Transcription activation by the siderophore sensor Btr is mediated by ligand-dependent stimulation of promoter clearance

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ABSTRACT

Bacterial transcription factors often function as DNA-binding proteins that selectively activate or repress promoters, although the biochemical mechanisms vary. In most well-understood examples, activators function by either increasing the affinity of RNA polymerase (RNAP) for the target promoter, or by increasing the isomerization of the initial closed complex to the open complex. We report that Bacillus subtilis Btr, a member of the AraC family of activators, functions principally as a ligand-dependent activator of promoter clearance. In the presence of its co-activator, the siderophore bacillibactin (BB), the Btr:BB complex enhances productive transcription, while having only modest effects on either RNAP promoter association or the production of abortive transcripts. Btr binds to two direct repeat sequences adjacent to the −35 region; recognition of the downstream motif is most important for establishing a productive interaction between the Btr:BB complex and RNAP. The resulting Btr:BB dependent increase in transcription enables the production of the ferric-BB importer to be activated by the presence of its cognate substrate.

INTRODUCTION

Transcription factors determine the global landscape of gene expression. Bacterial regulatory proteins most often act on transcription initiation, but the precise mechanisms vary. Transcription initiation is a complex, multistep process. In general, promoter recognition can be divided into an initial binding event (leading to a transient, closed complex, RP_C), isomerization to one or more intermediate (RP_i) complexes, and finally formation of the fully strand-separated open complex (RP_O) (1). Upon binding of initiating nucleotides, RNA polymerase (RNAP) forms an initial transcribing complex (RP_TTC) and commences synthesis of short (usually up to ~10–12 nt) RNA products which may or may not be released in a repetitive reaction termed abortive synthesis (2,3). Promoter escape is thought to be correlated with the loss of σ contacts to the promoter, release of σ from the elongating ternary complex, and conversion of RP_TTC into the highly stable and processive elongation complex (RP_E) (1,4).

Bacterial RNAP is directed to specific target promoters by an associated σ subunit which contacts DNA regions centered near −10 and −35 relative to the transcription start site (5,6). Repressor proteins bind near or overlapping the promoter recognition elements can impede RNAP binding or promoter escape. In many cases, simple occlusion of the promoter suffices for repression (7). In contrast, activation requires activator:RNAP interactions that increase the rate of the slowest (rate-limiting) step in initiation (8).

Mechanisms of activation have been studied, to various levels of refinement, for dozens of different bacterial activators. In most cases, activators bind adjacent to, and upstream of, RNAP and establish protein–protein interactions with either the α-subunit C-terminal domain (αCTD) or region 4 of the σ subunit (9,10). These interactions may serve to increase the affinity of RNAP for the promoter region (initial binding event; K_B) or the rate of the subsequent isomerization step(s) (k_i) leading to the formation of the transcriptionally competent open complex (8). Historically, activators that affect K_B versus k_i were often distinguished using abortive initiation assays in which the rate of product synthesis was monitored as a function of RNAP concentration (3). This assay, developed using Escherichia coli σ^70 holoenzyme, allows the early steps in transcription initiation to be separated into those that are dependent on RNAP concentration (K_B) and those that are independent (k_i). RNAP binding affinity can be independently monitored using direct methods such as the electrophoretic mobility shift assay (EMSA) or DNase I footprinting (11), while DNA

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The Q-modified RNAP efficiently escapes the pause to promoter and malT (which can release abortive products and revert to RPO) isomerization to RPO can be monitored using chemical (KMnO₄) sensitivity to monitor DNA melting (12). Depending on the details of the initiation pathway, the rate-limiting step assigned as kₑ may or may not correspond to DNA melting.

