RESEARCH ARTICLE

RNAi induced in mammalian and Drosophila cells via transfection of dimers and trimers of small interfering RNA

Kumiko Ui-Tei1,2*, Shuhei Zenno1, Aya Juni1,2 and Kaoru Saigo1

1Department of Biophysics and Biochemistry, Graduate School of Science, the University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. 2UPBSB, Department of Biophysics and Biochemistry, School of Science, the University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

*Correspondence to: Kumiko Ui-Tei, Email: ktei@biochem.s.u-tokyo.ac.jp, Tel: +81 3 5841 3044, Fax: +81 3 5841 3044

Journal of RNAi and Gene Silencing (2005), 1(2), 79-87
© Copyright Kumiko Ui-Tei et al

(Received 04 August 2005; Revised 22 September 2005; Accepted 26 September 2005; Available online 14 October 2005; Published 14 October 2005)

ABSTRACT

Twenty one base pair long small interfering RNAs (siRNAs) are widely in use in mammalian RNAi experiments. The present study assesses the capability of 43 and 63bp dsRNAs with two 2nt long 3’-overhangs to induce RNAi in mammalian and Drosophila cells. Human Dicer was found to cleave these dsRNAs from their ends to generate two or three monomeric siRNA units, each 21-22bp in length. When, in 43bp dsRNA, there was present a highly-effective siRNA sequence in frame with respect to the Dicer digestion, considerably high RNAi activity was noted to be induced in mouse embryonic stem E14TG2a, human HeLa, Chinese hamster CHO-K1 or Drosophila S2 cells. In contrast, RNAi depending on 63bp dsRNA, containing a highly effective siRNA sequence in frame with respect to Dicer digestion, varied considerably depending on cell lines used. While there was no appreciable RNAi in HeLa cells associated with relatively strong interferon response, a significant level of RNAi was noted in E14TG2a, CHO-K1 and S2 cells, in all of which interferon response induction was but slight, if at all. It would thus follow that siRNA oligomers with sequence of a highly functional siRNA monomer unit in frame with respect to dicer digestion should serve as a good RNAi agent in Drosophila and certain mammalian cells.

KEYWORDS: RNAi, siRNA, dsRNA, interferon response, mammalian cells, Drosophila

INTRODUCTION

RNAi is a double-stranded (ds)-RNA-dependent process of gene silencing in eukaryotes (Dykxhoorn et al, 2003; Meister and Tuschl, 2004; Mello and Conte Jr, 2004). In Drosophila, long dsRNAs of a few to several hundred bp in length, are presently in use as effective RNAi agents (Ui-Tei et al, 2000; Boutros et al, 2004). Long dsRNA introduced or expressed within cells are digested by Dicer, an RNase III-family member, into short interfering dsRNAs (siRNAs), 21-22bp in length (Bernstein et al, 2001; Ketting et al, 2001), which are incorporated into RNA-induced silencing complexes (RISC). Active RISC is considered to contain only the antisense strand of siRNA and recognizes target mRNA via hydrogen-bonding and eventually cleaves it at the position corresponding to the middle of the siRNA antisense strand (Elbashir et al, 2001a, 2001b, 2001c; Martinez et al, 2002). Ago protein performs a central role in mRNA processing (Hammond et al, 2001; Doi et al, 2003; Liu et al, 2004).

RNAi in mammals is quite similar, if not identical, in mechanism to RNAi in lower eukaryotes. However, most mammalian RNAi experiments are carried out using 21-22bp siRNA instead of long dsRNA to avoid possible interferon response (Elbashir et al, 2001b). siRNA-dependent RNAi in mammalian cells considerably varies depending on the sequence of the siRNA used (Holen et
Short Dicer-substrate dsRNA has recently been shown to be much more functional than 21-22bp-long siRNA, and maximal inhibitory activity of short dsRNA to be present at a duplex of 27 bp (Kim et al, 2005). RNA duplexes longer than 27 bp were found to show progressive loss of functional RNAi activity and by 40-45bp, to be entirely inactive at 1nM – this too being correlated with poor in vitro cleavage of the duplexes by Dicer (Kim et al, 2005).

