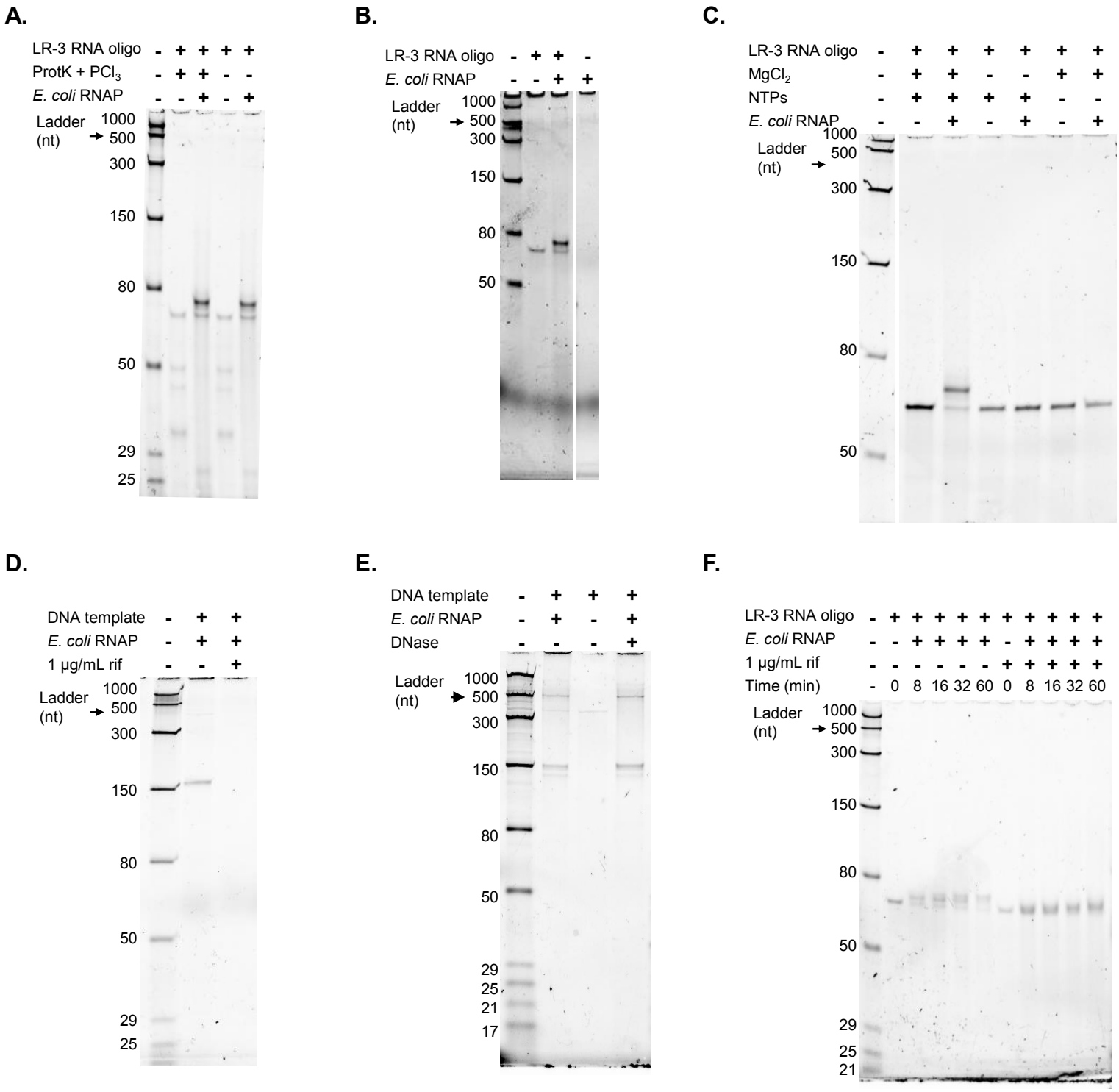
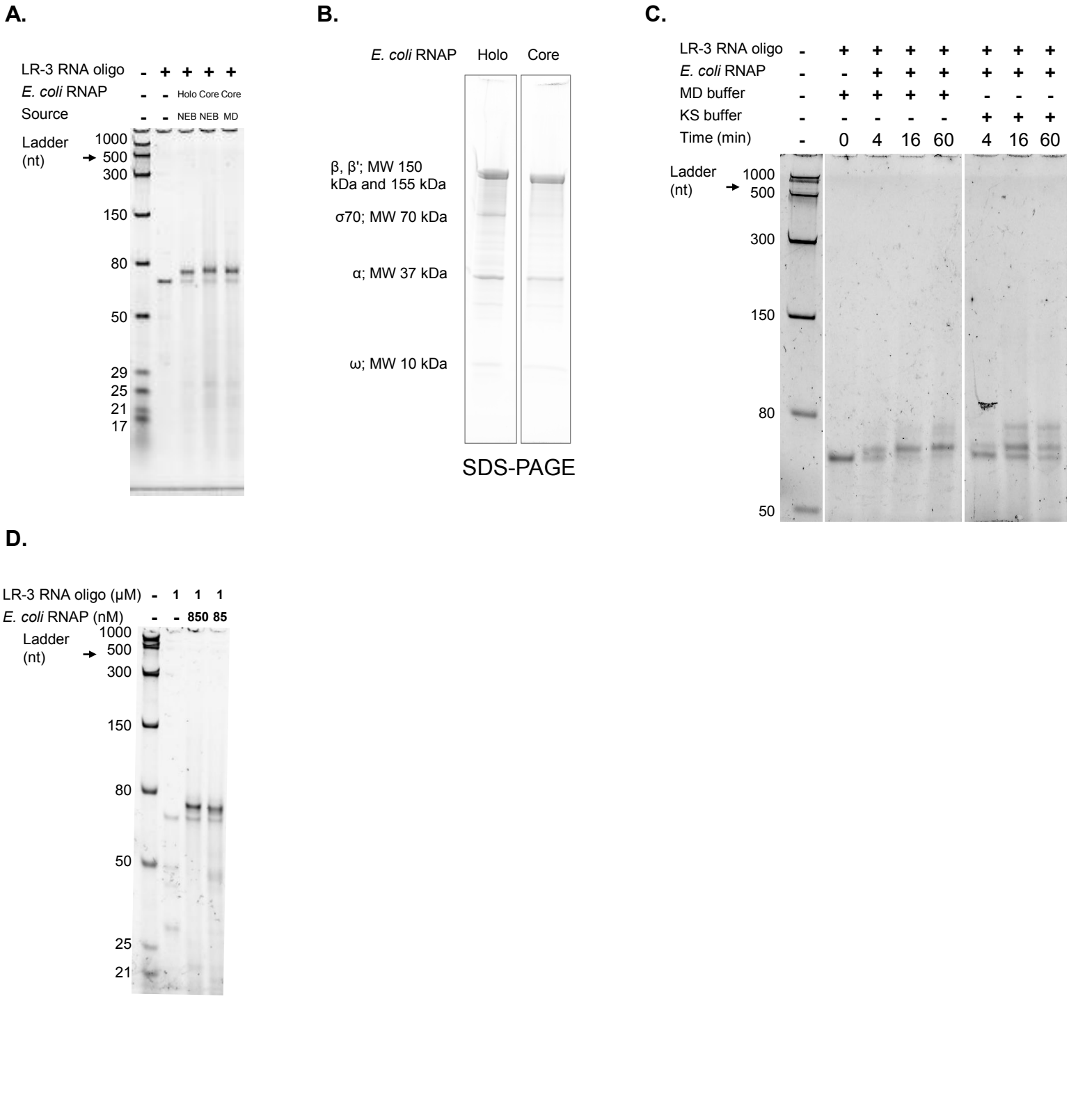
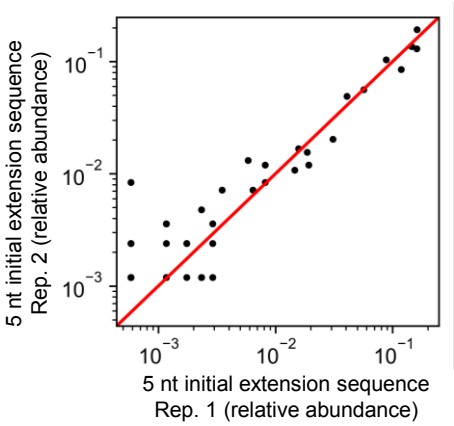


