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Characterization of Zebrafish Polymerase III Promoters for the Expression of Short-Hairpin RNA Interference Molecules

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Abstract

RNA interference (RNAi) is a powerful, sequence specific, and long-lasting method of gene knockdown, and can be elicited by the expression of short-hairpin RNA (shRNA) molecules driven via polymerase III type 3 promoters from a DNA vector or transgene. To further develop RNAi as a tool in zebrafish, we have characterized the zebrafish U6 and H1 snRNA promoters and compared the efficiency of each of the promoters to express an shRNA and silence a reporter gene, relative to previously characterized U6 promoters from pufferfish, chicken, and mouse. Our results show that the zebrafish polymerase III promoters were capable of effective gene silencing in the zebrafish ZF4 cell line, but were ineffective in mammalian Vero cells. In contrast, mouse and chicken promoters were active in Vero but not ZF4 cells, highlighting the importance of homologous promoters to achieve effective silencing.

Introduction

RNA interference (RNAi) based knockdown techniques are increasingly being used to knockdown or "silence" genes in a wide range of species.[1–5] Despite the amenability of the zebrafish to transgenic modification using various techniques and methods,[6,7] there has been a relative paucity of RNAi based technologies in this model organism. The use of anti-sense morpholino oligomers,[8] and the inability to consistently and reproducibly knock-down genes in the zebrafish using RNAi in either cell culture[9,10] or by microinjection of RNAi inducing molecules into the zebrafish embryo[9,11–15] has contributed to this paucity in the literature. However, recent studies *in vitro* have shown consistent, specific, and robust knockdown of exogenous viral mRNA in a variety of fish cell lines.[16–22] This has sparked renewed interest in RNAi technologies in zebrafish, as transgene-based RNAi constructs could potentially offer long-term heritable knock-down of gene targets as has been previously described in mice[23–25] and chickens.[26]

During the preparation of this manuscript, a further study into RNAi in zebrafish was published[27] in *Zebrafish*, expressing an shRNA from a pri-microRNA intron inside a CFP expression vector. The authors observed significant knockdown of *wnt5b* and *zDisc1* following generation of transient transgenic zebrafish at 24 hours and by using orthologous mRNA to rescue the original phenotype have provided the best evidence to date for specificity of action of RNAi in zebrafish.[27]

RNA interference (RNAi) is a conserved, sequence-specific, post-transcriptional silencing mechanism dependent on 19–21 nucleotide (nt) RNA duplexes described as small-interfering RNAs (siRNAs).[28,29] DNA vectors transcribing short hairpin RNAi precursor molecules (shRNAs) have been used to demonstrate specific RNAi silencing, in numerous cell lines[1,3,5,10,20,30] and animals[24–26] with greater efficiency and long-term effects than siRNAs. Furthermore, DNA-delivered RNAi allow the use promoters that are tissue specific,[31,32] inducible,[33] or modified for a particular application. Short-hairpin RNA molecules are transcribed from inverted complementary sequences separated by a loop sequence of between 4–25 nt.[1,34] Following transcription the RNA spontaneously folds into hairpin structures that are cleaved by the enzymes DICER and Drosha/DRG8 into mature siRNAs.[1,28,35]

Small-nuclear RNA (snRNA) polymerase III type 3 promoters such as U6, H1, or 7SK[1,4,5,22,36] are frequently used to express shRNAs from DNA vectors. These promoters have the advantages of a defined termination sequence of 4–5 thymidine bases and, unlike polymerase III type 1 and 2 promoters, the promoter sequence elements are contained entirely upstream of the transcription start sites.[37–39] U6

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promoters are frequently mentioned in the literature as they robustly express high levels of shRNAs. This level of expression may, however, induce cytotoxic effects, and weaker promoters that express less shRNA molecules such as the H1 or modified U6 promoters may prove to be a better option particularly *in vivo*.[13,40]

