Mycobacterial RNA Polymerase Requires a U-Tract at Intrinsic Terminators and Is Aided by NusG at Suboptimal Terminators

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ABSTRACT **Intrinsic terminators, which encode GC-rich RNA hairpins followed immediately by a 7-to-9-nucleotide (nt) U-rich "U-tract," play principal roles of punctuating and regulating transcription in most bacteria. However, canonical intrinsic terminators with strong U-tracts are underrepresented in some bacterial lineages, notably mycobacteria, leading to proposals that their RNA polymerases stop at noncanonical intrinsic terminators encoding various RNA structures lacking U-tracts. We generated recombinant forms of mycobacterial RNA polymerase and its major elongation factors NusA and NusG to characterize mycobacterial intrinsic termination. Using *in vitro* transcription assays devoid of possible mycobacterial contaminants, we established that mycobacterial RNA polymerase terminates more efficiently than *Escherichia coli* RNA polymerase at canonical terminators with imperfect U-tracts but does not terminate at putative terminators lacking U-tracts even in the presence of mycobacterial NusA and NusG. However, mycobacterial NusG exhibits a novel termination-stimulating activity that may allow intrinsic terminators with suboptimal U-tracts to function efficiently.**

IMPORTANCE **Bacteria rely on transcription termination to define and regulate units of gene expression. In most bacteria, precise termination and much regulation by attenuation are accomplished by intrinsic terminators that encode GC-rich hairpins and U-tracts necessary to disrupt stable transcription elongation complexes. Thus, the apparent dearth of canonical intrinsic terminators with recognizable U-tracts in mycobacteria is of significant interest both because noncanonical intrinsic terminators could reveal novel routes to destabilize transcription complexes and because accurate understanding of termination is crucial for strategies to combat mycobacterial diseases and for computational bioinformatics generally. Our finding that mycobacterial RNA polymerase requires U-tracts for intrinsic termination, which can be aided by NusG, will guide future study of mycobacterial transcription and aid improvement of predictive algorithms to annotate bacterial genome sequences.**

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Regulated expression of genes in compact bacterial genomes requires efficient and accurate initiation and termination of RNA synthesis by RNA polymerase (RNAP). Two types of mechanisms terminate transcription in bacteria: intrinsic (or Rho-independent) termination and factor-dependent (typically, Rho-dependent) termination (1, 2). Intrinsic termination is caused by RNA and DNA sequences and structures that directly destabilize elongation complexes (ECs). At intrinsic terminators, a weak RNA/DNA hybrid generated by a 7-to-9-nucleotide (nt) "U-tract" in the RNA causes an initial transcriptional pause, after which a stable stem-loop structure called the terminator hairpin forms in the nascent RNA directly adjacent to the U-tract (3, 4). Complete formation of the terminator hairpin dissociates the EC by pulling the RNA out of the EC accompanied either by hybrid shearing, if the U-tract is perfect or near perfect, or by forward translocation of the EC without new RNA synthesis when the 3′ portion of the hybrid contains one or more GC base pairs (2, 5, 6), although alternative models have been suggested (7).

These U-tract-dependent mechanisms of intrinsic termination fit nicely with the known structure and stability determinants of bacterial ECs. An RNA/DNA hybrid of at least 7 bp, and normally alternating between 9 and 10 bp in post- and pretranslocated ECs, respectively, is required for EC stability (8, 9). Full formation of a terminator hairpin appears to shorten the hybrid to 6 bp or less by melting the 5′ portion of the hybrid, which typically consists of all rU-dA base pairs (2, 5, 10). This same basic termination paradigm of RNA/DNA hybrid shortening also appears to underlie Rho-dependent termination, in which the hexameric ATP-dependent, RNA translocase Rho extracts RNA from RNAP (2, 11), and Mfd-dependent termination, in which the ATP-dependent DNA helicase Mfd drives forward translocation without RNA synthesis (12).

Intrinsic terminators can be predicted based on the presence of adjacent stem-loop and U-tract-encoding sequences and are widespread in bacteria (13–17). Although Rho protein is found in most bacteria, it is essential only in some species (e.g., Rho is

