Mechanism of Intrinsic Transcription Termination and Antitermination

W. S. Yarnell and J. W. Roberts*

Gene expression is modulated by regulatory elements that influence transcription elongation by RNA polymerase: terminators that disrupt the elongation complex and release RNA, and regulators that overcome termination signals. RNA release from *Escherichia coli* RNA polymerase can be induced by a complementary oligonucleotide that replaces the upstream half of the RNA hairpin stem of intrinsic terminator transcripts, implying that RNA hairpins act by extracting RNA from the transcription complex. A transcription antiterminator inhibits this activity of oligonucleotides and therefore protects the elongation complex from destabilizing attacks on the emerging transcript. These effects illuminate the structure of the complex and the mechanism of transcription termination.

Although the transcription elongation complex of the highly conserved multisubunit RNA polymerase is notably stable, it is disrupted by DNA termination sequences that are recognized either directly by the polymerase or by factors that mediate disassembly of the complex (*1–4*). The termination process and the elongation proficiency of the enzyme are the targets of regulatory proteins such as the phage λ gene *Q* and *N* transcription antiterminators (*4–6*) and the human immunodeficiency virus regulator Tat (*7*). Understanding how termination disrupts the transcription elongation complex should illuminate the function of these regulators and the construction of the complex.

We describe a method to simulate the RNA release function of intrinsic (rho-independent) terminators of *Escherichia coli* RNA polymerase (RNAP) using an annealing DNA oligonucleotide added in trans to substitute for the RNA hairpin that makes up part of the terminator. This method allows the steps of termination to be studied in static isolation and defines the function and sites of nucleic acid interactions by which the terminator mediates transcript release. We also show that the gene *Q* antiterminator of phage 82 acts by stabilizing the elongation complex against disruption by the RNA hairpin.

Terminators and the Structure of the Elongation Complex

The elements of intrinsic *E. coli* terminators are illustrated in Fig. 1A. The transcription elongation complex is maintained and stabilized by protein–nucleic interactions and by contacts between the DNA template and the growing RNA. RNAP makes a stabilizing interaction (sliding clamp) with about 8 nucleotide pairs of duplex DNA ahead of the nucleotide addition site (*8*). Just upstream of this site, the DNA template and RNA transcript make base pair–specific interactions for 8 to 10 nucleotides (*9–11*), forming a DNA/RNA hybrid within a protein binding site; the hybrid may be unlike stable DNA/RNA hybrid in free solution (*12*). Upstream of the RNA/DNA hybrid, RNAP binds or conceals 4 to 5 nucleotides of RNA product within the enzyme (RNA binding site) (*13–15*). Beyond about 13 nucleotides from the 3′ end, the RNA becomes accessible to probes, although it may be complexed with sites on RNAP (*16*).

Intrinsic terminators contain two sequence motifs that are required for RNA release (see Fig. 1A): an RNA stem-loop "hairpin" that alone can destabilize complexes stalled artificially (*17, 18*) and a segment of 8 to 10 nucleotides immediately downstream that encodes mostly uridine residues at the end of the released RNA (*19*). Early models of the elongation complex proposed a long RNA/DNA hybrid that would be unwound by formation of the hairpin, a process facilitated by the unstable, predominantly poly(rU·dA) hy-

