Supplemental Data

Passenger-Strand Cleavage Facilitates Assembly of siRNA into Ago2-Containing RNAi Enzyme Complexes

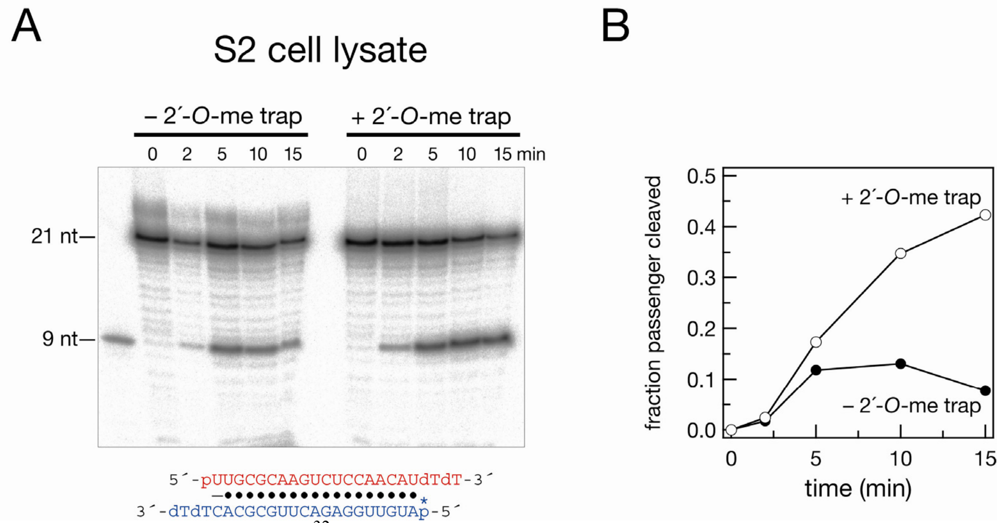
Christian Matranga, Yukihide Tomari, Chanseok Shin, David P. Bartel, and Phillip D. Zamore

Supplemental Experimental Procedures

Reverse-Phase HPLC Purification of Phosphorothioate Diastereomers

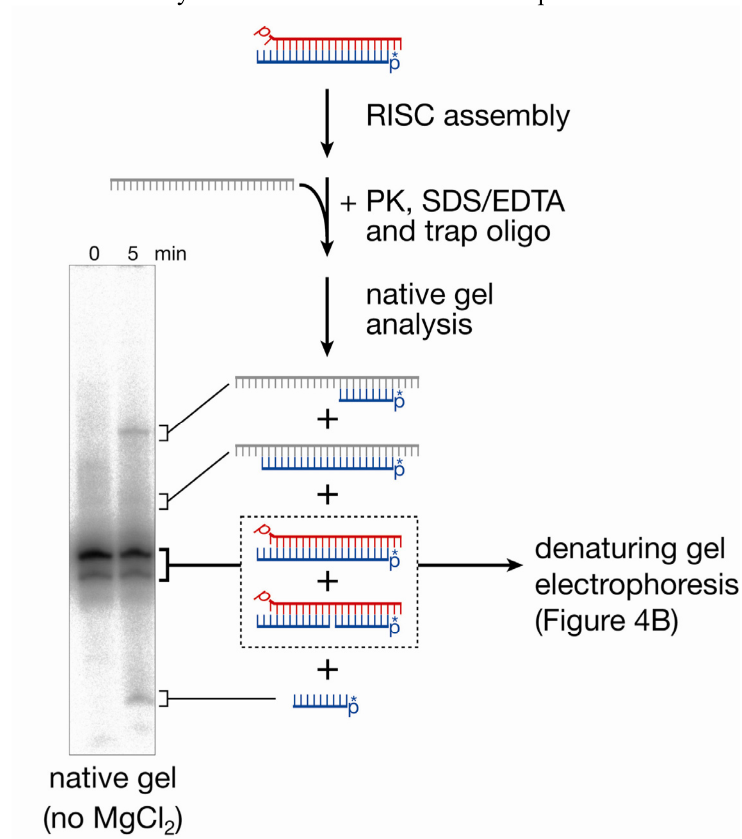
Twenty-one nt siRNA passenger strand containing a single phosphorothioate (PS) modification between nucleotides 9 and 10 was ethanol precipitated, resuspended in 0.1 M NH_4OAc (Buffer A), and 50 µg of RNA injected onto a Targa C18 HPLC column (Nest Group). The column was eluted with a 0-15% gradient of Buffer B (0.1 M NH_4OAc, 50% [v/v] acetonitrile) over 15 min followed by a 15-23% of Buffer B gradient over 45 min. Rp and Sp 21-mer retention times were approximately 24 min and 26 min, respectively. Purified diastereomer peak fractions were pooled, lyophilized, then redissolved in water and lyophilized again, for three cycles of lyophilization, flash-freezing in liquid nitrogen after each time dissolution in water. Sample recovery was about 50%. Diastereomer identity was established by nuclease P1 treatment. Samples were treated with 2 units nuclease P1 (US Biological) for 60 min at 37˚C, dephosphorylated with calf intestinal phosphatase (New England Biolabs), and then the resulting nucleosides were separated on a Targa C18 HPLC column (Nest Group), run first in 0-8% Buffer B over 25 min, followed by elution with a 8-30% Buffer B over 35 min. The characteristic nuclease P1-resistant Rp CU dinucleotide eluted from the column at 33 min. To confirm the identity of the dinucleotide, chiral dinucleotide standards were prepared by separating a racemic mixture of a PS-modified CU dinucleotide (Dharmacon) by reverse phase-HPLC. The identities of the two peaks were confirmed by their resistance (Rp) or sensitivity (Sp) to digestion with nuclease P1. Integrity of the diastereomerically pure RNA was confirmed by electrospray mass spectrometry (Keck Facility, Yale University).