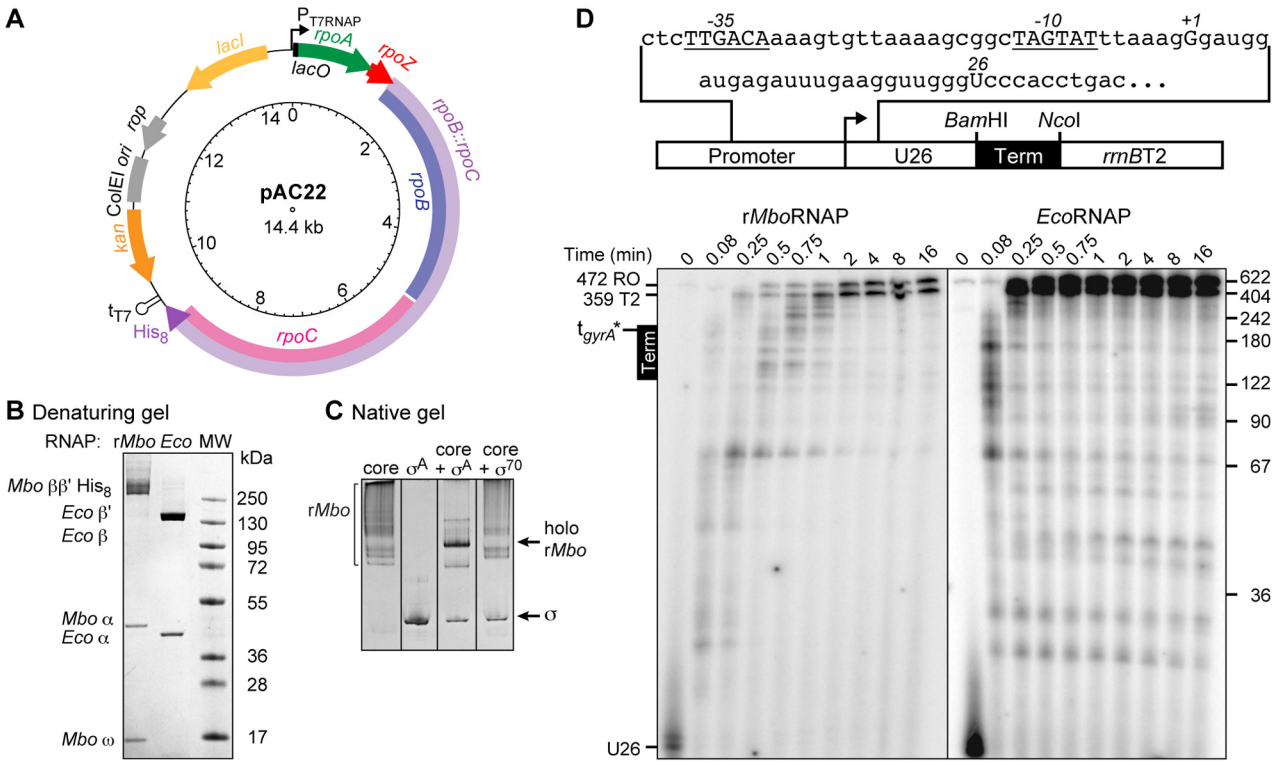


FIG 1 Overexpression and purification of r*Mbo*RNAP. (A) pAC22, containing *M. bovis rpoA*, *rpoZ*, *rpoB*, and *rpoC* genes (encoding the α, ω, β, and β′ subunits of r*Mbo*RNAP, respectively). β and β′ were fused with a 10-amino-acid (aa) linker (LARHVGGSGA) and a C-terminal His_8 tag. (B) Purified r*Mbo*RNAP and *Eco*RNAP analyzed by SDS-PAGE. Smearing of the β-β′ fusion polypeptide is likely an electrophoretic artifact. (C) Formation of r*Mbo*RNAP holoenzyme (holo) analyzed by nondenaturing PAGE. r*Mbo*RNAP core was incubated with *M. bovis* σ^{70} or *E. coli* σ^{70} at 37°C for 15 min, electrophoresed on a 4% to 15% gradient of native polyacrylamide gel, and stained with Imperial protein stain (Pierce). Core RNAP migrates as a collection of aggregates, as observed previously for *Eco*RNAP (55, 56). Incubation with *M. bovis* σ^{A}, but not with *E. coli* σ^{70}, converts most r*Mbo*RNAP core to a holoenzyme that migrates as a discrete, monomeric species. (D) Transcript elongation and lack of noncanonical intrinsic termination by r*Mbo*RNAP. Transcript elongation was assayed at successive times after addition of 100 μM concentrations of all 4 NTPs to halted U26 ECs on a linear DNA template from pAC74 containing *M. smegmatis* t_{gyrA}* (19) (Materials and Methods). Termination was evident at the *E. coli rrnB*T2 terminator (Term) near the end of the template but not at the proposed position of noncanonical termination in t_{gyrA}*. Position of runoff transcript is indicated (RO).

required for growth of *Escherichia coli* but not for growth of *Bacillus subtilis*) and use of intrinsic versus Rho-independent terminators is thought to vary among bacterial lineages. Intrinsic terminator-like sequences are largely absent in some bacterial genomes (14, 16–18). This paucity has led to suggestions that alternative intrinsic termination paradigms may operate at sequences lacking U-tracts (16, 19–21). Mechanistic studies (primarily using *E. coli* RNAP [*Eco*RNAP]) suggest that ≥3 Us directly adjacent to the terminator hairpin are necessary for intrinsic termination, with a longer U-tract required for efficient dissociation (22, 23). However, some cases of termination by purified RNAPs at noncanonical terminators lacking U-tracts have been reported, notably for mycobacterial RNAP (19, 24). It is unclear how termination at sites lacking a U-tract can be reconciled with the current mechanistic understanding of intrinsic termination.

Given the importance of understanding mycobacterial mechanisms of transcription termination to human health and the development of antimycobacterial drugs as well as to fundamental knowledge of bacterial RNAP structure and function, we sought to test whether purified mycobacterial RNAP terminates transcription at noncanonical terminators lacking U-tract sequences. A possible explanation for reports of termination at noncanonical sequences would be the presence of either contaminating nucleases that cleave RNAs produced *in vitro* or contaminating transcription factors that direct factor-dependent termination. Both Rho and Mfd are present in mycobacteria (25, 26), and the possible involvement of as-yet-uncharacterized factors cannot be excluded. Thus, to ensure that small amounts of mycobacterial transcription factors or nucleases did not contaminate our experiments, we devised a method to produce recombinant mycobacterial RNAP in *E. coli*. We then examined termination by the purified mycobacterial RNAP at both canonical and reported noncanonical terminator sequences alone and in the presence of recombinant mycobacterial NusA and NusG, the two universal bacterial elongation regulators.

RESULTS

Active, recombinant mycobacterial RNAP purified after overexpression in *E. coli*. To generate recombinant mycobacterial RNAP, we first assembled the overexpression plasmid pAC22 (Fig. 1A) in which the *rpoA*, *rpoB*, *rpoC*, and *rpoZ* genes from *Mycobacterium bovis* BCG were transcribed by phage T7 RNAP, a strategy that was previously successful with *Thermus aquaticus* RNAP (*Taq*RNAP), *Thermus thermophilus* RNAP (*Tth*RNAP),

and *Eco*RNAP (27, 28). Co-renaturation of individually purified subunits is also a viable route to recombinant RNAPs, including mycobacterial RNAP (29), but we used *in vivo* RNAP assembly to lessen the possibility of subunit or domain misfolding. We chose *M. bovis* RNAP (*Mbo*RNAP) because DNA encoding it was readily accessible and because its sequence is virtually identical to that of *M. tuberculosis* RNAP (*Mtu*RNAP). *Mbo*RNAP σ^A-holoenzyme contains a single β' R69P substitution relative to *Mtu*RNAP σ^A-holoenzyme (GenBank accession no. NC_008769.1 for *M. bovis* BCG strain Pasteur 1173P2 used to construct pAC22 and NC_000962.3 for *M. tuberculosis* strain H37Rv). To minimize the possibility that *E. coli* β' or β subunits would intermingle with *M. bovis* subunits in the purified *Mbo*RNAP, we fused the C terminus of *M. bovis rpoB* to the N terminus of *M. bovis rpoC* using a short linker. Similar fusions occur naturally in *Helicobacter* RNAP, can support growth of *E. coli*, and do not alter the transcriptional properties of *Eco*RNAP (30). To facilitate purification, we included a His_8 tag at the C terminus of *M. bovis rpoC*.

Recombinant *M. bovis* RNAP (r*Mbo*RNAP) was overexpressed by induction of T7 RNAP in *E. coli* BL21 λDE3 cells and then purified using Ni^{2+}-affinity chromatography and gel filtration to yield a preparation that was largely devoid of contaminating bands by SDS-PAGE (Fig. 1B). In initial tests, r*Mbo*RNAP failed to initiate transcription at a consensus *E. coli* σ^{70} promoter when incubated with *E. coli* σ^{70} (data not shown). Therefore, we overexpressed and purified *M. bovis* σ^A. When incubated with *M. bovis* σ^A or *E. coli* σ^{70}, r*Mbo*RNAP bound only *M. bovis* σ^A as judged by a nondenaturing electrophoretic gel mobility assay (Fig. 1C). r*Mbo*RNAP·σ^A initiated transcription at a consensus *E. coli* σ^{70} promoter, formed halted U26 ECs when CTP was withheld, elongated the nascent RNA when CTP was added (albeit more slowly than EcoRNAP, consistent with prior reports [see reference 31]), and gave patterns of pausing and termination that enabled *in vitro* study (Fig. 1D).