While most activators exert their primary effects on either initial binding or promoter isomerization, at some promoters later stages may be rate-limiting and therefore appropriate targets for activator proteins. For example, transcription activators may stimulate either the initiation or early elongation steps of RNA synthesis (regulators acting after the transition from Rₚₑ to Rₚₒ are classified instead as elongation factors; 13). Classic examples of late-acting activator proteins include E. coli CRP at the malT promoter and Q at Pᵣₑ. In the former case, CRP activates the production of full length transcripts while having little effect on either promoter binding or initiation (as measured using an abortive initiation assay) leading to the suggestion that CRP specifically increases the rate of promoter escape (14). Subsequent studies, however, support a model in which CRP acts early in initiation to favor formation of an escape-competent open complex (15,16). The Q protein acts on a promoter-proximal, paused elongation complex to stimulate promoter escape (17,18). Promoter escape, the conversion of the RₚₑTC (which can release abortive products and revert to Rₚₒ) into the stable and highly processive Rₚₒ complex, is generally accompanied by σ release although exceptions may be more prevalent than previously appreciated (19,20).

Recent studies suggest a correlation between the presence of σ factor recognition elements in the early transcribed region and stalling of RₚₑTC (promoterproximal pausing) (18,21). First recognized in the case of λ phage Pᵣₑ, recognition of a downstream promoter-like element by the still associated σ subunit leads to pausing of the RₚₑTC which provides a target for Q modification. The Q-modified RNAP efficiently escapes the pause to generate full-length transcripts (17). More generally, σ-dependent pausing during promoter clearance increases the association of σ during elongation and thereby modifies the properties of Rₚₑ, likely by competing for binding with elongation factors (20). In Bacillus subtilis, the GreA transcript cleavage factor has been proposed to associate with early paused elongation complexes to stimulate escape of RNAP from the promoter (22). Studies in E. coli suggest that promoter-proximal pausing may affect the transcription of a large fraction (estimated at >20%) of transcription units (20). Evidence for the widespread occurrence, and regulatory impact, of promoter-proximal pausing has also emerged in a variety of model eukaryotes (23,24).

Here, we have investigated the mechanism of transcription activation by B. subtilis Btr (25), an unusual member of the AraC family of transcription factors (26). Biochemical studies of AraC family activators are often challenging, due to the difficulties with both over-expression and purification (27,28). As a result, only a handful of AraC family proteins have been biochemically characterized (28–31). Btr contains an amino-terminal DNA-binding and dimerization domain appended to a carboxy-terminal ligand-binding domain structurally and functionally related to siderophore substrate binding proteins associated with iron import (25). Btr binds upstream of the promoter for the feuABC operon (P_feuA) encoding an ABC transporter for the import of ferric-bacillibactin (BB), a catecholate siderophore made by various Bacillus spp. In the presence of BB (and to a lesser extent, de-ferrated BB), the Btr:BB complex strongly activates transcription from P_feuA. The mechanism of activation is shown here to involve effects on multiple steps, but most prominently a large and ligand-activated increase in the formation of productive transcripts due to an increase in the rate of promoter escape.

MATERIALS AND METHODS

Strain construction and growth conditions

Bacillus subtilis was grown in Luria–Bertani (LB) medium or in a MOPS-based minimal medium, FS-MM (32). Unless otherwise indicated, liquid media were inoculated from an overnight preculture and incubated at 37°C with shaking at 200 rpm. For selection, antibiotics were added at the following concentrations: erythromycin (1 μg/ml) and lincomycin (25 μg/ml) [for selecting for macrolide-lincosamide-streptogramin B (MLS) resistance], spectinomycin (100 μg/ml), chloramphenicol (10 μg/ml), kanamycin (15 μg/ml) and neomycin (10 μg/ml). Routine molecular biology procedures were carried out using E. coli DH5α for DNA cloning as described in Ref. (33). Isolation of B. subtilis chromosomal DNA, transformation and specialized SPβ transduction were performed according to (34). Restriction enzymes, DNA ligase and DNA polymerases were used according to the manufacturer’s instructions (New England Biolabs).

To construct an feuA-lacZ transcriptional fusion, the feuA regulatory region (P_feuA) was amplified from genomic DNA by PCR and cloned as a HindIII-BamHI fragment into pJPM122 (35). The resulting construct was linearized with ScaI and used to transform strain ZB307A (36) to neomycin resistance by integration into the temperature sensitive SPβ prophage. An SPβ transducing lysate was prepared by heat induction (35) and used to transduce the resulting P_feuA-lacZ transcriptional fusion into strain HB8242 which lacks Fur and is Sfp⁺ (25). This strain constitutively expresses bacillibactin and therefore constitutively activates P_feuA. β-galactosidase activity was assayed in LB medium using a modification of the procedure of Miller (37) as described in Ref. (38).

