*Kidney International, Vol. 47 (1995), pp. 322–326*

# Highly efficient adenovirus-mediated gene transfer into renal cells in culture

HANGIL CHANG, TETSUO KATOH, MASAKUNI NODA, YUMI KANEGAE, IZUMU SAITO, SHIGETAKA ASANO, and KIYOSHI KUROKAWA

*Fourth and First Departments of Internal Medicine, University of Tokyo School of Medicine, and Laboratory of Molecular Genetics, Institute of Medical Science, University of Tokyo, Tokyo, Japan*

Nephrologists, studying the function of the kidney which is structurally so complex, have advanced their research analyzing more and more elementary substructures. They proceeded from the whole organ, through dissected tubules, to cultured renal cells. Information acquired in this way is essential in constructing, from physicochemical principles, our integrated view of the renal function. In this direction of research one further promising step would be to purposefully express the relevant molecules in cultured cells and evaluate their properties. Recent successes in cloning the molecules essential for renal function encourage us in attempting these types of experiments. For this purpose, cultured renal cells would be a suitable host by mimicking natural environment for those molecules, and we require a means for efficiently introducing foreign genes into these cells.

Adenovirus-based vector is a recently developed gene transfer system which has several advantages over the conventional methods such as calcium phosphate precipitation, DEAE dextran, electroporation, and lipofectin [1, 2]. Adenovirus vector: (1) is highly efficient in transferring foreign genes into cells and, unlike retrovirus vectors, can be applicable for non-replicating cells; (2) is easily multiplied in proper host cells; (3) accepts large DNA fragments (up to 7.5 kb); and (4) achieves steady, although transient, expression for weeks [2]. Regarding the safety of adenovirus-based vectors: (1) a replication-deficient adenovirus vector has been developed which lacks E1 and E3 regions from its genome, and can proliferate only in defined host cells such as 293 cells; (2) live vaccination of adenovirus has had been safely applied in humans [1]; and (3) most adult humans have acquired immunity against adenovirus.

This study was undertaken to determine whether adenovirus-based vector can direct efficient gene transfer into renal cells in culture. Recombinant adenovirus vector, carrying *E. coli lacZ* gene as the reporter gene, was constructed by homologous recombination and used for the experiments. Three types of renal cells were examined: one mesangial (primary cultured rat glomerular mesangial cells) and two tubular ($LLCPK_1$ and MDCK cells).

Received for publication February 22, 1994
and in revised form July 29, 1994
Accepted for publication August 2, 1994



## Methods

### *Cell culture*

Primary culture of rat glomerular mesangial cells was as described previously [3]. $LLCPK_1$ and MDCK cells were obtained from the Japanese Cancer Research Resources Bank. They were maintained with DMEM medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Trypsin-EDTA solution (0.5 g/liter trypsin and 0.2 g/liter EDTA · 4Na) was used to detach the cells for subculture.

### *Adenovirus vector*

The recombinant adenovirus Adex1EFLacZL, carrying *lacZ* gene which codes for a *E. coli* β-galactosidase, was constructed by homologous recombination between the expression cosmid cassette and the parental virus genome. The method was a modification of Saito et al [4] and the detailed procedure will be published elsewhere (Miyake et al, manuscript in preparation). Briefly, the expression cosmid cassette was constructed by inserting the expression unit, comprised of the elongation factor 1α (EF1α) promoter, *lacZ* coding sequence, and polyA sequence, into the SwaI site of pAdex1w. We used EF1α promoter because it is not only very potent but also effective in a wide range of cell types [5]. pAdex1w is a 42 kb cosmid containing 31 kb adenovirus type 5 genome lacking E1A, E1B, and E3 genes. The expression cosmid cassette and adenovirus DNA-terminal protein complex (DNA-TPC) was cotransfected into 293 cells by the calcium phosphate precipitation method. DNA-TPC is the viral genome with its terminal proteins covalently bound to its 5′ ends. The 293 cell, derived from human embryonic kidney, is transformed by incorporating adenovirus E1A and E1B genes into its genome; it allows proliferation of E1A and E1B deleted replication-deficient adenovirus vectors. DNA-TPC was purified from HeLa cells infected with Ad5-dlX [4], which is an adenovirus type 5 lacking E3 region, by the CsCl density centrifugation method. DNA-TPC was digested with EcoT22I, which cleaved the genomic DNA at seven sites, thus destroying the regions including E1A, E1B, and E2, but preserving the 21 kb right hand side of the genomic DNA. Incorporation of the expression cassette into the isolated recombinant virus was confirmed by comparing the DNA fragment pattern of the obtained adenovirus DNA and the expression cassette after digestion with appropriate restriction enzymes. The

