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MINIREVIEW

The Context-Dependent Influence of Promoter Sequence Motifs on Transcription Initiation Kinetics and Regulation

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ABSTRACT The fitness of an individual bacterial cell is highly dependent upon the temporal tuning of gene expression levels when subjected to different environmental cues. Kinetic regulation of transcription initiation is a key step in modulating the levels of transcribed genes to promote bacterial survival. The initiation phase encompasses the binding of RNA polymerase (RNAP) to promoter DNA and a series of coupled protein-DNA conformational changes prior to entry into processive elongation. The time required to complete the initiation phase can vary by orders of magnitude and is ultimately dictated by the DNA sequence of the promoter. In this review, we aim to provide the required background to understand how promoter sequence motifs may affect initiation kinetics during promoter recognition and binding, subsequent conformational changes which lead to DNA opening around the transcription start site, and promoter escape. By calculating the steady-state flux of RNA production as a function of these effects, we illustrate that the presence/absence of a consensus promoter motif cannot be used in isolation to make conclusions regarding promoter strength. Instead, the entire series of linked, sequence-dependent structural transitions must be considered holistically. Finally, we describe how individual transcription factors take advantage of the broad distribution of sequence-dependent basal kinetics to either increase or decrease RNA flux.

KEYWORDS RNA polymerase, gene regulation, kinetics, promoter motifs, transcription initiation

TRANSCRIPTION INITIATION OVERVIEW

The bacterial RNA polymerase (RNAP) core enzyme, composed of β, β', and ω subunits, along with an α dimer subunit, represents the catalytic machinery responsible for DNA-templated RNA synthesis (1, 2). For RNAP to initiate promoter specific transcription, it must first assemble with a σ factor to form RNAP holoenzyme (3, 4). Bacterial σ factors are classified into two families based on homology, called σ^{70} and σ^{54} (5). This review focuses on mechanisms specific to *Escherichia coli* σ^{70} (6); those of σ^{54} are distinct and require ATP-dependent remodeling by bacterial enhancer-binding proteins (7). Within the σ^{70} family, a further group classification is made based on the presence or absence of four structural domains ($\sigma_{1.1}$, σ_2, σ_3, and σ_4) (8, 9). Group 1 includes the essential housekeeping σ factor (σ^{70}) and contains all four structural domains; group 2 includes σ^{38} (σ^{S}) which lacks $\sigma_{1.1}$ and plays important roles in stress responses and survival but can also transcribe housekeeping genes (10–13); group 3 (usually containing σ_2, σ_3, σ_4) and group 4 (containing only σ_2 and σ_4) generally transcribe smaller sets of genes in response to specific stresses. In the case of group 4, these stress signals are often generated outside the cell, leading to their designation of extracytoplasmic function (ECF) σ factors (14–16). Expression of σ factors can be regulated based on environmental conditions either at the gene or protein level (13, 17), resulting in temporal concentration changes and permitting competition in binding of

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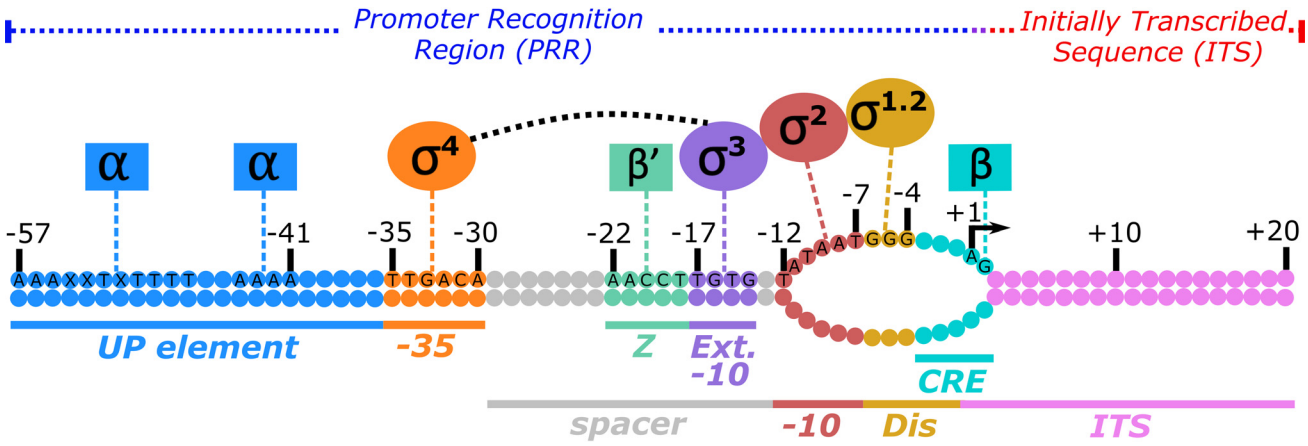

FIG 1 Promoter sequence elements and the RNA polymerase holoenzyme subunits that recognize them. Shown is a promoter under the control of the *E. coli* σ^{70} holoenzyme. The promoter recognition region (PRR) and the initially transcribed sequence (ITS) are delineated by the transcription start site (TSS) at nucleotide position +1 (arrow). While it varies depending upon NTP availability and promoter context, a bias for purines (A or G) is typically observed for the TSS (157, 228–231). An open DNA bubble is formed around the TSS, corresponding to positions −11 to +2. Specific sequences of the nontemplate strand are listed and correspond to either the optimal and/or consensus sequence for a given element. PRRs include (i) the full upstream (UP) element (A_{-57}AAXXTXTTTTnnAAAA$_{-41}$) (101), where X is an A or T nucleotide and n is no preference, contacting the carboxyl-terminal domain of the α-subunits (αCTDs, blue), (ii) the consensus −35 hexamer (T_{-35}TGACA$_{-30}$) contacting σ_4 (orange), (iii) the spacer region between −35 and −10 sites with an optimal length of 17 bp (gray) containing both (iv) the zipper (Z) element (A_{-22}ACCT$_{-18}$) (170) contacting β' (light green) and (v) an optimal extended (Ext) −10 element (T_{-17}GTG$_{-14}$) contacting σ_3 (purple), (vi) the consensus −10 hexamer (T_{-12}ATAAT$_{-7}$) contacting σ_2, where the −12 bp remains double stranded (red), (vii) an optimal discriminator (Dis) element (G_{-6}GG$_{-4}$) contacting $\sigma_{1.2}$ (yellow), and (viii) the core recognition element (CRE) (54) contacting β (teal). The CRE also makes up part of the ITS (pink), extending to the +2G position.

