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High-efficiency gene knockdown using chimeric ribozymes in fish embryos ☆

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Abstract

We report an effective gene knockdown technique in rainbow trout embryos using additional RNA components combined with ribozymes (R_zs). Chimeric R_zs (tR_zCs) containing $tRNA^{Val}$, R_z against GFP, and a constitutive transport element were microinjected into transgenic embryos. tR_zCs induced greater gene interference than R_zs alone. Control tR_zCs did not affect unpaired bases of target RNA, and the tR_zC did not interfere with non-relevant gene expression, suggesting that the tR_zC-mediated gene-interference effects were sequence-specific. Furthermore, the tR_zC-containing expression vector specifically suppressed target GFP expression in transgenic trout. tR_zCs enhance R_z cleavage and could therefore be powerful tools for studying unknown gene function in vertebrates.

Keywords: Constitutive transport element; Gene knockdown; Rainbow trout; Ribozyme; Transgenic fish; tRNA

Ribozymes (R_zs) are RNA molecules that possess specific catalytic RNA-cleavage activity for their target RNAs [1]. Hammerhead R_zs are the smallest and probably the most extensively used R_zs for gene-inactivation studies [2]. The hammerhead motif comprises 35–40 nucleotides (nts) that can cleave target mRNA through specific base pairing with the substrate [2,3]. Hammerhead R_zs suppress the expression of target genes, a phenomenon known as gene knockdown (GKD). R_zs are therefore useful tools for a range of applications, including treatments for viral infections [3], gene therapy [4], cancer therapeutics [5], and functional studies of particular genes [6]. Although the principle of R_z-mediated gene inactivation is relatively simple, several obstacles hinder the in vivo efficacy of this technique. GKD activity within the intracellular environment generally depends on the highly folded structure of the target mRNA, the presence of a sufficient concentration of the R_z, and the appropriate intracellular co-localization of the R_z and its target mRNA [7].

Cellular mRNAs tend to form a tight secondary structure, so the target cleavage sites can sometimes be inaccessible to R_zs. The identification of potential cleavage sites on the target RNA is therefore crucial for effective GKD. The selection of R_z binding sites has traditionally been based on the secondary structure of the target mRNA as predicted by computer simulations [8]. However, the estimated structures are often inaccurate because they fail to account for interactions between mRNAs and other cellular proteins. For example, in a previous study, only one of three potential cleavage sites predicted using computer modeling was found to produce effective GKD in zebrafish [9]. Several of the methods that have been developed to identify accessible R_z target sites utilize oligodeoxynucleotides (ODNs) in combination with RNaseH cleavage of the RNA in an

☆ *Abbreviations:* bp, base pairs; CTE, constitutive transport element; dpf, days post-fertilization; dsDNA, double-stranded DNA; EST, expressed sequence tag; GFP, green fluorescent protein; GKD, gene knockdown; nt, nucleotide; ODN, oligodeoxyribonucleotide; R_z, ribozyme; T7, T7 RNA polymerase.

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RNA–DNA hybrid [10] and the expression of an R_z library [11]. The requirement for either a trial-and-error experiment or a complicated search for accessible R_z target sites has limited the use of R_zs for GKD in most vertebrates, with the exception of mice.

Recently, a constitutive transport element (CTE) derived from the type D retrovirus was reported to bind to RNA helicase A [12]. The chimeric RNA motif, consisting of R_z and CTE, could shuttle from the nucleus to the cytoplasm and cleave the mRNA substrate irrespective of hidden sites. Furthermore, an expression vector using the RNA polymerase III promoter induced high levels of expression of chimeric R_zs and achieved specific GKD in mammalian cells [13]. However, the GKD effect of chimeric R_zs has yet to be confirmed in vertebrate embryos. Fish, especially the rainbow trout (*Oncorhynchus mykiss*), are useful models of the vertebrate embryo, as they produce large numbers of eggs and effective rearing systems have been established. Furthermore, the expression patterns of numerous genes have been characterized in rainbow trout and related species using expressed sequence tags (ESTs) [14–16].