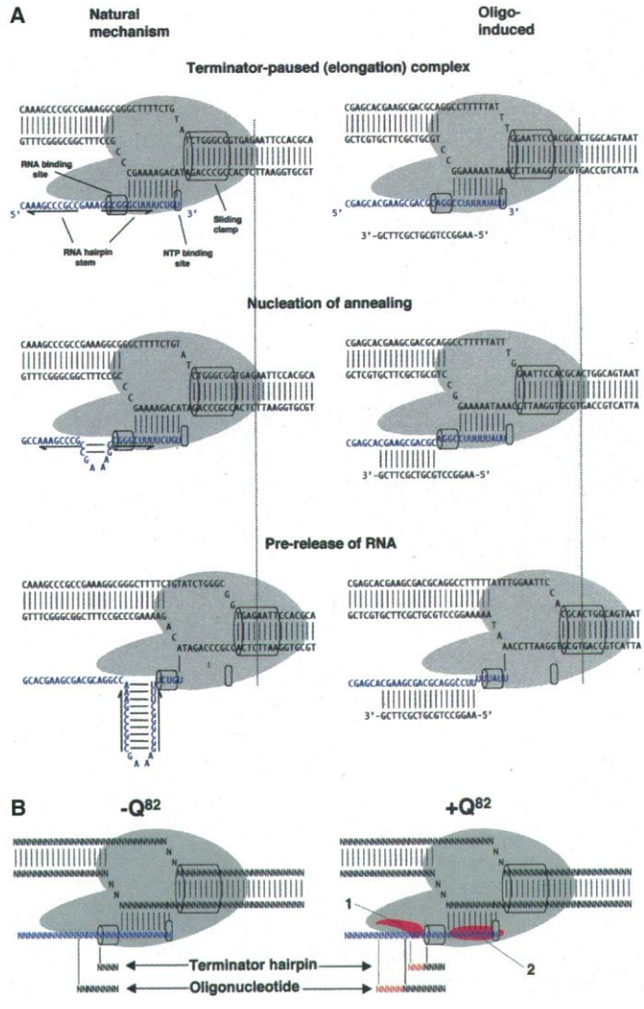

Fig. 1. (**A**) The translocation model of intrinsic terminator function (natural mechanism), and the release of RNA by an annealing oligonucleotide (oligo-induced). In the conventional model (not pictured), hairpin formation extracts RNA without movement of the polymerase and DNA bubble. (**B**) Minimal domain of transcript annealing by the upstream terminator stem half or by an oligo that can induce release in a paused complex with and without Q^{82} (gene *Q* transcription antiterminator of phage 82). The left boundary presumably marks the minimum sequence required for nucleation of annealing (Fig. 1A) and varies between hairpin and oligonucleotide; red segments designate the extended requirement in the presence of Q^{82}. The right boundary indicates the minimum annealing length required to destabilize the transcript. The red shapes on RNAP suggest alternative possibilities (labeled 1 and 2) for the site of Q^{82} action in the complex.

Section of Biochemistry, Molecular and Cell Biology, Biotechnology Building, Cornell University, Ithaca, NY 14853, USA.

*To whom correspondence should be addressed. E-mail: jwr7@cornell.edu

brid (*3, 19, 20*). A refined view reinterprets the role of the hairpin to be primarily extraction of RNA that originally occupied the enzyme RNA binding site (*12, 15*). In this model, the hairpin forms in an immobile complex and removes RNA from the binding site, so that the transcript is released as the weak 6- to 8–base pair (bp) rU/dA-rich terminal hybrid region dissociates. A variant, which we favor, posits release through forward translocation of RNAP concurrent with hairpin formation, facilitated by weakness of the rU/dA hybrid (Fig. 1A).

Besides its inherent instability as a DNA/RNA hybrid (*20*), a role for the U-encoding segment and surrounding sequences is implied by the activity of deletion variants of terminators tR2 (*21*) and t82 (see below) that lack the upstream hairpin stem half. These termination-inactive DNAs still specify transcription pauses at the sites of normal transcript release, suggesting that the U-encoding tract stalls RNAP at a position where the elongation complex is susceptible to disruption by formation of the RNA hairpin. The pause may reflect disfavored forward translocation after addition of a nucleotide (*22*). Eukaryotic RNA polymerases I, II, and III are also induced to pause or terminate by U-rich encoding segments (*23–25*).

Simulation of Terminator Hairpin Function with Oligonucleotides

If formation of the hairpin is its critical role in triggering RNA release from the paused complex—serving, for example, to extract the segment of RNA that becomes the downstream segment of the hairpin—then a DNA or RNA oligonucleotide (oligo) supplied in trans that can anneal to the downstream segment of the hairpin might substitute for the upstream half. Such an activity would provide direct evidence for the model described and would allow steps of termination to be studied in static isolation, so as to reveal how regulatory influences affect termination.