These findings may not necessarily mean dsRNA longer than 40 bp to be incapable of inducing functional RNAi in mammalian cells. Indeed, RNAi in mammals was initially noted to occur on transfection of long dsRNA. Wianny et al (1999) showed the expression of GFP gene introduced into mouse embryos to be effectively inactivated by cognate long dsRNA. Ui-Tei et al (2000) found the expression of exogenous firefly luciferase gene in CHO-K1 cells significantly abolished on transfection of long cognate dsRNA or expression plasmid encoding long hairpin RNA.

As a first step in the clarification of the molecular basis for long dsRNA-depending RNAi in mammals, examination was made as to whether dsRNAs equivalent in length to siRNA dimers or trimers were capable of inducing RNAi in mammalian and Drosophila cells and, if so, what conditions were required. Long dsRNA with two 2 nt 3'-overhangs was shown effectively cleaved 21-22 bp from its ends by Dicer and should the sequence of a highly-effective siRNA such as, class I (Ui-Tei et al, 2004a), be included in resultant siRNA monomer as the main Dicer digestion product, and if cells used for transfection were tolerant interferon induction, considerable RNAi induction may occur in mammals and Drosophila.

MATERIALS AND METHODS

dsRNA preparation

dsRNAs with two 2 bp long 3'-overhangs were enzymatically synthesized using the CUGA7 in vitro siRNA Synthesis Kit (Nippon Genetech, Japan) (Figure 1A). Three deoxyoligonucleotides, oligo 1, oligo 2 and oligo 3 were mixed in a 2:1:1 fashion and annealed. (N)41 or 61 in oligo 2 and (N)41 or 61 in oligo 3 are complementary in sequence to each other and serve as templates for dsRNA strand synthesis by CUGA7 polymerase. Annealing was carried out at 75°C for 5 min followed by 1 min incubation at room temperature. Annealed DNA solution thus generated (16 µl) was mixed with distilled water (14 µl), 5 x transcription buffer (8 µl), NTP solution (12 µl) and CUGA7 solution (2 µl). dsRNA synthesis was carried out by incubating the reaction mixture at 37°C for 2 hr. Resultant dsRNA contains an additional guanine residue at each 5’end. These guanine residues and template DNA were simultaneously eliminated by incubating at 37°C for 2 hr in the presence of DNase I, RNase T1 and BSA. See the manufacturer protocol for other details. dsRNA was purified by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation. siRNAs were chemically synthesized by Proligo and purified as described previously (Ui-Tei et al, 2004a).

In vitro Dicer cleavage

dsRNA was incubated with recombinant human Dicer (Stratagene) in the presence of RNase inhibitor (0.4 units/µl; Promega), 20 mM Tris-HCl (pH 8.0), 150 mM NaCl and 2.5 mM MgCl2 at 37°C. Digestion products were size-fractionated using 15% (w/v) non-denaturing polyacrylamide TBE gel, stained with SyberGreen II and analyzed by LAS-3000 (FUJIFILM). Digestion products were also size-fractionated using 15% (w/v) denaturing polyacrylamide TBE gel with 7M Urea and analyzed by FLA2000 imageanalyzer (FUJIFILM).

Cell culture

S2, CHO-K1, E14TG2a and HeLa cells were cultured as described previously (Ui-Tei et al, 2004a). T98G cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco BRL) at 37°C.

RNAi assay in cultured cells

One milliliter of S2 (1 x 10⁶ cells/ml), CHO-K1 (3 x 10⁶ cells/ml), E14TG2a (2 x 10⁵ cells/ml), HeLa (1 x 10⁵ cells/ml) or T98G (2 x 10⁵ cells/ml) cell suspension was carried out at 75°C for 5 min followed by 1 min incubation at room temperature. Annealed DNA solution thus generated (16 µl) was mixed with distilled water (14 µl), 5 x transcription buffer (8 µl), NTP solution (12 µl) and CUGA7 solution (2 µl). dsRNA synthesis was carried out by incubating the reaction mixture at 37°C for 2 hr. Resultant dsRNA contains an additional guanine residue at each 5’end. These guanine residues and template DNA were simultaneously eliminated by incubating at 37°C for 2 hr in the presence of DNase I, RNase T1 and BSA. See the manufacturer protocol for other details. dsRNA was purified by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation. siRNAs were chemically synthesized by Proligo and purified as described previously (Ui-Tei et al, 2004a).
Induction of functional RNAi by siRNA dimer or trimer transfection