Zebrafish U6 promoters have previously been shown to express snRNA genes and shRNA transcripts in cell culture.[10,15,41] Until now, the relative strengths of the promoters and the efficacy of knock-down have not been reported. This information has proven important in other species in avoiding or reducing cytotoxic effects resulting from the relatively high levels of expression of shRNAs, particularly from the stronger promoters.[13,23,36] High levels of shRNA and siRNA molecules have been shown to saturate elements of the microRNA processing pathway, with Exportin-5, RISC, and Argonaute 2 identified as particularly susceptible to saturation in mice[40] and zebrafish models.[13,42] In the zebrafish, a significant dose-dependent reduction in mature native microRNAs was observed following transfection of zebrafish embryos with siRNAs.[13,42] This observation provides some insight into the nonspecific effects observed in many of the trials of RNAi based technologies attempted in fish, including zebrafish.[11,12,30,43]

The H1 promoter transcribes a small nuclear RNA, ribonuclease P (RNase P) which post-transcriptionally cleaves tRNAs to generate mature 5′ terminals.[36,44,45] The H1 promoter is a pol III type 3 promoter and consists of a distal sequence element (DSE) comprised of an octamer motif (OCT) and a STAF binding site, a proximal sequence element (PSE), and finally a TATA motif.[36,44] The H1 promoter is generally considered a weaker promoter compared to the U6 promoters,[45,46] and only occurs once in the genome compared the multiple copies of the U6 genes. The human H1 promoter has been used frequently in a variety of species, including unsuccessfully in zebrafish,[9] and remains a standard promoter for shRNA expression in cell culture. However, for transgene-based *in vivo* work, using species-specific promoters is typically desirable.

Access to a set of wellcharacterized native promoters will increase the usefulness of DNA-based RNAi in zebrafish, and may help to prevent undesirable cytotoxic effects in transgenic animals. In this study, we compare the relative strength of knockdown mediated from three previously identified U6 promoters, two variants of a novel U6 promoter, and the H1 promoter in both ZF4 and Vero cells. This has provided insight into the relative strengths of these promoters and highlighted the importance of homologous promoters.

Materials and Methods

Cell culture and transfection

Zebrafish embryo ZF-4 cells (ATCC CRL-2050)[47] were cultured at 28°C in 5% CO_2 in DMEM/F12 Hams media (GIBCO 11330-057) with 10% FBS. They were trypsinized using 0.25% (w/v) trypsin (GIBCO 15050-065), as previously described.[47,48] ZF-4 cells were transfected using the Amaxa Nucleofector. Briefly, 1×10^6 cells were harvested and resuspended in 100 μL of nucleofection solution "V" with 5 μg of each plasmid. Cells were electroporated using a nucleofection program T-27, and then grown for 72 h in a 25 cm^2 culture flask (CORNING or NUNC).[33] African Green Monkey kidney epithelial Vero cells (CCL-81) were cultured at 37°C in 5% CO_2 in EMEM with 10% FCS and transfected using lipofectamine 2000 as previously described.[3]

Plasmid preparation and cloning

All shRNA expression vectors were constructed using the one-step PCR method described previously.[4,5] The shEGFP target sequence was described previously,[5] the pCH-U6-PB shRNA target sequence corresponds to the RNA-dependent RNA polymerase PB2 gene of influenza. The pZFIrrelevant shRNA targets the glyoprotein of viral hemorrhagic septicemia virus. The pFugu-shEGFP shRHA expression plasmid was generously donated by Professor Ki Hong Kim (Department of Aquatic Life Medicine, Pukyong National University, Korea). All oligonucleotides and primers used are indicated in Table 1. Primers were designed using either Primer 3[35] or Clone Manager 9 (SciEd Central).

Sequence management and bioinformatics

Zebrafish sequence information Zv9[49] was accessed via the National Centre for Biotechnology Information (NCBI) database and via Ensembl; U6 and H1 promoters were identified via the mega-Basic Local Alignment Search Tool (mega-BLAST).[50] Alignments were performed using ClustalW[51] and Clone Manager 7.

Detection of shEGFP expression by RNase protection assay (RPA)

Following transfection, cells grown on 25 cm^2 flasks were harvested using trypsin, then washed twice with PBSA. One-half of the cells were used for RNA extraction. RNA extractions were carried out using Trizol Reagent (Invitrogen) according to the manufacturer's instructions, except 10 μg of glycogen (Invitrogen) was added to the aqueous phase and an additional 80% ethanol wash step was introduced to enhance precipitation of small RNAs.

