OLIGONUCLEOTIDES 13:295–301 (2003)

Variations of the 3′ Protruding Ends in Synthetic Short Interfering RNA (siRNA) Tested by Microinjection in *Drosophila* Embryos

ALEXANDRA BOUTLA,[1,2] CHRISTOS DELIDAKIS,[1,2] IOANNIS LIVADARAS,[1] and MARTIN TABLER[1]

ABSTRACT

Short interfering RNAs (siRNAs) are the processing product originating from long double-stranded RNAs (dsRNAs) that are cleaved by the RNase III-like ribonuclease Dicer. As siRNAs mediate cleavage of specific single-stranded target RNAs, they are essential intermediates of RNA interference (RNAi). When applied in synthetic form, siRNAs likewise can induce the silencing process in the absence of long dsRNAs. Here, we tested variations of a conventional synthetic siRNA that had been used successfully to silence the *Drosophila Notch* gene. The variants had two 3′-terminal deoxynucleotides in their protruding single-stranded ends. In one case, the deoxynulceotides would match to the *Notch* mRNA, whereas the other variant had nonmatching deoxy-T residues, representing a widely used siRNA design. siRNAs with different combinations of sense and antisense strands were injected into *Drosophila* embryos at two different concentrations. We found that the all-ribonucleotide siRNA gave the best inhibition of *Notch* expression. The combination of two modified strands with 3′-terminal deoxynucleotides was effective, but if combined with a sense or antisense ribostrand, the efficacy dropped. The siRNAs with nonmatching 3′-terminal TT residues showed a reduced silencing potential, which became evident at low concentration. An siRNA with a nonmatching 3′-terminal ribonucleotide in the antisense strand retained most of its silencing potential in accordance with the hypothesis that primer extension for generation of ssRNA from single-stranded mRNA does not operate in *Drosophila*.

INTRODUCTION

IN MOST EUKARYOTES, double-stranded RNA (dsRNA) plays a key role in inducing a cellular process of sequence-specific RNA degradation. Although different in some details, this process is known as RNA interference (RNAi) in animals and posttranscriptional gene silencing (PTGS) in plants (for reviews, see Hammond et al., 2001; Matzke et al., 2001; Vance and Vaucheret, 2001; Vaucheret and Fagard, 2001; Voinnet, 2001, 2002; Baulcombe, 2002; Hannon, 2002; Hutvagner and Zamore, 2002; Plasterk, 2002). Recently, the uniform term RNA silencing has been suggested (Baulcombe, 2002). All models that have been proposed for the mechanism of RNA silencing agree that processing of dsRNA (regardless of its origin) into short dsRNA fragments is a key step in the process. This cleavage reaction is catalyzed by an RNase III-like enzyme, which in *Drosophila* and in most other organisms is called Dicer (Bernstein et al., 2001). The cleavage product is composed of two complementary RNA strands that base pair, with the exception of the

[1]Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology-Hellas, GR-71110 Heraklion/Crete, Greece.

[2]Department of Biology, University of Crete, GR-71110 Heraklion/Crete, Greece.

3′-terminal nucleotides that form an overhang (Hamilton and Baulcombe, 1999; Elbashir et al., 2001a,b,c). The length of the RNA strand varies slightly depending on the species. Typically, it ranges from 21 to 23 nucleotides, but in plants, two size classes of siRNAs have been described that may have different functions (Hamilton et al., 2002).

Tushl's group showed that chemically synthesized short RNA fragments (the first processing product derived from the long dsRNA) can likewise initiate the RNA silencing reaction (Elbashir et al., 2001a,c). Therefore, the term short interfering RNA (siRNA) was coined (Elbashir et al., 2001b). For the same acronym, the term small interfering RNA also is used (Elbashir et al., 2001a). Since then, a rapidly increasing number of successful examples of use of synthetic RNA in various species have been described, including *Caenorhabditis elegans* (Caplen et al., 2001), *Drosophila* embryos (Boutla et al., 2001), *Xenopus* embryos (Zhou et al., 2002), and mouse (Sorensen et al., 2003). Various applications in mammalian cells also have been described (Hamada et al., 2002; Heinonen et al., 2002; Hohjoh, 2002; Holen et al., 2002; Leirdal and Sioud, 2002; Martinez et al., 2002; McManus et al., 2002; Amarzguioui et al., 2003; Basu et al., 2003; Czauderna et al., 2003a,b; Harborth et al., 2003; Heidenreich et al., 2003; Kosciolek et al., 2003; Miyagishi et al., 2003; Vickers et al., 2003), including the inhibition of viruses (Gitlin et al., 2002; Jacque et al., 2002; Novina et al., 2002; Ge et al., 2003; Hamasaki et al., 2003; Jia and Sun, 2003; Randall et al., 2003; Wilson et al., 2003; Yokota et al., 2003).

