tion buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% NP-40 and 1 mM Pephabloc SC) and incubated for 5 min on ice. Each homogenate was centrifuged at 15 000 rpm for 5 min. The supernatant or the cytoplasmic fraction was subjected to Western blotting. The pellet was resuspended in nuclear extraction buffer and sonicated. The lysate was centrifuged at 15 000 rpm for 5 min and the supernatant was subjected to Western blotting as the nuclear fraction.

Concentrations of protein were determined with a Protein Assay kit (Bio Rad, California) following the manufacturer's protocol. Cell extracts were separated by SDS-PAGE and bands of protein were transferred to a Clear Blot™ Membrane-P (ATTO, Tokyo, Japan). The membrane was probed with various antibodies, as mentioned above, and then with peroxidase-conjugated second antibodies (Amersham Bioscience, New Jersey). Immunoreactions were detected with the ECL plus™ Western blotting system (Amersham Bioscience).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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Use of random ribozyme libraries for the rapid screening of apoptosis- and metastasis-related genes

Laura D. Nelson, Eigo Suyama, Hiroaki Kawasaki and Kazunari Taira

Apoptosis and metastasis are complex processes involving many gene products. Various biochemical, genetic and technological approaches are used to identify functional gene products, and are valuable for understanding basic biology and disease. However, now that sequence information is abundant and accessible through databases, there is a simpler, more definitive technology to identify genes that are the most relevant to biological phenomena of interest. A rapid screening system using libraries of randomized ribozymes has been developed that identifies key gene products involved in a defined phenotype using a functional gene 'knock-down' approach. Libraries of randomized ribozymes have been used to identify genes involved in pathways of apoptosis and metastasis, and there is great potential for this system to identify genes involved in other processes and diseases as well.

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▼ Observations of human cancers and animal models suggest that tumors develop through a process similar to evolution, in which a succession of genetic changes, each conferring a different type of growth advantage, leads to the progressive conversion of normal cells into cancer cells. The growth advantages obtained by cancer cells, the 'hallmarks of cancer', can be described by six essential changes in normal cell physiology that collectively promote malignancy: self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [1].

Complex cellular processes in malignant alterations of tumors

Apoptosis, or programmed cell death, is a normal cellular process that can be misregulated and cause various diseases, including cancer. In apoptosis, there are two pathways leading to the activation of caspases, proteases that produce morphological and biochemical changes in the affected cell that are distinct from necrosis [2]. The death receptor pathway is initiated by the ligation of transmembrane death receptors, such as CD95/Fas, TNF-R and TRAIL-R, to recruit and activate membrane-proximal activator caspases, which cleave and activate downstream effector caspases [3,4]. The mitochondrial pathway requires disruption of the mitochondrial membrane, and the release of mitochondrial proteins and cytochrome *c*. Mitochondrial membrane permeabilization is regulated by the opposing actions of pro- and anti-apoptotic Bcl-2 family members [5]. There is considerable cross-talk between the death receptor and mitochondrial pathways, resulting in the modification or amplification of the apoptotic signal, depending on the cell type. Overall, apoptosis is regulated at many levels, including initiation, transduction, amplification and execution. The efficiency of the entire process depends on the integrity of an elaborate molecular network. Disruption of the mitochondrial pathway is extremely common in cancer cells, but resistance to death-receptor-mediated apoptosis also occurs, albeit less frequently. Although chemotherapeutic drugs would ideally target only tumor cells, normal cells are usually susceptible to the toxic effects of these agents and undergo apoptosis as well. An increased knowledge of the molecular links between tumorigenesis and apoptosis would provide new opportunities for a more tailored approach to chemotherapy [2].

Apoptosis and metastasis

Metastasis is the process by which cancer cells migrate from a primary tumor to other

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sites in the body. It is by far the leading cause of death in cancer patients, but the molecular mechanisms are poorly understood. Metastasis involves many distinct, successive steps. Tumor cells must escape from the primary tumor site, enter the bloodstream or the lymph system, evade host defense systems, lodge in a spot that is conducive to growth and establish a colony by recruiting blood vessels for nourishment. Metastasis depends on the other five essential changes or 'hallmarks of cancer', but are there additional, unique genetic changes as well? Less than 0.01% of primary tumor cells actually develop into metastases, and there are two schools of thought concerning the origins and characteristics of these rare cells [6,7]. One hypothesis is that only a very small minority of cancer cells have the ability to successfully establish metastatic colonies, and that these cells have altered expression of genes that specifically promote or suppress metastasis. Another hypothesis is that the metastatic potential of tumors is already encoded in the majority of primary tumor cells. These two ideas are not mutually exclusive, as there could be two types of tumors, one of which has the metastatic phenotype [6].

Identifying genes involved in apoptosis and metastasis

Before the human genome was sequenced and high-throughput technologies were developed, methods to identify genes relevant to cellular processes and disease were slow and laborious. For example, to identify a gene, investigators would often define the phenotype or process, purify a protein of interest, do peptide sequencing, screen a cDNA library with a degenerate probe and clone the gene corresponding to the protein.

Now that sequence information is abundant and accessible through databases, some investigators are using methods such as gene microarrays and yeast two-hybrid screens to identify genes. The yeast two-hybrid system is a powerful method for detecting direct interactions between proteins *in vivo* and for the identification of genes whose products interact with proteins derived from a cDNA library. However, this method is limited in that the interactions do not always reflect a direct role in the process being studied and many false-positives often result. Differential expression microarrays and other chip technologies are also powerful methods to examine multiple genes simultaneously, but, as they identify all of the differentially expressed genes, they cannot specifically identify the primary genes that directly cause the phenotype from the other downstream genes that are indirectly affected. In addition, these methods fail to identify post-transcriptional or qualitative levels of control, such as mRNA stability, translational regulation, and protein phosphorylation and degradation. Results from these studies have suggested that mRNA abundance is a poor indicator of the levels of the corresponding protein, as it is the complete set of proteins and their level of activity in a cell that determine phenotype [8,9].

In a recent example, Golub and colleagues [10] set out to identify differences in gene expression patterns between primary tumors and metastases. They initially found an expression signature within 128 genes and then further refined it to 17 genes. The refined gene expression signature associated with metastasis contained eight upregulated and nine downregulated genes. None of these genes represented individual markers of metastasis; rather, it was the signature taken as a whole that seemed to contain predictive information. They concluded that there was a distinction between the expression profiles of primary tumors and metastases, but that the expression profile associated with metastases was also found in some primary tumors. Examining primary tumors with this metastasis-associated signature, they found that they were associated with a poor prognosis. Thus, their data are in accord with the idea that the average tumor cell in a metastatic colony has a stronger metastasis fitness, rather than with the idea that metastasis arises from rare metastatic cells [7]. However, it was unclear whether the expression profile was a cause or an effect, and they admitted that further study was required to determine whether any signature genes are mechanistically important in the cascade of events that drives metastasis [7,10]. Clearly there is a need for a simpler, more definitive technology to identify genes that are the most relevant to a phenotype of interest.

Randomized ribozyme libraries as a novel gene discovery tool

The Wong-Staal, Barber and Taira [11–21] laboratories developed a novel gene discovery system using randomized ribozyme libraries that were introduced into cells and produced a phenotypic change. Hammerhead and hairpin ribozymes are small, naturally occurring catalytic RNA molecules. They contain conserved sequences comprising structural and catalytic domains, as well as binding arms composed of variable sequences that determine target specificity (Fig. 1). Ribozymes recognize and bind their RNA targets by Watson–Crick base pairing, and then cleave these RNAs at specific triplet sequences in the target, depending on the type of ribozyme [22]. The use of ribozymes as a gene discovery tool involves randomizing the target recognition sequence (Fig. 2a), creating a library of ribozymes capable of potentially cleaving any mRNA that contains the triplet sequence recognized by the ribozyme

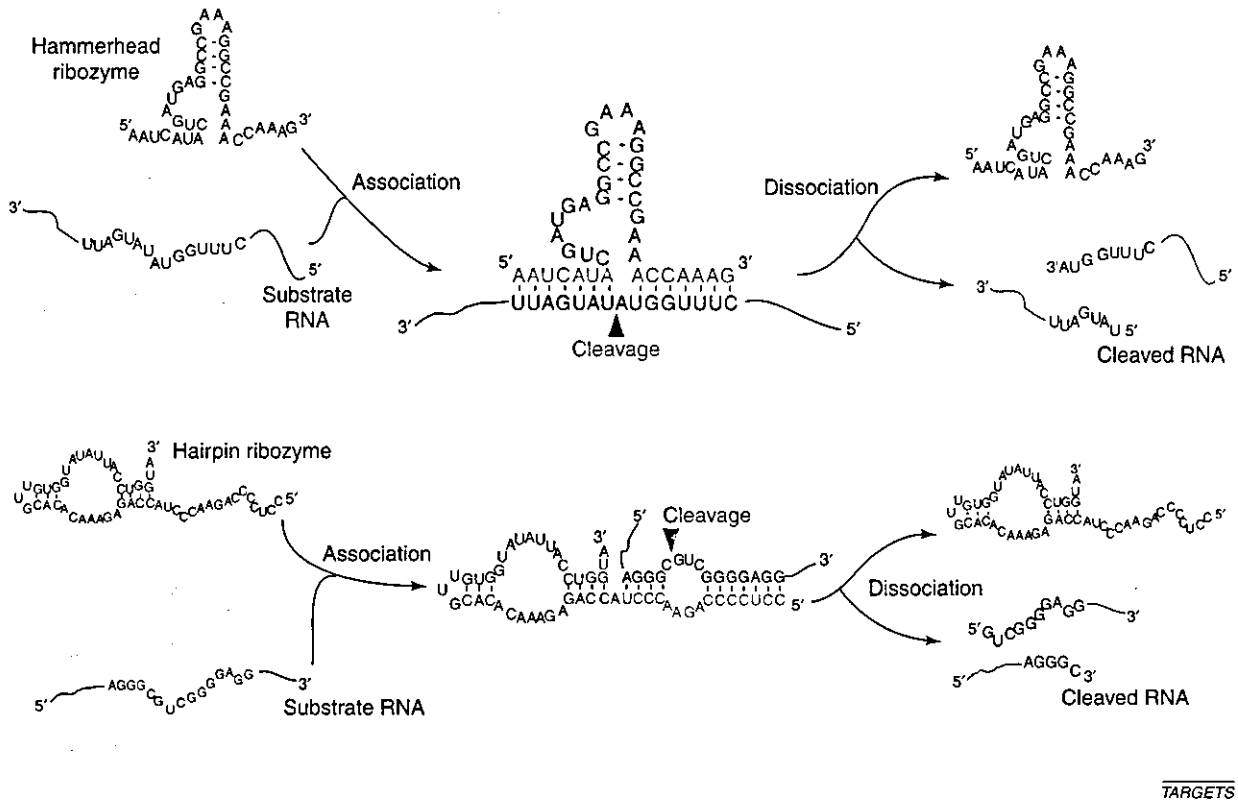

Figure 1. Ribozymes bind and cleave substrate RNAs in a sequence-specific manner. Hammerhead and hairpin ribozymes base pair with substrate RNAs using specific binding arms, shown in red, and contain conserved sequences of structural and catalytic domains, shown in blue. Upon dissociation, the substrate RNA is cleaved at a specific triplet sequence and its expression is 'knocked down'.