RNAP core to form σ-specific holoenzymes (18–21). This review will focus on mechanisms specific to group 1 σ-containing holoenzymes.

Initiation begins when the RNAP holoenzyme (R) binds to a fully duplexed promoter DNA sequence (P) to form a closed complex (RP_c). Sequence positions within a promoter, defined as a DNA segment essential for holoenzyme-specific initiation (22), are numbered relative to the transcription start site (TSS) occurring at +1 position. Sequences upstream of the TSS are negative and those downstream are positive. Promoter recognition is primarily driven by protein-DNA contacts between σ_2, σ_3, and σ_4 with the −10, the extended −10, and the −35 regions of the promoter, respectively (23–25). Contacts between the C-terminal domain of the α-subunits (αCTDs) and upstream (UP) elements in the DNA also contribute (26, 27) (Fig. 1). Contacts within the −10 region nucleate DNA unwinding around the TSS, resulting in an open complex (RP_o) (25, 28–30) after passing through a variety of structural intermediates. These intermediates involve conformational changes in the DNA (i.e., wrapping of upstream DNA and loading downstream DNA into the RNAP cleft) (31–35) and mobile RNAP structural elements that facilitate DNA opening and stabilize the RP_o (30, 36–41). Subsequent to DNA opening and binding of the initiating nucleotide, DNA template-directed nucleoside triphosphate (NTP) condensation reactions begin the stepwise polymerization of the nascent RNA transcript (42, 43). Within these initially transcribing complexes (RP_{itc}), holoenzyme contacts upstream of the TSS remain fixed, while downstream DNA is translocated toward the active site leading to DNA "scrunching" and an increase in the size of the DNA bubble (44, 45).

For RP_{itc} to enter into processive transcription elongation, the holoenzyme must break contacts made with the DNA promoter in a process termed promoter escape (reviewed in references 46 and 47). RP_{itc} complexes typically require synthesis of an RNA-DNA hybrid of at least nine nucleotides to escape and form a stable elongation complex (48). Variations in this length have been correlated with RP_o stability (49). Not all RP_{itc} complexes go on to escape the promoter but instead become stuck in abortive cycles, where short RNA transcripts are repeatedly generated and released (50–52). One major determinant that dictates the probability of escape versus abortive cycling is mobile region 3.2 of σ which makes contacts with the template DNA in RP_o but must be displaced for an RNA transcript longer than ~5 nucleotides to emerge (53–56). This steric

clash between $\sigma_{3.2}$ and the RNA can induce RNAP pausing/backtracking (57–59) and, along with conformational changes in the template strand during RP_{itc} scrunching (60, 61), can control the release of abortive transcripts or lead to $\sigma_{3.2}$ repositioning and escape (62–65). While the classic model dictates that σ dissociates during promoter escape (66), updates have been proposed to account for experimental observations of σ being retained throughout elongation (reviewed in reference 67). Such complexes may even reinitiate transcription following termination if the RNAP remains bound to the DNA and diffuses to the original or a new promoter (68, 69).

For more on mechanisms of bacterial transcription initiation, we direct the reader to published reviews (70–75). Taken together, the transcription cycle depicts the holoenzyme as a molecular isomerization machine under the regulatory control of the σ factor. The many conformations and intermediate states passed through on the way to the production of an RNA transcript provide ample opportunities for kinetic regulation.

QUANTITATIVELY MODELING TRANSCRIPTION INITIATION IN BACTERIA

From a biological perspective, the purpose of transcription is to generate a concentration of RNA suitable for downstream processes, including RNA-dependent regulation and protein translation. Cellular RNA concentration is dictated by the rate of production, the rate of degradation, and the cell volume, all of which are dynamic variables that can be affected by cell growth rate and division (reviewed in references 76 to 78). When considering the rate of RNA production, transcription initiation represents the kinetic bottleneck, where only one RNAP can initiate on a promoter at a time. This is in contrast to transcription elongation, where multiple polymerases act simultaneously on the DNA template.

In the simplest model of initiation, the process is described using Michaelis-Menten enzyme kinetics (79, 80), where RNAP is the enzyme, promoter DNA is the substrate, and full-length RNA transcript is the product (81). This analysis assumes a nonequilibrium steady state where a new RNAP-promoter complex is formed for each one lost by dissociation or escape, leading to a constant concentration of the RNAP-promoter complex and a constant velocity (V) or rate of RNA production. When DNA is present in large excess relative to RNAP and the free RNAP concentration is well below the K_m (defined as the concentration of RNAP that yields the half-maximal rate), V becomes independent of DNA concentration and proportional to the free RNAP concentration (82–84). Use of this model can differentiate between regulatory mechanisms of constitutively active and environmentally responsive promoters. Specifically, variations in transcript production in constitutive promoters arise from growth rate-dependent changes in the free RNAP concentration without changes in intrinsic promoter parameters K_m and V_{max} (i.e., the maximum velocity obtained at saturation), whereas the activity of environmentally responsive promoters depends on factors that effectively change K_m and/or V_{max} to increase or decrease transcript production under conditions of constant RNAP concentration (85, 86).

