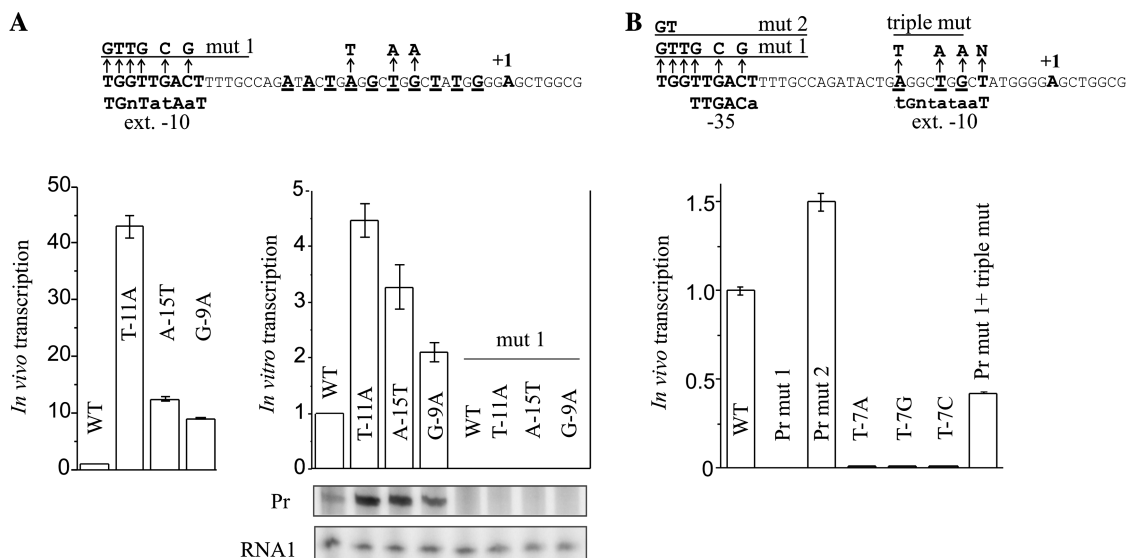


Figure 2. Scanning mutagenesis of the Pr promoter region identifies the -10 motif of Pr. (A) DNA sequence of the -38 to $+8$ region relative to the $+1$ of *dmpR* (upper). Bases that were subject to scanning mutagenesis (positions -21 , -19 , -17 , -15 , -13 , -11 , -9 , -7 , -5 and -3) are underlined with the three substitutions found to stimulate Pr output (A-15T, T-11A and G-9A) indicated. The multiple substitutions of mutation 1 (mut 1) of an apparent extended -10 promoter motif that renders the Pr promoter non-functional (21) are also shown. The graph (left) shows the stationary phase luciferase activities of *E. coli* MG1655 harbouring Pr -38 to $+8$ luciferase transcriptional reporter plasmids assayed as under Figure 1: pVI931 (WT), pVI1019 (T-11A), pVI1020 (A-15T) and pVI1021(G-9A). Data were normalized by setting the value for Pr WT as 1. The graph (right) shows the result of single-round *in vitro* transcription assays with 10 nM supercoiled wild-type (WT) or mutant DNA templates (-38 to $+8$ promoter region of Pr) and 25 nM σ^{70} -RNAP. Data are the average \pm SE of four independent experiments normalized by setting the levels of Pr WT as 1. Templates: pVI950 (WT), pVI1030 (T-11A), pVI1031 (A-15T), pVI1032 (G-9A), pVI951 (mut 1 only), pVI1033 (mut 1 + T-11A), pVI1034, (mut 1 + A-15T) and pVI1035 (mut 1 + G-9A). The autoradiograph shows transcripts from Pr promoter and an internal control transcript (RNA1) that is produced by all templates. (B) DNA sequence of the -38 to $+8$ Pr promoter region indicating an alternative potential -35 element and the substitutions in different luciferase transcriptional reporter plasmids used to obtain the data in the graph below. The graph shows the stationary phase luciferase activities of *E. coli* MG1655 harbouring different reporter plasmids assayed as under Figure 1: pVI931 (WT), pVI934 (mut 1), pVI1022 (mut 2), pVI1023 (T-7A), pVI1024 (T-7G), pVI1025 (T-7C) and pVI1026 (triple mut T-11A/A-15T/G-9A and the substitutions in mut 1).