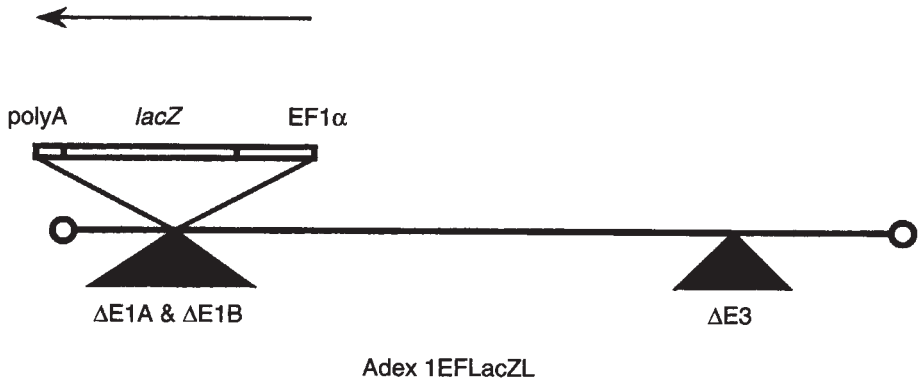



**Fig. 1.** *Diagram of the recombinant Adex1EFLacZL genome.* From wild type adenovirus type 5, genomic regions for E1A, E1B, and E3 were deleted (indicated by filled triangles labeled "ΔE1A & ΔE1B" and "ΔE3".) Expression cassette, consisting of promoter of elongation factor-1α (EF1α), *E. coli* β-galactosidase gene (*lacZ*), and polyA tail, was incorporated in place of E1A and E1B. Arrow indicates the transcriptional direction of the expression cassette.