(see below) and introducing them into cells with a certain phenotype (Fig. 2b). When a particular ribozyme in the library recognizes and cleaves a cellular mRNA directly involved in the phenotype, the target gene expression would be knocked down, the ribozyme could be recovered and sequenced, and the target gene identified. A particular ribozyme that targets a housekeeping gene would probably not be isolated because a cell containing these ribozymes would die and could not be assayed. Another advantage of using ribozymes is that, once a relevant ribozyme is recovered and sequenced, changing only one base can create a catalytically inactive mutant that should not produce the phenotypic change. This system allows rapid genetic screening that identifies genes involved in a particular phenotype without prior sequence information (Fig. 2a and 2b) [11–21].

Wong-Staal, Barber and colleagues [11–15] developed a randomized hairpin ribozyme library that recognizes a GUC triplet in the target mRNA. Kawasaki, Taira and colleagues [16–20] developed a randomized hybrid hammerhead ribozyme library that recognizes an NUX triplet (where N corresponds to any nucleotide, and X corresponds to adenine, uracil or cytosine) and contains a poly(A) sequence that recruits an endogenous RNA helicase. The advantage of the hybrid ribozymes is that accessibility to the target site is more efficient and is not dependent on an open conformation of the mRNA at the cleavable sequence.

Overall, the development of this technology is relatively simple compared to other high-throughput gene screening methods (Fig. 2b). There are approximately ten steps involved in the experimental design and procedure, which will be described in general here (refer to the original manuscripts for detailed technical information [11–21]).

1. After defining the phenotype of interest and choosing the cellular assay system, the design of the randomized ribozyme library must be considered. Both hairpin and hammerhead ribozymes have been used successfully, and a poly(A) sequence has been added to the hammerhead ribozyme to create a hybrid ribozyme. Both ribozyme libraries were under the control of a $tRNA^{Val}$ promoter used by RNA polymerase III to ensure a high level of expression and cytoplasmic localization of the ribozyme. The tRNA-attached ribozymes, without being processed in the nucleus, are recognized by exportin-t and efficiently transported to

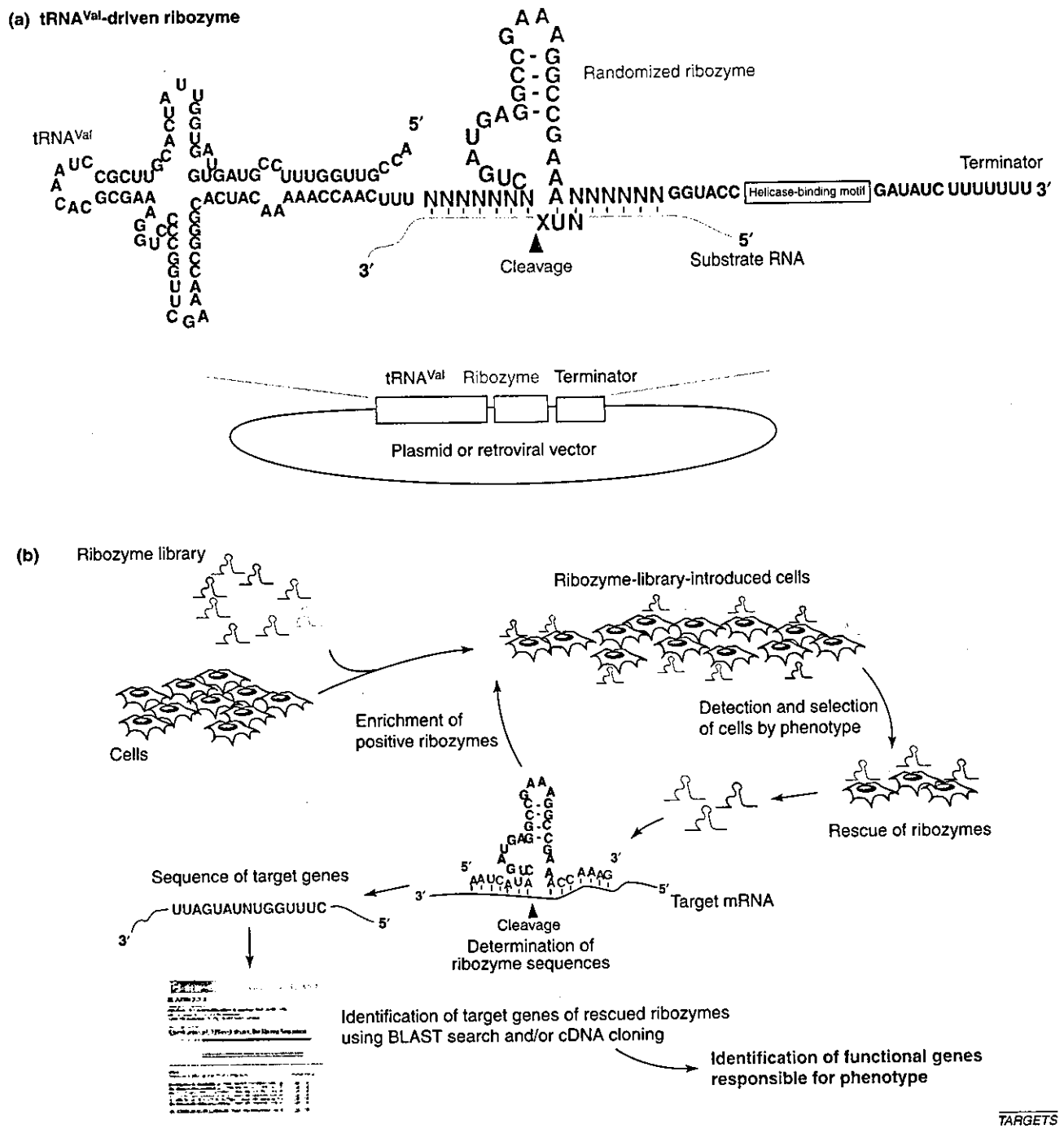

Figure 2. (a) Randomized ribozyme and its library. Randomized ribozymes contain a conserved structural and catalytic domain, and randomized substrate-binding arm sequences that can target potentially any substrate cellular RNA containing the specific triplet sequence. Ribozymes are expressed as tRNA^{Val}-fused RNAs in cells. **(b)** Gene discovery of your favorite phenotype using randomized ribozyme libraries. The application of a randomized ribozyme library as a gene discovery tool involves introducing the library into cells, growing and selecting ribozyme-containing cells, detecting and isolating cells that have a change in phenotype, recovering ribozymes from altered cells, cycling positive ribozymes by re-introducing them into fresh cells and assaying again, sequencing positive ribozymes and identifying the functional genes responsible for the particular phenotype.

the cytoplasm [23–26]. Important characteristics of a library are an appropriate degree of randomness and demonstrated cleaving ability.

2. Next, the expression vector and method of introduction into cells must be chosen, depending on the phenotypic change to be examined. Most often, stable expression

and integration of the ribozyme into the host cell genome is obtained with a retroviral vector. Adenoviral infection has not been found to be suitable because its expression is transient and it allows multiple copies to be introduced, complicating the signal and making the results hard to interpret. A plasmid vector allowing transient expression can be used if a short-term phenotype is assayed; cells usually do not lose the plasmid until ~60 h [18,19]. Repeated cycles of the transient expression of randomized hybrid libraries from plasmid vectors have been used to successfully identify genes involved in Fas-mediated apoptosis [19]. Phenotypic changes that require more than 48 h would need to be examined with a stable expression system. Any vector system would require an appropriate gene for antibiotic resistance for positive selection.

3. Perhaps the most important step in the design of this system is the assay used to monitor the phenotypic change. Because the system allows the identification of direct effectors, the most direct and simple assay system will produce the most interpretable results, and reduce the level of background and false-positives.

4. Once the assay has been performed, the effective ribozymes must be harvested in one of two ways: retroviral rescue, whereby the ribozyme-containing virus is induced to replicate, shed particles and is collected in the cell media (which can be used to transduce fresh cells), or isolating genomic DNA from the affected cells and performing PCR of the ribozyme sequence [11–15]. The latter procedure is preferable because it only recovers ribozyme sequences that have integrated into the host cell genome. It is highly advisable to repeat steps three and four one to three more times to enrich for relevant ribozymes and reduce the number of false-positives.

