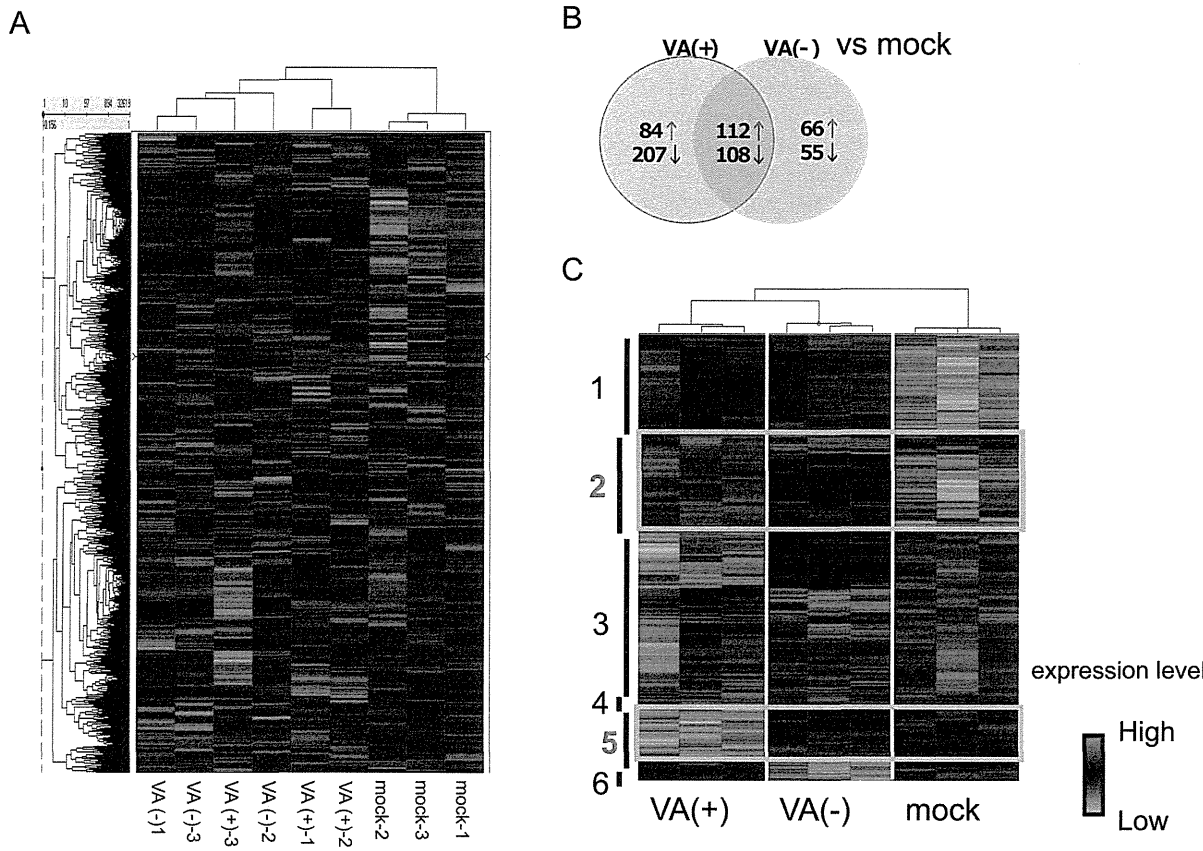

Figure 1. Microarray analysis. Global gene expression analysis of AdV infected A549 cells by Affymetrix microarray. Cells were harvested and total RNA was isolated after 24 h after infection. (A) Hierarchical clustering analysis using 32,619 genes of which expression was determined as "Present" in GCOS software in every sample. (B) The numbers of up or down regulated genes compared with mock infected group (Fold change >1.5, $P<0.01$). Red arrow indicates the numbers of up-regulated genes, and blue arrow indicates the numbers of down-regulated genes. (C) Identification and isolation of VA (+) specific gene clusters by hierarchical clustering analysis. The numbers of target genes were 2,800 genes, which were selected by ANOVA analysis in advance.
doi:10.1371/journal.pone.0108627.g001

All the data acquired by the microarray analysis were deposited in the NCBI Gene Expression Omnibus (NO. GSE58605).

Quantitative real-time PCR

The total RNA of the infected cells was extracted, and the amount of expressed target RNA and 18S-rRNA (correction standard) were quantified using reverse-transcription and real-time PCR (Applied Biosystems Villa7); the ratio of the target RNA to 18S-rRNA was then calculated. To quantify the AdV genome, the infected total cell DNA was prepared from cells using a previously described method [30,31] or a DNA preparation kit (TaKaRa Bio). Quantitative PCR (qPCR) was performed to detect the AdV genome using a probe for the pIX gene, as described previously [24]. The amount of chromosomal DNA was simultaneously measured to correct the Ct values of the viral genome per cell. The probes were derived from the sequence of the human β-actin gene for HeLa and HuH-7 cell lines. The qPCR reaction was performed according to the manufacturer's protocol: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min (Applied BioSystems).

Western blot analysis

Two days after transfection, 293 cells were harvested and the total protein was extracted using NP-40 lysis buffer [50 mM Tris-HCl (pH 8.0), 0.15M NaCl, 5 mM EDTA, 1% NP-40]. The lysates were mixed well in a rotator for 2 h at 4°C, centrifuged at 15,000 rpm for 5 min at 4°C, and the supernatants were collected. Western blotting was performed as described previously [32]. The membrane was incubated for 2 h at room temperature in the presence of anti-HDGF mouse monoclonal antibody (Bio Matrix Research, #BMR00572) diluted to 0.3 μg/mL with PBS-Tween, followed by incubation with peroxidase-conjugated goat anti-mouse IgG+IgM (Jackson Immunoresearch, #115-035-068) diluted to 1/10,000 with PBS-Tween. An anti-actin peptide goat polyclonal antibody (Santa Cruz Biotechnology, #sc-1616) diluted to 1/200 was also detected to show equal loading.

Results

HDGF gene expression was downregulated in FG-AdV infected cells

To determine whether VA RNAs expressed from FG AdVs disturb cellular gene expression, a microarray analysis was performed. We conducted a hierarchical clustering analysis using data for 32,619 genes, which were determined by GCOS software as being expressed in all the samples. The clusters were divided into two clear groups: namely, a mock group and an AdV-infected group (Figure 1A). Then, we conducted a pairwise comparison and drew a Venn diagram between the mock group vs. VA (−), i.e. VA-deleted AdV, and the mock group vs. VA (+), i.e. FG AdV,

according to the criteria of a 1.5-fold change (either an increase or a decrease) and $P<0.01$. In AdV-infected cells, more than 600 genes showed a significant increase/decrease against the mock cells in total. The numbers of VA-(+) specific genes and VA-(−) specific genes were found to be 300 and 100, respectively (Figure 1B). These results indicated that the VA RNAs expressed from AdV do not have a major impact on the expressions of whole genes. Using an ANOVA analysis ($P<0.01$) and a hierarchical clustering analysis, we isolated 6 gene clusters and 2,800 genes that showed different gene expressions between any of the group combinations. Among the 6 clusters, gene clusters 2 and 5 exhibited VA (+)-specific increases in gene expression and VA (+)-specific decreases in gene expression, respectively. According to this gene list (Table S1 in File S1) and literature survey, we finally selected several genes as targets for further research.

Next, we attempted to validate whether our microarray strategy was actually capable of identifying the targets of VA RNAs. We selected a subset of genes, all of which were upregulated or downregulated only after FG AdV infection and not after VA-deleted AdV infection, compared with the mock cells, and measured their transcript levels using quantitative RT-PCR (qPCR) in HeLa cells and HuH-7 cells. The results showed that the expression levels of some of these selected genes were actually changed in response to VA RNAs in both cell lines, except for the PTPRJ gene (Table 1). In contrast, we did not observe any significant changes in the transcript levels of TIA-1 (ENSG00000116001.11), which have been identified as a target for mivaRNA, a microRNA derived from VA RNA [15]. We chose the HDGF gene for further analysis since its transcript was remarkably decreased in both cell lines when FG AdVs were infected.

HDGF gene expression was suppressed by a lower level of VA RNAs than TIA-1

To examine whether the VA RNAs expressed from a plasmid also suppress HDGF gene expression, a VA-RNA expressing-plasmid, pVA41da [16], was transfected into 293 cells. Two days later, the total cellular RNA and protein were collected and HDGF expression was measured at the transcript level using qPCR (Figure 2A) and at the protein level using a western blot analysis (Figure 2B), respectively. The result showed that HDGF mRNA was significantly decreased, even in cells with a low level of VA-RNA transduction (Figure 2A, HDGF, 0.1 μg/well), in comparison with control plasmid-transduced cells (Figure 2A, HDGF, 0). In contrast, no significant change in TIA-1 expression was observed in the low VA RNA transduced cells (Figure 2A, TIA-1, 0.1 and 0.25), and it was suppressed only in the highest VA RNA-transduced cells (Figure 2A, TIA-1, 0.5). HDGF suppression mediated by VA RNA was also detected at the protein level (Figure 2B). The HDGF protein was significantly decreased in cells that had been transfected with the VA RNA-expressing plasmid (Figure 2B, lane 2, VA (+)), compared with the mock cells (lane 1, mock) or the control plasmid-transduced cells (lane 3, VA (−)). The suppression of HDGF transcript was also observed in VA RNA-expressing 293 cell lines named 293VA1 and 293VA42 [33], compared with that in the parent 293 cells (Table S2 in File S1). Therefore, VA RNAs suppressed HDGF expression under the conditions other than viral infection, and a smaller amount of VA RNA than TIA-1 was sufficient to suppress HDGF.