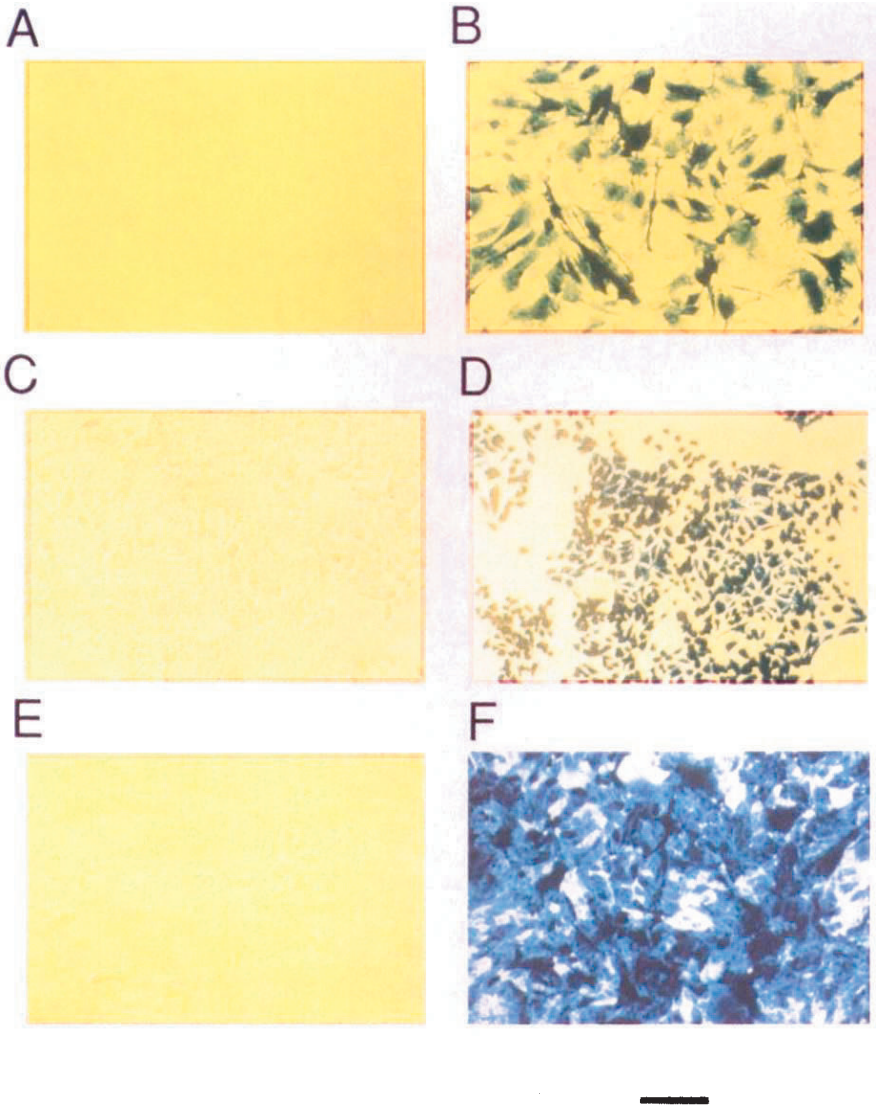



**Fig. 3.** *Dose dependency of* lacZ *gene expression on the relative number of Adex1EFLacZL applied.* **A.** LLCPK$_1$ cells infected with various concentrations of Adex1EFLacZL. Cells were seeded in 16 mm diameter culture well and infected with 18 μl of virus solutions. β-Galactosidase activity was detected 48 hours later. Concentrations of the virus solutions were (from the left column of wells to the right) 0, $0.8 \times 10^6$ (0.65 moi), $0.8 \times 10^7$ (6.5 moi), $0.8 \times 10^8$ (65 moi), and $0.8 \times 10^9$ pfu/ml (650 moi), respectively. B to F: Magnified view (100 × magnification) of the cells. B: 0 pfu/ml. C: $0.8 \times 10^6$ pfu/ml. D: $0.8 \times 10^7$ pfu/ml. E: $0.8 \times 10^8$ pfu/ml. F: $0.8 \times 10^9$ pfu/ml. Horizontal bar indicates 200 μm.

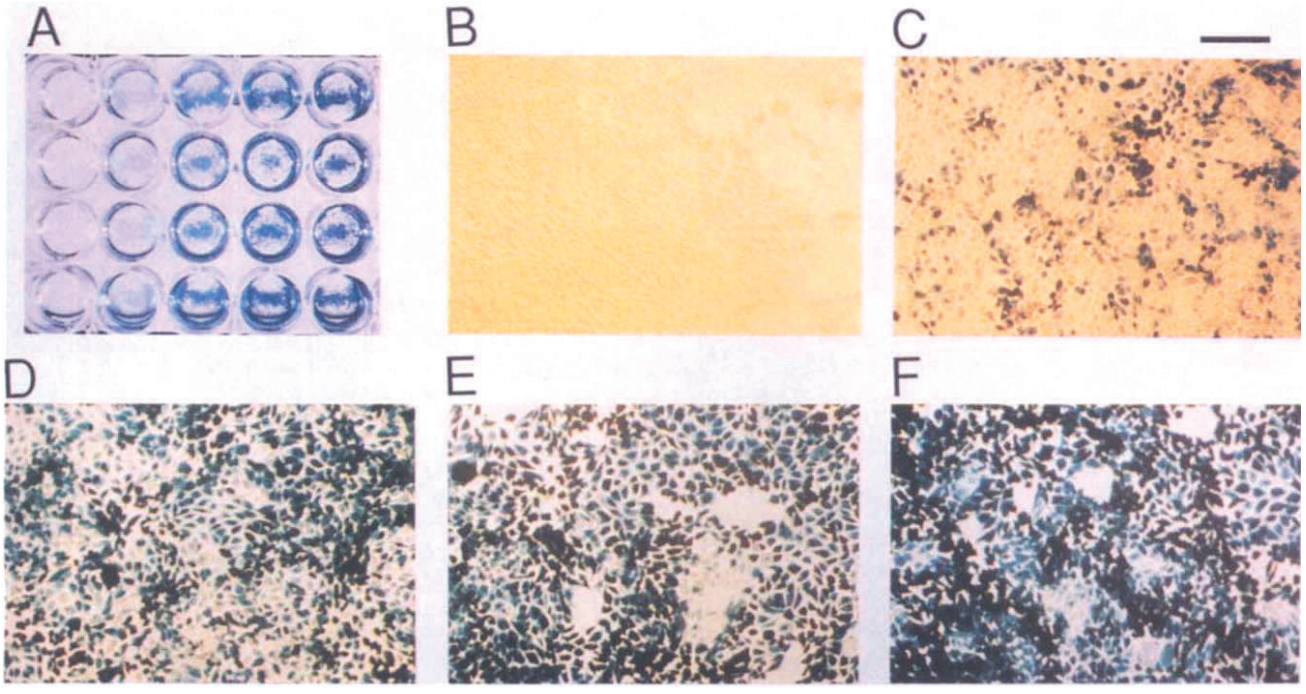
**Fig. 4.** *Time-course of* lacZ *gene expression.* Cells were seeded in 16 mm diameter culture wells and infected with 18 μl of virus solution ($0.8 \times 10^8$ pfu/ml). Cells were stained for β-galactosidase activity 12 hours (**B**), 24 hours (**C**), 48 hours (**D**), and 8 days later (**E**). Staining of the cells mock-infected with the vehicle is shown in **A**. Horizontal bar indicates 200 μm.

