

Supplemental Data for:

***In vitro* characterization of 6S RNA release-
defective mutants uncovers features of pRNA
dependent release from RNA polymerase in *E. coli***

Mariana Oviedo Ovando, Lindsay Shephard, and Peter Unrau.

Department of Molecular Biology and Biochemistry, Simon Fraser University,

8888 University Road, Burnaby, BC V5A 1S6, Canada

Alignment: Release-defective isolates DNA sequences.

Alignment Workspace of 6SfromScreening.meg ClustalW (Slow/Accurate, IUB)
Monday, September 10, 2012 10:10 AM

Page 1

		
	GGCGGGCCAGTCCCTGAGCC-GATATTTTCATACCACAA-GAATGTG-G-CG-CTC-CGC	
	10 20 30 40 50 60	
T1 SEQUENCE.seq	-GCGGGCCAGTCCCTGAGCC-GATATTTTCATACCACAA-GAATGTG-G-CG-CTC-CGC	53
MR9-8.seq	GGCGGGCCAGTCCCTGAGCC-GATTTTTGATACCAAA-GACTGTG-G-CG-CTC-CGC	54
MR9-1.seq	GGCGGGCCAGTCCCTGAGCC-GATTTTTCATCCCACCT-GAAAGTG-G-CG-ATT-CGC	54
MR9-11.seq	GGCGGGCCAGTCCCTGAGCC-AATATTGGATAGCACAA-GAAAGTG-G-CG-CTC-CGC	54
MR9-18.seq	GGCGGGCCAGTCCCTGAGCC-AATTTTTCATTTCCAAA-GAAGTTG-A-CG-CTC-CGC	54
MR9-24.seq	GGCGGGCCAGTCCCTGAGCC-AAAAATTCAACCATAA-GAATGTG-G-CG-CTC-CGC	54
MR9-33.seq	GGCGGGCCAGTCCCTGAGCC-GAATTTCTTTCCACAA-GAATGTA-G-CG-TTC-CGT	54
MR9-34.seq	GGCGGGCCAGTCCCTGAGCC-AATATCTCTTACCACCTC-GAATCTA-G-CG-CTA-CGC	54
MR9-44.seq	GGCGGGCCAGTCCCTGAGCC-GATTATTGATACCACAG-GAATGTG-G-GG-CTC-CGC	54
MR9-52.seq	GGCGGGCCAGTCCCTGAGCC-GAAACGTCATACCACAA-TAATGTG-G-CG-CTT-CGC	54
MR9-62.seq	GGCGGGCCAGTCCCTGAGCC-AATCTGTCAACCACAA-GAATGTG-G-CT-ATC-CGC	54
MR9-66.seq	GGCGGGCCAGTCCCTGAGCC-GTTTTATCATACCTCAA-GAGAGTG-A-CG-CTC-CGC	54
MR9-70.seq	GGCGGGCCAGTCCCTGAGCC-ATTTCTTCAAGCACAA-GAGTGTG-G-CG-CTT-CGC	54
MR9-71.seq	GGCGGGCCAGTCCCTGAGCC-GTTCTTTCATAACACAA-AAATGTA-G-CT-GTT-CGC	54
		
	GGTT-GGTGA-GCATG-CTCGG-TCCGT-CCGAG-AA-GC-CTTAA-AACTGCG-A-CG-	
	70 80 90 100 110 120	
T1 SEQUENCE.seq	GGTT-GGTGA-GCATG-CTCGG-TCCGT-CCGAG-AA-GC-CTTAA-AACTGCG-A-CG-	102
MR9-8.seq	GCTA-GGTGA-GCAAG-CTCGG-TCCGT-CCGAG-AA-GC-CTTAA-TAGTGCG-A-CT-	103
MR9-1.seq	GGTT-GGTGA-GCAAG-CTCGG-TTCGT-CCGAG-AA-GC-CTTAA-AACTGCG-A-CC-	103
MR9-11.seq	AGTT-GGTGA-GCATG-CTCGG-TCCGT-CCGAG-AA-GC-CTTAA-AACTGCG-A-CG-	103
MR9-18.seq	GGTT-GGTGA-GCATG-CTCGG-TCCGT-CCGAG-AA-GC-CTTAA-AACTGCG-T-CG-	103
MR9-24.seq	GGTT-GGTGA-GCAG-CTCGG-TCCGT-CCGAG-AA-GC-CTTAA-AACTGCG-A-CG-	103
MR9-33.seq	AGTT-GGTGA-GCAAG-CTCGG-TCCAT-CCGAG-AA-GC-CTTAA-AACTATG-A-CG-	103
MR9-34.seq	GGTG-GTTGA-GCATG-CTCGG-TCCGT-CCGAG-AA-GC-CTTAA-AACTGCA-A-CA-	103
MR9-44.seq	GGTT-GGTGA-GCATG-CTCGG-TCCGT-CCGAG-GA-GC-CTAAA-AACTGCG-A-CG-	103
MR9-52.seq	TGAT-GGTGT-GCATG-CTCGG-TCC-T-CCGAG-AA-GC-CTTAA-ATCAGCG-A-CG-	102
MR9-62.seq	GGTT-GGTGA-GCATG-CTCGG-TCCGT-CCGAG-AA-GC-CTGAA-AACTGCG-A-AG-	103
MR9-66.seq	AGTT-GGTGA-GCATG-CTCGG-TCCGT-CCGAG-AA-GC-CTTAA-AACTGCG-A-CG-	103
MR9-70.seq	GGTT-GGTGA-GCATG-CTCGG-TCCGT-CCGAG-AA-GC-CTTAA-AATTGCG-A-CG-	103
MR9-71.seq	GGTT-GGTGA-GCAG-CTCGG-TCCGT-CCGG-AA-GC-CTTAA-AGCCGCG-A-CG-	103
		
	A-CACATTC-ACCTTGAACCAAG-GGTTCAAGGGTTACAGCCTGC	
	130 140 150 160	
T1 SEQUENCE.seq	A-CACATTC-ACCTTGAACCAAG-GGTTCAAGGGTTACAGCCTGC	143
MR9-8.seq	A-CACATTC-ACCTTAAACAAG-GGTTCAAGGGTTACAGCCTGC	144
MR9-1.seq	A-CACATTC-ACCTTAAACAAG-GGTTCAAGGGTTACAGCCTGC	144
MR9-11.seq	A-CACATTC-ACCTTAAACAAG-GGTTCAAGGGTTACAGCCTGC	144
MR9-18.seq	T-CACATTC-ACCTTAAACAAG-GGTTCAAGGGTTACAGCCT	142
MR9-24.seq	A-CACATTC-ACCTTGAACCAAT-GGTTCAAGGGTTACAGCCTGC	144
MR9-33.seq	A-TTCATTC-ACCTTGAACCAAG-GGTTCAAGGGTTACAGCCTGC	144
MR9-34.seq	A-CAGATTC-ACCTTGAACCAAG-GGTTCAAGGGTTACAGCCTGC	144
MR9-44.seq	A-CACATTC-ACCTTGAACCAAG-GGTTCAAGGGTTACAGCCTGC	144
MR9-52.seq	A-CACATTC-ACCTTGAACCAAG-GGTTCAAGGGTTACAGCCTGC	143
MR9-62.seq	A-CACATTC-ACCTTGAACCAAG-GGTTCAAGGGTTACAGCCTGC	144
MR9-66.seq	A-TACATTC-ACCTTGAACCAAG-GGTTCAAGGGTTACAGCCTGC	144
MR9-70.seq	A-CACATTC-ACCTTGAACCAAG-GGTTCAAGGGTTACAGCCTGC	144
MR9-71.seq	A-CGATTC-ACCTTGAACCAAG-GGTTCAAGGGTTACAGCCTGC	144

Supplemental Figures

Fig. S1: A) *In vitro* selection scheme for release-defective 6S RNA mutants. A DNA pool having a diversity of 4×10^{12} sequences was constructed and selected for its ability to bind (and not release) $E\sigma^{70}$ for five rounds of selection. Residues 42–88 and 104–143 were initially mutagenized with a frequency of 10% (T7 RNAP promoter in green, mutagenized residues in red). An 8-fold excess of the RNA pool was incubated with $E\sigma^{70}$ and then incubated under rapid release conditions (250 μ M of each NTP and 4 mM $MgCl_2$, for 30 min). RNA capable of remaining in a complex was recovered from a native gel (red dotted box). These steps were repeated and mutants that remained bound to $E\sigma^{70}$ were excised from a second native gel prior to RT-PCR and transcription, ready for another round of selection. **B) The bound RNA Pool fraction increased with rounds of selection.** The blue bars show the fraction of T1 RNA control that remained bound after release was induced. The red bars show the fraction of RNA pool that remained bound after each round of selection.