Supplementary Figure 1: **Reaction components required for RNA extension.** A) RNA extension reactions were either digested with Proteinase K, extracted with phenol-chloroform and chloroform, and purified via spin column ("ProtK + PCl_3") or only purified via spin column. B) The sensitivity of RNA extension to lack of RNA template. C) The sensitivity of RNA extension to lack of *E. coli* RNAP, of Mg^{2+}, and of NTPs. D and E) DNA-templated transcription with *E. coli* RNAP. Rif = rifampicin. (E) used gel protocol 2 (see Methods), which allows visualization of the minor band <150 nt. F) Sensitivity of RNA extension to rifampicin. In several panels, nonessential lanes were cropped out and are indicated by white space; all samples were run on the same physical gel. Figs. S1A and S2D came from the same original gel. Figs. 2 (left panel) and S1C came from the same original gel.



Supplementary Figure 2: **Characteristics of RNAP requirements for RNA extension.** A) RNA extension with *E. coli* RNAP holoenzyme ("Holo"; with σ^{70}) or core enzyme ("Core"; without σ^{70}) made either by NEB (our default source unless otherwise specified) or by ourselves ("MD"). B) Analysis of protein composition of *E. coli* RNAP preparations from NEB via SDS-polyacrylamide gel electrophoresis (SDS-PAGE). C) RNA extension in a standard buffer typically used for *E. coli* RNAP-catalyzed transcription ("MD buffer"; see Methods) and in a standard buffer typically used for T7 RNAP-catalyzed transcription ("KS buffer"; our default RNA extension buffer unless otherwise indicated; see Methods). Note that this gel was inadvertently run at 280 V instead of 200 V. D) RNA extension with template in excess. In several panels, nonessential lanes were cropped out and are indicated by white space; all samples were run on the same physical gel. Fig. 1A (left panel) is a subset of Fig. S2A. Figs. S1A and S2D came from the same original gel.

A.



B.

Abundance:	Extension product:
4.5%	5'-CAAUGCUUCUCGAGAUACCCAGAUCAUAUGAAACAGCAUGAC$_U$ 3'-GGG$_{AAGCCCGUACCGUGAGAACUUU}$U
2.5%	5'-CAAUGCUUCUCGAGAUACCCAGAUCAUAUGAAACAGCAUGACUUUUUCAAGAGUGCCAUGC 3'-CGG$_{AAGC}$C
2.4%	5'-CAAUGCUUCUCGAGAUACCCAGAUCAUAUGAAACAGCAUGAC$_U$ 3'-UGGG$_{AAGCCCGUACCGUGAGAACUUU}$U
2.0%	5'-CAAUGCUUCUCGAGAUACCCAGAUCAUAUGAAACAGCAUGACUUUUUCAAGAGUGCCAUGCCC^{G}A 3'-CGGG$_A$
1.2%	5'-CAAUGCUUCUCGAGAUACCCAGAUCAUAUGAAACAGCAUGACUUUUUCAAGAGUGCCAUGCC^{C}G 3'-UACGG$_A$A

C.

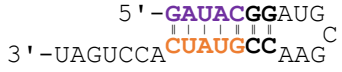
Abundance:	Extension product:
22.2%	5'-CAAUGCUUCUCGAGAUACCCAGAUCAUAUGAAACAGCAUGAC$_U$ 3'-...CUAUGGG$_{AAGCCCGUACCGUGAGAACUUU}$U
19.5%	5'-CAAUGCUUCUCGAGAUACCCAGAUCAUAUGAAACAGCAUGACUUUUUCAAGAGUGCCAUGCCC^{G}A 3'-...GUACGGG$_A$
18.4%	5'-CAAUGCUUCUCGAGAUACCCAGAUCAUAUGAAACAGCAUGACUUUUUCAAGAGUGCCAUGC 3'-...CUCACGG$_{AAGC}$C
13.3%	5'-CAAUGCUUCUCGAGAUACCCAGAUCAUAUGAAACAGCAUGACUUUUUCAAGAGUGCCAUGCCC^{G}A 3'-...UACGGG$_G$A
8.8%	5'-CAAUGCUUCUCGAGAUACCCAGAUCAUAUGAAACAGCAUGACUUUUUCAAGAGUGCCAUGCC^{C}G 3'-...GGUACGG$_A$A