Only a fraction of siRNA (highly effective siRNA or active siRNA) has been shown capable of inducing high RNAi activity in transfected mammalian cells (Ui-Tei et al, 2004a; Naito et al, 2004). Thus, RNAi due to dimer- or trimer-sized dsRNAs may be considered effectively induced in mammalian cells only when an active siRNA is produced in cells from transfected dimer- or trimer-sized cognate dsRNA as a major Dicer-digestion product. In Drosophila, most cognate siRNAs effectively induce RNAi (Ui-Tei et al, 2004a) and accordingly, nearly all long cognate dsRNA should be active. But on using for RNAi induction a hetero-oligomer of siRNAs, in which only one monomer unit is homologous in sequence to target mRNA, a similar phase problem of effective siRNA will have to be encountered even in Drosophila RNAi.

For the solution, HeLa cells (human), CHO-K1 (Chinese hamster) and E14TG2a (mouse embryonic stem (ES)) cells along with Drosophila S2 cells were transfected with D1 and D2, each containing M1, a highly functional class I siRNA sequence for luc inactivation (see the lower margin of Figure 2A, 2B). D1, the M1 sequence is situated in a terminal half and thus should be released intact from D1 through in vivo Dicer function (see the lower margin of Figure 2B). In all cell lines examined, D1 induced significant levels of RNAi, although RNAi in HeLa cells appeared less effective (Figure 2B). In contrast, in D2, the M1 sequence is situated in a region out of frame with respect to Dicer digestion (see the lower margin of Figure 2B) and thus, is broken in the central region by Dicer with no appreciable RNAi activity production (Figure 2B).

To determine whether siRNA-dimer-dependent RNAi occurs in individual cells and whether two active siRNAs presumed to be released from an identical dsRNA 43bp long are simultaneously functional, 4 types of siRNA heterodimers (D3-D6) were constructed, each consisting of two siRNA monomer sequences homologous to those of DsRed and EGFP genes (see the lower margins of Figures 3A, B). Using highly functional and non-functional siRNA sequences. The heterodimers were transfected into CHO-K1 and HeLa cells (Figure 3A2-6, B2). The specificity of siRNA-dimer-dependent RNAi appeared to depend on the capability of siRNA monomer units (Figure 3A1-4, B) and 5’→3’ direction of antisense strand of each component siRNA was not essential for RNAi induction (e.g., compare Figures 3A5 and A6). When two monomeric siRNA units were highly active, two target genes were simultaneously knocked down (Figure 3A5, 6).

Effective conversion of siRNA dimers and trimers to monomer units through Dicer cleavage from RNA ends with 2 nt-long 3’ overhangs

RNA duplexes with two 2 nt long 3’ overhangs and equivalent in length to siRNA dimers (D1,D2) and trimers (T1,T2) were synthesized following the CUGA7 system (Figure 1A). Resultant dsRNAs were terminally digested by Dicer by 4 hr incubation at 37°C to generate 21-22 bp-long dsRNA as the main reaction products (Figure 1B). Quantitative Dicer digestion analysis was also carried out using T1 as a substrate (Figure 1C). 63 bp signals were almost completely converted to 21-22 bp signals without generating any significant amount of possible intermediates. These findings may indicate Dicer to be considerably high in processivity, and 43 and 63 bp dsRNA to have been cleaved in two and three siRNA monomer units, respectively. To further confirm this, a 30 bp long dsRNA with two 2nt-long 3’ overhangs was chemically synthesized, end-labeled with 32P and subjected to Dicer digestion (Figure 1D). As anticipated, Dicer digestion generated two 32P-labeled products, 21-22 bp and 8-9 bp in length. Dicer thus appears capable of functioning as exonuclease to release successively 21-22bp siRNAs from the two ends of long dsRNA with two 2 nt long 3’ overhangs. As schematically shown in Figure 1E, any given siRNA monomer sequence in the Dicer substrate dsRNA is considered to be in either in-frame or out-of-frame configurations with respect to Dicer digestion; the frame-unit size is 21-22 bp.

