Supplemental Data for:

A transcription antiterminator constructs a NusA-dependent shield to the emerging transcript

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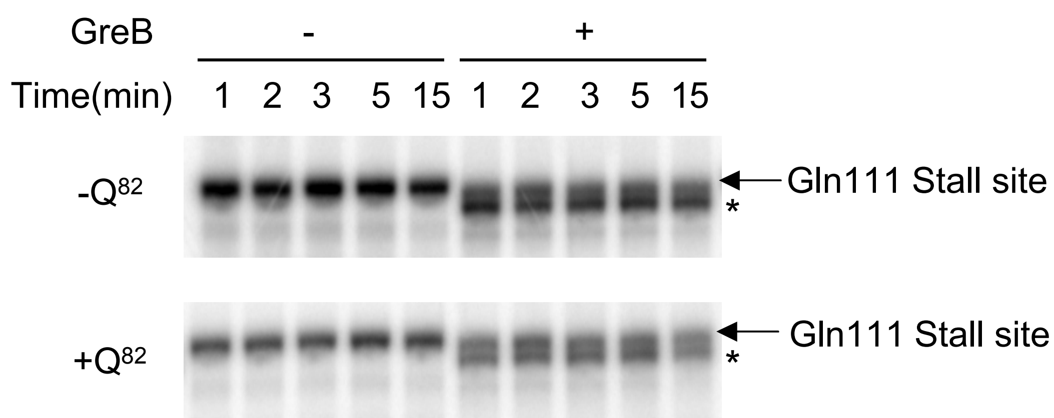
Preliminary characterization of the stalled transcription elongation complexes

As shown in Figure 1B, in the absence of termination mechanisms, the stalled elongation complexes are extremely stable and most of the RNA is retained in the pellet fraction (boxed), both in the absence and presence of Q^{82}; only about 5-10% of the RNA is released into the supernatant fraction (lanes 5,6,9,10). The stalled complexes may include some backtracked RNAPs, as determined by GreB cleavage, but the extent of backtracking is comparable both in the absence and presence of Q^{82} (Supplemental Figure 1). Therefore, the Q^{82}–modified and unmodified complexes are equivalent, in terms of their elongation states, for the assays described in the paper.



Supplemental Figure 1. Extent of backtracking of stalled Q^{82}-modified and unmodified complexes, as seen by GreB-mediated cleavage of the RNA; the cleaved transcripts are marked with the asterisk (*). Stalled complexes were made on template from pSS100 in the presence of NusA as described in the paper. The supernatant was removed and fresh buffer was added; the complexes were treated with 20 nM of GreB for the indicated time.

EXPERIMENTAL PROCEDURES

Templates and plasmids

Templates for transcription were made from plasmids by PCR amplification and gel purified. p82a contains the wild-type phage 82 late gene promoter followed by the intrinsic terminator t82 at position +82 (from the transcription start site). pSS100 is a derivative of p82a; starting 34 bp downstream of the intrinsic terminator t82, pSS100 contains a 43 nt long C-rich region for optimal Rho binding, followed by release sites I and II of the Rho-dependent λtR1 terminator, and an EcoR1 site. pAH100 is a derivative of p82 designed to lack cytidine residues before +29, allowing complex stalling at +29, and in addition containing a +2 change to guanine in order to avoid initial transcription slippage. pBY416 contains a modified phage 82 late gene promoter that contains A and C at position +1 and +2, followed by a C-less segment up to +25, such that initiation with ApC primer in the presence of ATP, GTP and UTP generates complexes stalled at the natural pause site at +25; the terminator t82 is at position +149 (Yarnell and Roberts, 1999). pSS418 is a derivative of pBY416, which contains nucleotide G at position +2 (instead of C) resulting in a completely C-less segment up to position +25, to enable 5' end labeling with γ–^{32}P-ATP; furthermore, it contains a U-less segment from position +25 to position +76. pXY312 contains the wild-type phage 82 late gene promoter followed by the λ tR1 Rho-dependent termination sites (Yang and Roberts, 1989).

