transcription more efficiently than do RNA polymerase II (Pol II) systems (Bertrand *et al*., 1997). In addition, Pol III promoters offer several advantages over Pol II promoters. For instance, the compact sequence of the Pol III promoter is easier to manipulate molecularly. Their transcription is initiated and terminated at precise positions, resulting in addition of very little extra sequence when fused to other genes. The promoters of Pol III genes (i.e., tRNA, 5S RNA) usually contain an intragenic control region (Carbon and Krol, 1991). In contrast, U6 small nuclear RNA (U6 snRNA) genes are unique because the cis-elements necessary for transcription are present in the 5'-flanking regions. U6 snRNA is an abundant nuclear RNA involved in RNA processing (reviewed in Das *et al*., 1987). Pol III-based shRNA expression constructs have recently become the method of choice to interfere with a gene of interest in mammalian cells. Stable expression using this shRNA approach has the potential to recapitulate traditional knockout phenotypes (Sui *et al*., 2002; Kwak *et al*., 2003).

Although the zebrafish genomic information has been completed and zebrafish are widely used for genetic research, U6 snRNA genes have not yet been fully characterized. So far, characterization of U6 promoters has been conducted in various vertebrates (Krol *et al*., 1987; Das *et al*., 1988; Kunkel and Pederson, 1988; Kudo and Sutou, 2005; Lambeth *et al*., 2005). In teleost fish, snRNA-type genes have been characterized in pufferfish, *Fugu rubripes* (Myslinski *et al*., 2004). However, the U6 promoters have been reported to only drive species-specific transcription (Das *et al*., 1987). In this study, we report the characterization of the U6 snRNA genes in zebrafish. Genomic organization and the expression of the U6 snRNA genes are documented. To provide sustained vector-based GKD approach, usage of putative the

U6 promoter for shRNA expression activity was also investigated in zebrafish and other teleost nuclear extracts.

RESULTS

DNA fragments approximately 550 base pairs (bp) (data not shown) in length containing the putative U6 promoter and a partial U6 snRNA coding region were amplified by nested PCR using zebrafish genomic DNA as the template. Sequencing these fragments revealed three different types of U6 snRNA genes. Three U6 snRNA genes were classified relying on the different contributions of sequences upstream and from within the RNA coding region. The three genes were randomly designated U6-1, U6-2, and U6-3 (Fig. 1). The complete sequences of the three U6 snRNA genes were also cloned.

Characterization of zebrafish U6 snRNA genes

The U6-1 gene consisted of 112 nucleotides of RNA coding sequence. The U6-2 and U6-3 genes contained 107 and 106 nucleotides of RNA coding sequences, respectively. All U6 snRNA genes have conserved sequence motifs homologous to an intragenic "box A," which is characteristic of the RNA polymerase III (Pol III) genes (reviewed in Das *et al*., 1987) (Fig. 1). The termination signals of the U6-1 gene consisted of four or five thymidines (Ts). The U6-2 gene contained five Ts, whereas the U6-3 contained four Ts. The coding sequences of U6-1 showed 85.7% and 86.6% homology to the coding regions of U6-2 and U6-3, respectively. The coding sequence of the U6-2 gene was 96.3% homologous to that of U6-3. The three zebrafish U6 snRNA genes had high identity with other known U6 snRNA genes (data not shown). Using the coding sequences of U6 snRNA as input, phylogenetic analysis indicated

that the U6-1 gene was closely related to the mammal U6 snRNA genes, while the U6-2 was more closely related to the *Drosophila* U6 snRNA gene than the other U6 snRNA genes. In addition, U6-3 has a close genetic relationship with the *Xenopus* U6 snRNA gene (Fig. 2).

Comparison of the putative promoter regions showed that the nucleotide sequences diverged significantly (Fig. 1). The nucleotide sequences of the 5'-flanking regions of all three U6 snRNA genes contained essential upstream elements such as "CCAAT boxes", the proximal sequence elements (PSE), and TATA box sequences. Compared to the location of the CCAAT in the U6-2 and U6-3 (-249 to -245) promoters, the CCAAT of the U6-1 promoter was located closer to the start site (-244 to -240). The predicted PSE consensus sequences, located from -79 to -48, showed two highly conserved regional ACCAC (-65 to -61) and AACAT (-52 to -48) sequences. The position of TATA boxes were conserved among the three U6 snRNA genes (-31 to -24), although the DNA sequences of the spacer were not.

Genomic organization and expression of U6 snRNA genes

In order to identify the location of each U6 snRNA gene on zebrafish chromosomes, a BLASTn analysis was performed against the zebrafish genomic database. The sequences of each of the U6 snRNA genes, which consisted of the PSE through the coding region and termination signal, were used as the input. Only perfect sequence identity between the input sequences and the genome database were identified. The result showed that the zebrafish genome contained 555 copies of the U6-1 gene, which were distributed across different loci on various chromosomes (Table 1). The U6-2 and the U6-3 genes were found to be single copy genes. To examine whether these three putative snRNA genes express detectable mRNAs, RT-

PCR was conducted with cDNA preparations from zebrafish organs including eyes, brain, gill, liver, digestive tract, ovary, and testis. All three U6 snRNA genes were expressed in all tissues examined (Fig. 3), demonstrating that these three U6 snRNA genes are constitutively expressed.

Another U6 snRNA gene was found when performing the genomic BLASTn search which was designated as U6-4. The coding sequence of U6-4 has totally identity to that of U6-3. However, the sequence of the upstream flanking region was 76 % homology to that of U6-3. The putative promoter region of U6-4 contains the core regulation elements which were identified in the upstream elements of the isolated U6 snRNA genes (Fig.1). The U6-4 was located as only one copy in chromosome 9 (Table 1).

Transcription of shRNA driven by U6 putaive promoters

We conducted *in vitro* transcription experiments using zebrafish cell extracts to confirm the utility of the putative zebrafish U6 promoters for vector-based RNA interference. Three types of shRNA expression cassettes (pU6-1shRNA, pU6-2shRNA, and pU6-3shRNA) were constructed. Each expression cassette consisted of the putative U6 promoter, the shRNA sequences and the termination sequences (Fig. 4). All three U6 snRNA promoters were able to express the shRNA (Fig. 5) in cell extracts prepared from zebrafish embryos. Two transcript signals were observed from each putative promoter expression vector, which may be produced by variable termination sequences of 4Ts and 5Ts. In addition, *in vitro* transcription experiments were performed using cell extracts from other fish species, including catfish (*Clarias garienpinus*), common carp (*Cyprinus carpio*), and Nile tilapia (*Oreochromis niloticus*). Fig. 5 shows the expression of the shRNA driven by all three of the U6 putative promoters in Nile tilapia cell extracts. None of the three putative U6

promoters were transcribed in cell extracts prepared from catfish or common carp (data not shown). The transcription experiments were also conducted using cell extracts from two shrimp species (*Penaeus monodon* and *Litopenaeus vannamai*). We found that the shRNA transcription products from the U6-1 and U6-2 putative promoters showed weak signals in shrimp cell extracts, whereas the U6-3 putative promoter transcribed no detectable signal (data not shown).

DISCUSSION

Three different U6 snRNA genes (U6-1, U6-2, and U6-3) were isolated and characterized from zebrafish, including some of the sequences of the putative promoters and the RNA coding regions. The DNA sequences corresponding to the 5'-flanking regions of all of the U6 snRNA genes were divergent, except for a few highly conserved upstream sequences. The core promoter elements that regulated the U6 snRNA genes were identified according to previous reports (Krol *et al*., 1987; Das *et al*., 1988; Kunkel and Pederson, 1988; Kudo and Sutou, 2005; Lambeth *et al*., 2005). Based on their conserved position in all U6 genes, TATA boxes and the PSE were characterized. A distal sequence element (DSE), which is an octamer motif (ATTTGCAT), has been found in U6 snRNA genes of human, mouse, cattle, chicken and *Xenopus* (Krol *et al*., 1987; Das *et al*., 1988; Kunkel and Pederson, 1988; Kudo and Sutou, 2005; Lambeth *et al*., 2005). However, this octamer motif was not present in the *Drosophila* U6 snRNA gene (Das *et al*., 1987). In zebrafish, instead of the octamer motif, all three types of putative promoters have upstream sequences homologous to the "CCAAT box" motif, which acts as a functional enhancer in several gene promoters (Ach and Weiner, 1991; Park and Levine, 2000). The position of the "CCAAT box" (≈ -240) was conserved compared to the location of the octamer

motif that is generally positioned around -250 nucleotides relative to the transcription start site. Thus, the "CCAAT box" might be identified as the DSE in the zebrafish U6 snRNA genes. In summary, the 5'-flanking sequences of the U6 snRNA genes among zebrafish, *Xenopus*, chicken, mouse, human, and *Drosophila* are not significantly conserved, although the nucleotide sequences corresponding to the U6 snRNA coding region reveals a high degree of sequence conservation.

In general, the U6 gene sequences, including the upstream PSE and TATA boxes and the 3' end T-rich terminator, were used to successfully synthesize U6 snRNA coding sequence (Das *et al*., 1988; Goomer and Kunkel, 1992). Therefore, we used each of the 5'-flanking regions up to -79, together with the complete U6 snRNA coding region, as the input for the BLASTn analysis of the zebrafish genomic database. Whereas the U6-2 and U6-3 genes were each present as a single copy gene, multiple copies of the U6-1 gene were located at various loci distributed throughout the zebrafish genome. Similar findings of multigene families of the U6 snRNAs have been previously reported by Hiyashi (1981) and Domitrovich and Kunkel (2003).

