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Gene silencing using micro-RNA designed hairpins

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ABSTRACT

During RNA interference (RNAi), long dsRNA is processed to ~21 nt duplexes, short interfering RNAs (siRNAs), which silence genes through a mRNA degradation pathway. Small temporal RNAs (stRNAs) and micro-RNAs (miRNAs) are ~21 nt RNAs that are processed from endogenously encoded hairpin-structured precursors, and function to silence genes via translational repression. Here we report that synthetic hairpin RNAs that mimic siRNAs and miRNA precursor molecules can target a gene for silencing, and the mechanism of silencing appears to be through mRNA degradation and not translational repression. The sequence and structural configuration of these RNAs are important, and even slight modification in structure can affect the silencing activity of the hairpins. Furthermore, these RNAs are active when expressed by DNA vectors containing polymerase III promoters, opening the possibility for new approaches in stable RNAi-based loss of function studies.

Keywords: dsRNA; H1 promoter; miRNA; RNAi; siRNA; T-cell

INTRODUCTION

RNA interference (RNAi) is a powerful technique used to study gene function in all major phyla (for recent review, see Bernstein et al., 2001b; Sharp, 2001; Vaucheret et al., 2001; Zamore, 2001). Mammalian RNAi was first described in mouse embryos using long dsRNA (Svoboda et al., 2000, Wianny & Zernicka-Goetz, 2000). Then, following the analysis of the structure of the intermediate in this process, short interfering RNAs (siRNAs) have been used to silence genes in mammalian tissue culture (Caplen et al., 2001; Elbashir et al., 2001a). Genes involved in RNAi are part of an evolutionarily conserved cellular pathway that processes endogenous cellular RNAs to silence developmentally important genes (Grishok et al., 2001; Hutvagner et al., 2001). Although the cellular functions of the RNAi pathway are not yet fully understood, the identification of a large class of miRNAs present in different organisms suggests that the extent of RNA-mediated gene regulation may be greater than previously thought (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee & Ambros, 2001).

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Although hundreds of miRNAs have been identified, only two miRNAs are well documented with respect to function. These are the *lin-4* and *let-7* miRNAs shown to be temporally expressed in *Caenorhabditis elegans*, and are therefore called stRNAs (Lee et al., 1993; Reinhart et al., 2000). The class of stRNAs and miRNAs cannot be distinguished by structure or sequence, and both appear to be transcribed as ~70 nt precursor hairpin RNA containing an ~4–15 nt loop. From this precursor hairpin structure, a single-stranded 21–23 nt miRNA is processed. Sometimes the miRNA forms a perfect duplex within the hairpin, but more often multiple bulges disrupt the perfect 21–23 nt duplex. Studies from the *lin-4* and *let-7* stRNAs suggest that miRNAs may function through translational repression via the 3′ untranslated region (3′ UTR) of genes (for recent reviews, see Ambros, 2001; Banerjee & Slack, 2002; Grosshans & Slack, 2002). Curiously, when the stRNA is hybridized to the mRNA, it does not exhibit perfect complementarity, a situation that is in contrast to siRNA-mediated degradation. In the existing models for hybridization, only 50–85% of the stRNA residues are base paired to the mRNA 3′ UTR.

One important feature of RNAi is the processing of long dsRNA into siRNAs of defined structure. This is accomplished by the evolutionarily conserved protein Dicer, an RNAse III related enzyme that processes precursor dsRNAs into 21–23 nt siRNAs that have an

~19 bp duplex containing 2-nt 3′ overhangs (Bernstein et al., 2001; Elbashir et al., 2001b). This has been shown to be the optimal structure of an siRNA and RNAs that deviate from this structure can be remarkably inefficient for RNAi (Elbashir et al., 2001c). Elbashir and colleagues demonstrated that nearly perfect duplexing between the target mRNA and the siRNA antisense strand is a requirement for mRNA cleavage. One possibility is that the stRNAs, because they do not completely duplex to the target mRNA, cannot activate the degradation pathway.

Similar to other RNAse III enzymes, Dicer is active at processing complex hairpin structures that can contain multiple mismatches in the helical stem (Hutvagner et al., 2001). Little is known about the structural determinants necessary for processing of stRNAs or miRNAs into 21-nt RNAs. Studies performed on RNAse III enzymes in other organisms have shown that dsRNA cleavage relies on antideterminants in the double-stranded stem, as well as sequence determinants in the terminal loop of the stem-loop RNA substrate (Zhang & Nicholson, 1997; Chanfreau et al., 2000; Wu et al., 2001). Comparison of the large class of miRNAs that are likely processed by Dicer has yet to reveal any obvious determinants or antideterminants that might guide Dicer recognition or processing.

