molecular pharmaceutics

BRIEF ARTICLE

pubs.acs.org/molecularpharmaceutics

Quantitative Analysis of the Leaky Expression of Adenovirus Genes in Cells Transduced with a Replication-Incompetent Adenovirus Vector

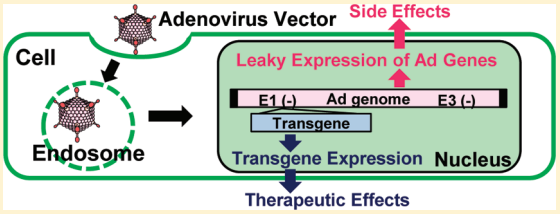
Kahori Shimizu,[†] Fuminori Sakurai,[†] Mitsuhiro Machitani,[†] Kazufumi Katayama,[†] and Hiroyuki Mizuguchi[*,†,‡,§]

[†]Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka, 565-0871, Japan

[‡]Laboratory of Stem Cell Regulation, National Institute of Biomedical Innovation, 7-6-8 Asagi Saito, Ibaraki, Osaka, 567-0085, Japan

[§]The Center for Advanced Medical Engineering and Informatics, Osaka University, 2-2 Yamadaoka, Suita, Osaka, 565-0871, Japan

ABSTRACT: Theoretically, adenovirus (Ad) genes should not be expressed following transduction with a replication-incompetent Ad vector because the E1A gene, which is essential for the expression of other viral gene, is deleted in a replication-incompetent Ad vector. However, leaky expression of viral genes is known to occur following transduction with an E1-deleted Ad vector, leading to an induction of cellular immunity against Ad proteins. To date, no detailed analysis of the leaky expression profiles of Ad genes has been performed. In this study, we systematically examined the expression profiles of Ad genes in cells following transduction with a replication-incompetent Ad vector (Ad-L2) at multiplicities of infection (MOIs) of 10 and 100 using real-time RT-PCR. Significant expression was found for the E4 and pIX genes following transduction with Ad-L2 in cultured cells. The expression levels of the E4 and pIX genes were approximately 30- to 600-fold lower than those of the transgene (firefly luciferase), and 50- to 5000-fold lower than those of the E4 and pIX genes following transduction at the same MOI with the wild-type Ad. Unexpectedly, expression levels of the major capsid proteins were approximately the same as, or even slightly above, the background levels (Ad gene expression levels in mock-transduced cells). This study provides valuable information for the design of a safe and efficient replication-incompetent Ad vector.



KEYWORDS: adenovirus vector, gene therapy, pIX, hexon, real-time RT-PCR, leaky Ad gene expression

INTRODUCTION

Replication-incompetent adenovirus (Ad) vectors are widely used in basic research and gene therapy studies because they are beneficial as a gene delivery vehicle enabling high-titer production and highly efficient gene transfer into a wide spectrum of dividing and nondividing cells *in vitro* and *in vivo*. In a replication-incompetent first-generation Ad vector, the E1A gene, which encodes an essential transactivator of the other viral genes, is deleted, in addition to the E1B gene. Therefore, theoretically, no other viral genes should be expressed following transduction; however, viral genes from the vector genome, including the E2A and E4 genes, are indeed expressed, which leads to an induction of cellular immunity against Ad proteins as well as to Ad protein-induced toxicity.[1−7] Such Ad protein-induced cellular immunity and toxicity frequently cause both an elimination of Ad vector-transduced cells and tissue damage, leading to short-lived transgene expression.[1−3,5,6,8,9] The administration of immunosuppressive agents has been shown to prolong Ad vector-mediated transgene expression, supporting the notion that cellular immunity against Ad proteins is largely involved in this type of short-lived transgene expression.[10] However, little is known about the leaky expression profiles of Ad genes in cells following transduction with a replication-incompetent Ad vector. Although the leaky expression of the Ad genes (e.g., E2A [DNA-binding protein], E4 gene, hexon, penton base, and fiber proteins) following transduction with a replication-incompetent Ad vector has been demonstrated,[1,4−7] Ad gene expression has been evaluated by Northern or Western blot analyses, both of which are less sensitive and quantitative than quantitative RT-PCR analysis. In addition, high titers of Ad vectors (≥500 MOI) were added to the cells in most previous studies,[4,6] while no systematic analysis of leaky expression has been performed. Quantitative analyses of the leaky expression of Ad genes following transduction will be crucial for designing an improved Ad vector that expresses prolonged transgene expression with minimal immunogenicity and toxicity.