Our sequencing data revealed that there are no additional types of U6 snRNA genes found in the products of nested PCR. However, the genomic BLASTn analysis showed another type of U6-4. The DNA sequences of U6-4 were taken from database. Comparison of the putative promoter regions showed that the 5'-flanking region of U6-4 contained essential upstream elements such as "CCAAT box", PSE and TATA box sequences. In addition, the coding region of U6-4 has totally identity to that of U6-3. Taken together, these data suggest that there are four U6 snRNA genes in the zebrafish genome.

In order to determine whether these three types of U6 snRNA genes were real genes or pseudogenes, we performed RT-PCR analysis with cDNA isolated from

various zebrafish tissues. The results showed that all three U6 snRNA coding sequences were expressed at detectable levels. Furthermore, these three genes were expressed in all tissues, suggesting these genes can be labeled as "house-keeping genes".

Previously, it was shown that U6 promoters that included the DSE could enhance transcription (Das *et al*., 1988). In humans, the DSE of the U6 snRNA gene contributes to the formation of preinitiation complexes (PIC) (Kunkel and Hixson, 1998). In this study, therefore, each shRNA expression cassette (pU6-1shRNA, pU6-2shRNA, or pU6-3shRNA) was generated to contain a "CCAAT box" in the 5'-flanking sequences. All three putative U6 promoters initiated expression of the cloned shRNA in cell extracts prepared from zebrafish. As demonstrated in Fig. 5, *in vitro* transcription reactions produced two transcripts, which most likely are the products that comprise 4 Ts or 5 Ts, revealing that both 4Ts and 5Ts are effective termination signals. Similar efficient termination of a T cluster is demonstrated in other known U6 snRNA genes (Krol *et al*., 1987; Das *et al*., 1988; Kunkel and Pederson, 1988; Kudo and Sutou, 2005; Lambeth *et al*., 2005).

We next sought to extend this finding to other fish species and shrimp cell extracts. The transcription efficiency from these putative U6 promoters in Nile tilapia cell extracts was similar to that from zebrafish cell extracts. However, these putative U6 promoters did not promote transcription in cell extracts prepared from catfish and common carp. The putative U6 promoters of the U6-1 and U6-2 genes promote very weak shRNA expression in shrimp cell extracts. Previous findings demonstrated variable transcription of the U6 snRNA genes across species. Das and colleagues (1987) reported that the *Drosophila* U6 snRNA genes could only be transcribed in *Drosophila* cell extracts and not in mammalian cell extracts, suggesting that the U6

snRNA genes promoted only species-specific transcription. Interestingly, the mouse U6 snRNA gene could be expressed in frog oocytes (Das *et al*., 1988). Moreover, commercially available human U6 promoter efficiently transcribed shRNA in chicken cell lines (Kudo and Sutou, 2005). The octamer motif was identified as the DSE in the U6 snRNA genes of *Xenopus*, chicken, mouse, and human, while this octamer motif was not found in the *Drosophila* U6 snRNA genes. Transcription efficiency across species may partially be explained by the fact that the core elements of the of the U6 snRNA genes promoters are homologous or nearly homologous across species.

In conclusion, our findings demonstrate that there are four different types of U6 snRNA genes. They are ubiquitously expressed throughout a variety of zebrafish tissues. One of the zebrafish U6 snRNA genes is present in multiple copies dispersed throughout the zebrafish genome. These U6 promoters could promote shRNA expression, suggesting that they have potential to be used for vector-based RNAi in zebrafish. A putative U6 promoter would provide a powerful tool for long-term GKD in zebrafish. Furthermore, these vector-based RNAi technologies might be applicable for some aquaculture-related species.

METHODS

Cloning of zebrafish U6 snRNA genes

The zebrafish U6 snRNA genes were cloned by nested PCR. Table 2 shows the sequence of the oligonucleotides used in this study. The putative promoter and partial U6 snRNA gene fragments were amplified using a zebrafish genomic DNA library as the template with λfix-F as the forward primer and reverse primers U6-r1 and U6-r2 as the primary and nested primers, respectively. The first PCR was carried out in a total

volume of 10 μl, consisting of 200 μM of each deoxynucleotide, 1 pmol of each primer (λfix-F and U6-r1), 1X EX Taq buffer and 0.25 U EX Taq (Takara Shuzo, Shiga, Japan). The PCR reaction was performed at 94°C for 3 min, then 35 reaction cycles were run, each consisting of 30 s at 94°C, 30 s at 58°C and 1 min at 72°C. The final elongation step was conducted at 72°C for 5 min. The resulting PCR product was used as the template in a second PCR reaction with λfix-F and U6-r2 as the primers. Another partial U6 snRNA gene fragment, including the termination signal, was amplified with U6-f1 and U6-f2 as the primary and nested forward primers and with the reverse primer λfix-R. DNA fragments were isolated using the Gelpure DNA Purification Kit (GeneMate, Kaysville, UT, USA). The PCR-amplified DNA fragments were cloned into pGEM T-Easy plasmid (Promega, Madison, WI, USA). DNA purification was performed using the FlexiPrep Kit (Amersham Pharmacia Biotech Ltd, Buckinghamshire, UK). All clones were sequenced using ALF express DNA Sequencer System with a Thermo Sequenase fluorescent labeled-primer cycle-sequencing kit and 7-deaza-dGTP (Amersham Pharmacia Biotech Ltd). A phylogenetic tree of the U6 snRNA coding region was generated using the neighbor-joining method (Saito and Nei, 1987). To locate the U6snRNA genes on the zebrafish chromosomes, the zebrafish genome resources (http:www.ncbi.nlm.nih.gov/genome) were used to perform a BLASTn search.

RT-PCR with zebrafish organs

To examine the expression of each U6-snRNA in various organs of the zebrafish, total RNA was isolated from tissue samples (brain, eyes, gill, liver, digestive tract, ovary and testis) using TRIZOL Reagent (Gibco BRL, Rockville, MD, USA) according to

the manufacturer's protocol and was treated with DNase I during RNA purification. First-strand cDNA was synthesized from 2 μg of total RNA using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech Ltd) with random hexadeoxynucleotides. PCR was conducted using ExTaq® (Takara) in 10 μl of the reaction solution. The conditions for PCR cycles were as follows: initial denaturation of 3 min at 94°C, followed by 35 cycles of 15 s at 94°C, 15 s at 58°C and 15 s at 72°C. The final elongation step was conducted at 72°C for 5 min. Three pairs of primers were designed to amplify the coding DNA of U6-1 (cU6-1f and cU6-1r), U6-2 (cU6-2f and cU6-2r) and U6-3 (cU6-3f and cU6-3r) (Table 2). The amplified products were separated by electrophoresis on a 15% nondenaturing polyacrylamide gel.

shRNA expression cassette construction

All U6 promoter-driven short-hairpin RNA (shRNA) vectors (pU6-shRNA) were constructed as follows. Plasmids containing the different types of the U6 putative promoters were cloned by nested PCR from zebrafish genomic DNA. Genomic DNA was extracted from the dorsal fin of zebrafish using Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol. PCR was performed using ExTaq® (Takara) at 94°C for 5 min, then 35 reaction cycles of were run each consisting of 45 s at 94°C, 45 s at 60°C and 45 s at 72°C. The final elongation step was conducted at 72°C for 5 min. For the first PCR reaction, three pairs of primers were designed to clone the putative promoters of U6-1 (pU6-1f and U6-r3), U6-2 (pU6-2f and U6-r3) and U6-3 (pU6-3f and U6-r3). The resulting PCR products of the putative promoter of U6-1, U6-2 and U6-3 were used as the template in the second PCR reaction with the specific primers (pU6-1f and pU6-1r), (pU6-2f

and pU6-2r) and (pU6-3f and pU6-3r), respectively. Because *Bam*H I and *Xba* I restriction sites were used in all expression cassettes to insert the DNA fragment encoding the test transcript, each nested reverse primer (pU6-1r, pU6-2r or pU6-3r) (Table 2) in the second PCR reaction was designed to add the restriction enzyme *Bam*H I and *Xba* I sequences downstream of the U6 putative promoter. Each PCR product was cloned into pGEM T-Easy plasmid (Promega) and verified by DNA sequencing. The plasmid containing the putative promoter of U6-1, U6-2 or U6-3 was designated as pU6-1, pU6-2 or pU6-3, respectively. Each plasmid was digested with *Bam* HI and *Xba* I, so two complementary oligonucleotides could be inserted.

Because we want to target the zebrafish golden phenotype (Lamason *et al*., 2005) in future studies, *slc*24a5-1 (GenBank accessions no. AY 538713) (nucleotide 385-405) was used to generate two oligonucleotides (ONs): slc-plus and slc-minus (Table 2). The two ONs were 5'-phosphorylated and hybridized in annealing buffer as described previously (Boonanuntanasarn *et al*., 2005). After annealing, the double-stranded DNA (dsDNA) fragment consisted of the 5'-*Bam*H I sticky end, the short hairpin RNA (shRNA) sense sequence, the 9-mer loop sequence, the shRNA antisense sequence, and the 5 attached thymidines at the 3'end, and the *Xba* I sticky end. The dsDNA was ligated into each of the *Bam* HI/*Xba* I digested pU6-1, pU6-2 and pU6-3 vectors to generate shRNA expression cassettes: pU6-1shRNA, pU6-2shRNA and pU6-3shRNA, respectively. All plasmid constructs were sequence-verified. The templates for *in vitro* transcription containing the shRNA driven by each U6 promoter (Fig. 4) were PCR-amplified with primers (pGEM-f and pGEM-r). Using pU6-1shRNA, pU6-2shRNA or pU6-3shRNA as a template, PCR was conducted using ExTaq® (Takara) at 94°C for 3 min, then 40 reaction cycles of were run each consisting of 45 s at 94°C, 45 s at 60°C and 45 s at 72°C. The final elongation step

was conducted at 72°C for 5 min. The amplified products were isolated using the QIA quick® Gel Extraction Kit (Qiagen, Hilden, Germany).

