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Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy

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RNA interference (RNAi) is the process of sequence-specific post-transcriptional gene silencing triggered by double-stranded RNAs[1–3]. In attempts to identify RNAi triggers that effectively function at lower concentrations, we found that synthetic RNA duplexes 25–30 nucleotides in length can be up to 100-fold more potent than corresponding conventional 21-mer small interfering RNAs (siRNAs). Some sites that are refractory to silencing by 21-mer siRNAs can be effectively targeted by 27-mer duplexes, with silencing lasting up to 10 d. Notably, the 27-mers do not induce interferon or activate protein kinase R (PKR). The enhanced potency of the longer duplexes is attributed to the fact that they are substrates of the Dicer endonuclease, directly linking the production of siRNAs to incorporation in the RNA-induced silencing complex. These results provide an alternative strategy for eliciting RNAi-mediated target cleavage using low concentrations of synthetic RNA as substrates for cellular Dicer-mediated cleavage.

RNAi is an evolutionarily conserved process by which specific mRNAs are targeted for degradation by complementary siRNAs. It has become the method of choice for mammalian cell genetics as well as for potential sequence-specific therapeutic approaches[1–3]. Suppression of gene expression triggered by double-stranded RNA (dsRNA) is well documented in a variety of systems[4–6]. Early attempts to use dsRNAs to trigger gene silencing in mammalian cells failed owing to activation of the interferon pathway genes, including the genes encoding PKR and 2′–5′-oligoadenylate synthetase[7,8]. In *Drosophila melanogaster*, dsRNAs of 150 base pairs (bp) or longer were shown to induce RNA interference efficiently, whereas shorter dsRNAs were less effective and 29- to 38-bp duplexes were inactive[9]. Long dsRNAs are degraded by the RNase III–class endonuclease Dicer into 21- to 23-nucleotide (nt) duplexes that have two-base 3′ overhangs[10,11]. Chemically synthesized exogenous 21-mer RNA duplexes suppress gene expression in mammalian cells without triggering interferon responses[12]. Studies in *D. melanogaster* embryo extracts have shown that 21-bp RNA duplexes with two-base 3′ overhangs were the most effective at triggering RNAi *in vitro*[13]. Duplexes of 20 or 23 bp were less potent, and duplexes of 25 bp were inactive; furthermore, duplexes with no overhang (blunt) or 5′ overhangs were less potent than duplexes with two-base 3′ overhangs. Subsequently, most investigators have adopted the use of 19- to 21-bp duplexes with two-base 3′ overhangs (21+2 or 'traditional siRNAs') as the preferred design when using synthetic RNA in RNAi applications in mammalian cells.

During investigations of the effects of different 5′ and 3′ end structures of dsRNAs made through bacteriophage T7 *in vitro* transcription[14], we observed that some seemed to have greater potency than synthetic 21-mer siRNAs directed to the same target site, and that this property seemed to correlate with length (data not presented). To further explore this phenomenon, we systematically studied the silencing properties of chemically synthesized duplex RNAs of different lengths and designs.

We synthesized a series of RNA duplexes specific to a variety of target genes (**Supplementary Table 1** online). Compounds are named according to target, length and overhang. For example, 'EGFPS1' refers to an RNA duplex targeting site, 'site 1,' in enhanced green fluorescent protein (EGFP); 27+2 denotes a 27-nt duplex with a two-base 3′ overhang; 27+0 denotes a 27-nt duplex with no overhang (blunt) and 27−2 denotes a 27-nt duplex with a 2-bp 5′ overhang. An expanded set of synthetic RNA duplexes of varying length containing 3′ overhangs, 5′ overhangs or blunt ends were tested for their relative potency in a model system targeting 'site 1' in EGFP[15]. We carried out our initial transfections using 50 nM of the various RNA duplexes (**Fig. 1a**). The real potency of the longer duplexes was shown only when transfections were done at subnanomolar concentrations. Using duplex RNA concentrations of 200 pM (**Fig. 1b**) and 50 pM (**Fig. 1c**), we observed that potency increased with length. Blunt, 5′-overhanging and 3′-overhanging ends were of similar potency. Increased potencies of the longer duplex RNAs were observed even in the NIH3T3 cells stably expressing EGFP (**Fig. 1d**). Duplexes longer than 27 nt were synthesized and tested for RNAi efficacy (**Fig. 1e**). Maximal inhibitory activity was seen at a duplex length of 27 bp. Longer duplexes showed progressive loss of functional RNAi activity and by 40−45 bp were wholly inactive at the tested concentrations, which also correlated with poor *in vitro* cleavage of these duplexes by Dicer (**Fig. 1e**).

