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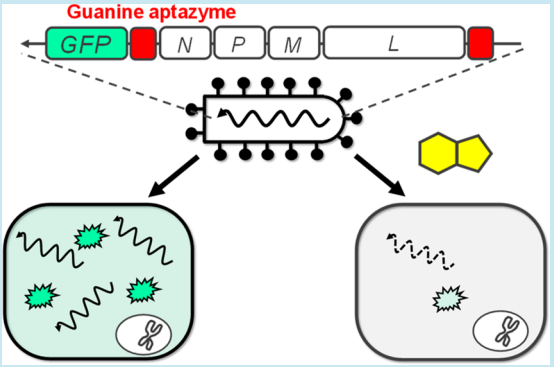
Reversible Gene Regulation in Mammalian Cells Using Riboswitch-Engineered Vesicular Stomatitis Virus Vector

Kei Takahashi and Yohei Yokobayashi*

Nucleic Acid Chemistry and Engineering Unit, Okinawa Institute of Science and Technology Graduate University, Onna, Okinawa 904 0495, Japan

Supporting Information

ABSTRACT: Synthetic riboswitches based on small molecule-responsive self-cleaving ribozymes (aptazymes) embedded in the untranslated regions (UTRs) allow chemical control of gene expression in mammalian cells. In this work, we used a guanine-responsive aptazyme to control transgene expression from a replication-incompetent vesicular stomatitis virus (VSV) vector. VSV is a nonsegmented, negative-sense, cytoplasmic RNA virus that replicates without DNA intermediates, and its applications for vaccines and oncolytic viral therapy are being explored. By inserting the guanine-activated ribozyme in the 3′ UTRs of viral genes and transgenes, GFP expression from the VSV vector in mammalian cells was repressed by as much as 26.8-fold in the presence of guanine. Furthermore, we demonstrated reversible regulation of a transgene (secreted NanoLuc) by adding and withdrawing guanine from the medium over the course of 12 days. In summary, our riboswitch-controlled VSV vector allows robust, long-term, and reversible regulation of gene expression in mammalian cells without the risk of undesirable genomic integration.



KEYWORDS: *RNA virus, RNA replicon, riboswitch, aptazyme*

Viral vectors are regaining attention as advances in gene therapy, cell therapy, vaccines, and regenerative medicine demand more efficient and safer methods to deliver genetic materials. Recombinant viruses can efficiently transduce targeted cells with genes that express various proteins transiently or stably for an extended period of time.[1,2] However, the majority of the existing viral vectors do not allow precise temporal and dosage control of gene expression and vector replication. Use of engineered transcription factor-based gene switches such as Tet-ON and Tet-OFF systems[3,4] in viral vectors is complicated because they involve optimization of multiple parameters (*e.g.*, expression level of the transcription factor, strength of the engineered promoter), and limited capacity of some vectors for exogenous genes.

A promising alternative strategy to control gene expression from viral vectors is to employ synthetic riboswitches. Riboswitches are chemically responsive gene switches that are composed exclusively of RNA. In mammalian cells, insertion of one or more allosterically regulated self-cleaving ribozymes (aptazymes) in the 5′ and/or 3′ untranslated region (UTR) of the gene of interest has been a well-established strategy to chemically control gene expression without protein factors.[5−10] Aptazymes are engineered by fusing a small molecule-binding RNA aptamer with a self-cleaving ribozyme in such a way that self-cleavage is either activated or inhibited by the aptamer ligand. Ribozyme cleavage in the UTR results in mRNA degradation and repression of protein expression, whereas ribozyme inhibition results in upregulation of protein expression. Riboswitches have several attractive features for applications in viral vectors. First, because no additional protein factors are needed, riboswitches add little or no immunogenic factors for *in vivo* applications. Second, aptazymes are typically 100−200 nt in size, and therefore leave relatively small genetic and metabolic footprints on the viral vectors and the host cells. Finally, riboswitches can potentially be adapted to respond to various chemical triggers by incorporating appropriate RNA aptamers as molecular sensors.[5−8]

To date, a number of major viral vectors have been engineered with aptazyme-based riboswitches to control gene expression in mammalian cells. Ketzer *et al.* first used theophylline riboswitches in adenoviral (AdV) and adeno-associated viral (AAV) vectors to control transgene expression as well as to control viral replication.[11] Riboswitches were also used to control gene expression from AdV and AAV vectors by Ketzer *et al.*,[11] Strobel *et al.*,[12] Zhong *et al.*,[13] and Reid *et al.*[14] While AAV and AdV are widely used and are attractive platforms for gene delivery and gene therapy applications, these DNA viruses pose safety concerns regarding possible genomic integration. AAV vectors also have limited capacity (~4 kb) for transgenes that can be integrated.[15]