Preparation of fish cell extracts

In order to investigate the expression of the putative U6 snRNA promoter, fish cell extracts were prepared from zebrafish. We also tested the transcription activities of all of the zebrafish U6 putative promoters across species in cell extracts prepared from common carp (*Cyprinus carpio*), Nile tilapia (*Oreochromis niloticus*) and catfish (*Clarias garienpinus*) obtained from the Fisheries Farm, Suranaree University of Technology Farm (Nakhon Ratchasima, Thailand) and marine shrimps. In fish, cell extracts were prepared from fry during swim-up stages. Marine shrimp in post-larvae stages 10-15 were also used to prepare cell extract samples. First, fish or shrimp were anesthetized in 300 ppm of phenoxy ethanol. All cell extracts, which contained the enzyme RNA Pol III, were prepared following a modification of the procedure described by Weil and colleagues (1979). Briefly, the samples were cut into 5 mm-pieces in ice-cold PBS. After washing three times in PBS, the samples were incubated in twice the volume of ice-cold hypotonic solution (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol) for 10 min and homogenized for 5 strokes. One-tenth volume of hypotonic stop solution (0.3 M HEPES-KOH, pH 7.9, 1.4 M KCl, and 0.03 M $MgCl_2$) was added. The homogenate was centrifuged at 100,000 x g at 4^0C for 1 h. The supernatant was used for protein determination using a Total Protein Kit, Micro Lowry (Sigma, MO, USA). Extracts with a protein concentration of 5 mg/ml were stored at -80^0C until they were needed for *in vitro* transcription.

***in vitro* transcription and northern blot analysis**

Transcription reactions were carried out essentially according to Fan *et al*. (2005) with slight modifications. The 300-µl reaction mixture contained 900 µg protein of each fish embryo extract, 20 mM HEPES-KOH, pH 7.9, 80 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol and 1.5 mg of each template DNA. In order to allow for preinitiation complex formation (PIC), the transcription mixtures were incubated at 28^0C for 30 min. Transcription reactions were initiated by adding 250 µM of rNTP and allowed to proceed for 1.5 h at 28^0C. The reactions were stopped by adding one-tenth volume of stop solution (5 % SDS, 100 mM EDTA, 1 M sodium acetate, pH 4.8). Subsequent to the transcription reaction, the mixtures were treated with DNaseI (RNase-free), extracted with phenol/chloroform and precipitated with ethanol. The precipitates were resuspended in loading buffer (95% formamide, 18 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol, 0.025% SDS), heat-denatured and loaded on a 15% denaturing polyacrylamide gel with 8 M urea. Following electrophoresis, the gels were electroblotted onto Hybond^{TM}-N^+ membranes (Amersham). After baking at 80^0C for 2 h, the membranes were hybridized overnight with the 3'dioxigenin (DIG)-labelled synthetic oligodeoxynucleotde (Sigma- Proligo, Singapore) complementary to the antisense shRNA strand. The membranes were then washed and incubated with anti-digoxigenin-AP antibodies (Roche, Mannheim, Germany). The signals were detected with NBT/BCIP kit (Roche) according to the manufacturer's protocol.

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Table 1 Chromosomal locations of U6 snRNA loci

U6 Locus	Chromosome	Number of loci	Number of copies	strand
U6-1	1	1	2	Minus
	3	2	56	Plus
	4	2	121	Plus and
	5	2	60	Minus
	12	1	6	Plus and
	14	11	260	Minus
	18	1	3	Minus
	20	1	1	Plus and
	23	1	15	Minus
	Not placed	3	31	Plus and
				Minus
				Plus
				Plus
				Plus and
				Minus
U6-2	21	1	1	Minus
U6-3	11	1	1	Plus
U6-4	9	1	1	Minus

The four U6 loci are numbered randomly. "Number of Loci" refers to the number of U6 snRNA locations on each chromosome. "Number of copies" refers to the total copies of each U6 snRNA gene on each chromosome.

Table 2 Primers and oligonucleotides used in this study

Primer	Sequence
λFix-F	5’-GAGCTCTAATACGACTCACTATAGG-3’
λFix-R	5’-GAGCTCAATTAACCCTCACTAAAG-3’
U6-r1	5’-TATGGAACGCTTCACGAAT-3’
U6-r2	5’-TGCGTGTCATCCTTGCGCAG-3’
U6-r3	5’-TGCGCAGGGGCCATGCT-3’
U6-f1	5’-GTGCTTGCTTCGGCAGCACA-3’
U6-f2	5’-TGGAACGATACAGAGAAGAT-3’
cU6-1f	5’-GTGCTCGCTACGGTGGCACA-3’
cU6-2f	5’-GTGCTTGCTTCGGCAGCACG-3’
cU6-3f	5’-GTGCTTGCTTCGGCAGCACA-3’
cU6-1r	5’-AAAACAGCAATATGGAGCGC-3’
cU6-2r	5’-AAAATGAGGAACGCTTCACG-3’
cU6-3r	5’-AAAAGATGGAACGCTTCACG-3’
pU6-1f	5’-TCCATATTGCTGTTTTAGTGCGTGG-3’
pU6-2f	5’-TGGCTTCAAGTCTCTCAGCG-3’
pU6-3f	5’-TCCGAGAGTCTGTGAATGTT-3’
pU6-1r	5’-TCTAGACTCGAGGGATCCGTGGACAGGCTCAGGGC-3’
pU6-2r	5’-TCTAGACTCGAGGGATCCAGAGCTGGAGGGAGAGC-3’
pU6-3r	5’-TCTAGACTCGAGGGATCCGGAGCCTGGAGGACTGC-3’
slc-plus	5’-GATCCGGGCGACATCGGCGTCAGCTTCAAGAGAGCTGACGCCGA TGTCGCCCTTTTTT-3’
slc-minus	5’-CTAGAAAAAAGGGCGACATCGGCGTCAGCTCTCTTGAAGCTGACG CCGATGTCGCCCG-3’
pGEM-f	5’-TAATACGACTCACTATAGGGC-3’
pGEM-r	5’-GGAAACAGCTATGACCATGA-3’

FIGURE LEGENDS

FIG. 1. Sequences of four zebrafish U6 snRNA genes.

The four U6 snRNA genes were numbered randomly. Note that the sequence of the U6-4 was taken from zebrafish genomic database (NW001514080.1). CCAAT boxes are indicated by a black box. The white and the gray boxes highlight the predicted PSE and TATA box elements, respectively. The start site of transcription, G, is shown as position +1. A dashed box indicates an intragenic control region "box A". Termination signals are underlined.

FIG. 2. Phylogenetic tree of the U6 snRNA coding sequences.

The tree was constructed based on the neighbor-joining method (Saito and Nei, 1987) using the DNA sequences listed below. GenBank accession numbers for the U6 snRNA genes included in the analysis are human (X07425), mouse[1] (X06980), mouse[2] (M10329), *Xenopus* (M31687) and *Drosophila* (AH004871). Note that the coding sequence of U6-4 is 100 % homologous to that of U6-3.

FIG. 3. RT-PCR of the three U6 snRNA transcripts in various zebrafish tissues.

cDNAs were synthesized from total RNA extracted from various tissues including liver (1), ovary (2), eyes (3), brain (4), gill (5), digestive tracts (6) and testis (7). Distilled water was used as the template in the negative control (N). Note that all three of the U6 snRNAs are expressed in all tissues examined.

FIG. 4. Schematic illustrations of the putative zebrafish promoter-driven plasmid vector for shRNA. See Fig. 1 for the PSE sequences. The shRNA fragment consisted of shRNA sense,
a 9-mer loop, shRNA antisense and termination sequences. These cassettes were PCR-amplified and used as the template for *in vitro* transcription.

FIG. 5. Northern blot analysis of each of the putative zebrafish promoter-driven shRNAs in cell extracts prepared from zebrafish and Nile tilapia. The structures of the shRNA expression cassettes are shown in Fig 4. U6-1, U6-2 and U6-3 denote the transcripts produced from the templates, which were PCR-amplified from pU6-1shRNA, pU6-2shRNA and pU6-3shRNA, respectively. Note that two bands were observed in each transcription assay, showing that the transcripts consisted of both 4 Ts and 5 Ts.