RNAi analysis of gene function in lower organisms has been greatly assisted by the development of in vivo systems that express long dsRNA hairpins (typically 500–1000 nt; Chuang & Meyerowitz, 2000; Kennerdell & Carthew, 2000; Smith et al., 2000; Tavernarakis et al., 2000). In mammals, this has been accomplished in mouse embryonic stem (ES) cells (Svoboda et al., 2001; Paddison et al., 2002a). However, attempts to develop a stable dsRNA hairpin-based expression system in differentiated somatic mammalian cells have been unsuccessful. This is possibly due to the nonspecific effects on gene expression resulting from long (>30 nt) dsRNA expression, which may activate the interferon-related pathways (Svoboda et al., 2001; Paddison et al., 2002a). Because siRNA-mediated RNAi is based on using dsRNA <30 nt, it bypasses the potent dsRNA-IFN response and evokes RNAi-type silencing (Bitko & Barik, 2001; Elbashir et al., 2001a). Given the above, it remains possible that small hairpin RNAs <30 nt may evoke a specific RNAi-type response. In the studies presented here, we investigate the potential for small hairpin RNAs to direct gene silencing.

RESULTS AND DISCUSSION

To determine whether silencing could be recapitulated with short (<30 nt) hairpin dsRNAs, two classes of hairpin RNAs were synthesized that targeted the stop codon region and 3′ UTR of the immunologically important HIV coreceptor CD4 and CD8α mRNAs, respectively (Fig. 1). Class I hairpins are based on an

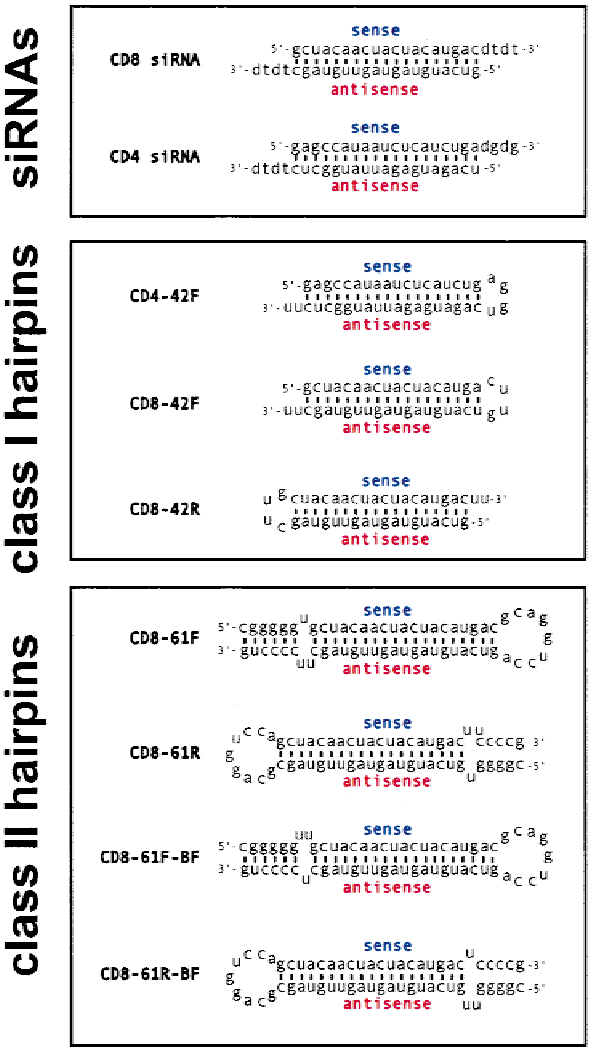

FIGURE 1. Sequence and design of RNAs. siRNAs and hairpin RNAs used in this study. Names at left identify each RNA.

siRNA that has been previously shown to be active (M.T. McManus, B.B. Haines, J. Chen, & P.A. Sharp, submitted), and consists of the covalent linking of two siRNA strands at the 5′ or 3′ end. Class II miRNA mimics are more reminiscent of a miRNA precursor, and contain a 12-nt loop length and one asymmetric stem-loop bulge. The loop sequence was chosen from the loop of the mir-26a gene, except that a single C residue was omitted to prevent a predicted alternative nonhairpin structure (Lagos-Quintana et al., 2001). The position and identity of the bulge residues were also adopted from several preexisting miRNAs, where bulges commonly flank the stRNA nested within the precursor hairpin (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee & Ambros, 2001). Because most of the stRNAs and miRNAs contain a 3–8 nt region of predicted perfect complementarity at the base of the stem, a 5-nt GC clamp was added to the miRNA structures that we synthesized. Both classes of hairpin constructs contain 19 nt of uninterrupted RNA duplex and 5′ hydroxyl termini.

These RNAs were tested in mammalian cell culture assays for the ability to silence transgenes or endogenous genes. Class I hairpins contained sequences based on an effective CD8α siRNA, and were transfected into mouse E10 T-cells as described (M.T. McManus, B.B. Haines, J. Chen, & P.A. Sharp, submitted). In these experiments, the level of expression of CD8α was monitored using flow cytometry. As a specificity control, cells were costained for CD4. The activity of CD8α class I hairpins differed depending on which end of the siRNA was linked (Fig. 2). When the linkage was present at the antisense 5′ end, the activity was significantly reduced as compared to siRNA (Fig. 2B); however, when the linkage was present on the antisense 3′ end, the hairpin was equally as effective as a siRNA (Fig. 2C). Similar to siRNAs, silencing was specific to CD8α, and did not nonspecifically alter the expression of a nontargeted gene, CD4 (Fig. 2A).