Mycobacterial RNAP undergoes intrinsic termination only at canonical sequences. Like *Eco*RNAP, r*Mbo*RNAP terminated transcription at a canonical intrinsic terminator present on our test DNA template (*E. coli rrnB* T2, ~50% efficiency; Fig. 1D). However, neither *Eco*RNAP nor r*Mbo*RNAP terminated at the *M. smegmatis gyrA** sequence on this template, which was previously reported to function as a noncanonical intrinsic terminator with ~85% efficiency (19). To investigate this discrepancy further, we compared the behavior of r*Mbo*RNAP to that of *Eco*RNAP using a set of known *E. coli* intrinsic terminators and predicted mycobacterial intrinsic terminators placed between the BamHI and NcoI sites of our *in vitro* DNA template (Fig. 1D; see also Fig. S1 in the supplemental material). Because the *M. smegmatis* t_{gyrA^*} sequence used previously (19) differed from the t_{gyrA} sequence in GenBank (CP000480.1), we tested terminators corresponding to both (t_{gyrA^*} [see reference 19] and t_{gyrA} [GenBank sequence]). Both r*Mbo*RNAP and *Eco*RNAP produced transcripts ending at the expected locations of known *E. coli* intrinsic terminators ($t_{Eco\text{-}rpoC}$, t_{trpA}, and t_{rrnBT1}), two synthetic terminators that we designed for use in mycobacterial expression vectors (t_{synA} and t_{synB} [see reference 32]), and the reported mycobacterial terminators t_{tuf}, t_{Rv1324}, and $t_{Rv1344c}$ (Fig. 2). However, neither RNAP produced a truncated product corresponding to the putative mycobacterial terminators t_{asnB}, t_{fadD23}, t_{gyrA^*}, and t_{gyrA}. r*Mbo*RNAP but not *Eco*RNAP appeared to terminate at $t_{Mtu\text{-}rpoC}$, although only at 14% efficiency. In general, r*Mbo*RNAP exhibited stronger apparent termination than *Eco*RNAP (Fig. 2B), as would be expected from its lower elongation rate.

We were particularly interested in the apparent termination for t_{tuf}, t_{Rv1324}, and $t_{Mtu\text{-}rpoC}$ because these sequences lack obvious U-tracts (19, 20). To investigate these cases further, we mapped the approximate 3′ ends of the apparent termination products by comparison to sequence ladders generated with 3′ deoxynucleoside triphosphates (deoxyNTPs) (see Fig. S2 in the supplemental material). Although electrophoretic compression precluded assigning precise 3′ ends, all three truncated RNAs unambiguously ended upstream from the previously predicted locations and within plausible U-tract sequences preceded by stem-loop structures (Fig. 2A; see also Fig. S2). Thus, mycobacterial t_{tuf}, t_{Rv1324}, and $t_{Mtu\text{-}rpoC}$ contain the same hairpin and U-tract elements as canonical terminators. Further, both t_{tuf} and t_{Rv1324} can also terminate *Eco*RNAP weakly, consistent with current models for intrinsic termination.

Termination events release RNA from *Mbo*RNAP. Termination assays that rely only on formation of truncated RNA products as evidence of termination are unable to distinguish true termination, involving dissociation of the EC, from long transcriptional pauses or arrest events that halt RNAP on the DNA template without EC dissociation. To test whether the mycobacterial terminators are capable of dissociating ECs, we immobilized r*Mbo*RNAP ECs on N^{2+}-nitrilotriacetic acid (NTA) beads via the His_8 tag at the C terminus of β'. For all 4 mycobacterial terminators at which we detected truncated products (t_{tuf}, t_{Rv1324}, $t_{Rv1344c}$, and $t_{Mtu\text{-}rpoC}$) and for the control *E. coli* intrinsic terminators ($t_{Eco\text{-}rpoC}$, t_{trpA}, and t_{rrnBT1}), essentially all the truncated products as well as the runoff and t_{rrnBT2}-terminated RNAs were found in the supernatant fraction when the beads were removed from the completed reactions (Fig. 2C). To ensure that the assay faithfully distinguished terminated RNAs from halted ECs, we performed control experiments in which r*Mbo*RNAP ECs were halted by a bound, noncleaving EcoRI protein on the templates that also bore the t_{tuf} and $t_{Mtu\text{-}rpoC}$ sequences (see Fig. S3 in the supplemental material). Halted ECs were retained on the beads, whereas the terminated RNAs were released, verifying that the mycobacterial terminators were true intrinsic terminators for r*Mbo*RNAP.

r*Mbo*RNAP tolerates fewer perfect U-tracts than *Eco*RNAP. The U-tracts of some functional mycobacterial terminators exhibited greater GC content than is typical for *E. coli* intrinsic terminators (e.g., t_{asnB}; Fig. 2). To investigate mycobacterial RNAP U-tract requirements, we constructed hybrid t_{asnB}-t_{rrnBT1} terminators (t_{rrnBT1} is an efficient *E. coli* terminator with a relatively U-rich U-tract and downstream sequence [Fig. 3A]). Even when the hairpin-proximal portion of the U-tract was converted to all Us ($t_{asnB\text{-}3}$) with the t_{asnB} downstream sequence, neither r*Mbo*RNAP nor *Eco*RNAP could terminate (Fig. 3B and C). However, when the AT-rich t_{rrnBT1} was combined with an improved U-tract ($t_{asnB\text{-}4}$) or the complete t_{rrnBT1} U-tract was present ($t_{asnB\text{-}5}$), r*Mbo*RNAP terminated with 40% to 55% efficiency (Fig. 3C). Thus, as with *Eco*RNAP (3, 6), AT-rich downstream sequences appeared to aid termination by mycobacterial RNAP on GC-containing U-tracts. In contrast, *Eco*RNAP terminated weakly or not at all on these hybrid templates (Fig. 3B), consistent with the greater termination susceptibility of r*Mbo*RNAP. To verify that r*Mbo*RNAP terminated in the U-tracts of $t_{asnB\text{-}4}$ and $t_{asnB\text{-}5}$, we mapped the approximate 3′ ends of the terminated RNAs. Compressions in the sequencing gels prevented precise assign-