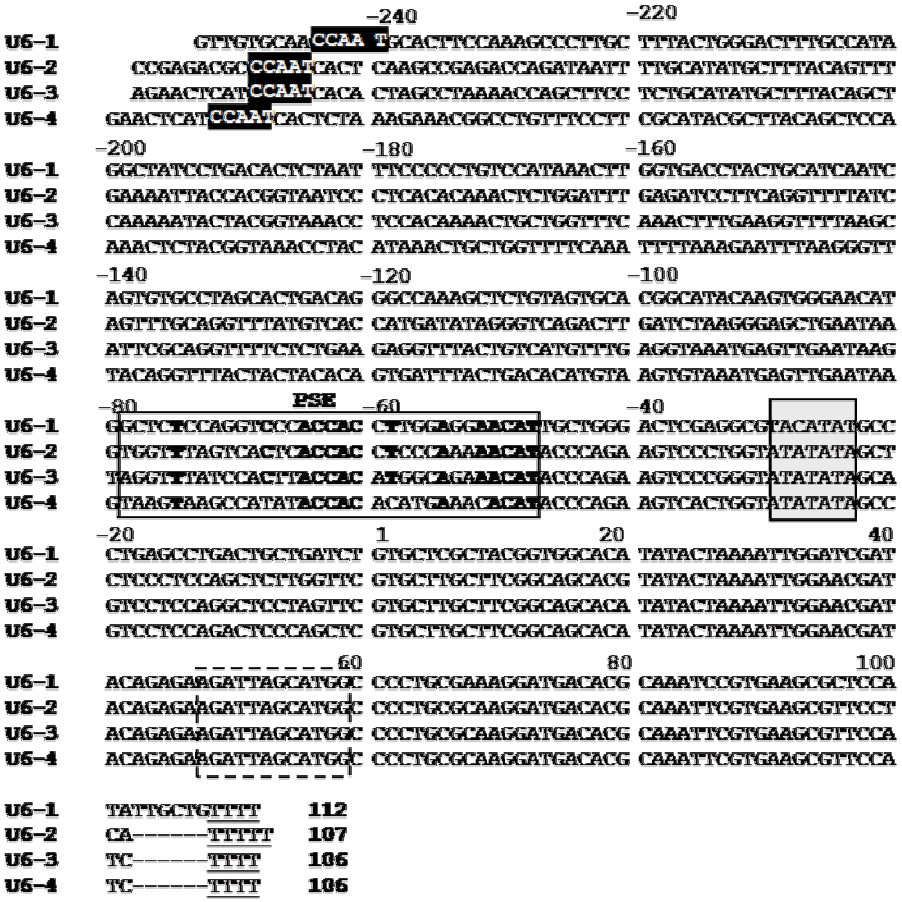

−240
−220
U6−1 GTTGTGCAACCAATGCACTTCCAAAGCCCTTGC TTTACTGGGACTTTGCCATA
U6−2 CCGAGACGCCCAATCACT CAAGCCGAGACCAGATAATT TTGCATATGCTTTACAGTTT
U6−3 AGAACTCATCCAATCACA CTAGCCTAAAACCAGCTTCC TCTGCATATGCTTTACAGCT
U6−4 GAACTCATCCAATCACTCTA AAGAAACGGCCTGTTTCCTT CGCATACGCTTACAGCTCCA
−200
−180
−160
U6−1 GGCTATCCTGACACTCTAAT TTCCCCCTGTCCATAAACTT GGTGACCTACTGCATCAATC
U6−2 GAAAATTACCACGGTAATCC CTCACACAAACTCTGGATTT GAGATCCTTCAGGTTTTATC
U6−3 CAAAAATACTACGGTAAACC TCCACAAAACTGCTGGTTTC AAACTTTGAAGGTTTTAAGC
U6−4 AAACTCTACGGTAAACCTAC ATAAACTGCTGGTTTTCAAA TTTTAAAGAATTTAAGGGTT
−140
−120
−100
U6−1 AGTGTGCCTAGCACTGACAG GGCCAAAGCTCTGTAGTGCA CGGCATACAAGTGGGAACAT
U6−2 AGTTTGCAGGTTTATGTCAC CATGATATAGGGTCAGACTT GATCTAAGGGAGCTGAATAA
U6−3 ATTCGCAGGTTTTCTCTGAA GAGGTTTACTGTCATGTTTG AGGTAAATGAGTTGAATAAG
U6−4 TACAGGTTTACTACTACACA GTGATTTACTGACACATGTA AGTGTAAATGAGTTGAATAA
PSE
−80
−60
−40
U6−1 GGCTCTCCAGGTGCCACCAC CTTGGAGGAACATTGCTGGG ACTCGAGGCGTACATATGCC
U6−2 GTGGTTTAGTCACTCACCAC CTCCCAAAAACATACCCAGA AGTCCCTGGTATATATAGCT
U6−3 TAGGTTTATCCACTTACCAC ATGGCAGAAACATACCCAGA AGTCCCGGGTATATATAGCA
U6−4 GTAAGTAAGCCATATACCAC ACATGAAACACATACCCAGA AGTCACTGGTATATATAGCC
−20
1
20
40
U6−1 CTGAGCCTGACTGCTGATCT GTGCTCGCTACGGTGGCACA TATACTAAAATTGGATCGAT
U6−2 CTCCCTCCAGCTCTTGGTTC GTGCTTGCTTCGGCAGCACG TATACTAAAATTGGAACGAT
U6−3 GTCCTCCAGGCTCCTAGTTC GTGCTTGCTTCGGCAGCACA TATACTAAAATTGGAACGAT
U6−4 GTCCTCCAGACTCCCAGCTC GTGCTTGCTTCGGCAGCACA TATACTAAAATTGGAACGAT
60
80
100
U6−1 ACAGAGAAGATTAGCATGGC CCCTGCGAAAGGATGACACG CAAATCCGTGAAGCGCTCCA
U6−2 ACAGAGAAGATTAGCATGGC CCCTGCGCAAGGATGACACG CAAATTCGTGAAGCGTTCCT
U6−3 ACAGAGAAGATTAGCATGGC CCCTGCGCAAGGATGACACG CAAATTCGTGAAGCGTTCCA
U6−4 ACAGAGAAGATTAGCATGGC CCCTGCGCAAGGATGACACG CAAATTCGTGAAGCGTTCCA
U6−1 TATTGCTGTTTT 112
U6−2 CA------TTTTT 107
U6−3 TC------TTTT 106
U6−4 TC------TTTT 106

Fig 1.

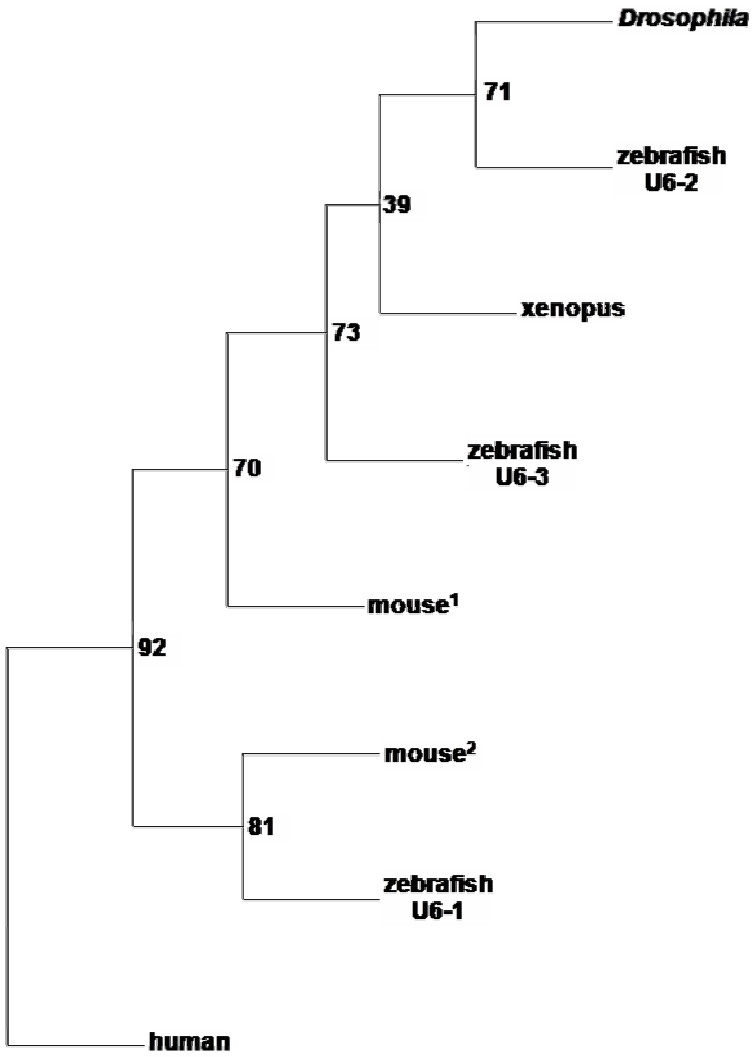

Drosophila
71
zebrafish
U6-2
39
xenopus
73
zebrafish
U6-3
70
mouse[1]
92
mouse[2]
81
zebrafish
U6-1
human

Fig 2.

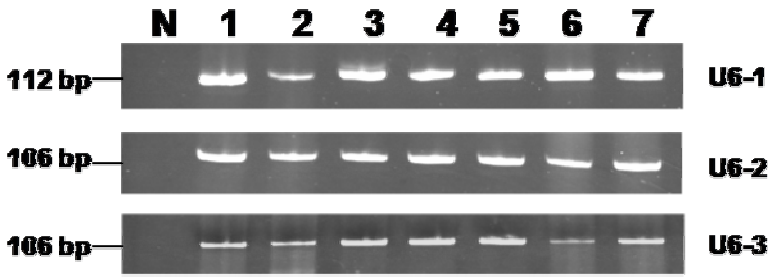
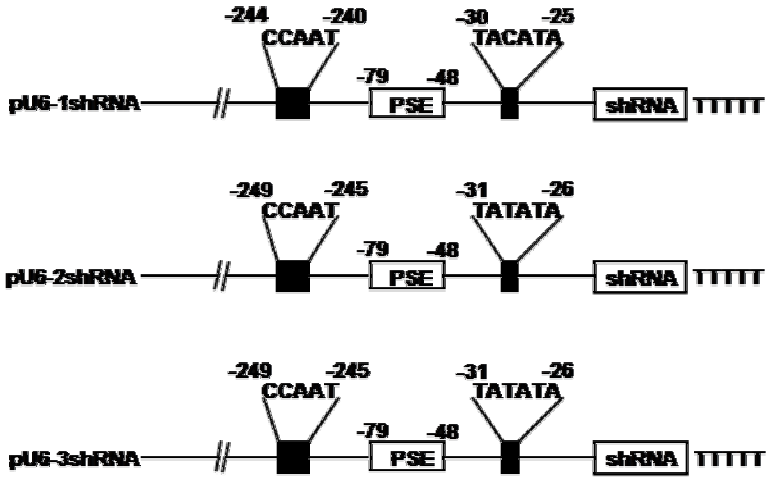


Fig 3.


