Published online 17 February 2014 *Nucleic Acids Research, 2014, Vol. 42, No. 8* ***5097–5108***
doi:10.1093/nar/gku147

Regulation of promoter-proximal transcription elongation: enhanced DNA scrunching drives λQ antiterminator-dependent escape from a σ70-dependent pause

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Received December 11, 2013; Revised January 29, 2014; Accepted January 30, 2014

ABSTRACT

During initial transcription, RNA polymerase remains bound at the promoter and synthesizes RNA without movement along the DNA template, drawing downstream DNA into itself in a process called scrunching and thereby storing energy to sever the bonds that hold the enzyme at the promoter. We show that DNA scrunching also is the driving force behind the escape of RNA polymerase from a regulatory pause of the late gene operon of bacteriophage λ, and that this process is enhanced by the activity of the Q^{λ} antiterminator. Furthermore, we show that failure of transcription complexes to escape the pause results in backtracking and arrest in a process analogous to abortive initiation. We identify a sequence element that modulates both abortive synthesis and the formation of arrested elongation complexes.

INTRODUCTION

The segment of DNA immediately downstream of a transcription start site frequently is an important site of regulation of transcription elongation, where the positive energies of the polymerization reaction encounter inhibitory forces that deter elongation. In one example, all multisubunit RNA polymerases (RNAPs) tend to abort early synthesis, presumably when polymerization fails to provide the energy to escape RNAP interactions with the promoter, thereby releasing abortive RNAs, typically 5–10 nt in length (1–4). Importantly, both eukaryotic and bacterial RNAPs can be restrained by specific protein interactions that make further elongation dependent on particular regulatory modifications. A prominent example is provided by eukaryotic RNAP II, which frequently stalls tens of nucleotides from the transcription start site due to the inhibitory activity of proteins like Dsif and Nelf, and then is rescued into elongation by the regulatory kinase pTefb in a reaction essential to transcription activation (5).

The energetic transactions in early transcription elongation are particularly exposed in the bacterial regulatory system of the bacteriophage λ gene *Q* antiterminator. Q^{λ} becomes a subunit of RNAP, allowing it to resist terminators through both antipausing and a structural rearrangement that inhibits terminator RNA function (6,7). Q^{λ} function requires an early transcription pause at +16 that is induced by a specific protein interaction, namely, binding of the σ^{70} initiation factor to a secondary −10-like sequence in the early transcribed segment (8,9). Q^{λ} helps RNAP escape this pause (as well as critical pauses at terminators downstream) (8), exhibiting an essential antipausing activity that may exemplify the way regulatory factors can overcome early transcription barriers.

The realization that initial transcribing complexes are formed through DNA ‘scrunching’ (10,11), namely, the unwinding of downstream DNA that then is incorporated as single-stranded DNA into the transcription complex, provides a structural context for understanding the nature of promoter-proximal pausing (e.g. σ^{70}-induced) and the reversal of pausing by antipausing factors (like Q^{λ}). This derives directly from the scrunching model of initial transcription and abortive initiation, as follows. A consideration of energies involved in synthesis and elongation of the initial transcribing complex suggests that scrunching stores energy that is used to break the σ^{70}-DNA bonds that stabilize the open promoter complex. Further energy is required to displace a protein linker between σ^{70} domains 3 and 4 (12,13); combined, these barriers oppose the elongation reaction and can lead at a significant rate to its failure, resulting in ‘abortive initiation’, the loss of the initial transcript from the complex. The extent and pattern of abortive transcript release

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*To whom correspondence should be addressed. Tel: +1 607 255 2430; Fax: +1 607 255 6249; Email: jwr7@cornell.edu depends both on the strength of σ^{70} binding in the promoter and the nucleotide composition of the initial transcribed sequence (ITS) (3,14,15).

A reasonable inference is that σ^{70}-dependent paused complexes (Figure 1) have the essential features of initial transcribing complexes, including the scrunched DNA structure (16,17), with the central difference that the RNA of ≥16 nt is not susceptible to abortive loss because it is stabilized by the interactions that also stabilize the elongation complex, i.e. enclosure of the RNA/DNA hybrid and binding of RNA in its exit channel (18). The paused complex is anchored by σ^{70} interacting with the non-template strand of the secondary −10 sequence (9) (Figure 1). However, the actual site of pausing, i.e. the extent of scrunching-based elongation after σ^{70} binds DNA, is determined by sequence elements that also likely determine the general features of elongation such as ubiquitous pausing (19–21).

Modification of the σ^{70}-dependent paused RNAP by Q^{λ} protein, which binds to both a specific DNA Q^{λ} binding site behind RNAP (22) and to elements of RNAP [including particularly the beta flap (23)], results in a stable complex of Q^{λ} with RNAP (24) that changes the RNAP elongation properties. Binding of Q^{λ} assists RNAP in escaping the pause, and the Q^{λ}-modified complex pauses less downstream, an essential element of the antitermination mechanism (25). Just as scrunching energy is proposed to break σ^{70} bonds with promoter DNA, we propose that Q^{λ} promotes further scrunching from the σ^{70}-dependent pause that provides the energy to break σ^{70} (and presumably Q^{λ}) bonds with DNA.

We have used a distinctive property of the σ^{70}-pausing/Q^{λ} system to provide evidence for this extended scrunching model of Q^{λ} function, and also to reveal important aspects of the sequence basis of early abortive transcript release. In addition to escaping into productive, antiterminated elongation, Q^{λ}-modified elongation complexes downstream of the σ^{70}-dependent pause site have a tendency to undergo arrest in a sequence-specific manner. We propose that this Q^{λ}-dependent arrest is analogous to a step of abortive release of early transcripts: it depends on the anchoring activity of σ^{70} (in combination with Q^{λ} in this case), and the RNAs are backtracked and sensitive to the action of GreB cleavage factor (Figure 1) (6), just as the formation and release of abortive initiation products are sensitive to GreB (26). The stability against dissociation of Q^{λ}-modified complexes, which are mature elongation complexes,

