

## Stability of a Recombinant Adenoviral Vector: Optimization of Conditions for Storage, Transport and Delivery

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**Recombinant viral vectors have been developed for use as therapeutic agents and for the introduction of exogenous genes into living cells. However, little is known about the viability and stability of such recombinant viruses during storage, transport and delivery under various conditions. We describe here an analysis of the stability of an adenoviral vector in crude solutions of cell lysates during freezing and thawing and during storage at various temperatures in the presence and in the absence of glycerol. For example, the titer of adenoviruses in crude lysates of infected cells was reduced only ten-fold or three-fold after two hundred rounds of freezing and thawing or after incubation at 28°C for 14 days, respectively. Our observations indicate that recombinant adenoviral vector was more stable than expected both during freezing and thawing and during storage at low temperatures. Our results confirm the importance of appropriate conditions for the delivery and transport of recombinant adenoviral vectors.**

Key words: Recombinant adenovirus — Banking — Gene therapy

Clinical trials of human gene therapy and the use of viral vectors in the laboratory have been reported with increasing frequency during the past decade.<sup>1–3)</sup> Recombinant adenoviral vectors have been exploited as gene-delivery vehicles in gene therapy, as well as in basic research, since high-titer preparations of replication-deficient recombinant adenoviruses with relatively large DNA inserts of up to 7.5 kbp can be generated without too much difficulty. Such recombinant adenoviruses infect a wide variety of dividing and nondividing cells.<sup>4)</sup> However, the viability of biological pharmaceuticals, such as recombinant viruses, might be expected to vary substantially from the time of production to the time of administration. Considerable efforts have been made to develop specifications for the characterization of viral vectors for large-scale production. However, little is known about the viability of recombinant viruses after storage under various conditions. The standard protocol involves storage of adenoviral vectors at –80°C. Under such conditions, adenoviral stocks with an adequate titer can be maintained, but the low temperature limits the shipment of such vectors over great distances. Dry ice is usually used to keep preparations of adenoviral vectors in a frozen state. The acidic pH of such an environment does, however, reduce the titer of infectious adenoviral vectors.<sup>5)</sup> Recently, Croyle *et al.*

reported a method for treating purified adenoviral vectors with polyethylene glycol (PEG) and the resultant preparations of adenoviruses were more stable at various temperatures than the untreated viruses.

We recently investigated the stability of unpurified replication-deficient adenoviral vectors under various conditions. We stored adenoviral vectors as crude lysates of infected cells rather than as purified preparations and examined the effects of freezing and thawing and the effects of temperature during storage on the recombinant adenoviruses. We used two independent parameters for the evaluation of a preparation of the recombinant adenovirus AxCANLacZ<sup>6,7)</sup>: the cytopathic effect (CPE)<sup>8)</sup> and the  $\beta$ -galactosidase activity associated with expression of AxCANLacZ. The CPE provides an indication of the ratio of infectious to non-infectious recombinant adenoviruses in a preparation. The  $\beta$ -galactosidase activity provides an indication of the efficiency of expression of the transgene in the adenovirus. The CPE of the adenoviral vector AxCANLacZ was reduced by about 80% [ $1.7 \times 10^8$  plaque-forming units/ml (pfu/ml)] after ten rounds of freezing and thawing as compared with that of the control ( $7.6 \times 10^8$  pfu/ml). Further treatment of infectious cells by freezing and thawing eliminated the CPE of the vector. When the AxCANLacZ vector in a cell extract was incubated for 14 days at 37°C, the titer fell by approximately forty-fold as compared with the titer of the vector after storage at –80°C ( $P < 0.001$ ). When the cell extract containing the recombinant adenovirus was stored at 4°C, the titer did not decrease significantly ( $P > 0.05$ ). Thus, it appears that recombinant adenoviruses in crude lysates of infected cells remain stable in preparations of adenoviral vectors when

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<sup>8</sup> <http://www.nih.gov/news/panelrep.html>. Orkin, S. and Motulsky, A. G. Report and recommendation of the panel to assess the NIH investment in research in gene therapy.

freezing and thawing is repeated no more than ten times. Moreover, these vectors should be stored below 4°C. Such conditions should ensure the stability of recombinant adenoviruses during transport, storage and delivery.

