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Supplemental Data

Inhibition of a Transcriptional Pause by RNA Anchoring to RNA Polymerase

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Supplemental Experimental Procedures

Measuring the Efficiency of Pausing and Termination

DNA at a concentration of 12 to 20 nM was combined with a 4-fold molar excess of RNAP in transcription buffer (TB; 20 mM Tris Glutamate (pH 8.0), 10 mM Mg Glutamate, and 50 mM K Glutamate) and incubated for 5 min at 37ºC. To initiate transcription, the following components were added at the same time: ApU to 350 μM, ATP, GTP, and UTP (each to a final concentration 100 μM), CTP (to 5 μM), [α - ^{32}P]CTP (to 0.1 – 0.5 μCi/μl), heparin (to 100 μg/μl), and oligonucleotides (if present) to 100 μM). After the indicated periods of time, transcription was stopped by addition of gel loading buffer (7 M urea, 50 mM EDTA). The RNA product was fractionated by polyacrylamide gel electrophoresis and quantified by Phosphoimager scan and ImageQuant software. For calculations, the intensities of the bands containing paused, terminated, and runoff transcripts were converted to molar equivalents according to their cytosine content.

$KMnO_4$ Footprinting of Roadblocked Complexes

Templates for transcription were cleaved with AgeI and purified by polyacrylamide gel electrophoresis. They were used to obtain elongation complexes containing a 16 nt long transcript (G16 complex) by incubating 20 nM template with 80 nM histidine-tagged RNAP in TB-KCl (50 mM Tris-KCl (pH 8.0), 10 mM $MgCl_2$, and 40 mM KCl) at 37ºC for 5 min. At this point, 350 μM of ApU, 10 μM each of ATP and GTP, 2 μM of CTP, and 0.5 μCi/μl of [α - ^{32}P]CTP were added, and incubation was continued for 5 more min at room temperature. (TB-

KCl was used instead of TB because the former did not interfere with binding of histidine-tagged RNAP to Ni-NTA agarose.) G16 complexes were mixed with Ni-NTA agarose beads (pre-equilibrated with TB-KCl buffer) for 10 min at room temperature, and the beads were washed 5 times with 1 ml of the buffer. Non-template DNA strands were labeled in the immobilized G16 complexes by treatment with 0.3 U/μl of Klenow fragment and 50μCi of [α -^{32}P] dATP for 10 min at room temperature. Labeled G16 was washed with TB-KCl buffer, and Lac repressor was added to a final concentration of 20nM followed by 10 min incubation at 37ºC. Transcription was resumed by adding 100 μM ATP, UTP, and GTP, and 5 μM CTP for 1 min to obtain roadblocked complexes, after which NTPs were removed by washing. $KMnO_4$ was added to a final concentration of 1 mM, and incubation was continued for 3 min at room temperature. Cleavage of modified residues was induced by piperidine and the products were separated by polyacrylamide gel electrophoresis as described (Komissarova and Kashlev, 1997b). Where indicated, repressor was removed by IPTG treatment after removal of NTPs .

Effect of *put* on Pausing

Supplementary figure 1 shows details of the effect of *put* on the kinetics of two pauses. One is the U-rich pause in its normal location, and the second is the his pause, inserted so that the pause position is 111 nt downstream of stem-loop 2 of *putL*. When *put* action was prevented by the β'-Y75N mutation in RNAP, we observed a reproducible increase in the probability but not the duration of the his pause.

Pausing and Backtracking Do Not Depend on the Roadblock

Supplementary figure 2A shows that the pattern of GreB cleavage of roadblocked ECs does not perceptibly change when the roadblock is removed and elongation is prevented by the absence of NTPs. Moreover, we observed a very similar pattern of GreB cleavage products when *put*$^-$ templates were transcribed with WT RNAP or *put*$^+$ templates were transcribed with Y75N RNAP in the absence of Lac repressor roadblock. Supplementary figure 2B shows that the presence of GreB during unobstructed transcription reduces the intensity of pausing.