Purification of RNAP, σₐ, Btr and BB

RNAP was purified from B. subtilis CU1065 cells by polyethyleneimidine precipitation followed by heparin affinity and size exclusion (Superdex 200 FPLC) column chromatography as previously described (39). The resulting RNAP is a mixture of core enzyme and σₐ holoenzyme. For reconstitution of σₐ-saturated holoenzyme, σₐ was purified after overproduction in E. coli using a DEAE-sepharose column, followed by a monoQ column as described in Ref. (39). His-tagged Btr protein was purified after overproduction in E. coli using Ni-NTA beads followed by size exclusion column chromatography.

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(Superdex 200 FPLC) as described in Ref. (25). Ferric-bacillibactin (here designated as BB) was purified from the supernatant of iron starved cultures of \textit{B. subtilis} strain (an Sfp\textsuperscript{+} strain) using a modification of published procedures (40) as described in Ref. (25).

**DNA binding (EMSA) and melting (KMnO\textsubscript{4}) assays**

\( P_{feuA} \) containing DNA fragments were amplified from \textit{B. subtilis} chromosomal DNA by PCR using a [\( \gamma^{32}\text{P} \)]-ATP labeled primer. To monitor Btr binding to \( P_{feuA} \) EMSA were done using \(<100\) pM of DNA as described previously (25) in a buffer containing: 20 mM Tris pH 8.0, 50 mM NaCl, 50 mM KCl, 5\%(v/v) glycerol, 5 \( \mu \)g/ml salmon sperm DNA and 2\,mM DTT. For RNAP EMISA, the buffer used was 20\,mM Tris–HCl (pH 8.0), 10\,mM Mg\textsubscript{2+}, 2\,mM DTT, and 50\,mM KCl, 3\,mM acetylated BSA, and 5\% (v/v) glycerol. A competitor oligonucleotide duplex containing a consensus promoter (\( P_{con} \)) was formed from oligonucleotide 5\textasciitildeGGCTTCGGAAAAATGTGAATGCTATC\tilde{G}TATTGTATGGAATGACAGAATTCGG3\textasciitilde and its complement. The two oligonucleotides were mixed together, heated at 95\,\degree C for 5 min and annealed at 55\,\degree C for 1 min followed by slow cooling to room temperature. DNA was combined with Btr and BB (where indicated) in the reaction buffer and incubated for 5\,min at room temperature. RNAP was added and the tubes were incubated at 37\,\degree C for 5\,min, followed by addition of A, G and U (0.25\,mM each) and further incubation for 10\,min. \( P_{con} \) was added to a final concentration of 1.5\,\mu M and the reaction was incubated for 5\,min (unless indicated otherwise) and loaded on a 5\% polyacrylamide native gel in TBE buffer.

To monitor promoter melting at \( P_{feuA} \). RNAP and regulatory proteins were added to negatively supercoiled plasmid (pJPM122 carrying \( P_{feuA} \)) and KMnO\textsubscript{4} probing and detection of DNA reactivity by primer extension was done as previously described (41). Sites of reactivity were indexed using an A+G chemical sequencing ladder generated from an end-labeled DNA fragment as size markers.