## MATERIALS AND METHODS

**Cell lines and infection with adenovirus** Adenovirus type 5 immediate early gene 1-transformed (Ad5-E1-transformed) human embryonic kidney (HEK) 293 cells<sup>9)</sup> and HeLa cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and the RIKEN Cell Bank (RCB0007; RIKEN, Tsukuba), respectively, and cultured according to each supplier's protocol. The adenovirus-mediated gene transduction was performed as described elsewhere.<sup>10)</sup>

**Preparation of recombinant adenovirus** The adenoviral vector AxCANLacZ<sup>6,7)</sup> was obtained from the RIKEN DNA Bank (RDB 1749; RIKEN). HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Invitrogen, Grand Island, NY), 2 mM glutamine (Invitrogen), 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen) in collagen-coated tissue culture dishes at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. When HEK293 cells were reached 80% confluence in 225-cm<sup>2</sup> flasks, they were infected with AxCANLacZ at a multiplicity of infection (m.o.i.) of 10. Four days later, after confirmation of the cytopathic effects of infection, we harvested the infected cells by pipetting. The cells were disrupted by sonication with a "Bioruptor" (20 kHz, 30 s, 200 W; Bioruptor UCD-200T; Tosho Denki, Yokohama). The suspension of sonicated cells was centrifuged at 3500 rpm at 4°C. The supernatant was divided into aliquots and stored at -80°C for use as crude lysates. The titers of viral stocks were determined by a limiting-dilution bioassay with HEK293 cells as described elsewhere.<sup>11)</sup>

**Protocols for freezing and thawing and for storage** Infected HEK293 cells and the culture medium containing 10% FCS were collected and samples were divided into two groups. Samples in one group were disrupted on ice by sonication. Aliquots of 500 µl of the resultant lysates were incubated separately at -80°C, -20°C, 4°C, 28°C and 37°C for 14 days. Samples in the second group were not subjected to sonication, but aliquots were incubated under the same conditions as the samples in the first group. The samples in the second group were sonicated before determination of the infectious virus titer. These experiments were repeated five times. Cell lysates were frozen in liquid nitrogen and thawed at 37°C at various times in the presence and also in the absence of glycerol, and stored at -80°C.

**Determination of cytopathic effects** HEK293 cells were seeded in 96-well plates at 5×10<sup>3</sup> cells/well. Twenty-four

hours later, the medium was removed by aspiration and replaced with 50 µl of medium that contained ten-fold serial dilutions of the vector used, from 1×10<sup>-2</sup> to 1×10<sup>-11</sup>. For controls, 50 µl of fresh medium was used instead of medium plus vector. The plates were centrifuged at 1000g for 10 min and then placed in an incubator. For determination of the CPE, the plates were incubated for a minimum of 10 days, during which time there was no evidence of a CPE in the control wells. Plates were examined daily for the CPE of the virus on the infected cells and the titer was calculated.<sup>10, 12)</sup>

**Cytochemical analysis** Aliquots of 3.3×10<sup>5</sup> HeLa cells were infected with recombinant adenovirus at an m.o.i. of 4 and an m.o.i. of 0.1. Then, 72 h after infection, the medium was removed by aspiration and the cells were fixed in 50 µl of 0.25% glutaraldehyde at 4°C for 10 min. After fixation, cells were rinsed five times with phosphate-buffered saline (PBS). The fixed cells were incubated in 5 mM K<sub>4</sub>[Fe(CN)<sub>6</sub>], 5 mM K<sub>3</sub>[Fe(CN)<sub>6</sub>], 1 mM MgCl<sub>2</sub>, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, and 0.1% halogenated indoyl-β-D-galactoside (Bluo-gal; Invitrogen) in PBS for 12 h at 37°C to allow complete color development. Blue cells were counted under a light microscope at low magnification. The titer (*T*) was calculated as follows:  $T=(A \times B)/C$ , where *A* is the number of blue cells per well, *B* is the dilution factor and *C* is the total volume of sample tested at that dilution.