**Fig. 2.** *β-Galactosidase activity in mesangial, LLCPK$_1$, and MDCK cells infected with Adex1EFLacZL.* Cells were seeded in 16 mm diameter culture well and infected with 18 μl of either Adex1EFLacZL ($0.8 \times 10^9$ pfu/ml) or DMEM with 5% FCS. After incubation at 37°C for 48 hours, β-galactosidase was detected by activity stain (blue pigmentation) using X-gal as substrate. **A** and **B**. Mesangial cells. **C** and **D**. LLCPK$_1$ cells. **E** and **F**: MDCK cells. A, C and E were mock-infected with DMEM with 5% FCS. B, D and F were infected with Adex1EFLacZL. Horizontal bar indicates 200 μm.

recombinant virus was subsequently propagated with 293 cells and viral solution was stored at −80°C. The titer of viral stocks, determined by plaque assay on 293 cells, was $0.8 \times 10^9$ pfu (plaque forming unit)/ml.

*In vitro infection*

We used subconfluent cells, seeded in 16 mm diameter culture wells, for in vitro adenovirus vector infection. After washing twice


A
B
C
D
E
F

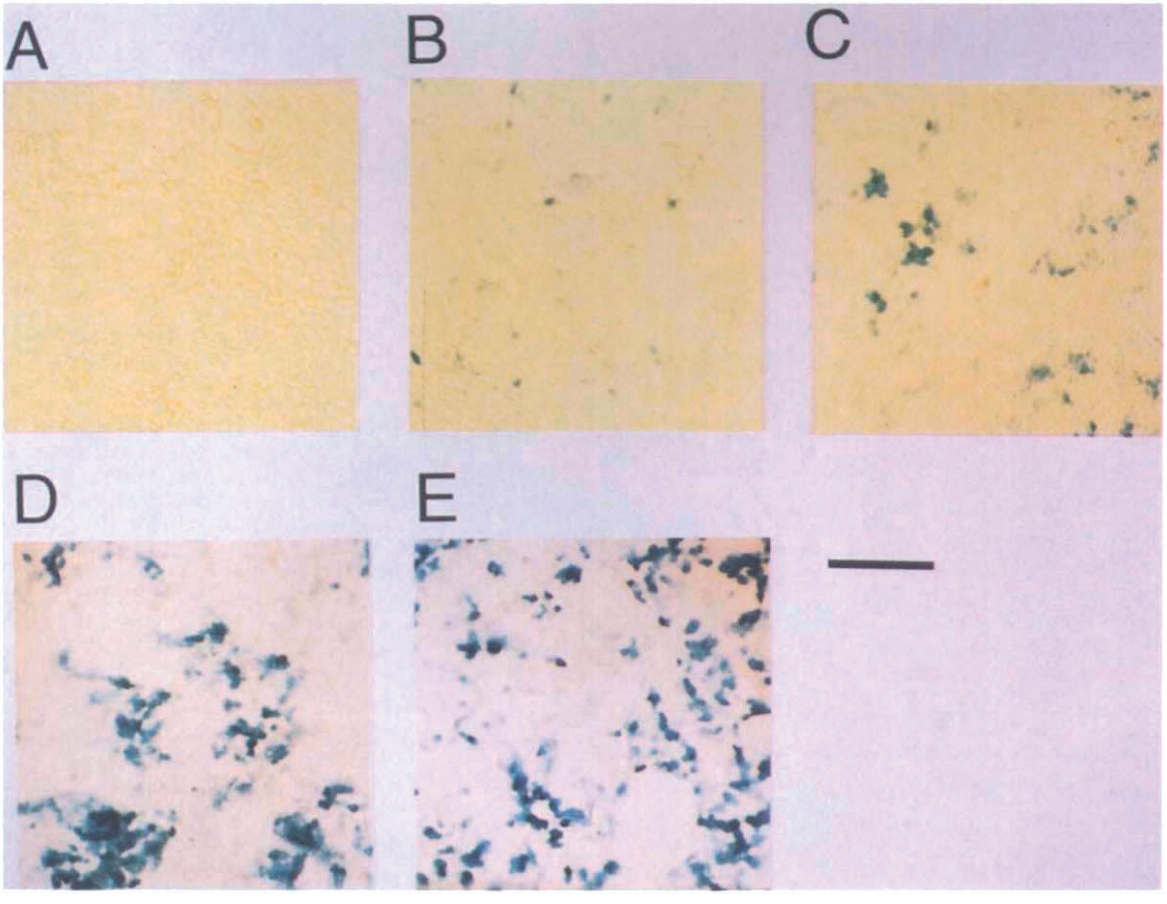

A
B
C
D
E

**Table 1.** Cell numbers of MDCK cells before and after virus infection

| | Before infection | After mock-infection[a] | After Adex1EFLacZ infection[b] |
|---|---|---|---|
| Experiment 1 | 2.7, 2.6 ($\times 10^4$/well) | 26.0, 30.4 | 31.2, 26.0 |
| Experiment 2 | 10.0, 13.0 | 44.0, 34.0 | 48.0, 41.0 |
| Experiment 3 | 3.3, 3.6 | 30.2, 33.4 | 39.0, 44.4 |

[a] Numbers of MDCK cells in a 16 mm diameter culture well 2 days after cells were mock-infected with 18 μl of control solution (DMEM containing 5% FCS)

[b] Numbers of MDCK cells in a 16 mm diameter culture well 2 days after cells were infected with 18 μl of $0.8 \times 10^8$ pfu/ml Adex1EFLacZL

with DMEM without FCS, cells were incubated in a viral solution (18 μl/well) at 37°C for one hour, shaken every 20 minutes, and cultured in growth medium for 12 hours to eight days, depending on the experiments, before histochemical stain. Various concentrations of viral solutions were prepared by diluting the stock solution ($0.8 \times 10^9$ pfu/ml) with DMEM containing 5% FCS.

### *Detection of β-galactosidase activity*

Histochemical staining of β-galactosidase was conducted according to MacGregor et al [6]. In brief, after washed twice with PBS, cells were fixed with 0.25% glutaraldehyde in PBS for 10 minutes at 4°C. After washed four times with PBS, they were incubated in staining solution for three hours at 37°C. The staining solution was comprised of 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6$ in 100 mM sodium phosphate buffer (pH 7.4). β-Galactosidase activity was detected as the development of blue pigmentation due to the enzymatic cleavage of X-gal.

All the experiments were repeated at least three times with consistent reproducibility.

## Results and discussion

### *Genomic structure of Adex1EFLacZL*

The genomic structure of Adex1EFLacZL is schematically illustrated in Figure 1. The insertion point of the expression cassette, as well as its transcriptional direction, are indicated. Adex1EFLacZL has the following characteristics: (1) originates from adenovirus type 5; (2) lacks E1A, E1B, and E3 regions from its genome; and (3) has expression unit (comprised of EF1α promoter, *lacZ* gene, and polyA tail) in place of E1A and E1B. The E1A region is involved in transcriptional transactivation of viral genes. The E1B region contributes to the shut-off of host protein synthesis. The E3 region is thought to be involved in retarding immune response in vivo, and is not required for viral replication in cultured cells (such as 293 cells). Lacking E1A and E1B, Adex1EFLacZL is expected to direct production of β-galactosidase under the EF1α promoter without unrestrained expression of viral proteins nor hindered synthesis of host proteins.