In addition to the direct delivery of synthetic siRNAs, different strategies of vector-based delivery systems have been developed (Brummelkamp et al., 2002; Donze and Picard, 2002; Lee et al., 2002; Paul et al., 2002; Kawasaki and Taira, 2003). According to the standard model of RNA silencing, siRNAs are incorporated in the RNA-induced silencing complex (RISC) (Hammond et al., 2000), of which only a part of its protein constituents have been identified to date. Guided by the antisense RNA strand of the siRNA, RISC will then cleave (slice) the single-stranded target RNA. Also a single-stranded antisense oligonucleotide may be incorporated into RISC and induce the silencing process (Martinez et al., 2002; Schwarz et al., 2002; Amarzguioui et al., 2003; Holen et al., 2003), but the efficiency is lower, and in some cases, no significant silencing was observed with an antisense RNA (Boutla et al., 2001). In view of their enormous potential, the design of siRNAs, the target site selection, and the use of chemically modified nucleotides are under detailed investigation.

For direct application of synthetic siRNA in gene suppression via RNAi, several rules have been developed, some of them primarily because they were copying the original successful example. In their first application in mammalian cells, Elbashir et al. (2001a) used siRNAs in which the two single-stranded nucleotides at the 3′-end were either UU or (deoxy) TT. The TT residues were chosen to reduce the cost of the RNA oligonucleotide and because the authors thought it possible that terminally located 3′-deoxynulceotides potentially could provide some protection against exoribonucleases. Moreover, in some cases, the two T residues did not even match to the target sense RNA. This original, more or less accidentally chosen design, which had proven successful, has meanwhile been transferred to many other examples, so that it developed almost into a rule for designing siRNAs. In this study, we wanted to test if there is experimental justification for such a TT rule. Moreover, we wanted to test siRNAs for their potential to induce RNAi against an endogenous gene rather than a reporter gene and in a whole organism rather than in a cell line.

Besides testing the siRNA design, we also assayed an all-ribonucleotide siRNA with a mismatch at the 3′-terminus of the antisense strand to test for potential spreading of silencing via a second pathway, causing siRNA-mediated degradation of the target RNA. According to that mechanism, the antisense strand of the siRNA is used for primer extension, causing synthesis of dsRNA on the single-stranded target RNA, which is used as template (Hutvagner and Zamore, 2002). It is suggested that the RNA synthesis is carried out by the RNA-directed RNA polymerase (RdRp), an enzyme that was shown by genetic screens to play an essential role in RNA silencing in plants (Dalmay et al., 2000; Mourrain et al., 2000). The resulting dsRNA product is then processed by Dicer or related enzymes to secondary siRNAs. By demonstrating the existence of transitive RNAi, that is, induction of silencing of a gene that is located 5′ to the initial target RNA, Sijen et al. (2001) could provide direct evidence that this reaction can proceed in *C. elegans*. For plants, similar mechanisms have been reported (Vaistij et al., 2002). For *Drosophila*, a similar spreading had been reported (Lipardi et al., 2001), but further experiments suggested that the siRNAs do not function as primers in insect and human systems (Schwarz et al., 2002).

As a basis for these studies, we took a synthetic siRNA composed solely of conventional ribonulceotides directed against the *Notch* gene, which is expressed in the early *Drosophila* embryo. Microinjection into embryos showed that this siRNA was able to induce RNAi, causing developmental defects (Boutla et al., 2001). This test system in a whole organism has the advantage that the RNAi effect can be quantified in two ways. First, the number of embryos showing mutant phenotypes will indicate the penetrance of the siRNA, and second, the kind of phenotype allows a conclusion about its expressivity (Fig. 1C). Because the less potent siRNAs also may work well if used in high concentrations, it is necessary to test the siRNAs in different concentrations to monitor their efficacy.