**Measurement of β-galactosidase activity** Aliquots of 3.3×10<sup>5</sup> HeLa cells were infected with recombinant adenovirus at an m.o.i. of 4. Then, 72 h after infection, the medium was removed by aspiration and the cells were collected and lysed by incubation in 20 mM sodium phosphate buffer (pH 7.2) that contained 50 mM β-mercaptoethanol and 1 mM MgCl<sub>2</sub>. Lysates were centrifuged at 13 000 rpm for 10 min at 4°C. Each supernatant was mixed with an equal volume of 80% glycerol. The concentration of total protein in each lysate was estimated with "ProteinAssay" (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol, with bovine serum albumin as the standard. Then, 10 µl of each lysate was mixed with 100 µl of reaction buffer [50 mM sodium phosphate (pH 7.8), 50 mM mercaptoethanol, 1 mM MgCl<sub>2</sub>] and 10 µl of 7.53 mg/ml *o*-nitrophenyl β-galactoside. After incubation at 28°C for 30 min, the reaction was stopped by addition of 50 µl of 0.52 M Na<sub>2</sub>CO<sub>3</sub>. Then the absorbance of the solution was measured at 415 nm and β-galactosidase activity was determined relative to that of control enzyme (Roche Diagnostics GmbH, Mannheim, Germany).

## RESULTS AND DISCUSSION

The viability of recombinant viruses varies substantially from production to delivery. We have shipped preparations

of recombinant adenoviruses with high titers of infectious virus to scientists all over the world, as part of the activities of the RIKEN DNA Bank. In an attempt to determine the stability of adenoviruses during transport, storage and delivery, we examined the effects of various conditions on the titer of recombinant adenoviruses and the expression of the encoded protein.

We initially examined the effects of freezing and thawing on the viability of adenoviruses in the presence and in the absence of glycerol. When crude lysates of cells infected with AxCANLacZ were frozen and thawed two hundred times in the absence of glycerol, the titer was reduced about ten-fold ( $7.6 \times 10^8$  to  $6.4 \times 10^7$  pfu/ml; Fig. 1B). This reduction in titer was partially overcome by the inclusion of glycerol (final concentration, 10% v/v). In the presence of glycerol, the final titer was  $2.0 \times 10^8$  pfu/ml. After four hundred rounds of freezing and thawing, the titer fell significantly to  $2.4 \times 10^7$  pfu/ml in the absence of glycerol. In the presence of glycerol, the final titer was slightly higher ( $3.2 \times 10^7$  pfu/ml). These results suggest that the addition of glycerol to cell lysates that contain adenovirus prevents decreases in titer to some extent, provided that freezing and thawing are not repeated too many times. Our results imply also that adenoviruses are unstable when subjected to many repeats of freezing and thawing. We next tested the effects on titer of one to ten rounds of freezing and thawing, which might more accurately reflect conditions for laboratory use.

As shown in Fig. 1A, the titer remained basically unchanged during ten round of freezing and thawing. Moreover, the addition of glycerol had no significant effect (Fig. 1A). Thus, it appears to be unnecessary to include glycerol in preparations that will be subjected to fewer than ten rounds of freezing and thawing.

We next examined the efficiency of expression of  $\beta$ -galactosidase encoded in the recombinant virus AxCANLacZ. We infected HeLa cells with AxCANLacZ at an m.o.i. of 4 and determined the relative activity of  $\beta$ -galactosidase in infected cells. The number of stained cells, which indicates the number of cells that express the transgene, was not significantly different after ten rounds of freezing and thawing from that of adenovirus-infected cells that were not subjected to freezing and thawing (Fig. 2). There was no significant reduction in  $\beta$ -galactosidase activity during each round of freezing and thawing (Fig. 3). The  $\beta$ -galactosidase activity of control cells was 0.32 units/ $\mu$ g total protein. The reduction in  $\beta$ -galactosidase activity of crude lysates of infected cells after freezing and thawing in the absence of glycerol was less than ten-fold as compared with that of cells infected with adenovirus that had been frozen and thawed in the presence of 10% glycerol. Thus, inclusion of glycerol in lysates of adenovirus-infected cells slightly, but not significantly, improved the efficiency of expression of  $\beta$ -galactosidase when lysates were frozen and thawed up to ten times.

