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Untemplated Oligoadenylation Promotes Degradation of RISC-Cleaved Transcripts

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In the best-characterized mechanism of RNA-mediated silencing, small interfering RNAs (siRNAs), incorporated into the RNA-induced silencing complex (RISC), guide the endonucleolytic cleavage of complementary RNAs (1). In *Drosophila melanogaster*, these RISC-generated products are eventually degraded by exonucleases: Xrn1, a 5'-to-3' exonuclease, and exosome, a 3'-to-5' multisubunit exonuclease (2). Interestingly, in *Arabidopsis thaliana* and in mammals, an oligouridine or oligoadenine [oligo(U/A)] tail is added to the 5' RNA fragments resulting from microRNA-directed cleavage (3). However, the biological role of this tail remains unclear.

An RNA interference-defective mutant strain of the alga *Chlamydomonas reinhardtii* provided some insight into this process. We had previously generated a strain (Maa7-IR44) that expresses an inverted repeat (IR) homologous to ~60 base pairs (bp) in the 3' untranslated region (UTR) of the *MAA7* gene (encoding tryptophan synthase β subunit) (fig. S1). In this line, the *MAA7* transcripts are present at reduced quantities compared with the amounts in the nontransgenic wild-type strain (CC-124) (Fig. 1A). In contrast,

in a mutant strain, Mut-68, obtained by insertional mutagenesis of Maa7-IR44, we detected increased amounts of the full-length *MAA7* mRNAs and a faint smear of RNA degradation products (Fig. 1A). Unexpectedly, Mut-68 also showed higher quantities of siRNAs than those of the Maa7-IR44 parental strain (Fig. 1B). This result suggested that Mut-68 was deficient in the decay of siRNA-targeted mRNAs.

DNA flanking the tagging plasmid was obtained by polymerase chain reaction (PCR), and Southern blot analyses revealed that Mut-68 contained a deletion at the *MUT68* locus that abolished mRNA expression (Fig. 1A). To confirm that this deletion was responsible for the observed phenotype, we used the *MUT68* coding sequence under the control of *PsaD* (encoding a photosystem I component) regulatory sequences to successfully complement the mutant strain [Fig. 1A, Mut-68(*MUT68*) lines].

The *MUT68* protein (*MUT68_p*) belongs to the noncanonical polyadenylate [poly(A)] polymerase family (fig. S1). Two other homologs, *Caenorhabditis elegans* RDE-3 and *Schizosaccharomyces pombe* Cid12, have recently been implicated in siRNA production and/or stabiliza-

tion (4, 5). However, *Chlamydomonas* Mut-68 is not defective in siRNA production. Thus, we examined whether *MUT68* might be responsible for the addition of an oligo(U/A) tail to RISC-generated RNA fragments. By using a reverse transcription PCR (RT-PCR) approach that relies on RNA circularization (3), we inspected the 3' ends of *MAA7* 5' RNA fragments resulting from siRNA-directed cleavage. The clones from Maa7-IR44 were quite heterogeneous at their 3' ends, and some terminated before the region of homology to the transgene-produced double-stranded RNA (dsRNA) (Fig. 1C), suggestive of 3'-to-5' degradation. Interestingly, nearly 45% of the examined clones contained an untemplated oligo(A) tail (Fig. 1D). In contrast, the clones sequenced from Mut-68 ended at fairly discrete sites, and none included untemplated nucleotides (Fig. 1C). The complemented strain, Mut-68(*MUT68*)-3, showed a clonal distribution similar to that of the Maa7-IR44 strain. We also transformed Maa7-IR44 with a second IR transgene designed to suppress the *CSL4* exosome subunit to test whether this would result in stabilization of the RISC-generated 5' RNA products. Indeed, in this strain (*Csl4*-IR2) most *MAA7* 5' RNA fragments had very discrete 3' ends, terminating at two sites within the region of homology to the *MAA7* dsRNA (Fig. 1C and fig. S1).

These results suggest that untemplated oligoadenylation by *MUT68*p stimulates the degradation of RISC-generated 5' RNA fragments by a 3'-to-5' exonuclease, most likely the exosome. This is reminiscent of the proposed role of the Trf4/Air2/Mtr4 polyadenylation complex in the decay of nuclear transcripts in *S. cerevisiae* (6). Moreover, *MUT68*p may also participate in the degradation of siRNAs, because their amounts are increased in Mut-68 and U and/or A addition to small RNAs has been detected in *Arabidopsis* (7).

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8. Supported by NIH (grant GM62915) and NSF (grant MCB-0544448). *MUT68* has GenBank accession number DQ989285.