In the present study, the expression profiles of Ad genes (i.e., E2A, E2B, E4, hexon, penton base, fiber proteins, and pIX) in cells following transduction with a firefly luciferase-expressing Ad vector were examined by real-time RT-PCR analysis, which enables sensitive quantification of mRNA expression levels. Following transduction with a replication-incompetent Ad vector in the cultured cells, it was found that E4 and pIX were mainly expressed, whereas the expression levels of the major capsid proteins (e.g., hexon, penton base, and fiber proteins) were quite low.

Received: March 12, 2011
Accepted: June 14, 2011
Revised: June 2, 2011
Published: June 17, 2011

EXPERIMENTAL SECTION

A Replication-Incompetent Ad Vector and Wild-Type Ad. An E1- and E3-deleted first-generation replication-incompetent Ad vector, Ad-L2, which possesses a cytomegalovirus (CMV) promoter-driven firefly luciferase expression cassette in the E1-deleted region in the reverse orientation, was previously prepared.[11] We confirmed by PCR analysis that none of the viral stocks used in this study contained detectable replication-competent virus.[12] A wild-type Ad was obtained from American Type Culture Collection (ATCC). Determination of infectious units (ifu) was accomplished using an Adeno-X Rapid Titer Kit (Clontech, Mountain View, CA).

Transduction with an Ad Vector and Wild-Type Ad. WI38 and SK HEP-1 cells were seeded at 0.6×10^5 and 1×10^5 cells per well, respectively, in a 12-well plate. On the following day, cells were transduced with Ad-L2 at MOIs of 10 and 100, or with a wild-type Ad at MOIs of 1 and 10 for 1 h. The medium was removed after 1 h incubation, and fresh medium was added to the culture. Cells were harvested at 2, 6, 12, 24, 48, 72, and 96 h after transduction.

Real-Time RT-PCR Analysis. After harvesting the cells, total RNA were extracted from the cells. The Ad gene mRNA levels were determined by real-time RT-PCR and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Thermal cycling conditions were subjected to 20 s at 95 °C, and 40 cycles of 1 s at 95 °C and 20 s at 63 °C. The plasmid encoding the Ad genome was used as a standard. The sequences of the primers and probes used in this study were as follows: E1A forward, 5′-TCC GGT CCT TCT AAC ACA CCT C-3′; E1A reverse, 5′-ACG GCA ACT GGT TTA ATG GG-3′; E1A probe, FAM-TGA GAT ACA CCC GGT GGT CCC GC-TAMRA; E1A amplicon, 71 bp; E1B forward, 5′-GAC AGG GCC TCT CAG ATG CT-3′; E1B reverse, 5′-TGG CTA CGT GAA TGG TCT TCA G-3′; E1B probe, FAM-ACC TGC TCG GAC GGC AAC TGT CA-TAMRA; E1B amplicon, 70 bp; E2A forward, 5′-CAC TAC GGT GCG AGT GCA A-3′; E2A reverse, 5′-GGT AGC TGC CTT CCC AAA AAG-3′; E2A probe, FAM-TCA AAG CCT GGG CAC GCG C-TAMRA; E2A amplicon, 61 bp; E2B forward, 5′-ACG CGC TAC CCG TAG AAA AG-3′; E2B reverse, 5′-CGG TAA GAG CCT AGC ATG TAG AAC T-3′; E2B probe, FAM-TGC GCC TAC CAG GCC GTC AAC-TAMRA; E2B amplicon, 73 bp; E4 forward, 5′-GGG ATC GTC TAC CTC CTT TTG A-3′; E4 reverse, 5′-GGG CAG CAG CGG ATG AT-3′; E4 probe, FAM-ACA GAA ACC CGC GCT ACC ATA CTG GAG-TAMRA (note that these E4 primer and probe are complementary to E4 orf6 and orf6/7 genes); E4 amplicon, 68 bp; hexon forward, 5′-ACG ATG ACA ACG AAG ACG AAG TAG-3′; hexon reverse, 5′-GGC GCC TGC CCA AAT AC-3′; hexon probe, FAM-CGA GCA AGC TGA GCA GCA AAA AAC TCA-TAMRA; hexon amplicon, 70 bp; penton base forward, 5′-GGC CGT CTA CTC CCA ACT CA-3′; penton base reverse, 5′-TCT CGG GAA AGC GAT TGA AC-3′; penton base probe, FAM-CCG CCA GTT TAC CTC TCT GAC CCA CG-TAMRA; penton base amplicon, 68 bp; fiber forward, 5′-GCG CCT ATC CGA ACC TCT AGT-3′; fiber reverse, 5′-AGA GGC CGT TGC CCA TTT-3′; fiber probe, FAM- ACC TCC AAT GGC ATG CTT GCG C-TAMRA; fiber amplicon, 65 bp; pIX forward, 5′-GCC CGC GGG ATT GTG-3′; pIX reverse, 5′-CGG GAA GCT GCA CTG CTT-3′; pIX probe, FAM-CTG ACT TTG CTT TCC TGA GCC CGC TT-TAMRA; pIX amplicon, 62 bp; luciferase forward, 5′-TCC TAT GAT TAT GTC CGG TTA TGT AAA-3′; luciferase reverse, 5′-TGT AGC CAT CCA TCC TTG TCA A-3′; luciferase probe, FAM-AAT CCG GAA GCG ACC AAC GCC-TAMRA; luciferase amplicon, 75 bp; GAPDH forward, 5′-GGT GGT CTC CTC TGA CTT CAA CA-3′; GAPDH reverse, 5′-GTG GTC GTT GAG GGC AAT G-3′; GAPDH probe, FAM-CAC TCC TCC ACC TTT GAC GCT GGG-TAMRA; GAPDH amplicon, 79 bp. We confirmed that the amplification efficiencies of the real-time RT-PCR reaction were close to 100%.

