RESEARCH ARTICLE

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

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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

al, 2002; Ui-Tei et al, 2004a). siRNA sequence preference rules have been previously reported (Ui-Tei et al, 2004a; Reynolds et al, 2004; Amarzguioui and Prydz, 2004).

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. $(N)_{41 \text{ or } 61}$ in oligo 2 and $(\underline{N})_{41 \text{ or } 61}$ 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 5 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 $MgCl_2$ 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 10^6 cells/ml), CHO-K1 (3 x 10^5 cells/ml), E14TG2a (2 x 10^5 cells/ml), HeLa (1 x 10^5 cells/ml) or T98G (2 x 10^5 cells/ml) cell suspension was inoculated into a 1.5-cm well 24 hr prior to transfection. For luc RNAi assay, cells were transfected with pGL3-Control DNA (1 µg, Promega) encoding firefly luciferase and pRL-SV40 DNA (0.1 µg, Promega) encoding *Renilla* luciferase, with or without dsRNA. The calcium phosphate precipitation method was used for transfection for S2, while Lipofectamine 2000 (Invitrogen), CHO-K1, E14TG2a, HeLa and T98G cells. Cells were harvested 24 hr after transfection and luciferase activity was measured using the dual-luciferase assay, in which firefly and *Renilla* luciferases served as target and reference, respectively. For EGFP or DsRed RNAi assay, cells were transfected with a 1:1 mixture of pCAGGS-EGFP DNA (Ui-Tei et al, 2004a; 0.2 µg) and pCAGGS-DsRed DNA (Ui-Tei et al, 2004a; 0.2 µg), with or without dsRNA, and the cells were observed under fluorescent microscope (Zeiss) 48 hr after transfection. For EGFP RNAi assay, DsRed served as an internal control and vice versa. For EGFP and DsRed double knockdown, EGFP and DsRed activity in cells untreated with dsRNA was used as an external control. Co-transfection efficiency of target and reference DNA was nearly 100% (see Figure 3A1, B1, C1) and more than 90% of target signals were eliminated upon active dsRNA cotransfection (see, e.g., Figure 3A3).

Detection of interferon response

Interferon response in human (HeLa and T98G) and mouse (E14TG2a) cells was examined using RT-PCR. As interferon-response markers, STAT1b, OAS1-3 and IFNMP2 were used. GAPDH was used as a control. Cells were transfected with pCAGIpuro-EGFP DNA (0.2 µg) encoding the EGFP and puromycin resistant genes irrespective of dsRNA treatment. Transfected cells were selected by a 24 hr puromycin (2 µg/ml) treatment initiated 24 hr after transfection. The total RNA was extracted and purified with TRIZOL (Invitrogen). RNA used was 0.5 µg. Other details are described in the manu-

facture's protocol. A 1 µl aliquot of the RT reaction mixture was PCR amplified using AmpliTaqGOLD (Invitrogen). After they preheated at 94°C for 10 min, the sample was subjected to PCR (95°C (15sec)-55°C (30sec)-72°C (60sec), 18-30 cycles). The following PCR primers were used:

hGAPDH
5'CAAGGCTGAGAACGGGAAGCT, and
5'GACACGGAAGGCCATGCCAGT
hSTAT1b
5'CAGAGCACAGTGATGTTAGAC, and
5'GTGATAGGGTCATGTTCGTAG
hOAS1
5'GGAACATGAGGTGGCTGTGCT, and
5'TTGGTACCAGTGCTTGACTAG
hOAS2
5'TCCTAGAGCAGATTGACAGTG, and
5'CCAGGCATAGATGGTGAGCAG
hOAS3
5'GCAACAGCATCAGCAGCTCTG, and
5'TGACCTCGAACTGCCGCTCCT
hIFNMP2
5'GGAACATGAGGTGGCTGTGCT, and
5'AGCCGAATACCAGTAACAGGA
mGAPDH
5'GCCTCGTCCCGTAGACAAAA, and
5'ACTGTGGTCATGAGCCCTTC
mSTAT1b
5'CAGAACACTGTGATGTTAGAT, and
5'GTAATAGGGTCGGGCTCATAG
mOAS2
5'TCCTAAAGCAGATCAAGGAAG, and
5'CCAGGCATACACGGTGAGCAG
mOAS3
5'GGGTCAGCAAGGGCGGCTCT, and
5'GGAGCATCACCTGCCGCCTCT.