To test this conjecture, we constructed variants of two terminators tR2 and t82 that lack sequences encoding the upstream half of the hairpin (designated ΔtR2 and Δt82), placed them in tandem after a strong promoter, and tested the ability of complementary oligos to release RNA from actively transcribing complexes (*26*). Release of terminated RNAs was assayed by removing complexed RNAs with magnetic beads attached to the template and then analyzing the supernatant and pellet fractions by gel electrophoresis (Fig. 2). The DNA templates with mutant terminators retained the ability to pause RNAP at the normal release site (lanes 1 to 6). For each terminator, the appropriate oligo released RNA from transcribing complexes at the U-encoding pause sites, exactly where RNA release normally occurs. The oligo t19 (targeted to site ΔtR2) released 59% of transcripts at ΔtR2 of template ΔtR2Δt82, but nothing over background from site Δt82 (lanes 7 to 9), whereas t18 (targeted to site Δt82) released no more RNA from ΔtR2 than buffer alone (1%) (lanes 10 to 12, compared to lanes 1, 3, and 5). Oligo t18 released transcripts from Δt82, although less efficiently, perhaps because the annealing segment is shorter and less G/C rich: 5.5% of transcripts over a buffer-only or oligo t19 background of 2.5% (lanes 10 to 12, compared to lanes 1, 3, and 5). A single base change in oligo t19 (oligo t19H1) reduced release to only 7.4% of transcripts from ΔtR2 of template ΔtR2Δt82 (lane 13); a compensating change in the template (ΔtR2–H1) allowed t19H1 to release 24% of transcripts (lane 14). (The latter template contains the unmodified t82 terminator.) Thus, the RNA hairpin functions only to engage the downstream stem half, and the hairpin structure per se is not required for termination (*27*).

Next, we challenged very slowly elongating or static complexes with oligos and found that the U-encoding segment is not required for release in these conditions. We first used the hairpin-deleted tR2 terminator to compare the domain of oligo-induced release activity to the destabilizing effect of the RNA hairpin (Fig. 3). Complexes were advanced to the beginning of the tR2 U-tract (same sequence as Fig. 2), washed extensively to remove nucleoside triphosphate (NTP) substrate, and either (i) slowly elongated, in the presence of oligo t2, t3, or t4 or buffer (Fig. 3), through the region surrounding the tR2 release site; or (ii) spread throughout the release region, washed, and then incubated with oligo t5 or buffer (Fig. 3). RNA release was assayed as in Fig. 2 (*28*).

Each oligo gives an extended region and distinct pattern of RNA release from slowly elongating or static complexes (Fig. 3). The downstream boundary of release is about 10 nucleotides downstream of the 5′ (rightward) end of each annealing oligo. The natural tR2 hairpin also induces instability 10 nucleotides downstream from the last position of annealing (the hairpin base) in static complexes (gray region of Fig. 3) (*18*). The consistent relation between the region of annealing and the window of transcript release by the terminator hairpin suggests that the oligo and hairpin act by analogous mechanisms.

The upstream-most complex destabilized by an oligo at these template sites occurred at a consistent distance downstream from the 3′ (leftward) end of oligos t2 and t5—about 18 nucleotides. (Release with t3 and t4 extended upstream of the first assayed complex.) As described (*29*), the position of first RNA release from static complexes by a terminator hairpin presumably reflects the availability of nascent RNA for nucleation of a hybrid at the hairpin stem (see Fig. 1A). For both the tR2 terminator and the unrelated terminator t500 (described below), with or without a U-tract, the distance from first annealing to initial RNA release is 14 nucleotides. The difference in this distance between terminator and oligos is likely due to sequence effects and to the extremely high local concentration of the upstream stem-half in the intact terminator