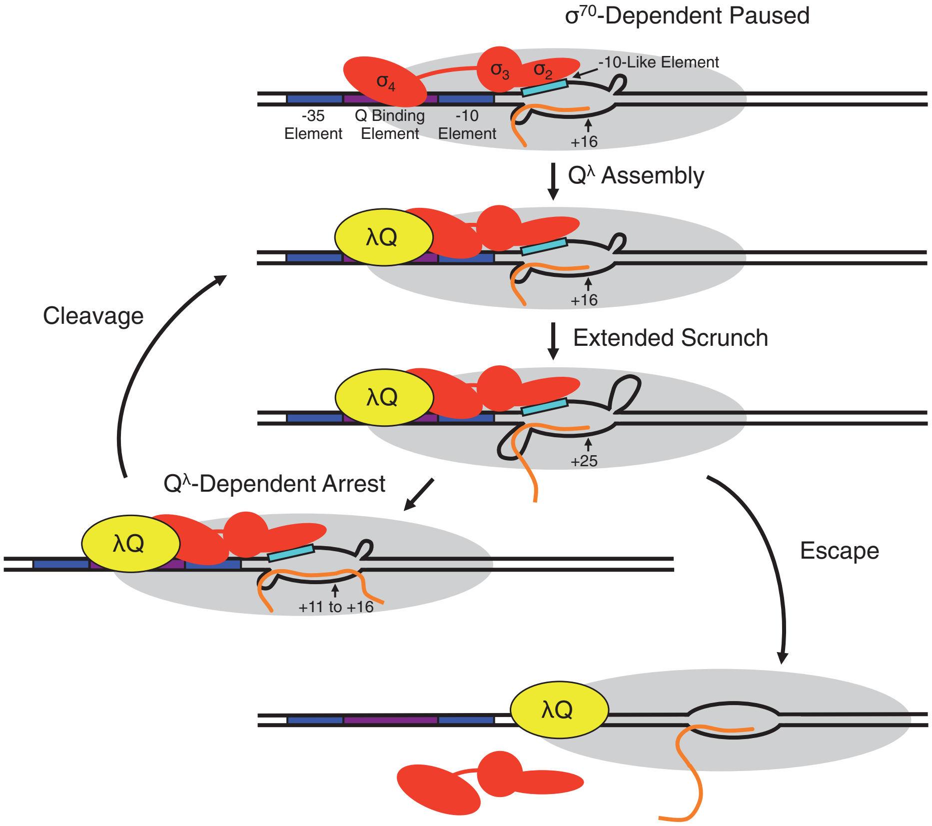

Figure 1. A model for Q^{λ}-stimulated escape from the λpR' σ^{70}-dependent pause. On engagement of the −10-like element by σ^{70} region 2, the transcription elongation complex undergoes a σ^{70}-dependent pause. The paused elongation complex is a substrate for modification by Q^{λ}, which engages the elongation complex through contacts with the Q binding element and with RNAP itself. Assembly of Q^{λ} into the elongation complex stimulates the resumption of transcription while σ^{70} is still bound to the −10-like element, resulting in an extended scrunch. The energy accumulated by DNA scrunching can be released in two ways: First, if the energy stored in the scrunch is insufficient to disrupt the interaction between σ^{70} and −10-like element, RNAP backtracks into an arrested state, termed the QAC, and cleavage of the backtracked RNA must occur in order to resume transcription. Second, if the energy stored in the scrunch is sufficient to disrupt the interaction between σ^{70} and −10-like element, the Q^{λ}-modified elongation complex disengages the pause site and resumes elongation. Presumably σ^{70} dissociates from RNAP following disengagement.

allows us to analyze their nature and sequence dependence. We discern important sequence elements that determine the efficiency and site of arrest of Q^{λ}-modified complexes, and we show explicitly that such sequences also determine the pattern of abortive transcript release from initial transcribing complexes. In particular, we identify a dinucleotide motif that determines a site and efficiency of major abortive RNA release. We further show that Q^{λ}-dependent arrest reflects backtracking to the site of the σ^{70}-dependent pause where Q^{λ} initially engages; this result implies that the mechanism of Q^{λ}-mediated release of RNAP from the σ^{70}-dependent pause involves further scrunching from the site of the σ^{70}-dependent pause. This result is consistent with the model that scrunching energy is stored in a stressed intermediate, which then functions in initiation to break the σ^{70}-promoter bonds, but which is proposed to act here to break both σ^{70} and Q^{λ} bonds to DNA and allow the modified complex to escape into elongation.

MATERIALS AND METHODS

Plasmids

pM650 (27), p'QE-30 (28) and pET-28a- σ^{70} (29) have been described. pES3 is *GreB*-6xHis in pET-28b(+). pES7 is $GreB_{D41A,\ E44A}$-6xHis in pET-28b(+). pSAN2 (a gift from L. Hsu) contains $pN25_{Anti.}$ pVS10 (a gift from I. Artsimovitch) contains all four core RNAP subunits. All mutants were constructed using Quikchange site-directed mutagenesis.

Proteins

RNAP (30), GreB and $GreB_{D41A,\ E44A}$ (31), σ^{70} (29) and NusA (32) were purified as described. Q^{λ} was purified as described (17), except that it is stored in 10 mM potassium phosphate, pH 6.5, 50% glycerol, 200 mM potassium chloride, 1 mM ethylenediaminetetraacetic acid (EDTA) and 7 mM tris(2-carboxyethyl)phosphine (T. Santangelo and J. Filter, unpublished data).

In vitro transcription

Reaction mixtures containing 2 nM template and 20 nM RNAP (20 nM core reconstituted with 100 nM σ^{70}) were incubated in transcription buffer (20 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 1 mM dithiothreitol and 50 mM KCl), 0.1 mg/ml bovine serum albumin and 200 μM ATP, GTP, CTP and 50 μM UTP (containing 0.5 μCi/μl α-^{32}P-UTP) and 150 nM NusA for 15 min at 37°C to form open complex. Supplementary Figure S3B used a nucleotide mixture containing 200 μM UTP, GTP, CTP and 50 μM ATP (containing 1.0 μCi/μl γ-^{32}P-UTP) and 10 nM template. Single-round transcription reactions were initiated by addition of magnesium chloride to 5 mM and rifampicin to 10 μg/ml. Total reaction volume was 25 μl. For abortive initiation assays (except that of Supplementary Figure S5B), open complex was formed as above, except that the NTP concentration was 200 μM ATP, UTP, GTP and 50 μM CTP (or a dilution of this NTP mix) and contained 0.5 μCi/μl α-^{32}P-CTP).

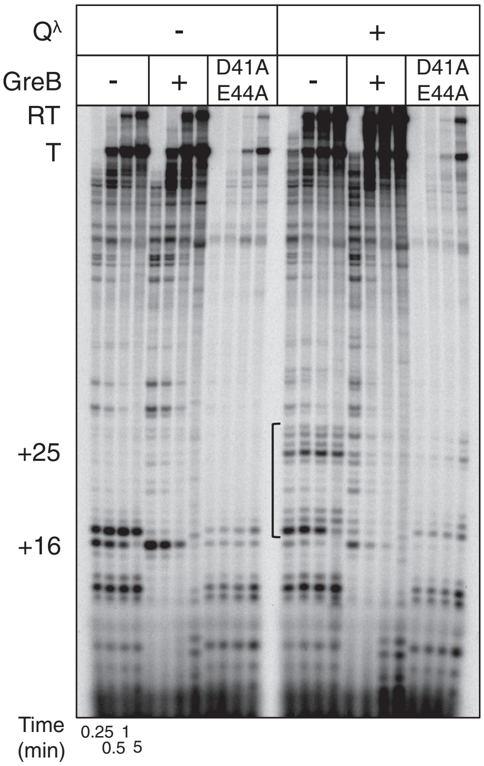

Figure 2. Q^{λ}-dependent arrest at λpR'. Single round *in vitro* transcription from λpR' in the presence and absence of Q^{λ}, GreB and $GreB_{D41A,\ E44A}$. Readthrough (RT), terminated (T), QAC and paused transcripts (+16, +17) are shown. The QAC are designated by a bracket. +17 is included in the QAC because complexes trapped at +17 chase by 5 min in the presence of Q^{λ}, suggesting that they are distinct from the complexes trapped at +17 in the absence of Q^{λ}. Percentage readthrough was determined by normalizing the intensity of the RT and T bands for U content and dividing the normalized RT by the sum of the normalized RT and T.

Figures 5D and 6B were repeated using three alternative NTP mixes (with ATP, UTP or GTP 50 μM, and the remaining NTPs at 200 μM) to ensure that the NTP mix did not bias the abortive pattern. Supplementary Figure S5B was performed using 20 μM UTP, GTP, CTP and 5 μM ATP (containing 0.5 μCi/μl α-^{32}P-ATP). Multiround transcription was initiated by the addition of magnesium chloride to 5 mM. When present, GreB was added to 100 nM before formation of open complex. In experiments of Figure 2 and Supplementary Figures S1A and B containing $GreB_{D41A,\ E44A}$, both GreB and $GreB_{D41A,\ E44A}$ were added to a concentration of 1 μM in their respective reactions. When present, Q^{λ} was added to a concentration of 250 nM after open complex formation and incubated at 37°C for 30 s before initiation. Reactions were stopped by adding 125 μl of stop solution (0.6 M Tris, pH 8.0, 12 mM EDTA, 0.16 mg/ml transfer RNA).