Generally, several GKD methods, including chemically modified antisense oligonucleotides [17,18] and small-interfering RNAs [19], are needed to elucidate the function of an unknown gene. R_z-mediated GKD using the cytoplasmic T7 RNA polymerase (T7)-expression system has been demonstrated in zebrafish. However, this method is complicated, because it requires co-microinjection of the T7 enzyme, and some of the potential cleavage sites are inaccessible to R_zs [9]. In the current study, we examined the efficacy of chimeric R_z–CTE motif-mediated GKD in transgenic rainbow trout embryos carrying the green fluorescent protein (*GFP*) gene.

Materials and methods

Construction and preparation of R_zs and chimeric R_zs. Two GUC sites at nucleotides (nt) 49–51 and 280–282 were selected as the target sites for R_z1 and R_z2, respectively. Two types of R_z were used as controls: one in which the substrate-recognition arms contained four base-mismatches (R_zM) and another in which they contained randomly scrambled sequences (R_zSc). The structures of the R_z, the $tRNA^{Val}$ [20], and R_z (tR_z) construct, the R_z and CTE [13] (R_zC) construct, and the $tRNA^{Val}$, R_z, and CTE (tR_zC) construct are shown in Fig. 1. The ODNs were obtained from the Espec Oligo Service (Ibaraki, Japan) and their sequences are shown in Table 1. The R_zs and chimeric R_zs were synthesized using T7 (Promega, Madison, WI). The templates for in vitro transcription were prepared as described below.

To produce each R_z (Fig. 1A), the sense ODN and its complementary strand were designed to contain T7 and R_z sequences for R_z1 ($T7R_z1$), R_zM ($T7R_zM$), and R_zSc ($T7R_zSc$). Double-stranded DNA (dsDNA) was hybridized in annealing buffer containing 20 mM Tris–HCl (pH 7.5) and 10 mM $MgCl_2$. The reaction mixture was heated at 85 °C for 5 min and then gradually cooled to room temperature. The resulting dsDNAs were used as templates for in vitro transcription.

The plasmids containing T7, $tRNA^{Val}$, each specific R_z, and the CTE sequences were designated as $pT7tR_zCs$ and were generated as follows. First, the plasmids containing T7, $tRNA^{Val}$, each specific R_z, and *Hin*dIII/*Xba*I sites ($pT7tR_z$ series) were constructed. dsDNA fragments for each R_z were then synthesized using a polymerase chain reaction (PCR) with two overlapping ODNs (T7tRNA-f and R_z1-r, R_zM-r, R_zSc-r or R_z2-r). PCR (1 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 68 °C, and 20 s at 72 °C) was performed in a total volume of 10 μl, consisting of 200 μM of each deoxynucleotide, 1 pmol of each primer, 1× *Ex Taq* buffer, and 0.25 U *Ex Taq* (Takara Shuzo, Shiga, Japan). The resulting PCR products, which had a length of 164 base pairs (bp), were isolated using the Gelpure DNA Purification Kit (GeneMate, Kaysville, UT), cloned into the pGEM T-Easy plasmid (Promega), and verified by DNA sequencing. Next, plasmids containing CTE and *Hin*dIII/*Xba*I sites were constructed. dsDNA encoding the CTE (196 bp) was generated using PCR with two overlapping ODNs (CTE-f and CTE-r) under the conditions described above, followed by ligation and DNA sequencing. In order to produce the $pT7tR_zCs$, a cleaved CTE fragment with *Hin*dIII/*Xba*I was ligated downstream into the $pT7tR_z$ series.

The template for in vitro transcription was prepared as follows. Using each $pT7tR_zC$ as a template, tR_z (Fig. 1B), R_zC (Fig. 1C), and tR_zC (Fig. 1D) were synthesized using PCR (1 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 68 °C, and 30 s at 72 °C) with each pair of primers: T7tRNA-f1 and R_z1-r, R_zM-r or R_zSc-r for tR_z; $T7R_z1$, $T7R_zM$ or $T7R_zSc$ and CTE-r1 for R_zC; and T7tRNA-f1 and CTE-r1 for tR_zC. Each PCR product was isolated using the Gelpure DNA Purification Kit (GeneMate) and was subsequently prepared for in vitro transcription. After transcription, the R_zs and chimeric R_zs were treated with RNase-free DNase I for 15 min and purified with phenol/chloroform.