Figure S1. Passenger-Strand Cleavage in Schneider 2 (S2) Cell Lysate



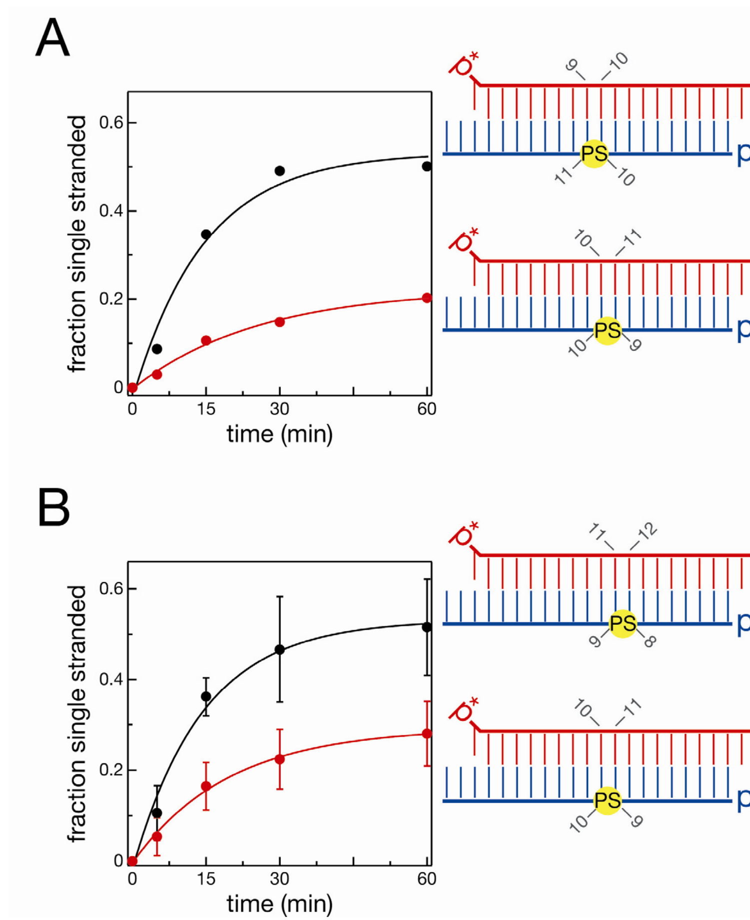
(A) The sod1 siRNA (Figure 1A), 5´-^{32}P-radiolabeled on the passenger strand, was incubated in an in vitro RNAi reaction prepared with *Drosophila* S2 cell lysate, in the absence or presence of a 2´-*O*-methyl oligonucleotide complementary to the passenger strand.
(B) Quantification of the data in (A).