Cleavage assays

Biotinylated template was bound to Promega Streptavidin MagneSphere Paramagnetic Particles. Transcription was initiated in the presence of Q^{λ} and allowed to proceed for 2 min to form the Q^{λ}-dependent arrested complexes (QAC), at which point the reactions were washed three times with T buffer + 0.1 mg/ml bovine serum albumin to remove nucleotides and magnesium chloride, halting the reaction. For nucleotide starvation cleavage, reactions were then resuspended in T buffer + 100 nM GreB, incubated at 37°C, and initiated by addition of magnesium chloride to 5 mM. To cleave backtracked RNAs and chase the 5′ cleavage products, reactions were resuspended in T buffer, 100 nM GreB and 200 μM NTPs, incubated at 37°C, and initiated by addition of magnesium chloride to 5 mM and rifampicin to 10 μg/ml. Reactions were stopped by adding 125 μl of stop solution.

Heteroduplex templates

Heteroduplex templates were constructed as described previously (33) and sequenced to confirm purity. Transcription was performed as described above.

Purification, fractionation and analysis of transcription reactions

Stopped transcription reactions were phenol extracted by addition of 150 μl of phenol/chloroform/isoamyl alcohol (25:24:1), vortexing, centrifugation and collection of the aqueous phase. Ethanol precipitation of RNA was performed by adding 450 μl of 100% ethanol to each reaction, followed by storage at −20°C overnight. Precipitated RNA was resuspended in transcription loading dye (1× T buffer, 80% formamide, 0.05% bromophenol blue and xylene cyanol). Reactions were fractionated by electrophoresis using 12 or 15% denaturing polyacrylamide gels containing 6 M urea. Reactive bases were detected by an Amersham Biosciences Typoohn 9400 Variable Mode Imager. Quantitation was performed using ImageQuant. '%Q^{λ}-modified' refers to the percentage of a subset of Q^{λ}-modified complexes out of all Q^{λ}-modified complexes (QAC + readthrough). '%Non-Abortive Transcripts' refers to the percentage of a particular transcript length out of all non-abortive transcripts, thus excluding abortive products.

RESULTS

Q^{λ} induces formation of a backtracked early transcription arrest

Inclusion of Q^{λ} protein during *in vitro* transcription from λpR' causes modification by Q^{λ} of 70% of the transcription complexes (Figure 2); of these, 52% appear as terminator readthrough owing to the antitermination property of Q^{λ}, and the remaining 48% are trapped in a backtracked state—termed the QAC. The QAC include species of 17–19 and 24–28 nt (Figures 1 and 2, bracket); they are eliminated by addition of the transcription cleavage factor GreB, resulting in all Q^{λ}-modified complexes appearing as readthrough (Figures 1 and 2). Although the QAC appear largely stable over 5 min (Figure 2), they disappear on longer incubation (Supplementary Figure S1A). We earlier surmised that the QAC are paused (16), implying an ability to resume elongation, but it is more likely that their disappearance results from intrinsic transcript cleavage (or possibly trace contamination by GreA or GreB) that rescues them into productive elongation. As further evidence that the QAC are backtracked and rescued by transcript cleavage, we used a catalytic mutant of *greB* that changes both essential carboxylates (D41 and E44) to alanine (34), resulting in a mutationally altered protein that inhibits the active center-dependent intrinsic cleavage reaction (Supplementary Figure S1B). Inclusion of $GreB_{D41A,\ E44A}$ slows the elongation rate substantially, but also traps 90% of the Q^{λ}-modified complexes as QAC (Figure 2 and Supplementary Figure S1A). Interestingly, $GreB_{D41A,\ E44A}$ also causes 97% of the total transcript to appear as abortive products (Supplementary Figure S2A and B).

Q^{λ} drives escape from the λpR' σ^{70}-dependent pause by stimulating DNA scrunching

The identity of the first 20 nt following a transcription start site (the ITS) is a major determinant of the pattern and quantity of aborted transcripts at a promoter (15). Because abortive initiation occurs from a scrunched complex, the ITS presumably affects the stability of the scrunched complexes as transcription proceeds. Similarly, if Q^{λ}-dependent arrest is analogous to abortive initiation, the sequence downstream of the σ^{70}-dependent pause should modulate the length and distribution of the RNAs in the QAC. Furthermore, RNAP in the QAC should remain stably bound at the σ^{70}-dependent pause, just as an initial transcribing complex would remain bound at the promoter following a failed attempt at promoter escape.

To determine the role of sequences downstream of the σ^{70}-dependent pause in Q^{λ}-dependent arrest, we replaced positions +17 to +36 of λpR' with the ITS of promoter $N25_{Anti}$ to create a template termed $\lambda pR'17N25_A$ (Figure 3A). $N25_{Anti}$ is a strongly abortive promoter known to form abortive transcripts up to 15 nt in length (26,35), making it an ideal model for studying our analogy. If the sequence downstream of the σ^{70}-dependent pause affects Q^{λ}-dependent arrest in the same way that the ITS affects abortive initiation, then substitution of this downstream sequence with an ITS that generates long abortive products should result in the formation of QAC that contain longer RNAs as a result of transcription further downstream before collapse into a backtracked state.

Figure 3B depicts single-round transcription reactions from both λpR' and $\lambda pR'17N25_A$ in the presence and absence of Q^{λ}. In the presence of Q^{λ}, elongation complexes transcribing from λpR' undergo Q^{λ}-dependent arrest after transcribing to a cluster of positions focused at +25, whereas elongation complexes transcribing from $\lambda pR'17N25_A$ undergo Q^{λ}-dependent arrest after transcription to a cluster focused at +30 and +31. The 30-nt RNA present in these QAC is exactly 14 nt longer than the σ^{70}-dependent pause product and thus corresponds in endpoint to a major 14-nt long abortive product of the $N25_{Anti}$ promoter. This result supports the interpretation that the $N25_{Anti}$ ITS alters Q^{λ}-dependent arrest in a way that reflects its abortive properties.