Expression vectors (Fig. 1E) were constructed containing each R_z and CTE driven by human $tRNA^{Val}$ promoter sequences [21]. Using each $pT7tR_zC$ as a template, PCR was performed with a pair of primers (tRNA-pro and CTE-r1) under the conditions described above. Each PCR

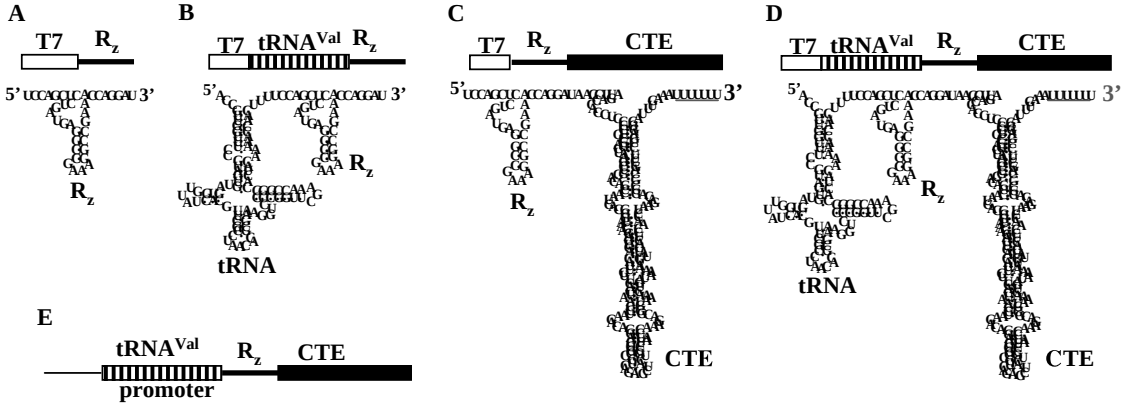

Fig. 1. Structures of R_z and chimeric R_z. (A–D) Upper: dsDNA templates for in vitro transcription. Lower: schematic structure of RNA. (A) R_z. (B) $tRNA^{Val}$ and R_z (tR_z). (C) R_z and CTE (R_zC). (D) $tRNA^{Val}$, R_z, and CTE (tR_zC). (E) Design of the expression cassette for tR_zC (ptR_zC). See Table 1 for the primer sequences.

Table 1
ODNs used for construction of the R_zs and chimeric R_z

Name	Sequence	Primer order
T7R_z1	5′-**TAATACGACTCACTATAGGG** TCCAGCTC CTGATGAGGCCGAAAGGCCGAA ACCAGGAT -3′	Sense
T7R_zM	5′-**TAATACGACTCACTATAGGG** TCGAGGTC CTGATGAGGCCGAAAGGCCGAA ACGAGCAT -3′	Sense
T7R_zSc	5′-**TAATACGACTCACTATAGGG** CGCGATAT CTGATGAGGCCGAAAGGCCGAA ACTCGACC -3′	Sense
T7tRNA-f	5′-**TAATACGACTCACTATAGGG**ACCGTTGGTTTCCGTAGTGTAGTGGTTATCACGTTCGCCTAACACGCGAAAG GTCCCCGGTTCGAAACCGGGCACTACAAAAACCAACTTT-3′	Sense
R_z1-r	5′-tctagaGTCAaagctt ATCCTGGT TTCGGCCTTTCGGCCTCATCAG GAGCTGGA AAAGTTGGTTTTTGTAGTGCCC-3′	Antisense
R_zM-r	5′-tctagaGTCAaagctt ATGCTCGT TTCGGCCTTTCGGCCTCATCAG GACCTCGA AAAGTTGGTTTTTGTAGTGCCC-3′	Antisense
R_zSc-r	5′-tctagaGTCAaagctt GGTCGAGT TTCGGCCTTTCGGCCTCATCAG ATATCGCG AAAGTTGGTTTTTGTAGTGCCC-3′	Antisense
R_z2-r	5′-tctagaGTCAaagctt GGCTACGT TTCGGCCTTTCGGCCTCATCAG CAGGAGCG AAAGTTGGTTTTTGTAGTGCCC-3′	Antisense
CTE-f	5′-TaagcttAGACCACCTCCCCTGCGAGCTAAGCTGGACAGCCAATGACGGGTAAGAGAGTGACATTGTTCACTAA CCTAAGACAGGAGGGCCGTCAGAGCTACTGCCTAATCCA AAGACGG-3′	Sense
CTE-r	5′-tctaga*AAAAAAA*TTTCAAATCCCTCGGAAGCTGCGCCTGTCTTAGGTTGGAGTGATACATTTTTATCACTTTTAC CCGTCTTTGGATTAGGCAGTAGCTC- 3′	Antisense
TRNA-pro	5′-TAGGACTAGTCTTTTAGGTCAAAAAGAAGAAGCTTTGTAACCGTTGGTTTCCGTAGTGTAGTG-3′	Sense
T7tRNA-f1	5′-**TAATACGACTCACTATAGGG**ACCGTTGGTTTCC-3′	Sense
CTE-r1	5′-tctaga*AAAAAAA*TTTCAAATCCCTCGGAAGCTGCGCC-3′	Antisense