RESULTS

Effective conversion of siRNA dimers and trimers to monomer units through Dicer cleavage from RNA ends with 2 nt-long 3’ overhangs

RNA duplexes with two 2 nt long 3’ overhangs and equivalent in length to siRNA dimers (D1,D2) and trimers (T1,T2) were synthesized following the CUGA7 system (Figure 1A). Resultant dsRNAs were terminally digested by Dicer by 4 hr incubation at 37°C to generate 21-22 bp-long dsRNA as the main reaction products (Figure 1B). Quantitative Dicer digestion analysis was also carried out using T1 as a substrate (Figure 1C). 63 bp signals were almost completely converted to 21-22 bp signals without generating any significant amount of possible intermediates. These findings may indicate Dicer to be considerably high in processivity, and 43 and 63 bp dsRNA to have been cleaved in two and three siRNA monomer units, respectively. To further confirm this, a 30 bp long dsRNA with two 2nt-long 3’ overhangs was chemically synthesized, end-labeled with 32P and subjected to Dicer digestion (Figure 1D). As anticipated, Dicer digestion generated two 32P-labeled products, 21-22 bp and 8-9 bp in length. Dicer thus appears capable of functioning as exonuclease to release successively 21-22bp siRNAs from the two ends of long dsRNA with two 2 nt long 3’ overhangs. As schematically shown in Figure 1E, any given siRNA monomer sequence in the Dicer substrate dsRNA is considered to be in either in-frame or out-of-frame configurations with respect to Dicer digestion; the frame-unit size is 21-22 bp.

Induction of functional RNAi by siRNA dimer or trimer transfection

Only a fraction of siRNA (highly effective siRNA or active siRNA) has been shown capable of inducing high RNAi activity in transfected mammalian cells (Ui-Tei et al, 2004a; Naito et al, 2004). Thus, RNAi due to dimer- or trimer-sized dsRNAs may be considered effectively induced in mammalian cells only when an active siRNA is produced in cells from transfected dimer- or trimer-sized cognate dsRNA as a major Dicer-digestion product. In Drosophila, most cognate siRNAs effectively induce RNAi (Ui-Tei et al, 2004a) and accordingly, nearly all long cognate dsRNA should be active. But on using for RNAi induction a hetero-oligomer of siRNAs, in which only one monomer unit is homologous in sequence to target mRNA, a similar phase problem of effective siRNA will have to be encountered even in Drosophila RNAi.

For the solution, HeLa cells (human), CHO-K1 (Chinese hamster) and E14TG2a (mouse embryonic stem (ES)) cells along with Drosophila S2 cells were transfected with D1 and D2, each containing M1, a highly functional class I siRNA sequence for luc inactivation (see the lower margin of Figure 2A, 2B). In D1, the M1 sequence is situated in a terminal half and thus should be released intact from D1 through in vivo Dicer function (see the lower margin of Figure 2B). In all cell lines examined, D1 induced significant levels of RNAi, although RNAi in HeLa cells appeared less effective (Figure 2B). In contrast, in D2, the M1 sequence is situated in a region out of frame with respect to Dicer digestion (see the lower margin of Figure 2B) and thus, is broken in the central region by Dicer with no appreciable RNAi activity production (Figure 2B).