Complexes that have undergone Q^{λ}-dependent arrest are backtracked, and thus are sensitive to the cleavage factor GreB (Figure 2). As expected, the $\lambda pR'17N25_A$ QAC are GreB-sensitive, indicating that they are

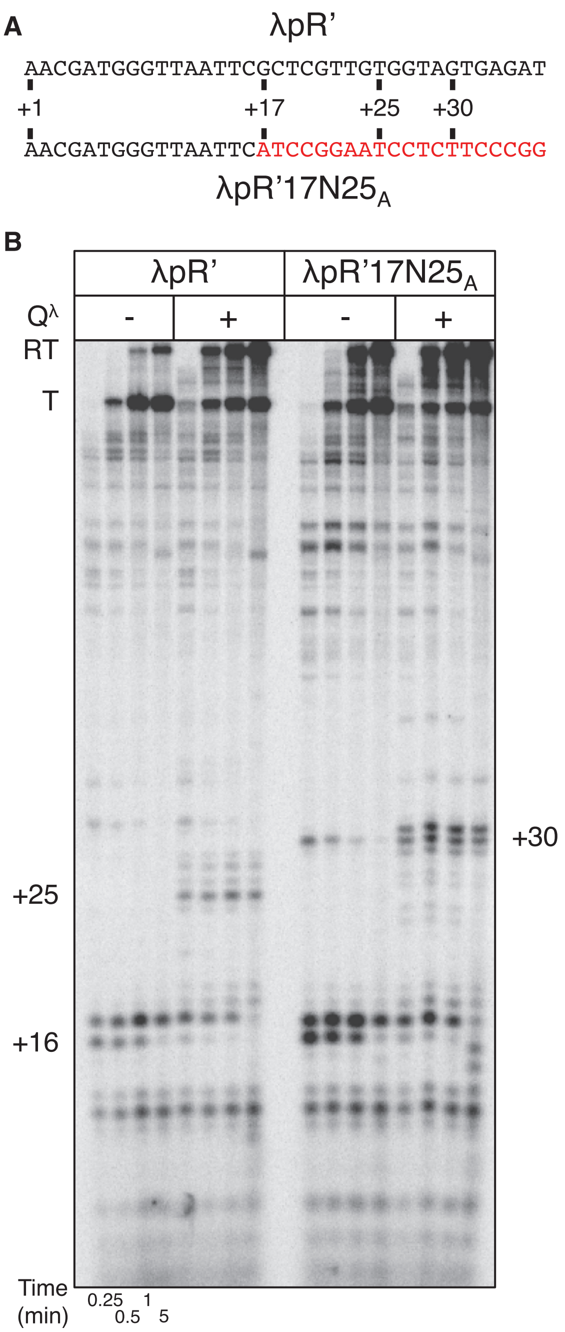

Figure 3. Sequence dependence of the QAC. (**A**) Sequence comparison of λpR' and λpR'17N25$_{A}$ from positions +1 to +36. (**B**) Single round *in vitro* transcription from λpR' and λpR'17N25$_{A}$ in the presence and absence of Q^{λ}.

backtracked (Supplementary Figure S3). It is noteworthy that in addition to the effects on Q^{λ}-dependent arrest, complexes transcribing λpR‘17N25$_{A}$ undergo a pause at +30 in the absence of Q^{λ} that is insensitive to GreB. The appearance of such a pause suggests that the N25$_{\mathrm{Anti}}$ ITS has an innate preference for pausing at this position, which may influence its abortive properties and its behavior in the fusion λpR‘17N25$_{A}$.

DNA scrunching requires that RNAP remain bound at a defined position while synthesizing RNA without translocation. In the context of initial transcription, failure to escape the promoter causes abortive release of the nascent transcript through a process thought to be similar to backtracking (15,26). In our model, the increase in DNA scrunching that results from modification of the σ^{70}-dependent paused complex by Q^{λ} occurs while σ^{70} is bound to the pause-inducing sequence; thus, collapse during further scrunching should result in a Q^{λ}-modified elongation complex still bound through σ^{70} interaction at the pause-inducing sequence, and arrested in a backtracked conformation. To determine the position to which the QAC of λpR' and λpR'17N25$_{A}$ backtrack, we first performed GreB-mediated cleavage in the absence of NTPs (Figure 4A). We generated QAC on template bound to magnetic beads, washed to remove NTPs and induced cleavage by incubating with GreB. In addition to an experiment in which RNAs were internally labeled, we performed a second nucleotide starvation cleavage experiment in which RNAs were end-labeled to allow for clear visualization of the 5′ cleavage products (Supplementary Figure S3B). Strikingly, both λpR' and λpR'17N25$_{A}$ QAC backtrack to a range of positions from +11 to +16, which is consistent with the previously established range of positions to which σ^{70}-dependent paused complexes backtrack on λpR' (16). Thus, the observation that the λpR' and λpR‘17N25$_{A}$ QAC backtrack to the same positions indicates that they remain bound at the σ^{70}-dependent pause regardless of the position to which they have transcribed.

To confirm our interpretation of the nucleotide starvation cleavage results, we examined the 3′ cleavage products that are generated when the QAC are treated with GreB: If λpR' and λpR‘17N25$_{A}$ QAC backtrack to positions from +11 to +16, then treatment with GreB should yield 3′ cleavage products of 9–14 and 14–20 nt, respectively. We generated QAC on template bound to magnetic beads, washed to remove NTPs and incubated in the presence of GreB and unlabeled NTPs to stimulate cleavage and chase the 5′ cleavage products into longer RNAs. As expected, cleavage of the QAC on λpR‘ yields RNA of ~9–14 nt in length, and cleavage of the QAC on λpR‘17N25$_{A}$ yields RNA of ~14–20 nt in length (Figure 4B). It is important to note that the long 3′ cleavage products are Q^{λ}-dependent, indicating that they originate from the QAC that contain long RNAs, thus, confirming the interpretation that the QAC backtrack to a cluster of positions from +11 to +16 regardless of the position to which they transcribe.

Together, the Q^{λ}-dependent nature of these complexes and the position to which they backtrack indicate that they result from Q^{λ}-modified elongation complexes that attempt and fail to escape from the σ^{70}-dependent pause. The observation that the λpR‘ and λpR‘17N25$_{A}$ QAC originate from the σ^{70}-dependent pause, yet transcribe to different positions before backtracking, establishes that the sequence composition immediately following the σ^{70}-dependent pause is a critical determinant of Q^{λ}-dependent arrest and is consistent with a mechanism of DNA scrunching.

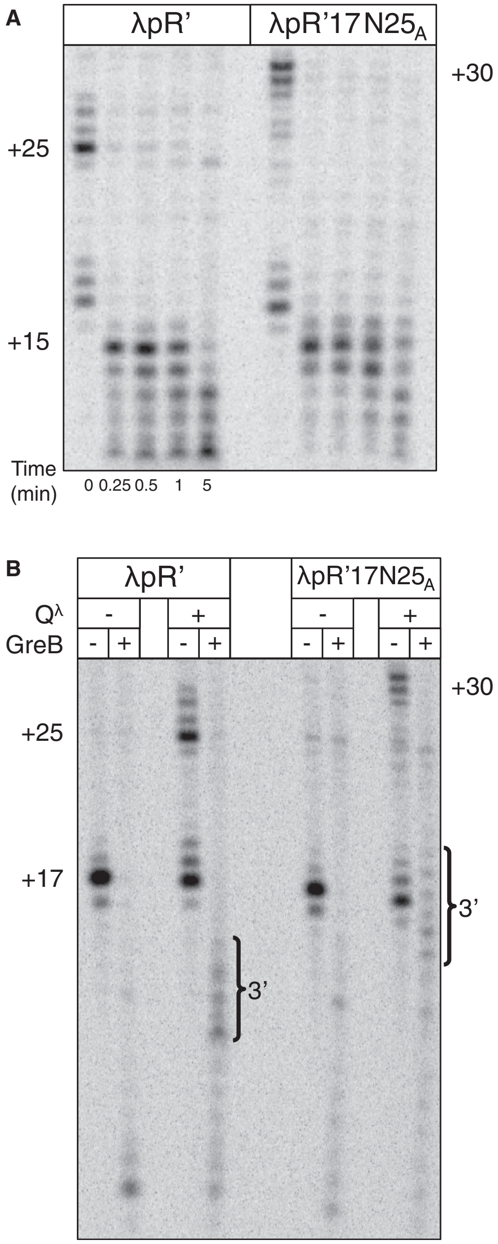

Figure 4. QAC backtrack to +15 regardless of the positions to which they have transcribed. (**A**) Cleavage of the λpR' and λpR'17N25$_A$ QAC. After formation of the QAC, NTPs were removed and GreB was added. Samples were incubated at 37°C and removed at the indicated times. 5′ and 3′ cleavage products were differentiated by the assay shown in (B) and Supplementary Figure S3B. (**B**) 3′ cleavage products of the λpR' and λpR'17N25$_A$ QAC. As in (A), except transcription was resumed in the presence of GreB and NTPs. The exact sizes of 3′ cleavage products are uncertain within about a nucleotide because they have 5′-monophosphate rather than 5′-triphosphate ends like the other transcripts. However, the approximate sizes of the λpR' and λpR'17N25$_A$ 3′ cleavage products are both distinct and within the expected ranges.