The 5' product of GreB cleavage can be the result of several successive cleavages from the 3' end of a progressively more backtracked EC (Lee et al., 1994; Feng et al., 1994). To see if we could detect such backtracking intermediates, we determined the 3' products of GreB cleavage. The complexes tested were not artificially halted by a roadblock, so this experiment also tests the effect of the roadblock on backtracking. The results show a prominent 8 nt 3' GreB cleavage product and minor *c*.9 and *c*.10 nt products that originate at the U-rich pause by two criteria: they are eliminated by pause mutants 1 and 2 and are suppressed by *putL* (Supplementary figure 3 and Supplementary table). These products can originate from complexes paused at either G93 or C94. Under our conditions, there should be roughly equal amounts of the two paused complexes (see Supplementary figure 2B). Additional GreB-dependent fragments can be seen, but they are unaffected or much less affected by mutations that inactivate either the U-rich pause or *putL*. They are probably the result of cleavages at other backtracking sites. We conclude that at the pause site the majority of unmodified ECs retreat by 8 to 10 nt as a result of spontaneous backtracking rather than as a result of backtracking induced by the roadblock or processive GreB cleavage.

Additional Evidence that *put* Suppresses Backtracking

Supplementary fig. 4A shows that a *put*-modified roadblocked EC resumed transcription more rapidly than an unmodified complex when repressor was removed and NTPs added. Supplementary fig. 4B shows that the initial rate of pyrophosphorolysis was higher for *put*-modified than for unmodified complexes roadblocked at the pause site. Together these experiments suggest that *put*-modified complexes exist predominantly in the pretranslocated state, while unmodified complexes exist predominantly in the backtracked state.

Probing the Secondary Structure of *putL*-G35A RNA with RNases T1 and V1

RNase T1 cleaves after G residues that are not stacked on adjacent bases, and RNase V1 prefers to cleave after base-paired residues without much base specificity. Comparison of RNase T1 and V1 cleavage of *putL*-G35A with that of wild type revealed no differences between the two (Supplementary figure 5), and we conclude that this mutation does not change the secondary structure of the RNA.

putL	Wild Type			Mutant	
Pause	WT	Mut 1	Mut 2	WT	Mut 1
8-mer band	0.21	0.03	0.02	1.0	0.002
9-mer band	0.01	0.07	0.14	1.0	0.005
10-mer band	0.32	0.5	0.46	1.0	0.26

Table S1. Production of 3' GreB Cleavage Products from the U-rich Pause

The relative intensities of the indicated bands of Supplementary figure 3, lower gel, are expressed as a fraction of the runoff plus pause transcripts (upper gel) normalized to the intensity of that found in mutant *putL* and U-rich pause template. (Raw intensities were corrected for the number of C residues in each transcript.) None of these bands could be detected when GreB was not added.