HDGF gene expression was suppressed during the early phase of viral infection

To determine the period during which HDGF was downregulated in the adenovirus life cycle, the VA-deleted AdVs and the FG-AdVs were used to infect 293 cells at an MOI of 5. Then, the cellular RNA was isolated to measure the HDGF transcript levels using qPCR at the indicated time points (Figure 3). The VA-deleted AdVs and the FG-AdVs are structurally identical except for their VA RNA expression, and these E1-deleted vectors are able to replicate in 293 cells because the E1 proteins are supplied *in trans*. The results showed that the transcript levels of HDGF started to decrease at 8 h after infection (early phase) in FG AdV-infected cells (Figure 3A, white circle). Interestingly, after VA-deleted AdV infection, the HDGF level clearly increased above the basal level at 8 h (Figure 3A, black square). This induction of HDGF expression after VA-deleted AdV infection was also observed under replication-deficient conditions in HuH-7 cells (Figure S1 in File S1, bars 1 and 3). In contrast, the TIA-1 mRNA level was similar to the basal level at 8 h and it obviously decreased to comparable level with HDGF only at 16 h (late phase) after FG-AdV infection (Figure 3B, white circle), whereas no significant upregulation was observed after VA-deleted AdV infection (Figure 3B, black square). Since the replication of the viral genome occurs at around 8 h after infection, these results showed that the suppression of HDGF and TIA-1 began during the early and late phases of viral infection, respectively. The results for TIA-1 suppression using AdVs were consistent with those of a previous report indicating that TIA-1 is downregulated during the late phase of infection with wild-type adenovirus [15]. We further examined the point that the HDGF level increased to more than 125% of the steady-state level at 8 h after VA-deleted AdV infection (Figure 3A), though the TIA-1 level did not (Figure 3B).

Table 1. Changes in expression levels of cellular genes in response to VA RNAs.

gene	ratio VA(+)/VA(−)	
	HuH-7	HeLa
PAPPA	1.23	1.60
PTPRJ	1.09	1.06
STS1-3	0.82	0.80
HDGF	0.43	0.65
TIA-1	1.10	1.09

Each mRNA level in the HuH-7 cells and HeLa cells was quantified using qPCR, and the ratio of the expression level in FG AdV infected cells (VA (+)) compared with that in VA-deleted AdV infected cells (VA (−)) was calculated.

doi:10.1371/journal.pone.0108627.t001

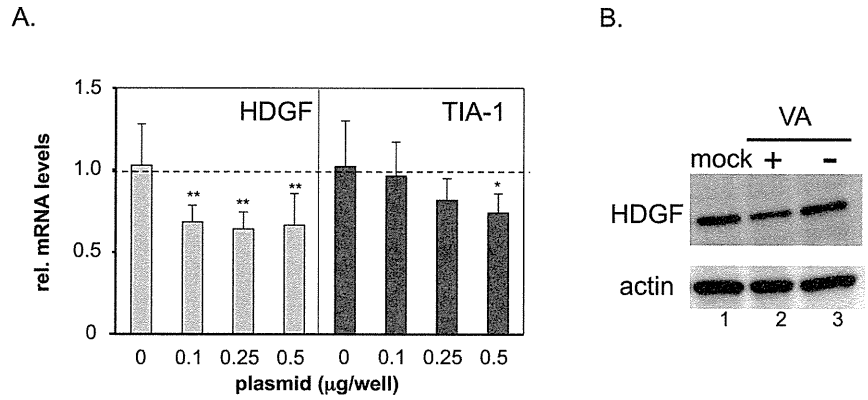

Figure 2. HDGF is downregulated in the presence of VA RNA. (A) HDGF and TIA-1 mRNA levels after VA RNA-expressing plasmid (pVAda41) transfection. RNA (A) and protein (B) were isolated after 48 h from 293 cells transfected with pVA41da or control plasmid. HDGF or TIA-1 and 18S rRNA were quantified using qPCR and plotted for comparison. The expression level in control-plasmid transfected cells was set at 1, and the ratio of the expression levels in all the cases was calculated. The error bars show the standard deviations of three different experiments. *$P<0.05$, **$P<0.01$ compared with mock cells (unpaired Student *t*-test). (B) HDGF and actin, used as a loading control, were evaluated using western blot analysis.
doi:10.1371/journal.pone.0108627.g002

VA RNAs suppressed the upregulation of HDGF gene expression during the early phase of viral infection

Although the replication of AdV genome starts 8 h after infection, the possibility that the infected cells at 8 h might contain cells reaching late phase cannot be ruled out. To examine the change in HDGF gene expression strictly during the early phase, VA-deleted and FG AdV-infected 293 cells were treated with AraC (cytosine β-D-arabinofuranosde hydrochloride), a nucleoside analog. AraC inhibits viral DNA replication and the transition from the early phase to the late phase; thus, AraC treatment amplifies the effect during the early phase. After the isolation of cellular RNA at 8 h and 24 h after infection, the transcript level of each gene was measured using qPCR and the relative mRNA level of each gene against the steady state level was calculated (Figure 4). The levels of both transcripts in AraC-treated cells at 8 h (white bars) was expected to be similar to those in untreated cells, since the replication of the viral genome had not yet started at this time point regardless of AraC treatment. Remarkably, the induction of HDGF after VA-deleted AdV infection against uninfected cells was detected at much higher levels at 24 h (bar 2) than at 8 h (bar 1). Since AraC amplifies the effect during the early phase, this result confirmed that HDGF gene expression is induced during the early phase of infection. Furthermore, after FG AdV infection with AraC treatment, no significant suppression was observed even at 24 h (bar 4), although without AraC the HDGF level was obviously decreased (Figure 3A, white circle). This result suggested that the increase in HDGF is offset by VA RNAs at 24 h in the presence of AraC, and the amount of VA RNA is not sufficient to decrease this high HDGF level below the basal level. In contrast, no significant change was observed in the TIA-1 level after VA-deleted AdV infection in AraC-treated cells (bars 5 and 6) and at 8 h after FG AdV infection (bar 7), as expected. However, the TIA-1 level was decreased at 24 h after FG AdV infection even in AraC-treated cells (bar 8), though AraC treatment inhibits transition to the late phase. The reason for this observation is unknown, but the amount of accumulated VA RNA might be sufficient for processing to mivaRNAs to suppress TIA-1 expression, which was not increased after AdV infection. Although VA RNAs suppressed both HDGF and TIA-1, the results shown here suggested that the suppression mechanism mediated by VA RNA is different from each other.

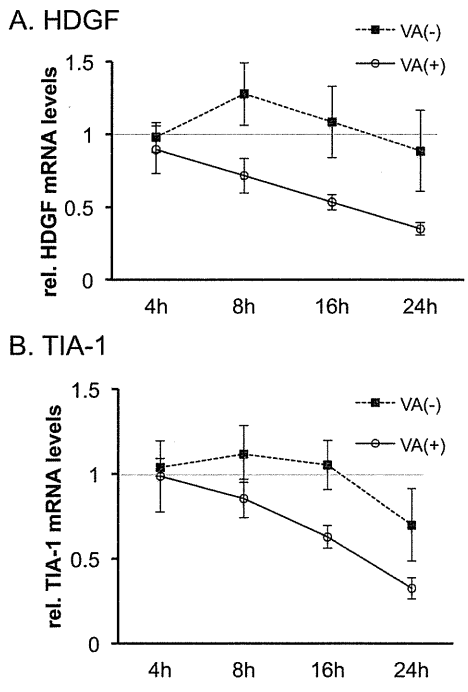

Figure 3. Suppression of HDGF begins during the early phase of viral infection. RNA was isolated from 293 cells infected with VA-deleted AdV (VA (−)) or FG AdV (VA (+)) after the indicated time periods. HDGF (A) and TIA-1 (B) mRNAs were quantified using qPCR. The expression level in uninfected cells was set at 1, and the ratio of the expression level in all the cases was calculated. The error bars show the standard deviations of three different experiments.
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Overexpression of HDGF gene inhibited VA-deleted AdV replication

Since the expression of the HDGF gene was increased after VA-deleted AdV infection during the early phase and, therefore, VA RNAs seemed to be responsible for the suppression of the increase in HDGF, we wondered whether HDGF affects viral growth. To test this hypothesis, HDGF-expressing VA-deleted AdVs and FG AdVs were constructed and used to infect 293 cells; the growth

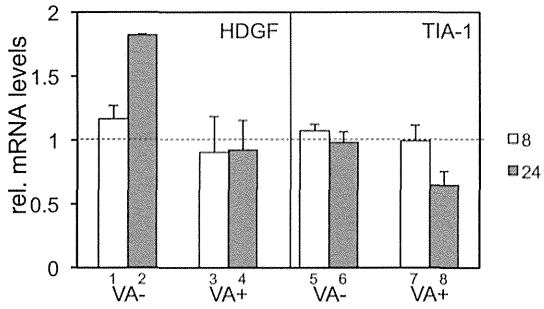

Figure 4. HDGF is upregulated during the early phase of viral infection in VA-deleted AdV-infected 293 cells. RNA was isolated from AraC-treated 293 cells infected with VA-deleted AdV (VA (−)) or FG AdV (VA (+)) after 8 h (white bars) and 24 h (gray bars), and each mRNA level was quantified using qPCR. The expression level in uninfected cells was set at 1, and the ratio of the expression level in all the cases was calculated. The error bars show the standard deviations of three different experiments.
doi:10.1371/journal.pone.0108627.g004

efficiency of each AdV was then determined using qPCR to measure the viral genome copy number (Figure 5). We applied an efficient method of generating the VA-deleted AdVs using a site-specific recombinase FLP [16]. A pre-vector, which contains VA RNA genes flanked with a pair of FRTs that are target sequences of FLP, was generated in 293 cells because it behaves as the same as FG AdVs. Subsequently, an obtained pre-vector with a high titer was used to infect 293hde12 cells [19], which are 293 cells expressing the humanized FLPe gene, to excise VA RNA genes out from the replicating AdV genome. For the efficient production of VA-deleted AdVs, a pre-vector was infected five-times more than for FG-AdV production. Under this condition, all cells are infected at once (one-step infection), and the amount of VA RNAs expressed from a pre-vector is sufficient to support the generation of HDGF-expressing VA-deleted AdVs. After AdV infection, the HDGF gene on the AdV genome is expressed exogenously under the control of a potent EF1α promoter. Therefore, the amount of HDGF protein is probably much higher than the endogenous level during AdV replication in 293 cells.