MATERIALS AND METHODS

The RNA oligonucleotides (listed in Fig. 1A) were custom synthesized by Xeragon/Qiagen (Valencia, CA). Injection of phosphorylated synthetic siRNAs into *Drosophila* embryos and scoring of the *Notch* phenotype were done as described previously (Boutla et al., 2001). Controls were mock-injected with buffer. In a typical experiment, about 250 embryos were injected, of which about 150–200 were fertilized and were included in the analysis (Fig. 1B).

RESULTS AND DISCUSSION

It was our intention to analyze systematically what influence the 3′ protruding single-stranded nucleotides of an siRNA may have, depending on whether they are ribonucleotide or deoxynucleotides and depending on whether they match to the target RNA or have nonmatching (deoxy) thymidine residues. As a basis, we took an siRNA consisting of the two conventional ribooligonucleotides R63 (sense polarity) and R61 (antisense polarity). This siRNA had been used previously to induce RNAi for *Notch* in *Drosophila* embryos (Boutla et al., 2001). We prepared several variants of these chemically synthesized RNAs. First, we replaced the two terminal GA ribonucleotides of the sense oligonucleotide R63 by two matching deoxynucleotides (R63–GA) or two nonmatching deoxy TT residues (R63–TT) (Fig. 1A). Similarly, in the antisense oligonucleotide R61, the two 3′-terminal ribonucleotides GU were replaced by either deoxy GT or TT (Fig. 1A).

The different synthetic sense and the antisense strands were phosphorylated at their 5′-termini and combined to several combinations of siRNA cassettes (Fig. 1B). The siRNAs were injected into *Drosophila* embryos at two concentrations. We used a starting concentration of 100 μM and a 10-fold dilution. The actual amount transferred to the *Drosophila* is roughly about 1% of the volume, so that the effective concentrations were about 1 μM and 0.1 μM, respectively. The number of *Drosophila* embryos with *Notch*-specific developmental defects was scored (Fig. 1B), and the expressivity was classified as described previously and as outlined schematically in Figure 1C.

The strongest RNAi effect was observed with the matching all-ribonucleotide siRNA at high concentration (97% of the animals), with a complete loss-of-function phenotype. In all the following experiments, the highest concentration of the siRNAs caused either complete loss of *Notch* function or no effect at all (compare Fig. 1C). However, the penetrance (frequency of occurrence) varied. If either the sense or the antisense strand was replaced by the variant with two 3′-terminal deoxynucleotides, the efficacy dropped to 85% and 81%, whereas the double-deoxy variant showed 90% of *Notch*-silenced embryos. In the variants with 3′-terminal TT residues, we observed a reduction to 75% and 67% when combined with an all RNA antisense or sense strand, respectively. Again the double combination with two strands having the 3′ TT termini caused restoration of silencing activity (91%). We previously have seen a similar reduction of the penetrance without influence on expressivity when the siRNA had a 5′-hydroxyl group or when there was a single mismatch in the center of the siRNA (Boutla et al., 2001).

Collectively, these data show that despite the fact that all combinations work generally well at high concentration, the combination of an all-ribonucleotide strand, regardless of whether sense or antisense, with a complementary strand that has either nonmatching residues or 3′-terminal deoxynucleotides causes some loss of silencing potential, especially in the case of the TT modification. The symmetrically modified siRNAs, having either two matching deoxynucleotides or even the TT modification, restore the silencing potential. One interpretation of this result is that an asymmetric siRNA (one strand all RNA, the other with a deoxy modification) is less efficiently incorporated into RISC.

Next, we tested the same siRNA combinations at 10-fold reduced concentration. These conditions were expected to reveal differences in the silencing potential depending on the chemical nature of the siRNA. In all cases, the penetrance of the siRNA was reduced along with lower expressivity of the *Notch* phenotype. As indicated in Figure 1B, the inhibitory potential of the siRNAs stayed in a narrow range unless the sense strand contained deoxy modifications, which caused lower expressivity. When the TT modification was used, the penetrance dropped noticeably to 36% and, if combined with the antisense TT modification, to 24%. We consider it unlikely that a reduced stability of the deoxy-modified siRNA is responsible for this effect. If 3′-terminal deoxynucleotides make the siRNA more vulnerable to degradation (unlike the original presumption that they would protect against 3′-exoribonucleases), the combination of the oligonucleotides R61-GT and R61-TT with the sense oligonucleotide R63 also should be affected. Therefore, we favor the idea that such unconventional siRNAs are less efficiently incorporated into the RISC complex.