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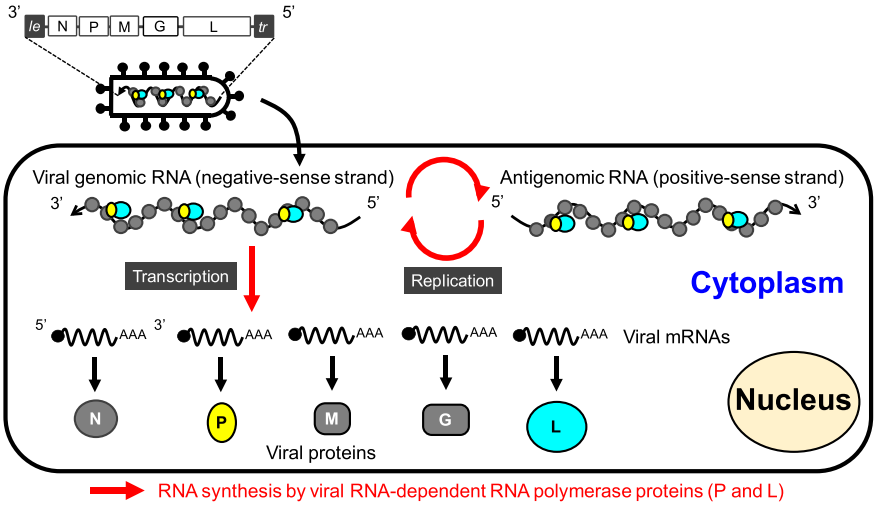

Figure 1. Life cycle of VSV. The viral particle penetrates the host cell through the receptor-mediated endocytosis. The single-stranded negative-sense genomic viral RNA composed of 5 genes flanked by the 3′ leader (*le*) and the 5′ trailer (*tr*) regions in the order 3′-N-P-M-G-L-5′ is released into the cytoplasm where the viral RNAs are synthesized. The 3′ *le* is thought to act as a promoter for transcription of the viral mRNAs and the positive-sense antigenomic RNA. The *tr* sequence is thought to act as a promoter for replication of progeny negative-sense genomes. The viral RNAs including the viral mRNAs and the antigenomic RNA are synthesized by the viral RNA-dependent RNA polymerase complex composed of P and L. The synthesized viral mRNAs are then translated into viral proteins by the cellular translational system. N: VSV nucleoprotein, P: VSV phosphoprotein, M: VSV matrix protein, G: VSV envelope protein, L: VSV RNA-dependent RNA polymerase.

RNA viruses that do not have DNA intermediates have minimal potential for unintended genomic integration, and therefore may provide a safer alternative to DNA virus-based vectors. Ketzer *et al.* demonstrated the use of theophylline aptazymes to control the expression of the viral fusion gene in the measles virus, thereby chemically controlling viral proliferation. The measles virus is a negative-sense, single-stranded RNA virus that replicates in the cytoplasm of the host cell without DNA intermediates, and is being studied for applications as oncolytic vectors.[11] Bell *et al.* later used theophylline-responsive riboswitches to control mammalian gene expression using an alphavirus-based vector which is a positive-sense RNA viral vector.[16] Riboswitches are of particular interest for these RNA viral vectors because canonical transcriptionally regulated switches (*e.g.*, Tet-ON/OFF) are not compatible with these vectors. However, riboswitch applications in cytoplasmic RNA viruses have been limited and additional investigations are needed to establish the utility of riboswitches for these vectors.

In this work, we investigated the use of a synthetic riboswitch to control gene expression from a vesicular stomatitis virus (VSV) vector. VSV is a nonsegmented, negative-sense, single-stranded RNA virus that belongs to the family *Rhabdoviridae*, genus *Vesiculovirus*. Once VSV enters the cell, the viral genomic RNA is released into the cytoplasm where the viral mRNA (positive-sense RNA) is synthesized by the viral RNA-dependent RNA polymerase protein (RdRp) contained in the viral particle. Also, the viral RNA polymerase synthesizes antigenomic RNA without cap structure and poly A tail to replicate its viral genome in the cytoplasm of the host cells (Figure 1).[17] As with other cytoplasmic RNA viruses, VSV does not go through a DNA intermediate during replication. Moreover, viral RNAs including the antigenomic RNA are synthesized in the cytoplasm and the viral RNAs never enter the nucleus of the host cell during the viral life cycle (Figure 1). Since VSV has no risk of genomic integration in the absence of retrotransposition, it has received increased attention as a promising vector for safer gene delivery. In fact, a VSV-based vector expressing interferon beta (IFN-β) which was designed to enhance its oncolytic activity has undergone preclinical trials to treat cancer.[18−20] Furthermore, although VSV infects a broad range of mammalian cells through its envelope protein VSV-G, it can be replaced with another viral envelope protein to confer different cellular tropism to the viral particle.[21] By taking advantage of this feature, many pseudotyped VSV-based vectors have been generated as vaccines against infectious diseases such as Ebola hemorrhagic fever.[22] Consequently, we sought to regulate transgene expression from the VSV vector using a riboswitch to enhance the utility of this emerging viral vector platform.