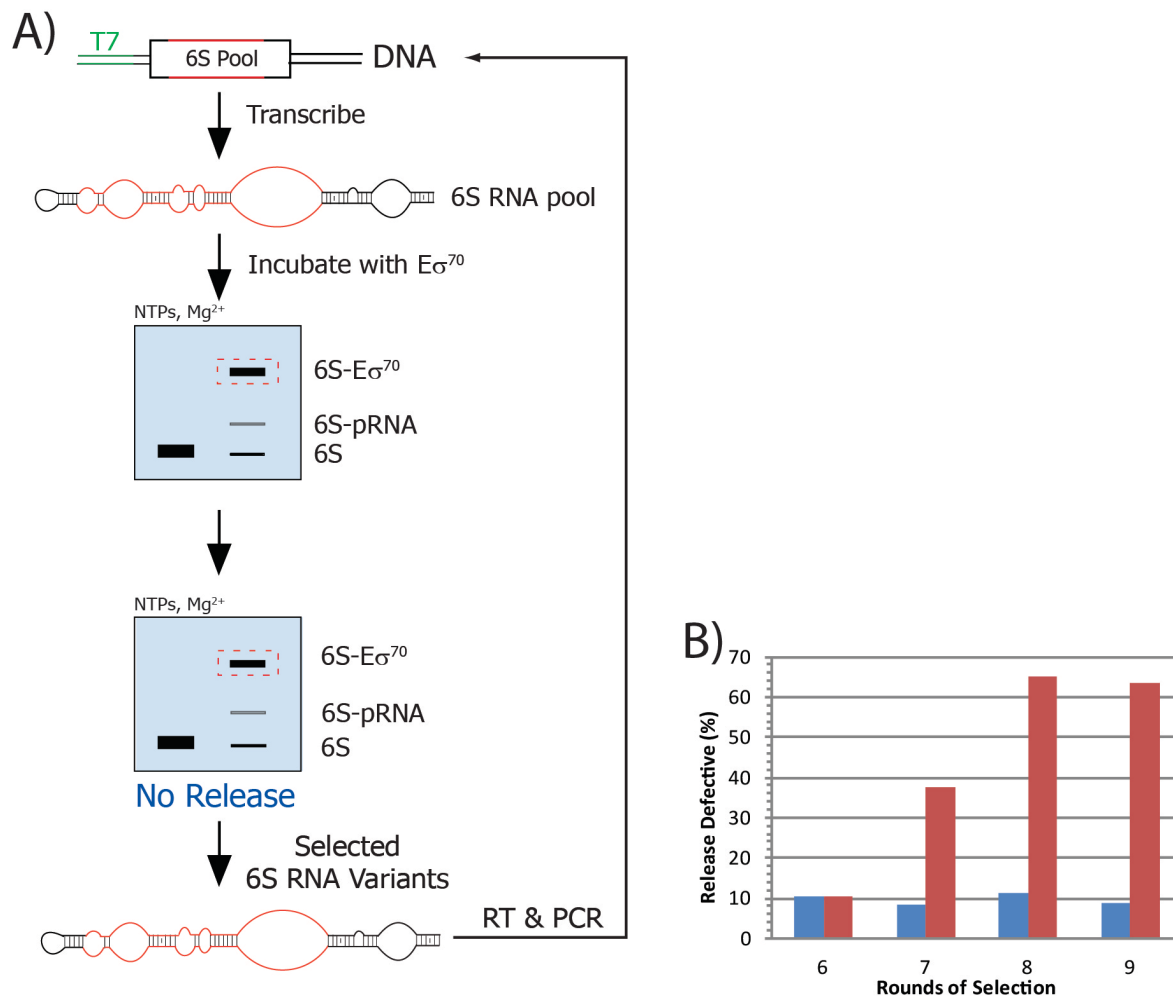


Figure S2: Round 9 release-defective 6S RNA consensus compared with γ -proteobacteria

phylogeny. **A)** The ‘-35’ sequence is conserved in 6S RNA release defective mutants in a pattern that is shifted with respect to the natural sequence (Shephard, Dobson and Unrau 2010). Significant changes away from the biological consensus were found in the immediate vicinity of the pRNA transcriptional initiation site (TSS, noted by arrow). Black residues were mutagenized during pool synthesis. Purple residues were found to be completely conserved after the selection. **B)** Consensus sequence of phylogenetically conserved 6S RNAs from several γ -proteobacteria (Enterobacteriaceae, Vibrionaceae, Pseudomonadaceae). Three main regions of highly conserved sequence are found among the γ -proteobacteria, namely the ‘-35’, ‘-10’ and transcriptional initiation and downstream regions (pink regions represent absolute conservation). Nucleotide variants are color coded according to the observed percentage of sequence variability.

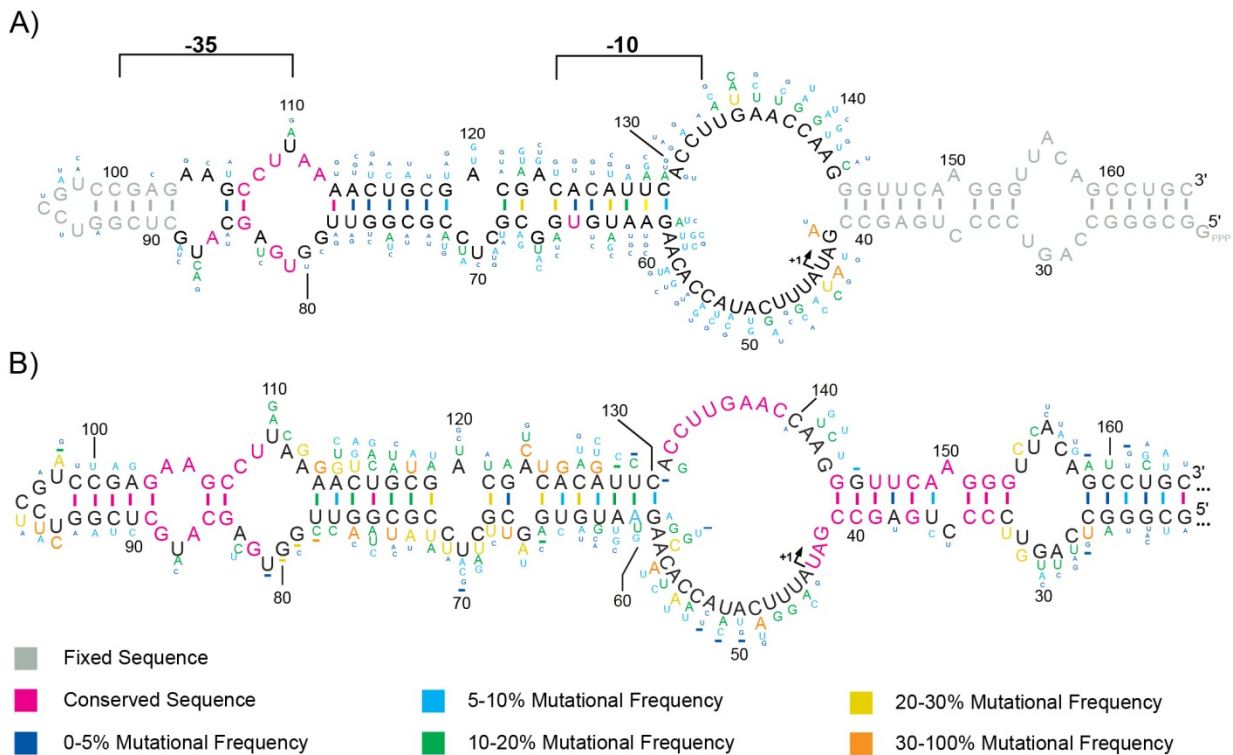


Figure S3: Sequence conservation among release-defective 6S RNA variants. Mutant 6S RNA sequences were compared to the wildtype sequence (T1 RNA in pink) using MegAlign software (DNASTAR). The 6S RNA variants were as expected, capable of efficient binding as summarized in the first column (B): +++, at least 75% of the mutant RNA bound to the $E\sigma^{70}$ or ++, between 75-60 % able to bind. Release rates are summarized in the R column and keyed to the table on the left).