In addition, there may be a second reason for the reduced efficiency, especially in siRNAs with mixed ribo/deoxy overhangs. According to the model for the mechanism of RNAi, the siRNA is incorporated into RISC and then unwound (both steps requiring ATP) (Nykänen et al., 2001). There is no indication of a preference for the sense or the antisense strand remaining in the active RISC complex, as synthetic siRNAs are able to

A

sense oligonucleotides	
R63:	5′ pACAAUGCUGCCUGCCACUAC**GA**
R63GA:	5′ pACAAUGCUGCCUGCCACUAC**dGdA**
R63TT:	5′ pACAAUGCUGCCUGCCACUAC**dTdT**
antisense oligonucleotides	
R61:	5′ pGUAGUGGCAGGCAGCAUUGU**UG**
R61GT:	5′ pGUAGUGGCAGGCAGCAUUGU**dTdG**
R61TT:	5′ pGUAGUGGCAGGCAGCAUUGU**dTdT**
R61A:	5′ pGUAGUGGCAGGCAGCAUUGU**UA**

B

Sense / Antisense	R63 All RNA		R63GA Matching 3′ dNTP		R63TT Non-matching 3′ dNTP	
	100μM	10μM	100μM	10μM	100μM	10μM
R61 All RNA	97 % +++++	64 % ++++	85 % +++++	48 % +++	75 % +++++	36 % +++
R61GT Matching 3′ dNTP	81 % +++++	65 % ++++	90 % +++++	70 % ++++		
R61TT Non-matching 3′ dNTP	67 % +++++	60 % ++++			91 % +++++	24 % +++
R61A All RNA Non Matching 3′	81 % +++++	61 % ++++				