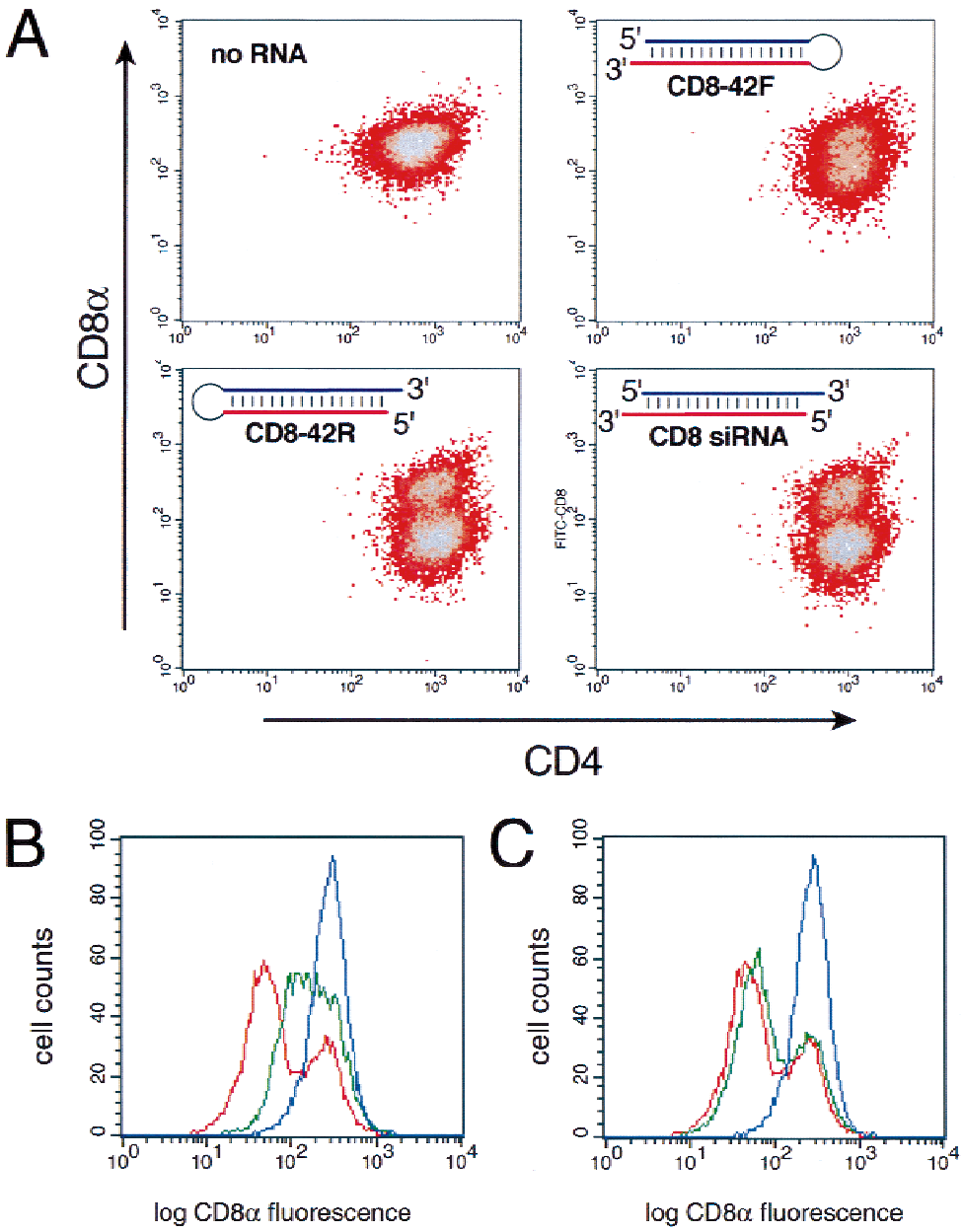

FIGURE 2. Activity of hairpin RNAs directed against CD8α in mouse E10 T-cells. Flow cytometry analysis of cells transfected with CD8α stem loops or siRNAs. **A:** Mouse E10 T-cells were stained for CD8α and CD4 (specificity control) at 36 h posttransfection and analyzed via flow cytometry. Dot plot of CD4 versus CD8 is shown; each dot represents one cell. Inset schematic depicts the overall structure of the transfected RNA; blue: sense strand; red: antisense strand. **B:** Histogram plot of the CD8α expression data from the class I RNA CD8-42F shown in **A**. **C:** Histogram plot of the CD8α expression data from CD8-42R configuration. Plots depict the number of cells (counts) versus the expression level of the CD8α marker (bottom axis). Blue: untransfected cells; red: siRNA transfected cells; green: hairpin RNA transfected cells.