-244
-240
CCAAT
-30
-25
TACATA
-79
-48
pU6-1shRNA
PSE
shRNA
TTTTT
-249
-245
CCAAT
-31
-26
TATATA
-79
-48
pU6-2shRNA
PSE
shRNA
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-245
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pU6-3shRNA
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shRNA
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Fig 4.

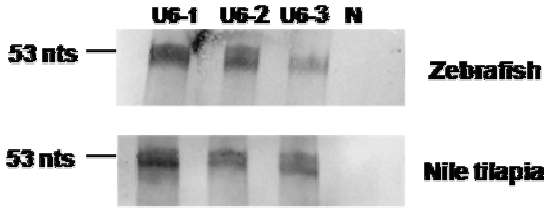

Fig 5.

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Gene Knockdown: A Powerful Tool for Gene Function Study in Fish

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Abstract

So far, there are a number of fish genome projects, including experimental and economically important fish that provide available DNA sequence information. However, the function of a gene cannot be deduced only by its DNA sequence. Therefore, a technique with which to investigate the function of the fish gene is needed. Gene knockdown (GKD), or antisense technology, is now being used as a powerful technique to study gene functions in living organisms. GKD effects result from the introduction of an antisense molecule into living cells. The antisense agents bind to target messenger RNA, thus inactivating the target gene expression. The appropriately spatial inhibitory effects on protein production from corresponding gene resulted in the phenotypic change. Therefore, the function of the gene can be understood. To date, there are a number of antisense molecules that can affect efficient GKD in fish. These include antisense oligonucleotides, small interfering RNA, and ribozyme. These antisense molecules cause specific gene inhibitor effects with different mechanisms. The various antisense mechanism types facilitate a number of GKD applications with various approaches in animals. In this review, we demonstrate the characteristics of each antisense molecule, its mechanism, and its application, especially for gene functional analysis in fish.

Recently, fish genome projects have been undertaken in experimental fish species, such as zebrafish, *Danio rerio*; medaka, *Oryzias latipes*; pufferfish, *Fugu rubripes* and *Tetraodon nugroviridis*; and stickleback, *Gasterosteus aculeatus*. In addition, aquaculture-related genome projects have been available for economically important species (http://www.animalgenome.org/aquaculture). Moreover, expression sequence tags (ESTs) databases have been providing comprehensive gene information from small pieces of complementary DNA (cDNA) sequences. ESTs have been studied in a number of fish and shrimp, which are useful for gene identification, expression, and full-length cDNA isolation. However, the functions of many of these genes cannot be deduced from their DNA sequences. These isolated genes cannot be used for any application if their functions are unclear. To complete our knowledge of genes, the development of efficient methods to investigate the role of genes is needed. Gene sequences information together with gene function studies would lead to a rapid advancement in biotechnological approaches for fish production.

Several efficient methods for the addition or inhibition of gene expression have been developed for studying gene functions. Production of transgenic fish offers a valuable means to study gene function because it permits the detection of phenotypes that have been altered by the addition of a function. The microinjected fish is typically mosaic and a so-called founder fish. Founder fish are bred individually to generate stable, independent lines of transgenic animals that generally exhibit variable expression characteristics. Thus, the transgenic approach is limited in that it is a rather time-consuming process to establish stable transgenics. Gene knockout is a technique for replacing wild-type alleles with mutant ones to produce heterozygous embryos with one to two mutant copies of a specific gene. Therefore, deleting gene products facilitates the comparison of phenotypes that have been changed by loss of function (Moreadith and Radford 1997; Lobe and Nagy 1998). However, creating generations of

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knockout animals that are necessary to study the role of such a gene *in vivo* is time-consuming, expensive, and labor intensive (reviewed in Stone and Vulchanova 2003). Gene knockdown (GKD) is an alternative technique that is now used for examining the function of a gene whose sequence is known. GKD or antisense technology is a technique to introduce an antisense agent that contains a complementary nucleotide (nt) sequence to target messenger RNA (mRNA) in living cells to inhibit target gene expression. Sequence alignment through nt base pairing of target mRNA antisense molecules leads to the specificity of inhibitory effects on gene expression. Consequently, the role of such a gene can be determined by phenotypic comparison. Therefore, GKD could mimic the loss of function method and be used to examine the function of a gene whose sequence is known. Although GKD may have some limitations, it seems to be the most suitable technique for fish because it requires no specialized facilities and temporary effects can be determined within a generation. The purpose of this review was to demonstrate GKD technology by using various antisense molecules, such as antisense oligonucleotides (AS-ONs), small interfering RNA (siRNA), and ribozyme. Recent studies using antisense agents are revealing, especially for fish. While various GKD approaches were reported to be efficient in mammalian cells, some limited use in fish has also been revealed. We propose to demonstrate that GKD can be a powerful technique to study gene function in fish and other applications, that is, to alter genetic traits of fish (Xie et al. 1997; Nasevicius and Ekker 2000) and to prevent viral diseases (Xie et al. 2005).

Antisense Oligonucleotides

The concept of AS-ONs was first demonstrated by simply using single-stranded DNA to arrest translation *in vivo* (Paterson et al. 1977). Because then, the GKD technology using AS-ONs has been rapidly developed as a powerful research tool with great potential for the study of gene functions. AS-ONs typically consist of 15–25 nts, which are designed in a complementary (antisense) orientation to their target (sense) mRNA (reviewed in Stone and Vulchanova 2003). Two major mechanisms contribute to their antisense effects when AS-ONs and target mRNA are hybridized in a strictly base pair (bp) specific. First, AS-ONs bind to the target mRNA and then are recognized by ribonuclease H (RNase H), thereby earning the name RNase-H-dependent antisense mechanism (Fig. 1A) (Galderisi et al. 1999a). RNase H is an enzyme that cleaves the RNA strand of a DNA–RNA duplex (Minshull and Hunt 1986). Second, AS-ONs that do not activate RNase H cleavage, but mediate gene silencing by only steric blockage of RNA (Fig. 1A) (Galderisi et al. 1999a; Summerton 1999). The mRNA–AS-ONs duplexes are thought to interfere with numerous RNA-processing steps. These are inhibition of 5′-capping, modulation of splicing, inhibition of 3′-polyadenylation, translational arrest, and disruption of critical RNA structure (Nasevicius and Ekker 2000; Karras et al. 2001; Vickers et al. 2001; Stone and Vulchanova 2003).

Native oligophosphodiester linkages have short half-lives in living cells because of the fact that cells contains a variety of exo- and endonuclease that can degrade native ONs (Wagner 1994). In zebrafish, the dechorinated eggs were incubated in native AS-ON with various concentrations from midblastula until 24-h postfertilization. Although these AS-ON treatments were dose-dependent, gene-interfering effects were limited before segmentation (Barabino et al. 1997). To date, many types of chemically modified AS-ONs have been produced to stabilize ONs against cellular nuclease, increase cellular uptake, enhance their target affinity to target mRNA, improve specific binding to target mRNA, and lower toxicity. In this review, we present only AS-ONs that have been widely reported for gene function analysis, especially in fish.

Phosphorothioate oligonucleotides (PS-ONs) are the main representatives of the first generation of AS-ONs that are best known and most widely used to date. The modification of ONs in which one of the nonbridging oxygen atoms in the phosphorodiester bond is replaced by sulphur to phosphorothioate bonds (Fig. 1B). This modification leads to an increased PS-ONs