To determine whether siRNA-dimer-dependent RNAi occurs in individual cells and whether two active siRNAs presumed to be released from an identical dsRNA 43bp long are simultaneously functional, 4 types of siRNA heterodimers (D3-D6) were constructed, each consisting of two siRNA monomer sequences homologous to those of DsRed and EGFP genes (see the lower margins of Figures 3A, B). Using highly functional and non-functional siRNA sequences. The heterodimers were transfected into CHO-K1 and HeLa cells (Figure 3A2-6, B2). The specificity of siRNA-dimer-dependent RNAi appeared to depend on the capability of siRNA monomer units (Figure 3A1-4, B) and 5’→3’ direction of antisense strand of each component siRNA was not essential for RNAi induction (e.g., compare Figures 3A5 and A6). When two monomeric siRNA units were highly active, two target genes were simultaneously knocked down (Figure 3A5, 6).
Figure 1. CUGA7-polymerase-dependent synthesis (A) and Dicer digestion (B-D) of 43, 63 and 30 bp dsRNAs with two 2 nt 3’ overhangs. (A) dsRNA synthesis procedure; N, X, Y = A, T, G or C. W, Z = A, T or G. Underline, complementary nucleotide. L-shaped arrows, Templates for dsRNA strands. (B) Nucleotide sequences of 43 bp (D1/D2) and 63 bp (T1/T2) dsRNAs are shown in the lower margins of Fig2B.C. dsRNA (200 ng/µl) was digested with Dicer (0.05 units/ml) for 4 hr at 37˚C. (C) Time course of Dicer digestion of T1. (D) Dicer (0.005 units/ml) digestion of 30bp-long dsRNA (0.05 pmol/µl) with two 2bp-long 3’ overhangs. Red and blue asterisks, respectively, show 32P-labeled and non-labeled ends. Presumed Dicer cleavage is shown in the lower margin. (E) Dicer cleavage model. Active monomer siRNA is presumed to be generated only when its sequence in long dsRNA is in frame with respect to Dicer digestion.
Figure 2. 43/63bp-dsRNA-dependent RNAi. Relative luc activity in S2, CHO-K1, E14TG2a and HeLa cells was examined. Dotted red lines in B and C, respectively, show dose-response curves for single transfection of M1 and M2 active siRNAs. (A) Control. siRNA-dependent RNAi. M1 and M2, active siRNAs. (B) 43bp-dsRNA-dependent RNAi. As 43bp dsRNA, D1 and D2 were used. The M1 sequence in D1 is in frame with respect to dicer digestion, while that in D2 is out of frame. D1 transfection induced considerable RNAi activity in all cell lines used, whereas D2 transfection induced no or little RNAi. (C) 63bp-dsRNA-dependent RNAi. The M2 sequence in T1 is in frame with respect to dicer digestion if 21bp is used as a frame-unit size. However, no 21-22bp active siRNA is produced should the frame-unit size be only 22bp (see green and red hooked lines). The M2 sequence in T2 is out of frame with respect to dicer digestion. T2 transfection produced no or little RNAi activity in all cell lines examined, whereas T1 transfection induced considerable levels of RNAi activity in S2 and E14TG2a cells but not in CHO-K1 and HeLa.
Figure 3. siRNA-dimer/trimer-dependent RNAi. siRNA dimers and trimers, whose sequences are shown in the lower margin of each panel triplet, were constructed using nucleotide sequences of active/inactive siRNAs for EGFP, DsRed and luc gene knock-down. The expression and nucleotide sequences of EGFP were colored in green, while those of DsRed were colored in red. The nucleotide sequences of 3’ overhangs, which may not be essential for gene knock-down, are not necessarily identical to those of original siRNAs. These are colored in black. Gene knock-down effects were observed 48 hours after transfection. Left-most panels, phase contrast pictures. Arrows, 5’→ 3’ direction of the antisense strand of monomer units. A1-6, CHO-K1 cells. A1, Mock. A2, D2 transfection. D2 is unrelated in sequence to targets. A3, D3 transfection. D3 = inactive EGFP siRNA + active DsRed siRNA. Only DsRed signals were reduced. A4, D4 transfection. D4 = active EGFP siRNA + inactive DsRed siRNA. Only EGFP signals were reduced.
two parts (see the lower panel of Figure 2C). On using E14TG2a and S2 cells for transfection, T1 but not T2 induced considerably high RNAi activity to eliminate luc (Figure 2C), EGFP and DsRed gene activity (Figure 3, C1-3). However, no appreciable RNAi activity could be detected in HeLa (Figure 2C). In CHO-K1 cells, although only a low level of luc RNAi was evident (Figure 2C), EGFP and DsRed expression was significantly abolished through T1 transfection (Figure 3C, 4-6), possibly suggesting T1 to cleave mainly 22 bp from ends through CHO-K1 Dicer (see green and red hooked lines in the lower margin of Figure 2C).

The above findings suggest that 43-63 bp long dsRNAs may be capable of considerable RNAi induction in certain mammalian and Drosophila cells, should a highly-effective siRNA sequences be present in frame with respect to Dicer digestion.