sequence as wild type. The activities of the different Pr variants of the library were screened in *E. coli* MG1655 using a luciferase plate test assay. Of 135 mutants screened, 43 were found to exhibit enhanced output from Pr in comparison to an equivalent control plasmid bearing the native -38 to $+127$ region. Of these, eight exhibit a hyperactive phenotype vastly increased promoter output. DNA sequencing allowed grouping of these eight hypermutants into five classes (A–E), in which each class carried identical multiple mutations. In addition to mutations common to each class, all possessed a unifying T \rightarrow A substitution at position -11 relative to the transcriptional start site (Supplementary Figure S1). To ascertain if the T \rightarrow A substitution at position -11 was responsible for the hyperphenotype of all the five classes, an otherwise identical transcriptional reporter plasmid bearing just the T-11A substitution was generated and compared with a representative from each class. Quantitative assays confirmed that the T-11A substitution alone can mediate a hyperpromoter phenotype, resulting in ~ 100 -fold higher output than its wild-type counterpart (Figure 1B). This greatly exceeds the ~ 5 -fold enhanced Pr output observed upon removal of the DNA encoding the 5'-UTR of *dmpR* (Figure 1B, compare + and $-5'$ -UTR controls). When the Pr T-11A-*luxAB* reporter plasmid was introduced into *P. putida* KT2440, it was found to be so powerful that it readily accumulated secondary

promoter-down mutations. Thus, all subsequent *in vivo* promoter activity assays were performed in the heterologous *E. coli* MG1655 host.

Targeted mutagenesis identifies additional hypermutants of Pr

The identification of the T-11A hypermutant suggested to us that this region of the core Pr promoter exerts a major constraint on Pr activity. To further investigate the contribution of the nucleotides around the -11 position, we generated a series of thirty additional plasmids in which the -38 to $+8$ region of *dmpR* controls transcription of the *luxAB* genes. These otherwise identical transcriptional reporter plasmids contain single substitutions of the 10 bases underlined in Figure 2A, which were each exchanged to all alternative bases. This scanning mutagenesis approach led to the identification of three independent substitutions that increased Pr output. The T-11A substitution was found to be the most potent of these: in this DNA context (i.e. lacking the influence of the 5'-UTR encoding DNA on Pr activity), the T-11A substitution increased output 42 ± 3 -fold as compared to the wild type. Two other substitutions, A-15T and G-9A, also markedly increased Pr output *in vivo* (11 ± 1 -fold and 9 ± 1 -fold, respectively, Figure 2A, left-hand graph).

We also analysed the effect of these substitutions *in vitro* using equivalent -38 to $+8$ Pr promoter variants cloned

into the *in vitro* transcription vector pTE103. Single round *in vitro* transcription assays using supercoiled templates (Figure 2A, right-hand graph) showed the same relative order of enhancement of Pr output (T-11A>A-15T>G-9A), although the differences between the variants were muted as compared with the *in vivo* result. The three stimulatory substitutions were each also combined with a previously defined Pr promoter mutation (mut 1, Figure 2A) that renders Pr inactive (21). In these cases, no transcripts were found, demonstrating that the three stimulatory substitutions mediate their effects through Pr activity, rather than creating independent promoters (Figure 2A). This conclusion is also supported by the identification of the same transcriptional start by primer extension analysis of RNA generated *in vitro* using the T-11A variant (Supplementary Figure S2).

Pr possesses a critical -35 element and an extremely suboptimal -10 region

Examination of the Pr core promoter region in the light of the three stimulatory mutations suggested that Pr might be a promoter that essentially lacks a -10 element (Figure 2B, upper). First, the region spanned by the substitutions of mut 1 incorporates a near perfect -35 element (-35 to -30 TTGACT, consensus TTGACA) that would be severely affected by three of the exchanges made in this mutation (-35 GTCAGT in mut 1). Secondly, although an appropriately spaced (17 bp) -10 element has only one of the six bases of the consensus (-12 to -7 CTGGCT, consensus TATAAT), all the three stimulatory substitutions would be predicted to improve functionality of this region: the T-11A and G-9A substitutions by exchanges towards consensus (from CTGGCT to CAGGCT or CTGGAT, respectively), while the A-15T substitution creates an extended -10 element (TGGCTGGCT, consensus TGnTATAAT). To further test if Pr has a -35 element and an essentially non-existent -10 region, we tested additional variants. In mutant 2 (mut 2, Figure 2B), just the potential extended -10 motif of the -38 to -30 region was destroyed, leaving the -35 motif intact. This would be predicted to have a drastic negative effect on Pr output if this region constituted an extended -10 element. However, rather than having a negative effect, the substitutions of mut 2 had a slight stimulatory effect, strongly supporting the idea that this region encompasses an essential -35 element. Next, we tested if substitution of the single consensus T (-7 T) of the potential -10 region influenced Pr output. Exchange to any other base abolished Pr activity, demonstrating the critical nature of this base for Pr output (Figure 2B). As a final test, we combined the three stimulatory -10 element substitutions (triple mut, Figure 2B) in the context of mut 1. Detectable activity was found with this derivative, despite the presence of the mut 1 substitutions that abolish Pr activity in the wild-type context. Hence, we conclude that Pr promoter has a critical -35 element (TTGACT, -35 to -30) that is associated with an extremely suboptimal -10 element (CTGGCT -12 to -7).