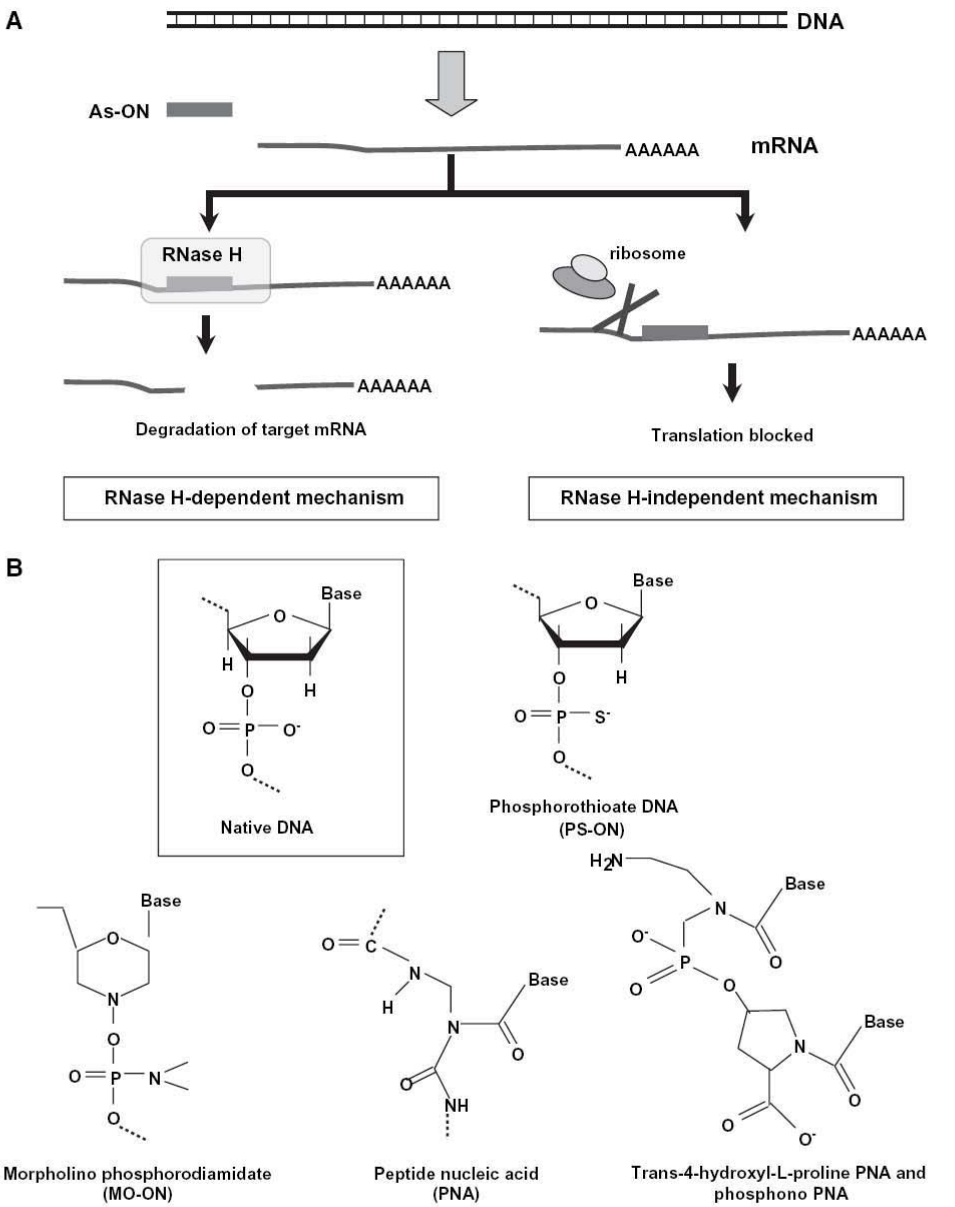

FIGURE 1. *Two mechanisms contributing gene knockdown effects by antisense oligonucleotide (AS-ON) (A). AS-ON binds to target messenger RNA (mRNA) and induces the degradation of target mRNA by ribonuclease H (RNase H). This mechanism is called RNAse-H-dependent mechanism. AS-ON hybridizes to the target mRNA and blocks the binding of ribosome to translate protein product. Because target mRNA is not degraded, this effect is called RNase-H-independent mechanism. Native oligonucleotide and various modified oligonucleotides that have been used in fish (B).*

resistance to cellular nuclease while preserving RNase-H-dependent properties (Galderisi et al. 1999a; Kurreck 2003; Stone and Vulchanova 2003). The main advantage of using PS-ONs for GKD studies is that the confirmation of GKD effects can be performed by evaluation of the amount of mRNA (Muller et al. 1998; Galderisi et al. 1999b). Theoretically, antisense RNase H cleavage would be effective against target sequences elsewhere in the target RNA transcript. Therefore, any gene segment that can be accessed by AS-ONs would have the potential to be an antisense site. The choice of target sequences should be determined with the intention of avoiding the secondary structure of target mRNA, which is identified in advance by a computer-based model of the structure (Zuker and Stiegler 1981). Nevertheless, many target sequences of PS-ONs, if not most, were designed near a translational start site (Galderisi et al. 1999b; Whitehead et al. 2003). PS-ONs also have several disadvantages. For instance, the cellular toxicity of using PS-ONs was caused by that PS-ONs bind to cellular proteins (unrelated target gene) (Brown et al. 1994) and growth factors (Guvakova et al. 1995). Another weakness in using PS-ONs is that the modification of the backbone of PS-ONs results in the reduction of the binding affinity between PS-ONs and target RNA, in comparison to their corresponding phosphodiester ONs (Stein et al. 1997). PS-ON (18-mers) was used to demonstrate memory function in gold fish, *Carassius auratus*. PS-ON-injected gold fish showed antisense effects on ependymin gene expression (Schmidt et al. 1995). In zebrafish, PS-ONs were also used to demonstrate gene function analysis for two hedgehog (*hh*) genes, sonic hedgehog (*shh*), and tiggy-winkle hedgehog (*twhh*). Three regions on two target mRNAs were chosen, such as regions near the N-terminus, the autoproteolytic site, and the C-terminus of each *hh* proteins. PS-ONs (20-mers) were designed to contain 55–80% guanosine and cytosine residues (GC) and verified to not have significant homology with any other known gene sequence. The cocktail of six PS-ONs was coinjected with lipofectamine into the embryo's head to obtain maximal GKD effects (Stenkamp et al. 2000). PS-ONs could be used for GKD research in fish if combined with an awareness of cellular toxicity and the need to carefully examine phenotype interpretation of gene function.

The second generation of AS-ONs has an alkyl modification at the 2′ position of the ribose sugar. The 2′-O-methyl and 2′-O-methoxyethyl RNAs are the most common. The major improvement was to reduce cellular toxicity. Additionally, these AS-ONs were developed to increase stability and nuclease resistance (Kurreck 2003). There are not many instances of the use of these AS-ONs in fish.

The third generation of AS-ONs has been developed to improve antisense properties (i.e., target affinity, nuclease resistance, and low toxicity to living cells). These DNA or RNA analogues are modified with phosphate linkages or riboses, as well as nts with a completely different chemical moiety replacing the furanose ring (Kurreck 2003). One group, morpholino phosphoroamidate oligonucleotides (MO-ONs), is widely used for GKD in fish. Additionally, there have been few reports that have shown the GKD effects in fish using peptide nucleic acids (PNA).

MO-ON has the riboside moiety of each subunit converted to a morpholine ring (morpholine = C_4H_9NO). It uses a phosphorodiamidate intersubunit linkage instead of phosphodiester linkages (Fig. 1B). MO-ONs have shown high stability in living cells, excellent solubility in any transfection or microinjection solution, low cellular toxicity, and even resistance to repeated freezing and thawing. However, MO-ONs are an antisense type that acts only by a steric block mechanism or RNase-H-independent property (Summerton 1999). In other words, MO-ONs only block the translation process. They are presumed to act by preventing binding of ribosomes. Therefore, for the study of gene functions, MO-ONs should be designed to be closely complementary to the start codon (5′ sequences at about the first 25 bases past the AUG translation start codon of an mRNA) (Summerton 1999). Gene-interfering effects using MO-ONs were first reported in zebrafish (Nasevicius and Ekker 2000). Because then, MO-ONs have been widely used for gene

function analysis in zebrafish in up to 300 publications. Significant GKD effects of MO-ONs were demonstrated during embryogenesis in transgenic rainbow trout, *Oncorhynchus mykiss* (Boonanuntanasarn et al. 2002). MO-ONs were also used to determine the function of two isozymes of tyrosinase in rainbow trout embryos (Boonanuntanasarn et al. 2004). In addition, a few studies on gene function were undertaken in medaka using MO-ONs (Candal et al. 2004; Yasutake et al. 2004; Yamamoto and Suzuki 2005). Further, the potential of MO-ONs for use as chemotherapeutic agents to inhibit the infectious hematopoietic necrosis virus in fish cell lines has been demonstrated. The MO-ON was conjugated with a membrane-penetrating peptide to deliver MO-ON across cellular membranes. This peptide-conjugated MO-ON was shown to inhibit virus replication (Alonso et al. 2005). In summary, MO-ONs offer potential use as antisense agents not only in experimental vertebrate but also in aquaculture-related fish species.

The limitation of MO-ONs for gene function studies has also been discussed. Because MO-ON elicits its effects solely by blocking translation, it reduces expression of the target protein without affecting mRNA levels (Boonanuntanasarn et al. 2002). Therefore, its antisense action needs to be confirmed at the protein level, using such methods as Western blotting or immunostaining. The limitation of these protein assays is that antibody is not always available. A strategy that was proposed to overcome this limitation is to block pre-mRNA splicing with MO-ON. Binding of an antisense MO-ON to one potential intron/exon junction could remove the pre-mRNA splicing step (Fig. 2). Consequently, the splice blocking reduced the amount of functional mRNA. GKD effects would be confirmed by evaluation of the amount of functional mRNA (Draper et al. 2001).

PNA is also attractive for gene function study in fish. The phosphodiester linkage of PNA is replaced with a polyamide or peptide (Hyrup and Nielsen 1996) (Fig. 1B). The mechanisms in which PNA acts as GKD agents are similar to that by which MO-ON does. PNA reveals excellent mismatch discrimination, nuclease resistance, and protease resistance but low water solubility. The negatively charged PNA, which is composed of trans-4-hydroxyl-L-proline nucleic acid monomers and phosphono PNA, exhibits higher water solubility. The antisense effects of negatively charged PNA were shown to be better than those of MO-ON in zebrafish. This negatively charged PNA (18-mers) exhibited a potency comparable to MO-ON (25-mers). In addition, the negatively charged PNA showed a stronger hybridization and a greater specificity to target mRNAs than did MO-ONs (Urtishak et al. 2003; Wickstrom et al. 2004; Duffy et al. 2005).