### *Highly efficient transfection by Adex1EFLacZL*

In all three types of cells, unequivocal β-galactosidase activity was detected when transfected with Adex1EFLacZL (Fig. 2, right column: B, mesangial cells; D, $LLCPK_1$ cells; F, MDCK cells). No such activity was seen in mock-infected cells (Fig. 2, left column: A, mesangial cells; C, $LLCPK_1$ cells; E, MDCK cells). These results show that: (1) Adex1EFLacZL successfully transferred *lacZ* gene, which was properly expressed to produce functional β-galactosidase molecule; and (2) EF1α promoter is potent in all three cell types. Remarkably, almost every treated cell exhibited β-galactosidase activity. It demonstrates the superior efficiency of adenovirus-based vector compared with the conventional transfection methods such as calcium phosphate precipitation and DEAE dextran, which are usually successful only in several percent of cells. Multiplicity of infection (moi), which is defined as the numerical ratio of the viruses to the cells at the time of infection, was in the range of 543 to 758 in these experiments. Assuming that several hundred moi assures infection of all the treated cells, we can expect to reliably introduce foreign gene into at least $10^7$ cells in usual experiments, as $10^{10}$ pfu Adex1EFLacZL was easily prepared from 293 cells.

Such high efficiency of adenovirus-based vector would be of special advantage in experiments investigating the cell function at the single cell level (for example, patch-clamp recording), as the experimenter can be confident that most of the cells examined express the gene of interest.

The result that both mesangial and tubular cells accepted adenovirus-based vector leads us to expect that this system would be useful in the wide range of nephrologic investigation, from studying mesangial cell function to exploring the molecular mechanism(s) of tubular transport. This was not entirely unexpected as adenovirus vector system has already been successfully applied to various cultured cell types including cultured B-lymphocyte [7], T-lymphocyte [8], myocyte [9], and hepatoma cell line [10].

### *Dose-dependency of Adex1EFLacZL transfection*

The β-galactosidase activity detected at 48 hours after infection was dependent on the number of vectors applied (that is, moi). As shown in Figure 3A, β-galactosidase activity in $LLCPK_1$ cells increased as moi was raised, from left to right, to 0, 0.65, 6.5, 65, and 650. Figure 3 B-F are the magnified view of these cells. In this representative experiment, moi as low as 6.5 was sufficient to infect nearly all cells treated. Furthermore, they show not only the number of β-galactosidase positive cells but also the level of β-galactosidase activity in each cell increased as the vector concentration was increased. This suggests that multiple copies of vectors are incorporated into these cells as the relative vector quantities are increased. Similar results were also obtained for mesangial and MDCK cells.

### *Time-course of Adex1EFLacZL transfection*

Figure 4 shows the time-course of expression of β-galactosidase in MDCK cells infected with Adex1EFLacZL (A, mock-infection; B, 12 hours after infection; C, 24 hours; D, 48 hours; E, 8 days). β-Galactosidase activity was already detectable as early as 12 hours after infection. The activity gradually increased until 48 hours, and persisted at least eight days (Fig. 4E) after infection. This prompt and stable expression of the introduced gene would be helpful for wide range of experiments.

### *Non-toxicity of Adex1EFLacZL*

Cellular toxicity of Adex1EFLacZL was examined by microscopic appearance and proliferative ability of the infected cells. Viruses sufficient to infect 100% of the cells (400 moi) was applied in these experiments. Microscopic appearance of the cells infected with Adex1EFLacZL was examined by phase-contrast microscopy and compared with untreated cells. In all three cell types,

morphological appearance of the infected cells was indistinguishable from untreated cells (data not shown). Proliferative ability of MDCK cells was examined by counting the cell number at 48 hours after infection of Adex1EFLacZL. Results of three independent experiments are presented in Table 1. Increases in cell numbers were quite similar between mock-infected and infected cells: mock-infected cells, 10.6-, 3.4- and 9.2-fold; Adex1EFLacZL-infected cells, 10.8-, 3.9- and 12.1-fold, in corresponding experiments. Preservation of microscopic appearance and proliferative ability shows that adenovirus vector does not greatly disturb the basic cellular functions.