\**In vitro transcription**

For \textit{in vitro} transcription, \( \sigma^{\text{A}} \)-saturated holoenzyme was reconstituted by mixing purified RNAP with purified \( \sigma^{\text{A}} \) (1:5 molar ratio) in transcription buffer (10\,mM Tris, 10\,mM MgCl\textsubscript{2}, 0.5\,mM EDTA, 1\,mM DTT, 10\,mM KCl, 50\,\mu g/ml acetylated BSA) and incubating on ice for 15\,min. The \textit{in vitro} transcription reactions contained 100\,ng (10\,nM) of \( P_{feuA} \) promoter fragment in transcription buffer with Btr or Btr:BB (as indicated) incubated for 15\,min at room temperature. For most transcription reactions, a 568-bp PCR product was amplified using primer 1 (5\textasciitildeGGGAGCTTTGTGGGATTAGGTTCCG3\textasciitilde) and primer 2 (5\textasciitildeGGGATCCCGTAATGGCACAATTGTCTC3\textasciitilde) to give a fragment that yields a 224-nt transcript. RNAP was added and the reactions were incubated for 10\,min at 37\,\degree C. Transcription was initiated by adding 0.25\,mM (final concentration) of each nucleotide and 25\,\mu Ci of [\( \gamma^{32}\text{P} \)]-UTP. After 7-min incubation, the reaction products were ethanol precipitated in the presence of 0.3\,M sodium acetate pH 5.2 and 3\,\mu g glycogen. The RNA pellet was washed with 70\% cold ethanol, dried and dissolved in formamide containing loading buffer and separated on a 6\% denaturing polyacrylamide sequencing gel with a DECADE RNA marker (Ambion). For single round transcription reactions, the same procedure was followed except that CTP was omitted from the NTP mixture. After 10\,min of incubation at 37\,\degree C, 0.25\,mM CTP and 20\,\mu g/ml heparin (final concentrations) were added.

For the abortive transcription assay, a similar protocol was used except that DNA was PCR amplified with primers 1 and 3 (5\textasciitilde-GTAAGAGAATCTTTTCTTAT-3\textasciitilde) to give a 417-bp fragment yielding a 55-nt transcript. Reaction products were precipitated in the presence of 0.3\,M sodium acetate with glycogen as a carrier and analyzed on a 23\% polyacrylamide gel as described in Ref. (42).

\**RESULTS AND DISCUSSION**

Btr binds to a direct repeat in the feuA promoter

We demonstrated previously that Btr protects an extended region in the \textit{feuA} promoter region (\( P_{feuA} \)) which contains a 9 base direct repeat that overlaps with the upstream portion of the \( \sim 35 \) element (25). Btr bound to ferric-bacillibactin (designated Btr:BB) has a high affinity (\( K_{d} \) of \(<16\,nM) for \( P_{feuA} \) \textit{in vitro} and strongly (\(<100\)-fold) activates transcription \textit{in vivo}. In contrast, in the absence of activating ligand, Btr binds DNA with \(<2\)-fold reduced affinity \textit{in vitro} and activates transcription \(<22\)-fold \textit{in vivo} (25). Thus, Btr is required for the basal level \( P_{feuA} \) expression (as seen in strains unable to synthesize BB).

Here, we have sought to define the sequence requirements for Btr binding and activation by introducing a series of mutations into the left and right conserved repeat sequences (Figure 1). Single mutations in any 1 of the 9-bp's of either the left or right arms of the direct repeat led to only modest decreases (\(<2\)-fold) in Btr:BB binding affinity as measured by electrophoretic mobility shift assay (EMSA; Figure 1B and Supplementary Figure S1). However, double mutations in both arms at positions 4, 6 or 7 greatly reduced Btr:BB binding affinity with only weak binding detected even at 120\,nM (Figure 1B and Supplementary Figure S1). Btr migrates as a dimer during gel exclusion chromatography and, by analogy with well-studied AraC family proteins (43,44), we propose that the two DNA-binding domains within each dimer recognize the two direct repeat sequences. Our results suggest that positions 4, 6 and 7 are critical for Btr:BB binding and that a high affinity DNA–protein complex can form despite mutations in one repeat, but not when both repeats are mutated.

One caveat with this analysis is that these DNA-binding experiments were conducted in the absence of RNAP. RNAP could increase the affinity of Btr:BB for DNA by favorable protein–protein interactions. Conversely, RNAP could decrease the affinity of Btr:BB for DNA by, for example, competing for a common DNA region such as the right arm of the Btr activator binding site.
which overlaps with the P_{feuA}−35 element. Therefore, the requirements for DNA-binding and transcription activation are not necessarily identical.