Several configurations of class II CD8α miRNA precursor mimics were tested in E10 cells and in a CD8α transient transfection HeLa cell assay. Similar to the class I hairpins, the loop was placed on the 5′ or 3′ end of the antisense strand. CD8α class II hairpins were nearly as effective as CD8α siRNAs, providing that the RNA loop was again placed on the 3′ end of the antisense strand (compare Fig. 3A and 3C). These structures contain an asymmetric bulge that is composed of a single uridine opposing a double uridine. The presence of this bulge structure adds an additional level of complexity that might contribute to the differences in the activities. To address the influence of this asymmetric bulge on silencing activity, the 2-nt bulge was flipped, so that the double uridine bulge would lie 5′ of the antisense strand. In this way, the influence of loop and bulge structures could be effectively uncoupled. The effect of this bulge swap significantly affected silencing activity (compare Fig. 3A and 3C and 3B and 3D). Taken together, these results indicate that the design of micro-RNA hairpin mimics is critical for the efficiency of silencing, and that even slight modifications in structure can have marked effects on silencing activity.

To further analyze the effectiveness of micro-RNA hairpins as silencing agents, we performed the CD4 and CD8α silencing experiments in HeLa cells. In several regards, HeLa cells are optimal for studying RNAi, as both siRNAs and plasmids can be transfected at practically 100% efficiency using cationic lipids. In these experiments, CD4 and CD8α expression vectors were

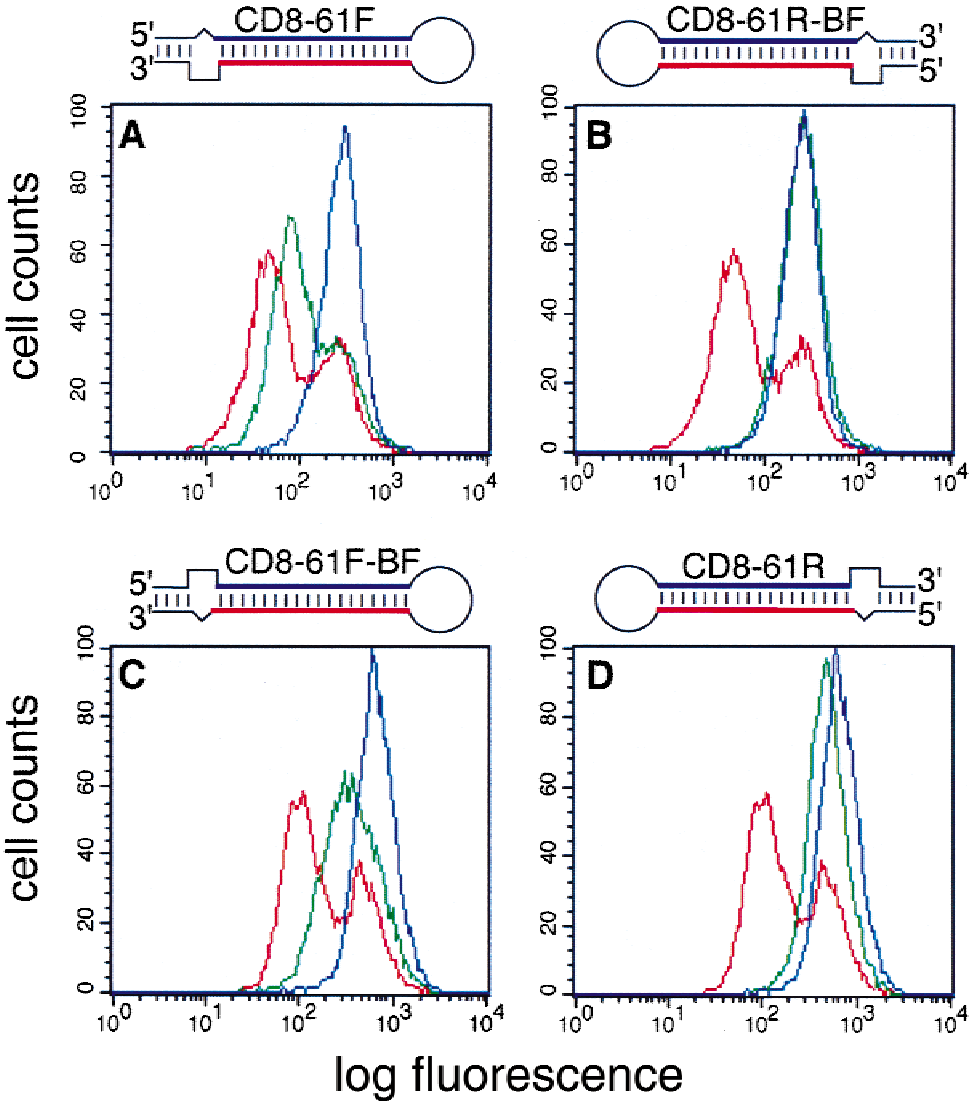

FIGURE 3. Activity of CD8α miRNA mimics in E10 mouse T-cells. Histogram plots showing activities of CD8α miRNA mimics in mouse E10 T-cells. Transfection and immunostaining were performed as in Figure 2. **A:** CD8α miRNA mimics were synthesized so that the RNA loop was again placed on the 3′ end of the antisense strand (**A** and **C**) or sense strand (**B** and **D**). RNAs **A** and **C** and RNAs **B** and **D** have switched stem bulges, which are depicted by a rectangle opposite a point. The point and the rectangle represent 1- and 2-nt bulges, respectively.