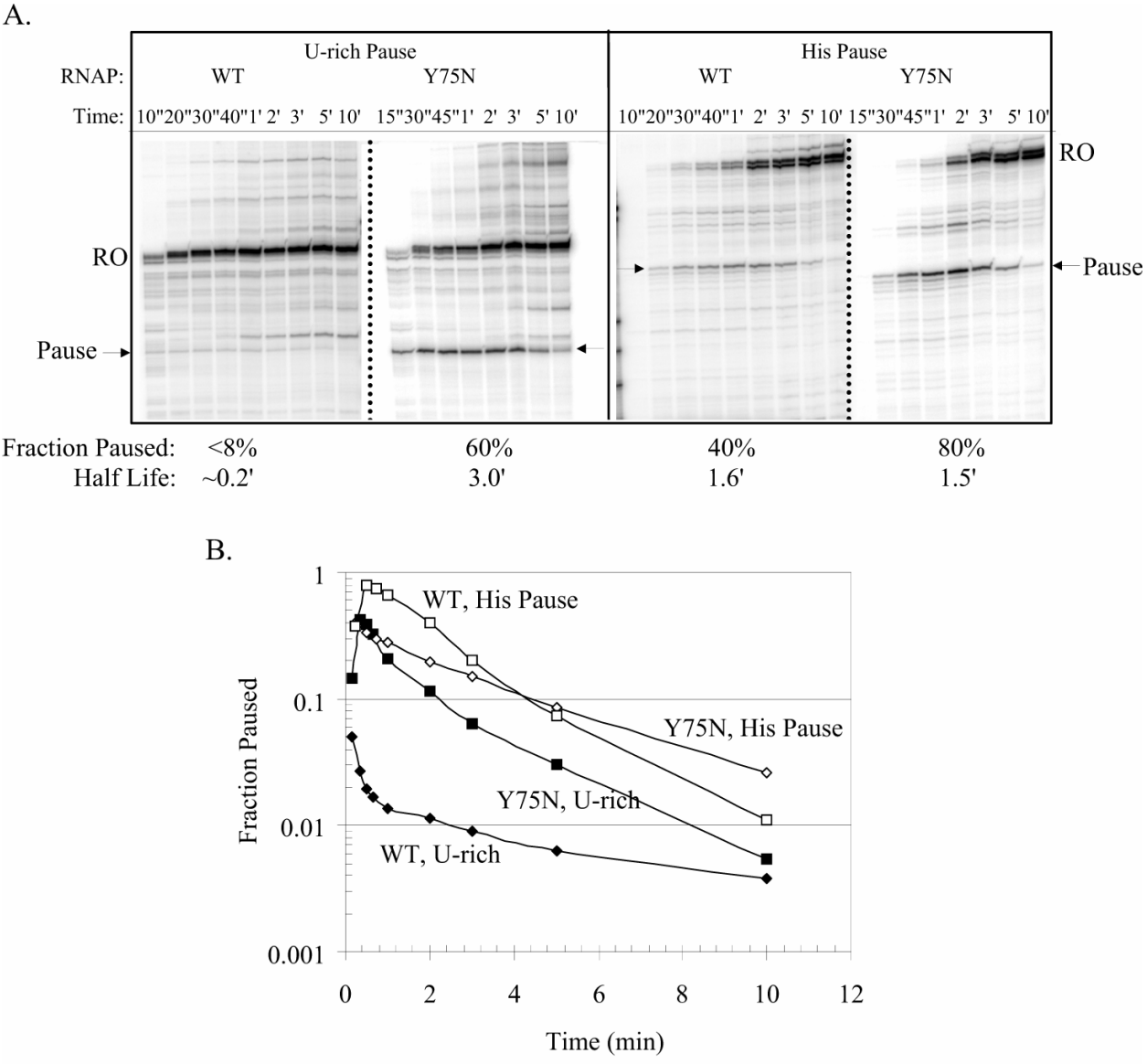

Figure S1. Kinetics of Pausing

A. The U-rich pause template was transcribed for the indicated times by WT or Y75N RNAP in the presence of 100 μM ATP, GTP, and UTP, 5 μM CTP, and 0.3 μM α-^{32}P-CTP. In the his pause template, the segment containing the U-rich pause was replaced with a fragment containing the his pause (Chan and Landick, 1989), and transcription was carried out in the presence of the same NTP concentrations. The his pause position was 111 nt from the P_L

promoter. B. The fraction of complexes paused and their half lives were estimated from the graph (Landick et al., 1996).