After RNA extraction and purification, an RPA was performed to detect expression of shEGFP hairpins using the LL91 probe (Table 1).[4] Preparation of probes was performed using the *mir*Vana Probe & Marker Kit (Ambion AM1554). Probes were incubated with γ-P^{32}-ATP (1.67pmol/μL) prior to hybridization. In addition to the LL91 probe used to detect the shRNAs, a second probe for miR-16 (Ambion) was used to confirm the presence of RNA in each sample analyzed.

Two μg each of total RNA were hybridized separately in solution with the miR-16 and shEGFP probes prior to RNase A/TI treatment, as per the manufacturers' protocol (Ambion *mir*Vana microRNA Detection Kit AM1552). Samples were run on 15%; (19:1) polyacrylamide:bis (8 M urea) gels which were exposed to x-ray films for up to 72 h at −80°C and developed using a FPM-100A X-ray processor (FUJIFILM).

Flow cytometry and EGFP knockdown assays

To prepare cells for flow cytometry, cells were harvested as described above, then washed once in PBSA, and then twice in FACS-solution (PBSA + 1% FCS) before final resuspension in FACS solution. Sampling and data acquisition was conducted using a FACScalibur (Becton Dickinson) fluorescence-activated cell sorter and CELLQuest software (Becton Dickinson). The EGFP knockdown was quantified as the reduction in normalized mean fluorescence intensity (MFI)

Table 1. Synthetic Oligonucleotides Used in This Study

Name	*Sequence*
PCR	
zU6-1 F	GAT TCC AAA TGG TGC ATG AG
zU6-1 R	AGA TCA GCA GTC AGG CTC AG
zU6-2 F	CTC GGT CAT TCA GTA AGA CG
zU6-2 R	GAA CCA AGA GCT GGA GGG AG
zU6-3 F	GTT TGA TGG GCC GTT GCT G
zU6-3 R	GAA CTA GGA GCC TGG AGG AC
zU6-4 F	GCT CTG CTC TAG TGA GAG CAG
zU6-4 R	GAG CTG GGA GTC TGG AGG AC
zH1 F	TCC TGC TAG GAC AGC GAG TG
zH1 R	CTG TTC ATG AGC GCT ACG
shRNA Construction	
zU6-1shEGFP	TCT AGA TTC CAA AAA AGC TGA CCC TGA AGT TCA TCT CTC TTG
	AAG ATG AAC GTC AGG GTC AGC AGA TCA GCA GTC AGG CTC AG
zU6-2shEGFP	TCT AGA TTC CAA AAA AGC TGA CCC TGA AGT TCA TCT CTC TTG
	AAG ATG AAC GTC AGG GTC AGC GAA CCA AGA GCT GGA GGG AG
zU6-3shEGFP	TCT AGA TTC CAA AAA AGC TGA CCC TGA AGT TCA TCT CTC TTG
	AAG ATG AAC GTC AGG GTC AGC GAA CTA GGA GCC TGG AGG AC
zU6-4shEGFP	TCT AGA TTC CAA AAA AGC TGA CCC TGA AGT TCA TCT CTC TTG
	AAG ATG AAC GTC AGG GTC AGC GAG CTG GGA GTC TGG AGG AC
zH1shEGFP	TCT AGA TTC CAA AAA AGC TGA CCC TGA AGT TCA TCT CTC TTG
	AAG ATG AAC GTC AGG GTC AGC GGG TTA TGA CGT AGT CG
RNA Probes	
LL91	GAU GAA CUU CAG GGU CAG C
miR-16	AUC GUC GUC CAU UUA UAA CCG

compared to the negative control which was either the pZFU6-shIrrelevant in ZF-4 cells or pCHU6-PB in Vero cells. Samples were analyzed in triplicate in each experiment and each experiment was performed in triplicate.

Statistics

Statistical significance of normalized MFI from each experiment was analyzed using one-way analysis of variance (ANOVA) [Prism, GraphPad Software and Minitab v16 (Minitab, Inc)] followed by Tukey's range test to compare all means. A difference was accepted to be significant when $p \leq 0.05$. Upper and lower confidence intervals are provided in Supplementary Table S1 (Supplementary Data are available online at www.liebertonline.com/zeb).