The T-11A substitution accelerates the rate of open complex formation

DksA and ppGpp co-stimulate transcription from Pr *in vivo* and *in vitro*, and DksA also has a ppGpp-independent stimulatory effect on Pr output (21). We compared *in vivo* transcription of the luciferase transcriptional reporters carrying either the wild-type Pr (-38 to $+8$) or the Pr T-11A variant in *E. coli* MG1655 and its otherwise isogenic ppGpp⁰ and DksA null counterparts (Figure 3A). Consistent with previous results in *P. putida* (21), lack of either of these two regulatory molecules decreased output from wild-type Pr. However, the response for the Pr T-11A variant was markedly different: output was still reduced in the strain lacking DksA, but lack of ppGpp resulted in increased, rather than decreased, output from the Pr T-11A variant. These results prompted us to investigate the effects ppGpp and DksA on transcription from wild-type Pr and Pr T-11A *in vitro*.

Single round *in vitro* transcription assays using supercoiled DNA templates showed the anticipated ppGpp-independent and ppGpp-dependent stimulatory effects of DksA with the wild-type Pr (-38 to $+8$) (Figure 3B, open bars). For the T-11A variant, however, only the ppGpp-independent effect of DksA was apparent (Figure 3B, hatched bars). These results suggest that the T-11A substitution by-passes a rate-limiting step for Pr output that is normally stimulated by the co-presence of ppGpp and DksA. Transcriptional initiation is frequently slower on linear templates, making differences in promoter activities easier to detect. Therefore, we also monitored the activities of Pr WT and Pr T-11A over time in single-round *in vitro* transcription assays using linear DNA templates. These linear templates (encompassing -265 to $+8$) result in much greater differences (~ 90 -fold) between the outputs from wild-type Pr and the T-11A variant (Figure 4A), which is in line with the large differences observed *in vivo* (Figure 1B). In these assays, the measurement of full-length transcripts represents the number of heparin-stable (transcription-competent) complexes present at each time point. Importantly, in addition to mediating vastly increased transcription, the T-11A substitution also markedly accelerated the rate of transcript formation at this RNAP concentration. This could occur through effects on initial binding or at subsequent steps leading to formation of heparin-stable complexes (Figure 4A and B).

Given the stimulatory effects of ppGpp and DksA on Pr output, we also monitored the effects of these regulatory molecules in similar assays. For Pr T-11A, the rates of heparin-stable complex formation were rapid in all cases, with maximal levels reaching a plateau after 5 min (Figure 4C). In this case, ppGpp and DksA independently and additively stimulated the maximal levels of transcripts achieved. For wild-type Pr, the effects of ppGpp and DksA on the kinetics were much greater (Figure 4D). While ppGpp and DksA independently enhanced output from Pr, the kinetics were still linear and did not reach a plateau within the time frame of the experiment. This contrasts the clear synergistic effects of co-addition of ppGpp and DksA (time points 0.3–5 min, Figure 4D), which

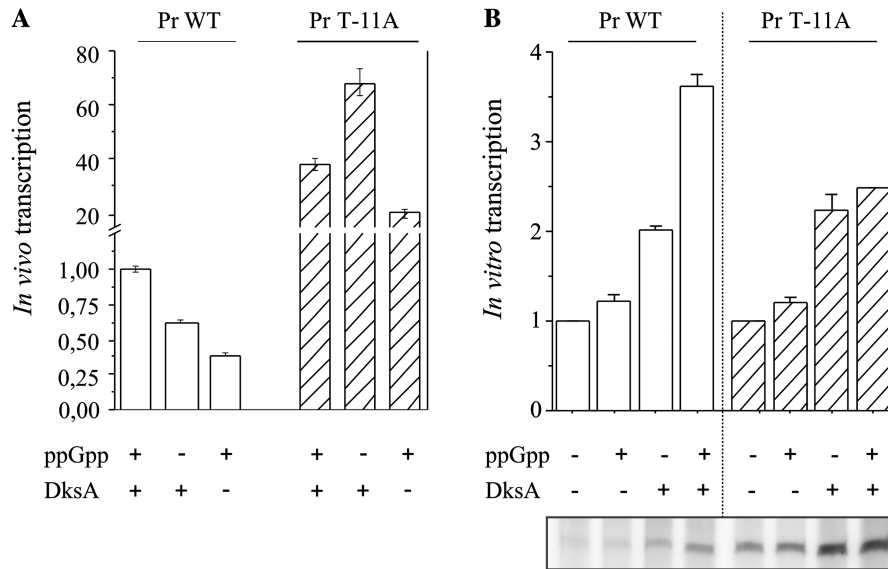


Figure 3. The T-11A substitution alters responses to ppGpp and DksA. (A) Stationary phase luciferase activities of Pr output from pVI931 (-38 to +8, Pr WT) or pVI1019 (-38 to +8, Pr T-11A) in LB-grown *E. coli* MG1655 or its otherwise isogenic ppGpp⁰ or DksA null counterparts. Assays, as under Figure 1, were normalized setting output from Pr WT in MG1655 as 1. (B) Single-round *in vitro* transcription assays on supercoiled DNA templates carrying Pr WT (-38 to +8, pVI950) or with an T-11A substitution (pVI1030). Assays were performed with 10 nM DNA template and 25 nM σ^{70} -RNAP, in the absence or presence of 400 μ M ppGpp, 3 μ M DksA, or both. Data are the average of two independent experiments, normalized by setting the transcript levels from each template in the absence of ppGpp and DksA as 1. The autoradiograph shows Pr transcripts from one of the experiments used to obtain the average.