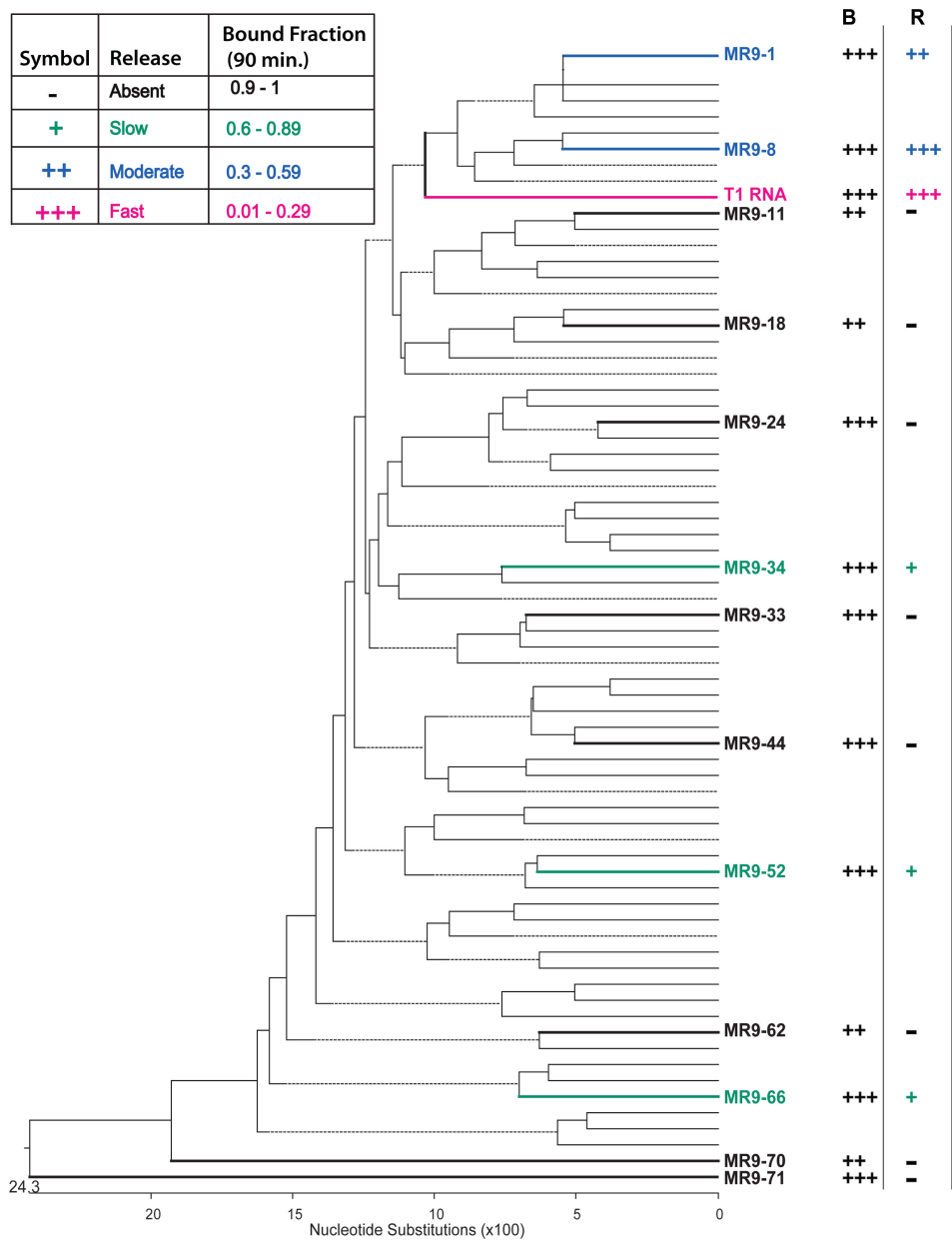
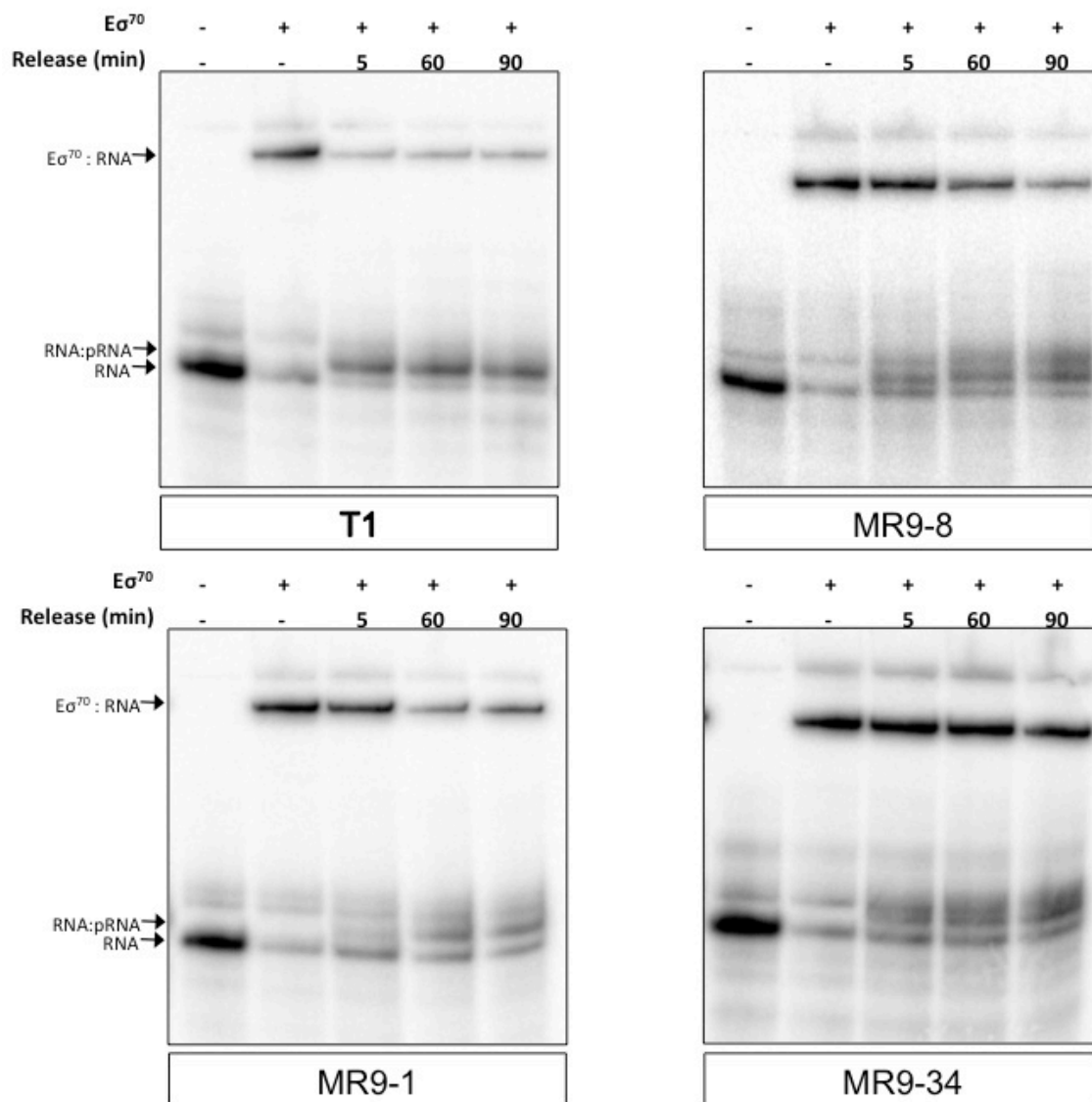
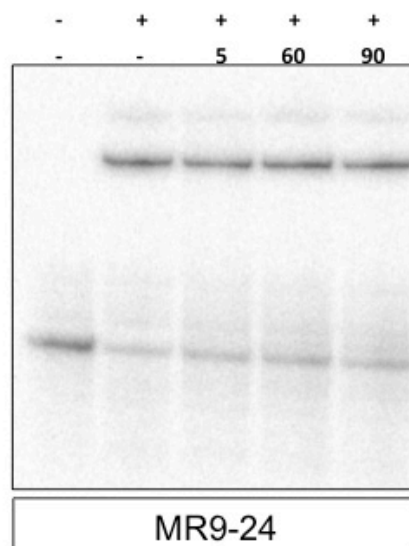
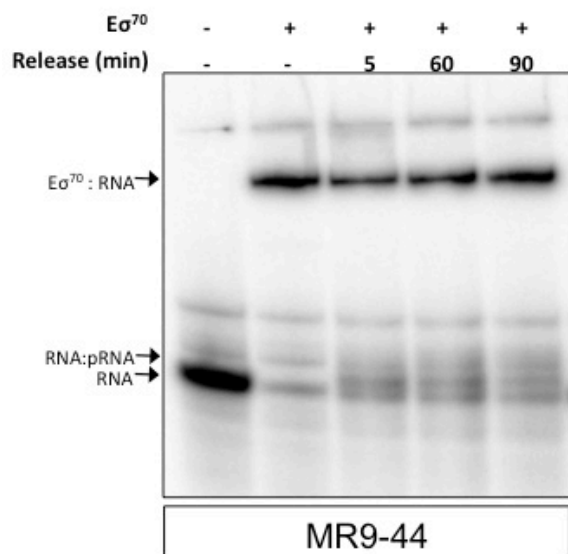
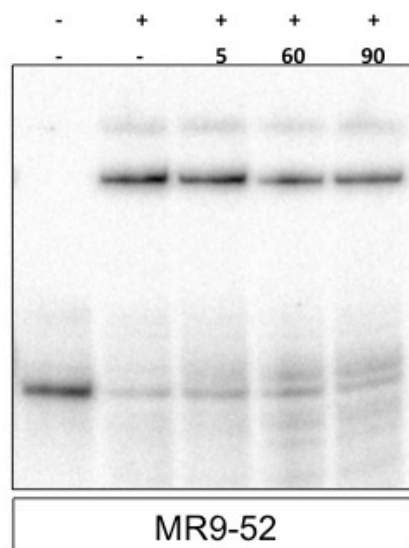
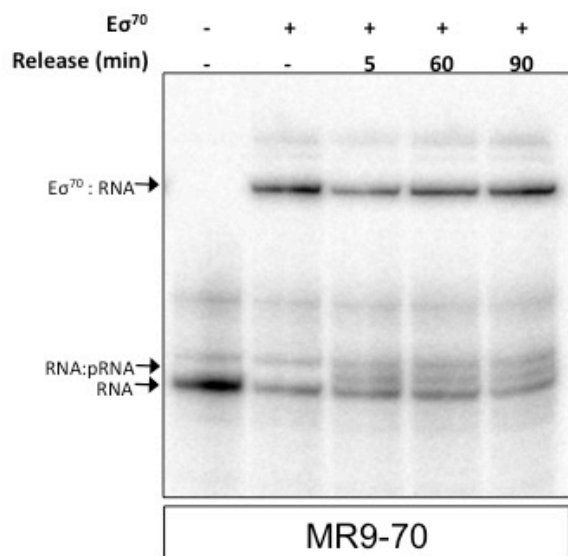
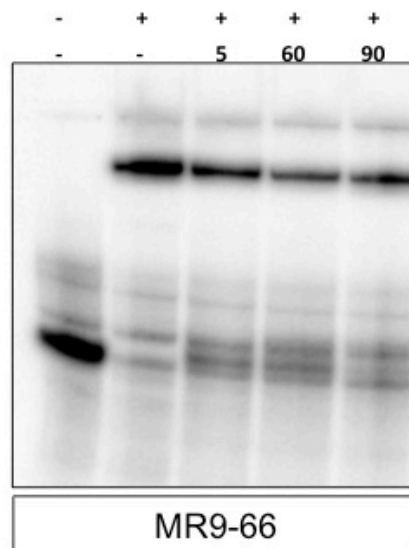
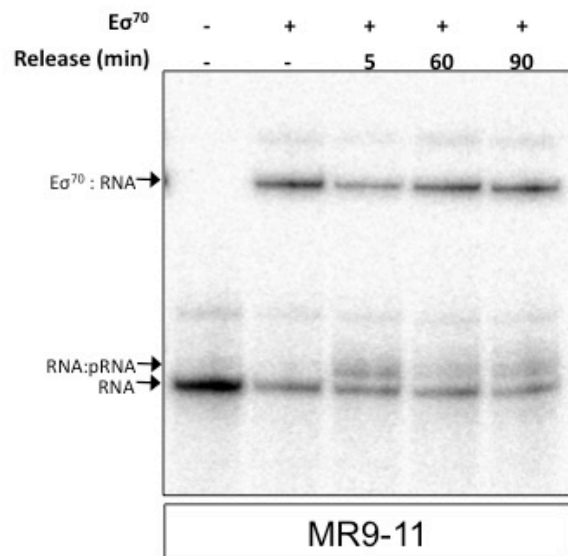