A systematic mutant scan reveals a sequence element responsible for the long abortive products of the N25$_{Anti}$ promoter and the extended Q-dependent arrest

The ability of the ITS to change the position to which Q$^\lambda$-modified elongation complexes transcribe before backtracking and arrest indicates that a sequence element within the N25$_{Anti}$ ITS is responsible for determining the extent of scrunching that occurs during escape from the σ^{70}-dependent pause and the N25$_{Anti}$ promoter. To reveal any such elements, we performed a systematic mutant scan that covered the positions +17 to +31 of the λpR'17N25$_A$ template (Figure 5A). The scan consisted of overlapping 3 bp substitutions in which we made A/C and G/T transversions. We then performed single-round *in vitro* transcription to assay for altered Q$^\lambda$-dependent arrest. Strikingly, two overlapping mutants showed a decrease in the amount of complexes arrested at +30 and the appearance of complexes at +24, 1 nt shorter than the wild-type position of +25 (Figure 5B). These mutants shared G to T mutations in positions +21 and +22 (positions +5 and +6 of the N25$_{Anti}$ ITS). To establish that the +21 and +22 nt are entirely responsible for the restoration of a shorter Q$^\lambda$-dependent arrest, we constructed λpR'17N25$_{A\text{-}TT}$, a λpR'17N25$_A$ mutant containing the +21 and +22 G to T mutations. As expected, in the presence of Q$^\lambda$, the majority of QAC clustered around position +24 rather than +30 (Figure 5C).

We then performed the mutant scan described above on λpR' from positions +16 to +25 to determine whether a similar sequence element existed within the wild-type λpR' sequence (Supplementary Figure S4A). The introduction of a G-rich region at positions near +21 and +22 shifted the primary position of arrest from +25 to +27, with some complexes appearing at +31 and +32 (Supplementary Figure S4B). Thus, introducing the GG sequence element from N25$_{Anti}$ is sufficient to make the arrest pattern of λpR' similar to that of λpR'17N25$_A$; in fact, no other portion of the N25$_{Anti}$ sequence has significant effect on the arrest, although the exact pattern of arrest sites varies slightly (Supplementary Figure S4C and D).

If there is a relationship between the effect of the N25$_{Anti}$ ITS on Q$^\lambda$-dependent arrest and the abortive properties of the N25$_{Anti}$ promoter, mutations that disrupt the extended Q$^\lambda$-dependent arrest of λpR'17N25$_A$ should affect the long abortive transcripts of the N25$_{Anti}$ promoter in a similar manner. To test this, we introduced G to T mutations at positions +5 and +6 of the N25$_{Anti}$ promoter. Multiround *in vitro* transcription revealed that the N25$_{A\text{-}TT}$ mutant primarily accumulates abortive products of 6, 7, and 8 nt and displays a dramatic reduction of 13 and 14 nt aborted transcripts (Figure 5D). The effect of the +5, +6 G to T mutations is enhanced at low nucleotide concentrations, but persists up to the standard concentration of our transcription reactions. This shift is analogous to the shift observed in the QAC of the λpR'17N25$_{A\text{-}TT}$ mutant, further indicating that the abortive properties of the N25$_{Anti}$ ITS are reflected in its effects on Q$^\lambda$-dependent arrest and thus supporting the model that scrunching is the mechanism by which Q$^\lambda$-modified elongation complexes escape the σ^{70}-dependent pause. In fact, a scan by overlapping 3-nt substitutions in the N25$_{Anti}$ ITS confirms that only the +5 and +6 doublet is important to the abortive pattern (Supplementary Figure S5A and B). [We ignore the transcripts of mutants 1, 2 and 3, which are obscured by

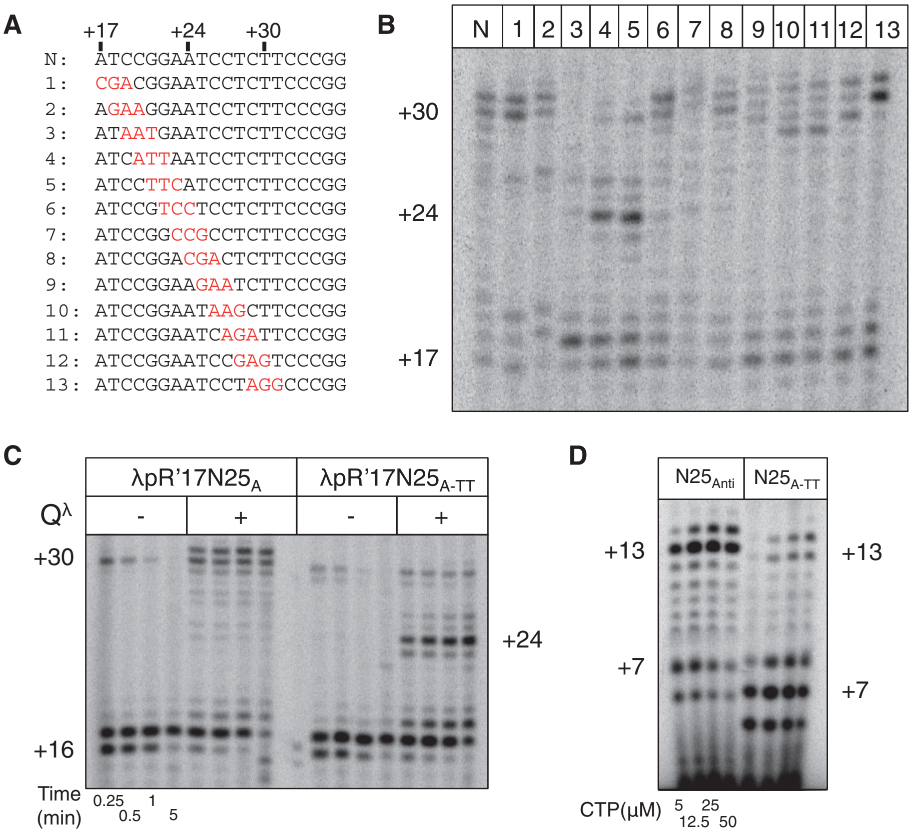

Figure 5. A dinucleotide motif is required for the abortive and arrest properties of the N25$_{\mathrm{Anti}}$ ITS. (**A**) Mutants used in the scan of λpR'17N25$_{\mathrm{A}}$. (**B**) Single round *in vitro* transcription from the mutants in (A). Mutant 3 displays increased arrest at +18, which may account for the decreased downstream QAC formation. Mutants 4 and 5 display an increased tendency to form QAC at +24. (**C**) Single round *in vitro* transcription from λpR'17N25$_{\mathrm{A}}$ and λpR'17N25$_{\mathrm{A\text{-}TT}}$ in the presence and absence of Q$^{\lambda}$. (**D**) Multiround *in vitro* transcription from pN25$_{\mathrm{Anti}}$ and pN25$_{\mathrm{A\text{-}TT}}$.

stuttering due to the T run in the beginning of the transcript (36)].