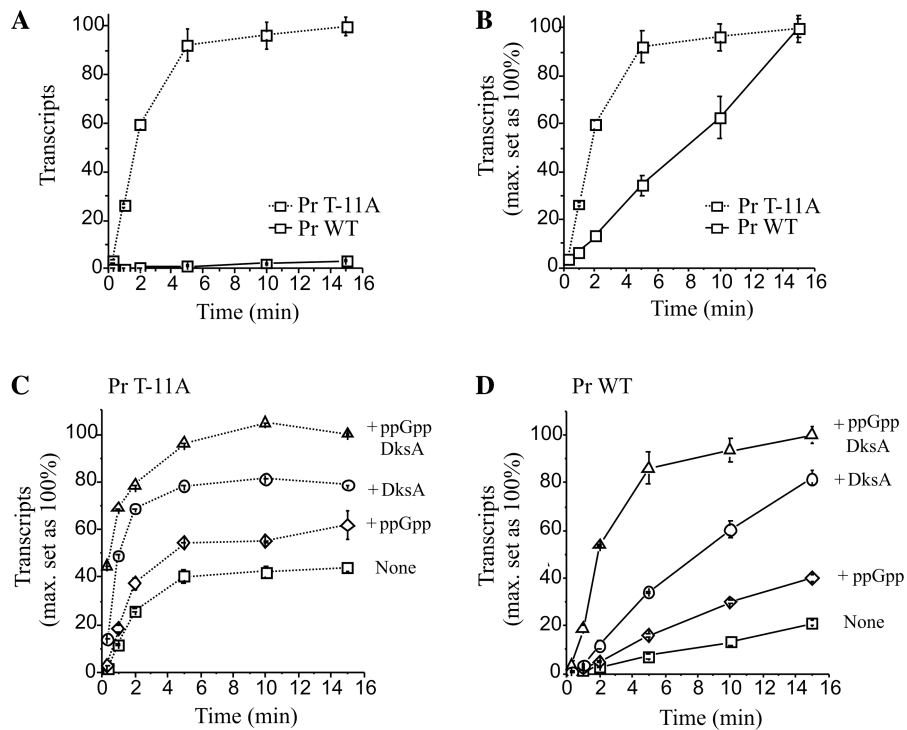


Figure 4. The T-11A substitution mimics the kinetics of ppGpp/DksA co-stimulation. (A) Single round *in vitro* transcription assays on linear DNA templates encompassing -265 to +8 of the Pr promoter region (Pr WT) or incorporating the T-11A substitution (Pr T-11A). Assays were performed with 10 nM template and 25 nM σ^{70} -RNAP. Relative transcripts (AU) from Pr WT (continuous line) and Pr T-11A (dashed lines) after different incubation times for heparin-stable complex formation (from 20 s to 15 min) are shown with the maximum transcript levels obtained from Pr T-11A set as 100. (B) As under panel (A), but setting the maximum transcript levels obtained from each promoter as 100% to illustrate the different kinetics. (C) As under panel (A), but with Pr T-11A in the absence (squares) or presence of 400 μ M ppGpp (diamonds), 3 μ M DksA (circles), or both (triangles). (D) As under panel (C), with Pr WT. Data are the averages of two independent experiments \pm SE.

result in kinetics similar to those of the Pr T-11A variant in the absence of ppGpp and DksA (compare Figure 4C and D). Hence, the T-11A substitution results in a promoter variant with kinetics that mimics those of co-stimulation by ppGpp and DksA. However, these experiments cannot distinguish if the same mechanistic step is being affected in each case. The data in Figure 4C and D are plotted to allow visualization of the kinetics, but it should be noted that even upon co-stimulation by ppGpp and DksA, maximal output from wild-type Pr is still much lower than for Pr T-11A (~45-fold at the 15-min time point). These data suggest that while the rate of heparin-stable complex formation is the step that is stimulated by co-addition of ppGpp and DksA, this parameter is not the only step that is altered by the T-11A substitution.

The T-11A substitution alters the nature of the promoter- σ^{70} -RNAP complexes

Initial test using Pr templates encompassing the -38 to +8 region in electromobility shift assays (EMSAs) demonstrated that specific binding of σ^{70} -RNAP to wild-type Pr could not be detected in the presence of competitor DNA (salmon sperm DNA). This contrasted the readily detectable binding to Pr T-11A, which was dependent on the critical -35 element that is disrupted in mut 1 (Supplementary Figure S3). Therefore, we performed DNase I footprinting assays in the absence of competitor DNA. A notable feature of the footprints shown in Figure 5A is that the T-11A substitution results in a longer σ^{70} -RNAP footprint than that observed with wild-type Pr. As summarized in Table 2, an extended footprint with the T-11A variant to encompass promoter downstream DNA was also found when probing the coding strand (Supplementary Figure S4A). Such extended footprints are typical of binary open complexes (RP_o) in which DNA melting extends downstream of the +1 to around +20, while the shorter protection observed for wild-type Pr is similar to those that have been described for closed (RP_c) or intermediate (RP_i) complexes (31,32).