Incubation of 21- to 27-bp RNA duplexes with recombinant human Dicer resulted in cleavage of the 23-mer, 25-mer and 27-mer duplexes, but not the 21-mer duplex (**Fig. 2a**). Determinations of relative efficiencies of Dicer cleavage were not possible under the *in vitro*

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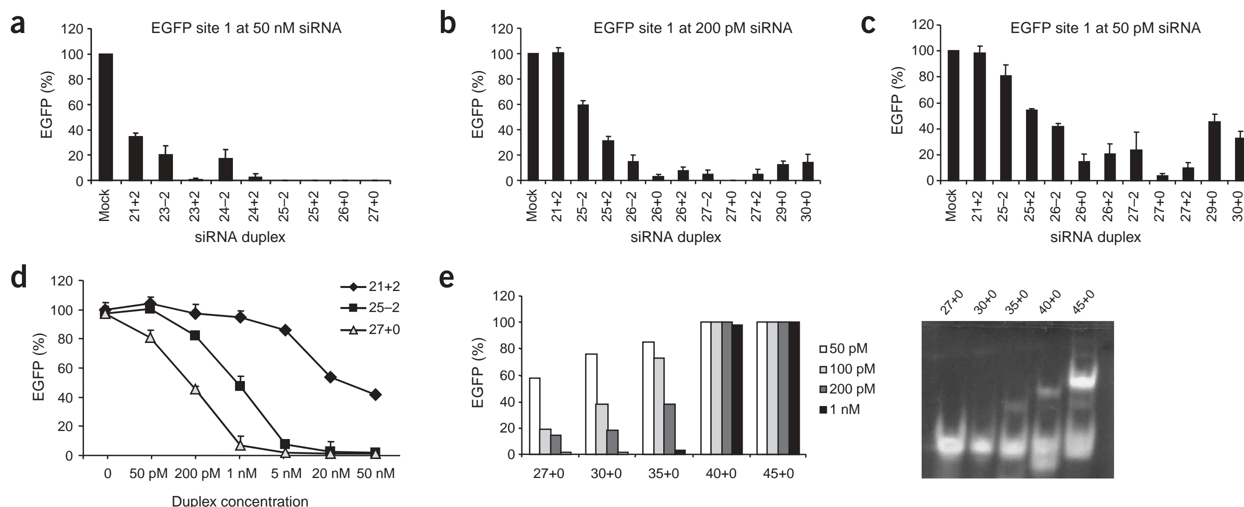

Figure 1 27-mer dsRNAs are more potent effectors of RNAi than a 21+2 siRNA. EGFP expression levels were determined after cotransfection of HEK293 cells with a fixed amount of EGFP expression plasmid and varying concentrations of dsRNAs. (**a**–**c**) Transfections were performed using (**a**) 50 nM, (**b**) 200 pM and (**c**) 50 pM of the indicated dsRNAs. (**d**) Dose-response testing of longer dsRNAs. Transfections were performed with the indicated concentrations of dsRNA. Right, depicts *in vitro* Dicer reactions with the same longer RNAs. Concentrations and conditions were as described in Methods. (**e**) Dose-response curve of dsRNAs transfected into NIH3T3 cells that stably express EGFP. Each graph point represents the average (with s.d.) of three independent measurements.

conditions recommended by the supplier owing to the slow kinetics of this reaction. Aside from the possibility that the dsRNAs longer than 30 bp may need to be preprocessed by Drosha, a micro RNA processing enzyme, to be good substrates for Dicer, we do not have an explanation for why these longer dsRNAs are both poor substrates for Dicer and poor triggers for RNAi.