Figure S4: Release kinetics for 6S RNA release-defective mutants. Binding and release capabilities were tested for different constructs and compared to the T1 RNA construct. Body labelled RNA (left lane) was incubated with $E\sigma^{70}$ (second lane of each panel), and release was induced by rapid release conditions. Time points were taken at 5, 60 and 90 minutes to assess the rate at which the RNA is being released from the $E\sigma^{70}$ (remaining lanes). Mutant type is shown at the bottom of each gel. Slower mobility bands were observed in some constructs that remained fixed in time and were interpreted as folding artefacts. We quantified those bands that changed intensity with time and ignored fixed bands (data not shown).





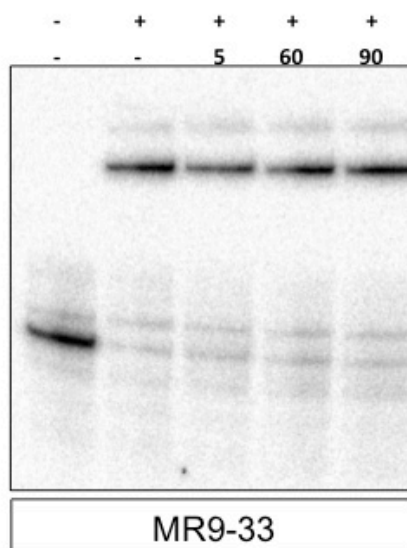
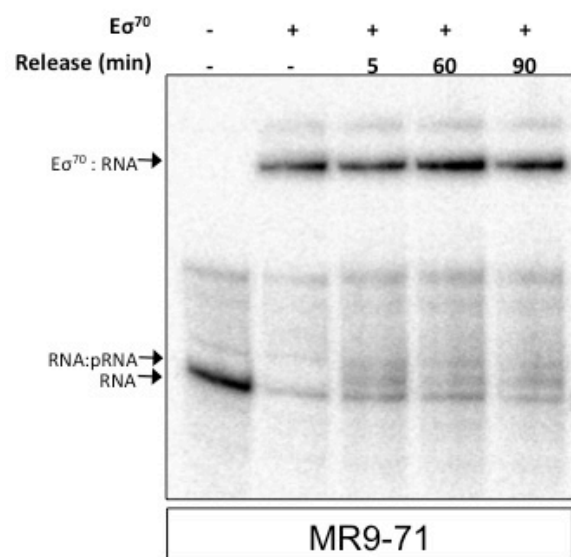
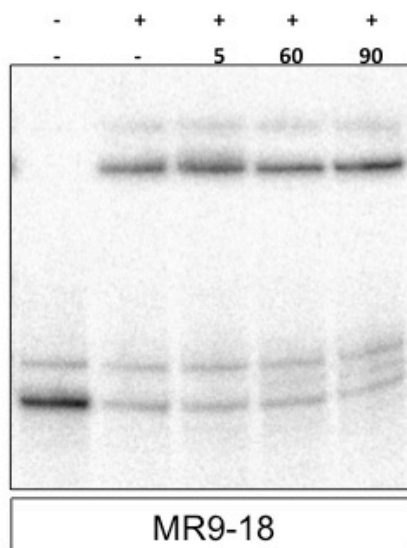
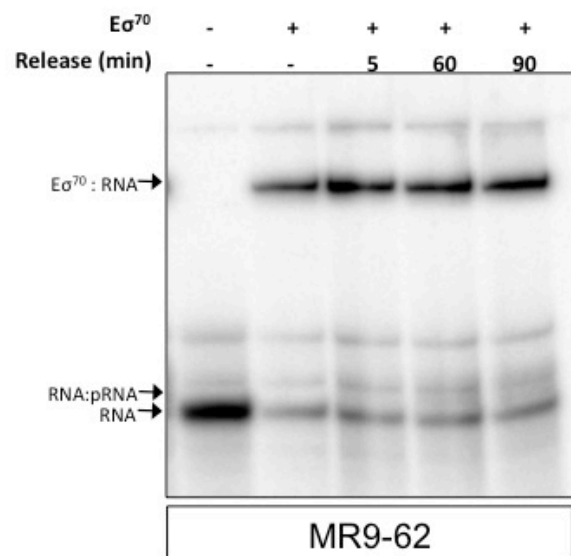


Figure S5: Rapid T1 RNA release kinetics. Binding and release capabilities were tested for the T1 RNA construct. Body labelled RNA (lane 1) was incubated with $E\sigma^{70}$ (lane 2) and release was induced by the addition of 250 μ M of each NTP and 4 mM $MgCl_2$. Time points were taken between 15 and 600 seconds to assess the rate at which the RNA is being released from the $E\sigma^{70}$ (remaining lanes). After 30 s there is no substantial change in the fraction of T1 RNA that remains bound to $E\sigma^{70}$.

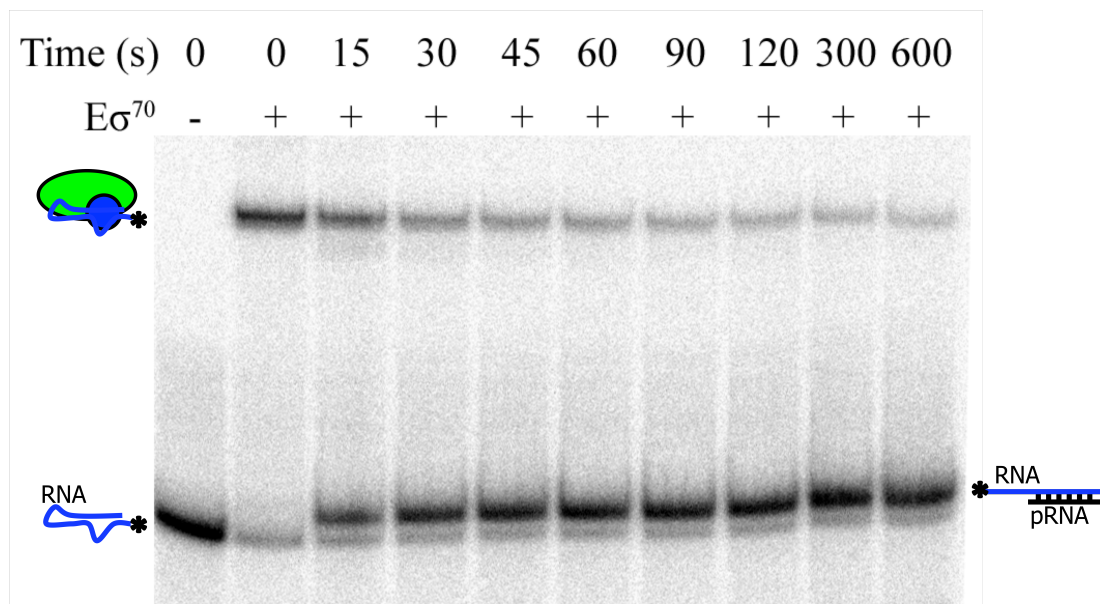


Figure S6: *In vitro* transcription using 6S RNA release-defective mutants as template (next page).

RNA (cold) was bound to $E\sigma^{70}$ and transcription was induced for 30 minutes as described in the Methods section. Control T1 RNA (truncated 6S RNA) and 12 mutant release defective RNAs were used as substrates. The newly synthesized RNAs were labelled using either $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ or $[\alpha\text{-}^{32}\text{P}]\text{-UTP}$. Radiolabeled short pRNA was resolved using denaturing 23% PAGE. The three marker lanes show from left to right are gel purified 13-nt long T1 pRNA, its hydrolysis ladder and a T1 RNase ladder. The letter A stands for $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ label, and U for the $[\alpha\text{-}^{32}\text{P}]\text{-UTP}$ label used in this assay. Note that the hydrolysis and T1 RNase ladders have faster mobilities than the uncleaved pRNA bands due to the presence of an additional 3' phosphate. On the right side of the gel, indicated with a star (*) symbol are the bands that come from contamination in the $[\gamma\text{-}^{32}\text{P}]$ isotopes used as resolved in Figures S8 and 9.

