About 53 and 79% of the SCL6-IV-related RNA detected 2 and 3 days postinfiltration, respectively, corresponded to the SCL6-IV(b) RNA product. Relatively small proportions of SCL6-IV mRNA were cleaved after coexpression with empty vector (Fig. 2B, top, lanes 4 and 5), IGR-mi39 (lanes 6 and 7), and 35S:AS-IGR-mi39 constructs (lanes 10 and 11), which can be explained by the basal or low levels of miRNA 39 in these samples. No cleavage products were detected with the SCL6-IV 5'-end probe (Fig. 2B), suggesting that the 5' fragment was less stable than the 3' fragment [SCL6-IV(b)]. Identical results were obtained when the SCL6-III mRNA was used as a target in coexpression assays (23).

To determine whether cleavage of SCL6-IV mRNA depends on perfect complementarity with miRNA 39, we introduced three mismatches into the sequence complementary to miRNA 39 in SCL6-IV RNA (construct 35S:SCL6-IV/mut39) (Fig. 3). Whereas the wild-type SCL6-IV mRNA was cleaved efficiently in the presence of the miRNA 39producing 35S:IGR-mi39 construct (Fig. 3, lanes 8, 9, 18, and 19), the SCL6-IV/mut39 mRNA was completely resistant to cleavage (lanes 10, 11, 20, and 21). Furthermore, the low level of cleavage of SCL6-IV mRNA in the presence of the empty vector construct was inhibited by the mutations (Fig. 3, lanes 4 to 7), confirming that this activity was due to low levels of endogenous miRNA 39 in N. benthamiana tissue.

The finding of miRNA-directed cleavage of several SCL mRNA targets in Arabidopsis indicates that there are at least two functional classes of miRNAs. Members of the small temporal RNA (stRNA)-like class, including C. elegans lin-4 and let-7 miRNAs, downregulate translation of target mRNAs but do not direct RNA target degradation or sitespecific cleavage (7, 8, 10, 11). The stRNA class members do not interact with perfect complementarity to their natural targets, which may explain why they do not exhibit siRNA-like activity (24). In contrast, members of a class represented by Arabidopsis miRNA 39 interact with perfect complementarity and appear to mimic siRNA function to guide cleavage. We propose that miRNA 39 incorporates into a RISC-like complex identical or similar to the RISC complex that mediates target cleavage during RNA silencing (21, 22). Support for this concept also comes from the finding that engineered miRNA-target combinations with perfect complementarity result in target RNA cleavage (25, 26). Finally, miRNA 39-guided cleavage of mRNAs has several possible consequences, including developmentally coordinated inactivation of SCL mRNAs. Internal cleavage might also generate RNA products with novel characteristics or coding potential for truncated SCL proteins. Given the numbers of miRNAs that were recently discovered in eukaryotes (4-6, 12, 13, 27, 28), additional members will likely be added to each class.

References and Notes

- 1. E. Bernstein, A. A. Caudy, S. M. Hammond, G. J. Hannon, *Nature* **409**, 363 (2001).
- 2. G. Hutvágner et al., Science 293, 834 (2001).
- R. F. Ketting *et al.*, *Genes Dev.* **15**, 2654 (2001).
 M. Lagos-Quintana, R. Rauhut, W. Lendeckel, T. Tusthe General **204**, 652 (2002).
- chl, *Science* **294**, 853 (2001). 5. N. C. Lau, E. P. Lim, E. G. Weinstein, D. P. Bartel,
- Science **294**, 858 (2001).
- 6. R. C. Lee, V. Ambros, Science 294, 862 (2001).
- R. C. Lee, R. L. Feinbaum, V. Ambros, Cell 75, 843 (1993).
- 8. B. Wightman, I. Ha, G. Ruvkun, Cell 75, 855 (1993).
- 9. E. G. Moss, R. C. Lee, V. Ambros, Cell 88, 637 (1997).
- 10. B. J. Reinhart et al., Nature 403, 901 (2000).
- 11. F. J. Slack et al., Mol. Cell 5, 659 (2000).
- 12. C. Llave, K. D. Kasschau, M. A. Rector, J. C. Carrington,
- *Plant Cell* **14**, 1605 (2002). **13.** B. J. Reinhart, E. G. Weinstein, M. W. Rhoades, B.
- Bartel, D. P. Bartel, Genes Dev. 16, 1616 (2002).
- 14. L. DiLaurenzio et al., Cell **86**, 423 (1996).
- A. L. Silverstone, C. N. Ciampaglio, T. Sun, *Plant Cell* 10, 155 (1998).
 C. Bolle, C. Koncz, N. H. Chua, *Genes Dev.* 14, 1269
- (2000).
- Y. Helariutta et al., Cell 101, 555 (2000).
 Materials and methods are available as supporting
- material on *Science* Online. 19. A. J. Hamilton, D. C. Baulcombe, *Science* **286**, 950
 - (1999).