Figure S2. Two-Dimensional Analysis of Double-Stranded siRNA Species



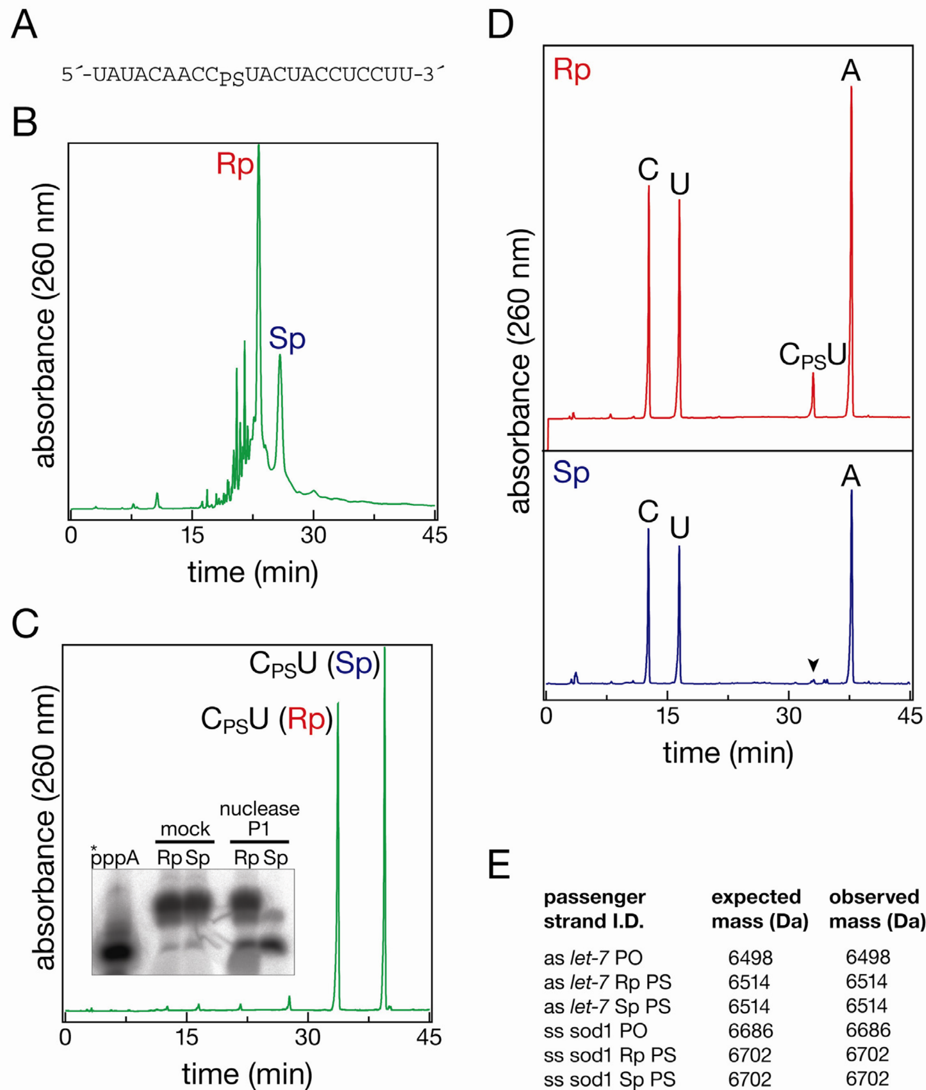
The sod1 siRNA, bearing a 5′-^{32}P-radiolabeled passenger strand, was incubated in a RISC assembly reaction with a 2′-*O*-methyl oligonucleotide complementary to the passenger strand. The identity of the bands was assigned by their comigration with synthetic RNA heteroduplexes as diagrammed. The species corresponding to double-stranded siRNA were excised from the gel and analyzed by denaturing polyacrylamide-urea gel electrophoresis. The denaturing gel analysis is shown in Figure 4B. Unlike that in Figure 3E, the native gel in this experiment did not contain Mg^{2+}. Consequently, the heteroduplex formed between the 2′-*O*-methyl oligonucleotide and the 9-nt passenger-strand cleavage product partially dissociates during electrophoresis, producing some free 9-nt, passenger-strand cleavage fragment.