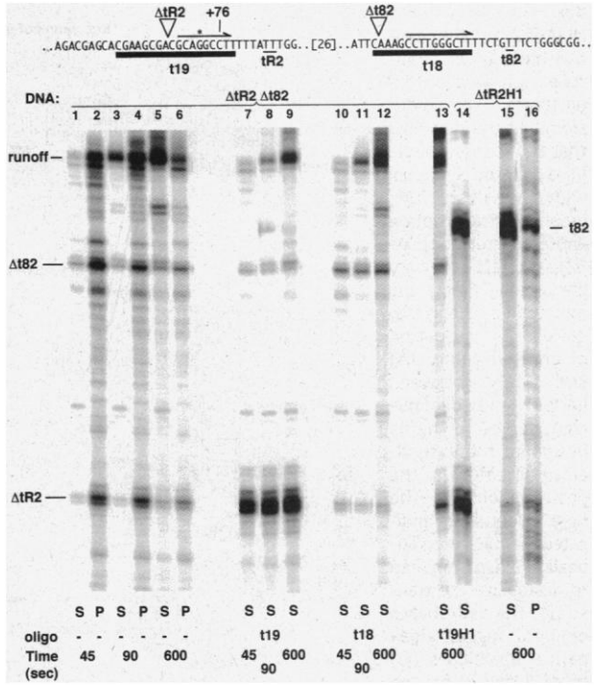

Fig. 2. Release of RNA from transcribing complexes by DNA oligonucleotides. A portion of template ΔtR2Δt82, which contains two truncated terminators, and the regions complementary to the oligos used (black bars) are shown; a rightward arrow indicates the original downstream stem half for each RNA hairpin, and the natural release sites are underlined. The sites at which the upstream hairpin halves were deleted are indicated. Pause sites for each terminator (Δt82 and ΔtR2) and the natural t82 release site are shown next to the gel. Released (supernatant, S) and complexed (pellet, P) RNAs were assayed at intervals during transcription in the presence of the indicated oligonucleotide. Template ΔtR2H1 has a single base change at the asterisk and an intact terminator t82; oligonucleotide t19H1 has a complementary change. In related experiments, DNA oligonucleotides were shown to target the transcript rather than complementary segments of DNA (*13, 51*).

relative to oligos in free solution.

In static complexes, release is induced in a region that is related to the boundaries of the oligo, but it does not require the U-encoding segment or other features of a terminator. Presumably, the indefinite time available to static complexes obviates the need for a transcription pause and allows the higher stability of non–U-encoding sequences to be overcome. The experiments below further generalize the oligo release activity to random sites in stopped complexes.

Modulation of Oligo-Mediated RNA Release by RNAP Mutations

To provide further evidence that oligo-mediated release reflects the natural termination process, we used RNAP altered in the β subunit by mutations that change the efficiency of termination at intrinsic terminators in vivo and in vitro (*30*–*32*). In conditions where wild-type RNAP terminates at 95% efficiency, the mutationally altered RpoB2 polymerase terminates only 25% (and pauses less), whereas RpoB8 terminates more efficiently than wild type (and pauses more) (*33*). We chose an oligo complementary to the transcript at a site unrelated to a terminator, and tested release on a dispersed set of stopped, washed complexes (*34*). This oligo released RNA from a region with a downstream boundary about 8 nucleotides from the 5′ end of the oligo (Fig. 4). The distribution and efficiency of RNA release differed for the three enzymes and correlated with their termination properties. RpoB2, which terminates less efficiently than wild type, also showed less efficient oligo-induced release. Furthermore, the pattern of release was different, slightly skewed toward the downstream boundary of the release window, similar to RpoB2 behavior at a terminator (*33*). The skewing suggests that oligo release activity is inefficient, relative to wild type, for minimal overlaps by the oligo 3′ (upstream) end. RpoB8 showed a distinctly extended region of release downstream, implying that complexes are destabilized with less overlap by the oligo of RNA within the enzyme than wild-type complexes require.

Stabilization by an Antiterminator Against Oligonucleotide Attack

The gene *Q* transcription antiterminators of phage λ (Q^{λ}) and its relatives bind and modify RNAP at a genome-specific site (*qut* λ) near the phage late-gene promoter (*35*). Through a pathway that involves binding to DNA within the promoter (*36*) and binding to RNAP at a promoter-proximal σ^{70}-dependent pause site (*37*), Q^{λ} becomes a subunit of RNAP, thereby changing its termination properties. For the close λ relative phage 82, Q^{82} remains firmly bound to the transcription complex: both the antitermination characteristics and the presence of 1 to 2 mol of Q^{82} per mole of RNAP (*38*) survived repeated washing of transcription complexes. Exposure of RNAP to Q^{82} downstream of the promoter-proximal site resulted in neither modification nor capture of the polypeptide (*38*).

Modification by Q both provides antitermination and inhibits pausing (*39*). Because the correlation of termination efficiency and pausing tendency also holds for the RNAP mutants *rpoB2* and *rpoB8* (*30, 40*), one conjecture has been that the basis of termination control is simply speeding RNAP through a termination release site before the mechanics of release are completed, without any intrinsic stabilization of the complex (*35*). On the other hand, if Q acts by stabilizing the complex against the action of the RNA hairpin so that an exclusively kinetic role need not be invoked, this might be evident in the accessibility of static, Q-modified complexes to oligos.