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 (Figure1B). Quantitative Dicer digestion analysis was also carried out using T1 as a substrate (Figure1C). 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 ^{32}P and subjected to Dicer digestion (Figure 1D). As anticipated, Dicer digestion generated two ^{32}P-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 Fig.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).

T1 is comprised of three active siRNA sequences targeting EGFP, luc and DsRed, all in frame with respect to Dicer digestion (see the lower panel of Figure 2C). But in T2, EGFP and DsRed siRNA monomer units are in an out-of-frame position and the luc siRNA sequence is divided into

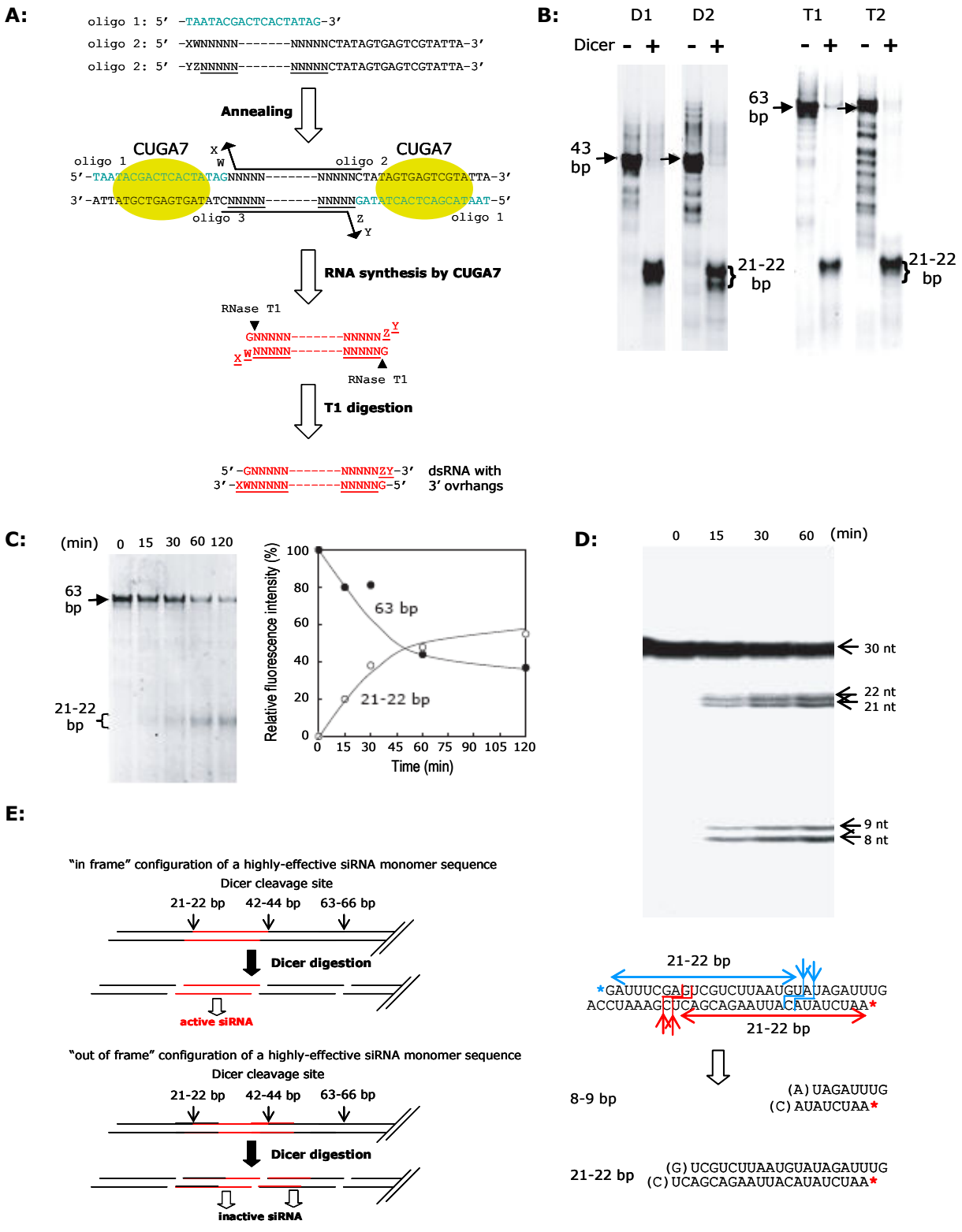

Figure 1. CUGA7-polymerase-dependent synthesis (**A**) and Dicer digestion (**B-D**) of 43, 63 and 30 bp dsRNAs with two 2 nt 3'overhangs. **(A)** dsRNA synthesis procedure; N, X, Y=A,T,G or C. W, Z = A,T or G. Underline, complementary nucleotide. L-shaped arrows, Templates for dsRNA strands. **(B)** Nucleotide sequences of 43bp (D1/D2) and 63bp (T1/T2) dsRNAs are shown in the lower margins of Fig.2B,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 ^{32}P-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.