- S. M. Elbashir, W. Lendeckel, T. Tuschl, *Genes Dev.* 15, 188 (2001).
- S. M. Hammond, E. Bernstein, D. Beach, G. J. Hannon, Nature 404, 293 (2000).
- S. M. Hammond, S. Boettcher, A. A. Caudy, R. Kobayashi, G. J. Hannon, *Science* 293, 1146 (2001).
- 23. C. Llave, J. C. Carrington, unpublished data.
- 24. G. Ruvkun, Science 294, 797 (2001).
- 25. M. T. McManus, C. P. Petersen, B. B. Haines, J. Chen, P. A. Sharp, *RNA* 8, 842 (2002).
- G. Hutvágner, P. D. Zamore, *Science* 297, 2056 (2002); published online 1 August 2002 (10.1126/ science.1073827).
- 27. Z. Mourelatos et al., Genes Dev. 16, 720 (2002).
 - 28. E. C. Lai, Nature Genet. 30, 363 (2002).
 - 29. We thank L. Johansen, M. Rector, and B. Shaffer for valuable comments and technical support. We are also grateful to J. Valkonen and J. Kreuze for helpful suggestions on 5'-RACE procedures and D. Olszyk for providing rice tissue. C.L. was the recipient of a postdoctoral fellowship from the Ministerio de Educación y Cultura (Spain). This work was supported by grant MCB-0209836 from the National Science Foundation, grants Al27832 and Al43288 from NIH, and grant NRI 2002-35319-11560 from the U.S. Department of Agriculture.

Supporting Online Material

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SOM Text Fig. S1 Table S1 References

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A microRNA in a Multiple-Turnover RNAi Enzyme Complex

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In animals, the double-stranded RNA-specific endonuclease Dicer produces two classes of functionally distinct, tiny RNAs: microRNAs (miRNAs) and small interfering RNAs (siRNAs). miRNAs regulate mRNA translation, whereas siRNAs direct RNA destruction via the RNA interference (RNAi) pathway. Here we show that, in human cell extracts, the miRNA *let-7* naturally enters the RNAi pathway, which suggests that only the degree of complementarity between a miRNA and its RNA target determines its function. Human *let-7* is a component of a previously identified, miRNA-containing ribonucleoprotein particle, which we show is an RNAi enzyme complex. Each *let-7*–containing complex directs multiple rounds of RNA cleavage, which explains the remarkable efficiency of the RNAi pathway in human cells.

Two types of 21- to 23-nucleotide (nt) RNAs are produced by the multidomain ribonuclease (RNase) III enzyme Dicer: small interfering RNAs (siRNAs) from long double-stranded RNA (1, 2) and microRNAs (miRNAs) from \sim 70-nt hairpin precursor RNAs whose expression is often developmentally regulated (3–8). siRNAs direct the cleavage of complementary mRNA targets, a process known as RNA inter-

ference (RNAi) (9). Target RNA cleavage is catalyzed by the RNA-induced silencing complex (RISC), which acts as an siRNA-directed endonuclease, cleaving the target RNA across from the center of the complementary siRNA strand (10, 11). Assembly of the RISC is adenosine triphosphate (ATP) dependent and precedes target recognition (10, 12). Unlike siR-NAs, miRNAs are single stranded and pair with target mRNAs that contain sequences only partially complementary to the miRNA and repress mRNA translation without altering mRNA stability (13-19). Although at least 135 miRNAs have been identified collectively from Caenorhabditis elegans, Drosophila melanogaster, and humans, none is fully complementary to