Each vector was used to infect 293 cells at an MOI of 0.5 and the infected cells were collected after 1 to 4 days. GFP-expressing AdV was used as a control. In this infection condition, only a

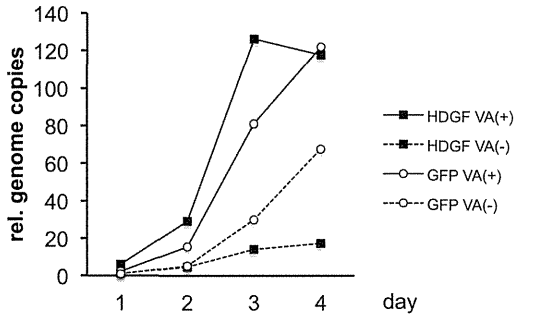

Figure 5. AdV growth in 293 cells. Total DNA was isolated from VA-deleted AdVs (VA (−)) or FG AdVs (VA (+)) infected 293 cells and each AdV genome copy was quantified using qPCR. The level of AdV genome in 293 cells after infection with GFP-expressing FG AdV on day1 was set at 1, and the ratio of the expression level in all the cases was calculated. Three independent experiments were carried out and representative results are shown.
doi:10.1371/journal.pone.0108627.g005

fraction of cells are infected and the uninfected cells are further infected by the newly produced AdVs (multistep infection). Although the growth of HDGF-expressing and GFP-expressing VA-deleted AdVs (dotted lines) was significantly lower than those of FG AdVs (solid lines), this finding was consistent with previous studies indicating a positive role of VA RNAs in viral growth [16,34]. The results clearly showed that the overexpression of the HDGF gene did not inhibit FG-AdV growth in comparison with control-FG AdV (solid lines). However, the growth of HDGF-expressing VA-deleted AdVs, the genome of which was only amplified to 20 copies on day 4, was much lower than that of GFP-expressing VA-deleted AdVs (dotted lines), which reached 70 copies. These results showed that the overexpression of the HDGF gene inhibited AdV replication as far as HDGF was not suppressed by VA RNAs.

Discussion

In this study, we demonstrated that adenovirus encoding VA RNAs suppressed HDGF gene expression. This finding revealed, for the first time, a partial role of VA RNAs in the early phase of viral infection.

The suppression of the HDGF level was observed even in cells infected with replication-deficient FG AdVs, which express a much smaller amount of VA RNAs than replicating viruses. The suppression was also detected during the early phase of viral infection in the AdV replication system, i.e., at 8 h after FG AdV infection in 293 cells. In contrast, we confirmed that TIA-1, which is suppressed by VA RNAs during the late phase of viral infection as reported by Aparicio *et al.* [15], was decreased only when VA RNAs were abundant or during the late phase of infection. Although both of these two genes, HDGF and TIA-1, were suppressed in response to VA RNAs, we revealed that the suppression of HDGF required a much smaller amount of VA RNAs than the suppression of TIA-1. This result led us to conclude that VA RNAs probably have different functions during each phase through the regulation of different gene expressions.

According to the adenovirus life cycle, the expression of E1A gene, which is a transactivator for DNA-polymerase II-dependent viral early-gene expression, starts in the immediately during the early phase. The transcription of VA RNAs mediated by DNA polymerase III is independent of E1A-regulated transcription and, therefore, starts almost at the same time as E1A. The amount of VA RNAs during the early phase is much lower than that during the late phase, since it depends on the number of genome copies, which increases to 100,000 copies per cell in the late phase. Actually, the level of VA RNAs during the early phase was about 200-times lower than that during the late phase (Table S3 in File S1). Therefore, the amount of VA RNAs expressed from replication-deficient FG-AdVs is also much smaller than that during the late phase of viral infection. It has been reported that VA RNAs are processed to microRNAs (mivaRNAs) through cellular RNAi machinery and that knockdown of Dicer using siRNA promotes the growth of VA-deleted adenoviruses [35]. However, mivaRNAs suppress TIA-1 expression only during the late phase [15] and have never been detected during the early phase of viral infection [34,36]. These findings strongly suggest that VA RNAs are processed to microRNAs only when the VA RNAs are abundant. Therefore, the suppression of HDGF gene expression by VA RNAs may not due to mivaRNAs.

In fact, our reporter assay using luciferase suggested that HDGF may not a target for mivaRNAs (Figure S2 in File S1). There is a putative target sequence for a mivaRNA in the 3′ UTR region of the HDGF gene, and we examined whether it is a target sequence

or not. As a result, no significant reduction in luciferase activity was detected when the putative seed sequence was cloned into the downstream of luciferase gene. The result suggested that, at least, the known mivaRNAs are not responsible for HDGF suppression. Together with the results shown in Figure 2 and 4, this finding indicated that the role of VA RNAs during the early phase differs from that during the late phase of viral infection, although further investigation is required to reveal the HDGF suppression mechanism mediated by VA RNAs.

The fact that the amount of VA RNA required for HDGF suppression differs from that required for TIA-1 suppression may explain why our microarray analysis did not detect the TIA-1 gene as a positive target. Aparicio *et al.* used cells transfected with a VA RNA-expressing plasmid for their microarray analysis [15]; however, it is difficult to introduce the same number of plasmid copies into 100% of the cells uniformly. Therefore, the results for cells with a high-copy number of plasmids, rather than those for cells with a low-copy number of plasmids, might be favored if a cell mixture containing both high-copy and low-copy number of plasmids is used for the microarray analysis. Consequently, they identified TIA-1 as a target of mivaRNA. In contrast, our microarray using AdVs for VA RNA transduction enabled us to introduce a small amount of VA RNAs into all the cells present in the dish in a uniform manner [18], allowing us to identify novel target genes of VA RNAs during the early phase of infection.

The E1A gene is essential for the adenovirus life cycle and viruses cannot replicate without E1A, such as AdV, which lacks the E1 genes and replicates only in E1-expressing 293 cells. Recently, the interaction of E1A with a cellular factor, CtBP (transcriptional corepressor C-terminal binding protein), has been reported to be required for the efficient E1A-mediated transactivation of early genes [37]. CtBP was initially discovered during screening for cellular factors binding to, and modulating the activity of E1A protein in Ras-mediated tumorigenesis [38]. CtBP was subsequently shown to play an important role in the regulation of cellular genes involved in growth and differentiation [39]. The C-terminal region of E1A interacts with CtBP, and an adenovirus containing the E1A mutation within the CtBP-binding motif, PLDLS, has been shown to decrease the level of early gene expression and, consequently, to inhibit viral growth.

HDGF has also been reported to be a CtBP-binding protein. Yang and Everett showed that HDGF functions as a transcriptional repressor of the SET and MYND domain containing 1 (SMYD1) gene through its interaction with CtBP using the same binding site as E1A [40]. HDGF is a transcription factor consisting of a nuclear protein with both mitogenic and angiogenic activity that is highly expressed in the developing heart and vasculature. HDGF contains an N-terminal PWWP domain and a C-terminal NLS signal. HDGF interacts with CtBP through a non-canonical binding motif (PKDLF), which is located within the PWWP domain, and represses target gene expression by binding to the promoter region leading to cell proliferation. Since both the HDGF and E1A proteins utilize the same binding site on the N-terminus of CtBP using PXDLS-like motifs [40,41], HDGF might compete with E1A to interact with CtBP. In other words, adenovirus may suppress the expression of HDGF, a cellular-CtBP binding protein, using VA RNAs so that E1A acquires an advantage for CtBP binding.

Our results showed that the upregulation of HDGF, compared with the steady-state level, was observed after infection with VA-deleted AdVs both during the early phase of the replicating condition and during replication-deficient conditions. Of note, some of other CtBP-binding proteins were also transcriptionally upregulated under the same conditions resulting in HDGF upregulation, and VA RNA suppressed these gene expressions, although the expression changes in these genes were not as noticeable as that of HDGF (Table S4 in File S1). These findings suggest that VA RNAs selectively suppress the induction in gene expressions, resulting in the expression of CtBP-binding proteins that may play a role in competitive inhibition with the E1A-CtBP interaction. Moreover, VA RNAs suppress the expression of these genes before the replication of the viral genome, i.e., during the early phase, because E1A-CtBP functions during this phase [37]. In other words, VA RNAs may act to prevent one of the host-defense mechanisms that lead to the inhibition of E1A function, which is essential for the initiation of viral replication.

In the case of AdV infection at a low MOI, which is similar to the condition for native viral infection, the growth of HDGF-expressing VA-deleted AdVs was much lower than that for GFP-expressing VA-deleted AdVs as well as FG AdVs (Figure 5). This result indicates that HDGF expression inhibits viral growth only when the replication starts from a small amount of virus. Our study using AdV at a low MOI may reflect actual viral infection during the very early phase, since a target cell does not express E1A protein before infection and viral infection does not occur at a high MOI. From this point of view, the suppression of the expression of CtBP-binding proteins mediated by VA RNAs might be advantageous for viral growth.