5. After the assay and ribozyme rescue have been repeated, the ribozyme can be sequenced, the complementary target mRNA deduced and its corresponding gene can be identified by database searches such as BLAST. In theory, for cleavage by hammerhead and hairpin ribozymes, wobbles and mismatches are somewhat allowed, although the cleavage efficacy reduces dramatically [22]. One might feel that this allowance complicates the identification of the key target genes. However, in practice, as long as hybrid ribozyme libraries are used, many candidate genes can be identified because of their high level of efficacy and thus we search for target genes with perfect complementarity. We also identify target genes using 5′ and 3′ RACE (rapid amplification of cDNA ends), a PCR-based technique that facilitates the cloning of full-length cDNA sequences when only a partial cDNA sequence is available.

6. The relevant ribozyme(s) should be re-introduced into fresh cells to confirm that they cause the phenotypic change. Additionally, a catalytically inactive mutant should be tested that should not effect the phenotype.

7. Next, the entire candidate gene should be cloned and sequenced, although many genes have already been cloned and their sequences are available.

8. Construct and test other ribozymes that target other cleavable sites within the target genes' mRNA, searching for the required triplet within the sequence [22]. Alternatively, siRNA (small interfering RNA) can be used to decrease the expression of the target gene [27–30].

9. Confirm the decreased expression of the target gene by RT-PCR or northern blotting. Confirm that housekeeping or unrelated gene expression is not affected.

10. Examine other cellular processes, such as proliferation, to see if they are affected, to ensure the phenotypic change is specific. If possible, an increase in target gene expression could be tested to obtain a reversal of the phenotypic change. Secondary phenotypic effects, such as cytokine or growth factor release, can be studied. Other cell types can be examined to determine the 'universality' of the effect.

Once the target gene(s) have been identified, they can be characterized individually for their specific involvement in the phenotype of interest, and/or the process can be refined and repeated. Additional genes in a pathway can be identified by slightly or moderately changing the selective assay and phenotype to be examined. As a gene discovery system, the randomized ribozyme library has broad applicability in the diverse cellular phenotypes that can be examined.

Using randomized ribozyme libraries to identify genes involved in pathways of apoptosis and metastasis

Using the hybrid hammerhead ribozyme library, our group identified genes involved in both Fas-mediated and TNF-α-mediated apoptosis (Fig. 3) [17–19]. In the Fas-mediated apoptosis study, four genes known to be involved in this process, FADD (Fas-associated death domain protein) and caspases 8, 9 and 3, as well as additional novel genes, were identified in HeLa Fas cells after surviving treatment with anti-Fas antibody [17]. Addition of the poly(A) tail to the ribozymes reduced the relative number of both false-positives and surviving cells that did not contain the hybrid ribozyme. In the absence of the poly(A) tail, FADD and caspase 8 were not identified with a conventional ribozyme library, demonstrating the efficiency of this system. In the TNF-α-mediated apoptosis study, TRADD (TNF receptor type I-associated death domain protein), caspases 2 and 8, RIP, RAIDD [receptor-interacting protein (RIP)-associated ICH-1/CED-3-homologous protein with death domain], TRAF 2 (TNF receptor

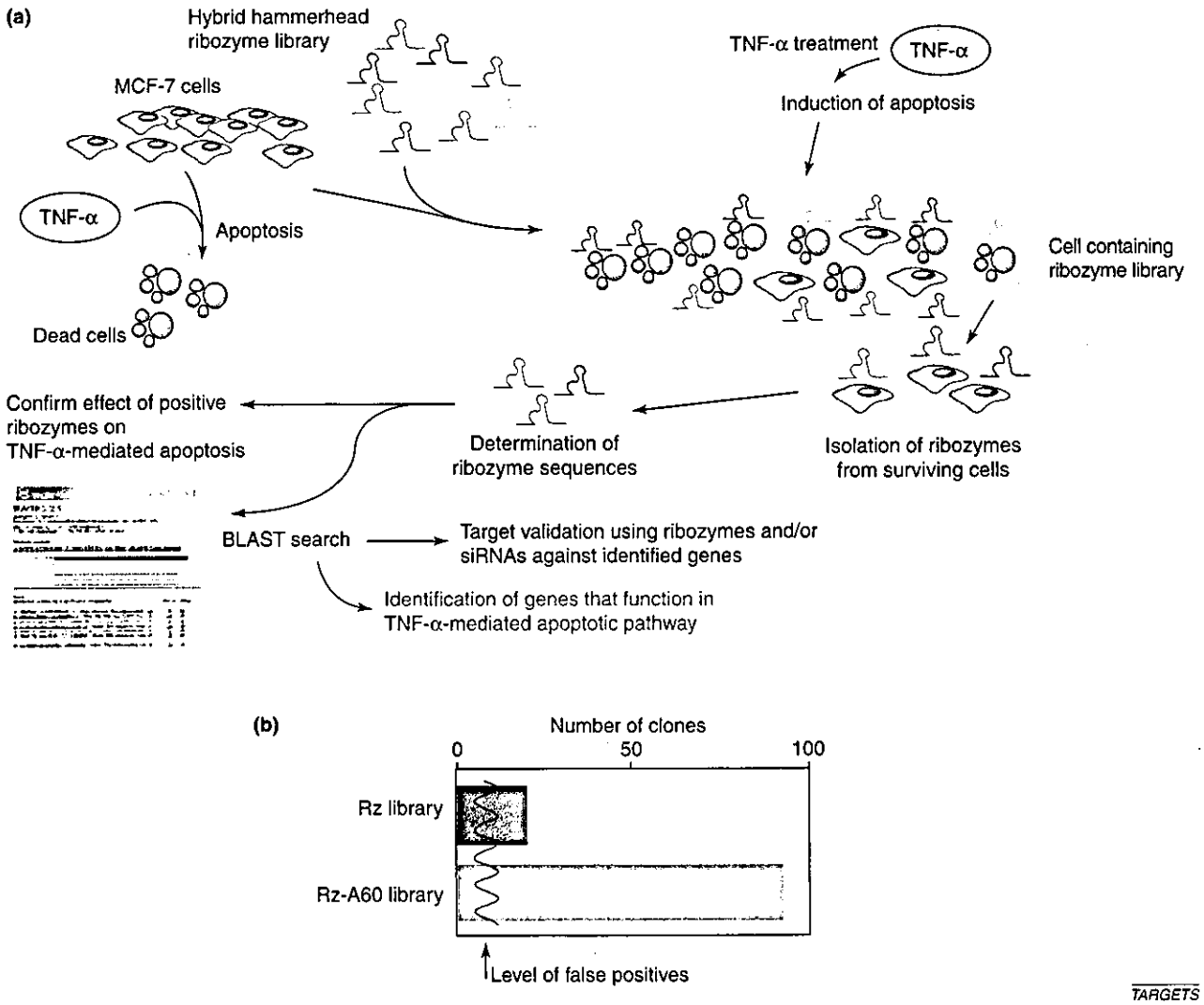

Figure 3. (a) The specific use of a hybrid poly(A)-linked hammerhead ribozyme library to identify genes involved in TNF-α-mediated apoptosis. After ribozymes are introduced into MCF-7 breast cancer cells, apoptosis is induced by TNF-α treatment, ribozymes are isolated from surviving cells and genes that promote TNF-α-mediated apoptosis are isolated. **(b)** The number of surviving clones after treatment of MCF-7 cells with TNF-α. The orange bar indicates results for the poly(A)-linked ribozyme library and the green bar indicates results for the conventional ribozyme library in an individual experiment in which 1×10^7 cells were transformed. The estimated level of false-positives is also indicated [18].

associated factor 2), Ets 1 and Bcl-2 family member Bak were identified when MCF-7 cells that lack caspase 3 were treated with TNF-α (Fig. 3a) [18]. In addition, partial sequences for 30 new target genes were also identified. We noted that the inhibition of apoptosis by the hybrid ribozymes was not necessarily complete; multiple pathways exist and the disruption of one factor or pathway usually causes a reduction in apoptosis, rather than complete inhibition.

Using the hairpin ribozyme library, Welch and colleagues identified and cloned the human homologue of *Drosophila ppan* (the Peter Pan gene) as an important factor in suppressing anchorage-independent growth of HF cells, a nontransformed revertant of HeLa cells that resulted after their exposure to the chemical mutagen EMS (ethyl methanesulfonate) (Fig. 4) [12]. When active ribozymes were introduced into the HF cells, the cells resumed anchorage-independent growth in soft agar, which is a capability of HeLa cells. By inactivating the target of a particular ribozyme enriched in these cells, the group has strong evidence that they identified a putative tumor suppressor gene that is important in regulating anchorage dependence (Fig. 4). Interestingly, when the group exogenously expressed human PPAN in HeLa cells, this resulted in a marked reduction of HeLa cell growth in soft agar compared to empty vector controls. By increasing PPAN mRNA