Results

Identification of zebrafish U6 and H1 polymerase III promoters

To identify native U6 promoters, the zebrafish genome was analyzed using BLAST for sequences homologous to the previously identified U6 sequences (U6 1–3),[41] from both zebrafish and pufferfish. This search yielded an additional novel zebrafish U6 promoter located on chromosome 9 (U6 4). These promoters were screened for the presence of known pol III promoter elements such as a TATA-box,[37] Octamer motifs,[52] PSE like,[53] SPH, and STAF[39] elements. All four zebrafish U6 promoters were found to possess a SPH element immediately upstream of the TATA box, which is different from the previously characterized mouse and chicken U6 promoters (Fig. 1B). Sequence analysis of relevant genomic DNA amplified from adult zebrafish and from the zebrafish ZF4 cell line confirmed that the Octamer, SPH, and TATA elements were highly conserved in all promoters against previously published consensus sequences. The zebrafish U6 4 promoter showed some variation in both the PSE and SPH elements, but most significantly a variant U6 promoter was identified (U6 4v2) with three base substitutions within the OCT motif (Fig. 1A).

The zebrafish snRNA RNA component of RNase P had also been previously identified.[54] The H1 promoter showed a more conserved make-up, each of the identifiable promoter elements was conserved when compared to the consensus sequences, and the order of the specific elements was also conserved (Fig. 2).

The zebrafish U6 and H1 promoters express shRNAs

To validate the ability of the zebrafish U6 and H1 promoters to express functional shRNAs, the putative sequences were used to construct six vectors pZFU6-1shEGFP, pZFU6-2 shEGFP,pZFU6-3 shEGFP, pZFU6-4v1shEGFP, pZFU6-4v2shEGFP, and, pZF-H1shEGFP, in which the pol III promoter mediates expression of a shRNA molecule targeting EGFP. Additional constructs using chicken and mouse promoters (pCH-U6shEGFP and pM-U6shEGFP, respectively) have previously been shown to strongly express shRNAs in a wide variety of cells,[3,5,22,31] the construct pFUGU-shEGFP has been shown to be an effective silencer of EGFP in Bluefin gill (BF-2) cells,[22] and more recently *Epithelioma papulosum cyprini* (EPC) cells and chinook salmon embryonic (CHSE-214) cells[17] were used as controls. Two irrelevant control vectors pZFU6-2 Irrelevant and pCHU6-4 NP were used to express an irrelevant shRNA as negative controls in ZF-4 and Vero cells, respectively. U6-2 was used to drive the irrelevant sequence as preliminary work showed that the U6 promoter was the most active in the cell line tested.