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any mRNA sequence in these organisms, which suggests that miRNAs do not function in the RNAi pathway because RNAi requires extensive complementarity between the siRNA and its mRNA target (20).

siRNAs and miRNAs have been proposed to act in distinct biochemical pathways, in part because distinct PPD (PAZ and Piwi domain) proteins are required in *C. elegans* for RNAi (21) and miRNA function (22). In this model, the specific PPD protein associated with an siRNA, miRNA, or miRNA precursor determines the pathway in which a small RNA functions. Unique features of miRNAs or their precursors might lead them to associate with miRNA-specific PPD proteins. Thus, the sequence or structure of a miRNA or its precursor would ensure that it functions as a translational repressor and not as a trigger of RNAi.

To determine whether an siRNA duplex in which one strand corresponded exactly to the miRNA let-7 (let-7 siRNA) could function in the RNAi pathway (Fig. 1A), we incubated the let-7 siRNA duplex with Drosophila embryo lysate in an in vitro RNAi reaction containing a 5' ³²P-radiolabeled target mRNA. This target RNA contained sequence fully complementary to let-7 as well as the sequence from the 3'untranslated region of the C. elegans lin-41 mRNA that mediates let-7-directed translational repression (Fig. 1B) (23). The let-7 siRNA directed cleavage of the target RNA only within the sequence that was fully complementary to let-7 (Fig. 1C). Thus, the intrinsic sequence of let-7 does not preclude its functioning in RNAi. No cleavage was observed at any other site, including within the lin-41 sequences. The lin-41 sequence differs from the let-7 complementary sequence at only 4 of the 19 positions that determine siRNA specificity (Fig. 1B). The lin-41 sequence was not refractory to RNAi, because an appropriately complementary siRNA directs its cleavage (Fig. 1D) (*lin-41* siRNA).

Nor does the structure or sequence of the let-7 precursor preclude entry of let-7 into the RNAi pathway. We added synthetic D. melanogaster pre-let-7 RNA (Fig. 1A) to an in vitro RNAi reaction mixture containing the let-7 complementary target RNA. Again, the target RNA was cleaved within the let-7 complementary sequences but not at any other site (Fig. 1C). Although pre-let-7 RNA promoted a lower level of RNAi than a let-7 siRNA duplex, it was surprising that RNAi occurred at all, because Dicer cleavage of pre-let-7 generates single-stranded let-7 in vivo and in vitro (3, 7, 22). It is not known how Dicer produces single-stranded miRNAs but double-stranded siRNAs. One model is that Dicer initially generates an siRNA-like, double-stranded intermediate whose nonmiRNA strand is then selectively destroyed. In this model, Dicer would produce the "prelet-7 siRNA" duplex shown in Fig. 1A. In an in vitro RNAi reaction, this siRNA duplex produced about the same amount of target cleavage as pre-let-7 itself (Fig. 1C). Only a small fraction (~5%) of the input pre-let-7 RNA (100 nM) is converted to mature let-7 in vitro (3). Thus, when produced from pre-let-7, \sim 5 nM mature *let*-7 entered the RNAi pathway as efficiently as 100 nM pre-let-7 siRNA duplex, which suggests that production by Dicer cleavage enhances entry of let-7 into the pathway. Dicer action may therefore be coupled to RISC formation, consistent with the interaction of Dicer with the RISC components Ago-2 in Drosophila (24) and Rde-1 in C. elegans (25).

These experiments suggest that the degree of complementarity between a miRNA and its target RNA is the sole determinant of its function, because single-stranded *let-7* can clearly act as an siRNA in vitro. Nonetheless, *let-7* might be precluded from entering the RNAi pathway in vivo. Therefore, we tested whether the endogenous *let-7* produced by cultured human HeLa cells (3) enters the RNAi pathway.