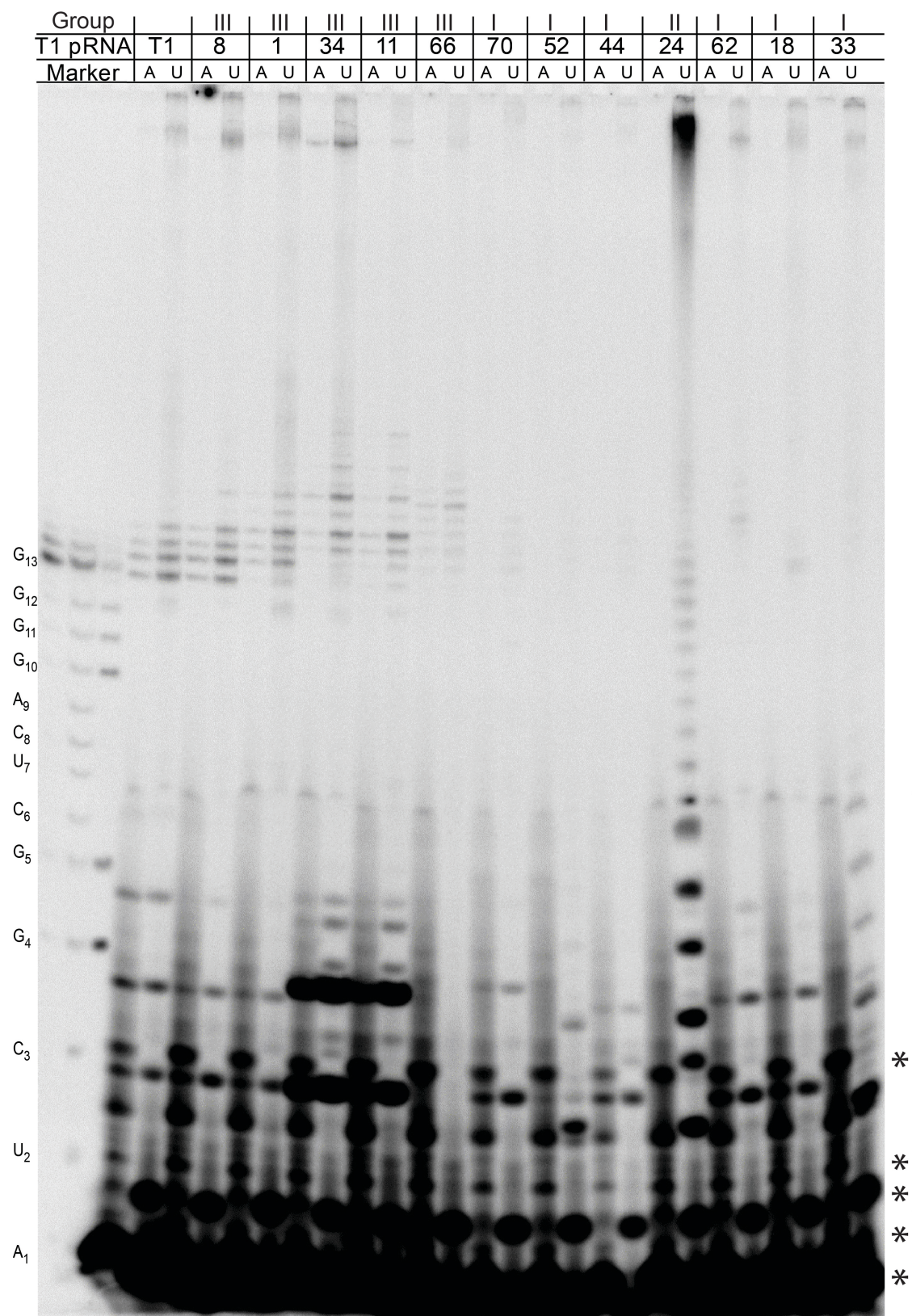


Figure S8: Time course for production of short RNAs and pRNA for R9-24 mutant RNA in presence of different combinations of NTPs. In the case of MR9-24 the long pRNAs were not observed in the presence of only UTP, other NTPs are required for polymerization to occur. The shortest and most abundant product transcribed from the T1 RNA was 13 nt long. Newly synthesized pRNA was labelled with [α - 32 P]-UTP. Samples were incubated in 250 μ M of each NTP and 4 mM MgCl₂ for 1, 5, 10, 20, 40, 80 min, and then analyzed by 23% denaturing PAGE. The first lane on the left is [α - 32 P]-UTP alone under the same buffer conditions and the next 5 lanes are a “No RNA template” control, where an extension reaction was set up in absence of template RNA. These two controls helped us resolve the origin of the lower bands in the gel and be confident that no polymerization occurs in absence of template RNA.

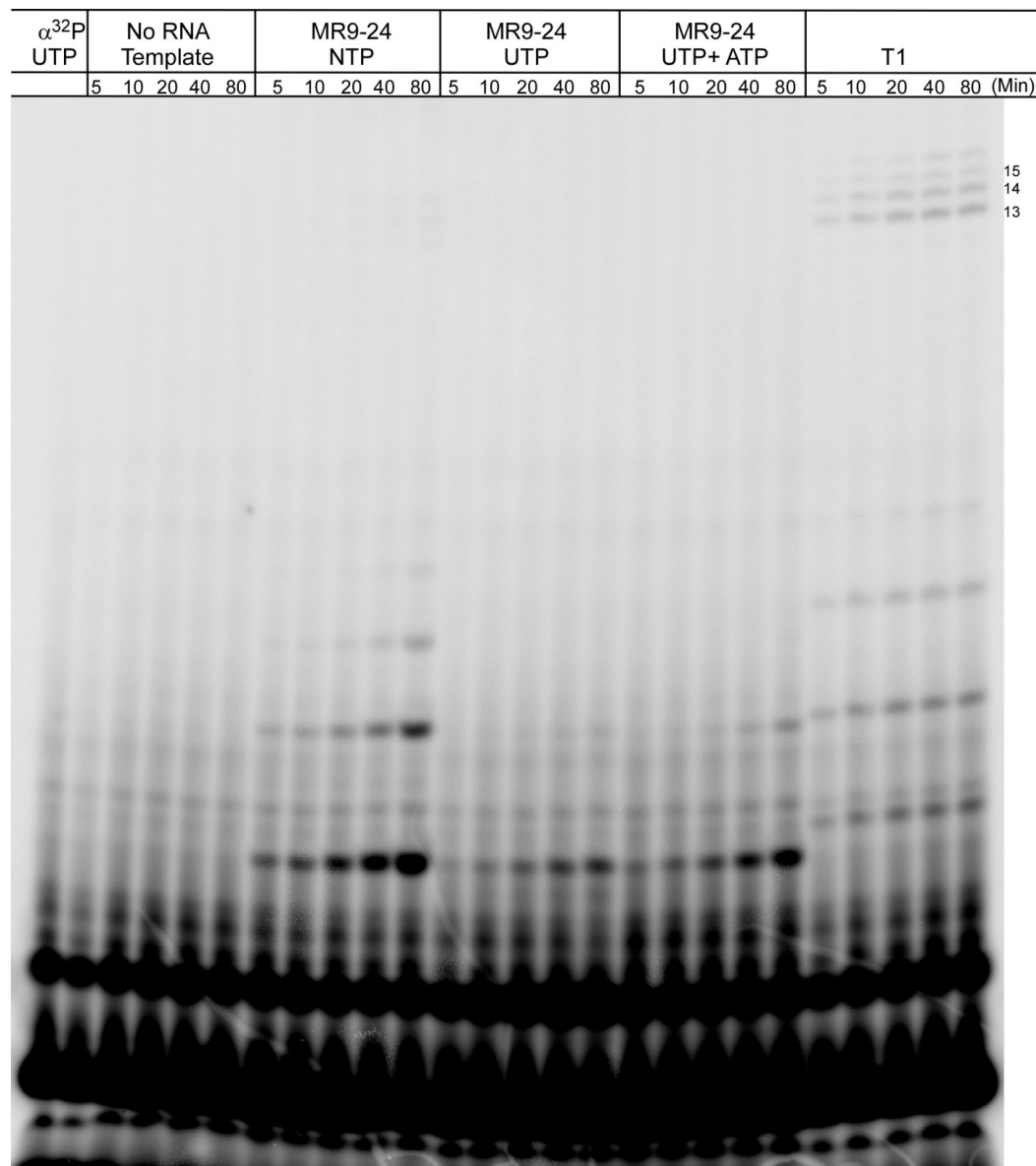


Figure S9: Time course for production of short RNAs and pRNA for release-defective RNAs. Newly synthesized pRNA transcribed from mutant RNAs was labelled with $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$. The pRNA transcribed from the T1 RNA was 13 nt long and was the only one labelled with $[\alpha\text{-}^{32}\text{P}]\text{-UTP}$. Samples were incubated in 250 μM of each NTP and 4 mM MgCl_2 for 1, 5, 10, 20, 40 and 80 min and analyzed by 23% denaturing PAGE. The right most lane is $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ alone under the same buffer conditions.