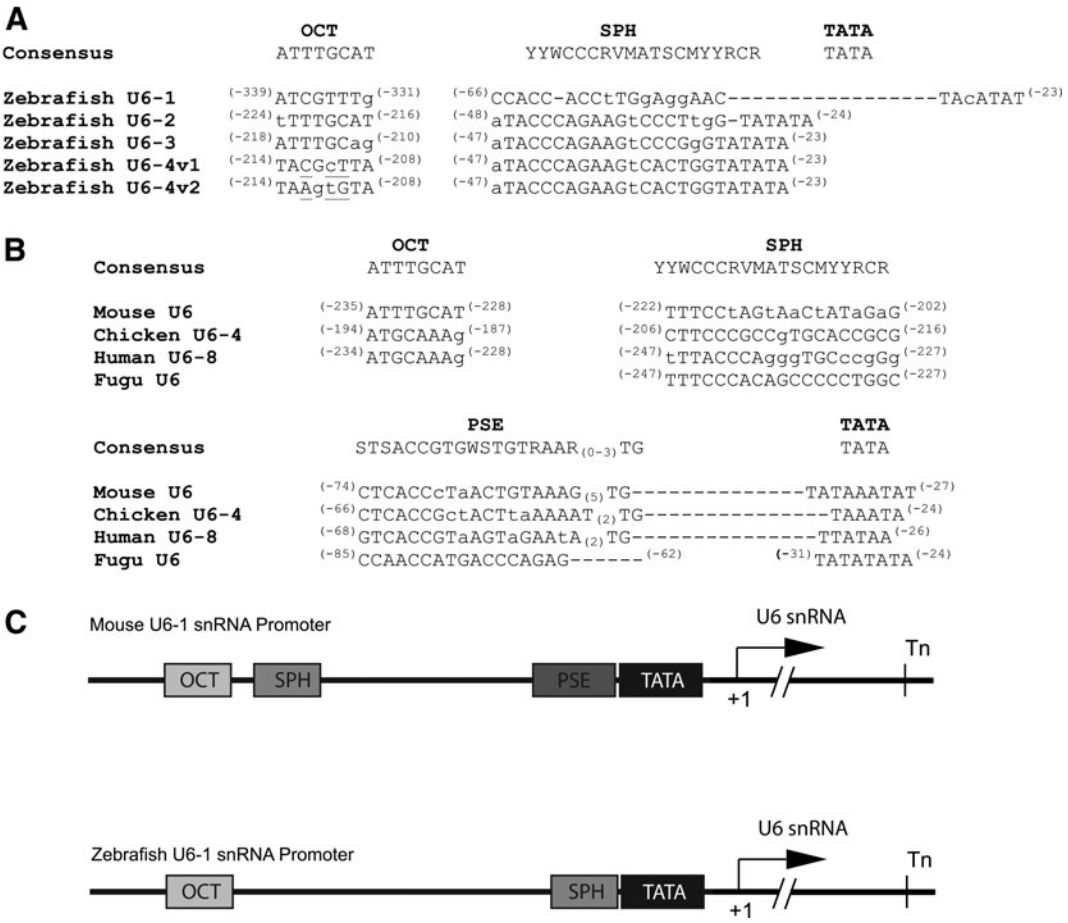

FIG. 1. **(A, B)** Sequence alignment of the distal (DSE) and proximal (PSE) sequence elements of zebrafish, and mouse, chicken, human, and fugu U6 promoters. For zebrafish **(A)**, the DSE is comprised of the OCT motif only and the PSE of the SPH motif and TATA sequence. For the other species **(B)**, the DSE consists of OCT and SPH motifs, except fugu that lacks the OCT element, and the PSE consists of the PSE motif and TATA sequence. Nucleotides that match the consensus sequence for SPH, OCT, PSE, and TATA sequence are shown in *uppercase* or in *lowercase* to identify mismatches. *Underlined letters* represent mismatches observed between variant U6-4 clones. *Dash* (–) represents spacing between PSE elements. Nucleotide positions are shown at the position relative to the predicted transcription start site (+1). The sequence of the amplified zebrafish H1 promoter region is also shown. **(C)** Graphical representation of interchanged PSE and SPH elements of the zebrafish and mouse U6-1 promoters.

RNA extracted from transfected ZF4 cells at 72 h and Vero cells at 48 h was probed for the expression of shEGFP using an RNase protection assay. A 19 nt band was detected in RNA samples from ZF4 cells transfected with all zebrafish U6 and H1 plasmids and the Fugu positive control. No shEGFP expression was detected in RNA samples from the negative control or from either the pCh-U6shEGFP or pM-U6shEGFP (Fig. 3). Detection of miR-16 as a loading control in all transfected and control RNA samples confirmed the presence of small RNAs in each case.