We made complexes in the presence and absence of the antiterminator protein Q^{82}, using a template with *qut82* and its associated promoter, spread them across ~100 nucleotides, washed away NTPs to make them static, and mapped the pattern of transcript release in the presence of various DNA oligos (Fig. 5) (*41*). The array of stopped complexes was challenged by five different oligos. RNAs were released by oligos from complexes made without Q^{82} (red bars and plot) consistently with the rules described above. Variances in the release profiles likely reflect different energetics of oligo hybridization

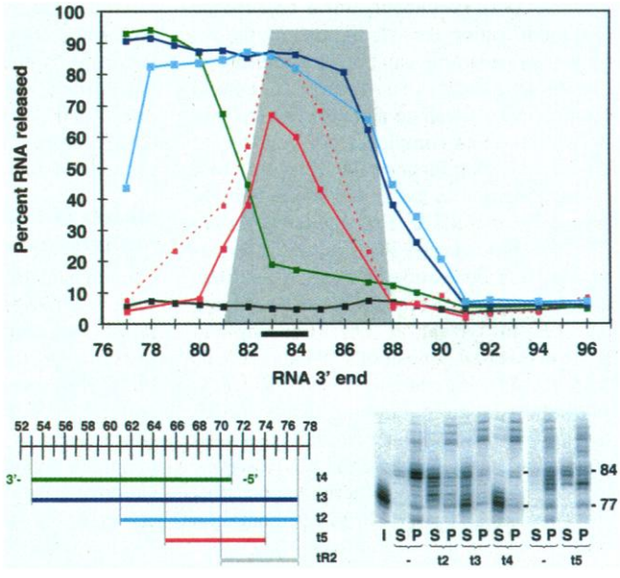

Fig. 3. Release of transcripts from static or very slowly elongating complexes by oligonucleotides. The template pBY416 contains a truncated tR2 derivative identical to that in ΔtR2Δt82. The fraction of RNA released during incubation with each of four complementary oligos (identified by color-coded bars below the graph), or with no oligo (black line in graph), is shown. Oligo t5 induces some arrest in stopped complexes, possibly by annealing to RNA extruded from backtracked complexes; the dashed red line is corrected for arrested complexes, which neither release nor chase with NTPs. Release sites (nucleotides 83 and 84 of the transcript) of intact tR2 during normal synthesis are marked with a black bar. The gray zone represents the instability of complexes halted in the region (*18*). The gray line below the graph indicates the stem of the wild-type tR2 hairpin. Released (S) and complexed (P) RNAs after incubation with oligo are shown, as are washed initial complexes used for release by oligos t2 through t4 (I).

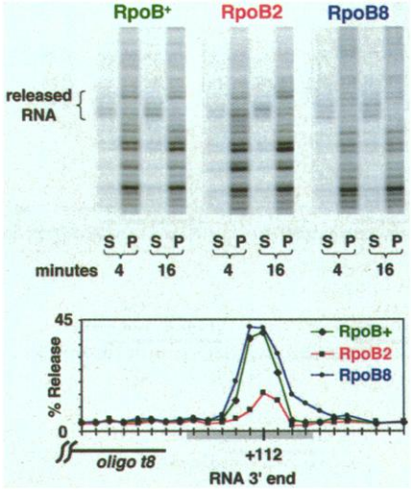

Fig. 4. Effect of termination-altering mutations in the RNA polymerase β subunit on the efficiency of oligonucleotide-induced release from static transcription complexes. The oligo t8 was the complement of transcript sequences from +105 to +86 (black bar). The gray shading below the graph indicates the zone of destabilization predicted by analogy to the tR2 hairpin stem (*18*), including sites 14 nucleotides from the oligo 3′ end through 10 nucleotides from the oligo 5′ end (omitting an oligo self-complementary region in t8 from +86 through +93). Time of incubation of the complex with the oligo is shown. Release at 4 min is graphed.

and, in the translocation model (see Fig. 1A), different energetics of DNA bubble movement. Modification by Q^{82} almost completely suppressed release by each of the five oligos (green markings). The Q^{82}-modified complexes allowed some release by oligos t14 and t15, but this was biased toward the downstream end of the distribution, positions at which the oligo extended relatively farther upstream of the complex. Thus, Q^{82} acts by stabilizing modified complexes against release (*42*).