Supplementary Figure 3: **Additional characterization of extension products**. A) For oligonucleotide LR-3' (a 1 nt variant of LR-3; see Fig. 4), the abundance of 5 nt initial extension sequences from two independent experiments (see Methods). Each point is one 5 nt initial extension sequence. Each axis is an independent RNA extension reaction and independent library preparation. The red line plots y=x. Note that counts for points on the lower left portion of the plot are relatively small (e.g., <10 for abundance $<10^{-2}$ for replicate 2); hence these points are subject to greater proportional variability. B) For the LR-3 oligonucleotide, table of the five most abundant extension products of less that 5 nt. We recognize that the interpretation of the origin of short extensions is challenging (because most 2 nt extensions simply by chance will be complementary to the 68 nt template). Note that the percentages reflect the abundance of a specific extension product relative to all extension products and not just extension products of less that 5 nt. See Table S2 for all extension products regardless of length and abundance. C) For the LR-3 oligonucleotide, table of the five most abundant 5 nt initial extension sequences. Note that the percentages reflect the abundance of a specific 5 nt initial extension sequence relative to all extensions of at least 5 nt (not including extensions of less than 5 nt). In (B) and (C), colors for the extension sequences are as in Fig. 3B.

A.

LR-4 extensions: Abundance:	LR-4 extension products:
11.5%	
4.2%	

DG-141 extensions: Abundance	DG-141 extension products:
17.1%	
14.1%	

DG-143 extensions: Abundance	DG-143 extension products:
7.7%	
2.5%	

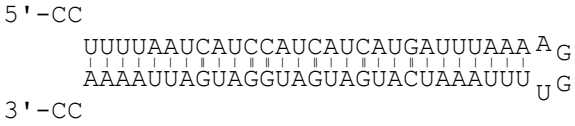
DG-144 extensions: Abundance	DG-144 extension products:
7.2%	
4.8%	