To examine the stability of recombinant adenovirus under extreme conditions, we incubated AxCANLacZ as a stock of crude lysates of infected cells at 37°C for up to 28 days (Fig. 4). The initial titer of the vector was  $2.6 \times 10^8$  pfu/ml. The titer of AxCANLacZ after the incubation of 7 days and 14 days at 37°C without sonication was reduced more than 10-fold (to  $1.2 \times 10^7$  and  $2.0 \times 10^7$  pfu/ml, respectively). The final titer was not determined because the CPE of the adenovirus after incubation for 28 days was undetectable.

Sonication of samples had a slight protective effect on the virus over the first five days of subsequent incubation at 37°C (the titer fell from  $2.4 \times 10^8$  pfu/ml to  $1.2 \times 10^8$

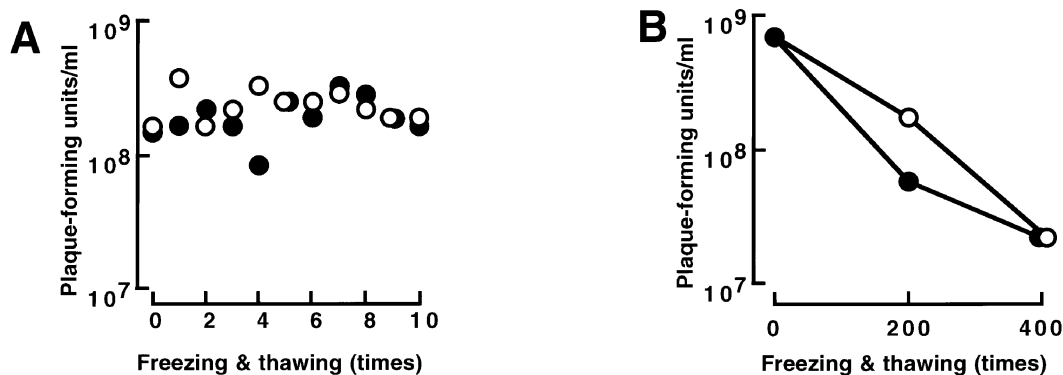


Fig. 1. Effect of freezing and thawing on the titer of AxCANLacZ. (A, B) The adenovirus AxCANLacZ was propagated in HEK293 cells supplemented with 10% FCS. Freezing and thawing were performed in liquid nitrogen and at 37°C in a water bath. Adenovirus AxCANLacZ in crude cell lysates was frozen and thawed in the presence and in the absence of glycerol (10%). Then titers were determined using HEK293 cells as described in "Materials and Methods." Open circles, presence of glycerol (10%); closed circles, absence of glycerol.