RESULTS AND DISCUSSION

In order to systematically examine the expression profiles of the Ad genes in cells following transduction with a replication-incompetent Ad vector, a firefly luciferase-expressing Ad vector, Ad-L2,[11] transduced WI38 and SK HEP-1 cells at multiplicities of infection (MOI) of 10 and 100. Subsequently, mRNA levels of the Ad genes, including the E2A, E2B, E4, hexon, penton base, fiber, and pIX genes, were determined by real-time RT-PCR analysis 2, 6, 12, 24, 48, 72, and 96 h after transduction. WI38 cells, which are a normal human lung diploid fibroblast, and SK HEP-1 cells, which are a human liver endothelial cell line, were used in this study in order to minimize the influence of Ad vector genome replication, which is often found in tumor cells,[13] on the leaky expression profiles of the Ad genes. Significant replication of the Ad vector genome was not found in SK HEP-1 cells.[13] A wild-type Ad was also added to the cells to compare the Ad gene expression profiles of a replication-incompetent Ad vector and a wild-type Ad.

In WI38 cells, apparent expression was found for the E4 and pIX genes following transduction with Ad-L2 (Figures 1a, 2a). Expression levels of the E4 and pIX genes were similar. Significant expression of the pIX gene was detectable from 12 and 24 h after transduction with 100 MOI and 10 MOI Ad-L2, respectively. The pIX expression levels obtained with 10 MOI Ad-L2 at the peaks (72 and 96 h after transduction) were more than 2500-fold lower than those obtained with the 10 MOI wild-type Ad. Moreover, E4 gene expression was first detected 12 and 24 h after transduction with 100 MOI and 10 MOI Ad-L2, respectively, whereby levels gradually increased, and reached a plateau 24 h after transduction. The E4 gene expression levels obtained with 10 MOI Ad-L2 at the peaks (48, 72, and 96 h after transduction) were 100-fold lower than those seen with the wild-type Ad at a MOI of 10. Expression levels of the E4 and pIX genes were approximately 60- to 600-fold lower than those of the luciferase gene, which was driven by the CMV promoter. Statistically significant expression of the E2A gene was detected starting 12 h after transduction with 100 MOI Ad-L2; however, the expression levels of the E2A were much lower than those of the pIX and E4 genes. Ad-L2 at a MOI of 10 did not exhibit any significant E2A expression. In addition, Ad-L2 at a MOI of 100 exhibited levels of E2A expression that were 3 orders of magnitude lower than those of the 10 MOI wild-type Ad at 48, 72, and 96 h after transduction. Expression levels of E2B and the major capsid proteins, including hexon, penton base, and fiber proteins, were almost the same as background levels in WI38 cells, although robust expression of the E2B and major capsid proteins was found for the wild-type Ad. E1A and E1B gene expression was detected starting at 12 or 24 h, respectively, in the case of the wild-type Ad.