Strand specificity of the N25$_{\mathrm{Anti}}$ +5/+6 Mutation

The template and non-template strands of a DNA duplex have distinct functions and contacts within a transcribing complex. Such asymmetry means that the effect of a mutation can be assigned to the template, non-template or both strands of DNA. It is possible to differentiate the activity of each strand by analyzing the behavior of heteroduplex templates. The availability of a 2-bp mutation that generates arrest (in the context of λpR'17N25$_{\mathrm{A}}$) and abortive (in the context of N25$_{\mathrm{Anti}}$) patterns that are easily distinguishable from those of the original N25$_{\mathrm{Anti}}$ sequence allowed us to create heteroduplex templates and evaluate the strandedness of the N25$_{\mathrm{Anti}}$ +5T, +6T mutant effects.

Single-round *in vitro* transcription was performed on λpR'17N25$_{\mathrm{A}}$, λpR'17N25$_{\mathrm{A\text{-}TT}}$ and the corresponding heteroduplexes. Figure 6A shows that the template strand dictates the position of Q$^{\lambda}$-dependent arrest: when λpR'17N25$_{\mathrm{A}}$ is in the template strand, Q$^{\lambda}$-dependent arrest is identical to that of the λpR'17N25$_{\mathrm{A}}$ homoduplex, whereas when λpR'17N25$_{\mathrm{A\text{-}TT}}$ is in the template strand, Q$^{\lambda}$-dependent arrest is identical to that of the λpR'17N25$_{\mathrm{A\text{-}TT}}$ homoduplex.

We then performed a heteroduplex analysis of N25$_{\mathrm{Anti}}$ and N25$_{\mathrm{A\text{-}TT}}$ to determine the strandedness of the +5T, +6T mutations in the context of abortive initiation (Figure 6B). Neither heteroduplex produces an abortive profile like that of the parental homoduplexes. Moreover, heteroduplexes containing a mutant non-template strand form a unique abortive profile, indicating that the ITS non-template strand is also a determinant of abortive transcription. The contribution of the non-template strand in the context of abortive initiation and the absence of such an effect in the context of Q$^{\lambda}$-dependent arrest suggests that while the underlying mechanism is shared, differences between a promoter-bound initial transcribing complex and a Q$^{\lambda}$-modified σ^{70}-dependent paused complex affect the ways in which ITS composition modulates escape.

Strengthening the interaction between σ^{70} and the −10-like element increases the quantity and alters the distribution of both Q$^{\lambda}$-dependent arrest and σ^{70}-dependent pausing

In addition to the ITS, the strength of the interaction between RNAP and the promoter elements plays a role in the formation of abortive products, with stronger promoters tending to yield more aborted transcripts (14). If the mechanism of Q$^{\lambda}$-dependent arrest is in fact analogous

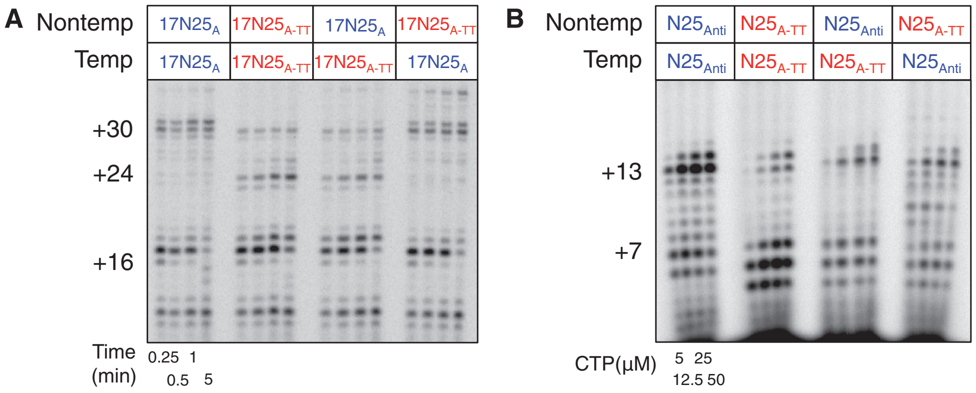

Figure 6. Strand specificity of the $N25_{Anti}$ +5/+6 Mutation. (**A**) Single round *in vitro* transcription from $\lambda pR'17N25_A$, $\lambda pR'17N25_{A\text{-}TT}$ and the corresponding heteroduplexes. (**B**) Single round *in vitro* transcription from $pN25_{Anti}$, $pN25_{A\text{-}TT}$ and the corresponding heteroduplexes.