Native Q^{82} purification

XL-1 Blue cells containing the plasmid pCWR82 expressing the native Q^{82} sequence with a C-terminal His-tag were grown to an A600 of 0.6. Protein expression was induced by the addition of 0.5 mM IPTG in the presence of 1% sorbitol and the cells were grown at 16°C overnight. Cells were lysed in 20 mM phosphate pH 7.5, 5% glycerol, 0.5 M NaCl, 1% Triton-X-100, 1 mM β-mercaptoethanol and 1 tablet of EDTA-free protease inhibitor cocktail (Roche). The cell lysate was loaded on a pre-equilibrated Ni-chelating column and washed successively with 5-10 times the column volume each of binding buffer (20 mM phosphate pH 7.5, 5% glycerol, 0.5 M NaCl, 1 mM β-mercaptoethanol) containing 0, 20, 40 and 60 mM imidazole. The protein was eluted with binding buffer containing 250 mM Imidazole, diluted five-fold to reduce the NaCl concentration to 0.1 M, and bound to a Hi-trap Heparin sepharose column and eluted with a salt gradient from 0.2 to 1 M NaCl. Q^{82} eluted at approximately 0.4 M NaCl. The fractions containing pure Q^{82} (as seen by SDS-PAGE analysis) were pooled and dialyzed into storage buffer (50 mM Tris-Cl, pH 8.0, 50% glycerol, 0.5 M NaCl, 0.1 mM EDTA, 1 mM DTT).

Other Proteins

The following proteins were purified as described: RNAP (Hager et al., 1990), NusA (Schmidt and Chamberlin, 1984), and Mfd (Selby and Sancar, 1993). His-tagged EcoR1-Gln111 was purified by affinity chromatography. Rho protein was a gift from M.Kainz and R.Gourse (University of Wisconsin, Madison). GreB protein was a gift of I. Artsimovitch (Ohio State University, Columbus).

RNA accessibility assays

Templates: For the RNase H and RNase A assays, Q^{82}-modified or unmodified stalled elongation complexes were made on template from pSS100; for the RNase I experiment in Figure 6B, the DNA template was from pSS418

RNase H assay: A schematic of this procedure is shown in Supplemental Figure 2. Q^{82}-modified or unmodified stalled elongation complexes were made as described in the paper on template from pSS100. The supernatant was removed and the beads resuspended in an equal volume of TB4 (with or without NusA). In order to make the complexes equivalent, 5 μM of the oligo anti-t82 was added back to the Q^{82}-modified complexes and all the complexes were treated with 0.25 U (per 25μl reaction) of RNase H (Invitrogen) for 10' at 37 °C in order to trim away RNA upstream of the t82 site; the resulting RNA is ~140 nt long and is referred to as the "uncut RNA" in the figures. The supernatant was removed and the beads were resuspended in fresh buffer containing 0.25 U of RNase H. 10 nM Q^{82} was also added back to the unmodified complexes (Figure 5) or to all reactions (Figure 7A, 7B) to ensure identical composition of the reactions. When present, the oligo probe was added to a final concentration of 1 μM. For the experiments in Figures 5, 7A and 7B, the digestion was allowed to proceed for 10'. For the experiment in Figure 7B, the DNA template was pre-incubated with either 20 nM of reconstituted wild type RNAP holoenzyme or 200 nM of reconstituted RNAP-αΔCTD, in the presence of NusA.

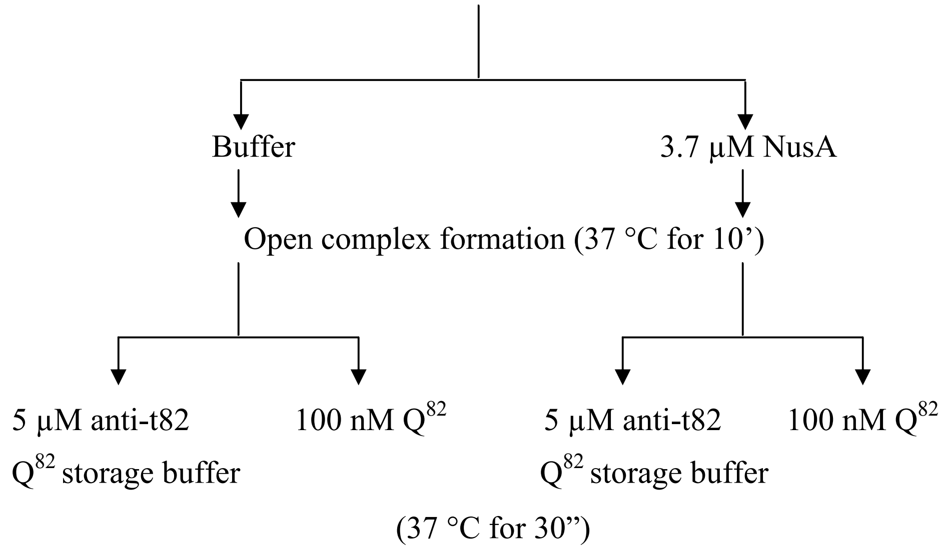
RNase A assay: For the experiment in Figure 6A, transcription was performed on a template from pSS100 as above except in the presence of 200 μM of all the four NTPs (non-radioactive). Once stalled, the complexes were washed extensively with and resuspended in TB4+NusA. In order to label the penultimate nucleotide (G) of the RNA in the stalled complexes, they were incubated at 37 °C for 5' with α-^{32}P-GTP, 5 μM UTP (terminal nucleotide) and 20 nM GreB. Since the sequence of the RNA in the hybrid contains only one G (UUUUCUGU), this should generate homogenously labeled RNA species in the stalled complexes. In order to isolate these complexes and remove any residual complexes at the promoter-proximal pause site containing 3' end-labeled RNA, they were washed with TB4+NusA, resuspended in NEB2 (+100 μg/ml BSA) and treated with restriction enzyme Xho1 at 37°C for 30', to remove DNA upstream of +54 of the transcribed segment (see schematic of template in Figure 1A). The supernatant was treated with 0.01 ng/μl of RNase A (Ambion) after 10 nM Q^{82} was added back to the unmodified complexes.