Small Interfering RNA

RNA interference (RNAi) describes a mechanism initiated by double-stranded RNA (dsRNA) that mediates the degradation of homologous mRNA. It is first observed in the nematode, *Caenorhabditis elegans*, that dsRNA was more effective at producing interference than individual antisense RNA strands (Fire et al. 1998). Later, dsRNA-mediated gene silencing was

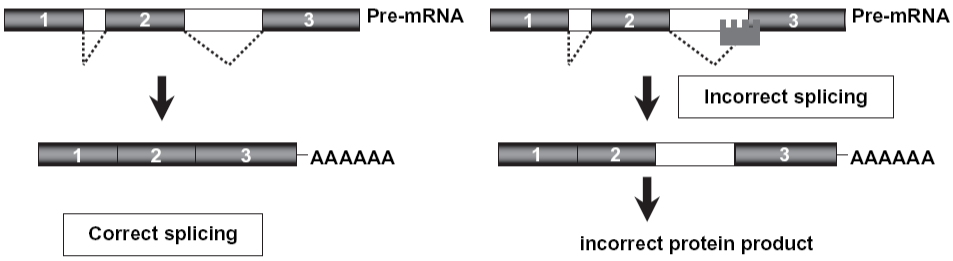

FIGURE 2. *Binding of antisense oligonucleotide at the splicing site leads to an incorrect splicing process and results in an incorrect translation product of the particular gene.*

intensively studied for its gene-interfering mechanism in many organisms and zebrafish (Wargelius et al. 1999). Functional genomic studies by long dsRNA can be undertaken in invertebrates. However, long dsRNA has been reported to cause nonspecific inhibition of gene expression in mammalian cells (Caplen et al. 2000). The mechanism of nonspecific inhibitory effects was explained as part of an antiviral response as many viruses generate dsRNA intermediates during their replication. These nonspecific effects have been attributed to the activation of dsRNA-dependent protein kinase R (PKR) and 2′-5′ oligoadenylate synthetase by dsRNAs longer than 30 bp (Fig. 3). Activated PKR inhibits general translation by phosphorylation of the translation factor eukaryotic initiation factor 2α (IF2α), leading to a generalized suppression of protein synthesis. Another dsRNA-response pathway involves the process in which 2′-5′-oligoadenylate synthetase causes a nonspecific reduction of mRNA (Minks et al. 1980; Clemens and Elia 1997; Tian and Mathews 2001). As a result, the activation of either, or both, of these pathways results in a nonspecific downregulation in gene expression. Recently, identification and characterization of PKR-like eukaryotic IF2α and IF2α have been investigated in their immunity responses to virus from zebrafish and rainbow trout, demonstrating that the immunity role of fish is conserved between vertebrates (Garner et al. 2003; Rothenburg et al. 2005). Thus, long dsRNA would not be able to cause specific GKD in fish. Indeed, the nonspecific inhibition of gene expression by long dsRNA in zebrafish was also reported (Oates et al. 2000; Zhao et al. 2001).

It has been revealed that the RNAi mechanism in vertebrates is related to the mobilization of transposon and a general defense against virus infection (Tijsterman et al. 2002). The dsRNA-mediated specific gene degradation is explained in two main steps (Fig. 3). First, long dsRNAs are recognized by an adenosine triphosphate (ATP)-dependent ribonuclease from the RNase III family, Dicer. Dicer cleaves the dsRNA into siRNAs of 19–25 nt containing 5′-phosphate and 3′-hydroxyl termini and 3′ overhangs of 2–3 nt. Second, these siRNAs are unwound in an ATP-dependent process and incorporated into a multicomponent nuclease complex, identified in *Drosophila* as the RNA-induced silencing complex (RISC). The siRNAs then guide RISC in the sequence-specific degradation of mRNA in the middle of the target region. The cleaved target RNA may be further degraded by a cellular exonuclease activity. Consequently, translation of the target gene is prevented. Thus, siRNA-mediated gene silencing is so-called posttranscriptional gene silencing (Yang et al. 2000; Zamore et al. 2000). This siRNA–RISC-mediated cleavage of target mRNA is sequence specific. Therefore, it is interesting to investigate the potential uses of siRNA in various strategies. Chemically synthesized duplexes of 21–23 siRNAs were used to examine their specific gene-interfering effects in *Drosophila*, *Cae. elegans*, and mammalian cell lines (Elbashir et al. 2001). siRNAs could bypass the nonspecific effects induced by long dsRNAs and enter the second steps of the RNAi mechanism. In agricultural biotechnology, the use of siRNA for GKD studies in chicken, pig, and cattle has been demonstrated (Karlas et al. 2004; Dai et al. 2005; Paradis et al. 2005).

In fish, the siRNA-mediated gene-inactivating technique was first demonstrated in rainbow trout embryos. Using transgenic fish carrying the green fluorescent protein (GFP) reporter gene, siRNA was able to interfere with transient and stable transgene expression during trout embryonic development. siRNA could also inhibit the expression of maternally inherited mRNA. In addition to transgenic fish, the silencing effect of siRNA was observed in endogenous tyrosinase gene expression (Boonanuntanasarn et al. 2003). In zebrafish, siRNA was shown to target the dystrophin gene, indicating that siRNA had the potential to assist in the investigation of the roles of multiple protein products expressed by a single gene. The results of siRNA-injected zebrafish embryos were similar to the use of MO-ON-targeting pre-mRNA (Dodd et al. 2004). siRNA was shown to have a potent antiviral purpose in a fish cell line, fathead minnow (FHM) cells (muscle cells of FHM). siRNA effectively inhibited the replication of an iridovirus (tiger frog virus) that causes severe disease in fish (Xie et al.

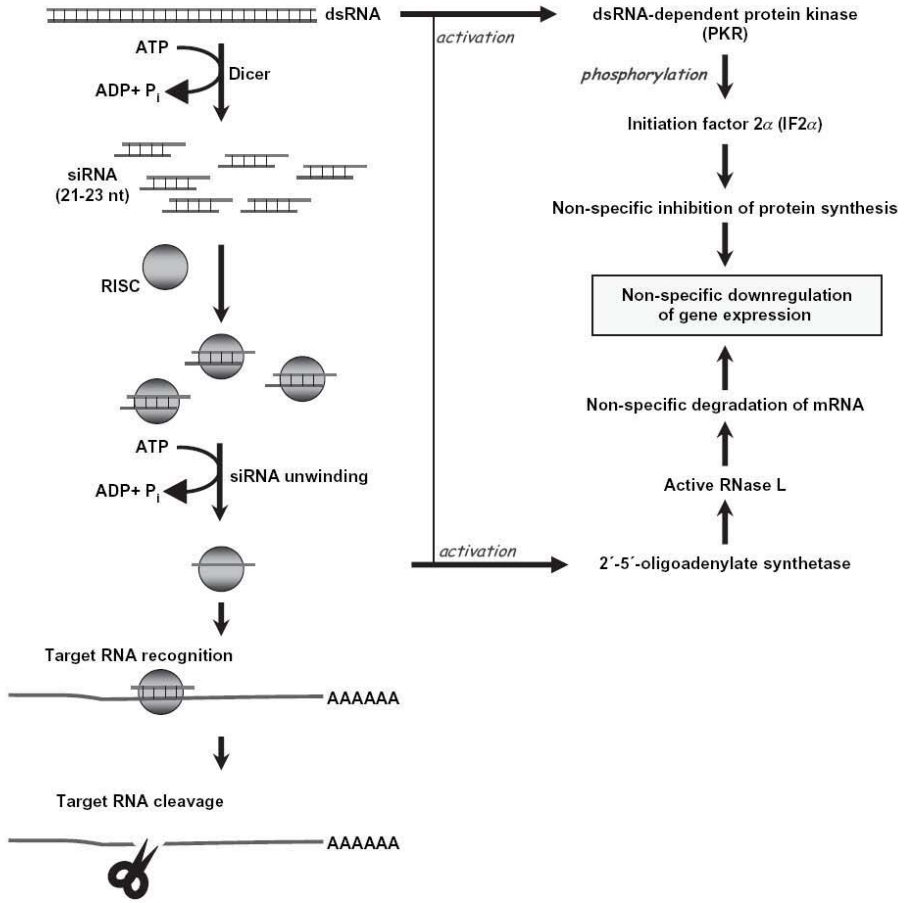

FIGURE 3. *Mechanism of RNA interference. The dicer cleaves the double-stranded RNA (dsRNA) substrate into a number of small pieces of RNA, called small interfering RNA (siRNA). RISC denotes RNA-induced silencing complexes. siRNA is recognized and unwound by RISC. Thereafter, siRNA guides RISC in the sequence-specific degradation of the target mRNA. Long dsRNA causes nonspecific inhibition of gene expression by activation of the two pathways. dsRNA activates protein kinase R (PKR) and 2′–5′-oligoadenylate synthease. A general suppression of protein synthesis is caused by PKR-inhibited phosphorylation of the translation initiation factor 2α (IF2α). Nonspecific mRNA degradation resulted from activation of RNase L by 2′–5′-oligoadenylate synthetase.*

2005). The efficient GKD effects of using chemically synthesized siRNAs were reported in fish up to 30 publications. The effects in fish of GKD from siRNA prepared by other sources have also been examined. siRNAs were recently prepared by *in vitro* digestion of long dsRNA using *Escherichia coli* RNase III, which is called esiRNA. esiRNA was able to efficiently interfere with gene expression in mouse embryos (Calegari et al. 2002). However, this approach was not found in zebrafish. esiRNA alleviated nonspecific defects and toxicity to zebrafish embryos. The explanation for these nonspecific effects was that these esiRNAs contained siRNA sequences that might be homologous with other gene expression (Liu et al. 2005). In addition, *in vitro* transcription of two strands of RNA by commercial RNA polymerase enzymes have been investigated to determine whether this can be an efficient technique. siRNA synthesized by

in vitro transcription with SP6 RNA polymerase was able to produce specific siRNA interference effects in zebrafish (Liu et al. 2005) and in fish cell lines (Xie et al. 2005).