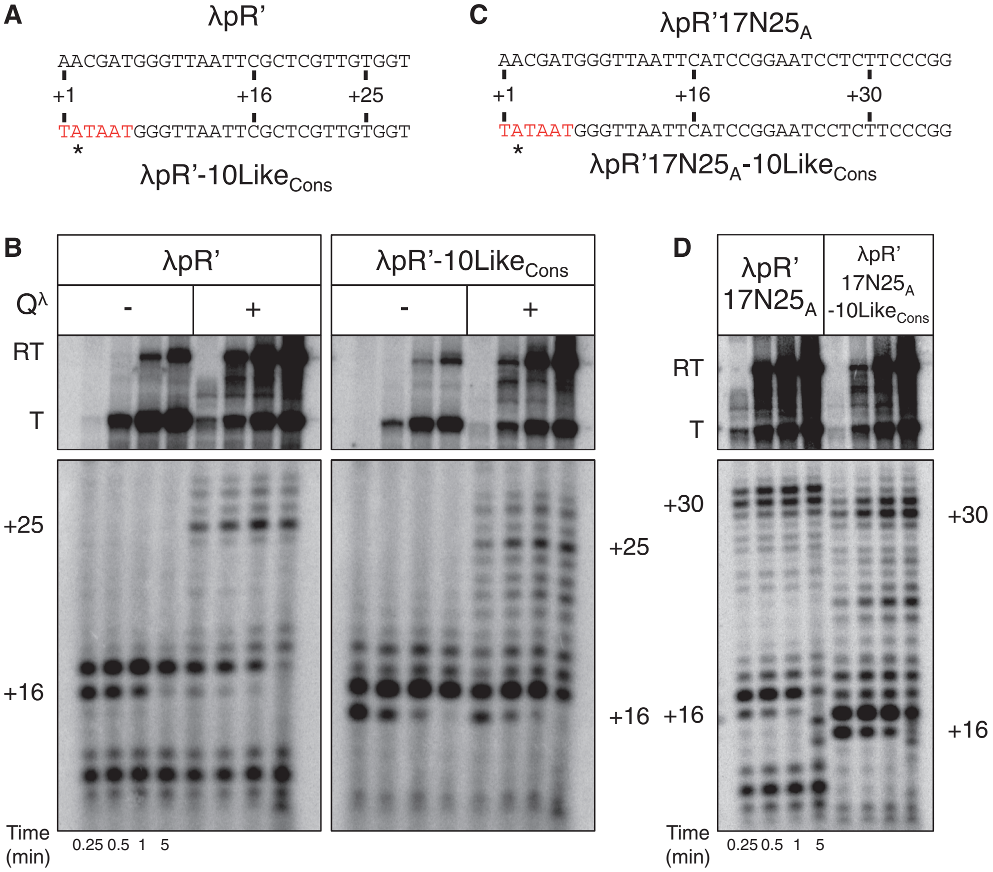

Figure 7. A Consensus -10-Like Element Expands the Formation of QAC. (**A**) Sequence comparison of λpR' and $\lambda pR'-10Like_{Cons}$ from positions +1 to +28. The $\lambda pR'-10Like_{Cons}$ alternate transcription start site is indicated by an asterisk. (**B**) Single round *in vitro* transcription from λpR' and $\lambda pR'-10Like_{Cons}$ in the presence and absence of Q^{λ}. Transcripts were labeled internally with α^{32}P-UTP and removed at the indicated times. Positions for λpR' are indicated on the left, and positions for $\lambda pR'\text{-}10Like_{Cons}$ are indicated on the right and are designated in reference to the wild-type start site. (**C**) Sequence comparison of $\lambda pR'17N25_A$ and $\lambda pR'17N25_A-10Like_{Cons}$ from positions +1 to +36. $\lambda pR'17N25_A-10Like_{Cons}$ alternate transcription start site is indicated by an asterisk. (**D**) Single round *in vitro* transcription from $\lambda pR'17N25_A$ and $\lambda pR'17N25_A-10Like_{Cons}$ in the presence of Q^{λ}. Transcripts were labeled internally with α^{32}P-UTP and removed at the indicated times. Positions for $\lambda pR'17N25_A$ are indicated on the left, and positions for $\lambda pR'17N25_A-10Like_{Cons}$ are indicated on the right and are designated in reference to the wild-type start site.

to abortive initiation, strengthening the interaction between σ^{70} and the −10-like element should increase the likelihood of Q^{λ}-modified transcription elongation complexes undergoing Q^{λ}-dependent arrest. To test this, we created a λpR' mutant, $\lambda pR'-10Like_{Cons}$, that contains the −10 element consensus sequence 'TATAAT' in place of the wild-type 'AACGAT' in positions +1 to +6 (Figure 7A). In λpR', substitution of a consensus −10-like element in place of the wild-type sequence creates an especially strong interaction with σ^{70} due to the presence of a GGG 'discriminator-like' sequence (37). Single-round *in vitro* transcription reveals that a consensus −10-like element increases the quantity and alters the distribution of both Q^λ-dependent arrest and σ^{70} pausing (in addition to shifting the ladder down 1 nt because initiation occurs at the A of position 2 relative to wild-type) (Figure 7B and Supplementary Figure S6A).

The effect of the λpR' − 10Like$_{\mathrm{Cons}}$ on Q^λ-modified elongation complexes is to broaden the range of species captured during Q^λ-dependent arrest from 17–19 to 24–28 so that QAC appear at all positions from 17 to 28 (Figure 7B and Supplementary Figure S6A). We propose that the expanded range of QAC on λpR' − 10Like$_{\mathrm{Cons}}$ is the result of the strengthened interaction between σ^{70} and the −10-like element trapping complexes that would normally bypass Q^λ-dependent arrest and causing them to collapse into a backtracked state. We constructed a −10-like consensus mutant of λpR'17N25$_{\mathrm{A}}$, termed λpR'17N25$_{\mathrm{A}}$ − 10Like$_{\mathrm{Cons}}$, to test whether strengthening the interaction between σ^{70} and the −10-like element would have similar effects on mutant QAC (Figure 7C). As expected, λpR'17N25$_{\mathrm{A}}$ − 10Like$_{\mathrm{Cons}}$ expands the QAC from 17–19 and 29–31 (on λpR'17N25$_{\mathrm{A}}$) to 17–34 (Figure 7D). It is striking that the pattern of Q-dependent arrest that λpR'17N25$_{\mathrm{A}}$ − 10Like$_{\mathrm{Cons}}$ displays from +17 to +30 (Figure 7D) is similar to the abortive release pattern of N25$_{\mathrm{Anti}}$ from +1 to +14 (Figure 5D), which is the same sequence of DNA; note particularly the peak of arrest at +23 of λpR'17N25$_{\mathrm{A}}$ − 10Like$_{\mathrm{Cons}}$, which corresponds to +7 in N25$_{\mathrm{Anti}}$.

In the absence of Q^λ, elongation complexes undergo σ^{70}-dependent pausing on both λpR' and the λpR' − 10Like$_{\mathrm{Cons}}$ mutant, as shown by bands at +16 and +17. λpR' − 10Like$_{\mathrm{Cons}}$ captures complexes with greater efficiency, as would be expected from a mutation that increases affinity for σ^{70} (Supplementary Figure S6B and C). In addition to an increase in pausing, λpR' − 10Like$_{\mathrm{Cons}}$ captures several-fold more complexes that have transcribed to +18 and +19 as well. This effect appears to be similar, albeit less dramatic, to the broadening of Q^λ-dependent pausing in the same mutant.

DISCUSSION

We have shown that two determinants of the efficiency and pattern of promoter escape and abortive initiation, namely, the nucleotide composition of the ITS and the strength of the interaction between σ^{70} and DNA, also underlie Q^λ-dependent escape from the σ^{70}-dependent pause at λpR' and the formation of the QAC. We conclude that both Q^λ-dependent escape from the σ^{70}-dependent pause and escape of initial transcribing complexes from the promoter share a common mechanism of advance: DNA scrunching. Thus, we have shown that DNA scrunching occurs in a context outside of promoter escape and, furthermore, can be modulated by a *trans*-acting factor, in this case the Q^λ-antiterminator. In addition, we identify a sequence element that is an important determinant of the pattern of abortive release of RNAs.

Mechanism of Q^λ-dependent release of paused complexes into elongation

At λpR', substitution of the N25$_{\mathrm{Anti}}$ ITS in place of the WT sequence from +17 to +36 changes the position to which Q^λ-modified complexes transcribe before undergoing Q^λ-dependent arrest, from a cluster focused at +25 to a cluster focused at +30 and +31. However, on both templates, RNAP remains bound to the −10-like element because the active center is found at +15 in both the WT and mutant QAC. Thus, the composition of the 20 bp immediately downstream of the σ^{70}-dependent pause site dictates the position of the 3′ end of the RNA bound within the QAC, but does not affect the position to which RNAP backtracks. These characteristics are consistent with a mechanism of scrunching, in which RNA is synthesized without movement of RNAP along DNA, but instead DNA is melted and drawn into the enzyme.

The role of the ITS in abortive synthesis is well defined, and it is clear that the base composition of positions +1 to +20 of transcribed DNA dictates the positions at which abortive transcripts are released. Similarly, sequences downstream of the σ^{70}-dependent pause determine where Q^λ-dependent arrest occurs; we suggest that in both processes, DNA sequence sets the transcription pattern by determining the stability of scrunched complexes. The relationship between the effects of the N25$_{\mathrm{Anti}}$ ITS on abortive synthesis and Q^λ-dependent arrest supports the interpretation that Q^λ promotes escape of RNAP from the σ^{70}-dependent pause as an advancing scrunched complex, accumulating energy of scrunching that is used to break σ^{70} or σ^{70} and Q^λ interactions with DNA and release modified RNAP into downstream elongation.