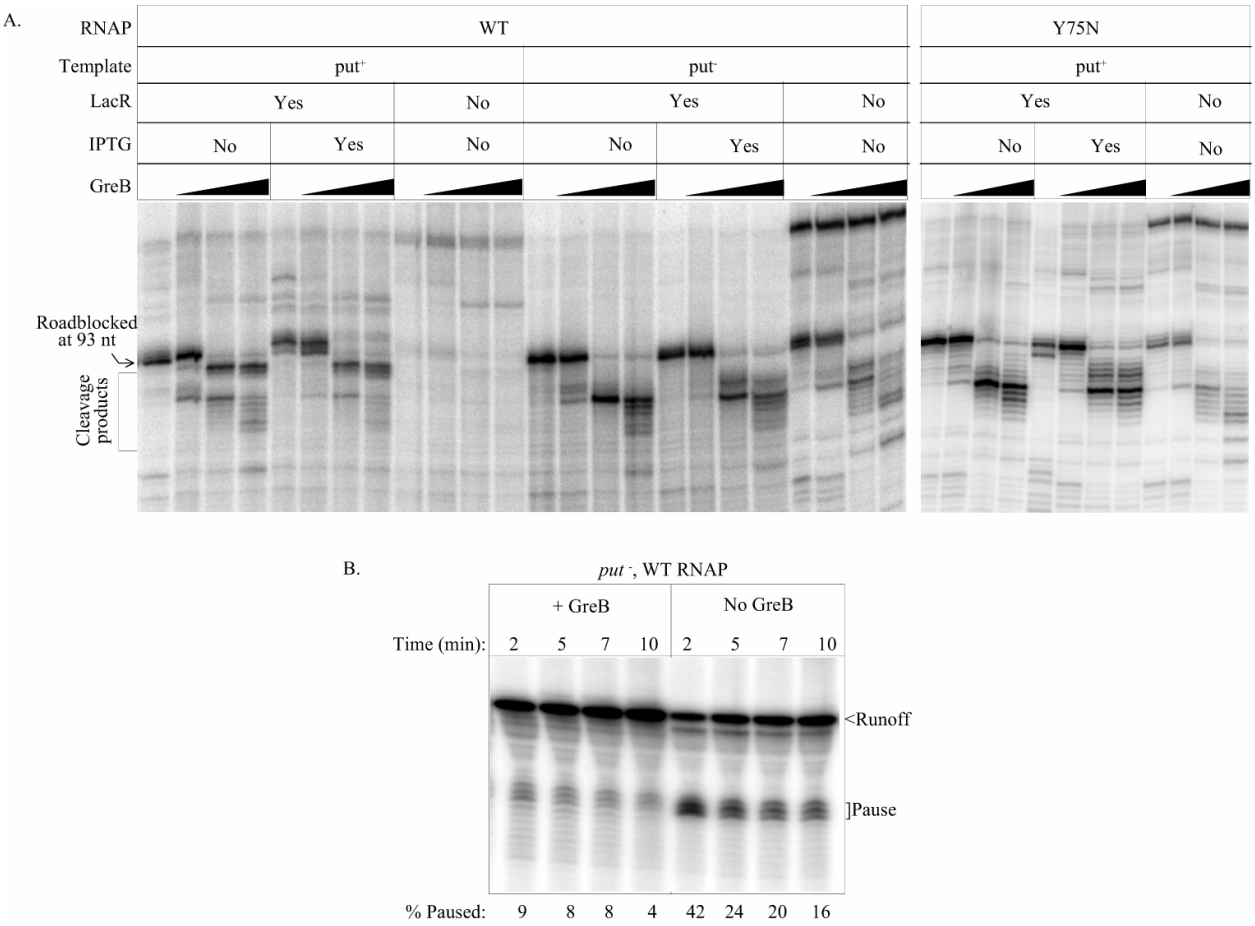

Figure S2. GreB Cleavage in the Presence and Absence of Lac Repressor

A. Some of these results were presented in Figure 3A, and the details of this experiment (concentrations of NTPs and GreB and times) are described in that figure. Where indicated, IPTG at a concentration of 1 mM was added to the washed roadblocked complexes and allowed to act for 10 min before addition of GreB. In experiments where no Lac repressor was present, GreB and NTPs were added to open complexes, and transcription was allowed to proceed for 3 minutes. (The reduced intensity of the bands in transcripts from *put*$^+$ templates with wild type RNAP in the absence of Lac repressor is the result of underloading, not poor transcription.) B. Wild type RNAP was allowed to transcribe a *put*$^-$ template to position G16 in the absence of

UTP (350 μM ApU and 5 μM each of ATP, GTP, and CTP). The complexes were then chased in the presence of heparin, 5 μM CTP, 0.3 μM α-^{32}P-CTP, 100 μM each of the other 3 NTPs, and, where indicated, 2 μM GreB for the indicated times. The products were fractionated on an 8% denaturing polyacrylamide gel. This experiment should not be directly compared to that of panel A because of differences in the experimental format (eg., solid state instead of liquid) and differing activities of the two GreB preparations.