**Btr:BB has stringent sequence requirements for activation of transcription**

Next, we monitored the effects of these same activator binding site mutations on the ability of Btr:BB to activate transcription *in vivo*. As anticipated, those mutations that greatly decreased the affinity of Btr:BB for P_{feuA} in *vitro* also prevented activation *in vivo* as monitored using a P_{feuA}-lacZ reporter (Figure 1C). Intriguingly, mutations in the right arm at position 4 or 6, or in either arm at position 7, eliminated activation *in vivo* (Figure 1C), despite the fact that these changes had little effect on Btr:BB affinity for DNA *in vitro* (Figure 1B).

These findings suggest that (i) Btr binds P_{feuA} in the context of a functional activation complex *in vivo*, (ii) this complex has more stringent requirements for formation than detected in an *in vitro* EMSA analysis (in the absence of RNAP) and (iii) that interaction with the right arm is especially important in the activation mechanism. Note that the right arm of the Btr binding site overlaps the −35 region recognized by σ region 4. This region of P_{feuA} lacks a strong −35 consensus sequence (TgtGt has only two matches to the TTGACA consensus). However, support for this assignment is provided by the effects of mutations in this region: mutation of the first (consensus) T to G eliminates activity (T8G in the right repeat; Figure 1C) and mutation of the second G to a consensus base (G9T in the right repeat; Figure 1C) increases activity. This suggests a model in which binding of the downstream protomer of the Btr dimer to the right repeat establishes contacts with RNAP and thereby compensates for a weak −35 element, as previously suggested for AraC (45). To determine which step(s) in transcription initiation are affected by Btr:BB, we next used biochemical analyses diagnostic for promoter binding, RPO formation, transcription initiation and promoter escape.

**Btr and Btr:BB enhance the formation of competitor resistant RNAP complexes at P_{feuA}**

We first used EMSA to analyze promoter binding. In initial experiments, we noted that RNAP alone forms a high molecular weight complex that was largely retained in the well thereby precluding quantification (data not...
shown). We therefore conducted subsequent EMSA experiments after addition of a consensus promoter duplex (P_{con}) that competes for non-specifically or reversibly bound RNAP (46) as shown in Figure 2A. Under these conditions, only a small amount of complex was detectable after 5 min of P_{con} competition (Figure 2B; lanes 2 and 8) with RNAP alone. This is consistent with the fact that most characterized *B. subtilis* RNAP promoter complexes remain competitor sensitive until after the formation of one or more phosphodiester bonds, presumably because open (RP_{O}) and closed (RP_{C}) complexes are in rapid equilibrium with each other and with free RNAP (47).

Interestingly, if RNAP was incubated with P_{feuA} in the presence of Btr or Btr:BB, a new complex was detected which migrated with a lower mobility (Figure 2B; lanes 3, 4, 9, 10). We designate this upper complex as C_{I} and suggest that it contains Btr (with or without BB), RNAP and P_{feuA} DNA. The lower C_{II} complex is similar in mobility to that seen with RNAP alone. Note that the major shifted band detected in these assays is the DNA:Btr complex and this complex increases with time after competitor addition (Figure 2C). Thus, competitor gradually sequesters RNAP from the activator-stabilized RNAP:P_{feuA} complexes.

We next monitored the effects of adding NTPs on the formation and stability of RNAP:P_{feuA} complexes. Note that in the absence of Btr, RNAP does not form competitor resistant complexes at P_{feuA} even in the presence of ATP, GTP and UTP (AGU) which can potentially allow the formation of nascent transcripts up to the 12-mer (Figure 2B; lanes 5 and 11). Indeed, after only 2.5 min of competition nearly all of the complexes are dissociated (free DNA predominates; Figure 2C). In contrast, AGU enhances the formation of the Btr-dependent C_{I} complex (Figure 2B; lanes 6, 7, 12 and 13) and these complexes are relatively stable against P_{con} competition with a measured half-life of >8 min (Figure 2C). At both concentrations of RNAP tested, only AGU enhanced the formation of competitor-resistant complexes whereas ATP and GTP, which can allow synthesis only up to the dinucleotide, was insufficient for complex stabilization (data not shown).