The expression patterns of the Ad genes following transduction with Ad-L2 in SK HEP-1 cells were similar to those in WI38

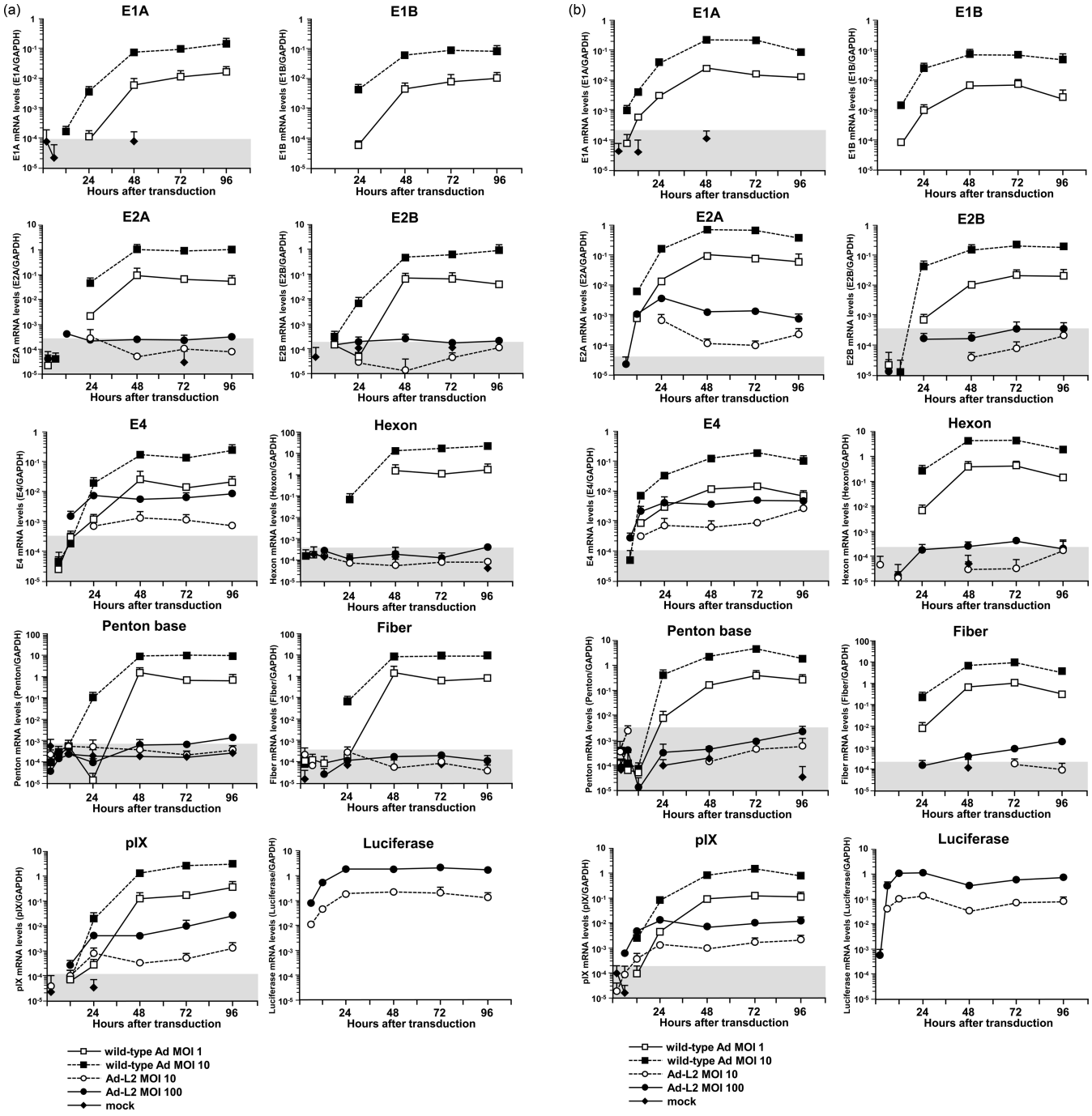


Figure 1. Time course profiles of Ad gene expression in WI38 and SK HEP-1 cells following transduction with a replication-incompetent Ad vector. WI38 (a) and SK HEP-1 (b) cells were transduced with Ad-L2 at MOIs of 10 (○) and 100 (●), with a wild-type Ad at MOIs of 1 (□) and 10 (■), or mock-transduced (◆) for 1 h, and the Ad gene expression levels were determined 2, 6, 12, 24, 72, and 96 h after transduction as described in the Experimental Section. The gray shaded boxes mean mRNA levels with no significant differences between gene expression in cells transduced with Ad-L2 and mock-transduced cells ($p > 0.05$). Expression of E1B and luciferase gene was undetectable by real-time RT-PCR analysis in mock-transduced cells. The data are expressed as the mean values ± SD ($n = 3–4$).