We tested both HeLa cytoplasmic (S100) and nuclear extracts for their ability to direct cleavage of the let-7 complementary RNA target (Fig. 1B) (26). In Drosophila embryo lysate, which contains no let-7, this target RNA is cleaved at the let-7 complementary site only upon addition of exogenous let-7 siRNA duplex. In contrast, HeLa cytoplasmic S100-but not nuclear-extract (Fig. 2A) directed target RNA cleavage within the let-7 complementary sequences in the absence of exogenous siRNA. Target cleavage in HeLa S100 occurred between nt 541 and 542, exactly the same cleavage site directed by the exogenous let-7 siRNA in Drosophila embryo lysate (Fig. 2B). No target cleavage occurred within the lin-41 sequences contained in the target RNA. These sequences do not pair with let-7 at positions 9 and 10 (Fig. 1B); mispairing between an siRNA and its target at these positions blocks RNAi (20) (27). These results suggest that the endogenous let-7 in the HeLa cytoplasmic extract is associated with RISC, the enzyme complex that mediates endonucleolytic cleavage in the RNAi pathway.

To test directly whether *let-7* was associated with RISC, we asked if the cleavage activity copurified with the protein eIF2C2, a member of the PPD family of proteins. PPD proteins are required for RNAi and posttranscriptional gene silencing in animals (21, 28, 29), plants (30, 31), and fungi (32). In flies, the PPD protein Ago-2 is a component of the RISC complex (24); in





sequence. The 5' 32 P radiolabel is indicated by an asterisk. UTR, untranslated region. (C) In vitro RNAi reactions with *Drosophila* embryo lysate using the triggers in (A) and the target in (B). (D) In vitro RNAi reactions as in (C) using *let-7–* and *lin-41–*specific siRNAs.



Fig. 2. Endogenous human *let-7* enters the RNAi pathway. (**A**) In vitro RNA reactions with *Drosophila* embryo lysate, human HeLa nuclear or S100 extract, using the target RNA described in Fig. 1B, with or without the *let-7* siRNA duplex described in Fig. 1A. (**B**) Denaturing gel analysis of the cleavage products from the RNAi reactions in (A).

Neurospora (Qde-2) (33) and C. elegans (Rde-1) (25), PPD proteins are associated with siRNAs in multiprotein complexes likely to correspond to RISC. A human RISC has not yet been characterized, but human eIF2C2 is associated with an RNA-protein complex, the miRNA ribonucleoprotein particle (miRNP), that contains miRNAs and is nearly the same size as Drosophila RISC (34). The let-7 paralog miR-98, which differs from let-7 at two positions, was previously shown to be in the miRNP (34). We immunoprecipitated eIF2C2 from HeLa S100 with monoclonal (8C7) or polyclonal (411-1) antibodies and tested the immunoprecipitates for their ability to cleave the target RNA at the let-7 complementary sequence (Fig. 2C) (35). Both let-7 (Fig. 2D) and the nuclease activity specific for let-7 complementary target sequences (Fig. 2C) copurified with eIF2C2. Therefore, we refer to this activity as a let-7-programmed RISC.

Next, we tested whether other components of the miRNP—Gemin4 and the putative DEAD-box RNA helicase Gemin3—were also components of the *let-7*—programmed RISC complex (34). Monoclonal antibodies to Gemin3 and Gemin4, but not the survival of motor neurons (SMN) protein, immunoprecipitated *let-7*—programmed RISC activity (Fig. 2C). SMN is a component of a Gemin3/Gemin4containing complex that restructures nuclear RNPs; this complex does not contain eIF2C2 (34). Consistent with the idea that *let-7* was preassembled into the miRNP before the HeLa cells were lysed, target cleavage in S100 was not enhanced by exogenous *let-7* siRNA (36).

(C) Immunoprecipitation of *let-7*–programmed RISC activity by antibodies (α) to components of the miRNP (eIF2C2, Gem3, Gem4) or to the SMN protein. (D) Relative amount of *let-7* RNA recovered, as determined by Northern hybridization, in the immunoprecipitates in (C).



Fig. 3. (**A**) In vitro RNAi reactions using the target RNA and HeLa S100. ATP-depleted reaction mixtures (–) contained about 1/100th the ATP of nondepleted reaction mixtures (+). (**B**) Northern hybridization analysis to measure the concentration of *let-7* in HeLa S100. (**C**) Quantification of (B). Hybridization signals are plotted for the synthetic *let-7* standards (filled circles), or for 3.6 μ l (open circle) or 7.2 μ l (open square) of HeLa S100.