Bold characters indicate T7. The white boxes show the substrate-recognition arms. The black boxes indicate mismatched bases. Underlining indicates overlapping sequences. The restriction enzyme sequences are shown as lowercase letters. Italic letters denote termination signals.

product was cloned into the pGEM T-Easy plasmid (Promega) and analyzed by DNA sequencing.

Fish and microinjections. Rainbow trout were reared at 10 °C at Tokyo University of Marine Science and Technology, Oizumi Research and Training Station (Yamanashi, Japan). The transgene used in this study contained *GFP* cDNA driven by the medaka β-actin promoter (pAG) [22]. Gamete collection and microinjection were performed 2–7 h post-fertilization, as described previously [17]. The transient transgene expression system in rainbow trout has previously been reported as a suitable model for investigating GKD [17,19]. During the early developmental stages, introduced DNA can exist extrachromosomally and is transiently expressed at extremely high levels after the mid-blastula transition. Transient transgenic embryos were produced by the microinjection of 50 pg pAG into wild-type embryos. In order to evaluate their effects on transient transgene expression, a 2-nl sample containing 5 ng R_z or chimeric R_z was microinjected with or without pAG in the blastodiscs of one-cell-stage embryos. Stable transgenic embryos were generated and the F_3 generation was used to study the long-term GKD effects. Each tR$_z$C expression vector (ptR$_z$C) was microinjected into F_3 transgenic embryos at the one-cell stage.

LacZ gene co-injection. In order to test whether the tR$_z$1C interfered with non-relevant gene expression, wild-type fertilized eggs were co-injected with 50 pg of plasmid containing the *LacZ* gene driven by the medaka β-actin promoter (pAZ) [23] with or without tR$_z$1C. The β-galactosidase activity in the blastoderm of 3-days post-fertilization (dpf) embryos was visualized by in situ staining, as described by Takagi et al. [23]. In addition, a β-galactosidase enzyme assay was carried out using the β-galactosidase Enzyme Assay System Kit according to the manufacturer's instructions (Promega).

RNA and protein isolation and quantification. Total RNA was extracted from pooled samples of three 15-dpf embryos. The RNA extraction was performed using the Trizol reagent (Gibco-BRL, Rockville, MD), according to the manufacturer's protocol. First-strand cDNA was synthesized from 2 μg of total RNA using Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Buckinghamshire, UK) with an oligo(dT) primer. Real-time reverse transcription (RT)-PCR was used to measure quantitatively the level of intact *GFP* target mRNA. The sequences of the sense and antisense primers of the *GFP* gene were 5′-GTGGTGCCCATCCTGGTCG-3′ and 5′-AGCTTGCCGTAGGTGGCAT-3′, respectively. SYBR green RT-PCR amplification was performed using an iCycler Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions, under the conditions described previously [19].

Protein extraction and Western blot analysis using antibodies against GFP and skeletal muscle protein were performed as described elsewhere [17].