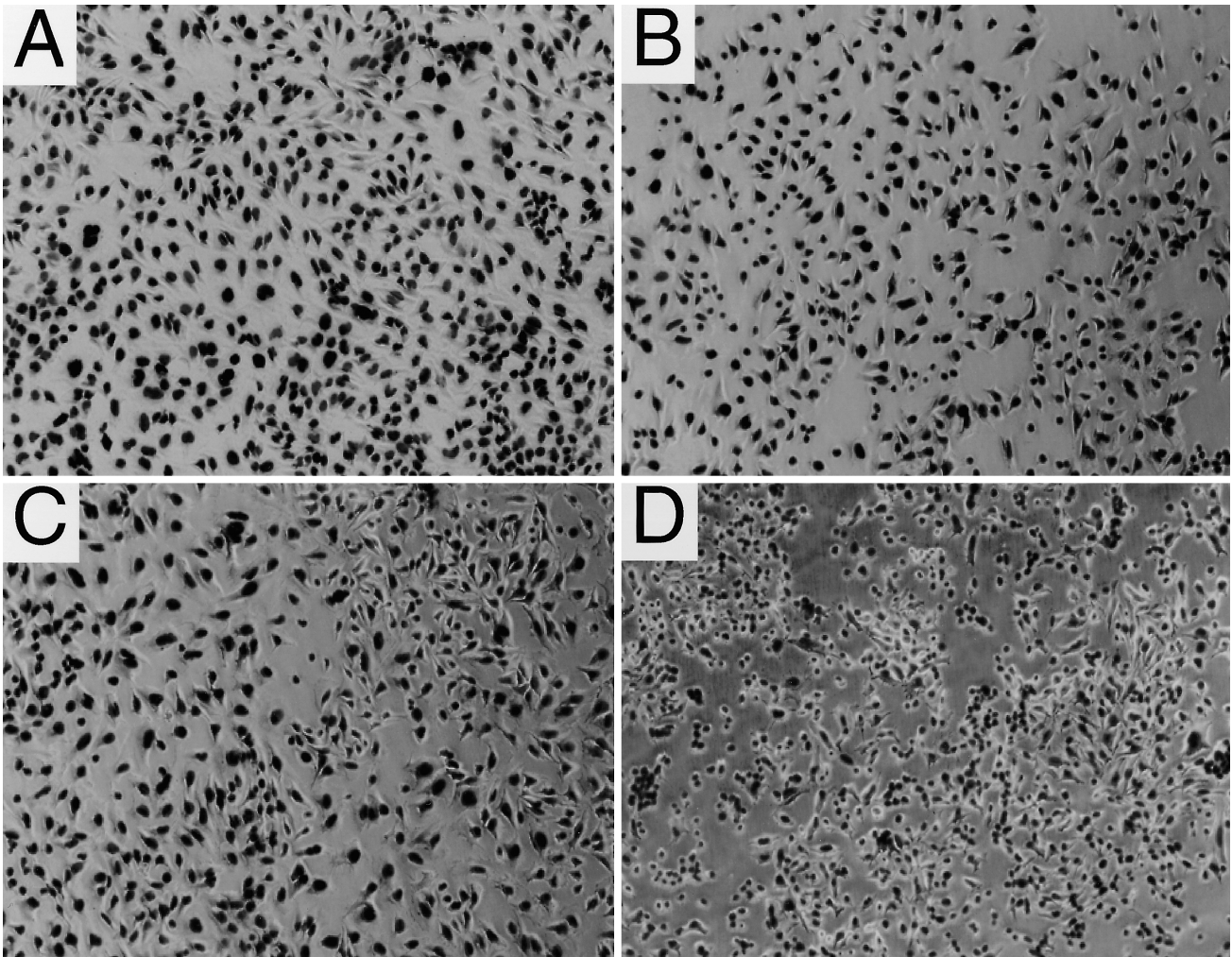


Fig. 2. Staining of HeLa cells for  $\beta$ -galactosidase activity after infection with adenovirus AxCANLacZ. Adenovirus AxCANLacZ in crude cell lysates was frozen and thawed as indicated. HeLa cells were infected with the vector at an m.o.i. of 4. Then, 72 h after infection, cells were stained as described in the text. (A, C) Incubation without addition of glycerol; (B, D) incubation with 10% glycerol; (A, B) infection with adenovirus AxCANLacZ in a cell lysate without freezing; (C, D) infection of adenovirus AxCANLacZ in a cell lysate after ten rounds of freezing and thawing.

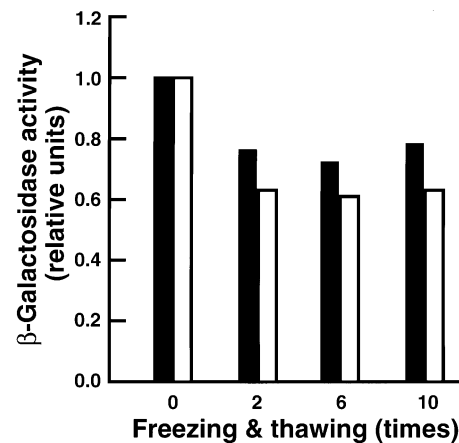


Fig. 3. Activity of  $\beta$ -galactosidase of HeLa cells infected by adenovirus AxCANLacZ. Adenovirus AxCANLacZ in a crude cell lysate was frozen and thawed as indicated. HeLa cells were infected with the vector at an m.o.i. of 4. Then, 72 h after infection,  $\beta$ -galactosidase activity was measured. Open bars, presence of 10% glycerol; closed bars, absence of glycerol in storage solutions.