**Poor induction of interferon response in ES cells**

dsRNA transfection-dependent change in interferon response in HeLa, T98G (human) and E14TG2a cells was examined by monitoring the expression of a number of interferon response marker genes such as, 2',5'-oligoadenylate synthetase (OAS)1-3, signal transducer and activator of transcription (STAT)1b and interferon-inducible transmembrane protein (IFNMP)2 (Sledz et al, 2003). dsRNA concentration for transfection was 0.6 µg/ml, which is equivalent to 50 nM in 21bp siRNA. Interferon response induction, however, varied considerably from cell line to cell line when transfected with D4 or T2. As previously reported, interferon response was greatest in T98G cells where RNAi may not be effectively induced (Sledz et al., 2003). HeLa cells transfected with D4 or T2 exhibited considerable interferon response but only slight, if any, increase in interferon response could be detected in E14TG2a cells transfected with D4 or T2 (Figure 4). In all cases, interferon response induced by monomer was very low, if any, and that induced by dimer was virtually the same as that by trimer.

**DISCUSSION**

The present findings indicate siRNA dimer and trimer capable of efficiently inducing RNAi, provided these oligomers possess two 2nt-long 3’ overhangs and contain an active monomer unit in frame with respect to Dicer digestion and suitable transfection cells are used.

Previous work demonstrated RNA duplexes 40-45bp in length not only to be poor substrates for Dicer processing but also to be incapable of inducing efficient RNAi in HEK293 cells (Kim et al, 2005). The present work demonstrated that 43 mers are good substrates for Dicer processing (Figure 1B) and inducers of efficient RNAi (Figures 2 and 3). These differences may be due to the fact that the duplexes employed in the Kim et al.’s work were blunt while those used in the present work possessed ends with 2 nt 3’-overhangs. Dicer is presumed to cleave off siRNAs from the termini of dsRNA substrates (Zhang et al, 2002), and to function through intramolecular dimerization of its two RNaseIII domains, assisted by the flanking RNA binding domains, PAZ and dsRBD (Zhang et al, 2004). Vermeulen et al (2005) have recently shown that 61mer dsRNA containing blunt and those with 3’ overhang ends are processed in distinctly different manners and pointed out the importance of interactions between dsRNA ends and the PAZ domain of Dicer for specificity and efficiency in dicing. Figure 1D shows that RNA duplexes with 2nt 3’-overhangs are specifically cleaved at points 21-22bp from the ends. During the preparation of this manuscript, Rose et al (2005) reported that blunt 27mer duplexes resulted in a wide variety of dicing patterns while major Dicer cleavage sites of 27 mers with 2 nt 3’-overhangs were located 21-22bp from the overhang. Thus, the presence of 2nt-long 3’ overhangs is critically important for sequential in-frame dicing of dsRNA.

In HeLa cells, interferon response significantly increased with transfection of siRNA dimer or trimer but not monomer (Figure 4). In contrast, in E14TG2a cells, dimer/trimer-dependent induction of interferon response was but slight, if at all (Figure 4). TLR7 or 8, Toll-like receptors, have recently been shown to be involved in the recognition of the immunostimulatory siRNA motifs, which is essential for induction of the interferon pathway (Hornung et al, 2005; Agrawal et al, 2005; Sledz et al, 2003; Judge et al, 2005; Sioud, 2005). Thus, difference in induction of interferon responses by different cell types and dsRNA lengths may be a reflection of interactions between TLR7/8 and immunostimulatory motifs of siRNA. Consistent with this M1 monomer lacking GU induced the lowest level of interferon response in all cells examined, while interferon response was increased in both HeLa and T98G cells with increasing the content of GU motifs (Figure 4; see also, Sioud, 2005). M2, D4 and T2, respectively, contain 3, 5, and 8 GU residues. However, in all four dsRNAs examined, neither 5’UGUGU (Judge et al, 2005) nor 5’GUCCUUCAA (Hornung et al, 2005) was found.