Results

The R_zs and chimeric R_zs combined with tRNA^{Val} and/or CTE (5 ng/embryo) were compared by co-injection with 50 pg pAG, to evaluate their interference effects on transient transgene expression (Fig. 2). When R_z1 and tR$_z$1 (Figs. 1A and B) were co-injected with pAG, the percentages of embryos strongly expressing *GFP* were similar to that in the control that received pAG alone. By contrast, co-injection with R_z1C (Fig. 1C) significantly reduced the percentage of embryos showing strong *GFP* expression. Moreover, co-injection with tR$_z$1C (Fig. 1D) caused the most significant reduction in the percentage of embryos strongly expressing *GFP*. The strong *GFP* expression induced by pAG and its suppression by tR$_z$1C are shown in Figs. 3A and B, respectively. tR$_z$2C, which was designed to target the second GUC site, also reduced the number of embryos strongly expressing *GFP* when it was co-injected with pAG (Fig. 2). In order to confirm the specificity of

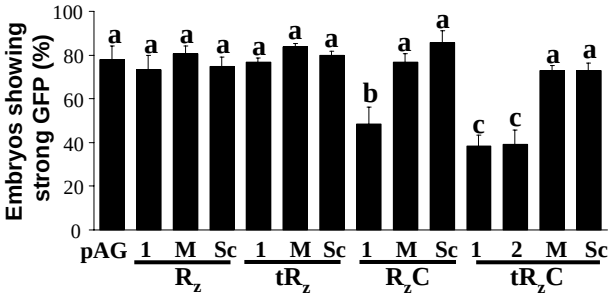

Fig. 2. Inhibition of transient GFP expression in 3-dpf embryos injected with R_z and chimeric R_z. Each bar represents the percentage of embryos showing strong green fluorescence (Fig. 3A). pAG denotes transgenic embryos receiving only pAG. The names of the tR$_z$Cs are detailed in Fig. 1 (1, R_z1; M, R_zM; Sc, R_zSc; 2, R_z2). The results are shown as means ± standard error (SE) of at least three independent experiments. A minimum of 20 embryos were used for each replicate. The letters denote statistically significant differences at $p < 0.01$.