The E1- and E3-deleted AdVs used in this study are widely applied for various studies including gene therapy. However, this vector has two concerns. One is that it, in fact, expresses viral genes, pIX and VA RNAs. It is known that AdVs cause severe immune responses, and we have reported that a main cause is aberrant expression of immunogenic, viral pIX protein, and the pIX protein is not produced when EF1α promoter is used for transgene expression [42]. In terms of VA RNAs, it has not been clear whether a small amount of VA RNAs transcribed via AdVs affects physiological responses in the infected cells or not. The study described here is the first report to show that the VA RNAs expressed from AdVs disturb cellular gene expressions including a transcription factor, HDGF. Our results strongly suggest that production of VA RNAs would be avoided, if possible, when AdVs are applied for gene therapy, since VA RNAs expressed from FG AdVs may affect various cellular signaling pathways. Disturbance of cellular gene expression caused by VA RNAs might also affect the data in the basic study using AdVs. Moreover, although AdVs are often applied for shRNA expression, VA RNA expressed from AdVs inhibits shRNA activity [33], since VA RNAs utilize cellular RNAi machinery for processing of mivaRNAs. The present study provided further evidence that VA-deleted AdVs are useful and might be substituted for FG AdVs.

Supporting Information

File S1 **Figure S1**, HDGF is suppressed after FG AdV infection in HuH-7 cells. **Figure S2**, HDGF mRNA is not a direct target of mivaRNAI-138. **Table S1**, Gene list for gene clusters 2 and 5. **Table S2**, HDGF and TIA-1 expression levels in 293 cell lines. **Table S3**, Amount of VA RNAs after FG AdV infection in 293 cells. **Table S4**, Ratio of expression levels of genes known to be CtBP-binding proteins after AdV infection in HuH-7 cells.
(PPT)

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Author Contributions

Conceived and designed the experiments: SK YK IS. Performed the experiments: SK KY MS. Analyzed the data: SK KY. Contributed reagents/materials/analysis tools: SK YK. Wrote the paper: SK YK IS.

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ORIGINAL ARTICLE

Preferable sites and orientations of transgene inserted in the adenovirus vector genome: The E3 site may be unfavorable for transgene position

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The adenovirus vector (AdV) can carry two transgenes in its genome, the therapeutic gene and a reporter gene, for example. The E3 insertion site has often been used for the expression of the second transgene. A transgene can be inserted at six different sites/orientations: E1, E3 and E4 sites, and right and left orientations. However, the best combination of the insertion sites and orientations as for the titers and the expression levels has not sufficiently been studied. We attempted to construct 18 AdVs producing GFP or LacZ gene driven by the EF1α promoter and Cre gene driven by the α-fetoprotein promoter. The AdV containing GFP gene at E3 in the rightward orientation (GFP-E3R) was not available. The LacZ-E3R AdV showed 20-fold lower titer and 50-fold lower level of fiber mRNA than the control E1L AdV. Notably, we found four aberrantly spliced mRNAs in the LacZ-E3L/R AdVs, probably explaining their very low titers. Although the transgene expression levels in the E4R AdVs were about threefold lower than those in the E1L AdVs, their titers are comparable with that of E1L AdVs. We concluded that E1L and E4R sites/orientations are preferable for expressing the main target gene and a second gene, respectively.

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INTRODUCTION

First-generation (E1 deleted) adenovirus vectors (FG AdVs), which lack the E1 and E3 regions, are popularly used in basic studies to elucidate gene functions, and have been employed for gene therapy.[1–4] Because the DNA fragments of up to about 7 kilobases (kb) in total can be inserted into the AdV genome, the AdVs are frequently used to produce two proteins simultaneously from two independent transgenes expressing both the target gene and the reporter gene, for example. In the studies using the cultured cells and in the animal experiments, the GFP and luciferase are used as the reporters. Recently, positron emission tomography has clinically been used in patients for diagnoses and in experimental animal models. Therefore, the AdVs containing both the therapeutic gene and the positron emission tomography reporter gene would be valuable in the gene therapy fields, because the therapeutic effects, the vector duration and distribution can simultaneously be monitored.[5–8] Probably one would wish for high-titer AdVs with the highest expression for the therapeutic gene and with the second highest for the reporter gene not causing any trouble, if the insertion sites and orientations in the AdV genome can be chosen. However, the titers and the expression levels of the AdVs may considerably be influenced by the sites and orientations of the transgenes. Such information may be very valuable for construction of the best vector, especially in the vector containing both the therapeutic gene and the reporter gene.

The simultaneous expression of two genes could be achieved by inserting the two genes into the E1 site under the control of a single prompter using the internal ribosomal entry sites or using porcine teschovirus-1 2A.[9,10] In the former approach, the expression of the second gene might be influenced by the sequences between internal ribosomal entry sites and its initiation codon, and in the latter, the manipulation is necessary to remove the stop codon of the first gene and to adjust the frames of the two genes. When two genes driven by the independent promoters are inserted into the E1 site, they might interfere with each other. However, when two independent expression units are inserted in different sites in the AdV genome, no interference occurs. Moreover, the advantage of this approach is that the main target gene can easily be changed using the AdV cassette that already contains the reporter gene.

There are three insertion sites and two orientations: a transgene can be inserted into the AdV genome by substitution of the E1 or E3 gene and by simple insertion at a position upstream of the E4 gene. Therefore, there are six different possible sites/orientations for any given transgene. Moreover, not only the potent promoters such as EF1α but also tissue-specific promoters such as α-fetoprotein (AFP) can also be employed. Although the studies examining which sites/orientations are superior to others are practically important, they have been very limited[11,12] and systematic analyses have not been reported so far.

As it is known that the expression level of a transgene varies considerably depending on the site in the cell chromosome of the human genome, the phenomenon is called the 'position effect'.[13,14] Although CG-methylation in the cell chromosome is clearly one reason, it is not observed in the AdV genome. Therefore, it would be of interest to examine whether the 'position effect' might also be observed similarly in the AdV genome for the potent promoter and for the tissue-specific promoter.

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FG AdVs retain almost all viral genes. They are normally not expressed in the target cells, because E1A protein, the essential transactivator for expression of all other viral genes, is not present. However, there is one report of splicing of aberrant mRNAs from the inserted foreign genes to a viral gene.[15] In this case, the aberrant mRNAs are transcribed by strong foreign promoters and produce transgene-viral gene fusion proteins, which elicit strong immune responses. However, it is not known whether the production of the aberrant gene product between the inserted transgene and viral gene is rare or not.

In this study, we examined the AdV titers and expression levels of an identical transgene inserted at the E1, E3 and E4 sites. We used three transgenes, namely, GFP, LacZ and Cre, and two promoters, namely, the potent EF1α promoter and the cancer-specific AFP promoter, and attempted to construct AdVs using all combinations, that is, 18 AdVs, and succeeded in constructing 17 of them. We found that insertion at the E1 and E4 sites yielded mostly high titers, whereas the one at the E3 yielded variable titers. Surprisingly, four aberrantly spliced mRNAs between the transgenes and viral genes were found in the vector obtained by insertion at the E3 site, which was probably the reason for the very low titers. As for the expression levels, clear differences were observed among the vectors obtained with insertion at the E1, E3 and E4 sites despite using the identical transgene, indicating that the position effect was certainly present for the AdV genome and that aberrant splicing may, at least in part, explain this effect. We also propose a strategy to avoid generation of the aberrantly spliced mRNAs.

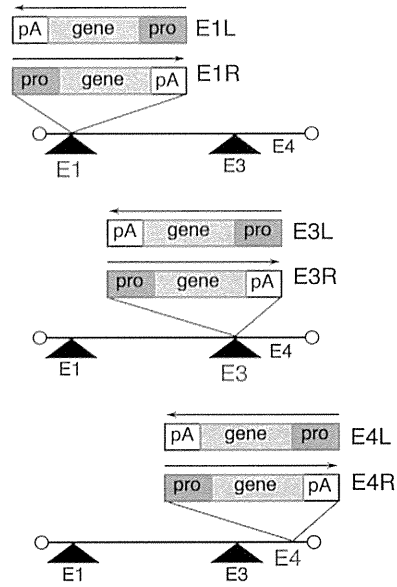

Figure 1. The FG AdV structures of six different site/orientations in all possible combinations. The box containing 'pro,' 'gene' and 'pA' represents the expression unit and the arrows show the orientation of transcription. 'pro,' EF1α and AFP promoter; gene, GFP, LacZ and Cre; pA, rabbit β-globin polyadenylation signal. For example, the vector containing the transgene at the E1 insertion site and in the left orientation is denoted as 'E1L.'

RESULTS

The vector titers were significantly influenced by the insertion sites and orientations of the transgene

We first examined whether the vector titers were influenced by the site/orientations of the transgenes containing a potent EF1α promoter. Towards this end, we attempted to construct six GFP-expressing (EF-GFP) and six LacZ-expressing (EF-LacZ) vectors in all possible combinations, that is, the E1, E3 and E4 insertion sites and the two orientations (Figure 1), and measured the vector titers (Figure 2a) (hereinafter, the vectors will be designated as per the following; the vectors containing the GFP gene and LacZ gene at the E1 insertion site and in the left orientation shall be denoted as G-E1L and Z-E1L vectors, respectively). Among the GFP-expressing vectors, high titers were obtained for G-E1L, G-E3L, G-E4L and G-E4R vectors (Figure 2a, bars 1, 3, 5 and 6), while the titer for the G-E1R vector was lower (bar 2). Notably, the G-E3R vector, that is, vector with the GFP transgene inserted in the E3 site in the rightward orientation, could not be obtained despite three independent attempts (bar 4, denote '×'). Therefore, although exactly the same EF1α-GFP expression unit was inserted in these vectors, the sites and orientations exerted considerable influence on the vector titers and even determined whether the vector was available or not. Similar results were obtained for vectors expressing LacZ: the titers of the Z-E1L, Z-E4L and Z-E4R vectors (bars 7, 11 and 12) were high, and that of the Z-E1R vector was also low (bar 8). However, the results of insertion at the E3 site differed for GFP and LacZ. The titer ratio of Z-E3L was significantly lower than that of G-E3L (compare bars 3 and 9, described later), and the Z-E3R vector was available, although its titer was extremely low (bar 10). Therefore, the GFP gene and LacZ gene themselves influenced the vector titers.