cells although the Ad gene expression levels in SK HEP-1 cells were higher than those in WI38 cells (Figure 1b, 2b). High levels of the leaky expression of Ad genes in SK HEP-1 cells were probably due to the greater efficiency of transduction with the Ad vectors in SK HEP-1 cells than in WI38 cells. Compared to WI38 cells, SK HEP-1 cells expressed a higher level of coxsackievirus and adenovirus receptor (CAR; data not shown). Significant expression of the E2A, E4, and pIX genes was detected in SK HEP-1 cells. Expression of the E2A gene was detected starting at 12 and 24 h following transduction with 100 MOI and 10 MOI Ad-L2, respectively. Expression levels of the E2A gene by 10 MOI Ad-L2 were more than 1500-fold lower than those observed with 10 MOI wild-type Ad 48, 72, and 96 h after transduction. The expression of the E4 and pIX genes was detectable starting at

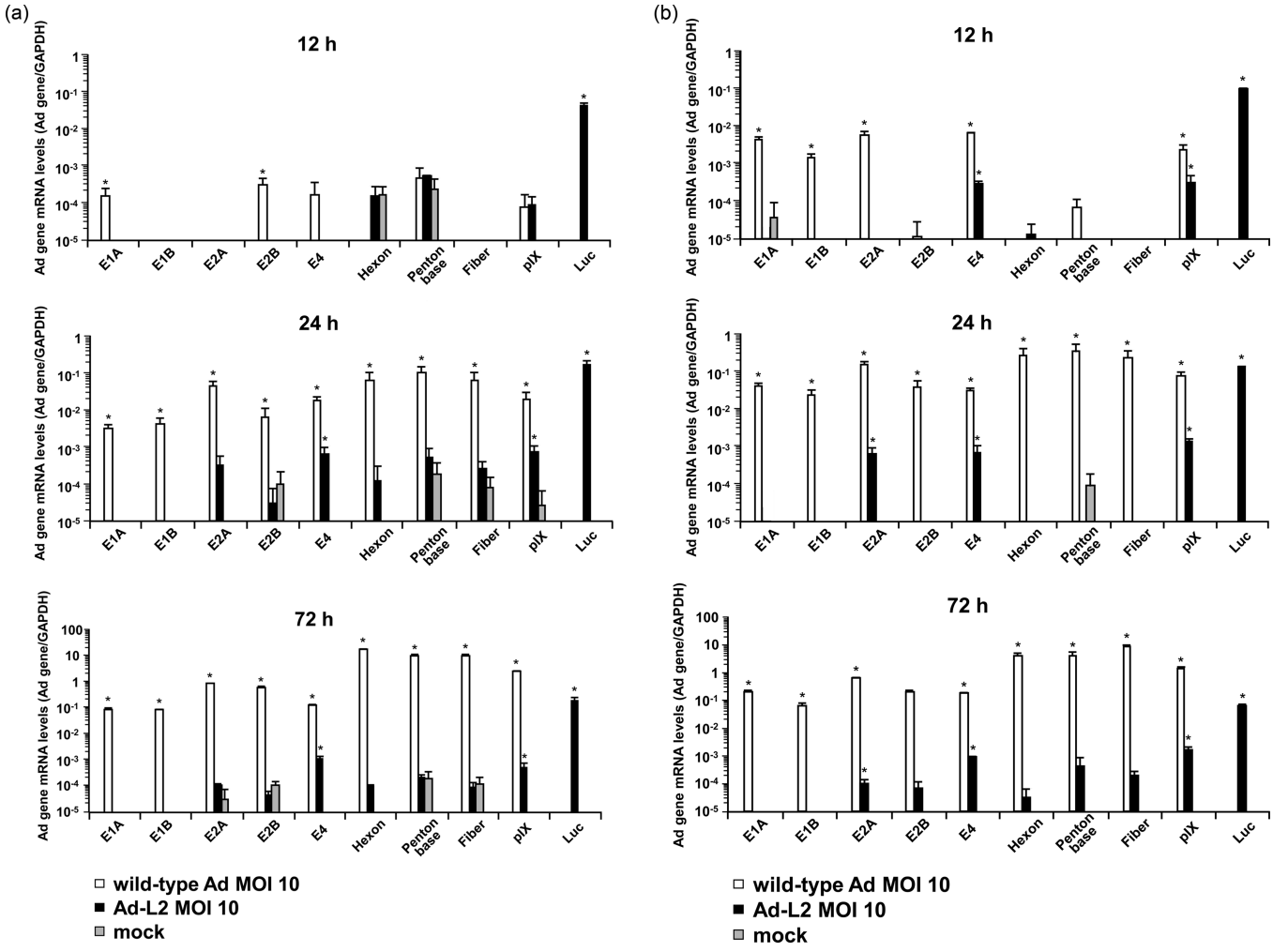


Figure 2. Comparison of Ad gene expression levels between a replication-incompetent Ad vector and a wild-type Ad at MOI 10. WI38 (a) and SK HEP-1 cells (b) were transduced with Ad-L2 (closed bar), the wild-type Ad at a MOI of 10 (open bar), or mock (gray bar), and the Ad gene expression levels were determined 12, 24, and 72 h after transduction as described in the Experimental Section. Significant difference, $^*p < 0.05$ compared with mock-transduced cells. The data are expressed as the mean values ± SD ($n = 3-4$).