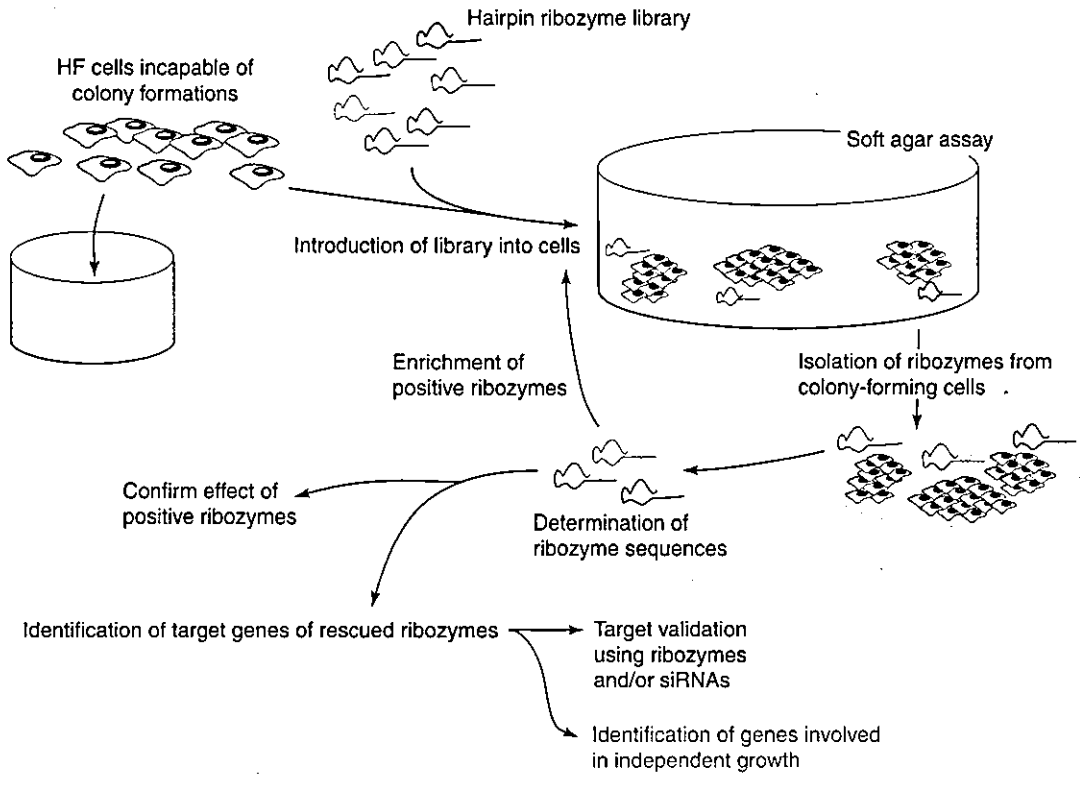

Figure 4. The specific use of a conventional hairpin ribozyme library to identify genes involved in anchorage-independent growth. After ribozymes are introduced into HeLa revertant HF cells, the cells are assayed for the renewed ability to form colonies in soft agar. Positive cells and ribozymes are recovered, and genes that suppress anchorage-independent growth (potential tumor suppressors) are identified [12].

levels in HeLa cells to the level normally found in HF cells, additional PPAN appeared to specifically induce toxicity in HeLa cells. This example shows the ability of the randomized ribozyme library to identify loss-of-function genes involved in cellular transformation. In addition, this example identifies how relatively small differences, perhaps threshold levels, in one gene's expression (~20% between HF and HeLa cells) can affect a phenotype such as anchorage-independent growth. Differential expression arrays would have missed this important data because of the noise in their assays and the focus on large differences in gene expression.

Additional studies by Suyama *et al.* in our laboratory have focused on identifying genes involved in cell migration and invasion of the extracellular matrix (ECM) (Fig. 5). In the first study, highly invasive HT1080 human fibrosarcoma cells were transduced with a plasmid vector containing a conventional randomized ribozyme library and subjected to an *in vitro* chemotaxis assay in transwell inserts with fibronectin as the chemoattractant [21]. Unaffected cells migrated to the lower chamber, but cells whose phenotype was changed remained in the top chamber and were collected. The target genes identified were ROCK1, a regulator of the reorganization of the actin cytoskeleton [31], myosin IXb, which acts both as a motor protein and as a GTPase-activating protein for the Rho family [32], and adducin, which is a substrate of protein kinase C and a mediator of the reorganization of actin filaments [33], as well as additional target genes (Fig. 5). The Rho-ROCK system had previously been implicated in cell mobility, migration and metastasis for a variety of cell types, and a specific ROCK inhibitor, Y-27632, blocked the invasive activity of rat MM1 hepatoma cells [34]. This adds validity to the identification of ROCK1 in cell migration assays by the randomized ribozyme system as a gene involved in promoting cell migration, possibly due to aberrant activation as a gain-of-function gene product (Fig. 5). Randomized ribozymes can be used for both positive and negative selection; cells with increased or decreased metastatic fitness can be isolated by the same assay using low or highly invasive cells, respectively.

In another study by Suyama and colleagues, hybrid ribozymes were isolated that enhanced the invasive properties

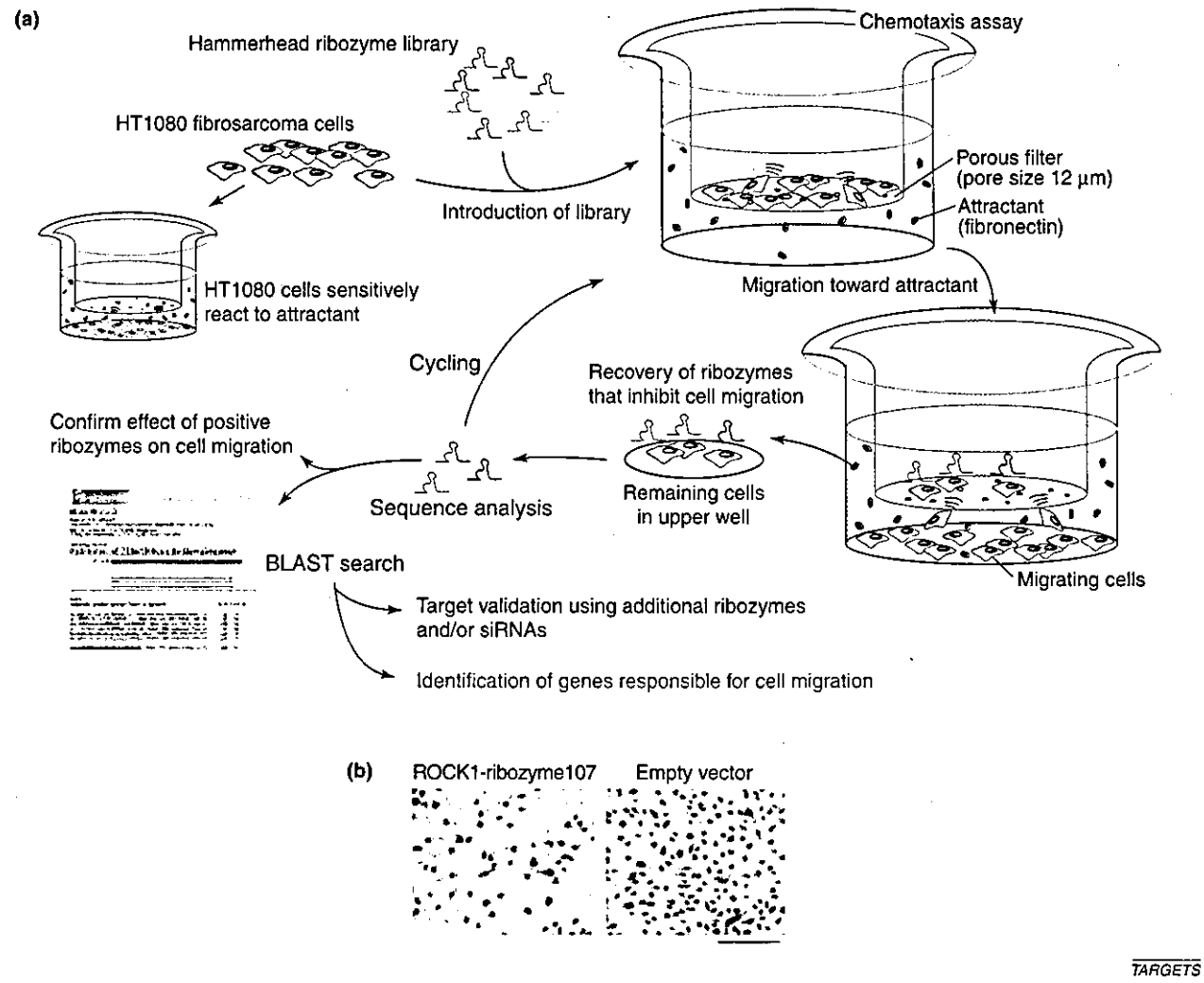

Figure 5. (a) The specific use of a conventional hammerhead ribozyme library to identify genes that promote cell migration. After ribozymes are introduced into highly invasive HT1080 fibrosarcoma cells, the cells are assayed for migration using a chemotaxis assay with transwells and fibronectin. Cells with reduced migration are isolated, positive ribozymes are recovered and sequenced, and genes that promote cell migration are identified. **(b)** Inhibition of cell migration by ribozymes specific for ROCK1 compared to empty-vector-containing control cells. Migrating cells on the microporous membrane were visualized by Giemsa staining. Scale bar, 200 μm [21].

of noninvasive NIH-3T3 cells in ECM-coated transwells to search for genes that could act as ECM invasion suppressors [20]. Genes identified included Gem GTPase, and uncharacterized genes that resemble myosin phosphatase and protein tyrosine phosphatases. The roles of these genes in cell migration and invasion are currently being investigated to further elucidate their functions in pathways that promote cancer metastasis. Randomized ribozymes are also currently being used in *in vivo* metastasis studies by injecting C57/Bl6 mice with B16/F0 low invasive melanocytes containing a ribozyme library.

It is noteworthy that, using the gene discovery approach, we could identify genes that are involved in cell differentiation and they turned out to be small RNAs (unpublished). It is likely that these microRNAs (miRNAs) play important roles in development. Indeed, our identification, for the first time, of a target of an miRNA in human cells strongly suggests that the miRNA regulates the expression of a gene at the post-transcriptional level and participates in neural differentiation in human cells [35].

Conclusions

The randomized ribozyme library is an effective tool for identifying genes involved in specific pathways in apoptosis and metastasis, as well as other pathways and phenotypes including transcription and translation regulation. For a summary of the pathways that have been investigated

Table I. Phenotypes and assays using the randomized ribozyme library as a gene discovery system.