The Q^{82} modification similarly restricts RNA release to a distal region when release is induced by the RNA hairpin of an intrinsic terminator. With wild-type terminators, we have observed no release by Q^{82}-modified complexes in the region downstream of the normal release site, even in extremely slow synthesis (*43*). Presumably this is because the Q^{82} modification persists throughout the region where a hairpin can potentiate release in natural terminators. To modify the natural termination reaction so as to observe release by Q^{82}-modified complexes, we constructed the artificial terminator t500 (derived from t82) that carries an extremely strong hairpin, braced by the RNA tetraloop GAAA (*44*) (Fig. 6). t500 is nearly identical to t82 downstream from nucleotide 96 (Fig. 6) and thus has the sequences responsible for inducing pausing. In typical in vitro transcription, RNA is released at positions 104 and 105, as with t82 (*33*), at greater than 98% efficiency. In synthesis at normal in vitro rates, with 20 μM to 200 μM NTP, Q^{82}-modified RNAP yields near 100% readthrough (*43*). However, in contrast to wild-type terminators, very slow synthesis allows t500 to overcome the effect of Q^{82} in a revealing way.

For the experiment of Fig. 6, Q^{82}-modified and unmodified complexes were staged at position 97 of t500 by UTP deprivation, and then moved through the region of RNA release at very low NTP concentration (*45*). RNAs were separated into pellet (P) and supernatant fractions (S) to identify released RNAs. Whereas unmodified complexes release efficiently beginning at position 103, Q^{82}-modified complexes mostly pass this position and begin to release at position 106. Because only about 50% of complexes typically become modified, it is likely that all modified complexes proceed at least to 106, even though the 1.5-min sample shows that they linger a significant time at positions 104 and 105. Note the general acceleration of transcription through this region by the Q^{82} modification.

Thus, the effect of Q^{82} on this strong terminator is similar in nature, but shorter in range, to its effect on oligo-induced release. We attribute the smaller domain of the Q^{82} effect, and the fact that Q^{82}-modified complexes can be released at all from t500, to the strength of the hairpin.

Models of Intrinsic Termination and Antitermination

The domain of oligo release activity from static complexes shows congruence with the inferred structure of the elongation complex. In particular, the minimum segment of transcript that must be annealed by the oligo closely matches the site of RNA binding or concealment by the enzyme and is also the minimum segment that must be withdrawn by hairpin formation in order to destabilize the complex (Fig. 1B).

How does withdrawal of an RNA segment from RNAP induce transcript release? If the transcript can be removed from its binding site in RNAP without RNAP translocation, then the terminal segment of ~5 to 8 nucleotides downstream of the hairpin might simply dissociate—the standard model. We suggest and illustrate (Fig. 1A) a variant in which release is a consequence of forward translocation of RNAP in the absence of transcript elongation (RNA synthesis); template DNA is rewound at the back and unwound at the front as the 3′ RNA end is pulled through the binding site and into solution (*46*). We offer several arguments for this mechanism: (i) The high energy yield of hairpin formation, along with the low energetic cost of breaking the predominantly rU/dA hybrid, would favor such movement at a terminator. The energetics of DNA bubble movement should affect release efficiency; consistently, changes in downstream duplex DNA affect termination, with AT-richness favoring termination (*47*). (ii) The ready interconversion of translocation states, including extended reverse translocation, implies substantial translational freedom (*10, 14, 22*). (iii) A DNA binding protein blocks RNA release at a terminator when it is placed so as to prevent forward RNAP movement (*21*); furthermore, the effect of similar blockage on oligo-mediated release from static transcription complexes suggests that polymerase must move at least 5 bp for release to occur (*43*). (iv) RNA release is inhibited by nontem-