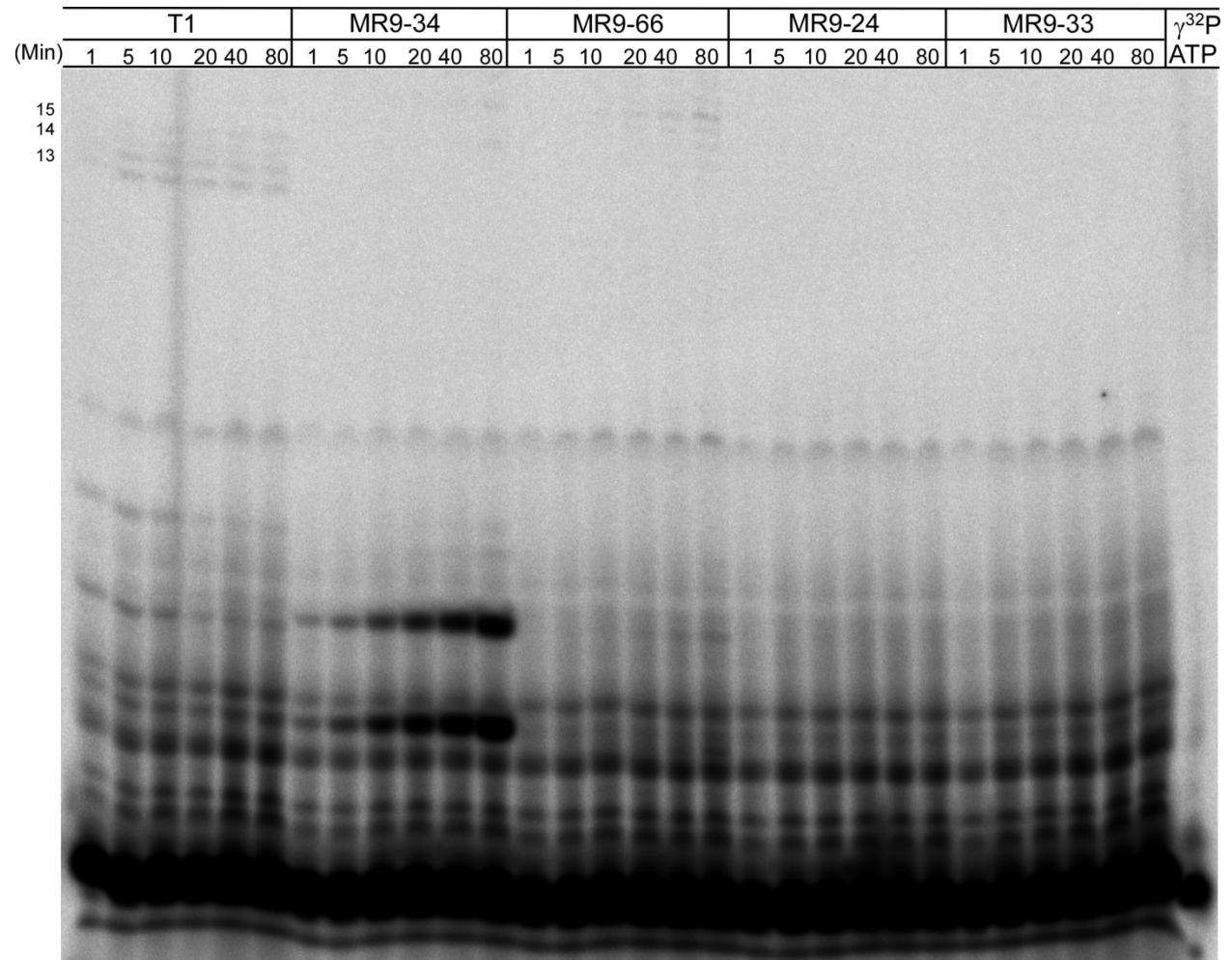


Figure S10: Cloning strategy to express mutant RNA in a pEcoli-Cterm 6x HN vector. The insert was produced by PCR reactions where the restriction enzyme sites, T7 promoter, *Lac* Operator, and terminator were introduced. Plasmid and insert were double-digested with *Sgr*A I and *Cla* I. The pEcoli-Cterm vector was treated with CIAP to avoid self ligation. Four constructs were used throughout the *in vivo* studies: Negative control does not have the insert illustrated below (no transcription should occur from it), pEcoli-T1 has a shortened version of the 6S RNA sequence that behaves like the full length 6S RNA, pEcoli-R9-33 is the selected release defective mutant sequence, pEcoli-LowBinder has the T1 sequence but 50% of it has been randomized and in consequence does not bind well to the $E\sigma^{70}$.

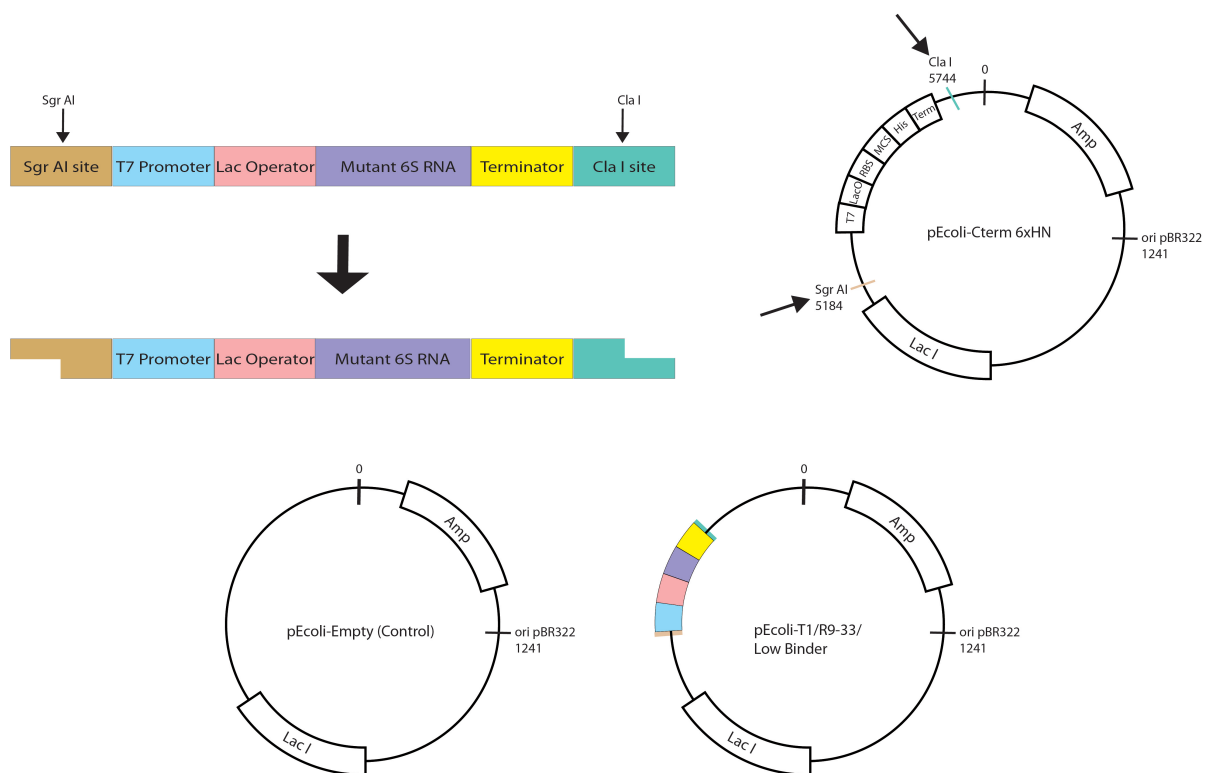
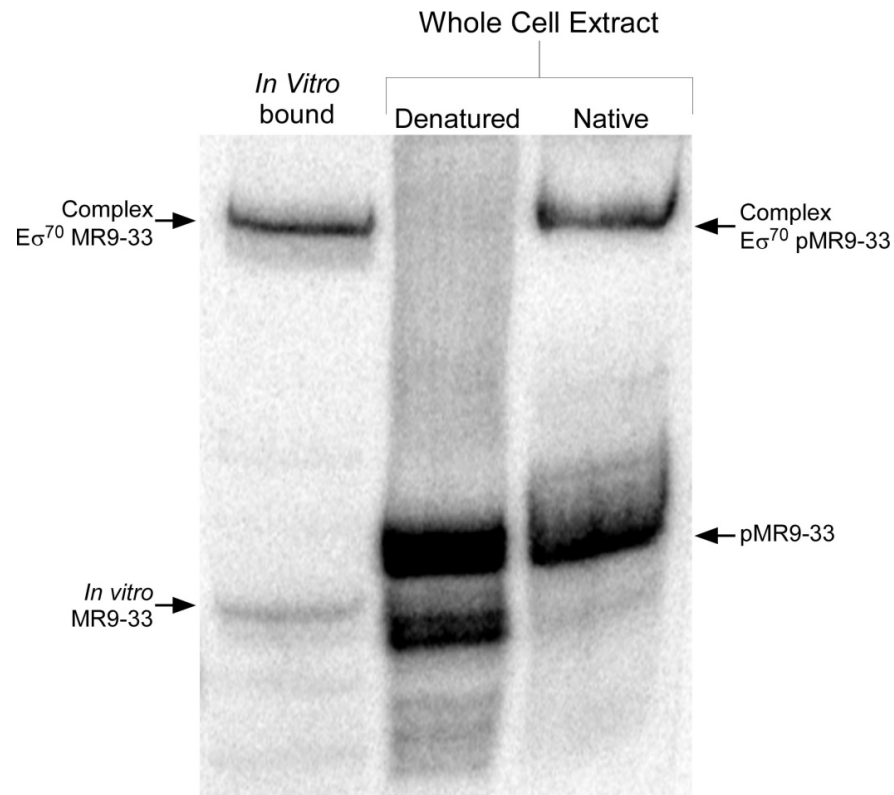


Figure S11: *In vivo* plasmid-derived RNA forms a shifted complex consistent with RNA polymerase binding. Native cell extracts were prepared from BL21 (DE3) pEcoli-R9-33 cells. Five OD₆₀₀ units were taken after 180 min of incubation with 5 mM IPTG. The RNA was run into a 5% native gel, blotted and then hybridized with a MR9-33 RNA probe. The first lane on the left is a positive control, *in vitro* transcribed RNA bound to commercially available E σ^{70} (see Methods). The middle lane is cell extract mixed with denaturing loading dye and heated for 3 min at 99°C. The third lane is the cell extract quenched with native loading dye.



Supplemental Tables

Table S1: Statistical analysis of the 71 clones sequenced. Mutations at each residue were quantified and the calculated frequency of mutation for each residue was compared to the expected mutational frequency (“Expected Freq per mut nt” in the table) expected from the pool design. In the last columns to the right, residues were tagged with three stars as being very interesting since the observed frequencies diverged largely from the null result of 0.033. On the “6S RNA residue” column, some residues are followed by a letter “F” (Fixed) that indicates that the nucleotide was left unchanged during pool design.