Phenotype examined	Ribozyme type	Vector	Assay	Refs
TNF-α-mediated apoptosis	Hybrid hammerhead	Retrovirus	TUNEL	[18]
Fas-mediated apoptosis	Hybrid hammerhead	Retrovirus	TUNEL	[17]
Fas-mediated apoptosis	Hybrid hammerhead	Plasmid	TUNEL	[19]
Cell invasion	Hybrid hammerhead	Retrovirus	ECM-coated transwell	[20]
Cell migration	Conventional hammerhead	Plasmid	Transwell and fibronectin	[21]
HCV-IRES-mediated translation	Conventional hairpin	Retrovirus	IRES-HSV-tk + ganciclovir	[13,15]
Regulation of BRCA1 expression	Conventional hairpin	Retrovirus	BRCA1-promoter-EGFP	[14]
Anchorage-independent cell growth	Conventional hairpin	Retrovirus	Soft agar growth	[12]
NIH-3T3 fibroblast transformation	Conventional hairpin	Retrovirus	Focus formation	[11]

using this technique, see Table 1. Additionally, this system can be used to study other aspects of basic cell biology and disease processes, such as aging, neurodegeneration [36], immortalization and differentiation. Libraries of ribozymes have great potential in elucidating cellular pathways and in the design of nucleic acid drug treatments [37]. Because many cellular pathways are redundant and have many levels of control, the benefit of various gene discovery systems is to compare, contrast and validate each other's results. No one system is guaranteed to be 100% successful, but when multiple systems coming from different perspectives are examined together, therein lies the strength of biotechnology to reveal the mechanisms of biology and disease.

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Short hairpin type of dsRNAs that are controlled by tRNA^{Val} promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells

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ABSTRACT

The post-transcriptional gene silencing in animals and plants is called RNA interference (RNAi). Guides for the sequence-specific degradation of mRNA are 21-nt small interfering RNAs (siRNAs) that are generated by Dicer-dependent cleavage from longer double-stranded RNAs (dsRNAs). To examine the relationship between the localization of dsRNA and the target cleavage of RNAi in human cells, we constructed five kinds of dsRNA expression vector that were controlled by tRNA^{Val} or U6 promoter. Transcripts of tRNA-dsRNA were consistently localized in the cytoplasm and were efficiently processed by Dicer. In contrast, transcripts of tRNA-dsRNA were not processed in cells that expressed Dicer-directed ribozymes. In addition, transcripts of U6-dsRNA were basically localized in the nucleus and were not significantly processed, unless the transcripts of U6-dsRNAs possessed a microRNA-based loop motif: in the latter case, U6-dsRNAs with a microRNA-based loop were transported to the cytoplasm and were effectively processed. Moreover, tRNA-dsRNA directed against a mutant k-*ras* transcript cleaved its target mRNA efficiently in assays of RNAi not only *in vitro* with a cytoplasmic extract but also *in vivo*. Therefore, it appears that RNAi in human cells occur in the cytoplasm. Importantly, the same tRNA-dsRNA did not affect the degradation of the normal k-*ras* mRNA *in vitro* and *in vivo*. Our tRNA-dsRNA technology should be a powerful tool for studies of the mechanism of RNAi and the functions of various genes in mammalian cells with potential utility as a therapeutic agent.

INTRODUCTION

RNA interferance (RNAi) is a process in which double-strand RNA (dsRNA) induces a sequence-dependent degradation of a cognate mRNA (1,2). The natural roles of RNAi might include defense against viral infection (3–7) and regulation of the expression of cellular genes (8,9). Genetic and biological studies have revealed that RNAi is a very complex process that involves many different proteins with mostly unidentified functions (3–11).

It has been demonstrated *in vitro* that dsRNA targets mRNA for cleavage in lysates of early *Drosophila* embryos and in extracts of cultured *Drosophila* S2 cells (12–14), and such reactions *in vitro* require ATP (14). The molecular basis for the requirement for ATP is due, in part, to a requirement for ATP in the initial processing of long dsRNA into the 21–25-nt small interfering RNAs (siRNAs) that serve as guides for targeted cleavage (14–18).

Recent studies with synthetic RNA duplexes demonstrated that the siRNA duplex must have 2- or 3-nt overhanging 3′-ends for efficient cleavage of its target (16). Such 3′ overhangs are characteristic of the products of cleavage reactions catalyzed by RNase III, and, in cultured *Drosophila* S2 cells, cleavage of dsRNA into siRNAs requires a multidomain RNase III, known as Dicer (17). Subsequently, siRNAs seem to associate with a multicomponent nuclease, identified in *Drosophila* and designated RNAi-induced silencing complex (RISC), and then they guide this enzyme so that it catalyzes the sequence-specific degradation of mRNA (13,17,19).

RNAi provides a method for inactivating genes of interest and, thus, provides a powerful tool for studies of gene function in *Caenorhabditis elegans, Drosophila melanogaster* and plants. Specific inhibition of gene expression also can be achieved by the stable or inducible expression of dsRNA in animals and plants (11). Inactivation of genes by dsRNA has been achieved in mouse embryonal carcinoma (EC) cells and embryonic stem (ES) cells (20,21), but elicitation of RNAi using long dsRNAs has generally been less successful in cultured mammalian cells. Such failures can be explained most readily by the activation of dsRNA-dependent protein kinase (PKR) and 2′,5′-oligoadenylate synthetase (2′,5′-AS) which are activated by long dsRNA (>30 bp; 22,23).

It was reported recently that 21-nt synthetic siRNAs (24) and siRNAs that were transcribed by U6 or H1 promoter (25–32) specifically suppressed the expression of endogenous genes in several lines of mammalian cells. These 21-nt siRNA

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duplexes were able to evade non-specific reduction of mRNAs and these findings suggested that RNAi or an RNAi-related system might exist in mammals. Some mammalian homologs of RNAi-associated proteins, such as Dicer, eIF2C2 and WRN have, indeed, been identified (17,33–36). However, details of the characteristics and mechanisms of RNAi in human somatic cells remain to be determined.

In this study, we constructed five kinds of dsRNA expression vectors that were controlled by $tRNA^{Val}$ or widely used U6 promoter in order to examine the relationship between the localization and a target cleavage of RNAi in mammalian cells. Transcripts of tRNA-dsRNA were localized in the cytoplasm and were processed efficiently by Dicer. In addition, a mutant k-*ras*-directed tRNA-dsRNA efficiently cleaved the targeted mRNA *in vitro* and *in vivo*. In contrast, the tRNA-dsRNA did not affect the expression of normal k-*ras* in HeLa cells. Therefore, these results indicated that RNAi in mammalian cells occurred in the cytoplasm and our tRNA-dsRNA expression system should be a powerful tool for studying mechanism of RNAi and other gene functions in mammalian cells with potential utility as a therapeutic agent.

MATERIALS AND METHODS

Culture and transfection of cells

SW480 human colon cancer cells were cultured in L-15 medium (ICN Biomedicals, Inc., OH) supplemented with 10% fetal bovine serum (FBS). HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Transfections were performed with the Effectin™ reagent (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. dsRNA-expressing SW480 and dsRNA-expressing HeLa cells were selected by incubation with puromycin for two weeks.

Construction of dsRNA expression plasmids

To construct vectors for expression of tRNA-dsRNA, we used the pPUR-tRNA plasmid that includes the chemically synthesized promoter for a human gene for $tRNA^{Val}$ (37) between the *Eco*RI and *Bam*HI sites of pPUR (Clontech, CA). Chemically synthesized oligonucleotides encoding mutant k-*ras*-directed dsRNA that included loop 1 (5′-GAG CTC GGT AGT TGG AGC TGT TGG CGT AGG CAA GAG AAA ATC TTG CCT ACG CCA ACA GCT CCA ACT ACC GGT ACC-3′) were amplified as double-stranded sequences by PCR. After digestion with *Sac*I and *Kpn*I, the fragments were cloned downstream of the promoter of the tRNA gene in pPUR-tRNA. The construction of vectors for expression of dsRNA from the mouse U6 promoter has been described elsewhere (38,39) except for the insertion step of dsRNA sequences. Chemically synthesized oligonucleotides encoding k-*ras*-directed dsRNA that included loop 1 (5′-GAA TTC GGT AGT TGG AGC TGT TGG CGT AGG CAA GAG AAA ATC TTG CCT ACG CCA ACA GCT CCA ACT ACC TCT AGA-3′) or loop 2 (5′-GAA TTC GGT AGT TGG AGC TGT TGG CGT AGG CAA GAC TTC CTG TCA TCT TGC CTA CGC CAA CAG CTC CAA CTA CCC TCG AG-3′) were amplified as double-stranded sequences by PCR with specific up primer and down primer which contained *Eco*RI and *Xho*I linker sequences, respectively. After digestion with *Eco*RI and *Xho*I, the fragments were cloned downstream of the promoter of the mouse U6 gene. In the case of a human U6 promoter, we used pUC-hU6 (40). Chemically synthesized oligonucleotides encoding k-*ras*-directed dsRNA that included loop 1 (5′-GTC GAC GGT AGT TGG AGC TGT TGG CGT AGG CAA GAG AAA ATC TTG CCT ACG CCA ACA GCT CCA ACT ACC TCT AGA-3′) or loop 2 (5′-GTC GAC GGT AGT TGG AGC TGT TGG CGT AGG CAA GAC TTC CTG TCA TCT TGC CTA CGC CAA CAG CTC CAA CTA CCT CTA GA-3′) were amplified as double-stranded sequences by PCR with specific up primer and down primer which contained *Sal*I and *Xba*I linker sequences, respectively. After digestion with *Sal*I and *Xba*I, the fragments were cloned downstream of the promoter of the human U6 gene.

Construction of Dicer-directed ribozyme expression plasmids

Chemically synthesized oligonucleotides encoding Dicer-directed ribozyme sequence (5′-TCC CCG GTT CGA AAC CGG GCA CTA CAA AAA CCA ACT TTC AAA GAA AGC TGA TGA GGC CGA AAG GCC GAA ACC CAT TGG GGT ACC CCG GAT ATC TTT TTT-3′) with a pol III termination sequence (TTTTTT) were converted to double-stranded DNAs by PCR. After digestion with *Csp*45I and *Pst*I, the fragments were cloned downstream of the tRNA promoter of pUC-dt (37,41,42). To generate poly(A)-connected ribozyme, we inserted a poly(A) sequence of 100 nt between the ribozyme and the pol III termination sequence (42–45).