C

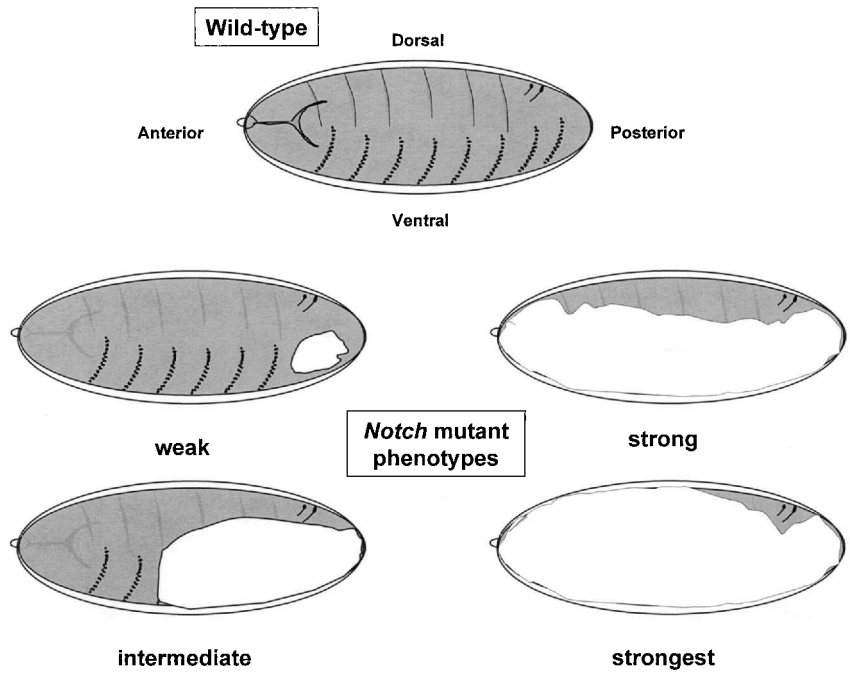

FIG. 1. The synthetic oligonucleotides used to generate siRNA cassettes and their effect on silencing of the *Notch* gene in *Drosophila* embryos. (**A**) Sequences of the sense and antisense RNAs are shown. Nucleotides that will form the 3′-terminal single-stranded end (overhang) of the siRNA are shown in bold. Deoxynucleotides are marked by a **d** prefix. (**B**) The sense and antisense oligonucleotides listed were combined to siRNA cassettes and injected into *Drosophila* embryos. The starting material had a concentration of 100 μM or 10 μM, as indicated. The percentage indicates the number of embryos with visible *Notch* mutant phenotypes. The boxes below the percentage indicate the expressivity. This code has been used previously (Boutla et al., 2001), wherein +++++ indicates that all or almost all embryos show the strongest possible *Notch* phenotype (compare **C**), ++++ indicates that the strongest or strong phenotypes are dominating and outnumber the intermediate phenotypes, +++ indicates that intermediate phenotypes are most frequent, accompanied by some embryos that have strong and weak phenotypes. (**C**) Schematic overview of *Notch* mutant phenotypes. In addition to counting the numbers, the effect of the siRNA can be the gauged by the extent of neural hyperplasia when examining the cuticle of a mature embryo. The more severe the hyperplasia, the larger is the amount of the missing cuticle (for actual photographs, see Figure 1 in Boutla et al., 2001). The top indicates a wild-type embryo in side view, and *Notch* phenotypes of different expressivity are given schematically. The strongest phenotype observed with siRNAs cannot be discriminated from genetic loss-of-function mutations. All embryos had been injected from the posterior side.

mediate cleavage of a sense as well as of an antisense target strand (Elbashir et al., 2001b). However, there might be a preference if one strand contains 3′ protruding ribonucleotides and the other strand deoxynucleotides. Assuming that preferentially the all-ribonucleotide strand is removed from the RISC complex charged with a mixed siRNA (also normally one of the two all-ribonucleotide strands is removed), this would explain the observation that at 10 μM mixed siRNA cassettes containing a deoxy-modified sense strand are much less active (Fig. 1B, top row). Here, an activated RISC would be formed containing preferentially a sense strand that could not cleave the single-stranded sense *Notch* mRNA target. In the opposite combination (Fig. 1B, left column), the sense RNA strand would be removed preferentially, leaving the active RISC with an antisense RNA, competent for *Notch* mRNA degradation. Although beyond the scope of this study, this prediction could be tested in an *in vitro* system that recapitulates the processes of RNAi.

In addition to analyzing the influence of terminal deoxynucleotides, we prepared the all-RNA oligonucleotide R61-A with a nonmatching 3′-terminal A residue, which could not be used in primer extension similarly to oligonucleotide R61-TT (Fig. 1A). The siRNA consisting of oligonucleotides R61-A and R63 was examined. No significant loss of inhibitory potential was observed (Fig. 1B), suggesting that this modification with the loss of the potential for primer extension does not play a role. This finding is in agreement with the observation that there is no indication for transitive and systemic pathways of silencing in *Drosophila* (Roignant et al., 2003).

In conclusion, our data show that replacement of the 3′ protruding single-stranded nucleotides of a synthetic siRNA by matching deoxynucleotides is possible without loss of silencing potential, although only if both strands are modified simultaneously. Replacement by nonmatching TT residues works fine, also under the provision that it is done simultaneously in both strands and used in a high concentration. At lower concentrations, however, the silencing potential of such an siRNA is significantly reduced. Because delivery of sufficient amounts of siRNA is a critical point, it is better to avoid the introduction of nonmatching TT residues.

ACKNOWLEDGMENT

This work was supported by a grant from the General Secretariat for Research and Technology of the Hellenic Ministry of Development (contract PENED 01ED325).

REFERENCES

AMARZGUIOUI, M., HOLEN, T., BABAIE, E., and PRYDZ, H. (2003). Tolerance for mutations and chemical modifications in a siRNA. Nucleic Acids Res. **31,** 589–595.

BASU, S., TOTTY, N.F., IRWIN, M.S., SUDOL, M., and DOWNWARD, J. (2003). Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14–3–3 and attenuation of p73–mediated apoptosis. Mol. Cell **11,** 11–23.

BAULCOMBE, D. (2002). RNA silencing. *Curr. Biol.* **12,** R82–84.