We conclude that, even in the presence of Btr:BB and with NTPs allowing synthesis of RNA (up to 12 nt in length), RNAP does not transition to a competitor resistant RP_{E} complex. Instead, we suggest that transiently formed RP_{ITC} (containing between 2 and 12 nt RNA transcripts) are in equilibrium with RP_{O} (by release of abortive products), with RP_{C}, and therefore also with free RNAP. This is consistent with prior studies of *B. subtilis* RNAP in which it has been noted that the early steps in promoter binding and initiation are typically reversible and therefore sensitive to competitors (47–51).

**Both Btr and Btr:BB enhance open complex formation**

In previous EMSA studies, *E. coli* RNAP at P_{lac} was observed to form two distinct promoter-bound RP_{O} complexes which, however, differed in their ability to escape from abortive initiation into productive elongation (promoter clearance) (52). We therefore wished to test whether C_{I} and C_{II}, as detected here, are also open complexes as detected using KMnO_{4} footprinting. In initial experiments, using end-labeled linear DNA fragments, we were unable to detect promoter melting at P_{feuA} by RNAP under any conditions tested including the presence or absence of Btr, BB and AGU (data not shown). These experiments suggest that even in the presence of Btr:BB and NTPs, RP_{O} and RP_{ITC} are transient intermediates in equilibrium with RP_{C}. The lack of
KMnO₄ reactivity suggests that only low equilibrium levels of RPO and RPITC are present at this promoter.

To increase the ability to detect RPO formation, we repeated these studies using a negatively supercoiled plasmid template. Even with negatively supercoiled DNA, RNAP was unable to form an open complex in the absence of Btr (Figure 3; lane 2). Addition of Btr or Btr:BB only slightly enhanced RPO formation. The level of KMnO₄ sensitivity detected at positions −8 to −6 was enhanced only ~2-fold (after normalization to control bands in the +18 to +21 region) compared to reactions in which Fur was present to prevent RNAP binding (Figure 3; lanes 3 and 4 versus lanes 5–7). Interestingly, both Btr and Btr:BB also led to an increase in KMnO₄ reactivity within the initial transcribed region (corresponding to the binding site of Fur). The Fur box sequence has similarity with the −10 consensus for σA (Figure 1A) and this might contribute to this extended pattern of reactivity.

We conclude that RNAP fails to efficiently establish a stable RPO complex at PfeuA, and that both Btr or Btr:BB have a modest, but measurable, impact on this step. Since stabilization of RPO appears to be largely independent of BB, we suggest that this may account for the previously reported requirement for Btr for the basal level transcription from PfeuA seen in strains unable to synthesize BB (25). However, full activation of PfeuA requires BB, suggesting that there is a rate-limiting step that is activated in a ligand (BB)-dependent manner in vivo.

BB greatly enhances the ability of Btr to stimulate productive RNA synthesis

We next used multiple round, in vitro transcription reactions to monitor the effects of Btr and Btr:BB on productive RNA synthesis. In this assay, Btr had a modest effect on RNA yield (<1.5-fold), whereas Btr:BB had a much stronger effect (>5-fold at RNAP concentrations of >50 nM) (Figure 4A). Importantly, this mirrors the BB-dependent transcription activation observed in vivo (25). In a time course experiment (Figure 4B), the rate of RNA accumulation was stimulated 3.4-fold by Btr alone and ~21-fold by Btr:BB compared to RNAP alone. The magnitude of the activation in this study was somewhat higher than noted in the RNAP titration study (Figure 4A), which may reflect the use of different preparations of both Btr and RNAP.

We reasoned that if Btr:BB function primarily to increase RNAP recruitment, their effects should be most pronounced at low concentrations of RNAP and there should be little if any stimulation at saturating RNAP concentrations. However, in several replicate experiments, the magnitude of stimulation of RNA synthesis by Btr:BB was similar at low and high concentrations of RNAP (Figure 4A and data not shown). This suggests that activation of transcription occurs largely at a concentration-independent step. Since, as noted earlier, RPITC and RPO are in rapid equilibrium at many B. subtilis promoters, both promoter melting (RPO formation) and early steps in initiation often partition into the concentration dependent steps in the RNA initiation pathway (47,49,50,53).