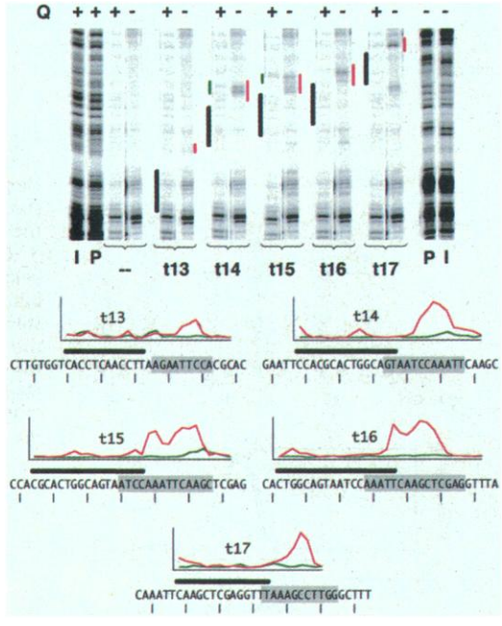

Fig. 5. Inhibition of oligonucleotide-mediated release of transcripts by Q^{82} modification. The lanes with oligos (t13 to t17) or no oligo show RNAs released (supernatants) after incubation; the surrounding panels show complexes before incubation (I) and the pellet fractions remaining after incubation with no oligo (P). Black bars designate the span of transcript complementary to each oligo, red bars and plots designate the region of RNA release in the absence of Q^{82} modification, and green bars and plots designate RNA release from Q^{82}-modified complexes. Release efficiency is plotted above each sequence; the vertical axes represent 60% release. The gray shading in the sequence designates predicted release by analogy to the tR2 hairpin, as in Fig. 4.

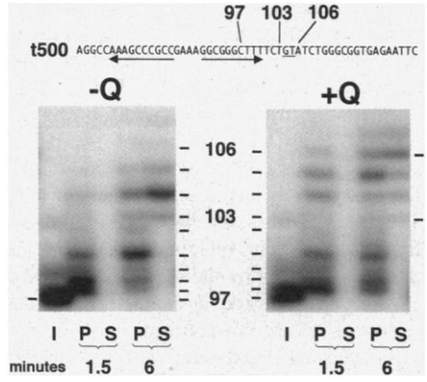

Fig. 6. Truncation of RNA release window at a synthetic terminator by Q^{82}. Complexes either modified by Q^{82} (+Q) or not (−Q) were advanced through the release segment of the synthetic terminator t500 in extremely slow synthesis for 1.5 or 6 min and assayed for release. The reaction with Q used a lower concentration of NTP, so the 50% fraction of unmodified complexes in this reaction advances more slowly than complexes in the reaction without Q. The major positions of release at low (104) and high (105) NTP concentration are underlined. The first lane in each set shows initial complexes, stopped by UTP deprivation at nucleotide 97. I, complexes at 97 before incubation; P, pellet fraction; S, supernatant (released) fraction. The arrows under the t500 sequence designate sequences encoding the terminator hairpin stem.

plate strand substitutions that create heteroduplex segments in the back of the bubble and thus remove the energy of DNA rewinding that would favor forward translocation (*48*). (v) There is a precedent for RNAP forward translocation when a hairpin forms at a pause site (*49*).

How is termination modified by the antiterminator Q^{82}, and by the *rpoB* mutations? Both affect the range as well as the efficiency of oligo-mediated RNA release. For Q^{82}, two possibilities are depicted by two regions colored red in Fig. 1B. (i) Q^{82} binds and directly occludes the region around and upstream of the RNA binding site, so that only longer annealing segments can access the RNA and dissociate the complex. (ii) Q^{82} affects the region of RNAP associated with the DNA/RNA hybrid and NTP binding, acting to strengthen interactions here so as to resist RNA extraction by the annealing of upstream RNA. The second mechanism is particularly attractive for the rifampicin-resistant *rpoB* mutations because rifampicin is thought to bind near the initiation site. In addition, both Q modification and *rif* mutations affect pausing and elongation rate. For a natural terminator, we propose that Q^{82} acts both by inhibiting hairpin formation while the enzyme is paused within the destabilizing domain of the hairpin and, possibly, by inhibiting pausing in the region of release. For a terminator like tR2, for example (Fig. 3), the Q^{82} effect would have to extend only through nucleotide 84, the natural limit of the pause. The *rpoB* mutations might similarly affect hairpin formation.

