

# Recombinant Adenoviruses with Large Deletions Generated by Cre-Mediated Excision Exhibit Different Biological Properties Compared with First-Generation Vectors In Vitro and In Vivo

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**In vivo gene transfer of recombinant E1-deficient adenoviruses results in early and late viral gene expression that elicits a host immune response, limiting the duration of transgene expression and the use of adenoviruses for gene therapy. The prokaryotic Cre-lox P recombination system was adapted to generate recombinant adenoviruses with extended deletions in the viral genome (referred to here as deleted viruses) in order to minimize expression of immunogenic and/or cytotoxic viral proteins. As an example, an adenovirus with a 25-kb deletion that lacked E1, E2, E3, and late gene expression with viral titers similar to those achieved with first-generation vectors and less than 0.5% contamination with E1-deficient virus was produced. Gene transfer was similar in HeLa cells, mouse hepatoma cells, and primary mouse hepatocytes in vitro and in vivo as determined by measuring reporter gene expression and DNA transfer. However, transgene expression and deleted viral DNA concentrations were not stable and declined to undetectable levels much more rapidly than those found for first-generation vectors. Intravenous administration of deleted vectors in mice resulted in no hepatocellular injury relative to that seen with first-generation vectors. The mechanism for stability of first-generation adenovirus vectors (E1a deleted) appeared to be linked in part to their ability to replicate in transduced cells in vivo and in vitro. Furthermore, the deleted vectors were stabilized in the presence of undeleted first-generation adenovirus vectors. These results have important consequences for the development of these and other nonintegrating vectors for gene therapy.**

Currently, recombinant adenoviruses are one of the more efficient viral vehicles for transferring genes into nondividing cells in vivo. First-generation E1-deficient adenoviruses were used for gene therapy on the basis of the premise that the deletion of E1a and part of E1b sequences should be sufficient to eliminate other viral gene expression and render the virus replication deficient (4, 25, 29, 44). However, recent extensive studies on adenovirus-mediated gene transfer, particularly to the lung and liver, have demonstrated a significant, E1a-independent expression of early and late viral gene products in mice (62, 63), cotton rats (13), and humans (14, 65). Expressed viral proteins contribute to the toxic effects shortly after intravascular adenovirus administration (6, 7). The production of immunogenic viral proteins in transduced cells leads to their loss by antigen-dependent cell-mediated immunity, causing chronic inflammation in the infected organ. The loss of virus infected cells occurs rapidly in the lung and liver over several weeks in immunocompetent animals (3, 6, 62). Readministration of adenoviruses to restore transgene expression is inefficient because of the production of neutralizing antiviral antibodies after the activation of B cells by capsid proteins of the input virus (61).

There have been promising attempts to modulate the antiviral host immune response in mice in order to make first-generation adenoviruses suitable for clinical gene therapy (31, 64). However, these strategies have numerous potential side effects and do not eliminate adenovirus early toxicity. Thus, further crippling of the adenovirus genome by deletions or

mutations in sequences coding for immunogenic or toxic viral proteins is of major importance.

In order to overcome some of the problems mentioned above, second-generation adenoviruses with additional lethal deletions or mutations in other genome regions have been developed. These include temperature-sensitive mutations in the E2a gene (15, 63) and partial deletions of the E4 (2, 33, 56) or the E2a (21) region. The deleted vectors can be propagated in cell lines which complement the E2a or E4 functions in *trans*. However, these viral vectors tend to have reduced titers compared with first-generation vectors. E1-deleted vectors in combination with E2a or E4 deletions seem to be less toxic; however, there is some uncertainty as to whether these vectors result in improved transgene persistence in animals (15, 66).

Another approach is to produce viral proteins necessary for viral replication and assembly by coinfecting helper virus together with a plasmid containing a minimal virus genome, in which all of the coding sequences are eliminated (17, 40). The propagation of high-titer viruses is difficult, and the risk of contamination with wild-type virus is high.

Heterologous, site-specific recombination systems have been widely used in mammalian cells and animals. The P1 bacteriophage Cre-loxP system includes the 38-kDa recombinase (Cre) and the 34-bp loxP target sequence (52). Cre-mediated recombination between two parallel lox sites (present in one DNA) results in excision of the intervening sequence, producing two recombination products, each containing one lox site. The reverse reaction, the intermolecular recombination between two lox sites on separate plasmids, resulting in integration, is much less efficient (46, 47).