6 h after transduction in the case of 100 MOI, and 12 h after transduction in the case of 10 MOI. A difference of approximately 200-fold in E4 expression by 10 MOI Ad-L2 and a 1000-fold difference in pIX expression levels by wild-type Ad were found 48 and 72 h after transduction. In SK HEP-1 cells, statistically significant expression of the fiber protein was seen 72 and 96 h after transduction with 100 MOI Ad-L2, although expression of the hexon and penton base remained at almost background levels at all time points examined.

In this study, we systematically examined the leaky expression profiles of Ad genes following transduction with a replication-incompetent Ad vector (Ad-L2) and a wild-type Ad. Among the Ad genes examined, the pIX gene was mainly expressed after transduction with Ad-L2. The pIX is a minor capsid protein crucial for the stability of Ad viral particles, especially thermostability, by associating with the hexons that make up the facets of the icosahedrons.[14−16] The promoter of the pIX gene contains an SP1 binding site and a TATA box and is remarkably similar to the E1B promoter.[17] Nakai et al. also demonstrated leaky expression of the pIX gene following transduction with a replication-incompetent Ad vector.[18] They also reported observing a higher amount of pIX was expressed when the transgene expression cassette was inserted into the E1-deleted region in the forward orientation, as opposed to in the reverse orientation. The Ad-L2 possessed used in that study a luciferase expression cassette in the reverse orientation; however, expression levels of the pIX gene were still the highest among those of Ad genes following transduction with Ad-L2. The pIX protein could be recognized as a main immune antigen of Ad proteins following *in vivo* transduction with a replication-incompetent Ad vector, even when the transgene expression cassette was inserted in the reverse orientation.

Previous studies have demonstrated leaky expression of the Ad major capsid proteins following transduction with a replication-incompetent Ad vector.[4−6] On the other hand, following transduction with 100 MOI Ad-L2, expression levels of the major capsid proteins (including the hexon, penton base, and fiber proteins) were similar to or slightly above those in the mock-transduced cells in this study. This discrepancy was probably due to differences in the Ad vector titers. In a previous study demonstrating leaky expression of the major capsid proteins, the cells were transduced with Ad vectors at an MOI of 500,[6] whereas in the present study, Ad-L2 transduced the cells at MOIs of 10 and 100. Moreover, previous reports have also noted the leaky expression of major capsid proteins (hexon, penton base, and

fiber protein) in A549 cells.[4,6] A549 cells have been demonstrated to possess E1A-like transactivating activity that supports the vector genome replication of E1-deleted adenovirus vectors.[13,19] In contrast, SK HEP-1 cells, in which the vector genome DNA did not replicate,[13] were used in this study. Thus difference between cells in terms of replication levels of the vector genome might lead to differences in levels of leaky expression of the Ad major capsid proteins.

The E4 gene encodes the products of open reading frame (ORF)1, ORF2, ORF3, ORF3/4, ORF4, ORF6, and ORF/7, which share the common 5′- and 3′-terminal sequences. The E4 gene products have several pleiotropic functions involved in transcription, apoptosis, cell cycle, DNA replication, and viral late mRNA accumulation.[6,20] The E4 promoter region includes the binding sites for ATFs (the activating transcription factors) and E4F.[21] Improved replication-incompetent Ad vectors in which the E4 genes were deleted from the genome have been developed to remove E4 gene expressions.[5,6,8] E1- and E4-deleted Ad vectors exhibited reduced host cellular immune responses and prolonged transgene expression.[5,22,23] In this study, the E4 genes were clearly expressed after transduction with Ad-L2, suggesting that the deletion of E4 genes from the vector genome would be a reasonable strategy for improving E1-deleted Ad vectors. In addition to the E4-deleted replication-incompetent Ad vectors, pIX-deleted Ad vectors have also been developed.[24] In these previous studies, the pIX gene was deleted to increase the insertion capacity for the transgene. It remains unclear whether deletion of the pIX gene improves cellular immunity against Ad proteins following Ad vector transduction; however, cellular immunity against Ad proteins might be reduced by deletion of the pIX gene. This study demonstrated significant leaky expression of the pIX gene following transduction, suggesting that cellular immunity against pIX might be induced in the manner described above.