For the 27-mer duplexes, we tested the effects of fluorescein end-modification on Dicer activity and the ability to trigger RNAi silencing. RNA oligonucleotides were synthesized for the EGFPS1 site with 6-carboxyfluorescein (6FAM) attached to the 5′ end of the sense (S) strand, 3′ end of the S-strand, 5′ end of the antisense (AS) strand and 3′ end of the AS strand. Pairwise combinations were used to make duplex RNAs (**Fig. 2b**). Duplex 3 was the unmodified wild-type EGFPS1 27+0 duplex (**Supplementary Table 1**). RNA duplexes were incubated for 24 h with recombinant human Dicer, separated by nondenaturing polyacrylamide gel electrophoresis (PAGE), stained and visualized by UV excitation (**Fig. 2c**). Unlike in earlier experiments, in which RNA duplexes were fully cleaved during a 24-h incubation (**Fig. 2a**), all of the modified duplexes showed some degree of resistance to cleavage by Dicer. Only the unmodified wild-type sequence (duplex 3) was fully cleaved in the *in vitro* Dicer reaction. The duplex bearing a 3′-6FAM on the S strand and a 3′-6FAM on the AS strand (duplex 5) was totally resistant to cleavage under these conditions. Functional potencies of these five duplexes were compared in EGFP cotransfection assays (**Fig. 2d**) using 200 pM RNA concentrations. Parallel to the patterns seen for *in vitro* Dicer cleavage, all of the 27-mer duplexes with 6FAM-modified ends were less potent than the unmodified duplexes in reducing EGFP expression. Duplexes 1, 2 and 4, which showed partial cleavage with recombinant Dicer, had three- to six-fold reduced RNAi activity. Duplex 5, which showed no cleavage with recombinant Dicer, had minimal RNAi activity, establishing a direct correlation between the relative effectiveness of *in vitro* cleavage by Dicer and RNAi in cell culture.

To confirm that the 27-mer dsRNAs are processed by Dicer *in vivo*, we transfected HEK293 cells with 10 nM of the duplex 3 (unmodified) or duplex 5 (both 3′ ends modified with 6FAM). After 14 h, total RNA was isolated and hybridized with a ^{32}P end labeled 21-mer probe oligo (S strand). RNA was separated by nondenaturing PAGE and visualized by autoradiography (**Fig. 2e**). Similar to the results seen with *in vitro* Dicer cleavage, in RNA prepared from cells transfected with duplex 3 (unmodified 27-mer), a smaller species was observed migrating with a 21-mer duplex marker, consistent with a Dicer cleavage product. This 21-mer species was not detected in RNA from cells transfected with duplex 5 (3′ end–modified 27-mer).

Cleavage of a 27-mer by Dicer could result in a variety of distinct 21-mers depending on where cleavage occurs; it is possible that one or a mix of these possible 21-mers is significantly more potent than the specific 21-mer that we used as our standard for comparison. To test this possibility we synthesized seven different 21-mers that could be derived from the EGFPS1 27+0 duplex, walking in single-base steps along the antisense strand, using the traditional 21+2 design. These seven duplexes were tested for RNAi activity in the HEK293 cell cotransfection assay individually and as a pool (**Supplementary Fig. 1** online). At concentrations of 50 or 200 pM, neither the individual 21-mer duplexes nor the pooled set of seven 21-mer duplexes showed activity comparable to the 27-mer duplex. *In vitro* Dicer cleavage of the 27-mers before transfection did not significantly enhance efficacy (**Supplementary Fig. 1**). As an additional control, we transfected a mutated EGFP 27-mer duplex (**Supplementary Table 1**, EGFPS1-27+0/mut) harboring four consecutive, centrally placed mismatched bases. The mismatches virtually eliminated any RNAi activity (**Supplementary Fig. 1**).

The species that are actually produced by incubation of the EGFPS1 27+0 duplex with recombinant Dicer *in vitro* were identified using electrospray ionization mass spectrometry (ESI MS) (**Supplementary Fig. 2** online). Calculated masses for each possible digestion product that could result from *in vitro* Dicer cleavage are shown in **Supplementary Table 2** online. The ESI MS analyses of the *in vitro* cleavage products are consistent with the known activity of this enzyme.