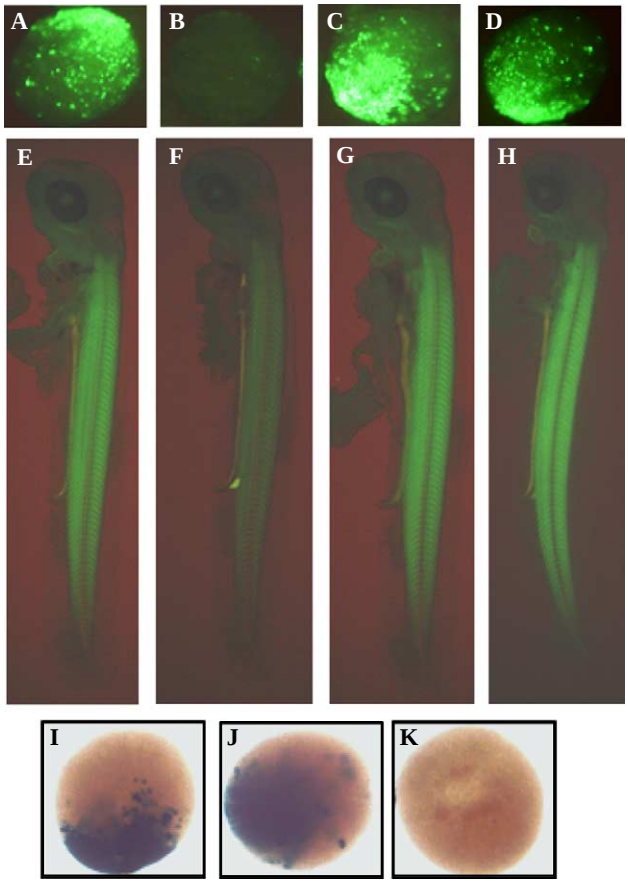

Fig. 3. Effects of tR_zC on targeted and non-targeted gene expression. (A) Strong *GFP* expression was observed in the blastoderm of 3-dpf embryos receiving pAG. (B) Co-injection with tR_z1C inhibited *GFP* expression. (C,D) Co-injection with control chimeric tR_zMC and tR_zScC, respectively, did not affect *GFP* expression. (E) Strong *GFP* expression was observed in F_3 transgenic embryos at 20 dpf. (F) GKD effects induced by expressing vector ptR_z1C. (G,H) No GKD effects were caused by expressing vectors ptR_zMC and ptR_zScC, respectively. Note that tR_zC interfered with target *GFP* expression while the control tR_zC had no such effect. (I) *LacZ* gene expression was observed in the blastoderm of 3-dpf embryos receiving pAZ. (J) tR_z1C did not affect non-targeted *LacZ* gene expression. (K) An uninjected embryo.

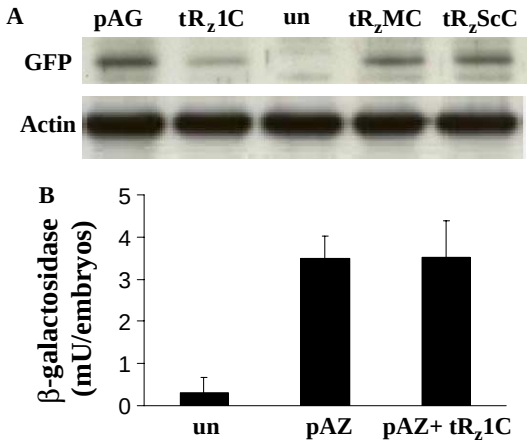

Fig. 4. tR_zC-mediated interference with the protein products of targeted and non-targeted genes in 3-dpf embryos. (A) Western blot analysis of GFP (top) and skeletal muscle actin (bottom). pAG denotes transgenic embryos carrying pAG. tR_z1C, tR_zMC, and tR_zScC denote transgenic embryos receiving tR_z1C, tR_zMC, and tR_zScC, respectively. un, uninjected wild-type embryos. Protein lysate equivalent to one (GFP) or one-third (actin) of a 3-dpf embryo was loaded onto each lane. (B) β-Galactosidase assay of embryos receiving pAZ alone and those receiving pAZ with tR_z1C. Note that tR_z1C decreased the target GFP protein, but did not affect the non-target actin and LacZ proteins.

these effects, we co-injected R_zM or chimeric R_zM (tR_zM, R_zMC or tR_zMC) with pAG. The results showed that *GFP* gene expression was not suppressed by any of these constructs (Figs. 2 and 3C). Similarly, neither R_zSc nor the chimeric R_zSc constructs (tR_zSc, R_zScC or tR_zScC) influenced *GFP* expression (Figs. 2 and 3D). Western blot analysis revealed that the GFP protein levels of embryos that were co-injected with pAG and tR_z1C were much lower than those of embryos receiving pAG alone (Fig. 4A). By contrast, the GFP protein signals in the tR_zMC- and tR_zScC-injected embryos were similar in intensity to those of the pAG-injected embryos.

To determine whether tR_zC interfered with the expression of non-relevant genes, we used Western blot analysis to evaluate the amounts of actin protein that were present. The results revealed no noticeable differences in the actin protein levels among the embryos receiving pAG with or without each tR_zC (Fig. 4A). In addition, the co-injection of tR_z1C with pAZ was performed in order to evaluate whether tR_z1C interfered with the expression of non-relevant *LacZ*. In situ staining and a β-galactosidase assay revealed that the pAZ-injected embryos and those co-injected with pAZ and tR_z1C showed similar patterns of *LacZ* gene expression (Figs. 3I–K and 4B, respectively).