transfected together with siRNAs or hairpin constructs. A class I hairpin targeting the stop codon region of CD4 was also tested in these assays (see Fig. 1 for structure). Flow cytometric analysis was performed 36–48 h posttransfection, and the specificity of silencing was confirmed by monitoring the cotransfected nontargeted CD4 or CD8α expression. For these experiments, differing concentrations of hairpin RNA were titrated into HeLa cells, and transgene expression analyzed using flow cytometry. For comparison, siRNAs of identical duplex sequence were titrated in parallel. The number of cells that displayed reduced CD4 or CD8α silencing activity were quantitated and normalized to 100%. These data are plotted on a semi-log lot in Figure 4A. From these data, it is apparent that the CD8-61F and CD4-42F have the capacity to silence as effectively as siRNAs. However, the overall curves are dissimilar, and the hairpin RNAs were less effective than the corresponding siRNAs at lower concentrations. If the input hairpin RNAs must be processed to be active, it is possible that only a fraction of the input material is used for silencing, and that at low doses, the amount of active processed RNA may be limiting. Such a scenario could be due to a saturatable nuclease that processes the hairpin structures. In separate experiments, both class I and II hairpins were synthesized for targeting firefly luciferase transgenes, and tested in HeLa cells. In these experiments, luciferase was specifically reduced to a level similar to that when siRNAs were used (data not shown). It should be noted that we have routinely found that transfection via electroporation requires a higher amount of nucleic acids as compared to transfection using cationic lipids. Therefore, it is difficult to draw conclusions about the actual amount of siRNA needed to produce the same level of silencing in these two systems.

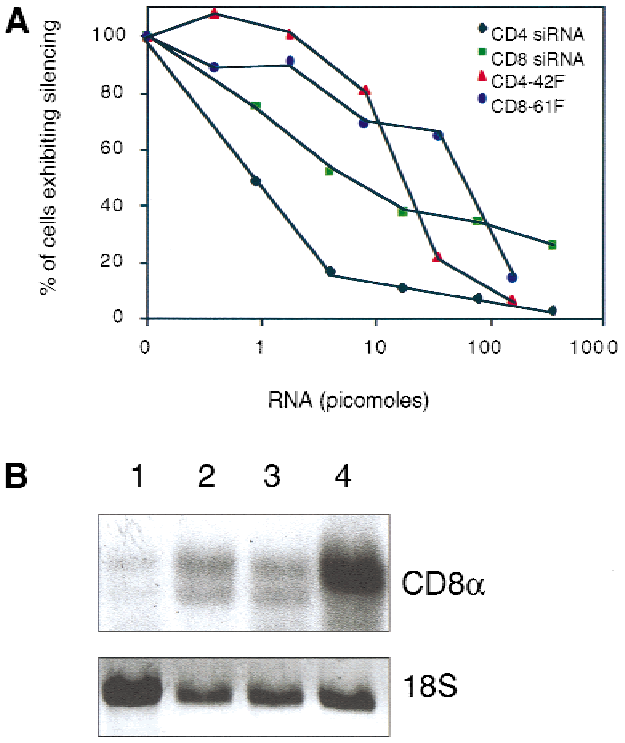

FIGURE 4. Hairpin RNAs can be as effective as siRNAs. **A:** Plot comparing the activities of CD4 and CD8α hairpin RNAs to siRNAs. CD4 and CD8α gene expressions were quantitated from HeLa cells transiently transfected with CD4 and CD8α expression vectors and the RNAs indicated (inset). The number of cells that exhibited silencing was quantitated and normalized to 100%. **B:** Northern blot of CD8α mRNA present in cells undergoing siRNA and hairpin silencing. Total RNA was extracted from cells 36 h posttransfection and subjected to northern blot analysis. The membrane was hybridized to a CD8α probe constituting the first 596 nt of CD8α mRNA. Lane 1: CD8-61F; lane 2: CD8-42R; lane 3: CD8 siRNA; lane 4: no treatment. 18S ribosomal is shown as a loading control.