RESULTS

We constructed a series of replication-incompetent VSV vectors expressing GFP in which the guanine-activated aptazyme GuaM8HDV was inserted in the 3′ UTRs of various viral genes involved in the viral RNA synthesis (N, P, and L, Figure 2A). GuaM8HDV was previously engineered by our group by inserting a guanine aptamer in the P4 stem of the HDV ribozyme which was shown to exhibit a high ON/OFF ratio (29.5) in transiently transfected HEK293 cells.[8] By removing the glycoprotein (G) gene from the viral genome, the viral particles constructed in cells transiently expressing the G protein can infect host mammalian cells, and the viral RNA can replicate in the cytoplasm for several days without forming infectious progeny VSV particles.

The recombinant VSV particles shown in Figure 2A were used to infect BHK-21 cells at multiplicity of infection (MOI) = 5. One hour postinfection (hpi), guanine was added to the wells by exchanging the inoculum to the medium with or without 500 μM guanine. GFP expression was measured 48 hpi using a microplate reader and the cells were imaged by fluorescence microscopy. VSVΔG(GFP) which does not contain an aptazyme in the viral genome showed no significant change in GFP expression in the presence or absence of guanine (Figure 2B). All VSV constructs containing

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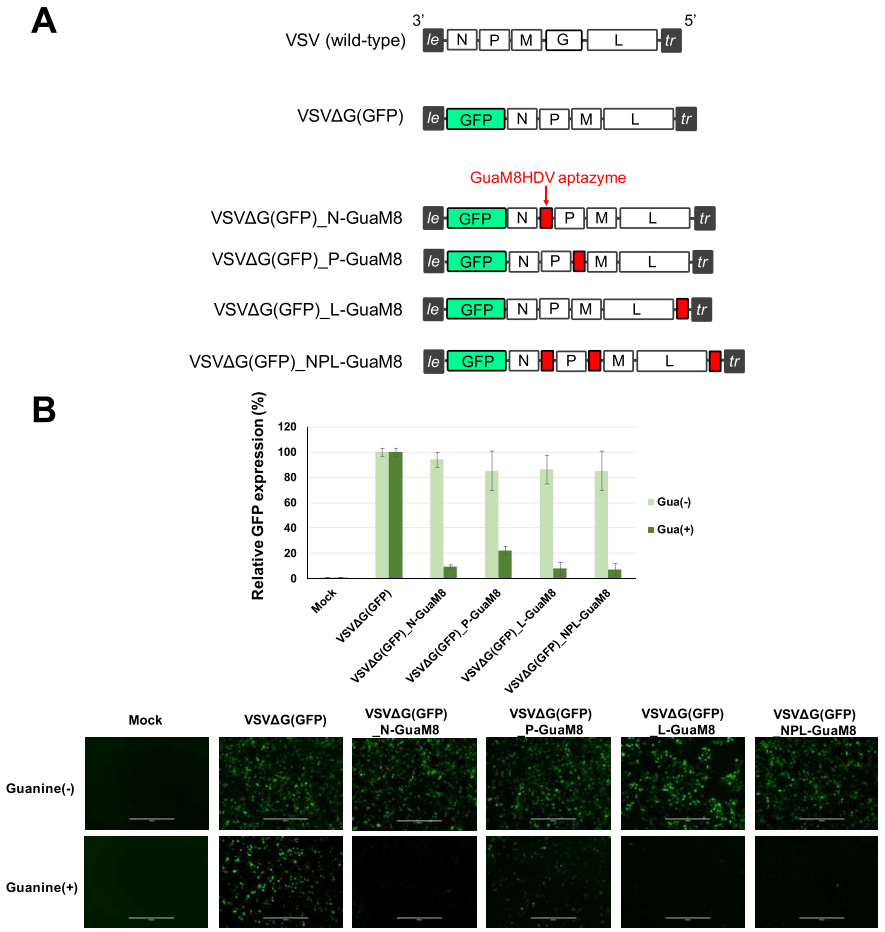

Figure 2. Regulation of exogenous gene expression by controlling viral genes with GuaM8HDV aptazyme. (A) A schematic illustration of the viral genome of the wild-type VSV and VSVΔG variants harboring the GuaM8HDV aptazyme. VSV-G was removed from the genome of the wild-type VSV, and GFP was inserted upstream of N to yield VSVΔG(GFP). GuaM8HDV was inserted in the 3′ UTR of the viral genes involved in the viral RNA synthesis (N, P, and L). One copy of the GuaM8HDV sequence is presented as a red box. (B) GFP expression of BHK-21 cells 48 hpi (MOI = 5) was measured by a microplate reader. The results are averages of three independent experiments and the error bars indicate ± SD. GFP expression was also confirmed by fluorescence microscopy (lower panel).