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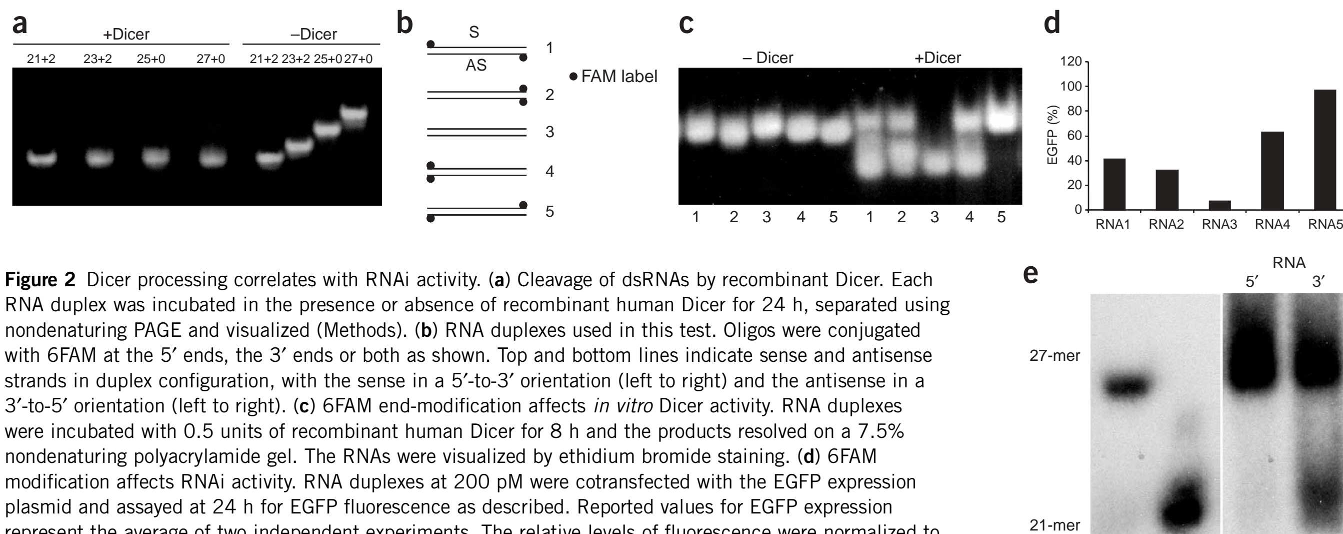

Figure 2 Dicer processing correlates with RNAi activity. (**a**) Cleavage of dsRNAs by recombinant Dicer. Each RNA duplex was incubated in the presence or absence of recombinant human Dicer for 24 h, separated using nondenaturing PAGE and visualized (Methods). (**b**) RNA duplexes used in this test. Oligos were conjugated with 6FAM at the 5′ ends, the 3′ ends or both as shown. Top and bottom lines indicate sense and antisense strands in duplex configuration, with the sense in a 5′-to-3′ orientation (left to right) and the antisense in a 3′-to-5′ orientation (left to right). (**c**) 6FAM end-modification affects *in vitro* Dicer activity. RNA duplexes were incubated with 0.5 units of recombinant human Dicer for 8 h and the products resolved on a 7.5% nondenaturing polyacrylamide gel. The RNAs were visualized by ethidium bromide staining. (**d**) 6FAM modification affects RNAi activity. RNA duplexes at 200 pM were cotransfected with the EGFP expression plasmid and assayed at 24 h for EGFP fluorescence as described. Reported values for EGFP expression represent the average of two independent experiments. The relative levels of fluorescence were normalized to those for luciferase. (**e**) 27-mer duplex RNAs are processed to 21-mers *in vivo*. Total RNA was prepared from cells transfected with duplex 3 and duplex 5 at 10 nM. RNA was hybridized with a 21-mer ^{32}P-labeled oligonucleotide probe. The hybridized samples were separated by nondenaturing PAGE and visualized by autoradiography. Size markers are ^{32}P end labeled 21-mer and 27-mer RNA duplexes.

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To further characterize the inhibitory properties of the 27-mer dsRNA in cells stably expressing the EGFP target, stably transfected NIH3T3 cells expressing EGFP were transfected with 21+2 and 27+0 dsRNA duplexes (both at 5 nM). To obtain a quantitative estimate of the duration of gene suppression, we carried out a time-course experiment, observing EGFP expression on days 2, 4, 6, 8 and 10 after transfection. Cell extracts were prepared and measured for EGFP fluorescence using a fluorometer (**Fig. 3a**). EGFP suppression lasted approximately 4 d using the 21+2 siRNA, consistent with previous observations[16], whereas inhibition obtained with the 27+0 dsRNA persisted up to 10 d. A class of 'hyperfunctional' 21+2 siRNAs has been reported showing a similar extended duration of silencing[17]; however, these sequences are rare and difficult to find or predict. Use of the 27-mer dsRNA design may permit longer, more potent RNAi to be achieved at a greater variety of target sites.