Preparation of the nuclear fraction and the cytoplasmic fraction of cells

SW480 or HeLa cells were grown to ~5 × 10^6 cells and were transfected with a tRNA-dsRNA or U6-dsRNA expression vector with the Effectin™ reagent (QIAGEN). Thirty-six hours after transfection, cells were harvested. For the preparation of the cytoplasmic fraction, collected cells were washed twice with PBS and then resuspended in digitonin lysis buffer (50 mM HEPES/KOH, pH 7.5, 50 mM potassium acetate, 8 mM $MgCl_2$, 2 mM EGTA and 50 µg/ml digitonin) on ice for 10 min. The lysate was centrifuged at 1000× *g* and the supernatant was collected as the cytoplasmic fraction. The pellets were resuspended in NP-40 lysis buffer (20 mM Tris–HCl, pH 7.5, 50 mM KCl, 10 mM NaCl, 1 mM EDTA and 0.5% NP-40) and held on ice for 10 min and the resultant lysate was used as the nuclear fraction.

Northern blot analysis

Cytoplasmic RNA and nuclear RNA were extracted and purified from the cytoplasmic fraction and the nuclear fraction, respectively, with ISOGEN reagent (Wako, Osaka, Japan). Thirty micrograms of total RNA per lane were loaded on a 15% polyacrylamide gel. After electrophoresis, bands of RNA were transferred to a Hybond-N™ nylon membrane (Amersham Co., Buckinghamshire, UK). The membrane was probed with synthetic oligonucleotides that were complementary to the sequences of the k-*ras* gene. The synthetic probe was ^{32}P-labeled by T4 polynucleotide kinase (Takara Shuzo Co., Kyoto, Japan).

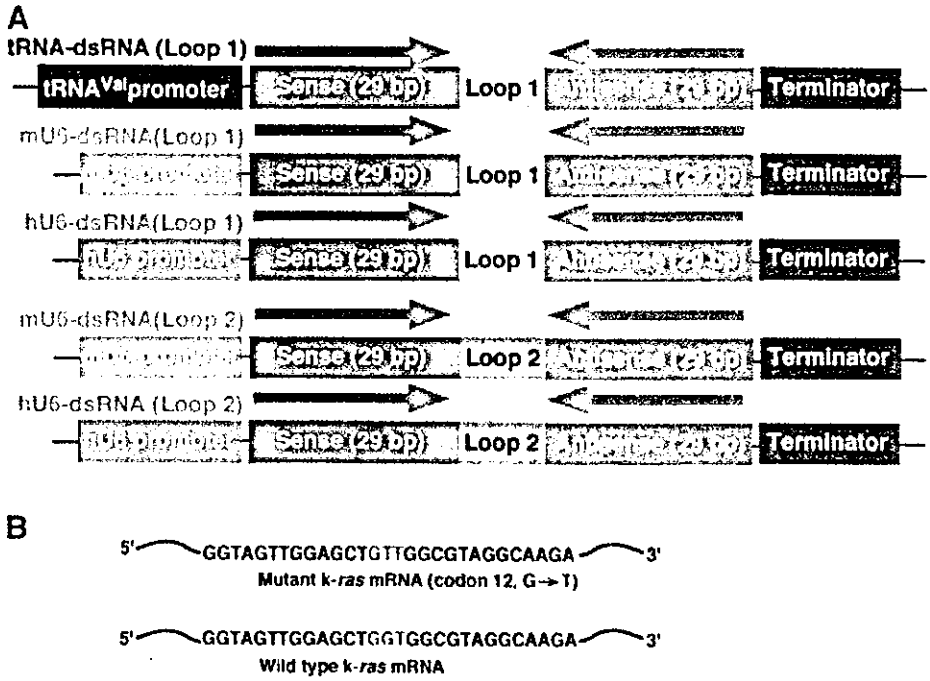

Figure 1. Construction of dsRNA expression plasmids. (**A**) The plasmid for expression of tRNA-dsRNA included the promoter sequence from a human gene for $tRNA^{Val}$ and a terminator sequence. The plasmid for expression of mouse U6- or human U6-dsRNA included a mouse or a human U6 promoter, respectively. The Loop 1 sequence (5′-GAAAA-3′) or loop2 sequence [miR-23 loop sequence (48): 5′-CUUCCUGUCA-3′] was inserted between a k-*ras* sense-strand sequence (29 bp) and an antisense-strand sequence (29 bp). (**B**) Sequences of mutant and normal k-*ras* mRNAs that were targeted by dsRNA. The mutant k-*ras* gene had a point mutation (codon 12 GGT→GTT).

RT–PCR analysis

RT–PCR was performed using an RNA PCR Kit ver. 2 (Takara, Kyoto, Japan) with dicer upstream (nucleotides 1–24) and downstream (nucleotides 435–459) primers or GADPH upstream (nucleotides 230–254) and downstream (nucleotides 442–466) primers as a control. The products of PCR were analyzed by electrophoresis on a 2% agarose gel.

Western blot analysis

SW480 or HeLa cells that had been transfected with individual dsRNA-expression vectors were harvested. Proteins were resolved by SDS–PAGE (10% polyacrylamide) and transferred to a polyvinylidene difluoride (PVDF) membrane (Funakoshi Co., Tokyo, Japan) by electroblotting. Immune complexes were visualized with ECL kit (Amersham Co., Buckinghamshire, UK) using specific polyclonal antibodies against K-Ras (UBI, CA) and Actin (Santa Cruz, CA).

Assay of RNAi *in vitro*

For cleavage of a target RNA, we synthesized a mutant k-*ras* template DNA (70 nt) and a normal k-*ras* template DNA (70 nt) using an automated DNA synthesizer. For preparation of k-*ras* mRNA substrates, we amplified template DNAs by PCR using a k-*ras*-specific up primer that included the T7 promoter sequence and a k-*ras*-specific down primer. The amplified k-*ras* DNA templates were transcribed by T7 polymerase. Transcribed normal and mutant k-*ras* mRNA substrates were purified by PAGE. These mRNAs were ^{32}P-labeled by T4 polynucleotide kinase. For detection of the cleavage of the target mRNA, target mRNA (5–10 nM) was incubated with a lysate of SW480 cells that expressed tRNA-dsRNA under standard conditions (14) for 2 h at 25°C. In the case of siRNA targeted to k-*ras* mRNA, 100 nM siRNA was incubated with target RNA (5–10 nM) in a lysate of SW480 cells under standard conditions (14) for 2 h at 25°C.

Detection of rates of cell proliferation

The rate of proliferation of each line of cells was measured with a Cell Proliferation Kit II (Roche Ltd, Switzerland) according to the manufacturer's instructions.

RESULTS

Construction of two kinds of dsRNA-expression plasmid with pol III promoters

It has not yet been determined whether a long dsRNA is processed by Dicer in the nucleus or the cytoplasm. Therefore, we used two kinds of pol III promoter (the promoter of a gene for human $tRNA^{Val}$, and mouse U6 or human U6 promoter) for expression of dsRNAs in mammalian cells. If designed appropriately, transcripts generated with the promoter of a human gene for $tRNA^{Val}$ are transported efficiently to the cytoplasm and we demonstrated previously that the cytoplasmic localization was important in improving ribozyme activities *in vivo* (41,46,47). In contrast, small RNAs transcribed under the control of the U6 promoter remained localized in the nucleus (37). Thus, we attached the gene for dsRNA (an extended stem–loop RNA; Fig. 1A) to the 3′ end of the promoter of a gene for $tRNA^{Val}$ (tRNA-dsRNA), to the 3′

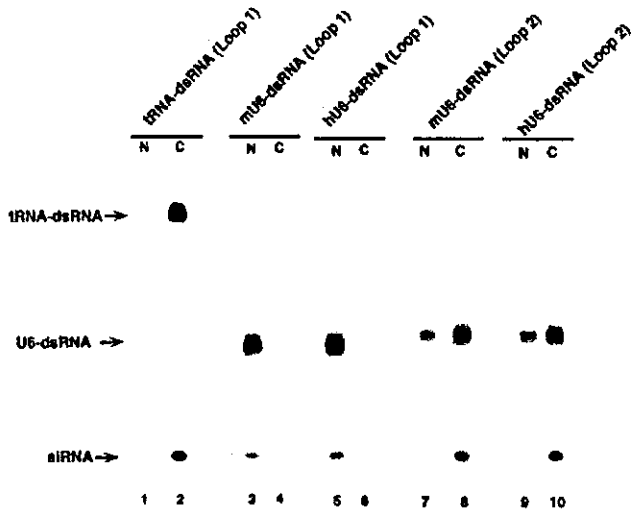

Figure 2. Detection of precursor dsRNAs and siRNAs. The presence of precursor dsRNAs and siRNAs was analyzed by northern blotting analysis. Plasmids encoding tRNA-dsRNAs or U6-dsRNAs were introduced into SW480 cells. After 48 h, the cells were collected and divided into cytoplasmic (C) and nuclear (N) fraction. Total RNA in each fraction was isolated and fractionated on a 15% polyacrylamide gel. Northern blotting analysis was performed as in the text. (Lane 1, nuclear fraction of cells that expressed tRNA-dsRNA; lane 2, cytoplasmic fraction of cells that expressed tRNA-dsRNA; lane 3, nuclear fraction of cells that expressed mU6-dsRNA (Loop 1); lane 4, cytoplasmic fraction of cells that expressed mU6-dsRNA (Loop 1); lane 5, nuclear fraction of cells that expressed hU6-dsRNA (Loop 1); lane 6, cytoplasmic fraction of cells that expressed hU6-dsRNA (Loop 1); lane 7, nuclear fraction of cells that expressed mU6-dsRNA (Loop 2); lane 8, cytoplasmic fraction of cells that expressed mU6-dsRNA (Loop 2); lane 9, nuclear fraction of cells that expressed hU6-dsRNA (Loop 2); and lane 10, cytoplasmic fraction of cells that expressed hU6-dsRNA (Loop 2).