The effect of BB on transcription activation by Btr is even more dramatic in single round transcription assays (Figure 4C). In this assay, RNAP was incubated (alone, with Btr or with Btr:BB) in the presence of AGU (which is necessary to stabilize the complexes against heparin) and the resulting complexes were challenged by simultaneous addition of heparin and CTP. Under these conditions, efficient transcription was only observed in the presence of Btr:BB (Figure 4C). Since both Btr and Btr:BB enhance the stable association of RNAP with PfeuA (e.g. the increased yield of C1 complexes; Figure 2), and both weakly activate RPO formation (Figure 3), we hypothesized that the nature and equilibrium distribution of the RPITC complexes formed with Btr:BB must differ from that formed with Btr alone. This could, for example, be explained by differences in the abortive initiation properties of the RNAP:Btr and RNAP:Btr:BB complexes.

To monitor abortive initiation products under reaction conditions allowing productive RNA synthesis, we performed transcription using a PfeuA fragment that produces a 55-nt run-off transcript to be able to resolve both full and abortive transcripts on the same gel.
(Figure 5). Surprisingly, both RNAP alone and in the presence of Btr produced abortive transcripts up to and including the U9 product (the 9-nt product terminating with U). However, in the presence of Btr:BB, RNAP produced longer abortive transcripts (up to 12 nt) and a 3.5- to 4-fold greater amount of the full-length 55-nt transcript, as expected (Figure 5; see inset). Chase experiments using high concentrations of NTPs did not affect the level of abortive transcripts, suggesting that these are not due to RNAP pausing (data not shown). The very similar levels of abortive transcription products seen in all three conditions (RNAP, RNAP plus Btr and RNAP plus Btr:BB) supports a model in which Btr:BB acts primarily on a late stage in transcription initiation to enhance the escape from abortive synthesis. In the single round transcription reactions, Btr:BB enables a transition from synthesis of abortive products of 9 nt or less (in the RNAP and RNAP plus Btr lanes) to a longer RPITC complex (up to 12 nt) that can efficiently escape the promoter to yield a full length product (Figure 4C).

Activator binding site mutations affect RNAP complex isomerization and productive transcription

Our mutational studies (Figure 1) suggest that the ability of Btr:BB to activate transcription requires a precise positioning of the activator relative to the RNAP:promoter complex. Next, we used single round transcription reactions to test the effects of these activator binding site mutations on RNA synthesis. Indeed, five of the six tested binding site mutations greatly decreased productive RNA synthesis even in reactions containing Btr:BB (Figure 6A). The one exception was a T4G mutation in the promoter distal (left) arm of the Btr binding site. Thus, there is an excellent correlation between the ability of the mutant activator binding sites to respond to Btr:BB in vivo and their ability to support conversion of RNAP to a heparin-resistant RPITC complex in vitro.

One trivial reason why these activator binding site mutations might prevent transcription activation would be an inability to bind Btr:BB. As noted earlier, the affinity for Btr:BB was measured in the absence of RNAP (Figure 1B). To test this idea, we monitored the effects of Btr:BB on RNAP complex formation at P_{fuA} using EMSA. As above, the binding reactions contained AGU (to allow formation of RP_{ITC}) and 50, 100, 150 or 200 nM of RNAP and the complexes were challenged with P_{con} (Figure 6B). The results indicate that RNAP can be efficiently engaged at the promoter by Btr:BB despite the activator binding site mutations, but the nature of the resulting complexes differs. While the CI complex forms at high levels with 50 nM RNAP on wild-type P_{fuA}, DNA containing the A7C mutation in the left arm [designated A7C(L)] formed mainly the CII complex and can only achieve 50% of CI complex even at 150 nM RNAP...
Similarly, the C6A(R) mutant DNA also required higher levels of RNAP to form the C1 complex when compared to the wild-type activator binding site (Figure 6B). Collectively, these results suggest that Btr:BB facilitates formation of a complex (C1) that more efficiently escapes from abortive cycling to enable productive RNA synthesis.