stRNAs and miRNAs represent a novel class of endogenous RNA regulators (Lee et al., 1993; Wightman et al., 1993; Reinhart et al., 2000; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee & Ambros, 2001). Similar to the stRNAs, the class I and II hairpin RNAs also target the 3′ UTR of developmentally expressed genes. Because these hairpin RNAs are also structurally similar to the stRNAs, it was important to test whether silencing proceeded through siRNA-degradation or stRNA-repressor pathways. Therefore, northern blot analysis was performed to examine the state of the CD8α mRNA in the silenced cells. Total RNA was collected from CD8α silenced and control CD8α-expressing HeLa cells 36 h posttransfection. The CD8α message was probed using the 5′-most 596 nt of the mouse CD8α gene. These blots revealed a significant reduction in CD8α mRNA levels in both siRNA and hairpin RNA transfected cells (Fig. 4B). The amount of CD8α mRNA reduction in the hairpin RNA-treated samples was comparable to that mediated by siRNAs, supporting a hypothesis that the hairpin RNAs are evoking silencing through an mRNA-degradative mechanism. However, we have not tested the alternative hypothesis that the hairpin RNAs or siRNAs are actually inhibiting transcription, but this seems unlikely given the transient nature of the silencing. Curiously, in the northern blot, an RNA species migrating faster than the predominant species was detected in both the siRNA and hairpin RNA-silenced cells (Fig. 4B, lanes 1–3). CD8α mRNA transcribed from the expression vector should contain 47 nt of native CD8α UTR sequence, plus an additional ~190 nt of pCDNA3 vector-encoded 3′ UTR. Based on size, the lower species on the northern blot is likely CD8α mRNA minus the ~190 nt of pCDNA3 vector-encoded 3′ UTR. The siRNA used here should direct cleavage of the CD8α 3′ UTR, leaving ~25 nt of native CD8α UTR sequence remaining on the mRNA.

If stRNAs silence through translational repression, why then do the 3′-UTR-specific miRNA hairpin mimics presented here target the mRNA for degradation? We favor the idea that because the 21-nt internal regions of these RNAs have the ability to perfectly duplex the

target mRNA, they are used by the cell as siRNAs. In this way, silencing would be effected by the degradative RNAi pathway, and not the stRNA translational repression pathway. An alternative possibility is that stRNA-type inhibition of translation could lead to mRNA degradation in some systems. In this hypothesis, inhibition of translation elongation by stRNAs would enhance the rate of targeted mRNA degradation, resulting in a reduced level of mRNA. Very little is known about the mechanisms for stRNA-mediated silencing, or siRNA-mediated silencing in mammalian cells. It is reasonable to speculate that once we know more "rules" about how stRNAs operate mechanistically, we might be able to design hairpin RNAs that silence in a similar way. This would add another technique in the toolbox of gene silencers.

If the hairpin RNAs are active at targeting mRNA degradation, then they might be processed to siRNAs or similar-sized dsRNAs, which then may mediate RNAi. To further examine this possibility, total RNA was again isolated from 36 h-silenced cells, and the presence of antisense RNA was probed in northern blots. As shown in Figure 5, the transfected hairpin RNAs were processed to smaller RNAs. Processing of the 42-nt class I hairpin RNAs resulted in the appearance of 21–23-nt-size RNAs, consistent with cleavages in the loop sequence. Class II hairpin RNAs were also processed to smaller RNAs, and produced RNAs in a broad distribution, ranging from 25 to 30 nt in length. This size class would again be consistent with cleavage within the hairpin loop region. These data do not indicate whether this processing occurs within the cell as part of the RNAi pathway or as the RNA is associated with the cells and exposed to the surrounding media. It should also be noted that these data represent the "majority" of fates of the synthetic RNAs delivered to the cells. Given the high doses of RNA that are delivered (and the requirement for a higher concentration of delivered hairpin than siRNA), it is possible that the interfering molecules might be a minor fraction of the total mixture of material that is derived from the hairpin in vivo. The results are consistent with a model whereby siRNAs or siRNA-sized dsRNAs are processed from the hairpins, and mediate silencing through an RNA-degradative mechanism.

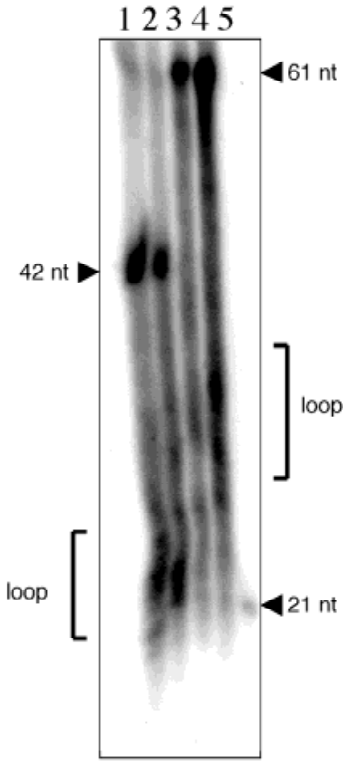

FIGURE 5. Processing of transfected hairpins in E10 thymocytes. Total RNA was extracted from cells 36 h posttransfection and subjected to small RNA northern blot analysis. The membrane was hybridized to a probe consisting of 5′-end-labeled sense siRNA strand. Lane 1: CD8-42F; lane 2: CD8-42R; lane 3: CD8-61R; lane 4: CD8-61F; lane 5: antisense siRNA strand (21-nt marker). Brackets indicate products that would result from cleavage in the hairpin loop.