A frequent problem in using RNAi as a tool to systematically inhibit the expression of any gene is that not all target sites are equally susceptible to suppression by siRNAs[18], necessitating complex design algorithms to predict effective sites[17,19,20]. We therefore asked whether the increased potency of the 27-mer dsRNA permits effective targeting at sites that are not active using traditional 21-mer siRNAs. Duplex RNAs were made having 21+2 and 27+0 designs to two sites in EGFP ('EGFP-S2' and 'EGFP-S3') both previously shown to be refractory to RNAi using standard siRNAs[15]. The duplexes were transfected into HEK293 cells using the cotransfection assay format (**Fig. 3b**) at 1 nM and 10 nM. At these doses, standard 21+2 siRNAs were ineffective at both sites, whereas the 27-mer dsRNAs reduced EGFP expression by 80–90% at EGFP-S2 and by 50% (1 nM) and 80% (10 nM) at EGFP-S3. Despite the increased potency of the Dicer substrate dsRNAs, empirical testing of target sites is still useful to find the most sensitive targets for RNAi. In this regard, it is important that the Dicer products of some 27-mers generated poorly functional siRNAs. By better understanding the Dicer substrate preferences, it should be possible to design substrate RNAs that will generate the desired 21-mers. We have observed that a two-base 3′ overhang on only one end of the 27-mer will preferentially guide Dicer to cleave 21–23 nt upstream of the two-base 3′ overhang (data not shown).

To ensure that the increased potency of the 27-mer dsRNAs was not an artifact of targeting a reporter construct, we targeted two endogenous transcripts. RNA duplexes were synthesized to target randomly chosen sites in the human hnRNP H mRNA[21] (analyzed by western blotting) and the mRNA encoding the La protein[22] (analyzed by northern blotting (**Fig. 3c,d**). For both targets the 27-mer duplex was more potent than the 21-mer siRNAs targeting these messages.

As a test for the sequence specificity of the 27-mer dsRNA, a series of 27+0 dsRNAs with one, two or three mismatches to the EGFP target mRNA were synthesized and tested at concentrations of 0 nM, 1 nM and 200 pM in the cotransfection assay (**Supplementary Fig. 3** online). At 200 pM, each of the mismatched sequences was less potent than the wild-type 27-mer dsRNA; the triple mismatch 27-mer dsRNA was completely ineffective at triggering RNAi at all concentrations tested. Similar results were obtained using a 27-mer dsRNA targeted to 'site 2' of EGFP (data not shown).

A potential problem in the use of the longer dsRNAs is activation of PKR and induction of interferons[23]. We therefore assessed whether a transfected 27-mer dsRNA activates interferon-α or interferon-β (**Supplementary Fig. 4** online). As a positive control for interferon induction, we transfected a triphosphate-containing single-stranded RNA which potently activated interferon-α and -β, as reported previously[14]. Neither cytokine was detected when either the 21+2 siRNA or 27+0 dsRNA was used. We have extended this observation to two other 27-mer sequences specific for the EGFP-S2 and EGFP-S3 sites (data not shown). PKR activation in cell lysates was also assayed as described previously[24]. The lysate was treated with the indicated RNAs, followed by immunoprecipitation. The positive control long dsRNA elicited PKR activation, but none of the shorter RNAs activated PKR (**Supplementary Fig. 4**).