Human Dicer protein has previously been shown to be localized to the cytoplasm (37). The experiments in Fig. 2 suggest that the rest of the human RNAi pathway is likewise cytoplasmic, because no RISC-associated let-7 was detected in the nuclear extract, nor could the nuclear extract be programmed with a let-7-containing siRNA to direct target cleavage. In contrast, an exogenous siRNA duplex complementary to firefly luciferase sequences successfully programmed the HeLa S100 to cleave the target (38). Although both the endogenous human let-7 and the exogenous luciferase siRNA triggered target cleavage, the two triggers differ in at least one respect: endogenous human let-7 is single stranded (7, 11, 19), whereas the siRNA was double stranded. Double-stranded siRNAs must be unwound in order to direct RNAi; this unwinding requires ATP (12). Once unwound, RISC-associated siRNAs can cleave their targets in the absence of high-energy cofactors (12). Because *let-7* is single stranded, target cleavage by HeLa *let-7* should not require ATP. To test this hypothesis, we depleted ATP (12) from HeLa S100 and then added the *let-7* complementary target RNA. The *let-7*-programmed RISC cleaved the target in the absence of ATP (Fig. 3A).

Therefore, as in *Drosophila* embryo lysates (12), RNAi in human HeLa cytoplasmic extracts does not require ATP for target cleavage. Accordingly, models for the RNAi pathway that invoke synthesis of new RNA as a prerequisite Fig. 4. Model for a common pathway in which miRNAs direct translational repression and siRNAs direct target RNA destruction (RNAi). The RNA duplex in brackets is proposed to be a short-lived intermediate. We propose that Dicer cleavage of both miRNA precursors and double-stranded RNA is coupled to the formation of a miRNP/RISC complex. RISC is envisioned to act stoichiometrically in repressing translation but catalytically in RNA destruction via RNAi.



for target RNA destruction (39) do not accurately describe the mechanism of RNAi in human cells. New RNA synthesis is thought to be an important step for RNAi in *C. elegans* and *Dictyostelium discoideum*, posttranscriptional gene silencing in plants, and quelling in *Neurospora crassa* (9). In each of these organisms, a member of a family of RNA-dependent RNA polymerases (RdRPs) is required for RNA silencing. In contrast, no such RdRP is encoded by the current release of the *Drosophila* or the human genome.

Why then is RNAi so efficient in flies and cultured mammalian cells? The concentration of *let*-7 in HeLa S100 is \leq 900 pM (Fig. 3, B and C) (40), and therefore the concentration of *let*-7–programmed RISC in our reactions is \leq 450 pM. Because the target RNA concentration in these experiments was ~6 nM and 70% of the target was destroyed in 2 hours (Fig. 2A), each *let*-7–programmed RISC must catalyze the cleavage of ~10 target molecules. (*let*-7 produced de novo during the reaction is negligible, because the concentration of pre–*let*-7 in HeLa

S100 is 1/10th to 1/20th that of *let-7*.) Thus, the *let-7*–programmed RISC is a true enzyme, catalyzing multiple rounds of RNA cleavage. It seems highly likely that all RISCs are multipleturnover enzyme complexes.

Our results suggest that human let-7 is in the enzyme complex that mediates RNAi, yet human cells do not contain mRNAs that could function as let-7 RNAi targets. Perhaps let-7 enters two separate complexes, one for RNAi and one for translational control. Such a model implies that a portion of let-7 enters a complex that serves no function in human cells. More likely is that the recently identified miRNP (34) is the human RISC (10, 24) and that this one complex carries out both target cleavage in the RNAi pathway and translational control in the miRNA pathway (Fig. 4). Such a view does not preclude miRNAs or siRNAs from also being associated with smaller complexes that contain only a subset of RISC components but that nonetheless are capable of target RNA cleavage (12).

At least one plant miRNA (miR171) is per-

fectly complementary to a potential regulatory target mRNA, raising the possibility that miR-NAs may naturally be used in plants for RNAibased regulation of gene expression (8, 41). We anticipate that, in plants, artificial miRNAs will direct translational control when they do not pair with their mRNA targets at the site of cleavage in the RNAi pathway. Conversely, we predict that, in animals, synthetic siRNA duplexes with only partial complementarity to their corresponding mRNA targets will repress translation of the mRNA without triggering RNA degradation.