Our data illustrate that the structure of the stem-loop RNA is important for effecting a silencing response. Because the majority of the processing observed here occurs in the loop sequences of the hairpin, it seems unlikely that Dicer is the catalytic nuclease. Genomes have the capacity to encode similar hairpin-structured RNAs, sometimes even in the context of an mRNA. Given the results of this study, it remains a possibility that gene silencing may be effected by RNAs other than stRNAs, and that nucleases other than Dicer may process such hairpin RNAs.

Gene silencing induced by siRNAs is a transient process that strongly depends on the structure of the siRNA (Elbashir et al., 2001b, 2001c). Time course analysis of CD8α silencing in the E10 cells indicates that the hairpin-mediated silencing evoked similar overall kinetics to that of siRNAs, and after a period during which there are eight cell doublings, CD8α expression returned to normal (data not shown). In lower organisms such as plants and *C. elegans*, the transient nature of RNAi can be overcome by expressing long hairpin RNA (usually ~500–1,000 nt) from integrated promoter constructs (for reviews, see Bernstein et al., 2001b; Sharp, 2001; Vaucheret et al., 2001; Zamore, 2001). When similar approaches were used in mouse ES cells (Svoboda et al., 2001; Paddison et al., 2002a), silencing disappeared upon in vitro differentiation of hairpin-containing ES cells. Thus, prospects for long-hairpin-mediated silencing in mammalian cells are unclear. As a first step towards this goal, we designed plasmid vectors expressing the CD8-61F RNA, which is capable of silencing CD8α. In this system, transcription is driven by RNA polymerase III promoter of the unusually compact human H1 RNA promoter (Myslinski et al., 2001), and transcriptional termination occurs at a stretch of

5-thymidine residues at the 3′ end of the hairpin (encoding Us in the RNA; Fig. 6A).

The polymerase III CD8α-hairpin vector was transiently transfected into HeLa cells, together with CD4 and CD8α expression vectors, and flow cytometry was performed as described above. The H1 RNA promoter silenced CD8α to nearly the same level as did 50 pmol of siRNA directed against the same region in the 3′ UTR of the CD8α transgene (Fig. 6B). Additionally, the silencing by the plasmids was gene specific, as there was no reduction in cotransfected CD4 expression (not shown), and control hairpin-containing plasmids lacking the promoter sequence or control plasmids lacking the hairpin could not elicit CD8α silencing (Fig. 6B). These results indicate that RNAi can be initiated from RNA hairpins that are transcribed by RNA polymerase III from transiently transfected plasmid DNA.

While these studies were in progress, we learned of the successful generation of siRNA-type silencing with a H1 promoter system (Brummelkamp et al., 2002). Subsequently, other studies have come to our attention where polymerase III-based vectors have been used to generate siRNA-type silencing (Lee et al., 2002; Paddison et al., 2002b; Paul et al., 2002; Sui et al., 2002).

Despite the lack of a mechanistic understanding of RNAi, gene silencing using siRNAs in mammalian cells is rapidly becoming a mainstream methodology for gene function studies. The studies reported here indicate that short hairpin RNAs can be effective silencers, and are likely to be processed to small RNA duplexes prior to silencing. The discovery that these RNAs may be transcribed from plasmid vectors and used to silence genes opens up the possibility for long-term stable silencing. Cell lines, and perhaps whole animals, could be generated in which stably expressing RNA hairpins silence expression of a targeted gene. It will be an important goal to further develop this tool as a methodology for silencing genes, and should be considered complementary to both siRNA-mediated knock-down and conventional gene-targeted knock-out methodologies.

MATERIALS AND METHODS

Cell culture

E10 is an immature double positive (DP) thymocyte line derived from TCR-α and p53 double mutant mice of a mixed 129/Sv $\times$ C57BL/6 background as described (Mombaerts et al., 1995). These cells, which proliferated vigorously, were maintained at a maximal concentration of 4×10^6 c/mL, and propagated in complete medium: Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μM 2-mercaptoethanol. HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FCS and 100 μg/mL penicillin/streptomycin.

Transfection

For electroporations, 2.5 μmol siRNA or hairpin RNA (Dharmacon) and/or 20 μg of pEGFP-N3 plasmid (transfection control) (CLONTEC, Palo Alto, California) were added to prechilled 0.4-cm electrode gap cuvettes (Bio-Rad, Hercules, California). E10 cells (1.5×10^7) were resuspended to 3×10^7 c/mL in cold, serum-free RPMI, added to the cuvettes, mixed, and pulsed once at 300 mV, 975 uF, 200 Ω with a Gene Pulser II electroporator (BioRad). Cells were plated