Although it was demonstrated that MO-ON is an excellent GKD agent in many fish species, a siRNA-mediated GKD technique may have some advantages. First, GKD effects can be achieved by using siRNA against a wide range of target mRNA strands, whereas MO-ONs should empirically target the region around the initiation codon. The use of MO-ONs is therefore limited to instances in which the location of the initiation codon of a gene has still not been determined. Recently, a number of genes from aquaculture-related species have been explored by ESTs analysis. ESTs provide information of the DNA sequence of small pieces of cDNA (usually 200–500 nt) that are expressed in target tissue under induced conditions. In cases in which there are many potential genes that are probably involved in any particular role, siRNA would be useful for preliminary screening of gene functions if the characterization of those cDNA is not yet complete. Second, the confirmation of MO-ONs-mediated GKD requires the protein level to be analyzed. This is complicated in some cases depending on the method to measure the protein. In contrast, siRNA-mediated gene inactivation causes a reduction of the level of target mRNA. The confirmation of the GKD effects by siRNA can be evaluated by measuring the target mRNA level, which is simpler.

In *Cae. elegans*, RNAi-mediated effects are stable and passed on to the progenies because the nematode contains RNA-dependent RNA polymerases that amplify siRNAs (Fire et al. 1998). In contrast, siRNA-mediated gene-inactivating effects in mammalian cells are transient. The effects of siRNA in mammalian embryos (including those of fish) are dependent on the amount of siRNAs delivered. These become progressively diluted as cells divide and are digested by cellular nuclease. As a result, a transgene vector for stable expression of siRNAs in vertebrate embryos would be applied for a long-term GKD technology in aquaculture-related species in combination with a transgenesis technique. In zebrafish, the transgene vector that contain express small hairpin RNAs (shRNAs) driven by the mouse U6 promoter was also observed to cause silencing effects in fish cell lines. However, the effects were less effective than those from the use of chemically synthesized siRNAs (Xie et al. 2005). Therefore, to improve stable GKD in fish, an expression vector that contains promoter, which can express the large amount of shRNA for fish cell, is needed.

Ribozymes

Naturally existing ribozyme has been found in plants, bacteria, and vertebrates (reviewed in Tanner 1999). Ribozyme (R_z) or catalytic RNA is an RNA molecule that specifically possesses catalytic RNA cleavage activity (McKee and McKee 1999). Catalytic RNAs are categorized into two types according to their size and reaction mechanisms. First, there are three types of large R_z consisting of RNase P and group I and group II introns. These molecules range in size from a few hundred nts to around 3000 nts. The catalytic reactions generate products with 3′-hydroxyls and 5′-phosphate groups. To date, the RNase P gene has been identified and has proved to be actively transcribed in zebrafish embryos (Eder et al. 1996). Nevertheless, there is no evidence in studies to demonstrate gene-targeting study by RNase P. Second, the small R_zs include the hammerhead, the hairpin, hepatitis delta, and Varkud Satellite (VS) RNA. These R_zs range in size from about 35 nts to around 155 nts. The cleavage reactions posses 5′-hydroxyls and a 2′,3′-cyclic phosphate groups (Forster and Symons 1987; Tanner 1999). Among them, the hammerhead R_z is the most extensively used for GKD in fish, although it has not been found to be naturally existing in fish.

The hammerhead R_z is the smallest of the naturally occurring R_zs that have been identified to date. Hammerhead R_z consists of at least three components of RNA helices. A model of the minimum structural requirements for R_z-catalyzed RNA cleavage is shown in Fig. 4. The cleavage reactions presumably result from hybridization of two substrate recognition

arms (catalytic arms) to target RNA substrate governed by complementary bases. This brings the reactive group (the conserved secondary structure maintained in R_z forms between two catalytic arms) close to the substrate RNA and then mediates specific cleavage of the target RNA at a conserved GUC target. In general, cleavage occurs after an NUH triplet, where N is any nt, and H is A, U, or C. The rate of cleavage is highest if the triplet is GUC, but other triplets will work nearly as well. Normally, the hybridizing arms of 6–8 bps are considered optimal (Haseloff and Gerlach 1988). Therefore, R_z is theoretically able to target any RNA molecule by altering the sequences of the hybridizing arms and retaining the conserved reactive sequences.

Although the principle of R_zs-mediated gene inactivating is simple, actual inactivation *in vivo* can be more complex. Because animal cells contain a variety of proteins, as well as intracellular mRNA, which usually form secondary structures, R_z might not be able to access it to cleave the target mRNA. The identification of potential cleavage sites on the target RNA is a key step toward achieving the most effective GKD. Several attempts have been made to achieve the gene silencing by R_z. For instance, R_z-accessible site selection is traditionally based on the prediction of the secondary structure of the target mRNA generated by a computer model (Zuker and Stiegler 1981). Nevertheless, the predicted structure of target mRNA is often inaccurate because of the interaction of mRNA with other cellular proteins. Xie et al. (1997) achieved three potential target sites on the target *no tail* (*ntl*) gene from zebrafish by computer software analysis. The three R_zs were designed to target against regions surrounding the GUC. However, only one R_z could show effective GKD in zebrafish embryos. Recently, a constitutive transport element (CTE), which is a retroviral nt, has been reported to facilitate the nucleocytoplasmic transport of mRNA. In addition, the CTE was capable of binding to RNA helicase A (Tang et al. 1997). The interaction of RNA helicase with CTE is attributed to the unwinding of the secondary structure of target mRNA (Warashina et al. 2001). The chimeric R_z comprising the hammerhead R_z and CTE was demonstrated to enhance the efficiency of GKD in trout embryos (Boonanuntanasarn et al. 2005). However, similar levels of GKD activity by hairpin R_z, with or without CTE, were demonstrated in the inhibition effects on Chronic Hepatitis C virus replicon RNA expression in human hepatoma cell line (Jarczak et al. 2005).

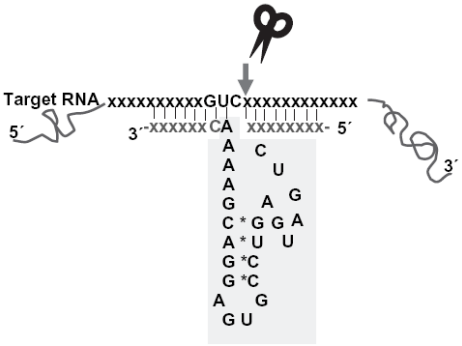

FIGURE 4. *Model of minimal structure of hammerhead ribozyme (R_z). Three structures of R_z consist of conserved GUC sequence in the RNA substrate, conserved sequences maintained in the R_z (grey box), and substrate recognition arms or catalytic arms (grey characters). The site of cleavage occurs immediately adjacent to the GUC-conserved sequences.*

The efficacy of R_z-mediated gene silencing also depends on the amount of stable R_z present in the same subcellular compartment. The R_z structure was incapable of export between the nucleus and the cytoplasm (Eckner et al. 1991). Maximum GKD effects will be achieved if R_z presents in both nucleus and cytoplasm. In addition, the cytoplasmic compartmentalization of R_z and target RNA was shown to be significant for R_z activity for viral therapy (Bertrand et al. 1997). To develop long-term GKD for R_z, it is necessary to have an expression vector that includes elements that express at high level of stable structure of small RNA molecules and that contributes to ("facilitates") the transportation of R_z between nucleus and cytoplasm. In general, the transcript resulting from RNA polymerase II promoter is capped and contains polyadenylated RNAs at the 3′ end. These

RNA polymerase II promoted transcripts are able to transport R_z into the cytoplasm. However, it expresses at low levels. In contrast, the RNA polymerase III system (i.e., transfer RNA [tRNA], U6 RNA, and adenovirus) naturally directs small RNA molecules at high level of expression. Thus, the RNA polymerase III system is more attractive to direct high-level expression of R_z in animal models. However, these promoter transcripts are limited mostly to the nucleus (Bertrand et al. 1997). In zebrafish, the GKD effects were carried out to target *ntl* mRNA in both the nucleus and the cytoplasm by using a dual promoter system to control R_z expression. This dual promoter system contains a strong T7 promoter that drives the cytoplasmic R_z expression and a human adenovirus type 2-associated VA_I (VA) gene promoter, which is an RNA polymerase III promoter that drives the nuclear R_z expression. This T7 cytoplasmic expression relies on the codelivery of vector DNA containing T7 RNA polymerase gene driven by the T7 promoter (pT7T7) and T7 RNA polymerase enzyme (Chen et al. 1994). This dual promoter system showed pronounced gene-silencing effects. However, no interfering effect was observed when fish were coinjected without the pT7T7 and T7 RNA polymerase, although there was expression of VA gene promoter. This suggests that most R_z-mediated gene-silencing effects occur in the cytoplasm (Xie et al. 1997). In an attempt to develop a suitable RNA pol III system, human $tRNA^{Val}$, which is an internal promoter and express small RNA molecules at a constitutive levels, were used. The human $tRNA^{Val}$ was fused at the 5′ end of chimeric R_z that contains hammerhead R_z and CTE. The chimeric R_z–CTE construct driven by human $tRNA^{Val}$ promoter showed GKD efficiency in trout embryos (Boonanuntanasarn et al. 2005). The $tRNA^{Val}$ promoter will transcribe the chimeric R_z at constitutive levels. The CTE facilitates the shuttles of chimeric R_z between the nucleus and the cytoplasm and probably attributed the accessibility of R_z to target mRNA in cytoplasm. This chimeric R_z expression strategy seemed to fulfill all criteria that are necessary for R_z-mediated GKD.

Conclusions

As a result of the discovery of antisense effects using diverse antisense agents, many applications of GKD have been rapidly developed in a variety of fish cell lines, experimental fish, and aquaculture-related fish. For obvious purpose, GKD has potential for studying gene function of many known genes. Further, GKD technology constitutes an important application to the biotechnological tools available for fish production. For example, sterile fish may be produced by interfering with a key gene for germ cell development. GKD of myostatin gene expression could be applicable for generation of increased muscle mass of fish. Viral GKD provides effective therapy for a number of viral diseases not only in commercially valuable fish but also in shrimp.

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