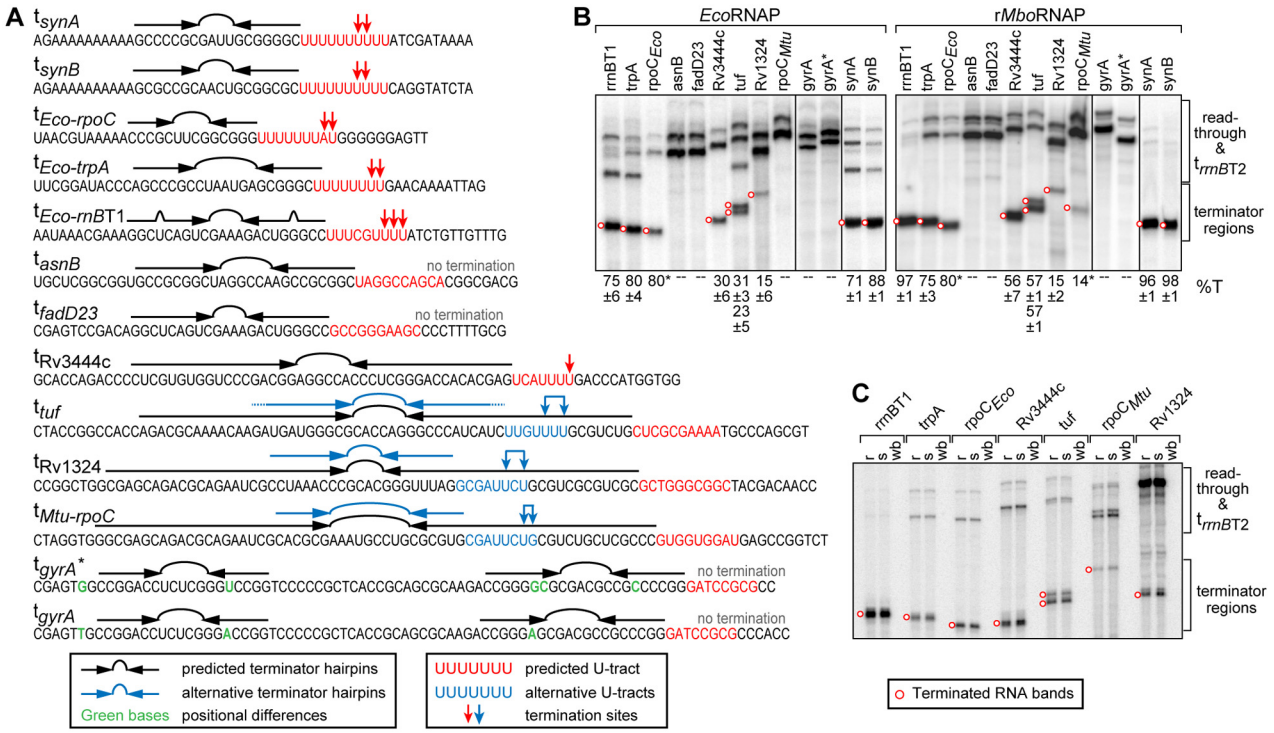
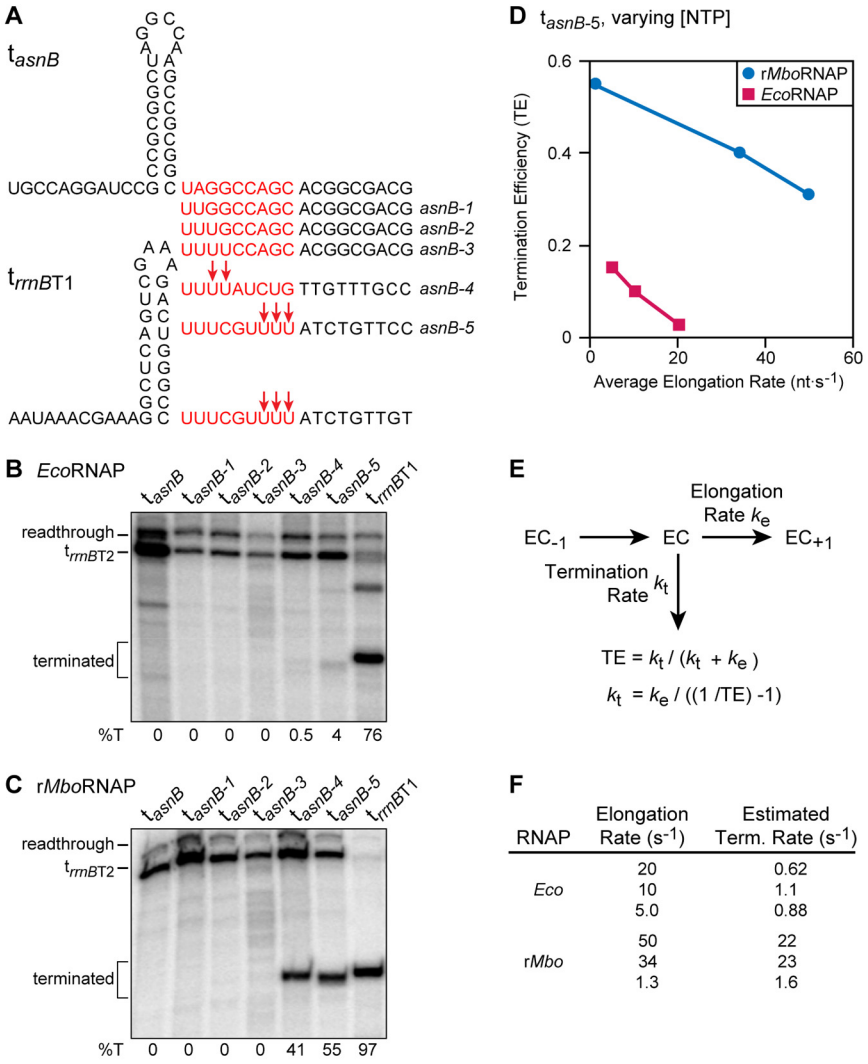


FIG 2 Termination by *Eco*RNAP and r*Mbo*RNAP at known and predicted intrinsic terminator sequences. (A) Terminator sequences are shown as RNA with horizontal arrows and half-ovals to indicate RNA stem-loop structures and vertical arrows to indicate points of termination. Alternative stem-loops and termination points mapped in this study are blue. The 8-to-10-nt segment immediately 3′ of the stem-loop, corresponding to the intrinsic terminator U-tract, is red (for previously proposed terminator hairpins) or blue (for alternative terminator hairpins). Sequence 3′ of termination points is shown as DNA. The *M. tuberculosis* t_{tuf} and t_{Rv1324} and *M. smegmatis* t_{gyrA}* sequences are reported to terminate transcription by *M. smegmatis* RNAP despite lacking canonical U-tracts (19). t_{gyrA} from GenBank accession no. CP000480.1 differs in sequence at positions colored green. *M. tuberculosis* $t_{Mtu\text{-}rpoC}$ and $t_{Rv3444c}$ and *M. bovis* t_{asnB} and t_{fadD23} are predicted to be noncanonical mycobacterial terminators (20). t_{synA} and t_{synB} are synthetic terminators designed for use in mycobacterial expression vectors (32). (B) *Eco*RNAP (left panel) and r*Mbo*RNAP (right panel) were used to transcribe DNA templates containing the indicated terminators or predicted terminators ligated between the BamHI and NcoI sites of the template depicted in Fig. 1D (see the sequence in Fig. S1 in the supplemental material). Transcription of 25 nM linear DNA by 50 nM RNAP was conducted at 37°C, and the products were separated on a 6% denaturing polyacrylamide gel (see Materials and Methods). Terminated products are marked with a red circle. Termination efficiencies with standard deviations (s.d.) from ≥3 experimental replicates are shown below the gel lanes (*, single determination with no error determination). (C) Detection of released RNA products in r*Mbo*RNAP reactions. In cases in which termination was detected, transcription reaction mixtures with RNAP immobilized on Ni^{2+}-NTA agarose beads were separated into supernatant (s) and washed-bead (wb) fractions for comparison to total reaction products (r) (Materials and Methods). Appearance of all RNAs in the supernatant fraction indicates transcript release, which was verified using halted ECs as controls (see Fig. S3 in the supplemental material).