Induction in interferon response and RNAi activity may not always be correlated to each other. Indeed, our results indicated that, in HeLa cells transfected with a siRNA dimer not only have considerable RNAi activity but also interferon response is virtually the same as that for a trimer dsRNA-transfected HeLa cells (Figures 2B, 3B and 4A). In CHO-K1 cells, which appear tolerant to long dsRNA transfection (Ui-Tei et al, 2000, 2004b), T1 transfection gave rise to only a marginal level of RNAi for luc (Figure 2C), but a considerable level of RNAi for EGFP and
**Figure 4.** siRNA-oligomer-dependent interferon response. Change in expression of interferon response markers (OAS1-3, STAT1b and IFNMP2) in E14TG2a, HeLa and T98G cells were examined using RT-PCR. GAPDH, control. Lane 1: plasmid DNA transfection. Lanes 2-5, respectively, corresponds to transfection of M1, M2, D4 and T2. Numerals in parentheses, Amplification cycles. In all cases except for T98G transfected with M2, monomeric siRNAs (M1 and M2) induced only marginal, if any, interferon response. The highest level of interferon response was observed in T98G cells transfected with D4 and T2, while that in HeLa cells was considerable and that in E14TG2a cells was very low, if any.

DsRed (Figure 3C). In T1, EGFP and DsRed siRNA sequences are terminally situated, but luc is in a central third. We consider that this position-dependent difference in T1-induced RNAi activity might be partly due to 1-2 bp ambiguity in framing by Dicer, which is capable of generating two-three distinct siRNA monomers in length (see the lower margin of Figure 2C).

One advantage of having a dimer or trimer dsRNA for gene silencing is that multiple siRNAs targeting one gene or multiple genes could be produced in all cells transfected with a single dsRNA. The simplest way to eliminate the off-target effect is considered to simultaneously introduce multiple siRNAs for a single target gene into cells (Jackson and Linsley, 2004). Simultaneous knock-down of the target gene and one of interferon response inducing genes would be required for future therapeutic application of RNAi. In *Drosophila* DNA encoding dsRNA, more than 500bp in length, is still capable of inducing highly functional RNAi to render UAS-constructs capable of generating double or triple mutant clones essential for conducting mosaic analysis or sophisticated analysis of development of various tissues.

**CONCLUSIONS**

As with 21bp siRNA, siRNA dimer or trimer, each containing a highly functional siRNA sequence as a monomer unit, was found capable of efficiently inducing RNAi in transfected cells.

**ACKNOWLEDGEMENTS**

We thank A. Tanaka and R. Numazawa for technical assistance. This work was partly supported by Special Coordination Fund for promoting Science and Technology to K.S., and grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan to K.S. and K.U.-T.

**STATEMENT OF COMPETING INTERESTS**

The authors declared no competing interests.

**LIST OF ABBREVIATION**

| nt: Nucleotide |
| dsRNA: Double-stranded RNA |
| RISC: RNA-induced silencing complex |
| ES: Embryonic stem |
| OAS: 2',5'-oligoadenylate synthetase |
| STAT: Signal transducer and activator of transcription |
| IFNMP: Interferon-inducible transmembrane protein |
| TLR: Toll-like receptor |

**REFERENCES**

Agrawal S and Kandimalla ER. 2005. Antisense and siRNA as agonists of Toll-like receptors. Nat Biotech, 22, 1533-1537.

Amarzguioui M and Prydz H. 2004. An algorithm for selection of functional siRNA sequences. Biochem Biophys Res Commun, 316, 1050-1058.

Bernstein E, Caudy AA, Hammond SM and Hannon GJ. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature, 409, 363-366.

Boutros M, Kiger AA, Armknecht S, et al. 2004. Genome-wide RNAi analysis of growth and viability in Drosophila cells. Science, 303, 832-835.

Doi N, Zenno S, Ueda R, Ohki-Hamazaki H, Ui-Tei K and Saigo K. 2003. Short-interfering-RNA mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors. Curr Biol, 13, 41-46.

Dykxhoorn DM, Novina CD and Sharp PA. 2003. Killing the messenger: short RNAs that silence gene expression. Nat Rev Mol Cell Biol, 4, 457-467.

Elbashir SM, Lendeckel W and Tuschl T. 2001a. RNA interference is mediated by 21 and 22 nt RNAs. Genes Dev, 15, 494-498.

Elbashir SM, Martinez J, Pathkaniowska A, Lendeckel W and Tuschl T. 2001b. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature, 411, 494-498.