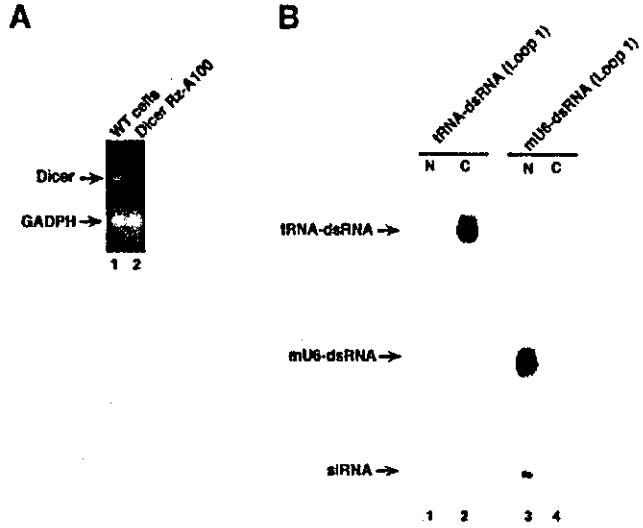

Figure 3. Effect of Dicer-ribozymes on processing of dsRNAs. (**A**) The levels of expression of dicer genes in cells that expressed poly(A)-connected Dicer-ribozyme (Dicer-RzA100). The dicer mRNA was detected by RT–PCR with primers specific for the dicer gene (see Materials and Methods). GADPH is an endogenous control. (**B**) Detection of precursor dsRNAs and siRNAs in cells that expressed the Dicer-RzA100. Lane 1, nuclear fraction of cells that expressed tRNA-dsRNA and Dicer-RzA100; lane 2, cytoplasmic fraction of cells that expressed tRNA-dsRNA and Dicer-RzA100; lane 3, nuclear fraction of cells that expressed U6-dsRNA and Dicer-RzA100; and lane 4, cytoplasmic fraction of cells that expressed U6-dsRNA and Dicer-RzA100.

end of the mouse U6 promoter (mU6-dsRNA) or to the 3′ end of the human U6 promoter (hU6-dsRNA).

We constructed dsRNA expression plasmids targeted to the mRNA for a mutant of K-Ras with a point mutation in codon 12 of the k-*ras* gene (Fig. 1B). The length of the double-stranded region within the dsRNA was kept at 29 bp because long dsRNAs (>30mers) induce non-specific reduction of mRNAs (22,23). In the case of U6-based constructs, we used two kinds of loop motif for stem–loop RNAs. One is a loop motif that consists of 5 nt (5′-GAAAA-3′), namely Loop 1 (Fig. 1A). The other is a microRNA (human *mir-23*; 48) loop motif, namely Loop 2 (Fig. 1A). It is believed that precursor microRNAs are transported to the cytoplasm and the processed microRNAs act post-transcriptional gene silencing (48–54).

dsRNA transcribed under the control of the tRNA^{Val} promoter was processed by a Dicer-complex in the cytoplasm

In RNAi, long dsRNAs are first processed to short RNA duplexes of predominantly 21 and 22 nt in length and with staggered 3′ ends in an RNase III-like reaction (17). To examine whether tRNA-dsRNA and U6-dsRNA might be processed by an RNase III complex in mammalian cells, we performed northern blotting analysis with a k-*ras* mRNA-specific probe. SW480 cells were transfected with plasmids that encoded dsRNA under the control of the tRNA^{Val} or U6 promoter. Forty-eight hours after transfection, cells were collected and separated into cytoplasmic and nuclear fractions. Total RNA in each fraction was isolated and fractionated on a 15% polyacrylamide gel. As shown in Figure 2, in cells that expressed tRNA-dsRNA, processed siRNAs were detected in the cytoplasmic fraction and not in the nuclear fraction. Moreover, the sequences of processed siRNAs were confirmed by cloning and sequencing of the siRNAs (data not shown). In contrast, in cells that expressed either mU6- or hU6-dsRNA (Loop 1), predominantly unprocessed precursor dsRNAs were detected in the nucleus and the nuclear fraction contained very little siRNAs in cells that expressed either mU6- or hU6-dsRNA (Loop 1). However, mU6-dsRNAs (Loop 2) and hU6-dsRNAs (Loop 2) were transported to the cytoplasm and were processed efficiently (Fig. 2). A mammalian Dicer has been detected mostly in the cytoplasm by immunostaining *in situ* (20), so it seems likely that tRNA-dsRNA, mU6-dsRNAs (Loop 2) and hU6-dsRNAs (Loop 2) that had been transported to the cytoplasm were processed by the Dicer-like RNase III complex.

To confirm whether tRNA-dsRNAs are processed by Dicer, we constructed a poly(A)-connected Dicer-directed ribozyme (Dicer-RzA100) expression plasmid (42–44). Then this plasmid was introduced into HeLa cells stably. Stable cell lines were obtained by neomycin selection. Next, to examine suppression of expression of dicer gene by Dicer-RzA100, we performed the RT–PCR analysis with specific primers for dicer mRNA. As shown in Figure 3A, the level of dicer mRNA in cells that expressed Dicer-RzA100 was reduced compared with that in WT-HeLa cells. The level of GADPH mRNA as a

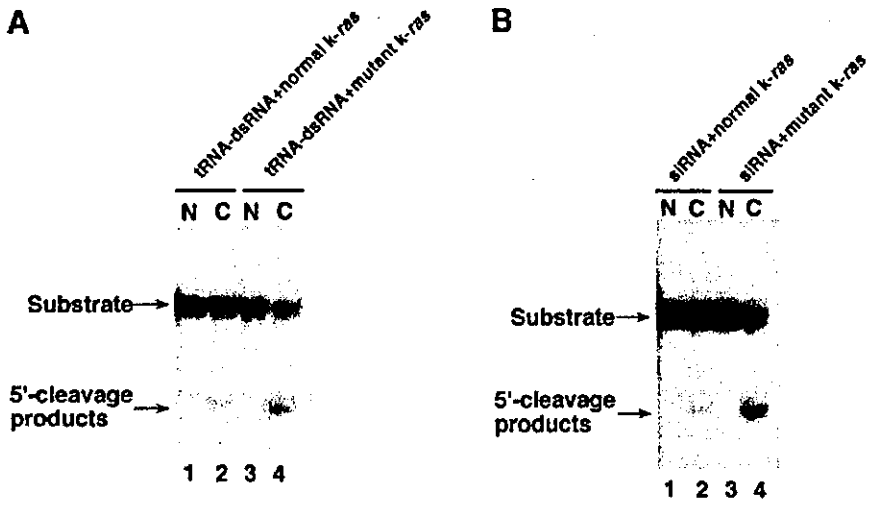

Figure 4. Detection of dsRNA- and siRNA-mediated cleavage of mutant k-*ras* mRNA. (**A**) Cleavage of mutant k-*ras* mRNA by cell extracts that contained tRNA-dsRNA. *In vitro* RNAi: the assay of RNAi *in vitro* was performed as described in the text. Lane 1, nuclear fraction (N) of cells that expressed tRNA-dsRNA and normal k-*ras* RNA; lane 2, cytoplasmic fraction (C) of cells that expressed tRNA-dsRNA and normal k-*ras* mRNA; Lane 3, nuclear fraction of cells that expressed tRNA-dsRNA and mutant k-*ras* mRNA; and lane 4, cytoplasmic fraction of cells that expressed tRNA-dsRNA and mutant k-*ras* mRNA. (**B**) Cleavage of mutant k-*ras* mRNA mediated by k-*ras*-directed siRNAs and extracts of SW480 cells. Lane 1, nuclear fraction of SW480 cells and normal k-*ras* mRNA; lane 2, cytoplasmic fraction of SW480 cells and normal k-*ras* mRNA; lane 3, nuclear fraction of SW480 cells and mutant k-*ras* mRNA; and lane 4, cytoplasmic fraction of SW480 cells and mutant k-*ras* mRNA.

control did not alter in both cell lines. These results indicated that the Dicer-RzA100 cleaved dicer mRNAs specifically.

To examine whether reduction of Dicer affects the processing of the tRNA-dsRNA, we performed northern blot analysis using total RNAs from cells that expressed the tRNA-dsRNA. As shown in Figure 3B, in the case of a total RNA from cells that expressed the Dicer-RzA100, siRNAs that are generated from tRNA-dsRNAs were not observed in both nucleus and cytoplasm. Therefore, these results suggest that tRNA-dsRNAs are processed by Dicer in the cytoplasm. In contrast, reduction of Dicer did not affect processing of mU6-dsRNA (Loop 1). Thus, it is possible that the processing of mU6-dsRNA (Loop 1) is dealt with another RNase III-like enzyme.

Degradation of target mRNA *in vitro* in a cell extract that contained tRNA-dsRNA, U6-dsRNA or synthetic siRNAs

To examine the cell compartment in which degradation of a target mRNA by dsRNA-mediated gene silencing occurs, we performed assays of RNAi *in vitro* using cell extracts that contained transcripts of tRNA-dsRNA. In these assays, we used mutant and normal k-*ras* partial mRNAs, which had been transcribed *in vitro* by T7 polymerase, as substrates. For cleavage of the target mRNA, each substrate was incubated for 2 h at 25°C with an extract of SW480 cells that had been transfected with the tRNA-dsRNA expression vector. The 5′-cleavage products were resolved on sequencing gels. As shown in Figure 4A, the mutant k-*ras* mRNA substrate was cleaved in the cytoplasmic fraction of cell extracts that contained tRNA-dsRNA (lane 4). In contrast, in the nuclear fraction of the cell extracts, the substrate was not cleaved (lane 3). These results support the earlier reports that the RISC is included in a ribosomal fraction (14,17,19).