The Ad vectors in which not only the E1 genes but also the E4 genes and/or the pIX gene are deleted have been developed by using packaging cell lines expressing the E4 genes and/or the pIX gene; however, it is difficult to make these packaging cells.[6,24,25] Furthermore, the total viral yields of the E1/E4-, the E1/pIX-, and the E1/pIX/E4-deleted Ad vectors were reduced compared those obtained with the E1-deleted first-generation Ad vectors.[6,24] The helper-dependent Ad (HD-Ad) vectors (also known as high-capacity, "gutless" or "gutted" vectors), which lack all viral coding sequences, have been developed. HD-Ad vectors show reduced inflammation and cellular infiltration, and persistent transgene expression.[26] However, the preparation of HD-Ad vectors suffers from low yield, especially when large amounts of HD-Ad vectors are required, partly due to the inefficiency of physical separation required for purification of the vector from the helper virus,[27] in spite of develoment of improved methods for HD-Ad vector production.[28] A simple and easy preparation system for Ad vectors exhibiting safer transduction properties would be necessary, and the profile of the leaky expression of Ad genes shown in this study provides important clues to prepare safer Ad vector easily.

In summary, we quantitatively analyzed the leaky expression profiles of Ad genes using real-time RT-PCR following transduction with a replication-incompetent Ad vector. The pIX and E4 genes were the most highly expressed among the Ad genes examined, and their expression levels were 1/50 to 1/5000 of the expression levels observed with the same MOI of the wild-type Ad. On the other hand, the expression levels of E2B and major capsid proteins (including the hexon, penton base, and fiber proteins) were almost the same as, or even slightly above, those observed in mock-transduced cells. These findings are expected to be quite useful for the design of safe and efficient Ad vectors for successful gene therapies. Based on the findings in this study, development of a novel Ad vector suppressing the leaky expression of Ad genes is currently underway.

AUTHOR INFORMATION

Corresponding Author

*Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka, 565-0871, Japan. E-mail: mizuguch@phs.osaka-u.ac.jp. Tel: +81-6-6879-8185. Fax: +81-6-6879-8186.

ACKNOWLEDGMENT

The authors would like to thank Koyori Yano (National Institute of Biomedical Innovation, Osaka, Japan) for her help. This work was supported by a Grant-in-Aid for Young Scientists (A) and Scientific Research (B) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan, by a grant from the Ministry of Health, Labour, and Welfare of Japan, and by a grant from the Takeda Science Foundation.

REFERENCES

(1) Yang, Y.; Nunes, F. A.; Berencsi, K.; Furth, E. E.; Gönczöl, E.; Wilson, J. M. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 4407–4411.

(2) Yang, Y.; Ertl, H. C.; Wilson, J. M. MHC class I-restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. *Immunity* **1994**, *1*, 433–442.

(3) Yang, Y.; Xiang, Z.; Ertl, H. C.; Wilson, J. M. Upregulation of class I major histocompatibility complex antigens by interferon gamma is necessary for T-cell-mediated elimination of recombinant adenovirus-infected hepatocytes in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 7257–7261.

(4) Gorziglia, M. I.; Kadan, M. J.; Yei, S.; Lim, J.; Lee, G. M.; Luthra, R.; Trapnell, B. C. Elimination of both E1 and E2 from adenovirus vectors further improves prospects for in vivo human gene therapy. *J. Virol.* **1996**, *70*, 4173–4178.

(5) Gao, G. P.; Yang, Y.; Wilson, J. M. Biology of adenovirus vectors with E1 and E4 deletions for liver-directed gene therapy. *J. Virol.* **1996**, *70*, 8934–8943.

(6) Lusky, M.; Christ, M.; Rittner, K.; Dieterle, A.; Dreyer, D.; Mourot, B.; Schultz, H.; Stoeckel, F.; Pavirani, A.; Mehtali, M. In vitro and in vivo biology of recombinant adenovirus vectors with E1, E1/E2A, or E1/E4 deleted. *J. Virol.* **1998**, *72*, 2022–2032.

(7) Sharma, A.; Bangari, D. S.; Tandon, M.; Pandey, A.; HogenEsch, H.; Mittal, S. K. Comparative analysis of vector biodistribution, persistence and gene expression following intravenous delivery of bovine, porcine and human adenoviral vectors in a mouse model. *Virology* **2009**, *386*, 44–54.

(8) Wang, Q.; Finer, M. H. Second-generation adenovirus vectors. *Nat. Med.* **1996**, *2*, 714–716.