DG-145 extensions: Abundance:	DG-145 extension products:
11.6%	
8.5%	

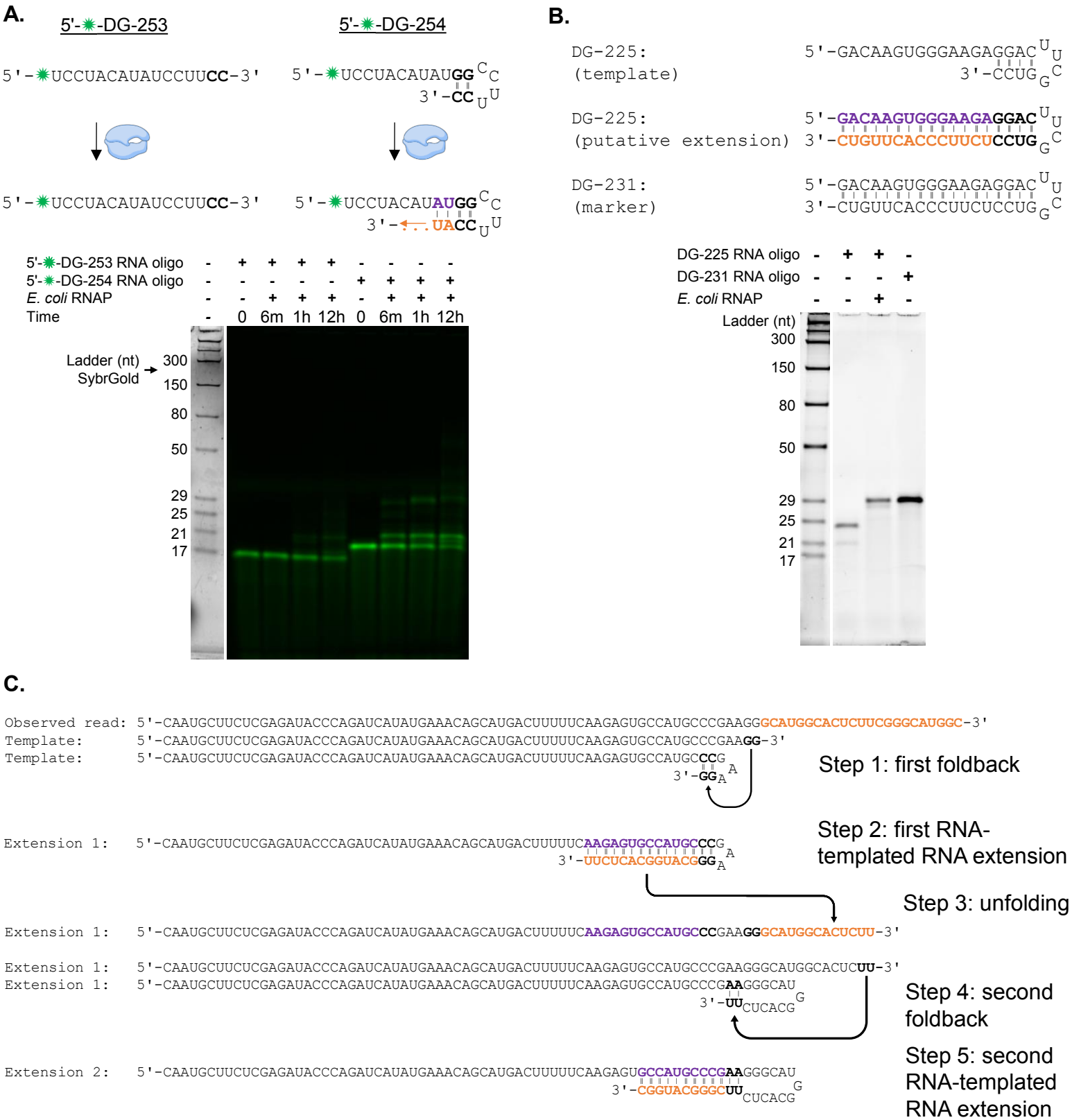
B.

Predicted secondary structure of JTG-20:

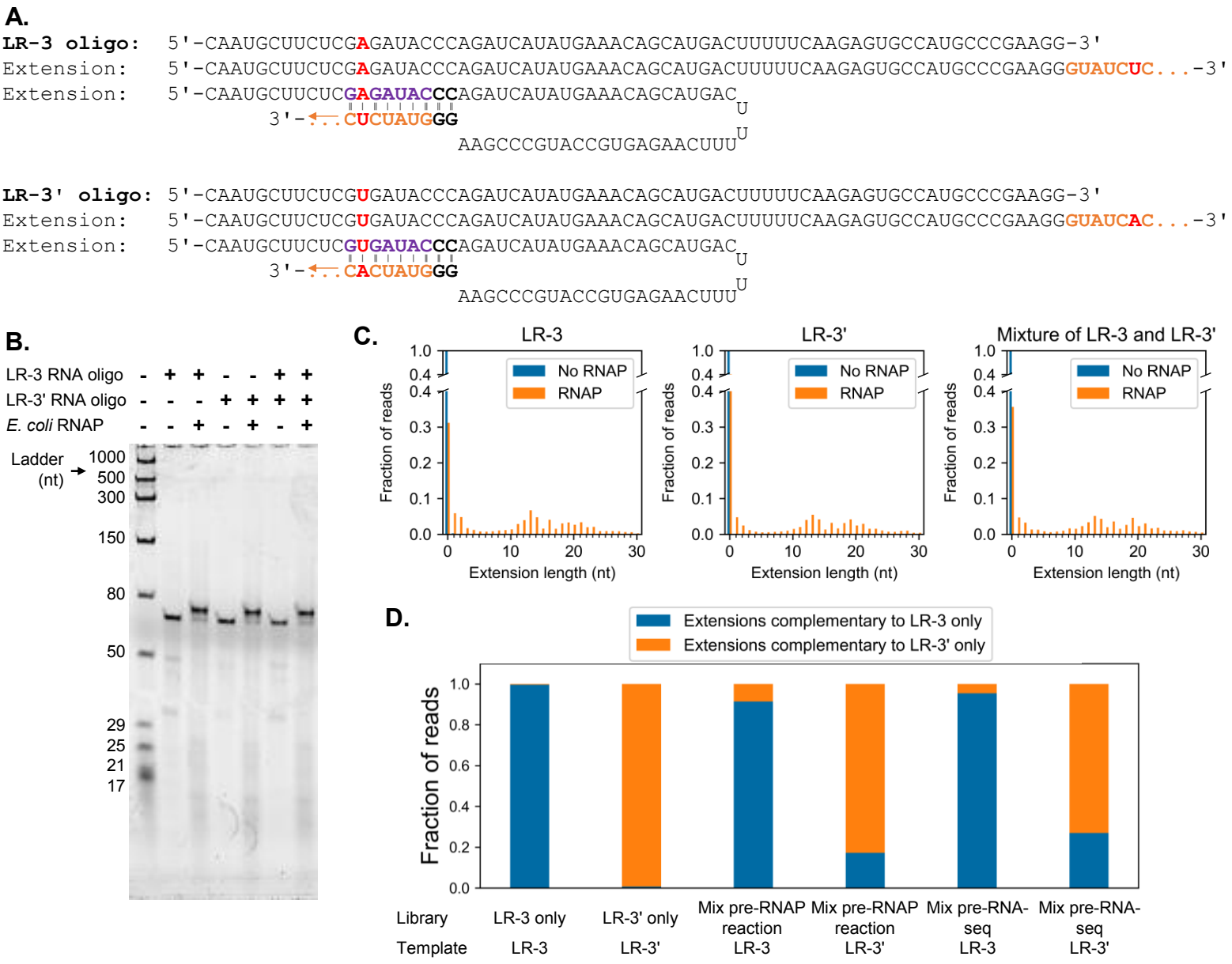
```
CCUUUUAAUCAUCCAUCAUCAUGAUUUAAAAGGUUUUAAAUCAUGAUGAUGGAUGAUUAAAACC
..((((((((((((((((((((((((((((....))))))))))))))))))))))))))))..
```



Supplementary Figure 4: **RNA extensions of diverse RNAs are templated**. A) For various oligonucleotides, tables of the two most abundant extension products of at least 5 nt. Colors are as in Fig. 3B. Note that the percentages reflect the abundance of a specific extension product relative to all extension products and not just extension products of at least 5 nt. See Table S2 for all extension products regardless of abundance. On shorter oligonucleotide templates (e.g., DG-143) on which extension readily reaches the 5' end of the template, we observed chimeric extension sequences in which additional bases were added. B) Predicted secondary structure of JTG-20, on which we did not observe RNA extension.



Supplementary Figure 5: **Templates for RNA-templated RNA extension**. A) Schematic of (top) and electrophoretic analysis of (bottom) two closely related oligonucleotides with and without priming bases. The samples were analyzed crude on a 15% gel with gel protocol 2 (see Methods). The gel is pseudocolored to reflect the 5' TYE 655 endlabel. The fluorophore was imaged first; the gel was then stained with SybrGold and reimaged (to visualize the ladder). B) Schematic of (top) and electrophoretic analysis of (bottom) oligonucleotides used to examine the lengths of *E. coli* RNAP-catalyzed RNA extensions. Gel is 15%. Note that the DG-225 extension product and the DG-231 marker oligonucleotide do not appear to migrate true to size, presumably due to their duplex nature. Figs. 2 (center) and S5B (bottom) came from the same original gel. C) Example of RNA-seq read consistent with an origin via multi-round RNA-templated RNA extension. In all panels, colors for the extension sequences are as in Fig. 3B.