Although RP_c and RP_i complexes are often short lived at 30°C, they can be accumulated within given temperature intervals. Additionally, depending on the promoter, intermediates can also differ in their sensitivity to heparin (RP_c and some unstable RP_i are heparin sensitive, whereas some stable RP_i and RP_o are heparin resistant) (31 and references therein). For the T7A promoter, the *lacUV5*, the *trp* and a hybrid *trp/lac* promoter, RP_c accumulate below 8°C, RP_i between 8°C and 21°C and RP_o above 21°C (31 and references therein). Therefore, we also performed DNase I footprinting assays at 4°C in the absence of heparin (Figure 5B and Supplementary Figure S4B) or in the presence of heparin (data not shown). At 4°C, footprints for the wild-type Pr and the T-11A variant were indistinguishable (Table 2), and in both cases the complexes were undetectable in the presence of heparin. In contrast, at 30°C, the extended footprint found with T-11A was resistant to heparin. Thus, the two promoter variants form apparently identical heparin-sensitive RP_c

(or unstable RP_i) complexes that in the case of the T-11A variant can rapidly convert to a heparin-resistant RP_o (or stable RP_i) complex.

The T-11A substitution *per se* promotes open complex formation with σ^{70} -RNAP

To test if σ^{70} -RNAP forms an open complex with Pr T-11A at 30°C, we performed KMnO₄ footprinting to detect reactive thymines within the region of DNA melting. Binary open complexes were only detectable with the Pr T-11A variant (Figure 5C), which exhibited reactive thymines at positions -11, -6 and +1 on the non-coding strand and -5 and -7 on the coding strand (Table 2). When mixtures containing binary open complexes were supplemented with NTPs (to allow progression through ternary initiating RP_{init} complexes to the elongating mode, RP_E) and NaCl (to eliminate remaining binary complexes), additional promoter downstream reactive thymines were detected irrespective of the presence or absence of heparin (Figure 5C, Table 2). Hence, we conclude that Pr T-11A spontaneously forms stable open complexes which rapidly progress to the elongation mode *in vitro*.

Although binary open complexes could not be detected with wild-type Pr, faint reactive thymine bands could be detected in the absence of heparin under conditions that allow progression to RP_E (Table 2, and data not shown). Presumably, this occurs by displacing the equilibrium of the low levels of unstable intermediates formed at this promoter to a more stable RP_E complex. Acceleration of open complex-formation to the elongation mode by ppGpp and DksA is clearly apparent in *in vitro* transcription assays (Figure 4); however, despite our efforts, we were unable to capture stimulation of this process by addition of ppGpp and/or DksA within these footprinting assays.

The T-11A substitution facilitates initial binding of σ^{70} -RNAP

Open complex formation is, to a large extent, dependent on the ability of the promoter to initially bind the holoenzyme. To test the effect of the T-11A substitution on binding of σ^{70} -RNAP, we performed EMSA analysis with linear DNA fragments encompassing -100 to +123 relative to +1 of *dmpR*. Reactions were performed at 4°C, where no DNA melting occurs and the Pr WT and Pr T-11A variant produce indistinguishable footprints (Figure 5B and Supplementary Figure S4B). Binding of σ^{70} -RNAP to Pr T-11A was more efficient and detectable at lower concentrations than binding to Pr WT (Figure 6A). As would be expected for closed promoter complexes, binding was heparin sensitive in both cases. These data strongly suggest that the T-11A substitution, at least in part, mediates its effects through the initial binding step of closed complex formation, and that binding of σ^{70} -RNAP to Pr is a major constraint for Pr output that likely contributes to the poor performance of this promoter both *in vivo* and *in vitro*.

Because DksA has previously been found to enhance binding of σ^{70} -RNAP to promoters (e.g. 9,33), we also