BERNSTEIN, E., CAUDY, A.A., HAMMOND, S.M., and HANNON, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature **409,** 363–366.

BOUTLA, A., DELIDAKIS, C., LIVADARAS, I., TSAGRIS, M., and TABLER, M. (2001). Short 5′-phosphorylated double-stranded RNAs induce RNA interference in *Drosophila*. Curr. Biol. **11,** 1776–1780.

BRUMMELKAMP, T.R., BERNARDS, R., and AGAMI, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. Science **296,** 550–553.

CAPLEN, N.J., PARRISH, S., IMANI, F., FIRE, A., and MORGAN, R.A. (2001). Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. Proc. Natl. Acad. Sci. USA **98,** 9742–9747.

CZAUDERNA, F., FECHTNER, M., AYGUN, H., ARNOLD, W., KLIPPEL, A., GIESE, K., and KAUFMANN, J. (2003a). Functional studies of the PI(3)-kinase signalling pathway employing synthetic and expressed siRNA. Nucleic Acids Res. **31,** 670–682.

CZAUDERNA, F., FECHTNER, M., DAMES, S., AYGUN, H., KLIPPEL, A., PRONK, G.J., GIESE, K., and KAUFMANN, J. (2003b). Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. Nucleic Acids Res. **31,** 2705–2716.

DALMAY, T., HAMILTON, A., RUDD, S., ANGELL, S., and BAULCOMBE, D.C. (2000). An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell **101,** 543–553.

DONZE, O., and PICARD, D. (2002). RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase. Nucleic Acids Res. **30,** e46.

ELBASHIR, S.M., HARBORTH, J., LENDECKEL, W., YALCIN, A., WEBER, K., and TUSCHL, T. (2001a). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature **411,** 494–498.

ELBASHIR, S.M., LENDECKEL, W., and TUSCHL, T. (2001b). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. **15,** 188–200.

ELBASHIR, S.M., MARTINEZ, J., PATKANIOWSKA, A., LENDECKEL, W., and TUSCHL, T. (2001c). Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. EMBO J. **20,** 6877–6888.

GE, Q., McMANUS, M.T., NGUYEN, T., SHEN, C.H., SHARP, P.A., EISEN, H.N., and CHEN, J. (2003). RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. Proc. Natl. Acad. Sci. USA **100,** 2718–2723.

GITLIN, L., KARELSKY, S., and ANDINO, R. (2002). Short

interfering RNA confers intracellular antiviral immunity in human cells. Nature **418,** 430–434.

HAMADA, M., OHTSUKA, T., KAWAIDA, R., KOIZUMI, M., MORITA, K., FURUKAWA, H., IMANISHI, T., MIYAGISHI, M., and TAIRA, K. (2002). Effects on RNA interference in gene expression (RNAi) in cultured mammalian cells of mismatches and the introduction of chemical modifications at the 3′-ends of siRNAs. Antisense Nucleic Acid Drug Dev. **12,** 301–309.

HAMASAKI, K., NAKAO, K., MATSUMOTO, K., ICHIKAWA, T., ISHIKAWA, H., and EGUCHI, K. (2003). Short interfering RNA-directed inhibition of hepatitis B virus replication. FEBS Lett. **543,** 51–54.

HAMILTON, A., VOINNET, O., CHAPPELL, L., and BAULCOMBE, D. (2002). Two classes of short interfering RNA in RNA silencing. EMBO J. **21,** 4671–4679.

HAMILTON, A.J., and BAULCOMBE, D.C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. Science **286,** 950–952.

HAMMOND, S.M., BERNSTEIN, E., BEACH, D., and HANNON, G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature **404,** 293–296.

HAMMOND, S.M., CAUDY, A.A., and HANNON, G.J. (2001). Post-transcriptional gene silencing by double-stranded RNA. Nat. Rev. Genet. **2,** 110–119.

HANNON, G.J. (2002). RNA interference. Nature **418,** 244–251.

HARBORTH, J., ELBASHIR, S.M., VANDENBURGH, K., MANNINGA, H., SCARINGE, S.A., WEBER, K., and TUSCHL, T. (2003). Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. Antisense Nucleic Acid Drug Dev. **13,** 83–105.