ments, but both $t_{asnB\text{-}4}$ and $t_{asnB\text{-}5}$ terminated RNAP within the U-tract as expected (see Fig. S4 in the supplemental material). $t_{asnB\text{-}4}$, which terminated *Eco*RNAP weakly if at all, appeared to halt r*Mbo*RNAP earlier in the U-tract than a typical intrinsic terminator. We conclude that mycobacterial RNAP requires less U-rich U-tracts than *Eco*RNAP and may terminate at noncanonical locations within the U-tract, although the precise 3′-end assignments are uncertain. Regardless, our results strongly indicate that the mechanistic pathways for intrinsic termination by mycobacterial RNAP are similar to those of *E. coli* RNAP. Mycobacterial RNAP can use weaker U-tract terminators more efficiently, and the effect of AT-rich downstream DNA supports dissociation via hairpin-driven forward translocation uncoupled from nucleotide addition (2, 5, 6).

r*Mbo*RNAP exhibited a greater termination proclivity than *Eco*RNAP. The reduced requirement of mycobacterial RNAP for Us in an intrinsic terminator U-tract as well as its apparently greater termination efficiency might be explained simply by an elongation rate that is lower than that seen with *Eco*RNAP. The U-tract plays a dual function in termination; it triggers transiently pausing RNAP at the prospective termination site and it destabilizes the EC to allow dissociation by the terminator hairpin (4). Termination can be considered a simple competition between the rate of pause escape and the rate of EC dissociation (or an irreversible commitment step preceding dissociation; see references 5, 33, and 34). To investigate whether a reduced rate of elongation (or pause escape) alone could explain the increased termination proclivity of r*Mbo*RNAP, we examined the termination efficiency of $t_{asnB\text{-}5}$ at various NTP concentrations with limiting UTP to generate different rates of elongation though the U-tract. Termination efficiency was consistently much higher for r*Mbo*RNAP than for *Eco*RNAP, even when the elongation rate of *Eco*RNAP was reduced to less than that of *Mbo*RNAP by using low NTP concentrations (Fig. 3D; see also Fig. S5 in the supplemental material). The rate of EC dissociation (or commitment) necessary to explain termination efficiencies over a range of elongation rates was relatively constant for *Eco*RNAP ($k_t = 0.9 \pm 0.2\ s^{-1}$; Fig. 3D and E). The higher elongation rates of r*Mbo*RNAP also gave similar pre-



RNAP	Elongation Rate (s^{-1})	Estimated Term. Rate (s^{-1})
Eco	20	0.62
	10	1.1
	5.0	0.88
r*Mbo*	50	22
	34	23
	1.3	1.6

FIG 3 r*Mbo*RNAP exhibits a greater termination proclivity than *Eco*RNAP at suboptimal U-tracts. (A) RNA structures and sequences of derivatives of the predicted *M. bovis* t_{asnB} terminator containing increasing numbers of U residues in the U-tract segment. The positions of termination are indicated by vertical arrows either as previously reported for t_{rrnBT1} (3) or as estimated by comparison on high-resolution denaturing polyacrylamide gels of terminated transcripts to RNA sequence ladders generated using 3′ deoxyNTPs (see Fig. S4 in the supplemental material and Materials and Methods). (B and C) Termination by *Eco*RNAP and termination by r*Mbo*RNAP were compared using the assay shown in Fig. 2. Data represent *Eco*RNAP (B) and r*Mbo*RNAP (C) transcription products of templates shown in panel A. Terminated products are marked with a red circle. (D) Termination efficiency (TE) for t_{asnB-5} was determined at various NTP concentrations to produce different rates of average transcript elongation (gel images for the six data points shown here are in Fig. S5 in the supplemental material). TE is plotted as a function of the average elongation rate for *Eco*RNAP and r*Mbo*RNAP. (E) Calculation of apparent termination rate (EC dissociation rate or rate at which ECs commit to the termination pathway) from average elongation rate and TE. (F) Calculated termination (Term.) rates. Except at very low NTP and elongation rates, r*Mbo*RNAP exhibited a significantly higher termination rate.

dictions for an EC dissociation rate of 22 ± 1 s^{-1}. However, the lowest elongation rate for r*Mbo*RNAP yielded a markedly lower predicted dissociation rate of 1.6 s^{-1}. It is possible that the reduced dissociation rate of r*Mbo*RNAP at a low elongation rate reflected backtracking in the U-tract that would inhibit terminator hairpin formation. Although this caveat, as well as the uncertainty of deducing U-tract elongation rates using overall elongation rates, makes our conclusions preliminary, all the predicted dissociation rates for r*Mbo*RNAP were higher than for *Eco*RNAP. Thus, our results suggest that r*Mbo*RNAP not only increases the kinetic window for termination by elongating more slowly through the U-tract but also dissociates faster than *Eco*RNAP. Together, these two differences may explain why r*Mbo*RNAP terminates efficiently at imperfect U-tracts.

***Mbo*NusG stimulates termination at imperfect terminators, but neither NusA nor NusG allows use of putative terminators lacking U-tracts.** Although r*Mbo*RNAP did not terminate at noncanonical intrinsic terminators *in vitro*, it is possible that the elongation regulators NusA and NusG might alter termination mechanisms in mycobacteria and account for the previous reports of intrinsic termination at noncanonical sequences. To test these possibilities, we overexpressed and purified recombinant *M. bovis* NusA and NusG (Materials and Methods) and compared their effects on termination to the effects seen with the previously char-