References and Notes

- E. Bernstein, A. A. Caudy, S. M. Hammond, G. J. Hannon, *Nature* 409, 363 (2001).
- 2. S. W. Knight, B. L. Bass, Science 293, 2269 (2001).
- 3. G. Hutvágner et al., Science 293, 834 (2001).
- 4. M. Lagos-Quintana et al., Science 294, 853 (2001).
- 5. N. C. Lau et al., Science **294**, 858 (2001).
- 6. R. C. Lee, V. Ambros, Science 294, 862 (2001).
- 7. R. F. Ketting et al., Genes Dev. 15, 2654 (2001).
- 8. B. J. Reinhart et al., Genes Dev. 16, 1616 (2002).
- 9. R. H. Plasterk, Science 296, 1263 (2002).
- 10. S. M. Hammond, E. Bernstein, D. Beach, G. J. Hannon, Nature **404**, 293 (2000).
- 11. S. M. Elbashir, W. Lendeckel, T. Tuschl, *Genes Dev.* 15, 188 (2001).
- 12. A. Nykänen, B. Haley, P. D. Zamore, *Cell* **107**, 309 (2001).
- 13. R. C. Lee, R. L. Feinbaum, V. Ambros, Cell 75, 843 (1993).
- 14. B. Wightman, I. Ha, G. Ruvkun, Cell 75, 855 (1993).
- 15. E. G. Moss, R. C. Lee, V. Ambros, Cell 88, 637 (1997).
- 16. P. H. Olsen, V. Ambros, Dev. Biol. 216, 671 (1999).
- 17. B. J. Reinhart et al., Nature 403, 901 (2000).
- 18. F. J. Slack et al., Mol. Cell 5, 659 (2000).
- 19. Y. Zeng et al., Mol. Cell 9, 1327 (2002).
- S. M. Elbashir, J. Martinez, A. Patkaniowska, W. Lendeckel, T. Tuschl, *EMBO J.* 20, 6877 (2001).
- 21. H. Tabara et al., Cell 99, 123 (1999).
- 22. A. Grishok et al., Cell 106, 23 (2001).
- 24. S. M. Hammond, S. Boettcher, A. A. Caudy, R. Kobayashi, G. J. Hannon, *Science* **293**, 1146 (2001).
- H. Tabara, E. Yigit, H. Siomi, C. C. Mello, *Cell* **109**, 861 (2002).
- HeLa extracts were used in place of Drosophila embryo lysate in a standard RNAi reaction, but they were incubated at 37°C.
- 27. D. Schwarz, P. D. Zamore, unpublished data.
- 28. M. Pal-Bhadra et al., Mol. Cell 9, 315 (2002).
- 29. R. W. Williams, G. M. Rubin, Proc. Natl. Acad. Sci. U.S.A. 99, 6889 (2002).
- M. Fagard et al., Proc. Natl. Acad. Sci. U.S.A. 97, 11650 (2000).
- 31. J. B. Morel et al., Plant Cell 14, 629 (2002).
- 32. C. Catalanotto et al., Nature 404, 245 (2000).
- 33. C. Catalanotto et al., Genes Dev. 16, 790 (2002).
- 34. Z. Mourelatos et al., Genes Dev. 16, 720 (2002).
- 35. Protein G agarose (25 μl) (Invitrogen) bound with 40 μg of monoclonal antibody (mAb) to eIF2C2 or 7 μl of polyclonal serum to eIF2C2, 5 μl of mAb to Gemin3, 5 μl of mAb to Gemin4, or 5 μl of mAb to SMN was incubated with 90 μl of HeLa S100 at 4°C for 30 min and washed three times with lysis buffer (12) containing 0.1% Nonidet P-40. Washed immune

agarose beads (2.5 $\mu l)$ were used in a standard RNAi reaction at 37°C.