While K_m and V_{max} can be empirically determined, they depend on the underlying kinetic rate constants that describe transitions between intermediates on the path to escape. As a result, mechanistic understanding of transcription initiation and its regulation is enhanced by the use of free energy models (87), where kinetic conversions are modeled using transition-state theory and scale with the free energy barrier between two states. We recently developed a computational resource that allows one to calculate the RNA flux (i.e., the steady-state rate or velocity of RNA transcript synthesis) as a function of varied initiation rate constants (88). This overall initiation rate is calculated in the context of a minimal three-state pathway used extensively in formative analysis of transcription initiation kinetics (89). The kinetic scheme is represented by one concentration-dependent equilibrium binding step that describes initial promoter recognition, one reversible transition that represents all interconversions required for formation of the RP_o, and one irreversible step representing promoter escape. In the free energy diagram, the stability of each intermediate is represented by an energy well,

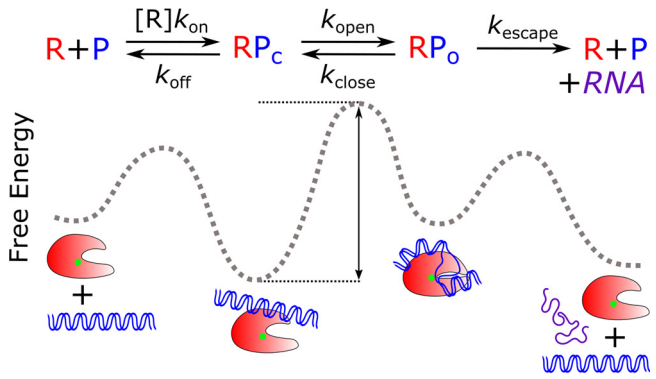

FIG 2 Kinetic scheme of initiation used for calculation of free energy landscapes. In this kinetic model, RNAP (R; red) and promoter DNA (P; blue) form a closed promoter complex (RP_c) with a concentration-dependent association rate (k_{on}) and dissociate with rate k_{off}. The equilibrium between the open promoter complex (RP_o) and RP_c is depicted by the composite forward and reverse isomerization rates, k_{open} and k_{close}, respectively. RP_o formation involves the wrapping of upstream DNA and loading into the RNAP cleft, coupled with RNAP conformational changes. The DNA is opened around the TSS, positioned near the active site (green dot). Promoter escape is modeled as an irreversible transition by rate k_{escape}, leading to RNAP dissociation from the promoter template and the generation of one full-length RNA transcript. These individual rate constants are used to calculate an overall initiation rate, which we use as a readout of the steady-state rate of RNA production. The stability of and transitions between these initiation intermediates can be depicted on a free energy reaction coordinate diagram. Here, the height of the barrier between an intermediate's energy well and its transition state (black arrow) determines the interconversion rates, and the depth of an intermediate's well determines its stability.

whereas the barrier height between an intermediate and its transition state (i.e., activation energy) determines the interconversion rates between two intermediates (Fig. 2). As a result, changes in interconversion rates brought on by a regulatory factor are represented by changes in the magnitude of the energy barrier and/or the stabilities of different intermediates (88). To be clear, in the model described in Fig. 2, these rates often do not represent single microscopic rate constants that would describe one molecular transition (i.e., DNA opening, closing, escape, etc.). Rather, they represent composite functions of all the intermediate kinetic steps involved within those given transitions (90, 91). As the number of significantly populated intermediates changes depending on the promoter, this model provides a useful representation of the general characteristics of all promoters rather than the most accurate depiction of an individual promoter's kinetics. Below, after describing the properties of promoter sequence motifs, we use this computational model to illustrate how promoter sequence can affect the kinetics of initiation and how these effects are dependent on the other rate constants within the initiation pathway.

PROMOTER SEQUENCE AND EFFECTS ON INITIATION KINETICS

Initiation rates are highly sequence dependent and vary 10,000-fold *in vivo*, partially explained by similar variations in RP_o formation rates and lifetimes measured *in vitro* (49, 81, 92). Diverse promoter sequences lead to different rate-limiting transitions during initiation, resulting in individual promoters being kinetically controlled at distinct steps. The kinetics at each step in the pathway are determined both by sequence-specific contacts between the promoter and the holoenzyme as well as nonspecific interactions made with the DNA phosphate backbone. In addition, purine-pyrimidine base preferences and nearest-neighbor effects dictate the energetic stabilities of both the duplex DNA and DNA/RNA hybrids (93–97) which can, in turn, exert effects independent of sequence motif conservation. Generally, DNA duplex stability affects RP_o formation (98), and DNA/RNA duplex stability and base stacking interactions between the incoming NTP and the 3′ end of the RNA affect escape kinetics (43, 99).

Promoter sequences can be functionally separated by location: sequences upstream of the TSS in the promoter recognition region (PRR) and those downstream of the TSS

in the initially transcribed sequence (ITS) (Fig. 1). Broadly speaking, promoter sequences upstream of and including portions of the −10 region affect initial binding, subsequent isomerizations, and rates of RP_o formation. In contrast, sequences in the −10 region and further downstream have larger effects on RP_o stability (72). Additionally, both the PRR and ITS can affect promoter escape rates. In the sections that follow, we summarize decades of work on how a promoter sequence affects initiation kinetics. All DNA sequences listed below refer to the nontemplate strand (5′ to 3′ direction) unless otherwise specified.

Promoter recognition region. (i) UP elements and αCTDs. The furthest sequence-specific contact upstream of the TSS is the recognition of the UP element by the αCTDs (reviewed in reference 27). UP elements are found at positions −60 to −40 relative to the TSS, where an AT-rich sequence is favorable for the interaction (26, 100). An optimal UP element sequence is able to increase transcript levels >300-fold *in vivo* (101) (Fig. 1), and further analysis identified two individual subsite sequences proximal and distal relative to the −35 region (102). Structurally, the αCTDs can adopt different conformations during UP element recognition (30, 103, 104), where transcriptional activation requires recognition by both αCTDs at the distal site but only a single αCTD is needed at the proximal site (102). Initial *in vitro* characterization on the rRNA promoter *rrnB*P1 indicated that the presence of the UP element increases both the association rate constant (k_{on}), approaching the theoretical diffusion limit, and the composite isomerization rate constant (k_{open}) (105). These effects have been understood as an energetic coupling between upstream DNA wrapping and the conformational changes required for loading DNA into the RNAP active-site cleft (reviewed in references 71 and 72) and not due to direct effects on the DNA opening step (34). In contrast, recent single-molecule work on *rrnB*P1 only observed the UP-dependent effect on k_{on} (106). Either way, by changing initiation kinetics, UP elements are an important determinant in the activation of rRNA transcription (107).