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26. Transcription complexes were made on template BY416-ΔXC3, which contains promoter *p82* (*50*) followed by a segment lacking C that allows complexes to be stopped at +25 (the natural pause site for Q engagement), followed by a 51-bp segment lacking U. RNAP (200 nM) and template (40 nM) bound through a terminal biotinylated nucleotide to streptavidin-coated magnetic beads were incubated 10 min at 37°C in TB1 [20 mM tris-Cl (pH 7.9), 0.1 mM EDTA, 1.0 mM dithiothreitol, 50 mM K^+ glutamate, 4 mM MgCl, 2 to 5% glycerol, bovine serum albumin (40 μg/ml)]. Complexes halted at position +25 were labeled by incubation for 10 min at 37°C with 75 μM initiating dinucleotide ApC, 100 μM GTP, 25 μM UTP, 25 μM ATP, and [α-^{32}P]UTP (2.3 μC/μl). The complexes were then washed twice by magnetic fixation in TB2 [TB1, except with 250 mM K^+ glutamate, 2.5 mM $MgCl_2$, and poly(dA-dT) (20 μg/ml)], resuspended in TB2 plus 100 nM NusA (*50*), divided into samples containing 10 nM complex, and supplemented with 150 μM oligo where added. After addition of 100 μM ATP and GTP, 10 μM CTP, and 50 μM UTP, samples were removed at indicated times during incubation at 37°C, divided into supernatant and pellet by magnetic partitioning, and diluted with stop buffer [final concentration 500 mM tris-HCl (pH 7.9), 10 mM EDTA, tRNA (50 μg/ml)]. After extraction with phenol/chloroform/isoamyl alcohol and ethanol precipitation, samples were resolved on a 7% polyacrylamide gel, imaged by Molecular Dynamics Phosphorimager, and quantified by ImageQuant software.
27. I. Artsimovitch and R. Landick (*51*) have shown release of RNA by an oligo at an attenuator-related pause site.
28. Complexes on template BY416 were labeled with ^{32}P and stopped at +25; the remaining open complexes and free RNAP were removed by incubation with TB3 [TB1, except with 350 mM NaCl in addition to K^+ glutamate, and poly(dA-dT) (200 μg/ml)] on ice for 5 min, followed by four washes in TB4 (TB2, except with 4 mM $MgCl_2$), followed by advance to +76 by incubation for 2 min at 37°C in TB4 plus 150 nM NusA and 25μM CTP, GTP, and ATP; most complexes advance to +77. The release of transcripts by oligos t2, t3, and t4 was measured during incubation of 8 nM complexes in TB4 plus 150 nM NusA for 5 min at 37°C with 100 μM oligo followed by the addition of 250 nM ATP, GTP, CTP, and UTP (which allowed very slow synthesis) for 15 min. For release with oligo t5, washed +76 complexes were spread downstream of +76 by incubation with 100 nM ATP and GTP and 2.5 μM UTP for 1.5 min, followed by four washes and incubation for 5 min with 200 μM oligo. Some complexes incubated without NTPs but with oligo were found to be arrested, failing to elongate upon further incubation with NTP (*10*, *14*, *17*), so release efficiency of the unarrested fraction also was calculated. Supernatant and pellet fractions were determined as before, and the percent transcript release was calculated.
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34. Complexes on BY416 were labeled during synthesis to +25 and advanced to +76 as described (*28*), except that ATP, CTP, and GTP were present at 150 μM during synthesis between +25 and +76 by RpoB8 RNAP. Complexes were washed four times in TB6 (TB1 lacking K^+ glutamate and $MgCl_2$), advanced to +91 in TB2 plus 150 nM NusA, 15 μM GTP, 15 μM ATP, and 15 μM UTP (or six times these concentrations of NTPs for RpoB8), and spread downstream of +91 by incubation for 6 min at 37°C in TB2 with 750 nM ATP and GTP, 250 nM UTP, and 40 nM CTP (six times these concentrations of NTPs for RpoB8). After five washes in TB6, complexes were resuspended in TB2 plus 150 nM NusA, incubated 0.5 min at 37°C, supplemented with 100 μM oligo t8 (complementary to the transcript from +86 to +105) or t8a (as t8, but mismatched at +99 and +101), and sampled at 4 and 16 min. There was no detectable release by t8a.
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45. Complexes were labeled to +25, modified by Q^{82} or not, and advanced to +97 in the absence of UTP on BY500 template affixed to magnetic beads. After four washes and resuspension in TB2 plus 150 nM NusA, reactions were incubated 0.5 min at 37°C before the addition of 1.5 μM GTP and ATP, 0.5 μM UTP, and 80 nM CTP (for the reaction without Q^{82}), or the addition of half these concentrations to the reaction with Q^{82}; bound and released RNAs were measured during incubation. An identical result was obtained with a t500 derivative lacking the U-rich segment.
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