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CTTCTCTTCC  AAACATTTTT  TATCCATTTG  AAAAATTTGG  CTACGTCACG
                                OCT           STAF
ATCAACTACC  CCAATCACTG  CGTACTCATT  TaCATAAAAA  CTTCCCtTAA
                              ATT   TGCAT       YYWCCCRNMA
TGttCGACCA  CGATTTGTGT  ACAATGCGTT  AAATAACAAA  AAACTGAAAT
TSCMYYRCRN
AACAGTGTAT  GAGGACAAAT  GAGCGGAGTT  GACCTGACAT  GTCTTCTGAG
                                          PSE
TTTACAATAC  TAAAATATCT  TAACTAGGTC  TATCACCATc  ACTGTgAAgc
                    TATA              TATCACCNTA  ANNNTAAAAT
ATGGTACAAG  TACAACCTAT  AaATAGATGA  CGCAGACTAC  GTCATAACCC
                    TAT  ATATA
GTA
```

FIG. 2. Sequence of the zebrafish H1 promoter region. The bolded sequences indicate the consensus OCT, STAF, PSE, and TATA motifs, with mismatches represented by *lowercase letters.*

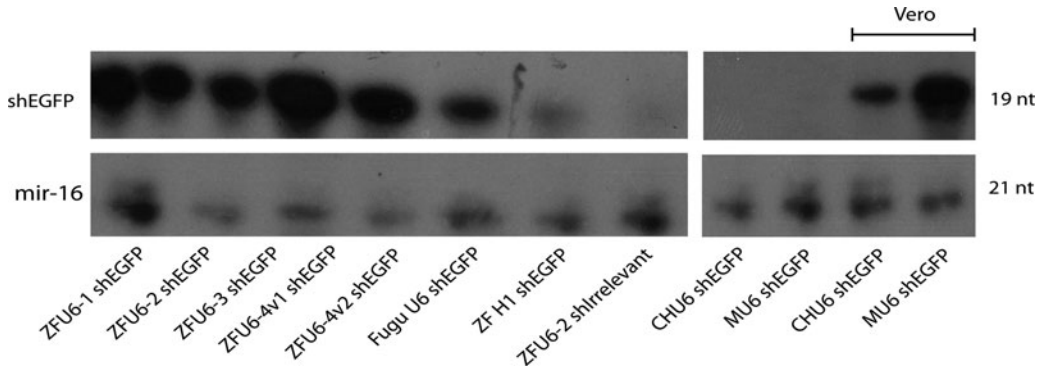

FIG. 3. Detection of expressed EGFP shRNAs from Pol III promoters in ZF4 cells. Zebrafish ZF4 cells were co-transfected by nucleofection; Vero cells were co-transfected with lipofectamine 2000 with the shRNA expression vectors and pEGFP-n1 as labeled. Small RNAs were isolated by Trizol™ at 72 h post-transfection; small RNA extracts were hybridized with a specific shEGFP γ-P32-ATP radiolabeled RNA oligonucleotide. In addition, a γ-P32-ATP radiolabeled RNA oligonucleotide corresponding to miR-16 was used as a loading control across all samples. The data for the chicken and mouse promoters also include RNA collected from Vero cells.

Comparison of zebrafish pol III induced EGFP knockdown in zebrafish ZF4 and mammalian Vero cells

We next compared the efficiency of EGFP silencing mediated via the various pol III promoters in both ZF4 and Vero cells, relative to the respective irrelevant controls. Fluorescence microscopy indicated that EGFP knockdown was comparable between pZFU6–1shEGFP, pZFU6–2shEGFP, pZFU6–3shEGFP, and pZFU6–4v1shEGFP, but no silencing was observed by either the pMU6shEGFP or pCHU6shEGFP plasmids (Fig. 4A). Statistical analysis of the mean fluorescence intensity (MFI) [Fig 4 B] indicated that in ZF4 cells there was a significant reduction in EGFP MFI following

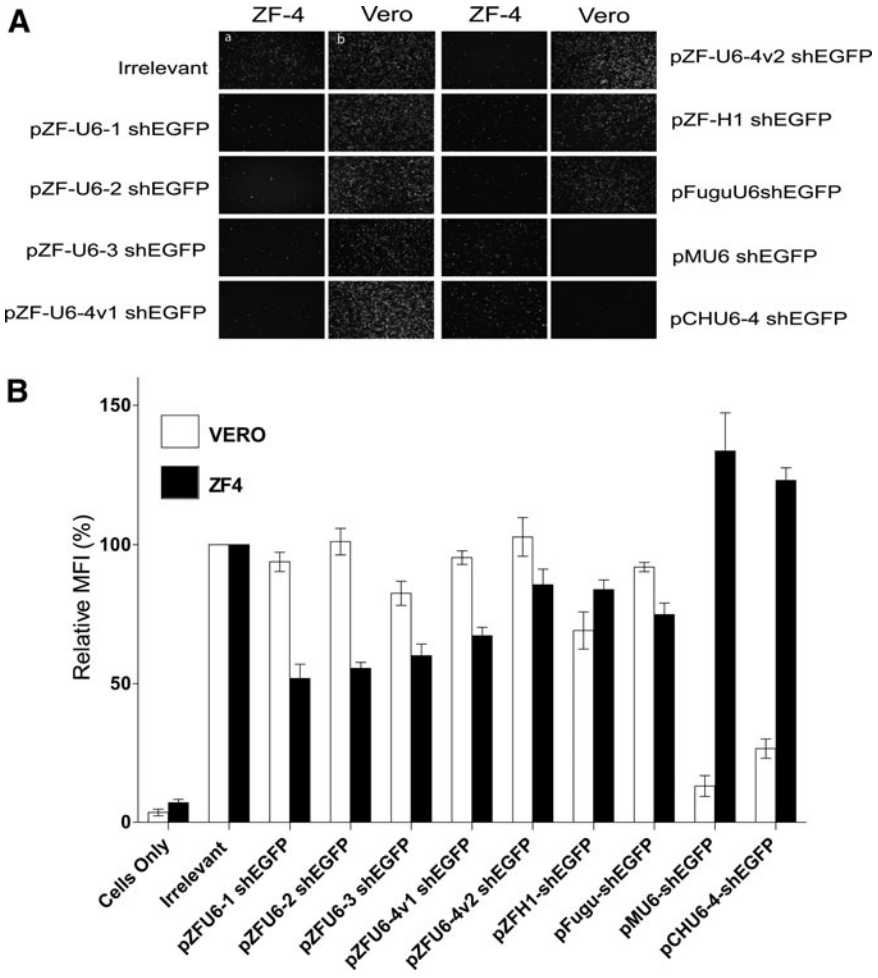

FIG. 4. shRNA knockdown of eGFP using zebrafish pol III promoters. **(A)** Flourescent microscope images of eGFP expression in zebrafish ZF4 and in Vero West African monkey kidney cells following co-transfection with shRNAconstructs and peGFP-N1. pZF-U62 Irrelevant and pCH-U6 Irrelevant represent nonsilencing vectors. Images are representative of triplicate transfections, **(a)** pZFU6-2 irrelevant, **(b)** pCH-U6 irrelevant. **(B)** eGFP knockdown in Vero and ZF4 cells. Flow cytometry results for eGFP knockdown in co-transfected Vero (*white*) and ZF4 (*black*) cells. eGFP knockdown was measured as the relative percentage decrease in mean fluorescence intensity (% MFI), normalized to the MFI of the respective irrelevant controls (100%) following transfection. *Error bars* are indicative of the standard error of the mean (SEM) calculated from duplicate FACS analysis of three separate transfection experiments. Statistical significance was measured by ANOVA with Tukey's post tests; $p \leq 0.05$ was considered significant.