(9) Chen, D.; Murphy, B.; Sung, R.; Bromberg, J. S. Adaptive and innate immune responses to gene transfer vectors: role of cytokines and chemokines in vector function. *Gene Ther.* **2003**, *10*, 991–998.

(10) Engelhardt, J. F.; Ye, X.; Doranz, B.; Wilson, J. M. Ablation of E2A in recombinant adenoviruses improves transgene persistence and decreases inflammatory response in mouse liver. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 6196–6200.

(11) Mizuguchi, H.; Koizumi, N.; Hosono, T.; Utoguchi, N.; Watanabe, Y.; Kay, M. A.; Hayakawa, T. A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob. *Gene Ther.* **2001**, *8*, 730–735.

(12) Suzuki, E.; Murata, T.; Watanabe, S.; Kujime, Y.; Hirose, M.; Pan, J.; Yamazaki, T.; Ugai, H.; Yokoyama, K. K. A simple method for the simultaneous detection of E1A and E1B in adenovirus stocks. *Oncol. Rep.* **2004**, *11*, 173–178.

(13) Nelson, J. E.; Kay, M. A. Persistence of recombinant adenovirus in vivo is not dependent on vector DNA replication. *J. Virol.* **1997**, *71*, 8902–8907.

(14) Parks, R. J. Adenovirus protein IX: a new look at an old protein. *Mol. Ther.* **2005**, *11*, 19–25.

(15) Vellinga, J.; Van der Heijdt, S.; Hoeben, R. C. The adenovirus capsid: major progress in minor proteins. *J. Gen. Virol.* **2005**, *86*, 1581–1588.

(16) Colby, W. W.; Shenk, T. Adenovirus type 5 virions can be assembled in vivo in the absence of detectable polypeptide IX. *J. Virol.* **1981**, *39*, 977–980.

(17) Babiss, L. E.; Vales, L. D. Promoter of the adenovirus polypeptide IX gene: similarity to E1B and inactivation by substitution of the simian virus 40 TATA element. *J. Virol.* **1991**, *65*, 598–605.

(18) Nakai, M.; Komiya, K.; Murata, M.; Kimura, T.; Kanaoka, M.; Kanegae, Y.; Saito, I. Expression of pIX gene induced by transgene promoter: possible cause of host immune response in first-generation adenoviral vectors. *Hum. Gene Ther.* **2007**, *18*, 925–936.

(19) Imperiale, M. J.; Kao, H. T.; Feldman, L. T.; Nevins, J. R.; Strickland, S. Common control of the heat shock gene and early adenovirus genes: evidence for a cellular E1A-like activity. *Mol. Cell. Biol.* **1984**, *4*, 867–874.

(20) Tauber, B.; Dobner, T. Molecular regulation and biological function of adenovirus early genes: the E4 ORFs. *Gene* **2001**, *278*, 1–23.

(21) Jones, C.; Lee, K. A. E1A-mediated activation of the adenovirus E4 promoter can occur independently of the cellular transcription factor E4F. *Mol. Cell. Biol.* **1991**, *11*, 4297–4305.

(22) Wang, Q.; Greenburg, G.; Bunch, D.; Farson, D.; Finer, M. H. Persistent transgene expression in mouse liver following in vivo gene transfer with a delta E1/delta E4 adenovirus vector. *Gene Ther.* **1997**, *4*, 393–400.

(23) Gorziglia, M. I.; Lapcevich, C.; Roy, S.; Kang, Q.; Kadan, M.; Wu, V.; Pechan, P.; Kaleko, M. Generation of an adenovirus vector lacking E1, E2a, E3, and all of E4 except open reading frame 3. *J. Virol.* **1999**, *73*, 6048–6055.

(24) Krougliak, V.; Graham, F. L. Development of cell lines capable of complementing E1, E4, and protein IX defective adenovirus type 5 mutants. *Hum. Gene Ther.* **1995**, *6*, 1575–1586.

(25) Caravokyri, C.; Leppard, K. N. Constitutive episomal expression of polypeptide IX (pIX) in a 293-based cell line complements the deficiency of pIX mutant adenovirus type 5. *J. Virol.* **1995**, *69*, 6627–6633.

(26) Kim, I. H.; Józkowicz, A.; Piedra, P. A.; Oka, K.; Chan, L. Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 13282–13287.

(27) Kochanek, S.; Clemens, P. R.; Mitani, K.; Chen, H. H.; Chan, S.; Caskey, C. T. A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 5731–5736.

(28) Palmer, D.; Ng, P. Improved system for helper-dependent adenoviral vector production. *Mol. Ther.* **2003**, *8*, 846–852.