HEIDENREICH, O., KRAUTER, J., RIEHLE, H., HADWIGER, P., JOHN, M., HEIL, G., VORNLOCHER, H.P., and NORDHEIM, A. (2003). AML1/MTG8 oncogene suppression by small interfering RNAs supports myeloid differentiation of t(8;21)-positive leukemic cells. Blood **101,** 3157–3163.

HEINONEN, J.E., SMITH, C.I., and NORE, B.F. (2002). Silencing of Bruton's tyrosine kinase (Btk) using short interfering RNA duplexes (siRNA) FEBS Lett. **527,** 274–278.

HOHJOH, H. (2002). RNA interference (RNAi) induction with various types of synthetic oligonucleotide duplexes in cultured human cells. FEBS Lett. **521,** 195–199.

HOLEN, T., AMARZGUIOUI, M., BABAIE, E., and PRYDZ, H. (2003). Similar behaviour of single-strand and double-strand siRNAs suggests they act through a common RNAi pathway. Nucleic Acids Res. **31,** 2401–2407.

HOLEN, T., AMARZGUIOUI, M., WIIGER, M.T., BABAIE, E., and PRYDZ, H. (2002). Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. Nucleic Acids Res. **30,** 1757–1766.

HUTVAGNER, G., and ZAMORE, P.D. (2002). RNAi: Nature abhors a double-strand. Curr. Opin. Genet. Dev. **12,** 225–232.

JACQUE, J.M., TRIQUES, K., and STEVENSON, M. (2002). Modulation of HIV-1 replication by RNA interference. Nature **418,** 435–438.

JIA, Q., and SUN, R. (2003). Inhibition of gammaherpesvirus replication by RNA interference. J. Virol. **77,** 3301–3306.

KAWASAKI, H., and TAIRA, K. (2003). Short hairpin type of dsRNAs that are controlled by tRNA(Val) promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells. Nucleic Acids Res. **31,** 700–707.

KOSCIOLEK, B.A., KALANTIDIS, K., TABLER, M., and ROWLEY, P.T. (2003). Inhibition of telomerase activity in human cancer cells by RNA interference. Mol. Cancer Ther. **2,** 209–216.

LEE, N.S., DOHJIMA, T., BAUER, G., LI, H., LI, M.J., EHSANI, A., SALVATERRA, P., and ROSSI, J. (2002). Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. Nat. Biotechnol. **20,** 500–505.

LEIRDAL, M., and SIOUD, M. (2002). Gene silencing in mammalian cells by preformed small RNA duplexes. Biochem. Biophys. Res. Commun. **295,** 744–748.

LIPARDI, C., WEI, Q., and PATERSON, B.M. (2001). RNAi as random degradative PCR. siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. Cell **107,** 297–307.

MARTINEZ, L.A., NAGUIBNEVA, I., LEHRMANN, H., VERVISCH, A., TCHENIO, T., LOZANO, G., and HAREL-BELLAN, A. (2002). Synthetic small inhibiting RNAs: Efficient tools to inactivate oncogenic mutations and restore p53 pathways. Proc. Natl. Acad. Sci. USA **99,** 14849–14854.

MATZKE, M., MATZKE, A.J., and KOOTER, J.M. (2001). RNA: Guiding gene silencing. Science **293,** 1080–1083.

McMANUS, M.T., HAINES, B.B., DILLON, C.P., WHITEHURST, C.E., VAN PARIJS, L., CHEN, J., and SHARP, P.A. (2002). Small interfering RNA-mediated gene silencing in T lymphocytes. J. Immunol. **169,** 5754–5760.

MIYAGISHI, M., HAYASHI, M., and TAIRA, K. (2003). Comparison of the suppressive effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. Antisense Nucleic Acid Drug Dev. **13,** 1–7.

MOURRAIN, P., BECLIN, C., ELMAYAN, T., FEUERBACH, F., GODON, C., MOREL, J.B., JOUETTE, D., LACOMBE, A.M., NIKIC, S., PICAULT, N., REMOUE, K., SANIAL, M., VO, T.A., and VAUCHERET, H. (2000). *Arabidopsis* SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell **101,** 533–542.