RNase I assay: For the experiment in Figure 6B, the DNA template was from pSS418. Transcription was performed as described except in the presence of 25μM ATP, 200 μM GTP, 200 μM UTP and 10 μCi of γ-^{32}P-ATP, in order to obtain complexes containing 5' end-labeled RNA stalled at position 25 (CTP deprivation). The complexes were washed with and resuspended in TB4+NusA. Q^{82} or Q^{82} storage buffer was added to the stalled complexes, which were then advanced to position 76 in the presence of 200 μM each of ATP, GTP and CTP (UTP starvation). After more washing, the mix of complexes stalled at 25 and 76 was treated with 0.4 U/μl RNase I (Ambion).

Supplemental Figure 2. Schematic of the experimental procedure used to show the role of NusA in Q^{82}–mediated RNA protection against RNase H digestion (Figure 7A).

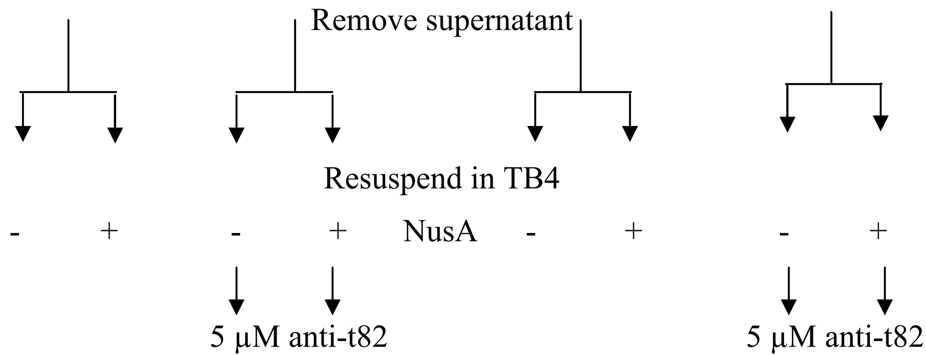
Master mix containing 2 nM template from pSS100, 20 nM RNAP holoenzyme, NTPs

(including α-^{32}P-UTP) and TB1, on streptavidin-coated magnetic beads



(37 °C for 30")

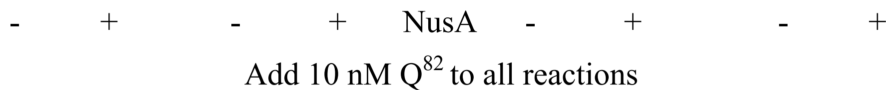
$MgCl_2$/ rifampicin mix to initiate transcription, elongation to stall site (37 °C for 15')

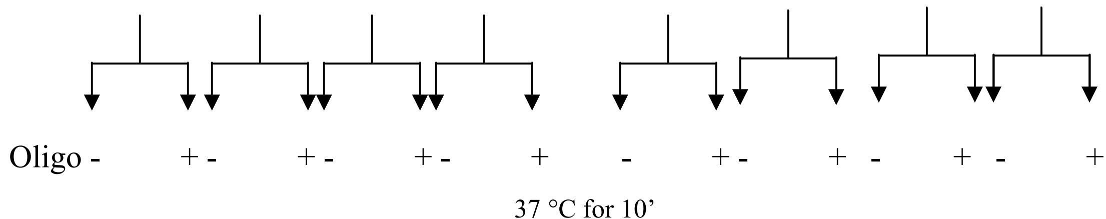


0.25 U RNase H treatment (37 °C for 10') to obtain 140 nt RNA

Remove supernatant

Resuspend in fresh TB4, containing 0.25 U RNase H





37 °C for 10'