	6S RNA residue						Expected Freq. per mut nt		0.033		
								Found mutational frequency for each nt (n.b. zero entered for wild type residue).			
Posit	Wt	A	T	G	C	Total	A	T	G	C	
42	G	26	1		1	28	0.366	0.014	0.000	0.014	***
43	A		4	1		5	0.000	0.056	0.014	0.000	
44	U	26		2		28	0.366	0.000	0.028	0.000	***
45	A		19		8	27	0.000	0.268	0.000	0.113	***
46	U	6			4	10	0.085	0.000	0.000	0.056	*
47	U	2		8	6	16	0.028	0.000	0.113	0.085	**
48	U			3	1	4	0.000	0.000	0.042	0.014	
49	C	4	1	10		15	0.056	0.014	0.141	0.000	**
50	A		5	6		11	0.000	0.070	0.085	0.000	*
51	U	6		1	4	11	0.085	0.000	0.014	0.056	
52	A		6	1	6	13	0.000	0.085	0.014	0.085	
53	C	4	3	4		11	0.056	0.042	0.056	0.000	
Posit	Wt	A	T	G	C						
54	C	1	2	2		5	0.014	0.028	0.028	0.000	
55	A		2	4	3	9	0.000	0.028	0.056	0.042	
56	C	6	5	1		12	0.085	0.070	0.014	0.000	
57	A		4	1	5	10	0.000	0.056	0.014	0.070	
58	A		4	4	4	12	0.000	0.056	0.056	0.056	
							0.000	0.000	0.000	0.000	
59	G	5	7		3	15	0.070	0.099	0.000	0.042	

60	A		3	3	1	7	0.000	0.042	0.042	0.014	
61	A		3	3	1	7	0.000	0.042	0.042	0.014	
62	T	8		5	3	16	0.113	0.000	0.070	0.042	**
63	G		2		1	3	0.000	0.028	0.000	0.014	*
Posit	Wt	A	T	G	C						
64	T	completely conserved				0		0.000	0.000	0.000	**
65	G	8	3		2	13	0.113	0.042	0.000	0.028	
							0.000	0.000	0.000	0.000	
66	G	6	9		6	21	0.085	0.127	0.000	0.085	**
							0.000	0.000	0.000	0.000	
67	C	4		5		9	0.056	0.000	0.070	0.000	*
68	G	3	4			7	0.042	0.056	0.000	0.000	
							0.000	0.000	0.000	0.000	
69	C	5	3	1		9	0.070	0.042	0.014	0.000	*
70	T				3	3	0.000	0.000	0.000	0.042	*
71	C	5	13			18	0.070	0.183	0.000	0.000	**
							0.000	0.000	0.000	0.000	
72	C	7	4	2		13	0.099	0.056	0.028	0.000	*
73	G	2	1			3	0.028	0.014	0.000	0.000	
74	C	2	3			5	0.028	0.042	0.000	0.000	
75	G	11	6		2	19	0.155	0.085	0.000	0.028	**
76	G		2		2	4	0.000	0.028	0.000	0.028	
77	U	1		1		2	0.014	0.000	0.014	0.000	*
78	U	1		1		2	0.014	0.000	0.014	0.000	*
							0.000	0.000	0.000	0.000	
79	G				1	1	0.000	0.000	0.000	0.014	**
80	G		2			2	0.000	0.028	0.000	0.000	*
81	U	completely conserved						0.000	0.000	0.000	**
82	G	completely conserved						0.000	0.000	0.000	**
83	A		8		3	11	0.000	0.113	0.000	0.042	*

							0.000	0.000	0.000	0.000	
84	G	completely conserved				0		0.000	0.000	0.000	**
85	C	3	1			4	0.042	0.014	0.000	0.000	*
							0.000	0.000	0.000	0.000	
86	A	completely conserved				0		0.000	0.000	0.000	**
87	U	11		2	8	21	0.155	0.000	0.028	0.113	***
88	G	5	1		1	7	0.070	0.014	0.000	0.014	
							0.000	0.000	0.000	0.000	
89	CF	completely conserved						0.000	0.000	0.000	
90	TF	completely conserved						0.000	0.000	0.000	
91	CF	completely conserved						0.000	0.000	0.000	
92	GF	completely conserved						0.000	0.000	0.000	
93	GF	1					0.014	0.000	0.000	0.000	
							0.000	0.000	0.000	0.000	
94	UF	completely conserved						0.000	0.000	0.000	
95	CF		3				0.000	0.042	0.000	0.000	*
96	CF		2				0.000	0.028	0.000	0.000	*
97	GF	4	2				0.056	0.028	0.000	0.000	*
98	UF	1			2		0.014	0.000	0.000	0.028	*
							0.000	0.000	0.000	0.000	
99	CF	completely conserved						0.000	0.000	0.000	
100	CF	completely conserved						0.000	0.000	0.000	
101	GF	completely conserved						0.000	0.000	0.000	
102	AF			3			0.000	0.000	0.042	0.000	*
103	GF	completely conserved						0.000	0.000	0.000	
							0.000	0.000	0.000	0.000	
104	A			3			0.000	0.000	0.042	0.000	*
105	A				1		0.000	0.000	0.000	0.014	
							0.000	0.000	0.000	0.000	
106	G	1	4			5	0.014	0.056	0.000	0.000	*

107	C	completely conserved				0	0.000	0.000	0.000	**
							0.000	0.000	0.000	
108	C	completely conserved				0	0.000	0.000	0.000	**
109	U	completely conserved				0	0.000	0.000	0.000	
110	U	13	1		14	0.183	0.000	0.014	0.000	**
111	A	completely conserved				0	0.000	0.000	0.000	**
112	A	completely conserved				0	0.000	0.000	0.000	**
							0.000	0.000	0.000	
113	A		1	1	2	0.000	0.014	0.014	0.000	
114	A		1	3	2	6	0.000	0.014	0.042	0.028
115	C	1	4	1	6	0.014	0.056	0.014	0.000	
116	U	3			4	7	0.042	0.000	0.000	0.056
117	G	4	3		7	0.056	0.042	0.000	0.000	
118	C		1		1	0.000	0.014	0.000	0.000	**
Posit	Wt	A	T	G	C					
119	G	3	5	1	9	0.042	0.070	0.014	0.000	
							0.000	0.000	0.000	0.000
120	A		6	6	12	0.000	0.085	0.085	0.000	
							0.000	0.000	0.000	0.000
121	C	2	2	3	7	0.028	0.028	0.042	0.000	
122	G	7	6	4	17	0.099	0.085	0.056	0.000	**
							0.000	0.000	0.000	0.000
123	A		2	4	3	9	0.000	0.028	0.056	0.042
							0.000	0.000	0.000	0.000
124	C		4	3	7	0.000	0.056	0.042	0.000	
125	A		1	2	3	0.000	0.014	0.028	0.000	
126	C		2	2	4	0.000	0.028	0.028	0.000	
127	A		5	1	1	7	0.000	0.070	0.014	0.014
Posit	Wt	A	T	G	C					
128	U	6		2	8	0.085	0.000	0.028	0.000	

129	U	13		5	1	19	0.183	0.000	0.070	0.014	***
130	C	7	2	2		11	0.099	0.028	0.028	0.000	
							0.000	0.000	0.000	0.000	
131	A		1	1		2	0.000	0.014	0.014	0.000	*
132	C	3	1	3		7	0.042	0.014	0.042	0.000	
133	C	4		1		5	0.056	0.000	0.014	0.000	*
134	U	2				2	0.028	0.000	0.000	0.000	*
135	U	10		2	4	16	0.141	0.000	0.028	0.056	**
136	G	11	14		8	33	0.155	0.197	0.000	0.113	***
137	A		8	3	4	15	0.000	0.113	0.042	0.056	**
138	A		7	3	6	16	0.000	0.099	0.042	0.085	**
139	C	4	6	8		18	0.056	0.085	0.113	0.000	**
140	C	5	4	10		19	0.070	0.056	0.141	0.000	**
141	A		6	5	2	13	0.000	0.085	0.070	0.028	
142	A		5	2		7	0.000	0.070	0.028	0.000	
143	G	1	1		8	10	0.014	0.014	0.000	0.113	

Table S2: DNA oligonucleotides used during PCR reactions, mutagenesis, and Northern blot hybridization.