co-transfection with pU6–1shEGFP (48.19 ± 5.92; $p \leq 0.01$), pU6–2shEGFP (39.95 ± 4.91; $p \leq 0.01$), pU6–4v1shEGFP (38.01 ± 4.67; $p \leq 0.01$), as well as a lesser but still significant reduction of EGFP MFI following co-transfection with pU6–3shEGFP (32.76 ± 4.02; $p \leq 0.05$), and with pFUGU-shEGFP (25.15 ± 3.09; $p \leq 0.05$). No other constructs induced significant silencing in ZF4 cells. We also transfected Vero cells with the Pol III shEGFP constructs, the previously characterized pMU6shEGFP and pCHU6shEGFP strongly and significantly $p \leq 0.01$ reduced EGFP MFI–pMU6shEGFP (86.95 ± 6.55, $p \leq 0.01$) and pCHMU6shEGFP (73.45 ± 6.05; $p \leq 0.01$). Additionally, the zebrafish pZFH1shEGFP construct also significantly reduced EGFP MFI (30.97 ± 11.65; $p \leq 0.05$). Finally, the variant U6 4 promoter U64v2 showed significantly less efficient knockdown of EGFP than the U64v1 promoter at (23.61 ± 5.24; $p \leq 0.05$; Fig. 4B).

Discussion

This study has characterized zebrafish pol III promoters. Specifically, a number of native zebrafish U6 promoters and the native H1 promoter were tested for their ability to silence an EGFP reporter by expression of an shRNA against this gene in both zebrafish ZF4 cell and Vero West African monkey kidney cell line. For comparison, mouse, fugu, and chicken U6 promoters were tested in parallel.

The zebrafish U6-1, U6- 2, U6-3, and U6 4 v1 promoters all strongly, and significantly, reduced EGFP expression in ZF4 cells. In contrast, the mouse and chicken U6 promoters did not reduce the expression of EGFP in this cell line. The zebrafish H1 promoter reduced the expression of EGFP slightly, although the reduction was not significant. In the mammalian Vero cell line, the reverse was observed, with none of the teleost U6 promoters able to reduce expression of EGFP, but both mouse and chicken U6 promoters strongly suppressed EGFP expression. The zebrafish H1 promoter weakly, but significantly, reduced EGFP expression in Vero cells. This is consistent with pol III promoter modification studies previously performed with the human H1 promoters to reduce the size of the promoter.[44] Modifications to the size of the regions between the DSE and PSE in the human H1 had no significant effect on the level of expression shRNAs expressed; however, modifications or deletions of either region reduced the expression from the promoters drastically and it is as expected that the differences between the promoter elements in the telost and other promoters prevent the function of the promoters in the respective cell cultures.[41,44]