Not all αCTD-DNA contacts are sequence specific. The presence of upstream DNA lacking a UP element sequence can also lead to increased promoter activity (108, 109). Work on *lac*UV5, which lacks a UP element, and λP_R, which contains a distal UP element, confirmed that, in both cases, the αCTD-DNA interactions increase k_{on} and k_{open} without affecting the dissociation rate of the complex (109, 110).

(ii) −35 hexamer and σ_4. The consensus sequence for the −35 hexamer is $T_{-35}TGACA_{-30}$, where the TTG motif is the most highly conserved (111–114) and is important for sequence-specific binding (115, 116). By assessing transcriptional output *in vitro* and *in vivo*, experimental studies established hierarchies of base preferences, where changes to the −30 base are the least detrimental to promoter activity (117, 118), and confirmed that a consensus −35 site yields the highest output on constitutive promoters (114). The −35 hexamer is recognized by a helix-turn-helix DNA binding motif within σ_4 (24), and these interactions, along with those of the UP element, represent the first sequence-specific interactions formed upon holoenzyme binding (30, 33, 119). Bending of the DNA occurs just upstream of the −35 site upon promoter recognition (104, 110) and is likely the result of conformational coupling with upstream DNA wrapping (see previous section) and/or effects of protein-protein interactions of σ_4 with an αCTD bound to a UP element proximal site (120, 121). This αCTD-σ_4 interaction primarily facilitates association kinetics (k_{on}) (120). The −35 site is not essential for RP_o formation, and strand separation can still occur in the complete absence of this motif (122).

(iii) Spacer region between −35 and −10. No consensus sequence of the spacer region has been identified (111), although nonrandom distributions of bases have been noted (123). While shorter lengths can be accommodated, a spacer length of 17 base pairs (bp) is structurally ideal for making both −35-σ_4 and −10-σ_2 interactions (23, 124). Accordingly, 17 bp is also the most common spacer length (111, 113, 114) and leads to the highest transcriptional output from a variety of promoters (125–128). Changes in both RNAP affinity (116, 127) and the isomerization rate (k_{open}) (127, 129) have been observed by altering the spacer length. Addition of an AT-rich sequence

just upstream of −10 (termed the −15 sequence) enhanced RNAP binding and subsequent RP_o formation (116, 130), consistent with studies that showed replacing the spacer region with G and C stretches leads to an inhibition of promoter activity (128) and those with T (131) and A (132) stretches increased promoter activity. This AT preference, like what is observed for UP elements, likely facilitates helix deformations (133) that have been proposed to link the binding energetics of −35 and −10 site recognition during promoter opening (134).

(iv) Extended −10 region and σ_3. It was first noted that the presence of −15T and −14G was important for a promoter designed to be constitutively active in the absence of an activator protein (135). Subsequent sequence analysis on a collection of 300 *E. coli* σ^{70} promoters identified that the extended −10 consensus sequence ($T_{-15}G_{-14}$) is present in ~20% of promoters (136) and is enriched in promoters with longer spacer lengths and less consensus in the −35 site (113, 137). On some promoters, the extended −10 can compensate for nonconsensus −35 or −10 motifs (reviewed in reference 138). The extended −10 region contacts σ_3 (23, 139, 140), where perpendicular α-helices insert into the major groove, causing the promoter to bend toward the σ factor (124, 141). Further addition of another TG motif directly upstream at −17/−16 contacts both core and σ subunits (140) and can lead to increased promoter activity (136, 137). The extended −10 motif has been reported to increase the association (k_{on}) and isomerization (k_{open}) rates as well as increase RP_o lifetime by slowing its dissociation (k_{close}) (142–144).

Some suggest that the definition of the extended −10 motif should be reevaluated as $T_{-15}GnT_{-12}$, termed the −15 motif (138) (note that this is different than the AT-rich −15 sequence discussed above). This change would account for the fact that the −12T (the first base in the −10 motif) remains double stranded (ds), whereas the rest of the −10 hexamer is opened to form the single-stranded DNA (ssDNA) bubble during promoter recognition (discussed below). This naming would split up the −10 hexamer to account for its different functional roles in binding and isomerization/RP_o lifetime (138, 145).

(v) −10 hexamer and σ_2. The consensus sequence for the −10 hexamer is $T_{-12}ATAAT_{-7}$, with −11A and −7T being the most highly conserved and the most sensitive to nucleotide substitution (111, 113, 114, 118, 146). Like the consensus −35, a consensus −10 region yields the highest transcriptional output on constitutive promoters (114). In RP_o, the −12 bp defines the upstream edge of the DNA bubble and is thought to be recognized as dsDNA, where conserved tryptophans in σ_2 bracket the −12T (25, 30, 54, 124, 140). Promoter contacts with this tryptophan "chair" are proposed to stabilize RP_o by preventing reannealing of the single strands, replacing the stacking interactions lost when −11A flips out of the DNA helix (25, 30, 140). However, an intriguing recent structural study indicates that the −12 bp transiently melts in the early steps of forming the stacking interaction (30), perhaps providing a rational for the conservation of the more easily melted A-T bp, and experimental studies that have suggested σ_2 interacts with a single-stranded −12 site (reviewed in reference 138). Bases in both the nontemplate and template strands of the ssDNA region of the −10 are flipped out (reviewed in reference 147), where the −11A and −7T bind within pockets of σ while the template strand −9T interacts with β subunit of RNAP (25, 30, 54, 124, 140). This −9T–β-protrusion interaction is thought to stabilize a pre-RP_o intermediate and allow for inhibition by the transcription factor TraR (30). Consistent with this hypothesis, the combination of DksA and ppGpp, which mimics the effects of TraR (148), represses transcription on promoters that show enrichment for the template strand −9T (149).