Primer Sequence	Modification Introduced	Primer Number	Template construct
GCA GGC TGT AAC CCT TGA ACC ttc taa tac gac tca cta tag GCG GGC CAG TCC CCT GAG CCG	T1 Control	21.48 42.12	
GCA GGC TGT AAC CCT TGA ACC CTG TGT TCG AGG TGA ATG	A135G, G140T, T141G	39.34	T1
GCA GGC TGT AAC CCT TGA ACC CTT GGT TCA AGG TGA ATG	G135A, T140G, G141T	39.35	R9-33
ttc taa tac gac tca cta tag GCG GGC CAG TCC CCT GAG CCG AAA TTT CTT TCC ACA AG	T44A, A50T, A52T	59.5	T1
ttc taa tac gac tca cta tag GCG GGC CAG TCC CCT GAG CCG ATA TTT CAT ACC ACA AG 39.34 + 59.5 39.35 + 59.6	A44T, T50A, T52A	59.6	R9-33
ttc taa tac gac tca cta tag GCG GGC CAG TCC CCT GAG CCG AAA TTT CAT ACC ACA AG	T44A	59.7	T1
ttc taa tac gac tca cta tag GCG GGC CAG TCC CCT GAG CCG ATA TTT CTT ACC ACA AG	A50T	59.8	T1
ttc taa tac gac tca cta tag GCG GGC CAG TCC CCT GAG CCG ATA TTT CAT TCC ACA AG	A52T	59.9	T1
ttc taa tac gac tca cta tag GCG GGC CAG TCC CCT GAG CCG ATA TTT CTT TCC ACA AG	A44T	59.10	R9-33
ttc taa tac gac tca cta tag GCG GGC CAG TCC CCT GAG CCG AAA TTT CAT TCC ACA AG	T50A	59.11	R9-33
ttc taa tac gac tca cta tag GCG GGC CAG TCC CCT GAG CCG AAA TTT CTT ACC ACA AG	T52A	59.12	R9-33
GCA GGC TGT AAC CCT TGA ACC CTT GGT TCG AGG TGA ATG	T135C	39.36	T1
GCA GGC TGT AAC CCT TGA ACC CTT TGT TCA AGG TGA ATG	C140A	39.37	T1
GCA GGC TGT AAC CCT TGA ACC CTG GGT TCA AGG TGA ATG	A141C	39.38	T1
GCA GGC TGT AAC CCT TGA ACC CTT TGT TCG AGG TGA ATG	T135C + C140A	39.39	T1
GCA GGC TGT AAC CCT TGA ACC CTG GGT TCG AGG TGA ATG	T135C + A141C	39.40	T1
GCA GGC TGT AAC CCT CGA ACC CTT GGT TCG AGG TGA ATG	T135C + A149G	39.41	T1
ttc taa tac gac tca cta tag GCG GGC CAG TCC CCT GAG CCG ATA TTT CAT ACC ACA AG + 39.41	T135C+A149G+T36C	59.13	T1
GCA GGC TGT AAC CCT TGA ACC CTT GGT TCG AGG TGA ATG 39.42 + 59.13	A149 G	39.42	T1
ttc taa tac gac tca cta tag GCG GGC CAG TCC CCT GAG CCG AAA TTT CTT ACC ACA AG	A149G+ U36C		T1
ttc taa tac gac tca cta tag GCG GGC CAG TCC CCT GAG CCG ATA TTT CAT ACC ACA AG	U44 A + A50 U	59.14	T1
GCA GGC TGT AAC CCT TGA ACC CTG GGT TCA AGG TGA ATG	A44 U + U50 A	59.15	R9-33
ttc taa tac gac tca cta tag GCG GGC CAG TCC CCT GAG CCG AAA TTT CAT ACC ACA AG	T140G + G141T	39.43	T1+6/ R9-33
ttc taa tac gac tca cta tag GCG GGC CAG TCC CCT GAG CCG ATA TTT CTT ACC ACA AG	T50A + T52A	59.16	T1+6/ R9-33
GCA GGC TGT AAC CCT TGA ACC CTT TGT TCG AGG TGA ATG	A44T + T52A	59.17	T1+6/ R9-33
GCA GGC TGT AAC CCT TGA ACC CTG GGT TCG AGG TGA ATG	G141T	39.44	T1+6/ R9-33
GCA GGC TGT AAC CCT TGA ACC CTT GGT TCA AGG TGA ATG	T140G	39.45	T1+6/ R9-33
GCA GGC TGT AAC CCT TGA ACC CTT GGT TCA AGG TGA ATG	C135T	39.46	T1+6/ R9-33
GCA GGC TGT AAC CCT TGA ACC CTG GGT TCA AGG TGA ATG	G135A + T140G	39.47	T1+6/ R9-33
GCA GGC TGT AAC CCT TGA ACC CTT TGT TCA AGG TGA ATG	G135A + G141T	39.48	T1+6/ R9-33
GCA GGC TGT AAC CCT CGA ACC CTT GGT TCA AGG TGA ATG	G135A + A149G	39.49	T1+6/ R9-33
GCA GGC TGT AAC CCT CGA ACC CTT GGT TCG AGG TGA ATG	A149G	39.50	T1+6/ R9-33
GAT GCC TGG CAG TTC CCT ACT CT	Nothern probe 5S rRNA	23.43	n/a
CTT GGT TCA AGG TGA ATG TG	Northern probe for T1/6S	20.117	n/a
AAC CCT GTG TTC GAG GT	Northern probe for R9-33	17.94	n/a
CAC CTG TGG CGC CGG TGA AAT TAA TAC GAC TCA CTA TAG GGG AAT TGT GAG CCG ATA ACA ATT CCG CGG GCC AGT CCC CTG AGC C	Adds T7 promoter, lacO, SgrAI site	85.4	n/a
GAC AGC TTA TCA TCG ATT TTC AGC AAA AAA CCC CTC AAG ACC CGT TTA GAG GCC CCA AGG GGT TAT GCT AGC AGG CTG TAA CCC TTG AAC C	adds T7 terminator, Cla I site.	91.1	n/a
GAG CAT GAA AAG GCT GCA GTT TAT CTC ACG GTC GCT TTC GTT CTT TAG TAC ACC CAA TAC ctc gga cgg acc gag cat gct cac caa ccg cg	LowBlinder	92.1	n/a

Table S3: Point mutations around the large bubble of T1 RNA act in a complex and highly synergistic way. The six mutations found in the LOB of MR9-33 were added to the T1 construct (44, 50, 52 bottom of LOB, 135, 150 & 141 top of LOB). In red the nucleotide sequence for control T1 RNA (Fig. 5B, circles).

Cosntruct Name	10 Upstream Substitutions	Position		Release time (min)*	Release from Eσ70 #	pRNA
		135, 140, 141	44, 50, 52			
T1	No	UCA	UAA	0.8	Yes	Yes +++
T1+6	No	CAC	AUU	30	No	Yes +
T1+U44A +A50U+A52U	No	UCA	AUU	1.5	Yes	Yes +++
T1+U135C +C140A +A141C	No	CAC	UAA	21	Yes	Yes +
T1+U135C	No	CCA	UAA	1	Yes	Yes ++
T1+C140A	No	UAA	UAA	0.9	Yes	Yes ++
T1+A141C	No	UCC	UAA	0.4	Yes	Yes +
T1+U44A	No	ACA	AAA	4.2	No	Yes +
T1+A50U	No	ACA	UUA	2	Yes	Yes ++
T1+A52U	No	ACA	UAU	0.3	Yes	Yes ++
R9-33	Yes	CAC	AUU	0.2	No	No
R9_33+C135U+A140C+ C141A	Yes	UCA	AUU	6	No	No
R9_33+A44U+U50A+U52A	Yes	CAC	UAA	1.2	Yes	Yes ++
R9_33-6 mutations in LOB	Yes	UCA	UAA	8.2	Yes	Yes +
*Time in minutes required for 50% of the mutant RNA bound to the Eσ70, to be released.						
# Full release is defined by at least 80% of the RNA recovering a fast mobility regardless of the presence of pRNA:6S RNA complex.						

Supplemental Methods

T1 RNase Digestions: T1 RNase (Fermentas) was serially diluted in 20 mM Sodium Citrate 6 M Urea and incubated with 5' end labelled RNA at 50 °C for 10 mins to find the optimal dilution. Reactions were quenched by adding 2X denaturing loading dye, snap frozen on liquid nitrogen and resolved in a 23% denaturing PAGE.

Alkaline Hydrolysis: 5' end labelled and gel purified pRNA was incubated with Sodium carbonate (Na_2CO_3) 50 mM and incubated at 80 °C for 10 minutes or until partial digestion is observed. Reaction was quenched by adding 2X denaturing loading dye, snap frozen on liquid nitrogen and resolved in a 23% denaturing PAGE.

Bacterial total RNA extraction: RNA was extracted under denaturing conditions (Rose, Winston and Hieter 1990). Ten millilitres of cell culture at $\text{OD}_{600} = 0.6$ or equivalent amount was harvested and centrifuged at 5,000 rpm for 10 min. The supernatant was discarded, next 2 ml of LETS buffer (0.1 M LiCl, 10 mM EDTA, 10 mM Tris-HCl pH 7.4, 0.2% SDS) and 2 ml of Phenol were added. The cell slurry was vigorously vortexed for 30 seconds and stored on ice for 30 seconds, for a total 6 min. The sample was then centrifuged for 10 min at 11,000 g. The cleared supernatant was transferred to a fresh tube, an equal volume of phenol-chloroform solution (50:50) was added and vortexed vigorously prior to centrifugation for 10 min at 11,000 g. The supernatant was recovered and precipitated with 300 mM NaCl and 2.5 equivalents of ethanol.

Cell extract preparation was adapted from Wassarman and Storz (Wassarman and Storz 2000). *E. coli* cells from 5 OD_{600} units (or equivalent) were centrifuged at 5,000 g for 10 minutes. The pellet was resuspended in 200 μl of lysis buffer (20 mM Tris-HCl pH 8, 150 mM KCl, 1 mM MgCl_2 , and 1 mM DTT) and mixed with an equivalent of 200 μl of 0.1 mm acid washed (concentrated HCl) glass beads before vortexing for 6 minutes alternating 30 seconds of vortex and 30 seconds of cooling on ice. An additional 200 μl of lysis buffer was added and samples were centrifuged at 16,500 g for 20 minutes at 4°C. The cleared extract was aliquoted, flash frozen and stored at -80°C prior to use.

References

- Rose, M.D., Winston, F., and Hieter, P. 1990. *Methods in yeast genetics: a laboratory course manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Shephard, L., Dobson, N., and Unrau, P.J. 2010. Binding and release of the 6S transcriptional control RNA. *Rna* **16**: 885-892.
- Wassarman, K.M. and Storz, G. 2000. 6S RNA regulates E. coli RNA polymerase activity. *Cell* **101**: 613-623.