Supplementary Figure 6: **RNA-templated RNA extension of LR-3 in *cis***. A) 7 nt extensions of the LR-3 oligonucleotide (top) and the LR-3' oligonucleotide (bottom). The single base difference between LR-3 and LR-3' is bolded in red; otherwise, colors for the extension sequences are as in Fig. 3B. B) Electrophoretic analysis of the extension of LR-3, LR-3', and an equimolar mixture of LR-3 and (continued on next page)

(continued from previous page) LR-3'. Note that for this experiment the samples were used for RNA-seq and were processed accordingly (see Methods). C) RNA-seq analysis of the length distributions of RNA extensions of LR-3, LR-3', and an equimolar mixture of LR-3 and LR-3'. Note that the plot for LR-3 is reproduced from Fig. 3A and shown here for comparison purposes. D) Oligonucleotides LR-3, LR-3', and an equimolar mixture of LR-3 and LR-3' ("mix pre-RNAP reaction") were extended with *E. coli* RNAP; the three extension reactions, as well as a mixture of the individual LR-3 and LR-3' extension reactions ("mix pre-RNA-seq") were sequenced. For each library and for each template, the fraction of extensions complementary to LR-3 alone (blue) or LR-3' alone (orange) was counted. Extensions complementary to both LR-3 and LR-3' were not counted here. Note that the LR-3 only, LR-3' only, and mix pre-RNAP reaction samples were plotted in Fig. 4B and are shown here for comparison purposes. E) 532 nm illumination of the gel from Fig. 4F (see legend to Fig. 4 for description of this experiment). The gel is pseudocolored to reflect the 5' Cy5 endlabel.

Supplementary Table 1: List of oligonucleotides used in this study.

Name	Sequence; for RNA oligonucleotides, predicted secondary structure and minimum free energy of the predicted secondary structure; source/notes.
LR-3	CAAUGCUUCUCGAGAUACCCAGAUCAUAUGAAACAGCAUGACUUUUUCAAGAGUGCCAUGCCCGAAGG .....((((....(((......))).........((((((((((....))))))..))))))..)))). (-8.90 kcal/mol) IDT 20 nmol RNA Ultramer. Corresponds to a fragment of the coding region of GFP.
5'-FAM LR-3	/6-FAM/ CAAUGCUUCUCGAGAUACCCAGAUCAUAUGAAACAGCAUGACUUUUUCAAGAGUGCCAUGCCCGAAGG .....((((....(((......))).........((((((((((....))))))..))))))..)))). (-8.90 kcal/mol) Genscript 10 nmol RNA oligonucleotide, high-performance liquid chromatography- (HPLC-) purified. Identical in sequence to LR-3.
DG-234	CCAGACCTAAAGACCAGAC IDT 25 nmol DNA oligonucleotide. Used for PCR amplification of DNA transcription template.
DG-235	GTGATTACATTTTCTCTTGAGGG IDT 25 nmol DNA oligonucleotide. Used for PCR amplification of DNA transcription template.
LR-4	UUCGGGCAUGGCACUCUUGAAAAAGUCAUGCUGUUUCAUAUGAUCUGGGUAUCUCGAGAAGCAUUGAA ....(((((((.....)))))))..((((.(((.(((.((....)).)))..))))))) (-14.40 kcal/mol) IDT 20 nmol RNA Ultramer. Corresponds to a fragment of the antisense of the coding region of GFP.
R-12	GACAAUGACAAAAAACACUCACACAGGAGAGGUGAGUCACUCCACACCCUCGUUUUGCUCCGAC .........................(((.((((.(......).)))))))..... (-7.90 kcal/mol) RNA product of *in vitro* transcription. Gift of I.N Zheludev.
JTG-20	CCUUUUAAUCAUCCAUCAUCAUGAUUUAAAAGGUUUUAAAUCAUGAUGAUGGAUGAUUAAAACC ..(((((((((((((((((((((((((....)))))))))))))))))))))))).. (-37.30 kcal/mol) IDT 100 nmol RNA oligonucleotide. The reverse of an RNA species observed to be replicated by T7 RNAP (Konarska and Sharp, 1990).
DG-141	CAAUGCUUCUCGAGAUACCCAGAUCGAAGG .........((((.........)))).. (-2.40 kcal/mol) Genscript 10 nmol RNA oligonucleotide. Internal deletion of LR-3.
DG-36	GUUCAGAGUUCUACAGUCCGACGAUC .... (0.00 kcal/mol) IDT 100 nmol RNA oligonucleotide. Nonrandomized 5' adaptor for RNA-seq (not used for sequencing; used only as an extension template).
DG-37	GAAUUCCACCACGUUCCCGUGG ........(((((....))))) (-5.90 kcal/mol) IDT 100 nmol RNA oligonucleotide. Stop oligonucleotide for RNA-seq.
DG-48	GUUCAGAGUUCUACAGUCCGACGAUCNNNN .... (0.00 kcal/mol) IDT 100 nmol RNA oligonucleotide, HPLC-purified. Randomized 5' adaptor for RNA-seq.
DG-143	GAUACGGAUGCGAACC ....((....)).... (-0.10 kcal/mol) Genscript 10 nmol RNA oligonucleotide, HPLC-purified.