Supporting Online Material

www.sciencemag.org/cgi/content/full/314/5807/1893/DC1

Materials and Methods

Fig. S1

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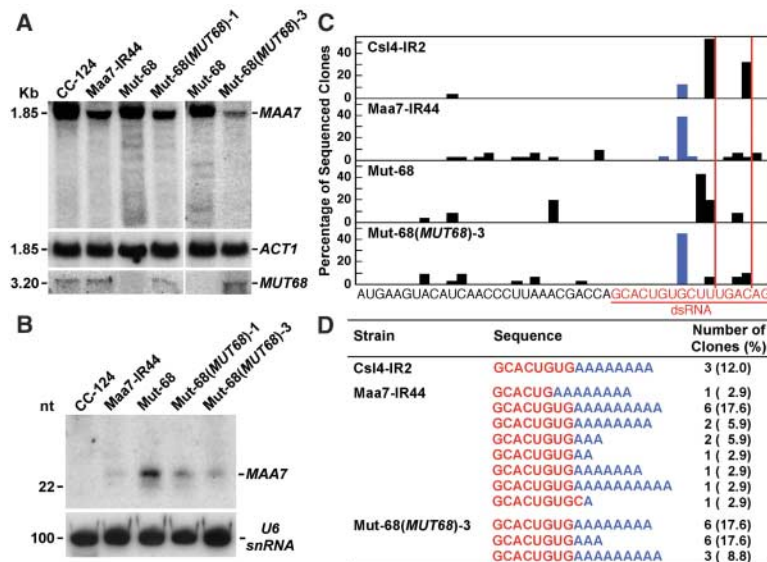


Fig. 1. (A) Northern blot analysis of the indicated strains using probes specific for the 5' end of the *MAA7* coding sequence, the *ACT1* 3' UTR sequence, and the *MUT68* coding sequence. (B) Detection of *MAA7* siRNAs by Northern hybridization. nt, nucleotide. (C) Frequency of circularized RT-PCR products ending at specific sites within the *MAA7* transcript (fig. S1). Clones containing untemplated adenines are denoted by blue bars, and the sites of RISC-directed cleavage (determined in the *CSL4*-suppressed strain) are shown by vertical red lines. (D) Sequence of circularized RT-PCR products displaying noncoded adenines (blue).



Supporting Online Material for

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This PDF file includes:

Materials and Methods
Fig. S1
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Supporting Online Material

Materials and Methods

Culture conditions, transformation, and selection procedures

Unless noted otherwise, *C. reinhardtii* cells were grown photoheterotrophically in Tris-Acetate-Phosphate (TAP) medium (S1). In order to generate transgenic strains, cells were transformed with linearized plasmid DNA by the glass beads procedure (S2). The construction of a *Ble* transgene containing a *MAA7* inverted repeat (IR) sequence in its 3' UTR has been previously described (S3). *Maa7-IR44* was generated by transformation of the wild type strain (CC-124) with this construct and selection on TAP medium containing 5 µg/ml zeocin (bleomycin) and 7 µM 5-fluoroindole (5-FI) (S3). A transgenic strain containing also an IR construct homologous to the *CSL4* gene was obtained by similar procedures (S3), using a vector conferring resistance to hygromycin B (S4). To identify RNAi-defective mutants, *Maa7-IR44* was mutagenized by transformation with plasmid DNA conferring resistance to paromomycin (S5). Antibiotic resistant transformants were then tested for their ability to grow in the presence of zeocin while dying in the presence of 5-FI.

RNA analyses

Total cell RNA was purified with TRI Reagent (Molecular Research Center), according to the manufacturer's instructions. The isolated RNA was separated by agarose/formaldehyde gel electrophoresis, blotted onto nylon membranes, and

hybridized with ^{32}P -labeled probes (S6-S8). In order to assay for the presence of small RNAs, TRI Reagent isolated total RNA was fractionated through Microcon YM-100 centrifugal devices (Millipore) to remove high molecular weight transcripts. Small RNAs were concentrated from the filtrate by ethanol precipitation, resolved in 15% polyacrylamide/7 M urea gels, and electroblotted to Hybond-XL membranes (S3). Blots were hybridized with ^{32}P -labeled DNA probes at 35°C for 48 hours using the High Efficiency Hybridization System (Molecular Research Center). Strand-specific riboprobes were generated by *in vitro* transcription with the Maxiscript kit (Ambion).