In this study we used the Cre-lox system to produce adenoviruses with extended genome deletions. Recombinant adenovirus with two parallel lox sites flanking the pIX expression unit

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and the entire E2 region were generated and amplified to high titers by standard techniques. This virus was then infected onto 293 cells stably expressing Cre recombinase, which mediated the efficient excision of the intervening 25-kb region, joining the left genome end, with the inverted terminal repeat (ITR), packaging signal, and human  $\alpha$ 1-antitrypsin (hAAT) expression cassette, and the right genome end, with the E4 region and right ITR, together. Assembly and packaging functions are provided by the genome that remains undeleted and possibly the excised region. The preparations of deleted virus were high in titer and in purity. The DNA and protein compositions of deleted vectors and the biological properties of cells after transduction in vitro and in vivo were analyzed and compared with those for undeleted viruses.

The 25-kb deleted region includes the pIX and E2 expression units. Protein IX, encoded in the E1b region with its own promoter, is a minor structural protein. The E2 region encodes proteins required for adenovirus DNA replication. The region corresponding to E2 on the other viral DNA strand contains the major late promoter and the L1 to L4 RNA family for penton (III and IIIa), hexon (II), and core (VII, pVI, VIII, etc.) proteins (reviewed in reference 54).

#### MATERIALS AND METHODS

**Vectors.** A 96-bp fragment containing the *loxP* site was amplified from pBS64 (45) by PCR with the following primers:

5' CGCTCGAGAAGCTTGGGCTG 3'  
*XhoI HindIII*

and

5' GGTCGGACCGGATCCGGAA 3'  
*BamHI*

The PCR product was digested with *XhoI* and cloned into the *SalI* site of pAd.RSV-hAAT-bPA (30) directly downstream of the expression cassette containing the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter, hAAT cDNA, and bovine growth hormone polyadenylation signal. Depending on the orientation of the *lox* site, the resulting plasmids were named pAd.hAAT-lox1 (Fig. 1) and pAd.hAAT-lox2. The cDNA for the secretory protein hAAT was used as a reporter gene so that protein levels could be easily monitored over time in vitro and in vivo. In order to insert the second *lox* site into the  $\Delta$ E3 region of pBHG11 (5) (Microbix Biosystems, Inc., Toronto, Canada), a packaging-deficient, circular adenovirus type 5 (Ad5) genome (34,304 bp) with a deletion in the E3 region ( $\Delta$ E3; bp 28133 to 30818), the PCR product containing the *lox* site was first cloned as an *XhoI-BamHI* fragment into the *SalI-BamHI* sites of pABS4 (Microbix Biosystems, Inc.). The *lox* site together with the kanamycin resistance gene were then cut out with *PacI* (1.4-kb fragment) and introduced into the unique *PacI* cloning site present in pBHG11 at position 29363. Recombinants were screened by double selection with kanamycin and ampicillin. The orientation of the insert was analyzed by *HindIII* digestion. The *lox* insert was sequenced with a primer specific for the kanamycin gene (5'TTTCCCGTTGAATATGGC TCATA3'). Next, the kanamycin gene was deleted by *SwaI* digestion and religation of the vector. The resulting plasmids were named pBHG11-lox1 (Fig. 1) and pBHG11-lox2.

Corresponding constructs based on pBHG10 were generated (5). All plasmids were purified by double CsCl centrifugation and transfected into 293 cells by Ca-phosphate coprecipitation in a 1:1 ratio as described earlier (30). Single plaques with recombinant adenovirus were isolated 13 days after cotransfection. Recombinant adenovirus was amplified in 293 cells and screened for hAAT expression. Adenovirus DNA was isolated from hAAT-positive viruses and digested with *HindIII* in order to confirm the correctness of all genome modifications. Vectors containing two *lox* sites were amplified in a large scale in 293 cells and purified by double CsCl gradient centrifugation. The virus titer was determined from plaque titers on 293 cells. Virus with a titer of  $5 \times 10^{11}$  PFU/ml was stored at  $-80^\circ\text{C}$  in 10 mM Tris-Cl (pH 8.0)-1 mM  $\text{MgCl}_2$ -10% glycerol. No growth abnormalities were observed with viruses modified with two *lox* sites in comparison with other recombinant adenoviruses based on pBHG11 or pBHG10. Tests for replication-competent virus were as described previously (3).

Clinical-grade Ad2/CMV $\beta$ gal-2 (AdA) and Ad2/CMV $\beta$ gal-4 (AdB) vectors were provided by Genzyme (Cambridge, Mass.). The former is a first-generation E1-deleted vector containing the CMV $\beta$ gal expression cassette in the E1 region, while the latter has a modified E4 region (2). Both preparations contain no detectable replication-competent adenovirus at a sensitivity of  $1$  in  $2.5 \times 10^9$  PFU.