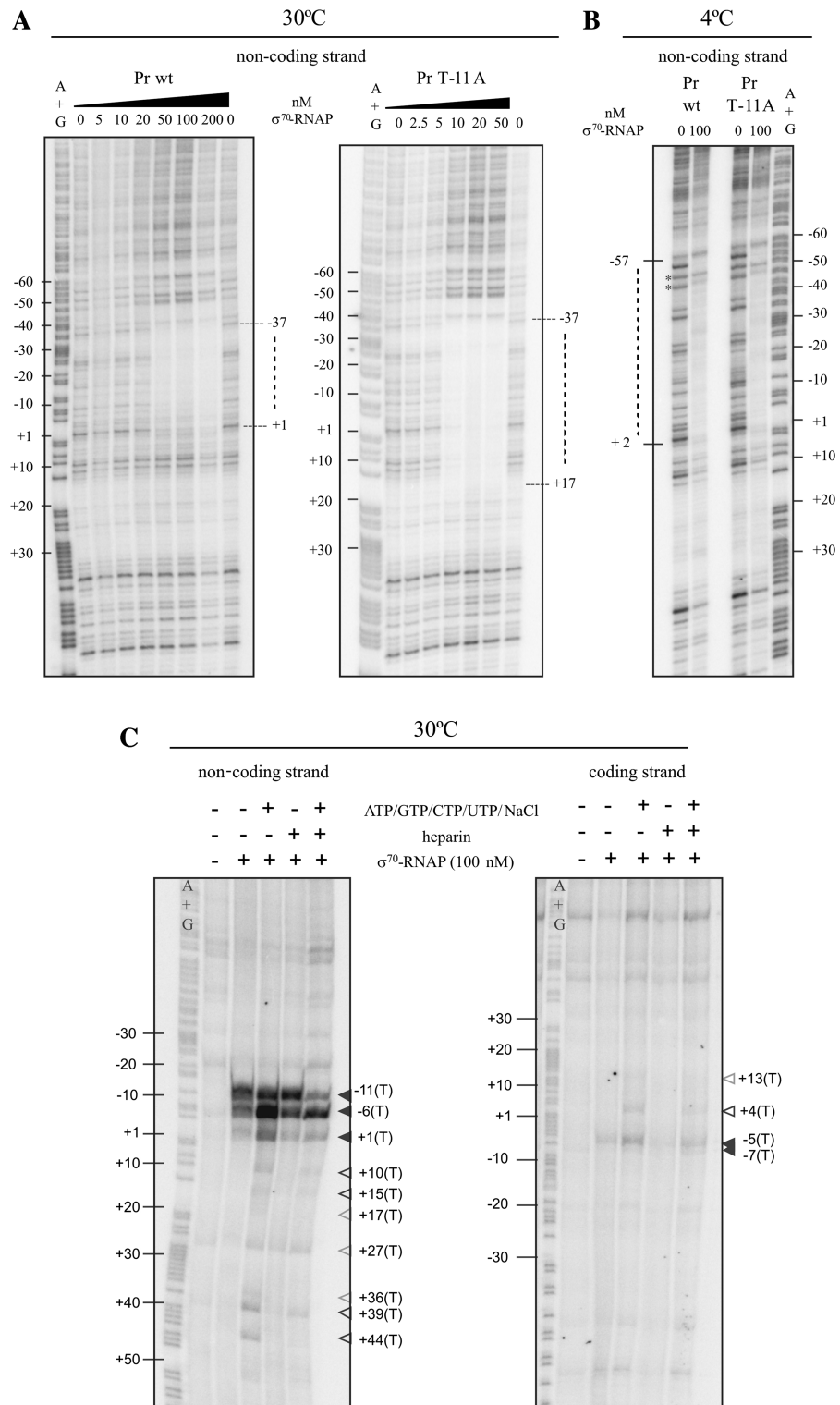


Figure 5. The T-11A substitution mediates detectable open complex formation. (**A** and **B**) DNase I footprinting of σ^{70} -RNAP binding to the non-coding strand (–265 to +126) of Pr WT or Pr T-11A at 30°C (for 30 min, panel A) or 4°C (for 60 min, panel B). The regions protected from DNase I cleavage are indicated between the dashed lines. (**C**) KMnO_4 footprinting assays. Binding reactions contained linear DNA encompassing the Pr promoter DNA bearing the T-11 A substitution (as under panels A and B), labelled either on the coding (right, Pr –112 to +126) or non-coding (left, Pr –265 to +126) strand. Binary open complexes and ternary elongating complexes were formed as described under ‘Materials and Methods’ section. The thymines that react to KMnO_4 are marked with closed (binary complexes) or open (ternary complexes) black triangles. Open grey triangles indicate faint minor reactive thymines that appear for the ternary elongating complexes.

Table 2. Summary of promoter-RNAP complexes formed on wild-type Pr and Pr T-11A

$$R + P \rightleftharpoons RP_c \rightleftharpoons RP_i \rightleftharpoons RP_o \xrightarrow{+NTPs} RP_{init} \xrightarrow{+NTPs} RP_E$$

σ factor

Footprinting	PrWT		Pr T-11A	
	Non-coding	Coding	Non-coding	Coding
DNase I 30°C	-37 to +1	-50 to +1	-37 to +17	-50 to +22
DNase I 4°C	-57 to +2	-50 to +3	-57 to +2	-50 to +3
KMnO ₄ binary	-	-	-11, -6, +1	-7, -5
KMnO ₄ ternary initiating	-	-	-11, -6, +1	-7, -5, +4
KMnO ₄ ternary elongating	(-6, +1, +36)	(-11, -7, -5, +4, +13)	-11, -6, +1, +10, +15 (+17, +27, +36, +39, +44)	-7, -5, +4, (+13)

For DNase I footprinting, the extent of the DNA protected by binding of σ^{70} -RNAP is given relative to the +1 transcriptional start of *dmpR*. In the case of KMnO₄ footprinting, numbers refer to the positions of reactive thymines, with those in brackets corresponding to faint bands. The upper schematic shows the different steps of transcriptional initiation. σ^{70} -RNAP (R), promoter DNA (P), closed complex (RP_c), intermediate complex(es) (RP_i), binary open complex (RP_o), ternary initiation complex (RP_{init}), ternary elongation complex (RP_E).