These harmless characteristics of Adex1EFLacZL would be explained by the following points. First, Adex1EFLacZL lacking E1A region, which is essential for the expression of viral genes, did not cause unrestricted production of viral proteins. Second, Adex1EFLacZL lacks E1B region, which is involved in the shut-off of synthesis of host proteins. Third, transfection procedure with Adex1EFLacZL requires only one hour of incubation with culture medium containing the vector, without altering the cellular environment very much. It contrasts with the conventional transfection methods, such as calcium phosphate precipitation, which usually need drastic change of cellular environment for hours.

### *Summary*

In summary, we have shown that adenovirus vector efficiently introduced foreign gene into cultured renal cells both of mesangial and tubular origin. Genes transferred were properly expressed to produce the molecules of expected function. It was possible to introduce the gene into nearly 100% of the cells treated. Expression of the gene began as early as 12 hours after the infection, increased until 48 hours and persisted at least up to eight days. Finally, the vector was non-toxic to the cells, as judged from simple toxicity tests.

Successful application of adenovirus vector enables for us to study function of pertinent molecules in suitable host cells and opens a new way for examining renal cellular physiology and pathophysiology.

## Acknowledgments

This study was in part supported by grants-in-aid of the Ministry of Education, Science and Culture, and of Welfare and Health of Japan.

*Reprint requests to Tetsuo Katoh, Department of Fourth Internal Medicine, University of Tokyo School of Medicine, 3-28-6 Mejirodai, Bunkyo-ku, Tokyo, Japan 112.*

## References

1. BERKNER K: Development of adenovirus vectors for the expression of heterologous genes. *BioTechniques* 6:616–629, 1988
2. KRIEGLER M: Adenovirus-based vectors, in *Gene Transfer and Expression: A Laboratory Manual*, New York, Freeman and Company, 1990, pp 31–34
3. OKUDA T, YAMASHITA N, KUROKAWA K: Angiotensin II and vasopressin stimulate calcium-activated chloride conductance in rat mesangial cells. *J Clin Invest* 78:1443–1447, 1986
4. SAITO I, OYA Y, YAMAMOTO K, YUASA T, SHIMOJO H: Construction of nondefective adenovirus type 5 bearing a 2.8 kilobase hepatitis B virus DNA near the right end of its genome. *J Virol* 54:711–719, 1985
5. KIM D, UETSUKI T, KAZIRO Y, YAMAGUCHI N, SUGANO S: Use of the human elongation factor 1$\alpha$ promoter as a versatile and efficient expression system. *Gene* 91:217–223, 1990
6. MACGREGOR G, MOGG A, BURKE J, CASKEY C: Histochemical staining of clonal mammalian cell lines expressing *E. coli* $\beta$ galactosidase indicates heterogeneous expression of the bacterial gene. *Somatic Cell Mol Genet* 13:253–265, 1987
7. RUETHER J, MADERIOUS A, LAVERY D, LOGAN J, FU S, CHEN-KIANG S: Cell-type-specific synthesis of murine immunoglobulin $\mu$ RNA from an adenovirus vector. *Mol Cell Biol* 6:123–133, 1986
8. VENKATESH L, ARENS M, SUBRAMANIAN T, CHINNADURAI G: Selective induction of toxicity to human cells expressing human immunodeficiency virus type 1 Tat by a conditionally cytotoxic adenovirus vector. *Proc Natl Acad Sci USA* 87:8746–8750, 1990
9. KIRSHENBAUM L, MACLELLAN W, MAZUR W, FRENCH B, SCHNEIDER M: Highly efficient gene transfer into adult ventricular myocytes by recombinant adenovirus *J Clin Invest* 92:381–387, 1993
10. YUASA T, KAJINO K, SAITO I, MIYAMURA T: Preferential expression of the large hepatitis B virus surface antigen gene by an adenovirus-hepatitis B virus recombinant. *J Gen Virol* 72:1927–1934, 1991