The −11A nucleates DNA unwinding (25, 29, 150, 151), although the exact mechanism, including the order of events and whether DNA is unwound before or after it is bent into the cleft toward the active site, has been subject of debate (reviewed in reference 74). In addition to the −11A, the −7T appears to play a critical role in the kinetics of RP_o formation, and as a result, it has been suggested that nucleation may be more delocalized within the −10 hexamer (98). RNAP binding affinity to forked-junction

templates is dependent on the entire −10 sequence, not just the −11A and the −7T (150, 152), and these conserved positions are not absolutely required for RP_o formation, as an AT-rich region lacking those specific bases also yields fast promoter melting kinetics (98). This later result is likely due to DNA duplex instability (93), as promoter melting activity directly correlates to the −11 bp stability (29), and a C-rich −10 region exhibits very little promoter melting (98).

Thus, the −10 region serves multiple functions in initiation, including promoter recognition of the dsDNA −12 bp, nucleating DNA unwinding, and specific ssDNA contacts maintained throughout RP_o formation. These effects combined can increase the association (k_{on}) and isomerization (k_{open}) rates and decrease k_{close} to facilitate an increase in RP_o lifetime, with the largest kinetic effects typically observed on the forward isomerization rate (29, 116, 144, 153–156).

(vi) Discriminator and $\sigma_{1.2}$. The discriminator sequence lies between the −10 and the TSS, with six-base discriminators being most common (157). The discriminator sequence can affect TSS selection (158), where purine-rich discriminators favor TSSs closer to the −10 than promoters containing pyrimidine-rich discriminators (159). Originally defined as a GC-rich region commonly found in rRNA promoters (160), it was later shown that a short sequence immediately downstream of the −10 (5′-GGG-3′) binds optimally to σ (161) and leads to an increase in RP_o stability, decreasing the reverse isomerization rate (k_{close}) (144, 162). Region 1.2 of σ interacts with this sequence (144, 162, 163), creating a binding pocket for the G positioned one base downstream of the −10 site (54). On *rrnB*P1, mutating the native C two bases downstream of the −10 to G leads to a large stabilization of RP_o (144). The presence of this C is common at rRNA promoters (160), contributing to their relatively unstable RP_o (144). This instability permits rRNA promoters to be regulatory targets of the initiating nucleotide, DksA/ppGpp, and TraR (144, 148, 149, 164–166).

Mechanistically, the discriminator is thought to drive a series of in-cleft and downstream conformational changes in the holoenzyme that stabilize RP_o (see the discussion in reference 39). Additionally, analogous to NTP-dependent scrunching that occurs during initial transcription (44, 45), NTP-independent scrunching in RP_o has been observed, leading to changes in the bubble size that correlate with variability in TSS position (158, 159, 167). The G two bases downstream of the −10-$\sigma_{1.2}$ interaction prevents RP_o scrunching, explaining the lack of this G at rRNA promoters, which require RP_o scrunching to accommodate their unusually long eight-base discriminator sequences (158). On promoters containing a C at this position, such as *rrnB*P1, the complementary G on the template strand forms a binding pocket the with β' lid, $\sigma_{3.2}$, and the neighboring base (104), likely representing a key interaction in the RP_o scrunched intermediate (168). In addition, it has been proposed that RP_o scrunching facilitates promoter escape by reducing the abortive pathway (158, 169). Consistent with this model, discriminator sequences affect the kinetics, length, and probability of abortive RNA production, where a more stable RP_o leads to longer abortive RNAs prior to escape (49). While the energetic costs of RP_o scrunching have been determined (167), it is unknown whether starting from a scrunched state favors subsequent nucleotide addition steps to bias the system toward the productive synthesis pathway (169). However, recent structural work has suggested that RP_o scrunching potentially reduces abortive synthesis by permitting one extra nucleotide to be incorporated into the nascent RNA before sterically clashing with $\sigma_{3.2}$ (104).

(vii) Promoter elements that interact with RNAP core—Z and CRE. Not all promoter elements interact with σ. For instance, the β' zipper region makes contacts with a "zipper or Z-element" corresponding to bases −22 to −18 within the spacer region (170) (Fig. 1). The Z-element facilitates RP_o formation and can serve as a substitute for −35 recognition by σ_4, although it is not clear whether this interaction with the core is sequence specific (170). Changes in the spacer conformation were suggested to be partially dependent on the presence of a −18T (171), and subsequent

structural studies indicated that highly conserved residues in the β' zipper make contacts at the −18 and −17 positions (30, 140).

Another RNAP core-promoter interaction is that of the β-subunit with the ssDNA of −4 to +2, termed the core recognition element (CRE) (54). Contacts are made with all but the −1 nucleotide, where the +2G is specifically bound in a pocket on the face of β, leading to an increased lifetime of the complex (54, 172). In the case of both CRE and Z, additional effects subsequent to RP_o have been observed. Both modulate pausing properties (170, 172, 173), and CRE can also modulate TSS selection (174) and promoter escape (99, 175). Lastly, as the polymerase changes register during elongation and termination, CRE-like sequences encountered at these positions can affect the rates of these processes as well (172).

Initially transcribed sequence. (i) "Downstream DNA." In the context of RP_o, the ITS is commonly referred to as downstream DNA. Nonspecific DNA contacts are made with RNAP mobile elements in β and β' that lead to stabilization of RP_o due to changes in both the forward (k_{open}) (37) and reverse (k_{close}) isomerization rates (37, 39, 176–178). While increasing the length of downstream duplex leads to higher RNAP affinity (179), the effects of downstream DNA in RP_o formation past +6 appear to be dependent on the sequence of the discriminator, being amplified in the context of a GC-rich sequence (180). Combined, these results suggest that the RNAP-downstream DNA contacts cannot be energetically decoupled from upstream PRRs. This is likely due to the coupled conformational changes required for DNA loading into the cleft (37, 71) instead of downstream DNA sequence-dependent effects on RP_o stability (99).