Stable expression of the *GFP* transgene was used as a model to confirm the effects of long-term GKD caused by ptR_zC. The transcription of ptR_z1C, ptR_zMC, and ptR_zScC expression vectors microinjected into transgenic eggs was confirmed in 15-dpf embryos by RT-PCR (data not shown). A significant reduction in the percentage of embryos showing strong *GFP* expression was observed in those that received ptR_z1C (Fig. 5A). By contrast, ptR_zMC and ptR_zScC did not affect the number of embryos strongly expressing *GFP*. The patterns of gene interference caused by ptR_z1C, but not by ptR_zMC or ptR_zScC, compared with the uninjected transgenic embryos (control) are shown in Figs. 3E–H. The level of target *GFP* mRNA was measured using real-time RT-PCR in 15-dpf embryos (Fig. 5B). The GFP mRNA level in the ptR_z1C-injected embryos was significantly lower than that in the control. However, the GFP mRNA levels of ptR_zMC- and ptR_zScC-injected embryos did not significantly differ from that of the control. Western blot analysis, which was used to quantify the amounts of GFP and actin protein (Fig. 5C), revealed weak or absent GFP protein signals in ptR_z1C-injected embryos compared with the controls. By contrast, the GFP protein signals of the control uninjected embryos and those injected with ptR_zMC or ptR_zScC were similar in strength. There were no significant differences in the actin protein signals among the groups.

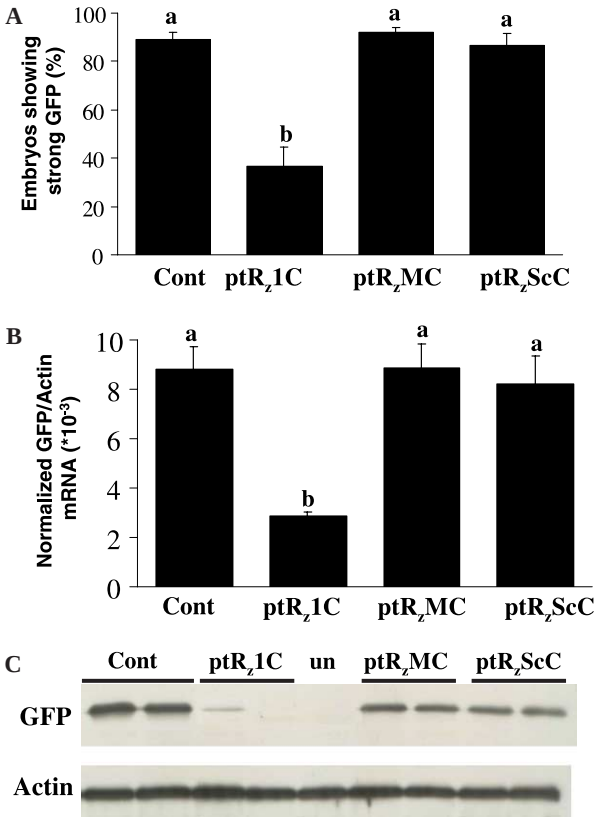

Fig. 5. Interference effects on the stable *GFP* transgene in 15-dpf embryos. Cont denotes F_3 transgenic embryos without injection. ptR$_z$1C, ptR$_z$MC, and ptR$_z$ScC denote F_3 transgenic embryos injected with ptR$_z$1C, ptR$_z$MC, and ptR$_z$ScC (each at 100 pg/embryo), respectively. (A) The percentage of embryos showing strong *GFP* expression was reduced when they received the ptR$_z$1C, but not ptR$_z$MC or ptR$_z$ScC, expression vector. The values are means ± SE from four independent experiments. At least 20 embryos were used in each replicate. (B) *GFP* mRNA levels in transgenic embryos receiving the tR$_z$C expression vector. Real-time RT-PCR was performed to measure the intact *GFP* mRNA level. The amount of *GFP* mRNA was normalized to the actin mRNA level. The values are means ± SE from at least three pooled embryos after duplicate PCR analysis. Values with different letters are statistically significant at $p < 0.01$. (C) Western blot analysis of GFP (top) and actin (bottom). Protein lysate equivalent to one-third (GFP) or one-ninth (actin) of an embryo was loaded onto each lane.

Discussion

In this study, we have described gene interference mediated by in vitro-synthesized chimeric R$_z$ transcripts and transgene-expressing chimeric R$_z$, using transient and stable transgenic GFP trout models, respectively. Our results showed that R$_z$C containing both R$_z$ and CTE efficiently interfered with target gene expression. Additionally, tR$_z$Cs consisting of tRNA^{Val}, R$_z$, and CTE produced the strongest interference, indicating that these components function synergistically to enhance the GKD effects. The suppression of target gene expression was induced during late embryogenesis by the introduction of the ptR$_z$1C construct, which expressed tR$_z$1C.