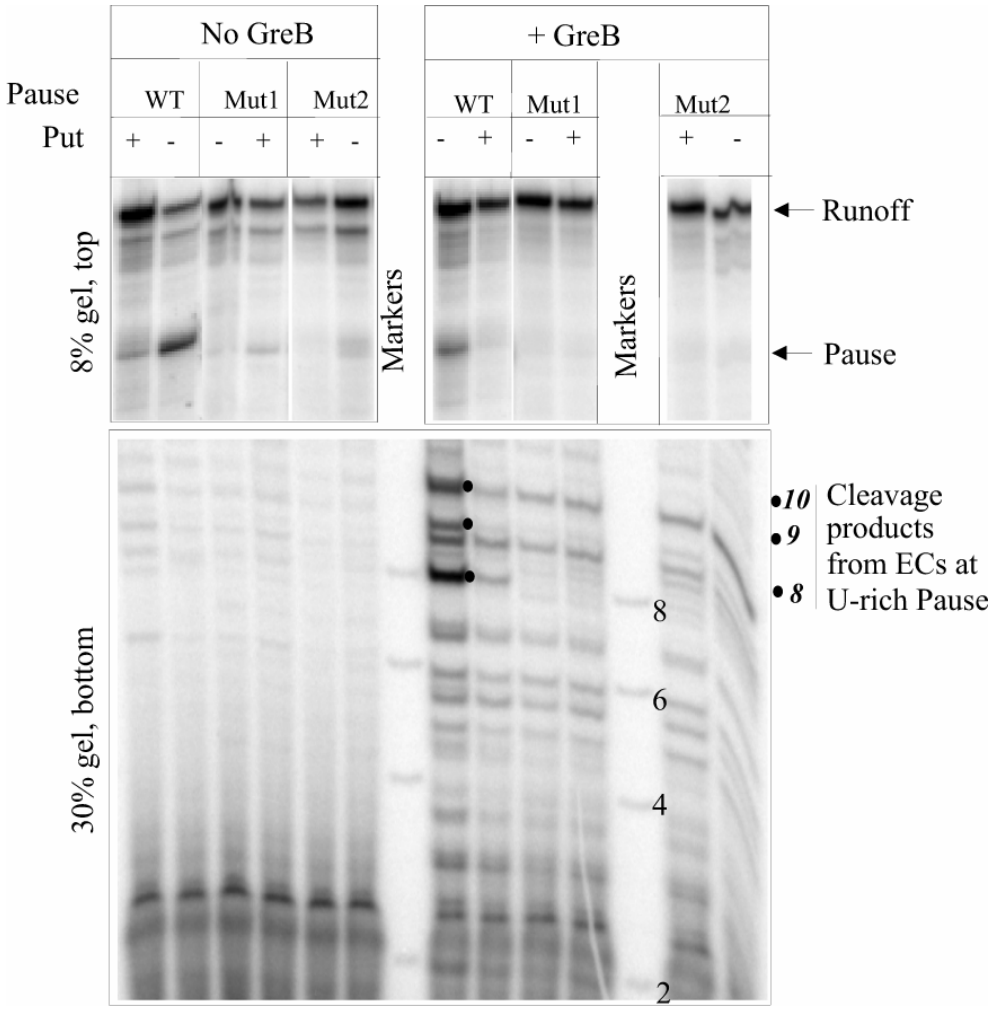

Figure S3

Unlabelled G16 complexes (see Supplementary figure 2 legend) obtained with wild type RNAP on templates containing wild type or mutant *putL* followed by wild type or mutant pause site were chased for 2 min in the presence of heparin, 5 μM α-^{32}P-CTP, 100 μM each of the other 3 NTPs, and, where indicated, 1 μM GreB. The products were fractionated on 8% and 30% (19:1 acrylamide to bisacrylamide) denaturing polyacrylamide gels and detected by autoradiography. The markers were pUpG, pUpUpUpG, pUpUpUpUpUpG, and pCpUpUpUpUpUpUpG, all labeled with ^{32}P at the 5' end. These sequences are those expected from cleavage of RNA

whose 3' end is at G93. The sizes of the "9 nt" and "10 nt" bands are estimates.