DG-144	GGCAAGUUCUGGACAC `.....` (0.00 kcal/mol) Genscript 10 nmol RNA oligonucleotide, HPLC-purified.
DG-145	GACAGGACUUCGGUCC `....((((....))))` (-4.40 kcal/mol) Genscript 10 nmol RNA oligonucleotide, HPLC-purified.
DG-35	/5Phos/TGGAATTCTCGGGTGCCAAGG/3ddC/ IDT 100 nmol DNA oligonucleotide, polyacrylamide gel electrophoresis- (PAGE-) purified. 3' adaptor for RNA-seq.
DG-3	GCCTTGGCACCCGAGAATTCCA IDT 100 nmol DNA oligonucleotide, PAGE-purified. Reverse transcription primer for RNA-seq.
DG-4	AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA IDT 100 nmol DNA oligonucleotide, PAGE-purified. PCR primer for RNA-seq.
Various	CAAGCAGAAGACGGCATACGAGAT[BARCODE]GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA IDT 100 nmol DNA oligonucleotide, PAGE-purified. 6 nt barcodes are from Illumina TruSeq small RNA kit. PCR primer for RNA-seq.
LR-3' (DG-140)	CAAUGCUUCUCGUGAUACCCAGAUCAUAUGAAACAGCAUGACUUUUUCAAGAGUGCCAUGCCCGAAGG `...((.(((..((((.....))))).)).)).))((((((((((....))))).))))).....` (-12.60 kcal/mol) Genscript 10 nmol RNA oligonucleotide. Contains 1 nt change (underlined) from LR-3.
DG-253	/5TYE665/UCCUACAUAUCCUUCC `.....` (0.00 kcal/mol) IDT 100 nmol RNA oligonucleotide, HPLC-purified. No predicted priming bases. Can prime in *trans* with DG-256. Spectrally distinct from DG-256.
DG-254	/5TYE665/UCCUACAUAUGGCCUUCC `.....` (0.00 kcal/mol) IDT 100 nmol RNA oligonucleotide, HPLC-purified. Contains GG insertion relative to DG-253 to allow priming in *cis*. Can prime in *trans* with DG-256. Spectrally distinct from DG-256.
DG-225	GACAAGUGGGAAGAGGACUUCGGUCC `.............((((....))))` (-4.40 kcal/mol) IDT 100 nmol RNA oligonucleotide.
DG-231	GACAAGUGGGAAGAGGACUUCGGUCCUCUUCCCACUUGUC `((((((((((((((((((....))))))))))))))))))` (-34.70 kcal/mol) IDT 100 nmol RNA oligonucleotide. Marker oligonucleotide for extension of DG-225 to its 5' terminus.
DG-256	/5Cy3/CAACAGAAGCAAGG `.....` (0.00 kcal/mol) IDT 100 nmol RNA oligonucleotide, HPLC-purified. No predicted priming bases. Can prime in *trans* with DG-253 and DG-254. Spectrally distinct from DG-253 and DG-254.