We conducted two further experiments to confirm the specificity of the GKD effects. The first investigated two control tR$_z$Cs, in which the substrate-recognition arms were substituted for four point-mismatched or randomly scrambled sequences. These control tR$_z$Cs had no significant effects on gene expression. The second experiment showed that tR$_z$1C did not inhibit the expression of two non-relevant genes: actin and *LacZ*. Taken together, these findings suggest that GKD mediated by tR$_z$C has great potential for the study of gene function in rainbow trout embryos and might also be applicable to other teleosts.

In rainbow trout, the transient transgene expression system has been reported as a suitable model for studying GKD [17,19]. In this study, rainbow trout embryos were reared at 10 °C, and naked RNA chimeric R$_z$s (5 ng/embryo) could cause significantly GKD effects. This successful GKD might be partly due to this low rearing temperature which will increase the stability of foreign RNAs.

Our results demonstrated that, while R$_z$1 did not suppress target gene expression, tR$_z$1C or tR$_z$2C, which included tRNA^{Val}, R$_z$1 or R$_z$2 (a catalytic domain), and CTE (a decoy domain), reduced this activity. As mentioned earlier, in a previous study, only one of the three R$_z$s predicted using a computer simulation showed effective GKD [9]. Gene interference was not achieved in this case, probably due to the inaccessibility of the target cleavage sites to the R$_z$s resulting from the complex structure of the mRNA substrate. The CTE was reported to bind to RNA helicase A proteins [12]. Thus, the chimeric motif could capture the proteins and unwind the intricate structure of the RNA substrate. Consequently, the tR$_z$C would be able to access the target cleavage site regardless of the structural complexity of the substrate. In addition, the CTE could shuttle between the nucleus and the cytoplasm [12]. This ability might contribute to the co-localization of the target mRNA and R$_z$. In addition, human tRNA^{Val} functions as a RNA polymerase III promoter system that expresses small RNAs at a relatively high level [20]. Furthermore, tRNA^{Val} facilitates the transport of tR$_z$C to the cytoplasm in order to obtain maximal GKD effects [20,24]. In fact, the adenovirus VA I promoter was used previously to express the R$_z$ for GKD, although this activity occurred only in the nucleus and was not transferred to the cytoplasm [9,25]. The use of the tRNA^{Val} promoter is preferable to the pol III promoter, because it facilitates the intracellular co-localization of the R$_z$ and substrate RNA.

Several other factors have been suggested to have important effects on R$_z$-mediated GKD, including the presence of an adequate amount of R$_z$, intracellular stability, co-localization of the R$_z$ and its target mRNA, and accessibility of the target cleavage site [7]. The current study demonstrated that tR$_z$C-mediated gene interference fulfilled all of these criteria. Indeed, chimeric R$_z$–CTE was reported to significantly enhance R$_z$ activity in cultured cells [13]. The present study is, to our knowledge, the first report of chimeric R$_z$ bound to CTE acting as a powerful tool for achieving GKD in vertebrate embryos.

The tR$_z$C-mediated gene-inactivation system described here has several advantages for use in studies of gene func-

tion, especially in the case of unknown genes. The CTE eases the unwinding of complex mRNA [12] and so GKD effects can be achieved by the selection of target cleavage sites that contain NUH (where N is any nt and H is A, C or T) [2,3] regardless of the in vivo complex mRNA substrate. This is of great benefit for the knockdown of unknown genes when only parts of the target sequences are available. Moreover, the tR_zCs interfere with the reduction of mRNA, so the GKD effects can be evaluated by measuring the target mRNA levels. Thus, this methodology is useful even when antibodies against the protein product of the target genes are not available. Our results confirmed that human $tRNA^{Val}$ is an effective promoter and revealed synergistic gene-inactivating effects in trout embryos. These findings suggest that this approach could be used to establish long-term GKD strategies using transgenic fish expressing tR_zCs.

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