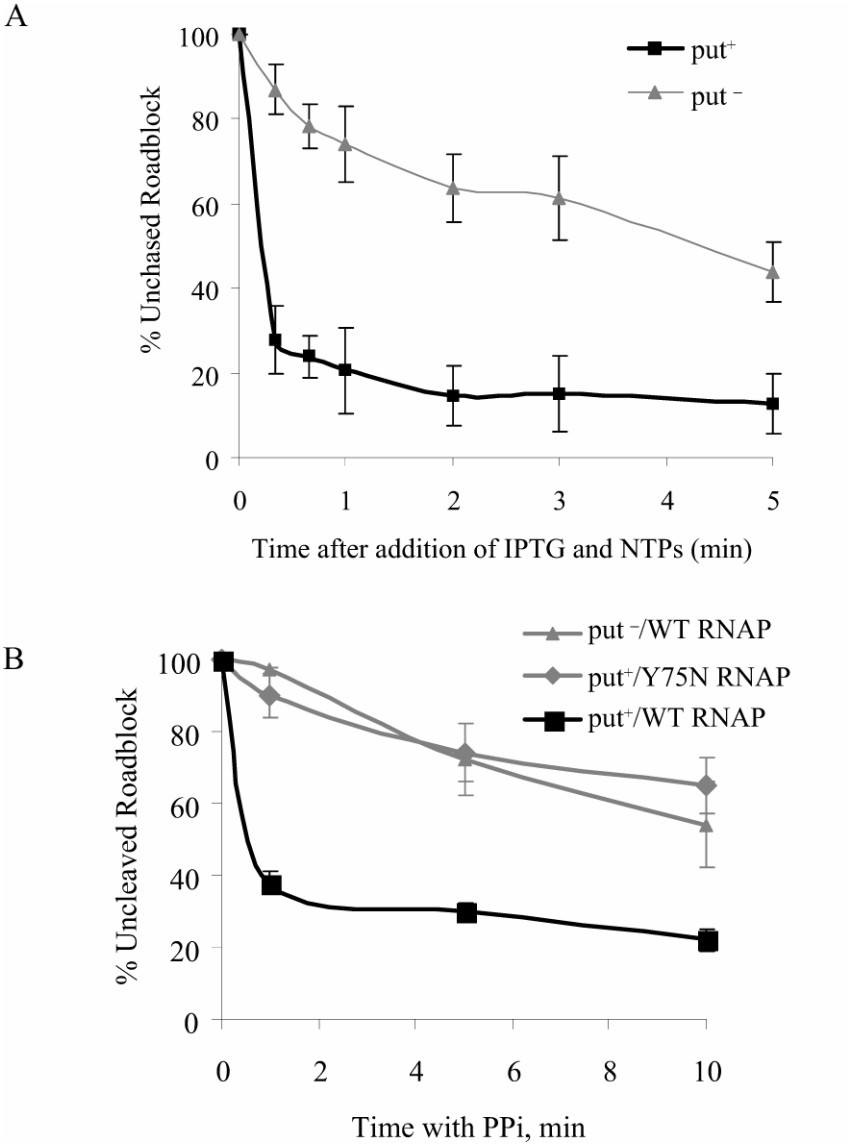

Figure S4

A. Resumption of transcription by immobilized ECs stalled at the U-rich pause. Roadblocked and washed ECs were formed at the U-rich pause site using an immobilized P_L-*put*$^+$ template and wild type or Y75N RNAP. Lac repressor was removed and transcription restarted by adding IPTG and 125 μM of all four NTPs. The reactions were sampled at the indicated times and fractionated on a denaturing gel. The fraction of roadblocked complexes that had escaped from

the pause is shown as a function of sampling time. B. Kinetics of pyrophosphorolysis. ECs that were roadblocked at the pause site and washed were treated with 20 mM sodium pyrophosphate for the indicated times, and the products were analyzed by gel electrophoresis and autoradiography. The fraction of roadblocked complexes that remained resistant to pyrophosphorolysis is shown as a function of sampling time.