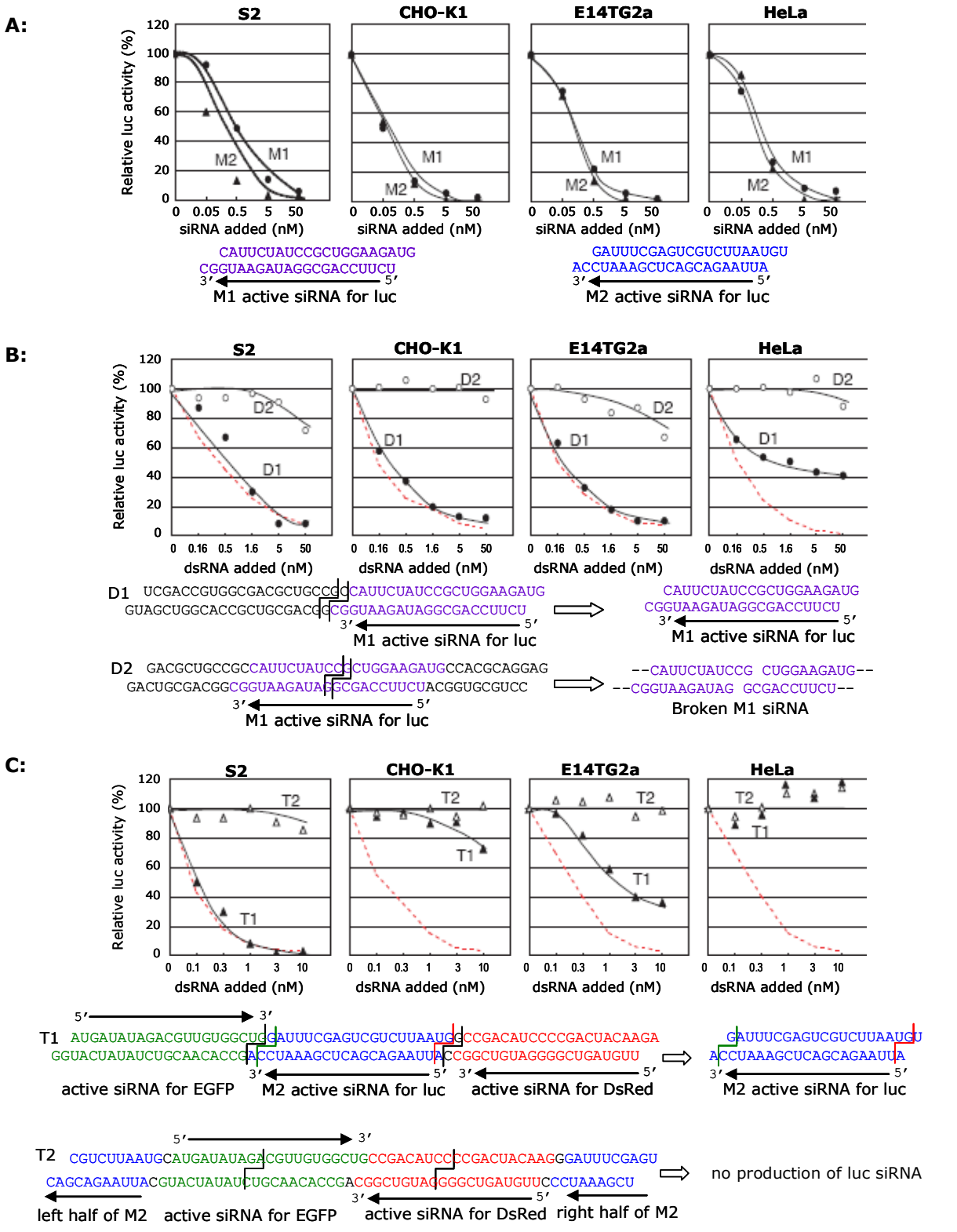

Figure 2. 43/63bp-dsRNA-dependent RNAi. Relative luc activity in S2, CHO-K1, E14TG2a and HeLa cells was examined. Dotted red lines in B and C, respectively, show dose-response curves for single transfection of M1 and M2 active siRNAs. **(A)** Control. siRNA-dependent RNAi. M1 and M2, active siRNAs. **(B)** 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.