(ii) "Escape region." In the context of escape, the ITS is the sequence that is transcribed during RP_{itc}. Originally defined as bases +1 to +20 (181), it is now known that this region is variable in length, where escape points occur between +3 (predicted for *rrnB*P1 [49]) to +19 on phage variant promoters (182, 183). Sequences within the ITS can affect both the propensity for abortive transcription and the escape rate (99, 181, 182, 184). These effects can in part be explained by the presence or absence of pause-inducing sequences. Pausing was originally observed during transcription elongation (reviewed in reference 185) and later confirmed during initiation, occurring both on- and off-pathway to productive transcription and, in some cases, inducing backtracking (57, 58, 186). The $Y_{-1}G_{+1}$ sequence (Y is T or C) was shown to be enriched for slow escape kinetics, especially when found as repeats (99). Pausing at this sequence occurs frequently when positioned at ITS positions +6 to +7, where the steric clash of the emerging RNA with $\sigma_{3.2}$ presents an additional energy barrier to escape (57–59). Subsequent *in vivo* studies defined an ITS pause sequence to contain a T two bases upstream of the $Y_{-1}G_{+1}$ motif (187), and this extended sequence has proved even more detrimental to escape kinetics (99). Observations on the effects of general base composition on escape propensity have also been made. A nontemplate purine-rich ITS favors productive transcription and fast escape kinetics, whereas a T-rich ITS promotes abortive transcription and slow escape (99, 184, 188, 189). Importantly, promoter escape can represent the rate-limiting step for some promoters (190), explained in part by ITS effects on abortive probabilities, escape kinetics, and pausing.

THE WHOLE IS GREATER THAN THE SUM OF ITS PARTS: USING SEQUENCE TO PREDICT TRANSCRIPTIONAL ACTIVITY

While we have presented an overview of how individual sequence motifs affect certain rate constants, using this information to predict transcriptional activity is not trivial. The first attempts to correlate activity to sequence only analyzed the steps up to RP_o formation, using the product of the initial binding equilibrium constant and the forward isomerization rate constant ($K_B k_2$). Here, a linear correlation was observed between the log of $K_B k_2$ and a promoter's similarity to consensus, focusing on the sequences around and including both the −35 and −10 regions and the length of the spacer region (191). However, it was not determined how either similarity to consensus or $K_B k_2$ correlates to overall RNA production, which would require accounting for

contributions of promoter escape. Subsequent studies that compared a promoter's association rate (k_{on}) measured *in vitro* to *in vivo* promoter strength (192, 193) found no correlation, suggesting that simply evaluating only part of the initiation pathway is not sufficient to predict transcriptional activity.

Advances in high-throughput methods have provided large data sets for modeling the effect of promoter and regulatory sequences on transcriptional activity (194–196). A first approximation to modeling protein-DNA interactions is based on an "additivity" approach, where the free energy contributions of each base pair within the binding site are treated independent of one another and added together (92, 195, 197–199). However, this approach is limited in that it does not account for multivalent binding interactions and thermodynamic linkage (200). Models that include multivalent binding between the −35 and −10 hexamers and that account for promoter context (i.e., background sequences outside the −35 and −10 regions, spacer length, presence/absence of UP elements, etc.) have been able to account for >90% of the sequence-dependent variance in transcriptional activity (196, 200). These results clearly demonstrate that, for an accurate prediction of transcriptional activity, the effect of a single sequence motif cannot be isolated from the rest of the promoter sequence.

THE EFFECT OF A SEQUENCE MOTIF DEPENDS ON PROMOTER CONTEXT

Studies evaluating the effect of a sequence motif in the context of different promoters show large variations in resultant changes to individual rate constants and overall transcriptional activity (49, 99, 137, 184, 201, 202). This calls into question if a generalized functional outcome can actually be prescribed to an individual sequence motif. Figure 3 illustrates this idea by presenting free energy diagrams for two hypothetical promoters: promoter number 1, where RP_c is more stable than RP_o, and promoter number 2, where RP_o is more stable than RP_c (Fig. 3A). These two promoters have individual rate constants that vary orders of magnitude, yet the overall initiation rates are similar, each generating ~5 RNAs min^{-1} (Fig. 3A). This example emphasizes the potential downfalls in using the stability of one individual intermediate to predict transcriptional activity. For instance, without consideration of the entire pathway, one would predict that promoter 1 would be more active based on the initial binding equilibrium constant and that promoter 2 would be more active based on RP_o stability, even when the rates of escape for both promoters are the same. Using the starting free energy diagrams from Fig. 3A as a representation of the kinetics in the absence of a sequence motif (−), we test how the addition of a sequence motif (+) might affect the overall RNA production rate. Here, we treat the added motif like a transcription factor and codify its effect in terms of fold changes to a specific rate constant(s). By applying the same fold changes in rate constant(s) to both promoters, we can see that the added motif can lead to different outcomes on RNA production simply due to differences in the starting (i.e., basal) kinetics for each promoter. For instance, the addition of a UP element or a consensus −35 region, modeled by increasing k_{on}, results in a larger change in RNA flux on promoter 2 as a result of it not already being near the diffusion limit like promoter 1 (Fig. 3B). In contrast, the addition an optimal discriminator, modeled by decreasing k_{close}, has a larger effect on promoter 1, which started with a relatively unstable RP_o (Fig. 3C). The addition of a pause sequence to the ITS, modeled as slowing k_{escape}, is also promoter specific (Fig. 3D), in line with experimental evidence that has indicated the $Y_{-1}G_{+1}$ sequence exerts promoter-specific effects (99). While often understood in qualitative terms, quantitively illustrating these three basic examples directly shows that sequence motifs have the largest effect on promoters that are rate limited at the kinetic steps they control.