Then, we constructed six vectors containing the AFP promoter and Cre gene (AFP-Cre) and measured their titers (Figure 2b). Although these vectors contained the AFP promoter and Cre gene, this transgene unit served as a nonfunctional DNA, because the AFP promoter, which is hepatocarcinoma-cell-specific, is not active in the 293 cells. The titers of the all six vectors were very similar (Figure 2b). Thus, the site/orientation does not always influence the vector titers, and it appeared that there may be some specific reasons why the titers were low for vectors containing the EF1α promoter expressing the GFP and LacZ genes.

Aberrant chimera mRNAs were produced in the vectors containing the expression unit at the E3 site

The E3 transgene is present within the large intron from the major late promoter (MLP) to the fiber gene (Figure 3a, except the first). We previously reported an aberrant splicing from a cryptic donor site present in the LacZ gene to the viral pIX acceptor site, which produces a LacZ-pIX fusion protein.[15] Therefore, we speculated that similar aberrant splicing might occur for the LacZ gene inserted at the E3 site.

Total RNA was prepared from the 293 cells infected with the E3R vector and reverse-transcribed to detect such aberrantly spliced mRNA spanning from the LacZ cryptic donor site to the possible fiber acceptor site, which is the only acceptor site present downstream of the LacZ donor site. In fact, we identified an aberrant mRNA spliced from this LacZ donor to the fiber acceptor (Figure 3a, second; Figure 3b, 0.7-kb band). The splicing donor site in the LacZ gene was identical to that of the reported LacZ gene inserted at E1 site to the viral pIX acceptor site, and the fiber acceptor site was the same as that normally spliced from the MLP donor site (Supplementary Table S1). This is quite abnormal because, in general, splicing occurs between only specific donor and acceptor sites, suggesting that an inserted transgene could disturb normal splicing.

We also examined whether any other aberrantly spliced mRNA upstream of the transgene was present or not. Surprisingly, we also detected an abnormal mRNA spliced from the donor site of the third exon of the viral MLP to the acceptor site of the second exon of the EF1α promoter (Figure 3a, third; Figure 3b, 1.2-kb band; the junction sequence is shown in Supplementary Table S1). These results mean that the normal splicing from the MLP donor to the fiber acceptor are doubly competed with aberrant splicing

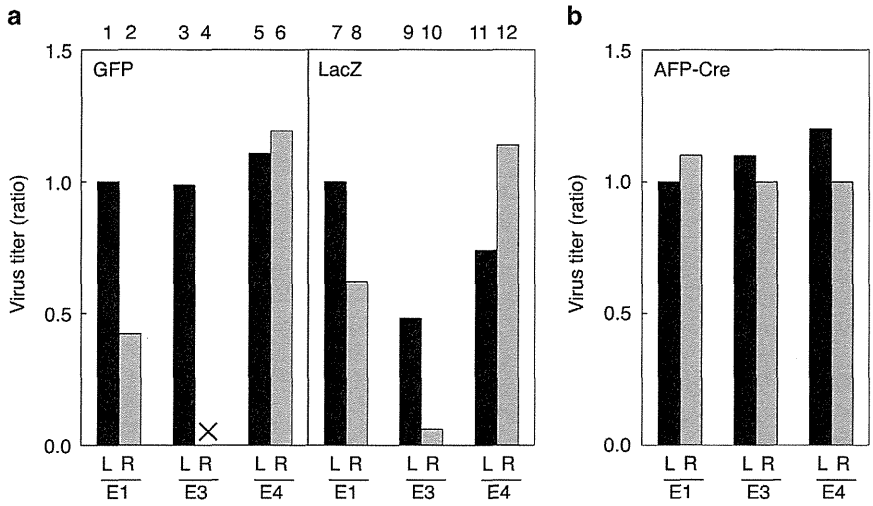

Figure 2. Titers of the virus vectors containing identical expression units. (**a**) Virus titers of the AdVs containing the EF1α promoter. The AdV genomes transduced into the HuH-7 cells were measured 3 days post infection. The virus titers were calculated relative to the copy numbers of the AdVs.[16] The titer of the E1L vector was set as 1; G-E1L, 8.3×10^8 relative virus titer (rVT)/ml, L-E1L, 5.0×10^9 rVT/ml. '×' indicates that G-E3R could not be obtained. (**b**) The titers of the virus vector containing Cre gene driven by the AFP promoter. E1L vector was used as the control. $^*P < 0.05$, $^{**}P < 0.01$.

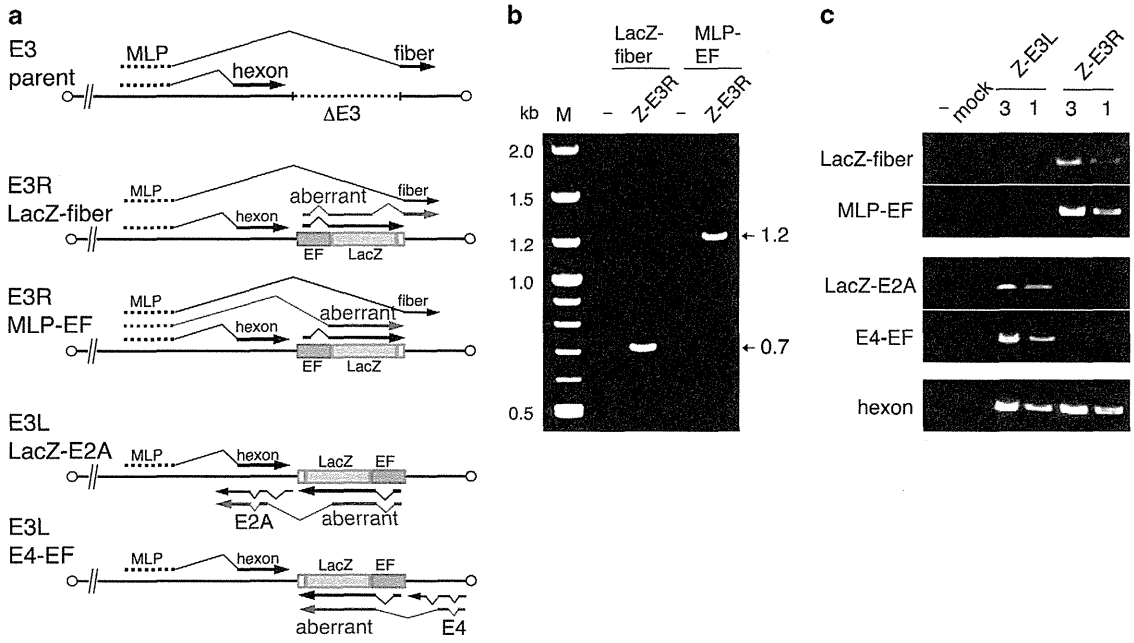

Figure 3. Structures of aberrant chimeric mRNAs. (**a**) Schematic representation of the aberrantly spliced mRNA and the expression unit in the E3 region. The LacZ expression units in the E3 region are shown. Aberrant mRNAs are shown in red. The bold lines and thin polygonal lines represent the exon and intron of the transcript, respectively. Arrow, orientation of the transcription; EF, EF1α promoter; LacZ, LacZ DNA. The 'parent' denotes the vectors before the insertion into the E3. 'LacZ-fiber,' 'MLP-EF,' 'LacZ-E2A' and 'E4-EF' indicate combinations of primers for detection of the chimeric mRNAs. (**b**) Detection of aberrant splicing by PCR. The 293 cells were infected with the Z-E3L and Z-E3R vectors, as indicated. The bands are generated from the chimera-specific mRNA between the viral gene and the inserted transgene. The primer sequences are shown in Supplementary Table S2. M, size maker; -, no DNA. (**c**) Specificity of the aberrant splicing. The 293 cells were infected with either Z-E3L or Z-E3R. The splicing from the MLP to hexon was used as the control. The bands of LacZ-fiber and MLP-EF in Z-E3R are the same as those described in (**b**) (0.7 and 1.2 kb, respectively). These bands were not detected in Z-E3L (lanes 3 and 1); threefold more DNA was loaded in lane 3 than in lane 1 to clearly show the semi-quantitative difference in the amount of cDNA. mock, mock infection of the 293 cells.

from the MLP donor to the EF1α acceptor and from LacZ donor to the fiber acceptor. We confirmed that these LacZ-fiber and MLP-EF aberrant mRNAs observed for the Z-E3R vector were not detected for the Z-E3L (Figure 3c, first and second rows).

We further examined whether such abnormal chimera mRNA was present for the Z-E3L vector of the opposite orientation. Actually, we detected two chimera mRNAs using the same PCR analysis; a viral E4 donor was spliced to the EF1α acceptor, and the

cryptic LacZ donor was spliced to a viral E2A acceptor (Figure 3a, fourth and fifth; Figure 3c, third and fourth rows. They were not detected for Z-E3R). The EF1α acceptor and LacZ donor were the same as those found in the E3L vector, and the sequences of the viral donor and the acceptor were identical to those found in the wild-type adenovirus, although the combinations were abnormal (Supplementary Table S1).

Then, we measured the amounts of fiber mRNA for the Z-E3R vector (very low titer) and compared them with those for the Z-E1L vector (high titer) and Z-E3L vector (medium titer) by conventional PCR and quantitative PCR (qPCR). These PCR and qPCR primers were designed to detect specifically the normal MLP-fiber mRNA, but not the aberrant mRNA, because they are prepared at the sequence junction: the forward and reverse primers are located in the MLP and fiber, respectively. The cDNAs from the 293 cells infected with Z-E1L, Z-E3L or Z-E3R were diluted from 10^0 to 10^{-3} before PCR (shown as '0' to '−3' in Figure 4a) for semi-quantitative detection (Figure 4a). The level of normal fiber mRNA of Z-E3L (middle titer) was lower and that of Z-E3R (very low titer) was much lower than that of Z-E1L (high titer), that is, the fiber mRNA levels and titers were well correlated. Notably, the amount of fiber mRNA of the Z-E3R vector was only 2% of that for the Z-E1L vector (Figure 4b, E3R). This may probably explain why the titer of Z-E3R was very low.