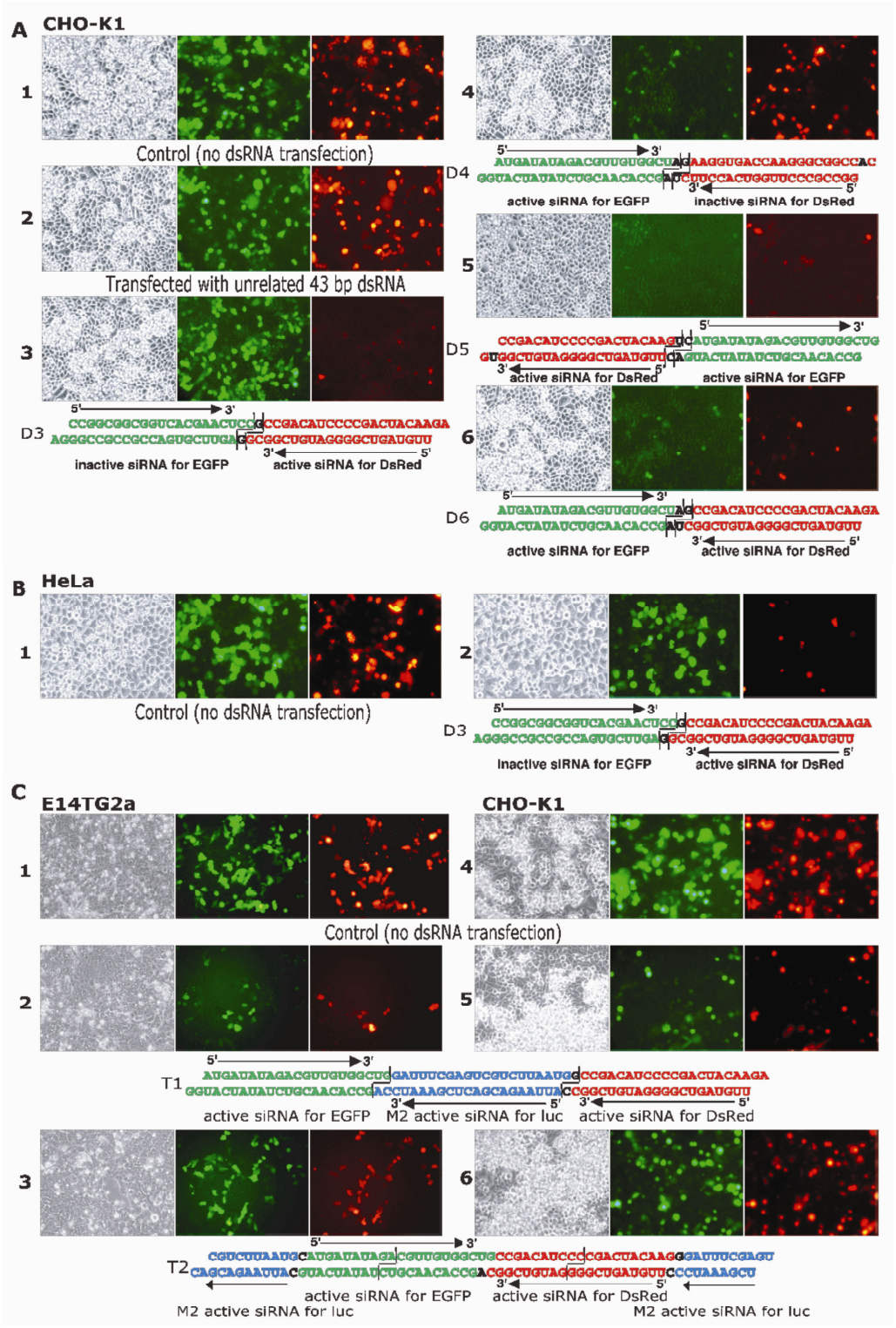

Figure 3. siRNA-dimer/trimer-dependent RNAi. siRNA dimers and trimers, whose sequences are shown in the lower margin of each panel triplet, were constructed using nucleotide sequences of active or inactive siRNAs for EGFP, DsRed and luc gene knock-down. The expression and nucleotide sequences of EGFP 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. **A**1-6, CHO-K1 cells. **A**1, Mock. **A**2, D2 transfection. D2 is unrelated in sequence to targets. **A**3, D3 transfection. D3 = inactive EGFP siRNA + active DsRed siRNA. Only DsRed signals were reduced. **A**4, D4 transfection. D4 = active EGFP siRNA + inactive DsRed siRNA. Only EGFP signals were reduced.

A5, D5 transfection. D5 = active EGFP siRNA + active DsRed siRNA. Both DsRed and EGFP signals were reduced. **A**6, D6 transfection. D6 = active EGFP siRNA + active DsRed siRNA. Both DsRed and EGFP signals were reduced. **B**1,2, HeLa cells. **B**1, Mock. **B**2, D3 transfection. Only DsRed signals were reduced. **C**1-3, E14TG2a