NOVINA, C.D., MURRAY, M.F., DYKXHOORN, D.M., BERESFORD, P.J., RIESS, J., LEE, S.K., COLLMAN, R.G., LIEBERMAN, J., SHANKAR, P., and SHARP, P.A. (2002). siRNA-directed inhibition of HIV-1 infection. Nat. Med. **8,** 681–686.

NYKÄNEN, A., HALEY, B., and ZAMORE, P.D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell **107,** 309–321.

PAUL, C.P., GOOD, P.D., WINER, I., and ENGELKE, D.R. (2002). Effective expression of small interfering RNA in human cells. Nat. Biotechnol. **20,** 505–508.

PLASTERK, R.H.A. (2002). RNA silencing: The genome's immune system. Science **296,** 1263–1265.

RANDALL, G., GRAKOUI, A., and RICE, C.M. (2003). Clearance of replicating hepatitis C virus replicon RNAs in

cell culture by small interfering RNAs. Proc. Natl. Acad. Sci. USA **100,** 235–240.

ROIGNANT, J.Y., CARRE, C., MUGAT, B., SZYMCZAK, D., LEPESANT, J.A., and ANTONIEWSKI, C. (2003). Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in *Drosophila*. RNA **9,** 299–308.

SCHWARZ, D.S., HUTVAGNER, G., HALEY, B., and ZAMORE, P.D. (2002). Evidence that siRNAs function as guides, not primers, in the *Drosophila* and human RNAi pathways. Mol. Cell **10,** 537–548.

SIJEN, T., FLEENOR, J., SIMMER, F., THIJSSEN, K.L., PARRISH, S., TIMMONS, L., PLASTERK, R.H., and FIRE, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. Cell **107,** 465–476.

SORENSEN, D.R., LEIRDAL, M., and SIOUD, M. (2003). Gene silencing by systemic delivery of synthetic siRNAs in adult mice. J. Mol. Biol. **327,** 761–766.

VAISTIJ, F.E., JONES, L., and BAULCOMBE, D.C. (2002). Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. Plant Cell **14,** 857–867.

VANCE, V., and VAUCHERET, H. (2001). RNA silencing in plants—Defense and counterdefense. Science **292,** 2277–2280.

VAUCHERET, H., and FAGARD, M. (2001). Transcriptional gene silencing in plants: Targets, inducers and regulators. Trends Genet. **17,** 29–35.

VICKERS, T.A., KOO, S., BENNETT, C.F., CROOKE, S.T., DEAN, N.M., and BAKER, B.F. (2003). Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. A comparative analysis. J. Biol. Chem. **278,** 7108–7118.

VOINNET, O. (2001). RNA silencing as a plant immune system against viruses. Trends Genet. **17,** 449–459.

VOINNET, O. (2002). RNA silencing: Small RNAs as ubiquitous regulators of gene expression. Curr. Opin. Plant Biol. **5,** 444–451.

WILSON, J.A., JAYASENA, S., KHVOROVA, A., SABATINOS, S., RODRIGUE-GERVAIS, I.G., ARYA, S., SARANGI, F., HARRIS-BRANDTS, M., BEAULIEU, S., and RICHARDSON, C.D. (2003). RNA interference blocks gene expression and RNA synthesis from hepatitis C replicons propagated in human liver cells. Proc. Natl. Acad. Sci. USA **100,** 2783–2788.

YOKOTA, T., SAKAMOTO, N., ENOMOTO, N., TANABE, Y., MIYAGISHI, M., MAEKAWA, S., YI, L., KUROSAKI, M., TAIRA, K., WATANABE, M., and MIZUSAWA, H. (2003). Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. EMBO Rep. **4,** 602–608.

ZHOU, Y., CHING, Y.P., KOK, K.H., KUNG, H.F., and JIN, D.Y. (2002). Post-transcriptional suppression of gene expression in *Xenopus* embryos by small interfering RNA. Nucleic Acids Res. **30,** 1664–1669.

Address reprint requests to:
Dr. Martin Tabler
Institute of Molecular Biology and Biotechnology
Foundation for Research and Technology-Hellas
P.O. Box 1527
GR-71110 Herablion/Crete
Greece

E-mail: tabler@imbb.forth.gr

Received July 1, 2003; accepted in revised form August 21, 2003.