GuaM8HDV in the 3′ UTR of N, P, L, or N/P/L, however, showed marked repression of GFP expression in the presence of guanine (Figure 2B). When normalized by GFP fluorescence of the cells infected with VSVΔG(GFP) in the absence of guanine, cells infected with VSVΔG(GFP)_N-GuaM8, VSVΔG(GFP)_P-GuaM8, VSVΔG(GFP)_L-GuaM8, and VSVΔG(GFP)_NPL-GuaM8, showed 9.6-, 3.8-, 17.8-, and 15.9-fold reduction in GFP fluorescence in the presence of guanine, respectively. These results show that controlling viral genes using riboswitch results in efficient regulation of the associated transgene (GFP) expression. However, while VSVΔG(GFP)_N-GuaM8 and VSVΔG(GFP)_L-GuaM8 exhibit higher ON/OFF ratios compared to VSVΔG(GFP)_P-GuaM8, insertion of aptazymes in the 3′ UTR of multiple genes (VSVΔG(GFP)_NPL-GuaM8) did not result in a significant improvement (Figure 2B).

To further improve the dynamic range of gene expression by the riboswitch-controlled VSV vector, GuaM8HDV was inserted in the 3′ UTR of the transgene (GFP) (Figure 3A). Addition of guanine (500 μM) repressed GFP expression in the cells infected with VSVΔG(GFP)_GFP-GuaM8 by 7.1-fold (Figure 3B). When GuaM8HDV was inserted in the 3′ UTR of both GFP and L (VSVΔG(GFP)_GFP/L-GuaM8), however, the infected cells showed 26.8-fold reduction in GFP expression in the presence of guanine (Figure 3B). Consequently, dual regulation of the transgene and a viral gene by riboswitches resulted in an additive effect by improving the ON/OFF ratio of transgene expression. Dose-dependent GFP expression of the cells infected with VSVΔG(GFP)_GFP/L-GuaM8 showed 100 μM guanine was sufficient to repress gene expression by 21.5-fold which is consistent with previous applications of GuaM8HDV (Figure 3C).[8]

Levels of the viral genomic RNA and the mRNAs encoding GFP and L were measured by quantitative reverse-transcription PCR (qRT-PCR). Addition of guanine to BHK-21 cells infected with VSVΔG(GFP)_GFP-GuaM8 resulted in 10-fold reduction in GFP mRNA level but did not affect L mRNA or the genomic RNA (Figure S1). However, GFP and L mRNAs as well as the genomic RNA were all downregulated in the presence of guanine in the cells infected with VSVΔG(GFP)_L-GuaM8 or VSVΔG(GFP)_GFP/L-GuaM8 (Figure S1). As expected, GuaM8HDV placed in the 3′ UTR of the transgene (GFP) only regulates its own mRNA level, but the riboswitch controlling the viral protein L affects both viral genomic RNA and mRNA levels.

Next, we examined long-term and reversible regulation of gene expression by the riboswitch-controlled VSV vector. We

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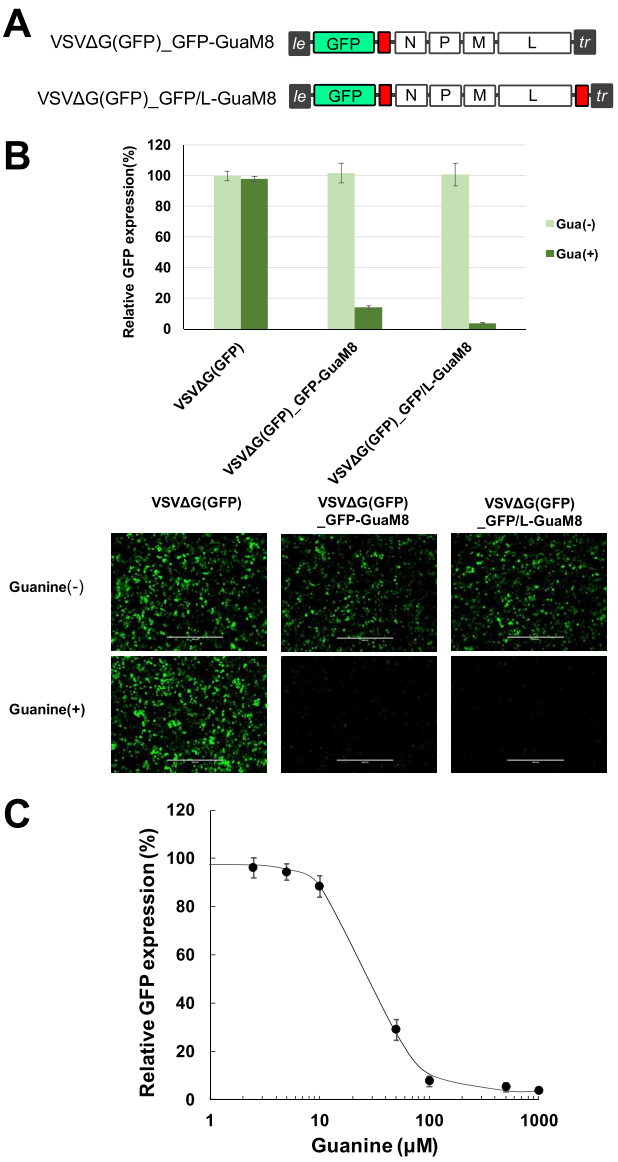