cells. **C**4-6, CHO-K1cells. **C**1,4, Mock. **C**2,5, T1 transfection. T1 = active EGFP siRNA + active luc siRNA + active DsRed siRNA. Note EGFP and DsRed signals were significantly reduced in both E14TG2a and CHO-K1. **C**3,6, abortive gene-silencing in cells transfected with T2, having no in-frame siRNA sequences for EGFP and DsRed knock-down.

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 2-nt 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

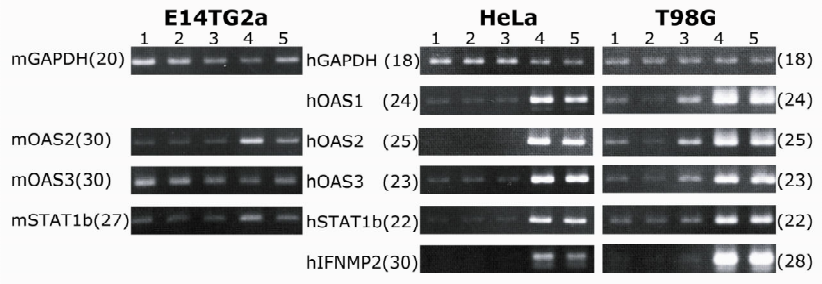

Figure 4. siRNA-oligomer-dependent interferon response. Change in expression of interferon response markers (OAS1-3, STAT1b and IFNMP2) in E14TG2a, HeLa and T98G cells were examined using RT-PCR. GAPDH, control. Lane 1; plasmid DNA transfection. Lanes 2-5, respectively, corresponds to transfection of 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.

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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

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