The expression of the E1 transgenes was higher than that of the E3 or E4 transgenes

The titers show amounts of infections virus particles produced by the vectors growing in the 293 cells, while expression levels are also important for the vector. The amounts of the produced gene products being influenced by the position of an identical expression unit on the cell chromosomes is referred to as the 'position effect' of gene expression.[13,14] To examine whether identical transgenes inserted into vector genomes are influenced or not by the 'position effect,' we infected the HuH-7 cells with the same numbers of active vector particles[16] expressing GFP under the control of the EF1α promoter at multiplicity of infection (MOI) 3 and 10 and measured the amounts of GFP mRNAs by qPCR (Figure 5a).

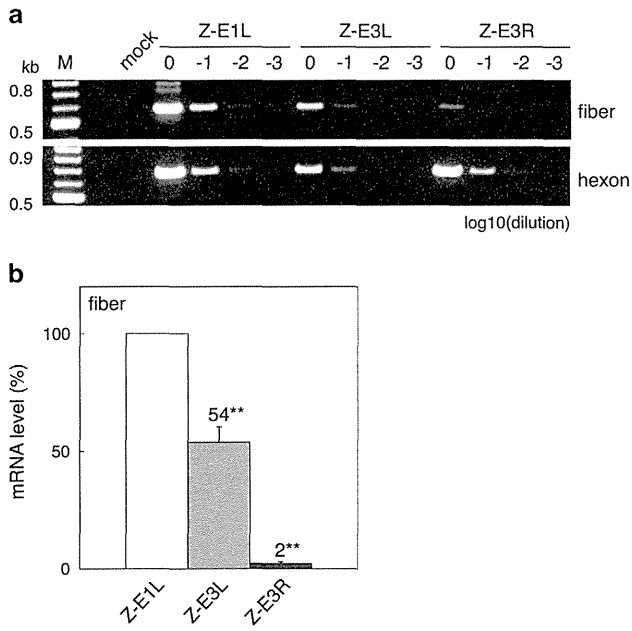

Figure 4. The relative levels of fiber mRNAs. The 293 cells were infected with the Z-E1L, Z-E3L and Z-E3R at MOI 5. (**a**) PCR detection of the fiber and hexon mRNAs. '0, -1, -2 and -3' mean '10^0, 10^{-1}, 10^{-2} and 10^{-3} dilution of the cDNAs', respectively. M, size marker; mock, mock infection of 293 cells. (**b**) The quantities of the fiber and hexon mRNAs determined by qPCR. Amount of fiber mRNA relative to those of each hexon and Z-E1L fiber are regarded as 100%. $n=3$; means ± s.d. ** $P<0.01$.

E1L and E1R vectors expressed much more GFP mRNA than the other three vectors, that is, the E3L, E4L and E4R vectors, both at MOI 3 and MOI 10 (bars 2 and 3 to 4, 6 and 7; bars 8 and 9 to 10, 12 and 13). In regard to the expression levels of these vectors, the mRNA amounts of the E4 vectors were about one-third of those of the E1 vectors. Similar results were obtained for the vectors expressing LacZ: E1L/R vectors expressed much more LacZ mRNA than all the E3 and E4 vectors, both at MOI 3 and MOI 10 (Figure 5b, bars 2 and 3 to 4–7; bars 8 and 9 to 10–13). Therefore, similar effects were observed using two different genes. These results might suggest that the position effect observed here was not dependent on the inserted transgene. It should be noted that about as much as a 50-fold more virus stock solution of Z-E3R vector could be needed than that of the Z-E1L vector to obtain the same expression level, because the titer of Z-E3R vector was about one-tenth and the expression level obtained was about one-fifth when the same volume of the virus stock solution is used for infection (Figure 2a, bars 7 and 10; Figure 5b, bars 2–5 and 8–11). We also measured the expressed protein levels of GFP and LacZ using fluorometry and β-galactosidase assay, respectively (Figure 6a and b). The G-E1 vectors produced significantly more GFP than the G-E4 vectors (Figure 6a, bars 2 and 3 to 6 and 7; bars 8 and 9 to 12 and 13), although the G-E3L vector expressed a similar level to that of the G-E1 vectors (bars 4 and 10). Also, the Z-E1 vectors expressed more LacZ than the Z-E3 and Z-E4 vectors (Figure 6b, bars 2 and 3 to 4–7; bars 8 and 9 to 10–13). These results in respect of the protein level confirm the mRNA expression levels measured by qPCR shown in Figure 5a and b.

To examine whether the position effects may also be observed for a tissue-specific promoter and for genes other than GFP and LacZ, the HuH-7 cells were infected with the vector expressing Cre under the control of the AFP promoter and the Cre expression levels were measured. The AFP promoter is specifically active in the HuH-7 cells derived from hepatocarcinoma in contrast to the case in the 293 cells. Because tissue-specific promoters, including the AFP promoter, are generally weak, the expressed Cre mRNA level was too low to measure quantitatively. Therefore, we used the method of 'excisional expression,' where the Cre enzyme driven by the AFP promoter switched on the potent EF1α promoter and specifically enhanced the expression level of dsRed by about 50-fold[17] (the strategy is shown in Supplementary Figure S1). The results were again very similar to those obtained using the EF1α promoter (Figure 6a and b): the AFP-E3 and E4 vectors expressed only about a half to one-fifth of dsRed mRNA than the AFP-E1 vectors (Figure 5c, bars 3 and 4 to 5–8, 9 and 10 to 11–14). Therefore, although the vector titers obtained using the AFP promoter were not influenced by their insertion sites (Figure 2b), the position effects at the E1, E3 and E4 sites showed very similar patterns to those of the EF1α promoter. Altogether, the E3L/R and E4L/R vectors expressed about two to fivefold less transgene products than the E1L/R vectors, not only when the potent EF1α promoter was used, but also when the tissue-specific AFP promoter was used, suggesting there may be a mechanism common to these promoters.

To examine whether the position effect of expression observed in the HuH-7 cells may also be observed in other cells, the HeLa cells were infected with the GFP-expressing vectors at MOI 3 and at MOI 10 (Figure 7a and b). The G-E1L vector expressed a significantly greater amount of mRNA than the G-E3 and G-E4 vectors (Figure 7a, bars 2 to 4, 6 and 7; bars 8 to 10, 12 and 13). However, the mRNA level of the E1R vector was not significantly different from those of the E3 and E4 vectors, because the

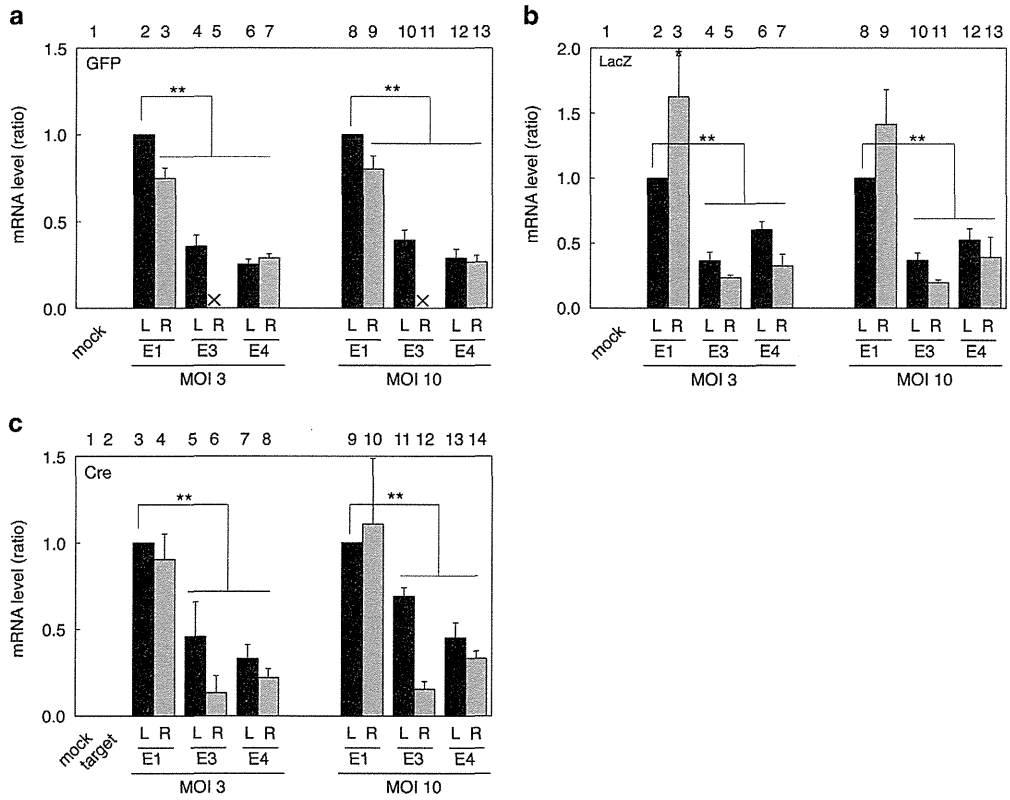

Figure 5. Expression levels of the transgene mRNA in the infected HuH-7 cells. The mRNA levels are shown relative to the mRNA level of the transgene in E1L-infected cells set as 1. (**a**) GFP mRNA levels. The cells were infected with the EF-GFP AdVs at the indicated MOIs. The GFP mRNA levels were quantified by qPCR, $n=3$. (**b**) LacZ mRNA levels. The cells were infected with the EF-LacZ AdVs. LacZ mRNA levels are shown in the same manner as that described in (**a**), $n=4$. (**c**) Cre mRNAs levels. The cells were co-infected with the AFP-Cre AdVs (switch vectors) and a target vector which expressed dsRed by Cre-mediated recombination (excisional expression). The dsRed mRNA levels were quantified in the same manner as that described in (**a**), $n=6$. Error bars indicate mean ± s.d; mock, HuH-7 cells without infection; target, HuH-7 cells infected with the target vector only; $^{*}P<0.05$, $^{**}P<0.01$. The other representations are the same as those in Figure 1.