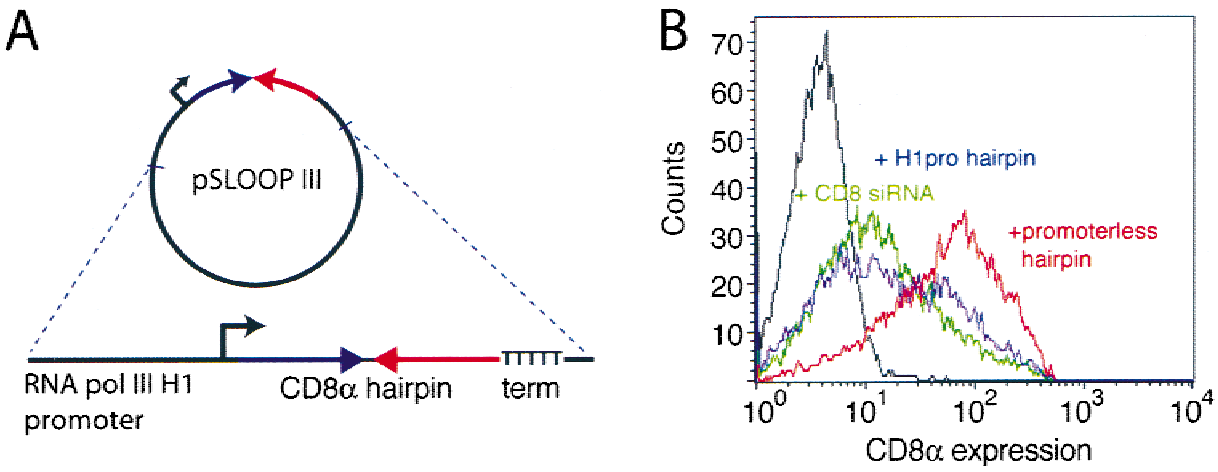

FIGURE 6. Hairpin RNAs transcribed from DNA constructs have the ability to silence CD8α in HeLa cells. **A:** Schematic depicting the polymerase III promoter construct (pSloop III) driving the expression of hairpin RNA CD8-61F. Transcription initiation begins at the first nucleotide of the hairpin RNA, with no intervening vector sequences transcribed. Termination at the stretch of 5 T residues is indicated by "term." **B:** Histogram plot showing silencing of CD8α in HeLa cells using pSloop III. Untransfected cells do not express CD8α (black line). Red line indicates cells transfected with the CD8α expression vector and a promoterless pSloop III-CD8-61F construct. The effect of the siRNA and pSloop III-CD8-61F treatment is shown by green and purple lines.

into six-well culture plates containing 8 mL complete medium, and incubated at 37 °C in a humidified 5% CO_2 chamber. Cell viability immediately after electroporation was typically around 60%. For cationic lipid transfections, 2 μg plasmid DNA and 100 nmol siRNAs were used per 10^6 cells, and transfection followed the manufacturer's recommended protocol. Transfection of HeLa cells was performed with Lipofectamine 20000 (Invitrogen) and performed as suggested by the manufacturer. For RNA transfections, 100 pmol of either siRNA or hairpin RNAs were typically used to transfect each well of a six-well dish. Hairpin-containing plasmids used in transfections were constructed in a single step, using overlap-extension PCR. Briefly, two DNA oligos containing H1 polymerase III sequence and hairpin sequence were annealed, PCR extended, and cloned into pCRII-TOPO vector (Invitrogen). These constructs were transfected using lipofectamine and using 5 μg/well of a six-well dish. To check for appropriate annealing and purity of the siRNAs and hairpin RNAs, gel analysis was performed on 15% nondenaturing acrylamide gels and UV shadowed.

Flow cytometry

HeLa cells or E10 cells (approximately 1×10^6) were washed once in FACs buffer (PBS supplemented with 2% FCS and 0.01% sodium azide), resuspended to 100 μL, and stained directly with phycoerythrin (PE)-conjugated anti-CD4 (clone RM4-5) and allophycocyanin (APC)-conjugated anti-CD8α mAbs, and in some experiments with PE- or APC-conjugated anti-mouse Thy-1.2 (clone 53-2.1) mAb. All mAbs were from PharMingen. The stained cells were washed once, then resuspended in 200 μL FACs buffer containing 200 ng/mL propidium iodide (PI). Unstained and singly stained controls were included in every experiment. 3A9, a T cell hybridoma line that had been infected with a MIGW GFP retrovirus (a gift from Dr. Luk Van Parjis), was included when GFP expression was analyzed. Cell data were collected on a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, California) and four-color analyses (GFP, PE, PI, and APC) were done with Cellquest software (Becton-Dickinson). All data were collected, but analyses were performed on 1×10^4 PI-negative events (viable cells).

Northern blot analysis

Cells were lysed in TRIzol reagent (Gibco-BRL), and total cellular RNA was purified according to the manufacturer's instructions. RNA (10 μg) was fractionated on a denaturing formaldehyde/agarose gel and transferred to a nylon membrane. Blots were hybridized overnight with uniformly ^{32}P-labeled CD8α (596 bp) DNA fragments or ^{32}P 5′-end-labeled 21-nt RNA oligo.

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