It is clear from recent studies that the effects of RNAi are not entirely specific and that undesired 'off-target' effects can occur of a magnitude dependent on the concentration of siRNA[16]. The new Dicer substrate dsRNA approach may facilitate use of lower concentrations of duplex RNA than are needed with traditional 21-mer siRNAs. It is clear from published data that 'off-target' effects can occur in certain cell lines using 21-mer siRNAs[16,25], but these also can

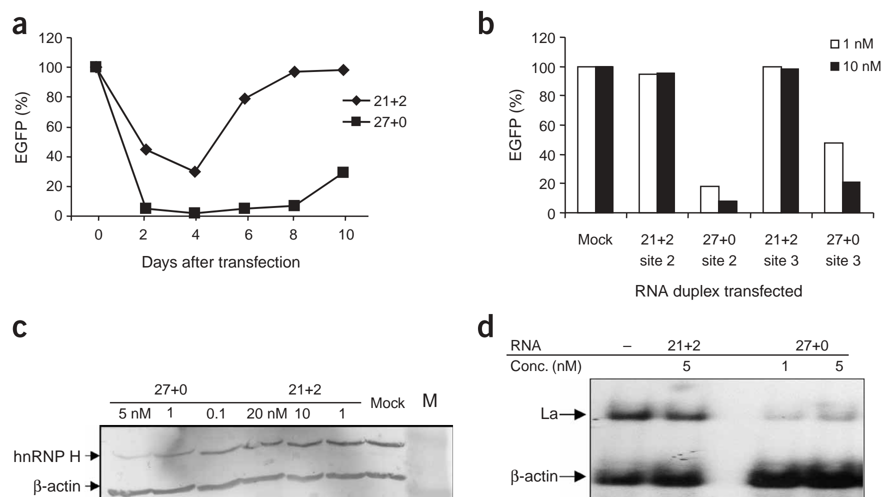

Figure 3 Features of 27-mer dsRNA in RNAi. (**a**) Enhanced duration of RNAi by 27-mer dsRNAs. Levels of EGFP were determined after transfection of 5 nM of a 21+2 siRNA or the 27-mer dsRNA into NIH3T3 cells stably expressing EGFP. Graphic representation of EGFP silencing mediated by a 21+2 siRNA as compared to the 27-mer dsRNA. Duplicate samples were taken on the indicated days and EGFP expression was determined by fluorometry. (**b**) 27-mer dsRNAs, targeting sites refractory to 21-mer siRNAs, can elicit RNAi. The dsRNAs were transfected along with the EGFP reporter construct, and EGFP expression was determined (Methods). Column 1, mock; column 2, 21+2 siRNA targeting EGFPS2; column 3, 27-mer dsRNA targeting EGFPS2; column 4, 21+2 siRNA targeting EGFPS3; column 5, 27-mer dsRNA targeting EGFPS3. (**c**,**d**) Comparison of 21-mer siRNA and 27-mer dsRNA in downregulation of endogenous transcripts. RNAs for a 21+2 siRNA and 27+0 dsRNA were designed to target sites in the hnRNP H mRNA (**c**) or La mRNA (**d**). HnRNP H knockdown was assayed by western blot and La knockdown by northern blot analyses. The dsRNAs were used at the indicated concentrations. β-Actin was used as an internal specificity and loading standard in both experiments.

be minimized by using reagents that have efficacy in the low to subnanomolar range[16]. To examine the potential for 'off-target' effects using Dicer substrate dsRNAs, we carried out microarray analyses comparing an siRNA 21-mer with the 27-mer, each targeting EGFP site 1. NIH3T3 cells that stably express EGFP were transfected with concentrations of siRNA that give effective target knockdowns (**Fig. 2a**, **Supplementary Fig. 4**). Total cellular RNAs were prepared from cells 24 and 48 h after transfection and analyzed by hybridization to an oligonucleotide microarray as described in **Supplementary Figure 4** online. Among the 16,282 mouse genes analyzed, only a small fraction showed upregulation or downregulation more than twofold above or below control values (**Supplementary Fig. 4**). The 27-mer and 21-mer gave comparable results at their effective RNAi concentrations. There was an increase in the number of transcripts upregulated when the 27-mer was used at the higher 25 nM concentration, but comparisons of the targets affected at 24 versus 48 h and at 5 nM versus 25 nM showed no overlap. Rather than specific 'off-target' effects, these changes are more consistent with statistical scatter among the 16,282 genes examined. The same assay was repeated using the EGFP-S2 27+0 duplex RNA with comparable results (data not shown).