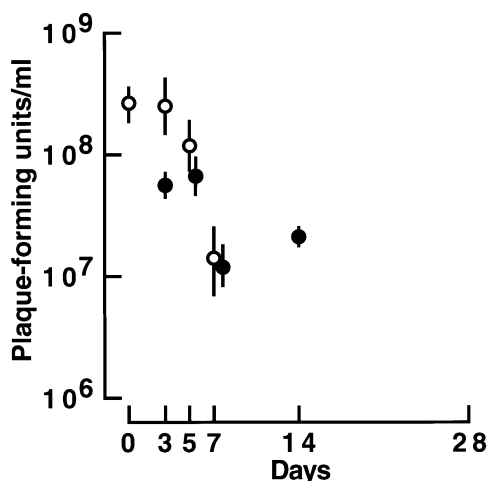


Fig. 4. Adenovirus AxCANLacZ in a crude cell lysate was sonicated before (open circles) or after (closed circles) incubation at 37°C. Each point and bar show the average and standard deviation of results from five independent experiments.

pfu/ml). Then the titer fell dramatically after a further two days ( $1.4 \times 10^7$  pfu/ml). On day 14, the CPE disappeared. The mechanism responsible for the resistance to loss of CPE of sonicated crude cell lysates during incubation at 37°C is not known. It is possible that precipitating aggregates of cells formed after sonication might be resistant to digestion by proteases in the short term.

To assess the effect of the storage temperature on the titer of samples, we incubated adenoviruses in crude lysates of infected cells at various temperatures for 3 days and 14 days with and without glycerol and/or sonication (Fig. 5, A and B). When adenoviruses in crude lysates of

infected cells were incubated at 37°C, the final titers of infectious adenovirus ranged from  $3.9 \times 10^6$  to  $3.9 \times 10^7$  pfu/ml and variance among treatments was apparent ( $P < 0.01$ , ANOVA). When the adenoviruses in crude lysates of infected cells were stored at  $-80^\circ\text{C}$ , the titers were  $2.4 \times 10^8$  to  $6.6 \times 10^8$  pfu/ml ( $P > 0.01$ , ANOVA). At other temperatures, the titers fluctuated, but there were no significant differences among treatments at the same temperature of incubation. Since the effects of glycerol and sonication were only evident when adenoviruses in lysates were incubated at 37°C, it was clear that such treatments would not affect the titer of adenoviruses under most storage conditions.

The titers of infectious vectors after incubation at 37°C were significantly lower than those after incubation at  $-80^\circ\text{C}$  with any of the additional treatments ( $P < 0.005$ , respectively), while only when preparations of vectors were sonicated and stored at 28°C in the presence of glycerol there was any significant effect of an additional treatment ( $P < 0.001$ ).

The rapid reduction in the titer of infectious adenovirus in crude lysates of infected cells during incubation at 37°C suggests that proteolytic or nucleolytic enzymes might degrade the adenovirus. The titer of adenoviruses remained close to  $2 \times 10^8$  pfu/ml at 28°C. Thus, transport of recombinant or wild-type adenoviruses in crude lysates of infected cells should be possible even without refrigeration if preparations are not exposed to extreme conditions.