Increasing the strength of the interaction between RNAP and the −10-like element of the λpR' σ^{70}-dependent pause, by substituting the wild-type sequence AACGAT with the consensus −10-element TATAAT, expands the region in which elongation complexes are captured, resulting in Q^λ-dependent arrest from +17 to +28 instead of the wild-type positions of +24 to +28. Similarly, when a consensus −10-like element is introduced into λpR'17N25$_{\mathrm{A}}$, Q^λ-dependent arrest occurs at all positions between +17 and +34, as opposed to arrest primarily at +17 to +19 and a cluster around +30 in the presence of the wild-type λpR' −10-like element. The changed pattern of arrest between wild-type and consensus −10-like elements gives insight into the natural process of Q^λ-dependent escape from the σ^{70}-dependent pause. We propose the following interpretation.

First, we note that the consensus −10-like element increases Q^λ-dependent arrest relative to the wild-type −10-like element in regions near the +16 pause, namely, +17, +18 and +19, and also invokes novel arrest sites in the region +20 to +23. What is the origin of this enhanced arrest? It must represent complexes that with the wild-type pause-inducing sequence would either continue scrunching or break the σ^{70} and Q^λ bonds and continue into productive Q^λ-modified elongation. However, it is implausible that the consensus −10-like element would disfavor continuing the scrunch because it should only increase the strength of the σ^{70}–DNA bond that must be maintained during the scrunch. Thus, it follows that the increase in arrest due to the consensus element must reflect complexes that, when bound to the wild-type-10-like element, have sufficient scrunching energy to release σ^{70} and Q^{λ} bonds with DNA and proceed into productive Q^{λ}-modified elongation. In the presence of a consensus −10-like element, the scrunching energy required for pause escape is increased so that these complexes no longer have sufficient energy to break the interaction between σ^{70} and the DNA, and instead, backtrack and arrest. Thus, the sites of enhanced arrest with the consensus −10-like element mark natural sites where σ^{70} dissociates from the −10-like element and where productive Q^{λ}-modified elongation begins.

On exceeding some limit of stability, the scrunched complex becomes prone to collapse and backtracks with high probability, whether the wild-type or consensus −10-like element is present, resulting in Q^{λ}-dependent arrest at RNA lengths 24–28. As evidenced above, this limit is sensitive to the nature of the 20 bp of sequence immediately following the site of the σ^{70}-dependent pause; for example, introduction of the $N25_{Anti}$ ITS changes the site of arrest to lengths around +30. Thus, these results reveal the natural process of Q^{λ}-dependent escape into elongation.

Nature of abortive initiation

In addition to the similarities between an initial-transcribing complex and a σ^{70}-dependent paused complex, there are also discrete differences, most notably the contacts between σ^{70} and the core, the presence of a mature RNA, and, when present, the contacts with Q^{λ}. The availability of a second system besides promoter initiation in which RNAP must break an interaction between σ^{70} and DNA to proceed forward provides a structurally distinct context in which to examine the elements that contribute to promoter escape. Such an analysis can clarify the role of these elements and may lead to a more general understanding of abortive synthesis.

The existence of abortive initiation has been attributed to two distinct phenomena: scrunching and displacement of the $\sigma^{3.2}$ loop by the emerging RNA product (10–13). A further barrier that could contribute to abortive initiation is the displacement of σ^{4} that occurs as the RNA reaches 14–15 nt in length (38). All of these processes would store energy as the initial transcript grows, and the failure of any to continue could lead to abortive loss of RNA. However, neither $\sigma^{3.2}$ loop displacement nor σ^{4} displacement can be involved in QAC formation because the RNA is too long, leaving scrunching as the only plausible process to account for QAC formation; therefore, our demonstration that the pattern of abortive release is reconstructed in the pattern of QAC formation, specifically in the case of $\lambda pR'17_{N25A} - 10Like_{Cons}$, suggests that scrunching is the dominant process that forms the pattern of abortive synthesis at this promoter.

Our investigation of the QAC has provided a pathway to understanding a sequence basis of the pattern of abortive transcript release. Previous work has shown, first, that stronger promoter consensus elements (e.g. −35, −10 and discriminator elements) increase the length and yield of abortive products (14), and, second, that the ITS determines both the pattern and level of abortive release (15). It was shown previously that there is a correlation between purine-richness of the ITS and abortive tendency (15), but no specific sequence elements have been identified. We have used a systematic scan across the $N25_{Anti}$ ITS to find elements that affect QAC formation, and then to make inferences about ITS function in abortive transcript release.

A pair of G to T mutations at positions +5 and +6 of the $N25_{Anti}$ ITS (+21 and +22 in $\lambda pR'17N25_{A}$) shifts the position to which RNAP transcribes before Q^{λ}-dependent arrest from a cluster at +30 to a cluster at +24, indicating that the identity of these 2 nt is essential to the properties of the $N25_{Anti}$ ITS. The critical nature of these positions is reflected by their function in abortive initiation in the $N25_{Anti}$ promoter: introduction of the +5, +6 G to T mutations into $N25_{Anti}$ alters abortive synthesis in a manner equivalent to the effect of the corresponding changes in $\lambda pR'17N25_{A}$ on Q^{λ}-dependent arrest. The bulk of $N25_{Anti}$ abortive products are 7, 13 and 14 nt in length, with minor abortive products occurring at all positions between +2 and +15. The mutant $N25_{A-TT}$ promoter displays strongly decreased abortive transcripts at +13 and +14, and instead accumulates many more abortive transcripts of 6, 7 and 8 nt in length. A reasonable conjecture is that the uridine-richness at +5, +6 near the end of the 6, 7 and 8 transcripts destabilizes the DNA/RNA hybrid and promotes dissociation. The decrease in 13 and 14 nt aborted RNAs in $N25_{A\text{-}TT}$ is likely the result of increased abortive release at +6, +7 and +8 reducing the number of transcripts that proceed to +13 and +14; the ratio of transcripts at +12 to +15 to full-length transcripts, i.e. the probability of abortive stalling at +12 to +15 is constant across the overlapped set of 3-nt substitutions that drastically changes the ratio of +12 to +15 to smaller abortive transcripts. The abortive pattern is sensitive to NTP concentration: low NTP concentrations favor the accumulation short abortive products, whereas high NTP concentrations facilitate the formation of longer abortive products and partly restore the 13 and 14 nt abortive transcripts in $N25_{A\text{-}TT}$. This trend implicates the rate of transcription as a key determinant of abortive synthesis and suggests that other influences on transcription rate, e.g interaction with DNA-bound factors, influence promoter escape and abortive synthesis.

It is not surprising that the differences between these complexes would produce subtle variations in the way DNA scrunching is modulated by the ITS. This is evident in a heteroduplex analysis of the $N25_{Anti}$ and $N25_{A\text{-}TT}$ ITSs in both contexts. Transcription on $\lambda pR'17N25_{A}$ and $\lambda pR'17N25_{A\text{-}TT}$ heteroduplexes reveals that the effect of ITS composition on formation of the QAC is solely dependent on the template strand. However, in the context of initial transcription at $N25_{Anti}$ and $N25_{A\text{-}TT}$, neither heteroduplex produces an abortive pattern identical to either of the homoduplexes from which they are derived. Thus, the effect of ITS composition on the QAC is clearly attributable to the template strand, whereas both the template and non-template strands modulate abortive initiation. It is plausible that this difference is a reflection of the numerous structural distinctions between a σ^{70}-dependent paused complex and an initial-transcribing complex that affect the interactions between RNAP and the DNA template.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors thank Ann Hochschild for criticizing the manuscript and Lillian Hsu and Irina Artsimovitch for providing materials.

FUNDING

National Institutes of Health (NIH) [GM21941]. Funding for open access charge: Research funds.

Conflict of interest statement. None declared.

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