Given the increase in potency of the 27-mer dsRNAs relative to 21+2 siRNAs, it is of interest that this observation has not been previously reported. Although others have used dsRNAs of up to 27 bp for RNAi studies in mammalian cells[26,27], no differences in efficacy were reported as compared with traditional 21+2 duplexes. This discrepancy between previous studies and our own may simply be due to differences in the concentration of dsRNAs tested. 'Good' sites for 21-mer siRNAs can have potencies in the nanomolar range[17]. When 'good' sites are tested at high concentrations of transfected RNA, differences between 21-mer siRNAs and 27-mer dsRNAs will most likely be small and easily overlooked. Marked differences in potency are best shown by testing at low nanomolar or picomolar concentrations, something that is not routinely done in most laboratories.

Thus far, the 27-mer dsRNA design has shown increased RNAi potency relative to 21+2 siRNAs at every site examined. Within the set of 27-mers studied here, however, a range of potencies is nevertheless seen between different target sites within the same gene (**Fig. 3b**). We have shown that, even in the absence of fully optimized design rules, use of the Dicer substrate dsRNA approach can increase RNAi potency relative to traditional 21+2 siRNAs. Additionally, the use of 27-mer dsRNAs allows targeting of some sites within a given sequence that are refractory to suppression with traditional 21-mer siRNAs. Use of Dicer substrate dsRNAs to trigger RNAi should result in enhanced efficacy and longer duration of RNAi at lower concentrations of RNA than are required for 21+2 applications. Consistent with our results linking Dicer cleavage to enhanced RNAi efficacy, it has recently been shown that chemically synthesized hairpin RNAs that are substrates for Dicer are more potent inducers of RNAi than conventional siRNAs and, moreover, that a two-base 3′ overhang directs Dicer cleavage[28].

METHODS

Chemically synthesized siRNAs. All chemical siRNAs described in this study were synthesized and HPLC-purified by Integrated DNA Technologies or by the oligo synthesis facility at the City of Hope. The RNAs used in this study are listed in **Supplementary Table 1**.

RNAi EGFP suppression assays. HEK 293 cells were split in 24-well plates to 60% confluency in DMEM medium 1 d before transfection. After adding the aliquot of each RNA, 50 μl of Opti Media containing the reporter vectors was added. Next, 50 μl of Opti Media containing 1.5 μl of Lipofectamine 2000 (Invitrogen) was mixed and incubated for 15 min. The cells were then added in 0.4 ml of DMEM medium. To normalize for transfection efficiency, each assay included cotransfection of the target and/or duplex RNAs with either firefly luciferase or a red fluorescent protein (RFP) reporter plasmid (all other assays). For the luciferase assay, the Steady Glo Luciferase assay kit was used according to manufacturer's instructions (Promega). For RFP cotransfection, the indicated amount of EGFP reporter plasmid (pLEGFP-C1 vector, Clontech) was transfected with 20 ng of RFP reporter plasmid (pDsRed2-C1, BD Sciences). After 24 h, RFP expression was monitored by fluorescence microscopy. Only experiments where transfection efficiency was $>90\%$ (as assessed by RFP expression) were evaluated. EGFP expression was measured 24 h later. EGFP expression was determined either from the median number of EGFP-fluorescent cells determined by FACS (live cells) or by fluorometer readings (cell extracts). For EGFP assays using NIH3T3 cells stably expressing EGFP, measurements were determined using a VersaFluor Fluorometer (Bio-Rad) using excitation filter D490 and emission filter D520. Before transfections, cells were seeded to approximately 30% confluency in a 24-well plate. On day 0, cells

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were transfected as described above and the medium was changed on day 1 after transfection. The first EGFP assay was carried out on day 3. For extract preparation 1×10^5 cells were taken and 1×10^4 cells were further propagated for the day 6 EGFP assays. On days 6 and 9 the same procedure was repeated.

Extract measurements of EGFP were obtained as follows: 1×10^5 cells were suspended in 300 µl of PBS and sonicated for 10 s followed by a 2-min microcentrifugation. The supernatants were used for fluorescence measurements. Percentages of EGFP expression were determined relative to extracts prepared from untreated NIH3T3 cells.

***In vitro* Dicer cleavage assays.** RNA duplexes (100 pmol) were incubated in 20 µl of 20 mM Tris pH 8.0, 200 mM NaCl, 2.5 mM $MgCl_2$ with 1 unit of recombinant human Dicer (Stratagene) for 24 h. A 3-µl aliquot of each reaction (15 pmol RNA) was separated in a 15% nondenaturing polyacrylamide gel, stained with GelStar (Ambrex) and visualized using UV excitation.