Figure 3. Characterization of VSVΔG(GFP)_GFP/L-GuaM8. (A) A schematic of the viral genome of VSVΔG(GFP)_GFP-GuaM8 and VSVΔG(GFP)_GFP/L-GuaM8. (B) GFP expression levels in BHK-21 cells infected with VSVΔGvariants. The results are averages of three independent experiments and the error bars indicate ± SD. GFP expression was also confirmed by fluorescence microscopy (lower panel). (C) Dose-dependent repression of GFP expression from VSVΔG(GFP)_GFP/L-GuaM8. The data shown are mean ± SD from triplicate samples. The curve is shown to guide the eye only.

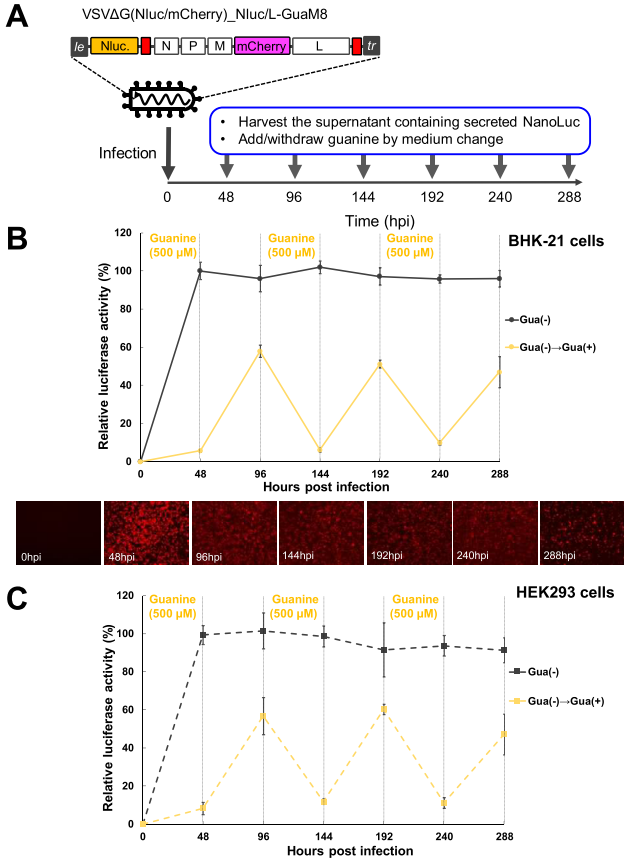

Figure 4. Reversible control of gene expression by VSVΔG(Nluc/mCherry)_Nluc/L-GuaM8. (A) Experimental schedule. BHK-21 cells were inoculated with VSVΔG(Nluc/mCherry)Nluc/L-GuaM8 (MOI = 5) and incubated in the medium without (Gua (−)) or with 500 μM guanine (Gua (+)) for the first 48 h. After 48 h incubation, the cells were detached from the dish and plated onto a fresh dish and cultured in Gua (−). (B) Relative luciferase activity secreted from BHK-21 cells infected with luciferase-expressing VSV vector at each time point. Relative luciferase activity was calculated as described in Methods. The data shown are means ± SD from triplicate samples. mCherry expression in the cells was monitored with fluorescent microscopy at each time point (lower panel). (C) Relative secreted luciferase activities from HEK293 cells infected with luciferase-expressing VSV vector. The data shown are means ± SD from triplicate samples.