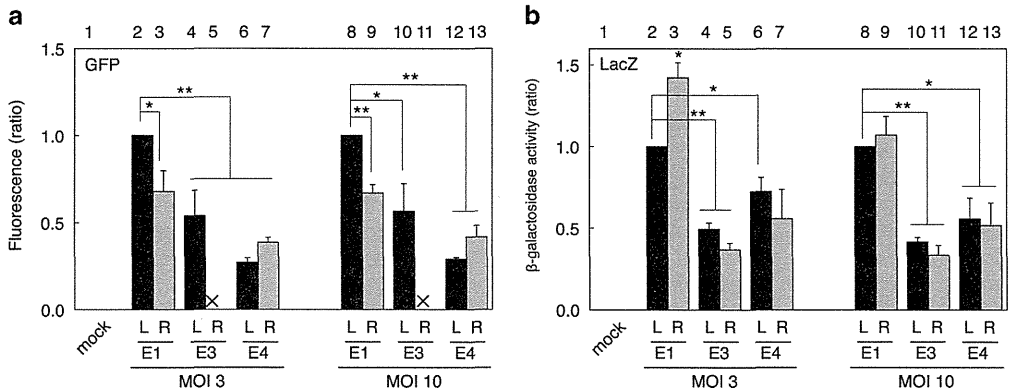

Figure 6. Protein expression levels in the infected HuH-7 cells. (**a**) Fluorescence of GFP. The cells were infected with the EF-GFP AdVs at the indicated MOIs. The fluorescence of GFP was quantified by Ascent fluorometry. The fluorescences are shown relative to the fluorescence level in E1L-infected cells set as 1, $n=4$. (**b**) Activities of β-galactosidase. The cells were infected with the EF-LacZ AdVs. β-galactosidase activities were evaluated by the β-gal assay, $n=3$. Error bars represent ± s.d.; mock, mock infection of HuH-7 cells; $^{*}P<0.05$, $^{**}P<0.01$. The other representations are the same as those in Figure 1.

expressed mRNA level of the E1R was lower than that of the E1L (bars 3 to 4, 6 and 7; bars 9 to 10, 12 and 13). Similar results were obtained using GFP fluorometry: the G-E1L vector exhibited significantly more fluorescence than the other G-E3 and G-E4 vectors (Figure 7b, bars 2 to 4, 6 and 7), whereas the E1R vector expression was not statistically significant (bars 3 to 4, 6 and 7). These results were confirmed by fluorescence microscopy (Supplementary Figure S2a). Moreover, the same results were obtained using the CV-1 cell line derived from monkey fibroblasts (Supplementary Figure S2b). Therefore, very similar position effect

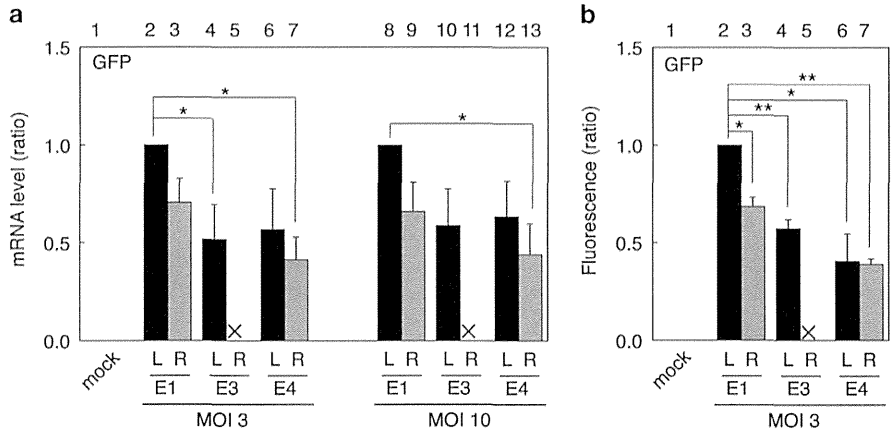

Figure 7. Expression levels of the transgene mRNA and protein in the infected HeLa cells. (**a**) GFP mRNA levels. The HeLa cells were infected with the EF-GFP AdVs at the indicated MOIs. The other representations are the same as those in Figure 4a, $n=3$. (**b**) Fluorescence of GFP. The HeLa cells were infected with EF-GFP AdVs at MOI 3. The other representations are the same as those in Figure 5a, $n=4$. Error bars indicate mean ± s.d.; mock, mock infection of HeLa cells; $^{*}P<0.05$, $^{**}P<0.01$.

among the E1, E3 and E4 insertion sites are obtained at least for the G-E1L vector.

DISCUSSION

We demonstrated in this study that the inserted sites and orientations for a given transgene greatly influenced the vector titers and expression levels. Especially, when the transgene was inserted in E3R, the GFP-expressing vector could not be obtained and the LacZ-expressing vector titer was extremely low. Also, the titer of E3L AdV was lower than that of E1L AdV. Because the aberrantly spliced mRNAs from the transgenes to a viral gene have been reported for the E1R site/orientation[15] (described below), and similar aberrant splicing might have occurred for E4L site/orientation in the same mechanism. Therefore, considering the titers, aberrant splicing and expression levels, E1L and E4R sites/orientations were preferable for the main target gene and the second gene, respectively, in the simultaneous expression. As for the titer, the information might be useful not only for FG AdVs but also for the replication-competent AdVs containing E1A gene under the control of a cancer-specific promoter, because they both are prepared using the 293 cells.

We have demonstrated that the vectors containing transgene at E1L/R showed higher titers and expression levels than other vectors. E1L/R is the most frequently used, probably because the E1 site is required or convenient for the major methods of AdV construction which are now commonly used.[18–20] For example, for the method established by the Graham's group, the use of the E1 site is essential because it exploits the viral packaging sequences partially overlapping with the E1 region. Consequently, we think that the E1 site, found to be the best in this work, might have been chosen in the currently popular methods. However, there seems to be one concern with E1R: aberrant splicing has been reported to occur to viral pIX gene from the cryptic donors present not only in the LacZ gene but also in the herpes thymidine-kinase (TK) gene, which is used for positron emission tomography as a reporter gene and for suicide gene therapy. Consequently, TK-pIX and LacZ-pIX fusion proteins were produced, and the pIX protein evokes strong immune responses.[15] For this reason, we always adopt the leftward orientation for the E1 site.

Currently, in the simultaneous expression of the target gene and the reporter gene, the E3 sites are mostly employed for the reporter gene.[21–25] However, as described here, when the LacZ-expressing transgene driven by the EF1α promoter was inserted at E3R, the vector titer and expression level were very low, probably because of the aberrant splicing, and the G-E3R AdV could not be obtained. In our experiences, the E3R AdV containing GFP gene driven by SRα promoter[26] could also not be obtained. In contrast, however, the E3R AdV containing GFP driven by CMV promoter could be obtained. The reason of such difference is unclear, but it might be related to the fact that both EF1α and SRα promoters contain the splicing unit including the splicing acceptor site in their promoters, which might produce the aberrant mRNA spliced from the MLP donor, but the CMV promoter contain no splicing unit. Because aberrant splicing was detected even at E3L, both E3R and E3L were problematic and to be avoided, if possible.

The E4 site has not frequently been used.[17,19,27–29] The E4 insertion position, *Sna*BI site, is located 162 nucleotides (nt) from the right end of the AdV genome. Vectors containing a transgene at the E4 site showed high titers, although their expression levels were lower than those of the E1 vectors. The titers and expression levels do not significantly differ between E4L and E4R vectors. However, the E4L might produce aberrantly spliced mRNAs as observed for the E3L/R. The E4R site/orientation was successfully used as the position of the second transgene[17] where Cre was expressed under the control of the AFP promoter. The expressed Cre turned on the potent promoter present at the E1L and the high-level expression of the target gene was obtained, while maintaining strict sepecificity. Interestingly, it was also recently reported that the E4L, not the E4R, is better than the E1 site for short hairpin RNA expression.[27] The difference may be related to the use of the RNA polymerase III promoter for the short hairpin RNA production, whereas the polymerase II promoter is used for the protein production. If so, the E4L may be advantageous not only for the production of short hairpin RNA, but also of guide RNAs used for the CRISPR/Cas9 system.[30,31] The reported results might not contradict with the results described here, because the RNA polymerase III expression is not involved with splicing.

Altogether, therefore, the E1L and E4R sites/orientations appear to be the best for use in AdVs for the simultaneous expression of the target gene and reporter gene, respectively. Importantly, the occurrence of aberrant splicing sometimes yields a viral-transgene fusion protein, which may induce strong immune responses caused by the viral encoding region.[15] The probability is one-third to coincide the coding frame of the transgene with that of the viral gene. The coding regions of the four aberrant mRNAs described in this report were, by chance, connected out-of-frame, yielding no transgene-fusion protein, but only a truncated LacZ protein composed of several amino acids. However, in a previous study,[15] LacZ-pIX and TK-pIX fusion proteins were produced under the control of potent promoters. Thus, the production of such fusion proteins by aberrant splicing is not a rare event.

In general, use of the same promoter may cause troubles by the homologous recombination in the simultaneous recombination. We have experienced that, when the identical promoters in the E1L and E4R sites/orientations were applied, a rearrangement through the homologous recombination occurred, whereas the AdVs in the E1L and E4L was stable. Therefore, use of different promoters would be preferable.