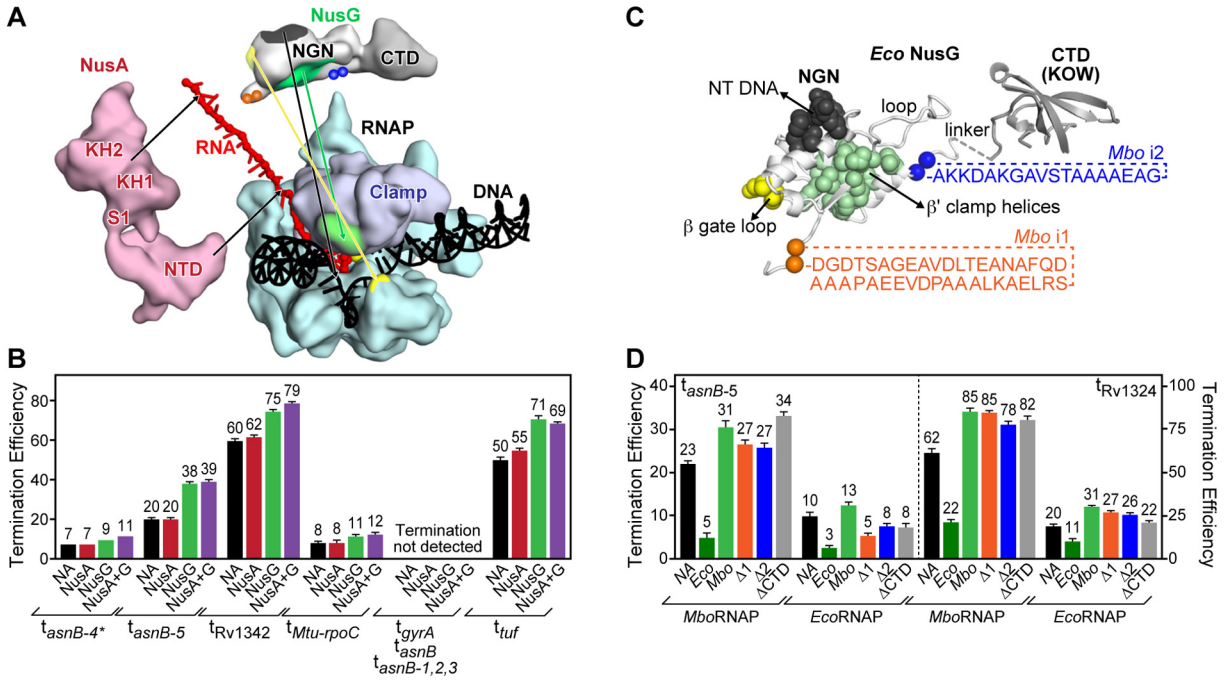


FIG 4 NusA and NusG effects on termination by r*Mbo*RNAP. (A) Structures of *Mtu*NusA (pdb 1k0r; see reference 36), TthRNAP EC (adapted from pdb 2o5i; see reference 9), and *Eco*NusG (pdb 2k06 and 2jvv; see reference 41). Key domains are labeled, and known interactions are indicated with arrows and, for NusG, color coded on the surface of NusG and the EC (clamp helices, green; β gate loop, yellow; nontemplate DNA strand, black). (B) Effects of mycobacterial NusA and NusG on termination by r*Mbo*RNAP at a set of possible terminators. NA, no addition. NusG, but not NusA, consistently enhanced TE, but neither NusA nor NusG allowed termination at possible noncanonical terminator sequences. A representative gel of the termination assay for t_{Rv1342} is shown in Fig. S6 in the supplemental material. (C) Structure of *Eco*NusG colored as described for panel A with the backbone shown as a ribbon, key residues shown as spheres, and insertions present in mycobacteria shown as orange (*Mbo*i1) or blue (*Mbo*i2) sequences. (D) Effect of *Eco*NusG, mycobacterial NusG, and deletion proteins derived from mycobacterial NusG on TE at t_{asnB-5} and t_{Rv1324}. NA, no addition.

acterized *E. coli* NusA and NusG proteins. Mycobacterial NusA resembles *Eco*NusA, which is known to stimulate termination at intrinsic terminators through interactions of its N-terminal domain (NTD) with the nascent RNA and RNA exit channel (35) (although mycobacterial NusA lacks the C-terminal AR domains of *Eco*NusA involved in λN antitermination). An *Mtu*NusA crystal structure (Fig. 4A) reveals interaction of the KH1-KH2 domains with a "boxA" nascent RNA sequence that may be involved in *rrn* antitermination (36, 37). *Eco*NusG typically suppresses pausing with little effect on termination through contacts with the RNAP clamp domain and "gate loop" (Fig. 4A), although *B. subtilis* NusG (*Bsu*NusG) and *Tth*NusG can stimulate rather than suppress pausing (38, 39).

Consistent with earlier studies (35), we observed that *Eco*NusA enhanced termination by *Eco*RNAP at t_{Rv1324} (data not shown). We observed a similar effect of EcoNusA on r*Mbo*RNAP, suggesting that *Eco*NusA is capable of similar interactions with both *Eco*RNAP and r*Mbo*RNAP (see Fig. S6A in the supplemental material). However, *Mbo*NusA failed to alter termination by either *Eco*RNAP or r*Mbo*RNAP significantly. We confirmed the lack of *Mbo*NusA effect on r*Mbo*RNAP using multiple terminators (Fig. 4B). To investigate whether *Mbo*NusA interacted with r*Mbo*RNAP despite its lack of effect, we reasoned that bound *Mbo*NusA might block the effect of *Eco*NusA and therefore tested mixtures of *Mbo*NusA with *Eco*NusA. *Mbo*NusA eliminated *Eco*NusA enhancement of termination by r*Mbo*RNAP (Fig. S6B). We conclude that mycobacterial NusA does not allow mycobacterial RNAP to terminate at noncanonical intrinsic terminators and, in contrast to *Eco*NusA, does not significantly enhance termination at canonical intrinsic terminators.

However, *Mbo*NusG did enhance termination at t_{Rv1324} and at several other intrinsic terminators that we tested, either alone or in combination with *Mbo*NusA (Fig. 4B; see also Fig. S6A in the supplemental material). This enhancement of termination contrasted with the effect of *Eco*NusG, which suppressed termination by *Eco*RNAP to a limited extent and by r*Mbo*RNAP more dramatically (Fig. 4B; see also Fig. S6A). Although pause enhancement by NusG has been observed previously (38, 39), the strong enhancement of intrinsic termination by *Mbo*NusG appears to be unprecedented. Importantly, *Mbo*NusG stimulated termination by both r*Mbo*RNAP and *Eco*RNAP at terminators with suboptimal U-tracts but did not cause observable termination at putative noncanonical terminators lacking U-tracts (e.g., t_{gyrA}, t_{asnB}, or $t_{asnB1-3}$; Fig. 4B).

Termination enhancement by *Mbo*NusG is not caused by lineage-specific insertions or the NusG CTD. The conserved core of NusG (and Spt5 in archaea and eukaryotes) is a two-domain protein whose NusG N-terminal (NGN) domain interacts with RNAP and the DNA at the upstream edge of the transcription bubble and whose C-terminal KOW (Kyprides, Ouzounis, Woese) motif-containing domain interacts with other transcription factors (Rho or ribosomal protein S10, NusE, in *E. coli*; see references 40 and 41). The NGN domain of *Mbo*NusG diverges from *Eco*NusG by an N-terminal extension (*Mbo*i1) and a larger inter-

nal loop (*Mbo*i2; Fig. 4C). To test whether one of these features explained the termination enhancement by *Mbo*NusG, we prepared proteins lacking the C-terminal domain (CTD) or lacking *Mbo*i1or *Mbo*i2. The NGN domain of *Mbo*NusG alone or NGN derivatives lacking *Mbo*i1 or *Mbo*i2 enhanced termination similarly to full-length *Mbo*NusG (Fig. 4D). Thus, termination enhancement appears to be a property of the core structure of the *Mbo*NusG NGN domain.

Despite their strong effect on intrinsic terminators containing suboptimal U-tracts, neither *Mbo*NusG nor *Mbo*NusG fragments nor a combination of *Mbo*NusG and *Mbo*NusA allowed r*Mbo*RNAP to terminate transcription at the noncanonical t_{asnB}, t_{fadD23}, t_{gyrA^*}, and t_{gyrA} sequences. We conclude that r*Mbo*RNAP is incapable of intrinsic termination at sequences that lack canonical stem-loop and U-tract features even in the presence of the universal elongation regulators NusA and NusG, even though *Mbo*NusG possesses novel termination-stimulating activity at canonical intrinsic terminators.