The stability of adenoviruses during transport has been examined by other researchers.<sup>5, 13</sup> The acidic pH that develops in the presence of dry ice appeared to decrease the titer of infectious adenoviruses, but the mechanism responsible is unknown. The formation of aggregates might occur as a result of the loss of charge from side-

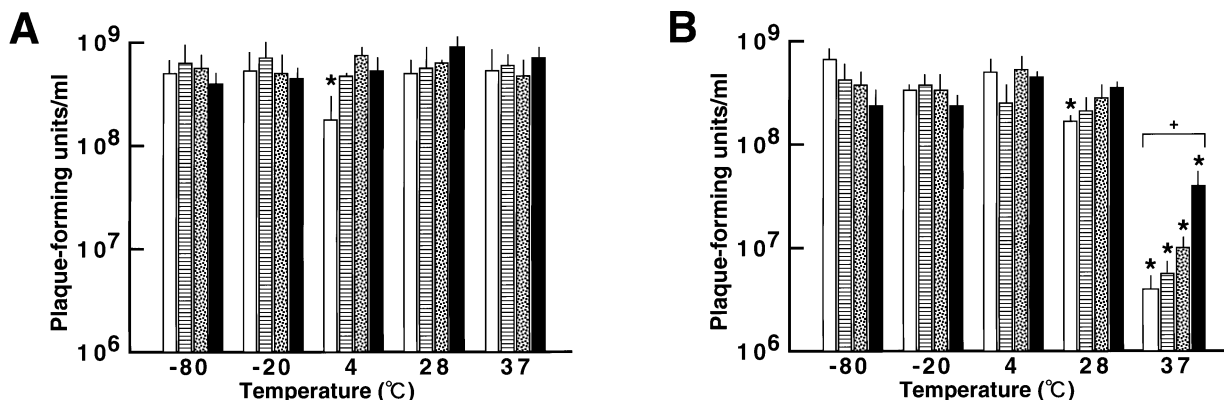


Fig. 5. Adenovirus AxCANLacZ in crude cell lysates was incubated at  $-80^\circ\text{C}$ ,  $-20^\circ\text{C}$ ,  $4^\circ\text{C}$ ,  $28^\circ\text{C}$  or  $37^\circ\text{C}$ . Each column and bar show the average and standard deviation of results of three independent experiments. (A) Incubation for 3 days. (B) Incubation for 14 days. Open bars, incubation after sonication with glycerol; hatched bars, incubation after sonication without glycerol; dotted bars, incubation before sonication with glycerol; closed bars, incubation before sonication without glycerol. \*,  $P < 0.005$  (*t*-test); +,  $P < 0.01$  (ANOVA).

chain carboxyl groups of proteins at an acidic pH.<sup>5)</sup> Croyle *et al.* reported a method for modification of purified adenoviruses that improved their stability, using activated monomethoxypoly (ethylene) glycols. They reported that the titer of unconjugated adenovirus fell ten-fold after storage at 4°C for 8 h and that infectious virus was undetectable after storage for seven days at 4°C. By contrast, we found that the storage of adenovirus at 4°C for 14 days had no significant effect on the titer of infectious viruses. The discrepancy between the results might be due to the conditions for preparation of viral stocks. Croyle *et al.* used adenovirus that had been purified on CsCl gradients while we used crude solutions of adenoviruses from culture medium and cell lysates. Loss of infectious activity might result from the degradation of viral capsids and/or formation of aggregates of viruses. The considerable reduction in titer at 37°C suggests that enzymatic processes might be involved in this phenomenon. However, the mechanisms responsible for the rapid degradation of viral capsids in solutions of purified virus at 4°C are unknown. Although our preparations of adenovirus are not suitable for direct therapeutic application, our convenient method for preparation of adenovirus is suitable for the transport of the vectors even when refrigeration is not available.

In summary, we examined several methods for the generation of preparations of a recombinant adenovirus in

crude lysates of infected cells and investigated the stability of the adenovirus. Our studies indicate that recombinant adenovirus should, optimally, be transported at temperatures from 4°C to –80°C. In some cases, a sonicated sample is preferable for maintenance of stability at room temperature or at 37°C for 3 to 5 days. Freezing and thawing of samples should not be repeated more than ten times. The addition of glycerol (final concentration, 10%) improves the viability of adenovirus to a certain extent. The conditions that we recommend should help to maintain high titers during the transport and delivery of adenoviral vectors for use in the laboratory and in a clinical setting.

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