The harmful aberrant splicing can be avoided using the technique described herein. The aberrant splicing can be identified by PCR analysis as reported here. The preferred sequences of the splicing donor site are AGGT/AAGT (slash denotes the exon-intron junction), where the underlined GT dinucleotides are definitely required for splicing. Nucleotide mutations of these sequences, especially the GT nucleotides, can be introduced without changing the amino acid encoded by the therapeutic gene, and successful disruption of the aberrant splicing can be easily confirmed by a subsequent PCR using the same primer set. As an example, the change from T to C, G or T (Gly) in the aberrant splicing present in the TK gene removes the splicing while keeping the amino acid sequence intact, and this mutated TK gene is expected to be safer than the original TK gene for suicide gene therapy and for positron emission tomography analyses using TK gene as a reporter.

The results described here demonstrated that position effects were evident for the expression of transgenes present on the AdV genome. The mechanisms of the position effect in the AdV genome are unknown. We think that viral enhancers, silencers or other *cis*-acting sequences near to the foreign transgene promoter might influence its expression. It might be related to the fact that similar results were obtained for a strong EF1α promoter and a cancer-specific AFP promoter. In contrast to the expression levels, the vector titers were probably not influenced by the position of the transgene, because similar and high titers were obtained using a tissue-specific promoter, which does not produce the transgene mRNA in the 293 cells (Figure 2b). The results might suggest that the vector titers were mainly influenced by the combination of a strong promoter and aberrant splicing between the transgene and viral genes (Figure 2a). These results are probably valuable for efficient and safe gene therapy using FG AdVs.

In this work, the range of the E3 deletion is between two *Xba*I sites in the E3 region (nt position 28,592–30,470). Because the lengths of the E3 deletions are slightly different among the commercially available AdV construction kits, the differences might influence the results obtained here. We surmise that these differences might not influence the conclusion described here as far as the same transgene is used, because the same splicing event is essentially expected to occur by the same mechanism. However, the possibility cannot be ruled out.

The cassette plasmids (also used as cosmids) are available on request in a collaboration basis, which bear the full-length AdV genome containing the unique *Swa*I site at the E1 site and the unique *Cla*I site at the E4 site (pAxw4cit2) and that inversely containing the *Cla*I site at the E1 site and *Swa*I at the E4 site (pAxc4wit2). The method to insert a transgene into these sites is described in the Materials and Methods section.

MATERIALS AND METHODS

Cells and virus titration

Human 293,[32] HeLa and HuH-7 cell lines are derived from the human embryonic kidney, human cervical carcinoma and human hepatocellular carcinoma, respectively. The CV-1 cell line is derived from African green monkey kidney. The cells were cultured in Dulbecco's Modified Eagles Medium supplemented with 10% fetal calf serum. The 293 cells constitutively express adenoviral E1 genes and support the replication of E1-substituted AdVs. After infection with AdVs, the cells were maintained in Dulbecco's Modified Eagles Medium supplemented with 5% fetal calf serum without geneticin. FG AdVs were titrated using the method described by Pei *et al.*[16] Briefly, the copy numbers of a viral genome that was successfully transduced into the infected target cells were measured by real-time PCR (relative virus titer). HuH-7 cells were used as the target cells. The titer of the standard virus was determined using the copy number of serially diluted plasmid DNA. When FG AdVs are used, the relative virus titer (copies per ml) normally corresponds to about one-fifth of the Tissue Culture Infectious Dose 50 titer, when the gene product is not deleterious; the probable reason for this difference is that the transduction efficiency of the 293 cells is exceptionally high as compared with that of the other cells. The sequences of the TaqMan probes for the titration are derived from adenovirus-5 (Ad5) pIX gene: forward primer, 5′-TGTG ATGGGCTCCAGCATT-3′; probe, 5′-ATGGTCGCCCCGTCCTGCC-3′; reverse primer, 5′-TCGTAGGTCAAGGTAGTAGAGTTTGC-3′.

Vector construction

All the AdVs described here were constructed using the cosmid cassette pAxcwit2 containing the full-length AdV genome.[19,33] The GFP/LacZ-expression unit were under the control of the EF1α promoter[34] and the Cre-expression unit was driven by the AFP promoter; the AFP promoter used here was the (AB) 2S6 AFP promoter.[35] All the expression units were inserted into the *Swa*I cloning site at the E1 substitution region as E1L or R vectors. All the E3L and E3R vectors possessed the same expression units at the *Xba*I site in the E3 region of pAxcwit2; the *Xba*I site is originally generated by deletion between the two *Xba*I sites in the Ad5 genome (nt position 28,592-30,470). The E4 cloning site represented by the *Sna*BI site (nt position 35770) located in the E4 region at 165-nt downstream from the right end of the Ad5 genome.[17]

Conventional PCR

The PCR experiments were essentially performed by the standard method.[36] Typically, the 293 cells in the 6-well plate were infected at MOI 5 and 16 h post infection, total RNAs were prepared and reverse-transcribed using oligo(dT) primer; the resultant cDNAs were amplified by PCR with Tks Gflex DNA polymerase (Takara Bio, Shiga, Japan) and a PCR system (ProFlex PCR system, Applied Biosystems, Foster City, CA, USA). The PCR cycling conditions were in accordance with the manufacturer's protocol (Takara Bio): 94 °C for 1 min, followed by 30 cycles at 98 °C for 10 s, 60 °C for 15 s, and 68 °C for 30 s. The primer sets are described in Supplementary Table S2. The PCR products containing each splice junction were subjected to agarose gel electrophoresis.

Quantitative real-time PCR

The sequences of the GFP primers and the dsRed primers have been described previously.[17,37] The sequences of the LacZ primers were as follows: forward primer, 5′-ATCAGGATATGTGGCGGATGA-3′; probe, 5′-CGG CATTTTCCGTGACGTCT-3′; reverse primer, 5′-TGATTTGTGTAGTCGGTTTA TGCA-3′. The primer sequences of the normal splicing junctions of MLP-fiber and MLP-hexon were as follows: for MLP-fiber detection forward primer in MLP third exon, 5′-AAAGGCGTCTAACCAGTCACAGT-3′; probe in the fiber gene, 5′-AGCGCGAAGACCGTCTGAAGATACC-3′; reverse in the fiber gene, 5′-CCGCTTTCCGTGTCATATGG-3′; and for MLP-hexon detection forward primer in MLP third exon, 5′-TCTAACCAGTCACAGTCGCAAGA-3′; probe in the hexon gene, 5′-CGCGCCCGCTTTCCAAGATG-3′; reverse in the hexon gene, 5′-CACTGCGGCATCATCGAA-3′.

The mRNA levels were calculated as described by Maekawa *et al.*[37] Briefly, the total RNA of the infected cells was extracted, and the amounts of the expressed target RNAs and 18S-rRNA (correction standard) were quantified using reverse transcription (TaqMan Reverse Transcription Reagents, Roche, Basel, Switzerland) and real-time PCR (Applied Biosystems Prism 7000); the ratio of the target RNA to 18S-rRNA was then calculated. To quantify the AdV genome, the infected total cell DNA was prepared from the cells using a previously described method[29,38] or a DNA preparation kit (Macherey-Nagel, through Takara Bio). Quantitative PCR was performed to detect the AdV genome using a probe for the pIX gene, as described above.[17] The amount of chromosomal DNA was simultaneously measured to correct the Ct values of the viral genome per cell, and the corrected Ct is shown throughout. The probes were derived from the sequence of the human β-actin gene for the HeLa and HuH7 cell lines. The qPCR reaction was performed using the following cycling conditions according to the manufacturer's protocol (Applied BioSystems): 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

Measurement of the expressed GFP and LacZ

The HuH-7, HeLa and CV1 cells were infected at MOI 3 or MOI 10 of each vector in a 24-well plate in triplicated experiments. Three days after the infection, the infected cells were washed twice with phosphate-buffered saline.The cells in the three wells were then fixed with 4% paraformaldehyde to quantify the GFP fluorescence using Labsystems Fluoroskan Ascent FL (GMI, Ramsey, MN, USA) or by fluorescence microscopy. The cells infected with the LacZ vectors were harvested for the quantification of β-galactosidase (β-gal) (β-gal assay kit, Invitrogen, Carlsbad, CA, USA). To quantify the β-gal activity, the infected cells were disrupted by sonication and the lysate was subjected to a color reaction assay using o-Nitrophenyl β-D-galactopyranoside. The stained color standard was determined using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

Insertion of a transgene into the cassette plasmid (cosmid) containing both the E1 and E4 sites

The method of inserting a given transgene into the *Swa*I site of pAxw4cit2 and pAxc4wit2 is followed by the protocol of Takara Bio (Adenovirus Dual Expression Kit). Briefly, the plasmid containing the transgene DNA fragment is treated with the appropriate restriction enzymes and treated with the DNA polymerase I Klenow fragment, followed by agarose gel electrophoresis. The isolated transgene fragment of about 50 ng is ligated with 1–2 μg of the cassette cosmid at a volume of 15 μl at 15 °C for overnight. The ligated DNA is cleaved with *Swa*I to remove the self-ligated parent plasmid, and then transformation or lambda *in vitro* packaging is performed. The latter method is highly efficient and removes the deleted plasmids smaller than 38 kb. To insert a given transgene DNA to the *Cla*I site, the DNA fragment is treated with the Klenow polymerase and ligated with a DNA linker of *Bsp*T104I (Takara Bio, reaction temperature is 37 °C) or *Bst*BI (New England Biolabs, Ipswich, MA, USA, 65 °C), 5′-GGTTCGAACC-3′ (the underline shows the recognition sequences), for example, and digested with either enzyme. Because the termini produced with this enzyme can be ligated with *Cla*I-cleaved DNA and the ligated DNA cannot be cleaved with either enzyme. Therefore, after ligation of the transgene and *Cla*I-cleaved cassette, the self-ligated parent plasmid can be removed by *Cla*I digestion. Alternatively, both *Swa*I and *Cla*I sites can be converted to I-*Ceu*I, I-*Sse*I or both by insertion of the cleavage-site oligonucleotides.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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