DISCUSSION

Although the sequences at intrinsic terminators that govern pathways of transcription complex dissociation remain under study, nearly universal agreement exists about the need for two conserved features: a stem-loop, terminator hairpin structure and a U-tract that abuts the terminator hairpin. Nonetheless, these terminator features occur significantly less frequently in some bacterial genomes, including mycobacterial genomes (14, 16–18), making reports that mycobacterial RNAP is susceptible to an alternative intrinsic termination mechanism of considerable interest (16, 18, 19). Alternative mechanisms are possible, at least for the single-subunit, phage T7 RNAP (42), but have not been rigorously tested for multisubunit RNAPs. The results of our study of intrinsic termination by recombinant mycobacterial RNAP suggest that the mycobacterial enzyme can use terminators with weak U-tracts that have little effect on *E. coli* RNAP and that mycobacterial NusG assists intrinsic termination. However, even in the presence of the conserved elongation regulators NusA and NusG, intrinsic termination by mycobacterial RNAP at sites lacking a recognizable U-tract was not observed. Thus, our results suggest that these previously described structures either do not trigger intrinsic termination or encode functional intrinsic terminators for which the cognate U-tract was overlooked (Fig. 2).

Two important points follow from these findings. First, the highly conserved structure of multisubunit, bacterial RNAPs appears to recognize a single class of intrinsic termination signal, albeit one that tolerates variation in sequence, efficiency of termination, and pathway of EC dissociation. The intrinsic termination signal reflects the energetics of the EC's exceptional stability; reversing this stability requires both weakening the RNA/DNA hybrid with the U-tract sequence and disrupting both RNA-DNA and nucleic acid-protein interactions with the energy derived from formation of the terminator hairpin.

Second, the presence of sequences encoding RNA structures at the ends of genes and operons does not necessarily mean that these sequences terminate transcription. Inverted repeats play many roles in gene regulation and nucleic acid metabolism. Most obviously, RNA structures inhibit RNA degradation (43, 44). Thus, some 3′-untranslated-region (3′-UTR) structures in mycobacteria may exist to stabilize RNAs or to define RNA 3′ ends formed by 3′ → 5′ exonucleolytic processing of RNAs generated by factor-dependent termination sites further downstream. For example, the combined actions of downstream Rho-dependent termination and RNase trimming form the mature ends of the *E. coli trp* operon mRNA (45) and of *E. coli* $tRNA^{Leu}$ (46). Further, intragenic elements called repetitive extragenic palindromic (REP) elements in *E. coli* encode RNA structures that are not dissimilar from some of the RNA structures predicted for mycobacteria (18). REP elements may stabilize mRNAs against exonucleolytic degradation (47, 48). In *Rhodobacter*, Rho is actually a component of the degradosome (49), making models for the combined action of Rho-dependent termination and exonucleolytic processing even more plausible. Finally, intragenic palindromes that bear some resemblance to sequences encoding terminator hairpins have also been proposed to function as DNA uptake elements in some bacteria (13). Thus, several functions other than intrinsic termination could explain sequences encoding RNA structures near the ends of mycobacterial genes and operons.

We found that r*Mbo*RNAP terminated more efficiently than *E. coli* RNAP at all intrinsic terminators tested and could terminate at sequences that did not support termination by *E. coli* RNAP provided that at least a suboptimal U-tract was present (e.g., $t_{Mtu\text{-}rpoC}$; Fig. 2). Further, the increased efficiency of r*Mbo*RNAP was not readily explained simply because it elongates more slowly but appeared to reflect a reduced stability of ECs that gave rise to more-rapid dissociation (Fig. 3D to F). The greater termination of mycobacterial RNAP at suboptimal U-tract terminators may reflect greater reliance on EC dissociation via hairpin-driven forward translocation without nucleotide addition (6). Unlike the hybrid-shearing pathway that predominates for perfect or near-perfect U-tracts, forward translocation becomes favored when non-rU-dA base pairs in the U-tract disfavor shearing (2, 5). It is possible that mycobacterial RNAP has evolved to uncouple forward translocation from nucleotide addition more readily and thereby lessen the requirement for near-perfect U-tract terminators. It is also possible that Rho-dependent termination plays a much more significant role in mycobacterial transcription, making intrinsic termination less important. Although the essentiality of Rho in mycobacteria has not been determined, mycobacterial Rho exhibits a robust release activity *in vitro* (25).

Mycobacterial termination also appears to differ from the well-studied *E. coli* model system in the stimulatory effect of mycobacterial NusG. In *E. coli*, NusA stimulates termination through interactions with the RNA exit channel and terminator hairpin, whereas NusG suppresses pausing and has little effect on termination. In mycobacteria, NusA has little effect on intrinsic termination even though it can displace *E. coli* NusA from RNAP, and NusG significantly stimulates intrinsic termination. Mycobacterial NusA binds a *rrn* antitermination signal (37); thus, it is possible that mycobacterial NusA has become a more specialized regulator relative to diverse roles of *E. coli* NusA.

The effect of mycobacterial NusG on intrinsic termination has implications both for the mechanism of NusG action in diverse bacteria and for the use of intrinsic terminators with imperfect U-tracts in mycobacteria. The termination-stimulating effect of mycobacterial NusG requires only the N-terminal domain of NusG (Fig. 4D) and does not involve the two lineage-specific insertions in NusG (Fig. 4). Although untested, it seems probable that the pause-enhancing effects of *Bsu*NusG and *Tth*NusG (38, 39) would be similarly mediated by the core NGN sequences in the NTD. The NusG NTD is thought to contact both sides of the main

cleft of RNAP, thereby both promoting transcript elongation and inhibiting nascent RNA hairpin formation by inhibiting opening of the RNAP clamp domain (50, 51). To create an apparently opposite effect of stimulating termination, mycobacterial NusG presumably uses a different set of contacts to exert effects on the EC. Although further work will be needed to determine these contacts and effects, among the most attractive possibilities are putative contacts to the nontemplate DNA strand (39) and the apparent ability of NusG to promote forward translocation (52). In particular, promotion of forward translocation might stimulate termination at imperfect U-tract terminators, where the hypertranslocation pathway of intrinsic termination may predominate.

It is not surprising that elongation regulators in bacteria with lifestyles markedly different from those of model organisms studied to date modulate transcription in different ways. Regulatory mechanisms are known to be subject to much greater evolutionary plasticity than other cellular functions (53, 54). However, this plasticity means that details of transcription-regulatory mechanisms must be determined by careful biochemical studies of diverse bacterial lineages, even when the broad outlines of mechanisms such as intrinsic termination remain constant. The huge growth in microbiome-based bioinformatics makes the need for such studies acute.

MATERIALS AND METHODS

Bacterial strains, plasmids, and proteins. *E. coli* strains DH10b and BL21 λDE3 pRARE2 (see Table S1 in the supplemental material) were used for DNA manipulations and protein overexpression, respectively. Plasmid construction and protein purification are described in the supplemental material.