Elbashir SM, Martinez J, Pathkaniowska 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.

Hammond SM, Boettcher S, Caudy AA, Kobayashi R and Hannon GJ. 2001. Argonaute2, a link between genetic and biochemical analyses of RNAi. Science, 293, 1146-1150.

Holen T, Amarzguioui M, Wiiger MT, 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.

Hornung V, Guenthner-Biller M, Bourquin C et al. 2005. Sequence-specific potent induction of IFN-α by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat Med, 11, 263-270.

Jackson AL and Linsley PS. 2004. Noise amidst the silence: off-target effects of siRNAs? Trends Genet, 20, 521-524.

Judge AD, Sood V, Shaw JR, Fang D, McClintock K and MacLachlan I. 2005. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Nat Biotech, 23, 457-462.

Ketting RF, Fischer SEJ, Bernstein E, Sijen T, Hannon GJ and Plasterk RHA. 2001. Dicer functions in RNA interference and in synthesis of small developmental timing in C. elegans. Genes Dev, 15, 2654-2659.

Kim DH, Behlke MA, Rose SD, Chang M-S, Choi S and Rossi JJ. 2005. Synthetic dsRNA dicer substrates enhance RNAi potency and efficacy. Nat Biotech, 23, 222-226.

Martinez J, Patkaniowska A, Urlaub H, Lührmann R and Tuschl T. 2002. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell, 110, 563-574.

Meister G and Tuschi T. 2004. Mechanisms of gene silencing by double-stranded RNA. Nature, 431, 343-349.

Mello CC and Conte Jr D. 2004. Revealing the world of RNA interference. Nature, 431, 338-342.

Naito Y, Yamada T, Ui-Tei K, Morishita S and Saigo K. 2004. siDirect: highly effective, target-specific siRNA design software for mammalian RNA interference. Nucleic Acids Res, 32, W124-W129.

Reynolds A, Leake D, Boese Q, Searinge S, Marshall WS and Khvorova A. 2004. Rational siRNA design for RNA interference. Nat Biotech, 22, 326-330.

Rose SD, Kim D-H, Amarzguioui M, Heidel JD, Collingwood MA, Davis ME, Rossi JJ and Behlke MA. 2005. Functional polarity is introduced by Dicer processing of short substrate RNAs. Nucleic Acids Res, 33, 4140-4156.

Sioud M. 2005. Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localiziation. J Mol Biol, 348, 1079-1090.

Slecz CA, Holko M, de Veer MJ, Silverman RH and Williams BRG. 2003. Activation of the interferon system by short-interfering RNAs. Nat Cell Biol, 5, 834-839.

Ui-Tei K, Zenno S, Miyata Y and Saigo K. 2000. Sensitive assay of RNA interference in Drosophila and Chinese hamster cultured cells using firefly luciferase gene as target. FEBS Lett, 479, 79-82.

Ui-Tei K, Naito Y, Takahashi F, Haraguchi T, Ohki-Hamazaki H, Juni A, Ueda R and Saigo K. 2004a. Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Res, 32, 936-948.

Ui-Tei K, Ueda R, Zenno S, Takahashi F, Doi N, Naito Y, Yamamoto M, Hashimoto N, Takahashi K, Hamada T, Tokunaga T and Saigo K. 2004b. RNA interference induced by transient or stable expression of hairpin structures of double stranded RNA in Drosophila and mammalian cell. Mol Biol, 38, 276-287.

Wianny F and Zemicka-Goetz M. 1999. Specific interference with gene function by double-stranded RNA in early mouse development. Nat Cell Biol, 2, 70-75.

Vermeulen A, Behlen L, Reynolds A, Wolfson A, Marshall W, Karpilow J and Khvorova A. 2005. The contributions of dsRNA structure to Dicer specificity and efficiency. RNA, 11, 674-682.

Zhang H, Kolb FA, Brondani V, Billy E and Filipowicz W. 2002. Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. EMBO J, 21, 5875-5885.

Zhang H, Kolb FA, Jaskiewicz L, Westhof E and Filipowicz W. 2004. Single processing center models for human dicer and bacterial RNase III. Cell, 118, 57-68.