constructed VSVΔG(Nluc/mCherry)_Nluc/L-GuaM8 by replacing GFP gene in VSVΔG(GFP)_GFP/L-GuaM8 to secreted NanoLuc luciferase gene (Nluc, Promega) and inserting the mCherry gene between M and L genes (Figure 4A). GuaM8HDV controls expression of both Nluc and L genes. Secreted Nluc was used because GFP would be too stable to observe ON to OFF transition of the riboswitch regulation while mCherry would serve as a marker of cells infected with the VSV vector. BHK-21 cells infected with VSVΔG(Nluc/mCherry)_Nulc/L-GuaM8 were cultured for 48 h in the presence of guanine (500 μM). After 48 h, Nluc expression was measured by sampling the supernatant, the cells were harvested, and 50 000 cells per well were seeded in fresh medium without guanine. Similarly, Nluc expression was measured every 48 h while exchanging the medium with or without guanine up to 288 h (12 days). As a control, parallel cultures without guanine were maintained throughout the experiment (Figure 4B). We observed robust and reversible regulation of Nluc expression during the time course of the experiment. Similar results were obtained with HEK293 cells (Figure 4C).

DISCUSSION

Chemical regulation of gene expression in cytoplasmic RNA viral vectors and RNA replicons could have practical implications in gene therapy and vaccine applications. For example, dynamic regulation or fine-tuning of therapeutic protein expression may reduce unwanted side-effects, or regulation of vector replication can be used as an emergency kill-switch. Temporal regulation of antigen expression may lead

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to the development of a novel priming and boosting strategy for vaccine applications.[16] Riboswitches are of particular interest for RNA viral vectors because transcription factor-based gene switches are not compatible with these vectors due to their lack of DNA genomes.

Ketzer *et al.* first demonstrated the use of a theophylline-activated aptazyme to control the measles virus, a negative-sense RNA virus which have been investigated for oncolytic virus applications.[11] In this work, the authors inserted an aptazyme in both the 5′ and 3′ UTR of the viral fusion gene which enables the fusion of the viral and the host cell membranes and subsequent viral entry. They demonstrated that addition of theophylline significantly reduced the production of infectious viral particles compared to the control viruses with inactivated aptazymes. No regulation was observed with only one aptazyme embedded in either 5′ or 3′ UTR. Also, regulation of a transgene expression was not demonstrated. Another example of controlling mammalian RNA viral vector by a riboswitch was reported by Bell *et al.*, who inserted theophylline-responsive aptazymes in the UTRs of the subgenomic RNA of the positive-sense alphavirus-based RNA replicon.[16] In this work, the authors first demonstrated that they can activate a reporter gene expression by 3.1-fold by inserting a theophylline-inactivated aptazyme in the 3′ UTR, and by 47-fold when two copies of the aptazyme were inserted. They also demonstrated that they could modulate the type I interferon (IFN) response in mouse embryonic fibroblast (MEF) cells by theophylline, indicating that riboswitches may be used as a tool to optimize immune response triggered by RNA viral vectors. While these pioneering reports demonstrate the potential utility of riboswitches to regulate RNA viral vectors, further demonstration of riboswitch function in additional vectors would be desirable. Moreover, long-term, and reversible regulation of mammalian gene expression by riboswitches has not been demonstrated.

In this work, we focused on the VSV vector which is a negative-sense RNA viral vector similar to the measles virus. Among the viruses that have been engineered for the development of effective gene delivery system so far, VSV is one of the promising viruses because of its unique biological properties. First, the pathogenicity of VSV to humans is quite low compared to other RNA viruses such as the measles virus.[23] Although measles virus is naturally pathogenic to humans, VSV has been associated with only mild symptoms in a small group of the agricultural workers who have been infected. In fact, the safety of VSV has been proven in preclinical studies and VSV has been in preclinical trials for oncolytic viral therapy.[18,24] Second, VSV has been successfully developed as a vaccine against pathogenic infectious diseases. Various types of pseudotyped VSV have been generated as vaccines against infectious diseases by replacing the envelope protein and the gene from VSV-G to another viral envelope protein, and the safety and efficacy of these vaccines have already been proven in a test using nonhuman animals.[25] Third, the tropism of VSV can be easily modified by replacing VSV-G with another viral envelope protein. Although one advantage of VSV as a viral vector is its broad range of cells that it can infect, it is also possible to restrict the scope of targeted cells by exchanging the viral envelope protein, if desired.

Regulation of GFP by GuaM8HDV resulted in only 7.1-fold repression in the presence of guanine (Figure 3B) which is lower compared to the previously reported 29.5-fold repression in cultured HEK 293 cells observed by plasmid transfection.[8] The discrepancy may be due to the context dependence of the aptazyme activity. Alternatively, the extremely high mRNA level of VSV transcripts may have increased the baseline expression level. Nevertheless, combination of GuaM8HDV regulation of GFP and L resulted in an additive effect on the switch performance, yielding an ON/OFF ratio up to 26.8 (Figure 3B). However, simultaneous control of N, P, and L by GuaM8HDV resulted in only 15.8-fold repression of gene expression and did not result in an improvement from the single-riboswitch constructs (Figure 2B). A possible explanation for this observation is that the reduced level of the major viral polymerase component L became the limiting factor in viral replication, and further reduction in other viral proteins had less influence on the overall viral RNA level. Therefore, we found that dual regulation of the transgene and a viral gene resulted in the optimal dynamic range of gene expression.