Figure S3. A Single Racemic Phosphorothioate Modification on the Passenger Strand Inhibited siRNA Unwinding



Production of single-stranded guide siRNA, a measure of active RISC assembly, was monitored using a 5′-^{32}P-radiolabeled guide strand (red) annealed to a passenger containing a racemic phosphorothioate at the linkage between nucleotides 9 and 10 or between nucleotides 10 and 11 (A) or between nucleotides 8 and 9 of the passenger strand (B). Data in (B) correspond to the average of three trials ± standard deviation. The rates of unwinding of the siRNAs bearing racemic phosphorothioate modification between passenger strand nucleotides 10 and 11 or 8 and 9 were essentially indistinguishable from that of the all-phosphodiester siRNA (see Figure 5E).

Figure S4. Purification of the Phosphorothioate-Modified siRNA Diastereomers by Reverse-Phase HPLC



passenger strand I.D.	expected mass (Da)	observed mass (Da)
as *let-7* PO	6498	6498
as *let-7* Rp PS	6514	6514
as *let-7* Sp PS	6514	6514
ss sod1 PO	6686	6686
ss sod1 Rp PS	6702	6702
ss sod1 Sp PS	6702	6702

(A) The sequence of the phosphorothioate (PS) -substituted *let-7* siRNA passenger strand.
(B) The diastereomers of the passenger strand were resolved by reverse-phase, HPLC on a C18 column. The identity of the Rp and Sp peaks were confirmed by sensitivity to nuclease P1 (D) and by mass spectrometry (E).
(C) Chiral dinucleotide standards were prepared by separating a racemic mixture of a PS-modified $C_{PS}U$ dinucleotide by reverse phase-HPLC. The identities of the two peaks were confirmed by their resistance (Rp) or sensitivity (Sp) to digestion with nuclease P1 (inset).
(D) The HPLC-purified, 21-nt passenger-strand diastereomers were treated with nuclease P1, and then with calf intestinal phosphatase to remove 5´-monophosphates, and, lastly, resolved by reverse phase-HPLC of the resulting nucleosides. The Rp phosphorothioate dinucleotide prepared in (C) was used as a marker to confirm the identity of the $C_{PS}U$ peak in (D). The early eluting peak in (B) corresponds to the Rp diastereomer, because it contains a $C_{PS}U$ dinucleotide refractory to nuclease P1. The late eluting peak in (B) was fully converted to nucleosides by treatment with nuclease P1 followed by phosphatase and must therefore be the Sp diastereomer. A, adenosine; C, cytidine; U, uridine. Antisense *let-7* contains no guanosine.
(E) The purified 21-nt RNA diastereomers were analyzed by negative ion electrospray mass spectrometry to confirm their integrity.

Figure S5. Phosphorothioate Modification of the Scissile Passenger-Strand Phosphate Inhibited Cleavage for the *let-7* siRNA

(A) Diastereomers of the *let-7* siRNA passenger strand were purified for the *let-7* siRNA.
(B) Passenger cleavage was detected for the Rp diastereomer only in the presence of supplemental Mn^{2+}. No passenger-strand cleavage was observed for the Sp siRNA.

Figure S6. Phosphorothioate Modification of the Scissile Phosphate of the Passenger Strand Dramatically Inhibited Active RISC Assembly in HeLa S100, Relative to Modification of the Adjacent Linkages

Target cleavage activity was monitored using sod1 siRNA with racemic phosphorothioate modification (PS) between nucleotides 8 and 9, 9 and 10 (the scissile phosphate), or 10 and 11 of the passenger strand. The sod1 siRNA with an all-phosphodiester (PO) passenger strand was used as a control.