The zebrafish H1 promoter was identified and compared to those in mouse, chicken, and human. The zebrafish H1 promoter contained each of the conserved sequence elements identified in the other H1 promoter sequences, with all promoter elements present in the same order in each species. The zebrafish H1 promoter was the only promoter able to reduce EGFP expression in both the zebrafish and mammalian cell cultures. Two previous comparisons of mammalian U6 and H1 promoters, first in mice brains and endothelial cells,[45] and second in Vero cell cultures,[46] highlighted that the U6 promoter as a much more effective promoter than the H1 promoter. Our research indicates that the zebrafish is no exception.

This study identified an additional zebrafish U6 promoter (U6 4) to the three previously characterized in this organism. Genomic analysis indicated the presence of a common variant (U6 4v2) with 3 nucleotide alterations in the OCT motif, which was much less effective than the U6 4v1 promoter at reducing EGFP expression. This result is consistent with previous work that indicated that either the complete or partial removal of the OCT motif resulted in reduced expression of the H1 snRNA gene in mice and HeLa cells.[44] This information may be useful if attenuation of the promoters is required.

Interestingly, both pMU6 shEGFP and pCHU6-4 shEGFP appeared to significantly increase the expression of EGFP above the level observed in the pZFIrrelevant control in ZF4 cells. As neither of these constructs showed evidence of expression of shRNAs following co-transfection, it remains possible that the pZFIrrelevant construct is causing a moderate nonspecific knockdown of expression from the pEGFP-N1 plasmid, consistent with other studies suggesting that high levels of RNAi molecules have a nonspecific inhibitory affect on cells normal functions.[13,23]

Conclusion

In this study, we identified and isolated a functional zebrafish H1 and a new zebrafish U6 promoter, along with a variant of the latter. We have shown expression of shRNAs from these and three other previously characterized zebrafish U6 promoters in a zebrafish cell line. We observed that the newly identified U6 promoter showed similar levels of activity to previously characterized zebrafish U6 promoters and the H1 promoter showed a lower level of efficacy as measured by EGFP MFI reduction following co-transfection with pEGFP-N1. We demonstrated that species-matched promoters are required for effective expression of, and knockdown by, shRNAs, supporting previous evidence that there are inherent differences between mammalian and avian U6 promoters compared with their teleost counterparts.

This work provides important insight into the relative promoter strengths of the zebrafish pol III promoters. This information will be important in developing viable RNAi-induced knockdown models in zebrafish, which will further enhance the application of this organism to model adult disease and pathology.

Disclosure Statement

No competing financial interests exist.

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