This observation was corroborated by quantification of viral mRNAs and genomic RNA (Figure S1). While ribozyme cleavage in the 3′ UTR of the transgene (GFP) only reduces its own mRNA level, cleavage within the 3′ UTR of L mRNA affects no only the viral mRNAs but also the viral genomic RNA. Consequently, the ON/OFF ratio of the riboswitch controlling L expression is amplified by a negative feedback loop. Although further reduction in GFP mRNA level in the dual-riboswitch construct VSVΔG(GFP)_GFP/L-GuaM8 is unclear (Figure S1A) possibly due to the detection of cleaved but not degraded mRNA, it can be expected that direct cleavage of the GFP mRNA in the presence of guanine would exhibit an additive effect on the final GFP expression level.

Due to the low solubility of guanine and the relatively high concentration that is required to control the riboswitch, guanine is not an ideal ligand for *in vivo* applications. In fact, the lack of sensitive and low-toxicity ligand-riboswitch systems applicable *in vivo* is a major bottleneck for the applications of mammalian riboswitches. Efforts to develop new aptamers suitable for biological applications have been reported which may lead to improved riboswitches for *in vivo* applications.[26−28]

Applications of riboswitch-controlled viral vectors for gene therapy and vaccines are likely to involve long-term treatments and should offer reversible activation and repression of gene expression during the course of the administration. However, previous reports on riboswitch applications in mammalian cells have been limited to durations of few days, with limited ON/OFF transitions.[6,9] Here, we used the GuaM8HDV-engineered VSV vector (VSVΔG(Nluc/mCherry)_Nluc/L-GuaM8) to control a secreted luciferase gene to demonstrate robust reversible regulation of gene expression of up to 3 cycles over 12 days (Figure 4). To our knowledge, such repeated cycles of long-term reversible gene expression by mammalian riboswitches have not been reported. Robust, long-term, and reversible gene regulation by riboswitch-engineered VSV vectors shown in this work provides a foundation for safer and more effective viral vectors for applications in gene therapy and vaccines.

METHODS

Plasmid Construction. All plasmids were constructed by standard recombinant DNA techniques. The recombinant plasmids were transformed into NEB Stable Competent *E. coli* (New England Biolabs, NEB) cells and cultured at 30 °C. All plasmids containing parts of or the whole viral genome were

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derived from pVSV GFP dG (Addgene, plasmid #31842). To facilitate the cloning process, the full-length viral genome was divided into 3 fragments (GFP/Nluc-N, P-M(-mCherry), and L), and the fragments were subcloned into the parental vector (p*BR*322) using In-Fusion HD Cloning Kit (TaKaRa). The plasmids encoding the recombinant viral genomes were assembled using NEBuilder HiFi DNA Assembly Master Mix (NEB) from appropriate fragments amplified using Q5 High-Fidelity 2X Master Mix (NEB). The plasmids expressing the viral proteins and T7 RNA polymerase from the CAG promoter were obtained from Addgene: pCAG-VSVN (plasmid #64087), pCAG-VSVP (plasmid #64088), pCAG-VSVL (plasmid #64085), pCAG-VSVG (plasmid #35616), and pCAG-T7pol (plasmid #59926). The secreted Nluc gene was subcloned from pNL1.3 (Promega). Nucleotide sequences of the plasmids are provided in Supporting Information.

Cell Culture. Human embryonic kidney 293 T (HEK293T) cells and baby hamster kidney 21 (BHK-21) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wako) supplemented with 10% fetal bovine serum (FBS) (Gibco) and penicillin-streptomycin solution (Gibco). The cells were incubated in 5% CO_2 at 37 °C.

Production of Replication-Incompetent VSV Vector by Reverse Genetics. The replication-incompetent VSVs harboring GuaM8HDV were generated and rescued as previously described.[29] Briefly, 1.0×10^6 cells of HEK293 cells were seeded onto a well of 6-well plate 1 day before transfection. The next day, 5 μg of the plasmid expressing the positive-strand viral genome was transfected to the HEK293 cells using *Trans*IT-293 reagent (Mirus Bio) along with appropriate amounts of the plasmids expressing the viral proteins and T7 RNA polymerase (1.5 μg pCAG-VSVN, 2.5 μg pCAG-VSVP, 0.5 μg pCAG-VSVL, 4 μg pCAG-VSVG, and 5 μg pCAG-T7 RNA polymerase), and the cells were cultured. Two days after transfection, the supernatant containing the replication-incompetent VSV particles were collected and inoculated into BHK-21 cells transfected with pCAG-VSVG to amplify the viral particles. The supernatant was harvested 2−3 days after inoculation and stored at −80 °C. Stock virus titers were determined by counting the number of GFP-expressing BHK-21 cells or HEK293 cells after infection. For VSVΔG(Nluc)-Nluc/L-GuaM8, the viral titer was determined by counting the number of mCherry expressing cells using BHK-21 and HEK293 cells.