36. G. Hutvágner, P. D. Zamore, unpublished data.

- E. Billy, V. Brondani, H. Zhang, U. Muller, W. Filipowicz, Proc. Natl. Acad. Sci. U.S.A. 98, 14428 (2001).
- 38. D. Schwarz, G. Hutvágner, P. D. Zamore, Mol. Cell, in press.
- 39. C. Lipardi, Q. Wei, B. M. Paterson, *Cell* **107**, 297 (2001).
- Synthetic ¹/₂ ¹/₂ ²/₂ ¹/₂ ¹/₂
- a probe. The concentration of twice gel-purified target

RNA was determined by absorbance at 260 nm and confirmed by estimating the yield of ³²P-labeled RNA after the second gel purification step.

- C. Llave, K. D. Kasschau, M. A. Rector, J. C. Carrington, *Plant Cell* 14, 1605 (2002).
- 42. We thank D. Bartel and members of the Zamore laboratory for discussions and comments on the manuscript. We also thank M. Moore for the generous gift of HeLa extracts and G. Dreyfuss for kindly providing antibodies. G. H. is a Charles A. King Trust

A Role for CD40 Expression on CD8⁺ T Cells in the Generation of CD8⁺ T Cell Memory

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The delivery of CD4 help to CD8⁺ T cell responses requires interactions between CD40 and CD40 ligand and is thought to occur through antigen-presenting cell (APC) activation. Here we show that generation of memory CD8⁺ T cells displaying an enhanced capacity for cell division and cytokine secretion required CD4 help but not CD40 expression by the APCs. Activated CD4⁺ and CD8⁺ T cells expressed CD40; and in the absence of this protein, CD8⁺ T cells were unable to differentiate into memory cells or receive CD4 help. These results suggest that, like B cells, CD8⁺ T cells receive CD4 help directly through CD40 and that this interaction is fundamental for CD8⁺ T cell memory generation.

Interactions between CD40 and CD40 ligand (CD40L) play a major role in direct CD4⁺ T cell–B cell collaboration, and the absence of

Fig. 1. The role of CD4 help in CD8⁺ T cell responses. Mice carrying either Tg cells or Tg cells and CD4⁺ T cells were immunized in vivo with male cells expressing the Ly5.1 marker (16). (A) Elimination of Ly5.1 male cells by Tg cells in the presence or absence of CD4⁺ T cells, 2 months after priming. (B) Tg cells, labeled with 5- (and 6-) carboxyfluorescein diacetate succininyl ester (CFSE), 3 days after immunization with male cells. Dotted lines represent CFSE labeling of Tg cells in mice that were not immunized. By day 5 after immunization, all cells had lost CFSE labeling. (C) The number of Tg cells recovered at different times after immunization in one of three experiments. (D) Interferon- γ (IFN- γ) secretion by purified Tg cells (naïve, or recovered at different days after immunization). Purified Tg cells (naïve, or recovered 2 months after priming) were stimulated in vitro with male APCs: (E) optimal [³H]thymidine incorporation and (F) cytokine secretion. CPM, counts per minute; IL-2, interleukin-2.

these molecules results in a failure of germinal center formation, memory B cell activation, immunoglobulin class switching, and fellow of the Medical Foundation. P.D.Z. is a Pew Scholar in the Biomedical Sciences and a W. M. Keck Foundation Young Scholar in Medical Research. Supported in part by a grant to P.D.Z. from the National Institutes of Health (GM62862-01).

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somatic hypermutation (1-3). The CD40 and CD40L deficiencies also impair CD8⁺ T cell responses, suggesting an important role for these molecules in CD4⁺ and CD8⁺ T cell collaboration (4-6). This role has been confirmed by experiments showing that monoclonal antibodies (mAbs) to CD40 could substitute for the delivery of CD4 help to CD8⁺ T cells in vivo (7, 8). Because CD40 is expressed on antigen-presenting cells (APCs) (9), it has been assumed that CD4 help to CD8⁺ T cells is indirect. Thus, CD4⁺ CD40L⁺ T cells would first activate APCs by engaging CD40, allowing activated APCs to become competent to drive CD8+ T cell responses (7, 8, 10). Other experimental data, however, have suggested dissociation of APC activation from CD4-CD8⁺ T cell collaboration. For example, CD8⁺ T cells can activate APCs without CD4⁺ T cells (11). In addition, CD4⁺ T cells can activate APCs through a CD40-independent pathway (12),