The above-mentioned examples are simplified, where addition of a motif only affects one rate constant. For instance, while generally thought to affect early steps in initiation such as binding and isomerization, PRRs have also been shown to have effects on promoter escape and abortive transcript production (99, 182, 183, 201, 203–205). This effect is not direct *per se*, as new sequence contacts are typically not formed during escape, although exceptions have been noted during the generation of

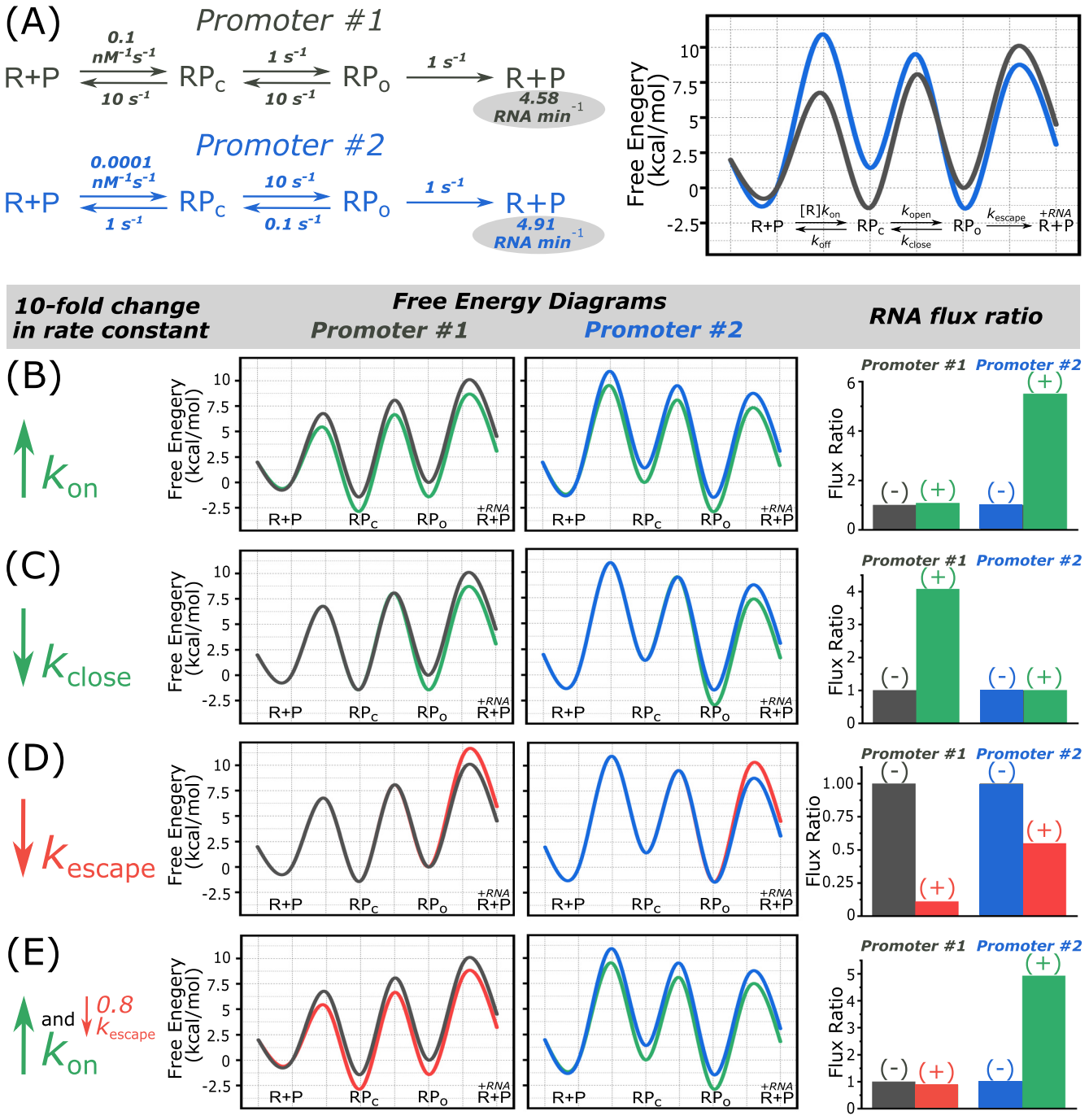

FIG 3 The effect of a sequence motif on RNA flux is promoter context dependent. (A) Two different promoters are used to illustrate how changes in rate constants due to the addition of a sequence motif affect transcription. Using the rate constants listed at an RNAP concentration of 1 μM, free energy diagrams and the resultant steady-state rate of RNA synthesis in units of RNA per minute were calculated with an online resource we developed (88), available at https://egalburt.github.io/transcript-flux-calculator/fluxcalc.html. Using these sets of rate constants, promoter 1 forms a more stable RP_c but less stable RP_o than promoter 2, but both promoters yield similar RNA production rates. To simulate the addition of different consensus motifs, RNA flux was calculated for both promoters by applying a 10-fold increase in rate on k_{on} (B), a 10-fold decrease on k_{close} (C), a 10-fold decrease on k_{escape} (D), and a 10-fold increase on k_{on} in addition to a 0.8-fold (20%) decrease on k_{escape} (E). In each panel, the gray and blue diagrams represent those calculated in panel A, and the free energy diagrams obtained by increasing or decreasing the rate constant(s) are plotted in green when leading to an increase in RNA flux or red when leading to a decrease in RNA flux. The resultant changes in flux upon changing a rate constant(s) (+) are plotted as a ratio of the flux values obtained in panel A (−), such that no change yields a ratio of 1, an increase in transcript rate yields a ratio greater than 1, and a decrease in transcript rate yields a ratio less than 1.