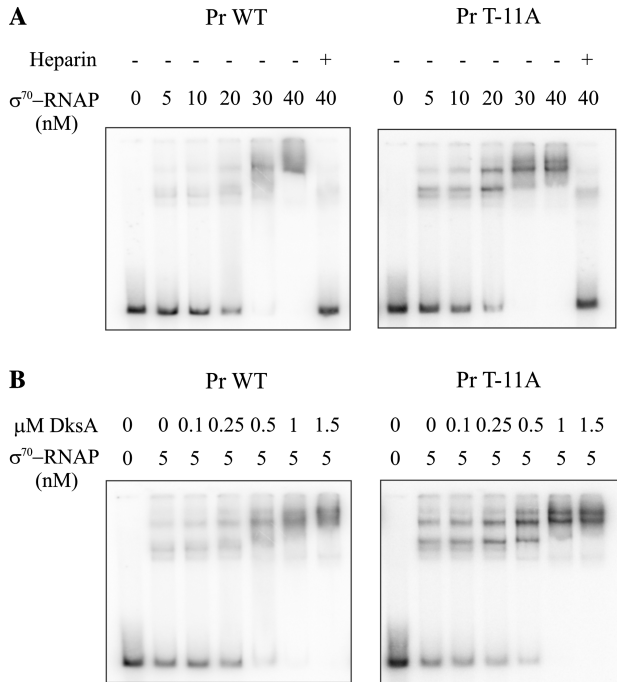


Figure 6. The T-11A substitution enhances binding of σ^{70} -RNAP. (A) EMSA assays of linear DNA probes (2 nM) encompassing the -100 to +123 Pr wild-type region (Pr WT) or a variant introducing the T-11 A substitution (Pr T-11A) and different concentrations of σ^{70} -RNAP (0, 5, 10, 20, 30 or 40 nM). The binding reactions were performed for 60 min at 4°C in the absence of any competitor DNA. Where indicated (+), heparin (final concentration 0.15 mg/ml) was added during the last 5 min. The results are representative of three independent experiments. (B) EMSA assays as under panel (A) but in the presence of 5 nM σ^{70} -RNAP and increasing concentrations of DksA (0, 0.1, 0.25, 0.5 or 1.5 μ M).

tested if DksA facilitated binding of σ^{70} -RNAP to Pr. Titrations of DksA enhanced binding of σ^{70} -RNAP to both Pr WT and Pr T-11A (Figure 6B). Addition of ppGpp had no additional stimulatory effect on binding

of σ^{70} -RNAP in EMSA assays, either when added alone or in the presence of DksA (data not shown). Therefore, we conclude that the ppGpp-independent stimulatory effect of DksA in *in vitro* transcription assays is likely a consequence of improved binding of σ^{70} -RNAP to the Pr promoter DNA.

DISCUSSION

The σ^{70} -Pr promoter controls expression of the aromatic sensor-regulator DmpR that is strictly required for transcription from the *dmp*-operon promoter, and thus formation of the (methyl)phenol catabolic enzymes. The Pr promoter is intrinsically weak in the absence of stimulation by ppGpp and DksA. Here, we show that the poor performance of the σ^{70} -Pr promoter is attributable to its possession of a markedly suboptimal -10 element that compromises both σ^{70} -RNAP binding and the rate of formation of heparin-stable complexes—the process that is stimulated by the co-action of ppGpp and DksA. Our evidence for this configuration of the Pr promoter is the following: (i) an almost perfect -35 element is located between positions -35 and -30 of the core Pr promoter and disruption of this motif (but not disruption of an overlapping potential extended -10 motif) totally abolishes any detectable transcription; (ii) hypermutants of Pr map to an optimally spaced -10 element that naturally has only one out of six of the bases of the consensus -10 hexamer, and either result in an additional consensus base (in the case of the T-11A and G-9A mutations) or expand the motif to that of an extended -10 promoter (in the case of the A-15T mutation); (iii) the hypermutations do not create new promoters, rather they—like wild-type Pr—are dependent on the critical -35 region for activity; (iv) when combined, the three hypermutations allow detectable transcription in the absence of the otherwise essential -35 element; and (v) substitution of the single -10 element consensus base (-7T) to any other base abolishes

detectable transcription even in the presence of the critical -35 element.