Virus Infection. Replication-incompetent VSV was inoculated into BHK-21 or HEK293 cell monolayers at MOI = 5 in 10% FBS-DMEM and cultured. One hour postinfection, the inoculum was removed and 10% FBS-DMEM containing 500 μM guanine was added to the wells and cultured for 47 h. The expression levels of GFP were determined by measuring the fluorescence intensity the cells by a fluorescence microplate reader (M1000 PRO, Tecan) at 484 nm excitation and 510 nm emission. Background cellular fluorescence of the mock transfected cells was subtracted from each measurement, and it was then normalized by the cellular fluorescence value of the cells infected with VSVΔG(GFP) in the absence of guanine.

Reversible Gene Regulation Using the VSV Vector. One day before infection, BHK-21 cells were seeded in a 12-well plate at a concentration of 1.2×10^5 cells/well in 10% FBS-DMEM. The next day, the cells were inoculated with replication-incompetent VSV harboring NanoLuc (VSVΔG-(Nluc)_Nluc/L-GuaM8) at MOI = 5 in 10% FBS-DMEM. The inoculum was removed 1 hpi and the cells were cultured in the absence or presence of guanine (500 μM). The supernatant was harvested 48 hpi and stored at −20 °C until the measurement. After the sampling, the cultured cells were detached from the well by trypsinization and suspended with guanine-free 10% FBS-DMEM and seeded onto a fresh 12-well plate with at 5.0×10^4 cells/well and cultured for another 48 h. After these 2 days of culturing, the supernatants were sampled and stored at −20 °C until measurement. The cells were again detached and seeded onto a fresh 12-well plate at the same concentration as described above and cultured in the absence or presence of guanine (500 μM) for additional 48 h. The process was repeated up to 288 h (12 days) from the first inoculation. After collecting all samples, the activity of the secreted NanoLuc in the supernatant was measured using Nano-Glo Luciferase Assay Reagent (Promega) according to the manufacturer's instructions. NanoLuc activities were normalized to that of the supernatant from the untreated (no guanine) culture 48 hpi.

Quantification of Viral RNAs. The mRNAs encoding GFP and L, and the viral genomic RNA were quantified by qRT-PCR. BHK-21 cells were infected with the VSV vector (MOI = 5) and incubated in the medium without (Gua (−)) or with 500 μM guanine (Gua (+)). The total RNAs were extracted from the cells using Direct-zol RNA Kit (Zymo Research) 48 hpi. Reverse transcription was performed with an oligo(dT) primer by using the extracted RNAs as the template, and real-time PCR was conducted by using the gene specific primer set with Power SYBR Green Master Mix (Applied Biosystems) on a StepOnePlus instrument (Applied Biosystems). GFP-specific primers: 5′-AAGCTGACCCTGAAGTTCATCTGC-3′ and 5′-CTTGTAGTTGCCGTCGTCCTTGAA-3′, L-specific primers: 5′-TGATACAGTACAATTATTTTTGGGAC-3′ and 5′GAGACTTTCTGTTACGGGATCTGG3′, viral genomic RNA-specific primers (a region corresponding to the L gene): 5′-TGATGATGCATGATCCAGCTCT-3′ and 5′-ACACACCTCCAATGGAAGGGT-3′, β-actin-specific primers; 5′-CGACAACGGCTCCGGCATGT-3′ and 5′-TCACGCCCTGGTGCCTAGGG-3′. The level of each viral RNA was normalized by the level of β-actin mRNA in the same sample and then further normalized by the RNA level of the cells infected with VSVΔG(GFP) in the absence of guanine. The data shown are means ± SD of triplicate samples.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.9b00177.

Sequence information on the VSV constructs described. Measurement of the viral RNA levels (Figure S1) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: yohei.yokobayashi@oist.jp.

ORCID

Yohei Yokobayashi: 0000-0002-2417-1934

Author Contributions

K.T. and Y.Y. conceived the project and designed experiments. K.T. conducted all experiments and analyzed the data with critical input from Y.Y. The manuscript was written by K.T. and Y.Y.

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Notes

The authors declare no competing financial interest.

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