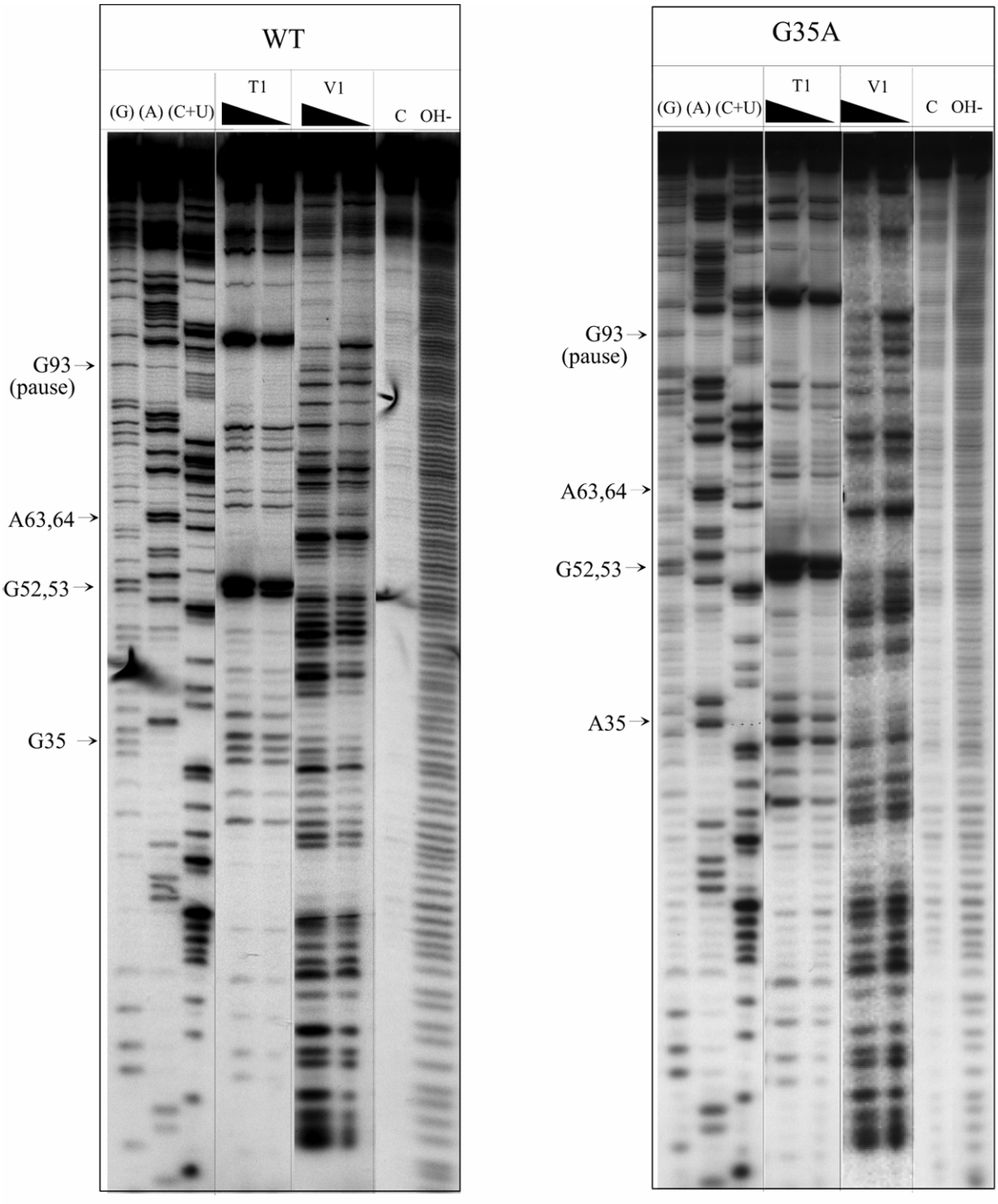
WT
(G) (A) (C+U)
T1
V1
C
OH-
G93
(pause)
A63,64
G52,53
G35
G35A
(G) (A) (C+U)
T1
V1
C
OH-
G93
(pause)
A63,64
G52,53
A35

Figure S5: Structure Probing of *putL*-G35A

The lanes marked T1 and V1 are partial digests of 5'-end labeled wild type and G35A mutant *putL* RNAs with RNases T1 and V1, respectively. The experimental conditions and the results of probing wild type RNA were described in (Banik-Maiti et al., 1997). The three lanes to the left of each panel are sequencing lanes and the two lanes to the right are undigested and a partial alkali digest of the same RNAs.

Supplemental References

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