We found that the T-11A substitution of the Pr promoter was the most potent of the hypermutations, mediating vastly increased output from the Pr promoter both *in vivo* (Figures 1 and 2A) and *in vitro* (Figure 4A). The $-11A$ (along with $-12T$ and $-7T$) is one of the three most highly conserved residues of the -10 element (34, Figure 7). This element interacts with the σ_2 domain as double-stranded DNA within the closed complex (via subregion 2.4) and as single-stranded DNA during melting to the open complex (via subregion 2.3). The presence of the consensus $-11A$ in the T-11A mutant greatly increases binding of σ^{70} -RNAP to the Pr promoter (Figure 6), which is consistent with previous work on other σ^{70} -promoters where mutations from the $-11A$ consensus decrease affinity for σ^{70} -RNAP (35 and references therein). Binding of σ^{70} -RNAP to the wild-type Pr promoter is so weak that it cannot even be detected in the presence of non-specific competitor DNA (Supplementary Figure S3). Hence, low affinity for σ^{70} -RNAP is one major attribute that constrains the output from Pr. Although the T-11A substitution increases binding of σ^{70} -RNAP to Pr, binding is still further enhanced by DksA (Figure 6B), which likely underlies why transcription from even this powerful promoter variant is decreased in a DksA null strain (Figure 3A). DksA mediates long-range structural changes within RNAP that alter its interactions with the -6 to $+6$ region at σ^{70} -promoters (12–14). However, the molecular details of how DksA stimulates binding of σ^{70} -RNAP to promoters remain to be resolved.

In addition to binding of the holoenzyme to the promoter DNA, the $-11A$ residue plays a crucial role in the formation of open promoter complexes. The T-11A substitution within Pr radically accelerates the kinetics of open complex formation to match and even exceed those observed with the wild-type Pr promoter upon co-addition of ppGpp and DksA (Figure 4C and D). The kinetic changes mediated by the T-11A substitution lead to rapid accumulation of heparin-stable open complexes at this promoter variant, which is in marked contrast to wild-type Pr where such complexes cannot be detected (Figure 5C). Work from different laboratories has underscored the importance of the $-11A$ residue in the

nucleation of promoter DNA melting (36 and references therein). During this process, $-11A$ is rotated out of the DNA helix and captured by Y430 within region 2.3 of σ^{70} (37). Because substitution of the consensus $-11A$ to other bases is deleterious to nucleation of DNA melting and results in slower rates of stable open complex formation (35), nucleation of promoter melting represents a likely step that is stimulated by co-action of ppGpp and DksA at Pr.

Besides lack of homology to consensus within the -10 element, another particular feature of the Pr promoter is the G-rich region between the -10 element and the $+1$ (4Gs in positions -1 to -4 , see Figure 7). GC-rich regions are typical of discriminators that are characteristically associated with promoters that are negatively (stringently) regulated by ppGpp (38). The discriminator region interacts with σ region 1.2 at many, if not all, σ^{70} -dependent promoters (39). Suboptimal discriminator- σ 1.2 contacts, in part, underlie the notorious instability of the open complexes of rRNA promoters, which are further destabilized by ppGpp and DksA (40). However, the effects of discriminator sequences are promoter specific (41), and suboptimal -35 elements and spacing between the -35 and -10 also contribute to negative stringent regulation at different promoters (e.g. 42,43). At the *tyrR* promoter, GC content of its discriminator region is related to its inability to form stable open promoter complexes, and mutational analysis suggests that this region imposes kinetic resistance to DNA untwisting and/or DNA melting (44). Although it remains to be experimentally tested, similar constraints mediated through the -1 to -4 G-rich region of Pr and/or suboptimal contact with σ region 1.2 may contribute to the inability of this promoter to form detectable stable open complexes.

The promoter alignment shown in Figure 7 highlights the two conspicuous features of the Pr promoter that are not apparent in other ppGpp/DksA co-stimulated promoters, namely lack of homology within the -10 element and the G-rich region in the melt region adjacent to $+1$. Although examples of σ^{70} -dependent promoters that lack a -35 element are relatively common, such promoters have an extended -10 element that is readily identified through *in silico* analysis. It is worth noting that one out of six matches to the consensus -10 element, as found in Pr, is below random chance. We have been unable to identify any other verified promoter with so little identity in its -10 element. This lack of identity with the consensus -10 element leads to miss-predictions or non-detection of the Pr promoter using online promoter search engines. Hence, it is perfectly plausible that other σ^{70} -promoters that essentially lack a -10 element exist but have eluded detection. The identification of the extremely suboptimal -10 element of Pr and the constraints it imposes on both binding of σ^{70} -RNAP and the rate of open complex formation should greatly facilitate future work aimed to elucidate the mechanisms by which divergent transcription and the presence of the 5'-UTR modulate the activity of this unusual promoter.

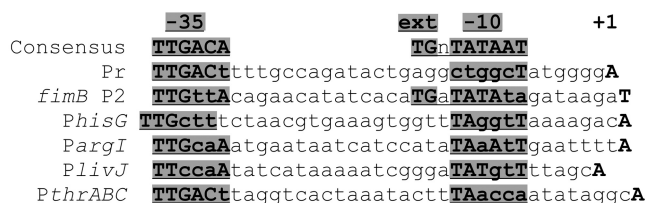


Figure 7. Alignment of Pr with other ppGpp/DksA co-stimulated σ^{70} -promoters. Sequences of σ^{70} -promoters, which have been documented to be directly co-stimulated by ppGpp and DksA (8,9,21), are aligned relative to their -10 elements. Sequences of the -35 and -10 elements [and where present extended (ext) -10 element] are shown underlined and highlighted in grey. Similarity to consensus is emphasized by uppercase bold letters, as are the $+1$ start sites.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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