long abortive transcripts (183). Rather, it is an energetic effect linked to the relative stability of RP_o (204, 205), the intermediate preceding nucleotide incorporation. As a result, a sequence motif that may be functionally characterized as being important in binding, such as a UP element, can indirectly encode effects on subsequent rate constants, where the overall effect on flux depends on a promoter's coupled transitions (201). To illustrate this, we use the same case presented in Fig. 3B, where increasing k_{on} 10-fold models the effects of adding a motif important for the initial binding step, decreasing the free energies of both RP_c and RP_o. As a more stable RP_o frequently leads to a lower rate of promoter escape (204, 205), we now assume that addition of this sequence motif also affects escape kinetics. A 10-fold increase in k_{on} coupled with a 20% decrease in k_{escape} still leads to activation on promoter 2 but actually leads to repression on promoter 1, a promoter that already contained optimal binding kinetics (Fig. 3E). While we only observe an ~10% decrease in RNA flux, this overall effect can be magnified by slowing escape kinetics even further. Consistent with this idea, nucleotide changes in the ITS have the largest effect for promoters containing consensus PRRs, which are rate limited at escape (184, 206). Thus, the repressive effect on escape outweighs the activating effect on association in the context of promoter 1, leading to an overall reduction in transcription. This result illustrates that promoter context cannot only dictate the magnitude but also dictate the direction (i.e., up or down) of a change in flux resulting from the addition of a sequence motif.

THE REGULATORY EFFECT OF A TRANSCRIPTION FACTOR DEPENDS ON PROMOTER CONTEXT

Analogous to how the effect of a sequence motif is dependent on the entire promoter sequence (Fig. 3), the extent of regulation encoded by transcription factors is determined by the kinetic variations made possible by an individual promoter sequence. Many transcription factors regulate initiation by associating directly with a specific DNA sequence to either cooperatively recruit or competitively occlude RNAP binding to the promoter (reviewed in references 207 and 208). However, not all transcription factors directly recognize a specific DNA sequence (here termed DNA site independent) and are instead recruited to initiation complexes through protein-protein interactions with σ, RNAP core, or both (reviewed in references 70 and 209 to 211). In *Mycobacterium tuberculosis*, two essential DNA site-independent transcription factors called CarD and RbpA are recruited to promoter regions via interaction with RNAP holoenzyme to regulate transcription (reviewed in references 75, 211, and 212). RNA-sequencing experiments suggest that *in vivo*, both CarD and RbpA can activate transcription on some promoters but repress transcription on others (213, 214). *In vitro* kinetic experiments indicate both factors increase the forward isomerization rate and, in the case of CarD, decrease the reverse isomerization rate, leading to an increase in RP_o stability (215–218). While these kinetic effects by themselves would be a mechanism for transcriptional activation, we also observed CarD and RbpA to slow promoter escape kinetics (219). As in Fig. 3E, where differential changes in multiple rate constants can lead to activation or repression depending on the basal kinetics of the promoter, we have proposed a model where these factors can activate transcription at promoters rate limited at RP_o formation but can repress transcription at promoters rate limited at escape (88, 219).

The prototypical examples of DNA site-independent transcription factors are *E. coli* DksA, in combination with ppGpp, and its homolog TraR that bind the RNAP secondary channel (reviewed in reference 220). Recent structural studies indicate that these factors induce conformational changes in RNAP that may facilitate bubble nucleation and/or σ ejection from the RNAP channel—a mechanism for activation (30, 221)—but also may stabilize DNA contacts within the channel of an intermediate prior to RP_o, promote a clash with the position of the DNA template strand near the active site, and/or promote DNA melting outside the RNAP cleft—a mechanism for repression (30, 104, 221, 222). Kinetically, these structural changes have been linked to increasing the forward isomerization rate but also reducing the lifetime of RP_o by increasing the reverse

rate. Combined, these kinetic changes activate amino acid promoters that form RP_o slowly but have long RP_o lifetimes while inhibiting rRNA promoters that form RP_o quickly but have short RP_o lifetimes (148, 166, 221, 223, 224). Here, the same RNAP contacts that lead to the same fold changes in rate constants can lead to different regulatory outcomes depending on the rate-limiting step of a given promoter (88).

WHAT IS THE CONSENSUS ON CONSENSUS SEQUENCES?

A promoter sequence containing each motif in its consensus form, as depicted in Fig. 1, deviates significantly from real promoters found in the genome. A study tracking promoter evolution from randomized sequences indicated that recently evolved promoters primarily contain only −35 and −10 consensus-like motifs (225). This suggests that loss of consensus sequence and the addition of sequence motifs is driven by adjustments in initiation kinetics that result in increased fitness. DNA site-independent transcription factors may further exploit these kinetic variations to confer differential regulation. Alternatively, as newly evolved promoters lack transcription factor binding sites (225), promoter sequence evolution could be driven by a pressure to adjust basal promoter kinetics to take advantage of the effects of existing DNA site-independent factors.

Not all promoters require both the −35 and −10 motifs (122), where a "mix-and-match" approach has been taken by nature with regard to which PRRs are used for RNAP recruitment (reviewed in reference 138). In fact, the bacterial RNAP can bind and initiate transcription from PRRs that are far from consensus (225), perhaps explaining pervasive transcription (226) and antisense transcription following secondary initiation (68). Furthermore, experimental studies have indicated bringing a promoter closer to consensus, either through sequence mutation or entire addition, can actually result in lower transcriptional output (196, 201, 227), likely explained by the "over"-stabilization of initiation intermediates and the slowing of escape (88, 200) (Fig. 3E). Combined, these results suggest that fully consensus promoters are likely not favorable due to "self-inhibition" brought on by inefficient promoter escape and that promoters evolve to have specific kinetics that allow them to be subject to regulation.

CONCLUDING REMARKS

Here, we have summarized how PRRs often have redundant functional roles with regard to the specific initiation rate constant(s) they affect, permitting variation of motif combinations for active promoters (138). These motifs often affect more than one rate constant, and the outcome of those effects (both in magnitude and in direction) is dependent on the entire sequence context of the promoter. As a result, quantitative predictions about how sequence affects kinetics require consideration of the entire kinetic landscape. As a final thought, we caution against use of broad statements such as "the presence of consensus motif X facilities transcription" or "transcription factor X represses transcription." Without specifying the promoter context, these statements will not always hold true.

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