Circularization reverse transcriptase polymerase chain reaction (cRT-PCR)

Total cell RNA was treated with DNase I, de-capped with tobacco acid pyrophosphatase, and circularized with T4 RNA ligase as previously described (S9-S10). First strand cDNA was synthesized with the Maa7-R1 primer (5'-CTTCAGGATAGCCTCCATCTCC-3') following a published protocol (S11). Two semi-nested PCRs were then performed, first with primers Maa7-R2 (5'-GATCAGGGTCTCAGGCACGTA-3') and Maa7-F2 (5'-ACGTCAACAACGCCATGAAGT-3'), and then with primers Maa7-R3 (5'-AGCAGCAATGCGAACAGTAGAC-3') and Maa7-F2. PCR was carried out with high fidelity Taq polymerase (Roche) for 30 cycles at 93°C for 30 s, at 55°C for 30 s, and at 71°C for 60 s (S3, S8). The amplified PCR products were cloned into the pSTBlue-1 vector (Novagen), by standard procedures (S7), and sequenced with T7 and SP6 primers annealing at regions flanking the multiple cloning site.

Supplemental Figure S1. (A) Schematic representation of the *MAA7* transcript. The regions of homology to the transgene-produced dsRNA and to the probes used for northern blot hybridization are indicated by black lines. Part of the *MAA7* sequence is shown underneath the diagram, with the ~60 bp region homologous to the dsRNA trigger highlighted in red and underlined. The putative sites of RISC-mediated cleavage (determined in a strain suppressed for the CSL4 exosome subunit) are indicated by black arrows and the sites of untemplated oligo(A) addition are shown with blue arrowheads. The forward primer used for circularized RT-PCR is also shown. (B) Northern blot analysis of the indicated strains. The same filters were sequentially hybridized with probes specific for the 3' end of the *MAA7* coding sequence and the *ACTIN1* 3' UTR sequence. A faint smear of *MAA7* RNA degradation products was detected in Mut-68 with the probe hybridizing to the 5' end of the coding sequence (Fig. 1A). However, this smear was much less obvious with the probe specific for the 3' end of the *MAA7* coding sequence, suggesting that the mutant was defective in the 3'-to-5' exonucleolytic degradation of the RISC-generated 5' RNA fragments. CC-124, non-transgenic wild type strain; Maa7-IR44, transgenic strain with RNAi-suppressed *MAA7*; Mut-68, insertional mutant of Maa7-IR44; Mut-68(*Mut68*)-1 and Mut-68(*Mut68*)-3, Mut-68 mutant strains complemented with a *MUT68* cDNA.. (C) Schematic representation of MUT68p, including conserved motifs, and sequence alignment of the poly(A) polymerase (PAP/25A) core domain of MUT68p and of several members of the polymerase β nucleotidyltransferase

superfamily. The sequences correspond to *C. reinhardtii* MUT68p (XXXXXX); *A. thaliana*, At2g39740 (AAV59264) and At3g45750 (AAP40443); *H. sapiens* PAPD4 (AAH26061); *D. melanogaster* CG5732 (NP_651012); *C. elegans* RDE-3 (NP_491834); *S. cerevisiae* Trf5 (NP_014100); and *S. pombe* Cid12 (CAA20372). Residues identical in most proteins are highlighted in black and conservative amino-acid substitutions are shaded in gray. A highly conserved active site motif is boxed and the aspartic-acid residues of the predicted catalytic triad are indicated with asterisks. **(D)** Neighbor-Joining (S12) tree indicating the phylogenetic relationship of *Chlamydomonas reinhardtii* MUT68p (red) to polymerase β nucleotidyltransferases from other eukaryotes. Non-canonical, putative poly(A) polymerases implicated in RNAi in other species are shown in blue. Sequences corresponding to the PAP/25A core domain were aligned by using Clustal X (S13) and the tree was drawn with MEGA3.1 (S14). Numbers indicate bootstrap values, as percentage, based on 1000 pseudoreplicates (only figures >60% are shown). Species are designated by a two-letter abbreviation preceding the name of each protein: At, *A. thaliana*; Ce, *C. elegans*; Cr, *C. reinhardtii*; Dm, *D. melanogaster*; Hs, *H. sapiens*; Sc, *S. cerevisiae*; and Sp, *S. pombe*. Accession numbers of proteins used to draw the tree: At2g39740, AAV59264; At2g45620, AAK64074; At3g45750, AAP40443; CeF31C3.2b, CAE54892; CeF43E2.1, AAK70648; CeGld-2, AAB70342; CeK10D2.3, NP_498099; CeRDE-3, NP_491834; CrMUT68, XXXXXX; DmCG5732, NP_651012; HsAK055546.1, BAB70951; HsAK055948.1, BAB71052; HsKIAA0191, BAA12105; HsPAPD1, NP_060579; HsPAPD4, AAH26061;

OsOJ1020_C02.27, XP_463923; OsP0446B05.30-1, BAD81828; OsP0562A06.26, XP_483772; ScTrf4, NP_014526; ScTrf5, NP_014100; SpAC821.04c, CAB57438; SpBC1685.06, CAA20054; SpCid-12, CAA20372; and SpCid-14, CAI79317.

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