A model for Btr:BB-dependent transcription activation

Here, we define the binding site required for activation of transcription by Btr:BB and propose a model for the ligand-stimulated activation of PfeuA transcription. The dimeric Btr:BB binds to a 9 base direct repeat in which the right (promoter proximal) element is juxtaposed to the PfeuA/C35 element. In EMSA studies, the simultaneous mutation of both arms of the direct repeat (at positions 4, 6 or 7) was required to substantially impair Btr binding (Figure 1B). In contrast, single point mutations in the right arm at any of these positions was sufficient to prevent Btr:BB activation of feuA expression in vivo (Figure 1C) and in vitro in single round transcription reactions (Figure 6A). We interpret this to suggest that Btr:BB must be precisely positioned relative to RNAP for activation of transcription. This is supportive of a model in which the downstream Btr protomer must make specific contacts with RNAP (perhaps with the σ subunit) that serve to compensate for the weak C35 element.

EMSA analyses reveal that RNAP can form two complexes with either Btr or Btr:BB at PfeuA, C1 and C11 (Figure 2). Btr:BB enhances the formation of the C1 complex (Figures 2B and 6B), productive transcription (Figure 4C), and the formation of longer abortive transcripts (Figure 5). The correlation between the formation of a relatively stable C1 complex and productive transcription is supported by the analysis of mutant activator binding sites: mutations that reduced the fraction of C1 complexes (Figure 6B) also reduced transcription activation both in vivo (Figure 1C) and in vitro (Figure 6A). Although the precise nature of the C1 complex remains to be established, we suggest that Btr:BB specifically
alters the nature of the RP₀/RPₜₜc complexes to favor promoter escape and productive transcription. Thus, ligand (BB) responsive transcription activation results primarily from enhanced promoter clearance (Figure 7). In contrast, either Btr or Btr:BB can help stabilize RNAP binding to the promoter (Figure 2B and C), and both may weakly stimulate RP₀ formation (Figure 3). These effects may account for the previously noted requirement for Btr for basal level transcription of P_{feuA} as seen in strains unable to synthesize BB (25).

Comparison with other AraC activators

Members of the AraC family typically contain two domains: an ~100 amino acid C-terminal DNA binding domain (54) and an additional N-terminal domain that mediates dimerization and substrate binding (55). These proteins regulate diverse functions including sugar catabolism, responses to stress and virulence (55). Btr, in contrast, has an AraC-like domain (comparable in size to most full length AraC proteins) which is joined to a C-terminal BB binding domain. The BB binding domain is homologous to the substrate-binding protein (FeuA) involved in ferric-BB uptake and confers ligand responsiveness (25).

Mechanisms of activation by AraC family members include RNAP recruitment and open complex stimulation as shown in E. coli for the regulation of araBAD by AraC (44,56). AraC family proteins often bind adjacent to or overlapping the −35 region and interact with RNAP. Indeed, E. coli AraC and σ recognize overlapping nucleotides (45). Escherichia coli RhaR and RhaS directly contact domain 4 of σ (57–59), whereas activation of Vibrio cholerae tcpA by ToxT requires the alpha C-terminal domain of RNAP (60). At least partly because of the difficulty in purification of AraC family proteins, detailed biochemical insights into the mechanisms of activation have been slow to emerge.

Transcription activation by Btr also appears to involve a precise positioning of the activator protein immediately upstream of bound RNAP. While we do not yet know the molecular details of the interactions between Btr and RNAP, we have here defined the functional consequences of this interaction. Our results suggest a primary, ligand-dependent mechanism that involves an increase in RNAP escape from an abortively transcribing RPₜₜc to a productive elongation complex. While it is well documented that many activators stimulate transcription by RNAP recruitment (61) or by enhancing open complex formation (62,63), activation of promoter clearance has rarely been documented. One recent example is the phage Mu transcription activator C which contacts the β subunit of RNAP to reduce abortive transcription and thereby facilitate promoter clearance at the Mu mom promoter (64). Given the prevalence of AraC-like regulators it will be interesting to determine if activation of promoter clearance is a common activation mechanism within this family of proteins.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figure 1.

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