In contrast to these results, in the case of the normal k-*ras* mRNA substrate, significantly smaller amounts of 5′-cleavage products were detected and these were found only in the cytoplasmic fraction of cell extracts that contained transcripts of tRNA-dsRNA (Fig. 4A, lane 2). These results are in accord with the report that synthesized siRNAs with one mismatched base pair in the middle of the siRNA was reduced in efficiency of cleavage of a target RNA in an assay of RNAi *in vitro* with lysates of *D.melanogaster* (55). We confirmed that the siRNA generated from the tRNA-dsRNA formed a mismatched base pair with normal k-*ras* mRNA in the middle of the siRNA by sequencing analysis (data not shown). We obtained similar results with synthesized siRNAs targeted against the mutant k-*ras* mRNA (Fig. 4B; 36). Thus, our results suggested that degradation of a target mRNA by dsRNA-mediated gene silencing occurs in the cytoplasm and that siRNAs are capable of recognizing one mismatched base pair in a middle position.

Efficiency and specificity of RNAi by tRNA-dsRNA in colon cancer cells

To examine the efficiency and specificity of RNAi by tRNA-dsRNA in human colon cancer SW480 cells, we introduced tRNA-dsRNAs and four kinds of U6-dsRNAs expression plasmids into SW480 and HeLa cells. The mutant k-*ras* gene was expressed in SW480 cells (56). We used HeLa cells that expressed a normal k-*ras* gene as controls. We generated stable lines of cells that expressed tRNA-dsRNAs or each U6-dsRNA by selection in the presence of puromycin. We examined levels of K-Ras protein in cells that expressed tRNA-dsRNA or each U6-dsRNA by western blotting with K-Ras-specific antibodies. For quantitation, intensities of bands were analyzed by densitometry using the NIH Image Analysis. As shown in Figure 5, the level of K-Ras protein in SW480 cells that expressed tRNA-dsRNA, mU6-dsRNA (Loop 2) or hU6-dsRNA (Loop 2) was significantly lower

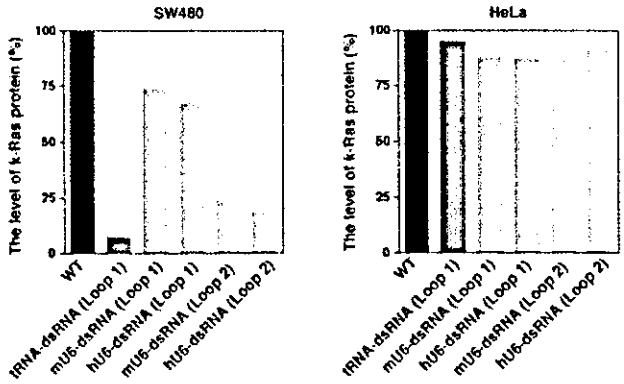

Figure 5. Efficiency and specificity of RNAi by tRNA-dsRNA *in vivo*. The level of K-Ras protein in cells expressed that tRNA-dsRNAs or U6-dsRNAs was analyzed by western blotting analysis with specific k-Ras antibodies. Mutant k-*ras* gene is expressed in SW480 cells. In contrast, normal k-*ras* gene is expressed in HeLa cells. For quantitation, intensities of bands were analyzed by densitometry using the NIH Image Analysis.

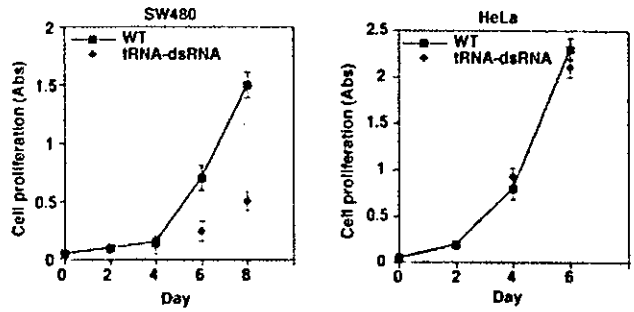

Figure 6. Effect of proliferation of cells by tRNA-dsRNA. Rates of proliferation of cells that expressed tRNA-dsRNA. Rates of proliferation were determined as described in the text. Values are means ±SD of results from three replicates in each case. SW480 cells that expressed tRNA-dsRNA proliferated significantly more slowly than wild-type SW480 cells. In contrast, the rate of proliferation in HeLa cells that expressed tRNA-dsRNA was the same as that of wild-type SW480 cells.

than that in wild-type SW480 cells, whereas the level of K-Ras protein in SW480 cells that expressed mU6-dsRNA (Loop 1) or hU6-dsRNA (Loop 1) was reduced only slightly compared with that in wild-type SW480 cells. The level of actin, chosen as an endogenous control, remained constant in these cell lines. Moreover, in HeLa cells that expressed tRNA-dsRNA, the level of K-Ras protein was similar to that in wild-type cells and in cells that expressed respective U6-dsRNA. These results demonstrated clearly both the efficiency and specificity of the tRNA-dsRNA in human cancer cells.

To examine the phenotype of cells that expressed tRNA-dsRNA, we analyzed the rates of proliferation of various lines of cells. As shown in Figure 6, SW480 cells that expressed tRNA-dsRNA proliferated significantly more slowly than wild-type SW480 cells. In contrast, the rate of proliferation in HeLa cells that expressed tRNA-dsRNA was the same as that of wild-type SW480 cells. These results indicated that the reduced rate of proliferation of SW480 cells that expressed tRNA-dsRNA was correlated with the reduction in the level of K-Ras protein in the cells. Thus, our tRNA-dsRNA targeted to the mutant k-*ras* gene appears to have potential utility as a therapeutic agent.

DISCUSSION

The discovery that dsRNA could induce gene silencing in animals and plants has raised the possibility that RNAi might be a nearly universal mechanism of gene silencing. An outline of the processes involoved in RNAi and several components have been identified in *C.elegans*, *D.melanogaster* and plants, but details of the mechanism and many of the necessary participants in RNAi in mammalian cells remain unclear. In this study, to clarify the relationship between the localization and the target specificity of RNAi in mammalian cells, we constructed two kinds of dsRNA expression plasmid, namely, tRNA-dsRNA and U6-dsRNA expression plasmids. We demonstrated that tRNA-dsRNA which was localized in the cytoplasm was efficiently processed by the RNase III complex. An initial step in RNAi is the cleavage by Dicer, which is localized in the cytoplasm (20), of long dsRNAs. Although short dsRNAs are cleaved less effectively by Dicer *in vitro* (16,17), our tRNA-dsRNA was processed with significant efficiency in mammalian cells (Fig. 2). In addition, U6-dsRNAs that had a microRNA-based loop motif were transported to the cytoplasm and were processed. Although transcripts from the U6 promoter are generally localized in the nucleus, the microRNA-loop motif promotes the transport of dsRNAs to the cytoplasm. Thus, cytoplasmic localization of dsRNAs is important for processing by Dicer.

The degradation step of mRNA is a very interesting aspect of RNAi-mediated gene silencing. Although two groups proposed that an RNA-directed RNA polymerase (RdRP) chain reaction with siRNA amplifies the interference caused by a small amount of 'trigger' dsRNA in *C.elegans* (57), the mechanism is unclear in mammalian cells because an RdRP homolog does not exist in mammalian cells and siRNAs function as guides, not primers, in the *Drosophila* and human RNAi pathways (58). In this study, we demonstrated that a cytoplasmic fraction from cells which expressed tRNA-dsRNA had mRNA degradation activity (Fig. 4A). In addition, synthetic siRNAs mixed with the cytoplasmic fraction also had mRNA degradation activity (Fig. 4B; 36). Moreover, it was recently reported that the level of HIV RRE-containing mRNA in the nucleus was not affected by siRNAs (59). Thus, it is likely that a siRNA-associated silencing complex (SASC) including Dicer or -Slicer- is localized in the cytoplasm of mammalian cells.

RNAi has been shown to be a powerful tool for studies of gene function in *C.elegans*, *D.melanogaster* and plants. However, in mammalian cells, a long dsRNA causes the non-specific reduction in expression of many genes. Thus, it was believed initially that RNAi could not be used for gene inactivation in mammalian cells. However, Tuschle's group demonstrated that siRNA could specifically suppress the expression of a target gene specifically (24). Exploitation of RNAi in mammalian cells requires evasion of non-specific reduction of mRNAs. In addition, since the putative SASC is located in the cytoplasm, it is important that dsRNA

transcripts be localized in the cytoplasm. If properly designed, tRNA-dsRNAs (with a short hairpin structure) can be transported to the cytoplasm and can escape the non-specific reduction of mRNAs. Indeed, we found that PKR was not activated in cells that expressed tRNA-dsRNA (data not shown).

Although our U6-dsRNA with a general loop motif that consisted of five nucleotides was not transported to the cytoplasm and we could detect only marginal RNAi by our U6-dsRNA (Figs 2 and 5), transcripts of U6-dsRNAs (Loop 2) that had a microRNA-based loop motif were transported to the cytoplasm and were processed by Dicer (Fig. 2). In addition, they induced RNAi-mediated gene silencing (Fig. 5).

When alternative conditions were used, other U6-dsRNAs prepared by several independent groups (length of dsRNA and the size and the sequence of the hairpin-loop in these constructs were different from those of our U6-dsRNA) could support RNAi in mammalian cells (25–30). In one case, a microRNA motif was used as the loop motif of dsRNA and the efficacy could have been enhanced by a microRNA pathway (32). Thus, the length and the nucleotide sequence of the loop in hairpin types of dsRNAs are important for construction of effective hairpin types of dsRNAs. Now we are examining effects of both different length and structure of the loop on the localization and RNAi-mediated gene silencing by hairpin types of dsRNAs in detail.

Taken together, our results indicate that RNAi in mammalian cells occurs in the cytoplasm and is very specific in mammalian cells. Our tRNA-dsRNAs should be powerful tools for studies of the mechanism of RNAi and the functions of specific genes in mammalian cells, and they might also be useful as therapeutic agents.

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