DNA templates for *in vitro* transcription assays. Linear transcription templates for *in vitro* reactions were prepared by PCR on plasmids containing a consensus σ^{70} promoter fused to transcription terminators (Fig. 1 and 2; see also Fig. S1 and Table S1 in the supplemental material) using the following primers: 5′-GAAGCATTTATCAGGGTTATTG TCTC (no. 6372) and 5′-CAGGGTTTTAAGGAGTGGTTCATAG (no. 6273). The resulting 686-to-722-bp DNAs were purified after separation in low-melting agarose gels using QIAquick gel extraction reagents (Qiagen) followed by phenol extraction, ethanol precipitation, and storage in Tris-EDTA at −20°C.

Single-round *in vitro* transcription assays. *In vitro* transcription reactions were initiated by incubating 25 nM linear template and 50 nM *Eco*RNAP or r*Mbo*RNAP holoenzymes in transcription buffer (40 mM Tris-HCl [pH 7.9], 10 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 0.1 mM EDTA, 25 μg bovine serum albumin [BSA]/ml) at 37°C for 15 min. Halted ECs were generated by addition of NTPs at 10 μM ATP, 10 μM GTP, and 2 μM [α-^{32}P]UTP followed by incubation for 10 min at 37°C. Transcription was restarted by adding all 4 NTPs at the concentrations described in the figure legends and heparin at 25 μg/ml. Reaction mixtures were removed at the indicated time points and mixed with an equal volume of 2× STOP buffer (8 M urea, 1× Tris-borate-EDTA [TBE] buffer, 50 mM EDTA, bromophenol blue and xylene cyanol [0.01 mg/ml each]; 1× TBE is 98 mM Tris-borate [pH 8.3]–2.5 mM Na_2EDTA). RNA products were resolved by separation by 8% PAGE containing 0.5× TBE and 8 M urea and quantified using a Typhoon Phosphorimager and ImageQuant software (GE Healthcare).

***In vitro* termination assays.** To assay termination, reactions were performed by incubating 25 nM linear template and 50 nM *Eco*RNAP or r*Mbo*RNAP holoenzymes in transcription buffer at 37°C for 15 min. Halted complexes were generated by addition of 10 μM ATP, 10 μM GTP, and 2 μM [α-^{32}P]UTP, followed by incubation at 37° C for 10 min. Transcription was restarted by adjustment of all 4 NTPs to 200 μM, or as described in the figure legend, followed by addition of heparin at 25 μg/ml. Reactions were stopped after 30 min by addition of an equal volume of a 2× STOP buffer. The RNA products were separated and quantified as described above except that a 6% polyacrylamide 8 M urea gel was used. The efficiency of termination was calculated as a percentage of the terminated transcript relative to the sum of terminated and read-through transcripts.

Transcription assays using immobilized RNAP. Halted ECs were formed as described above and mixed with 30 μl of nickel agarose beads (Sigma) preequilibrated with low-salt buffer (LSB; 20 mM Tris-HCl [pH 8.0], 20 mM NaCl, 10 mM $MgCl_2$, 2% glycerol, 1 mM β-mercaptoethanol, 20 μg acetylated BSA/ml) and incubated for 30 min at room temperature (RT) with gentle tumbling. The beads were washed 6 times using 2 bead volumes of LSB to remove unbound substrates and resuspended in the same buffer supplemented with tRNA competitor (50 μg/ml final concentration). Transcription was started by addition of rNTPs (200 μM ATP, 200 μM GTP, 200 μM CTP, 10 μM [α-^{32}P]UTP). Heparin was then added at 25 μg/ml. After 30 min of incubation at RT with tumbling, samples (10 μl) containing the whole reaction mixture or (after separation by centrifugation) the supernatant only or the beads washed with LSB were collected. The samples were then mixed with an equal volume of 2× STOP buffer. Samples were heated to 90°C and analyzed as described above using a 10% polyacrylamide 8 M urea gel.

Transcription assays using *Eco*RIQ111 as a roadblock. A linear DNA template (10 nM) containing biotin at the 5′ end of the nontemplate strand and 25 nM *Mbo*RNAP holoenzyme were combined with 10 μl of streptavidin-coated agarose beads (Sigma) and incubated for 15 min at 37°C in RB (30 mM Tris-HCl [pH 7.5], 80 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.025% Triton X-100, 2% glycerol, 0.05 mg BSA/ml). *Eco*RIQ111 (500 nM) was added to complexes and incubated for 10 min at 37°C. Transcription was started by addition of 200 μM (each) ATP, CTP, and GTP, 2 μM [α-^{32}P]UTP, 0.05 mg/ml tRNA, and $MgCl_2$ at 10 mM, followed by addition of heparin at 25 μg/ml to ensure single-round transcription. After 30 min, samples containing the whole reaction mixture, supernatant only, or washed beads were mixed with 2× formamide STOP buffer (125 mM Tris-HCl [pH 8.0], 15 mM EDTA, 333 mM NaCl, 1.25% SDS), extracted with phenol-chloroform (1:1), ethanol precipitated, dissolved at 90 for 1 min in formamide loading dye (95% formamide, 15 mM Tris-HCl [pH 7.9], 5 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol), and electrophoresed on a 5% polyacrylamide–0.5× TBE–8 M urea gel and quantified as described above.

Termination assays with NusA and NusG. Transcription assays were performed as described above with 25 nM halted EC formed at 37°C with 10 μM ATP, 10 μM GTP, and 2 μM [α-^{32}P]UTP. NusA or NusG or both proteins (1 μM each) were then added. After incubation for 10 min at 37°C, transcription was restarted by addition of all 4 NTPs at 200 μM each and heparin at 25 μg/ml. After 15 min at 37°C, the reactions were stopped with an equal volume of 2× STOP buffer and analyzed on an 8% polyacrylamide 8M urea gel as described above.

RNA 3′-end mapping. To map the precise positions of termination, halted ECs were first generated as described above. To generate sequence ladders, transcription was restarted by addition of ATP, CTP, and GTP at 100 μM each, 10 μM [α-^{32}P]UTP (0.1 μCi/μl), and one 3′-deoxyNTP in each of 4 separate reactions at 120 μM dGTP, 150 μM dATP, 175 μM dCTP, or 200 μM dUTP, followed by addition of heparin at 25 μg/ml. After incubation for 1 h at 37°C, samples were mixed with an equal volume of 2× STOP buffer and electrophoresed on a 5% polyacrylamide 8 M urea gel next to transcription products obtained by regular *in vitro* transcription on terminator templates. RNA products were visualized as described above.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00931-14/-/DCSupplemental.

Text S1, PDF file, 0.1 MB.

Table S1, PDF file, 0.1 MB.

Figure S1, PDF file, 0.5 MB.

Figure S2, PDF file, 0.6 MB.
Figure S3, PDF file, 0.6 MB.
Figure S4, PDF file, 0.3 MB.
Figure S5, PDF file, 2.9 MB.
Figure S6, PDF file, 0.6 MB.

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