Electrospray-ionization liquid chromatography mass spectroscopy (ESI-LCMS) of duplex RNAs before and after treatment with Dicer were done using an Oligo HTCS system (Novatia), which consisted of ThermoFinnigan TSQ7000, Xcalibur data system, ProMass data processing software and Paradigm MS4 HPLC (Michrom BioResources). The liquid chromatography step used before injection into the mass spectrometer (LC-MS) removes most of the cations complexed with the nucleic acids; some sodium ions can remain bound to the RNA and are visualized as minor +22 or +44 species, reflecting the net mass gain seen with substitution of sodium for hydrogen.

Assay for intracellular processing of 27-mer RNAs. RNA duplexes were transfected as described above into HEK293 cells in a six-well plate at 10 nM. After 14 h, total RNA was prepared as described below. First, 20 µg of total RNA was heated for 10 min at 75 °C and mixed with ^{32}P 5′-end-labeled oligonucleotide probe (5′- ACCCTGAAGTTCATCTGCACC-3′) and hybridized in 150 mM NaCl, 50 mM Tris-HCl, pH. 7.4, 1 mM EDTA) at 24 °C. Samples were loaded on 7.5% nondenaturing polyacrylamide gel and separated at 200 V for 3 h at 4 °C , and the gel was exposed to X-ray film. ^{32}P-end-labeled 27-mer and 21-mer duplex RNA oligos were used as size standards.

RNAi assays against hnRNP H and La. HEK293 cells were plated to 30% confluency in a six-well plate. The next day, the cells were transfected with the indicated amount of dsRNA, and the medium was changed on the following day. The cells were harvested in 300 µl PBS 72 h after transfection. Extracts were prepared as described above for the EGFP assays. For western blots, 2 µl of cell extract was loaded on a 10% SDS-PAGE gel. Endogenous hnRNP H was detected using a rabbit polyclonal anti–hnRNP H antibody[21] and anti-rabbit antibody conjugated to alkaline phosphatase (Sigma). β-Actin was detected with a mouse-derived anti-actin antibody (Sigma) and anti-mouse antibody conjugated to alkaline phosphatase (Sigma). For northern blot analyses, harvested cells were mixed with RNA STAT-60 (Tel-Test B) and total RNA was extracted according to the manufacturer's protocol. RNA was electrophoresed in a 6% denaturing polyacrylamide gel, transferred to a nylon membrane and probed with ^{32}P-end-labeled oligos (La, 5′-CCAAAGGTACCCAGCCTT-CATCCAGTT-3′; β-actin, 5′-GTGAGGATGCCTCTCTTGCTCTGGGCCTCG-3′). Hybridizations were carried out in 10 ml of hybridization solution (1 ml 50× Denhardt's, 3 ml 20× SSPE, 0.5 ml 10% SDS) for 3 h at 37 °C. After hybridization, the blot was washed three times with 2× SSPE at 37 °C.

Interferon and PKR activation assays. After transfection of 293 cells with 20 nM of each RNA as described previously, medium was collected after 24 h and used for ELISA assays of interferon α and β as previously described[14]. The PKR activation assay was done as previously described[24]. PKR in the lysate was first activated by co-incubation of the indicated RNAs and radiolabeled by its auto-kinase reaction. The radiolabeled PKR was then immunoprecipitated for analysis. To determine the activity of PKR in the cell lysate without prior activation, dsRNA was omitted. The reaction was incubated at 30 °C for 20 min. PKR from the reaction was immunoprecipitated using polyclonal antibody. The polyclonal antibody was added to the reaction, which was then placed on ice for 1 h, followed by addition of 50 µl of 10% protein A–Sepharose in IPP500 (10 mM Tris, pH 8, 500 mM NaCl, 0.1% Nonidet P-40). This mixture was rocked for 30 min at 4 °C. The protein A–Sepharose beads were washed with 1 ml IPP100 buffer (10 mM Tris, pH 8, 100 mM NaCl, 0.1% Nonidet P-40) five times. After the last wash, the beads were boiled in protein sample buffer and loaded onto an SDS-polyacrylamide gel followed by autoradiography.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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