**Generation of 293-cre cells.** Although the 38-kD Cre protein is small enough to pass through the nuclear membrane without a nuclear localization signal (NLS), it was demonstrated that an NLS-modified Cre is more active in recombination between *lox* sites in genomic DNA (22). Because adenovirus DNA replication takes place in the nucleus, Cre modified with the simian virus 40 T-antigen NLS was used for the excision strategy. pCMV-nlscre was generated by replacing the *XhoI-EcoRV* fragment of pBS185 (612 bp of the 5' end of *cre*) (46) by the *XhoI-EcoRV* fragment from pICK-NCREK (a gift from C. Wilson, University of Washington) with the NLS-modified 5' end of *cre*. The *EcoRV* junction was sequenced with a *cre*-specific primer. pCMV-nlscre was cotransfected into 293 cells with pSV2neo (49) (20:1). After selection for neomycin phosphotransferase-expressing clones for 4 weeks with 500  $\mu\text{g}$  of G418 per ml, single clones were isolated, propagated, and analyzed for *cre* RNA by in-solution hybridization of 10  $\mu\text{g}$  of total RNA with a *cre*-specific oligonucleotide (position 1070, AAC ATTCTCCACCGTCAGTACGTGAGAT) as described previously (36). The subsequent cell line used was designated 293-creB1. *cre* mRNA was quantified on the basis of a concentration standard curve with in vitro-transcribed *cre* RNA. As a template for T7 polymerase, in vitro transcription of pGCre (1.1-kb *XhoI-BamHI* fragment cloned into pGEM1 [Promega]) was used. Transient *cre* RNA concentrations at day 3 after transfection were about 30 times higher than levels in most clones with stable *cre* expression 7 weeks postselection, indicating that high levels of NLS-Cre may be toxic in 293 cells.

**Generation of deleted virus.** Cells ( $2.5 \times 10^8$ ) from clones with different *cre* RNA levels were infected with Ad.hAAT(*lox*)<sub>2</sub>-UD at a multiplicity of infection (MOI) of 10. Infected cells were collected 36 h after infection, and the virus was released by four cycles of freezing and thawing. The first ultracentrifugation (2 h, 35,000 rpm, SW41 rotor) was performed in a CsCl step gradient (0.5 ml at 1.5 g/cm<sup>3</sup>, 2.5 ml at 1.35 g/cm<sup>3</sup>, 4.0 ml at 1.25 g/cm<sup>3</sup>, and 5 ml of cell lysate in phosphate-buffered saline [PBS]) in 12-ml ultraclear tubes (Beckman). Three clearly separated bands with viral material were obtained. Fractions were collected by puncturing the tube. The yields of deleted and undeleted virus in each fraction were quantified on the basis of viral DNA analysis in agarose gels. Fractions containing the deleted virus were combined and centrifuged at 35,000 rpm overnight in an equilibrium gradient with a CsCl density of 1.32 g/cm<sup>3</sup>. The deleted virus was dialyzed against 10 mM Tris-Cl (pH 8.0)-1 mM  $\text{MgCl}_2$ -10% glycerol and stored at  $-80^\circ\text{C}$  at a titer of  $10^{11}$  transducing particles per ml (see below). The recombination conditions with the Cre recombinase were adjusted to maximize the ratio of deleted to undeleted DNA. An Ad.hAAT(*lox*)<sub>2</sub>-UD MOI of 50 per 293-creB1 cell gave the best ratio and the largest total amount of deleted vector. With a higher MOI, the amount of undeleted virus increased considerably. At a lower MOI, the total output particle number was reduced. The optimal time postinfection for harvesting the cells was determined to be 30 to 32 h. Longer culture times decreased the ratio of deleted to undeleted DNA, perhaps because this corresponded to a suppression of *cre* mRNA expression due to viral infection, as detected by RNA in-solution hybridization. To calculate the adenovirus particle number, the  $A_{260}$  was determined, where 1.0 optical density unit was estimated to be  $10^{12}$  particles per ml. The titers of undeleted and deleted viruses were determined on HeLa cells. HeLa cells ( $10^9$ ) were infected with dilutions of  $10^2$  to  $10^5$  viral particles, and 40 hours later the cells were subjected to immunofluorescence analysis with hAAT- and E4-specific antibodies. The number of positive cells was scored and used to express the titer in transducing units. Recombinant deleted and undeleted viruses based on pBHG10 or pBHG11 had identical properties. The presented transduction studies were performed with pBHG11 derivatives.

**Analysis of adenovirus DNA.** To isolate DNA,  $10^5$  to  $10^6$  293 or 293-creB1 cells with full cytopathic effect resuspended in 100  $\mu\text{l}$  of PBS or  $10^9$  CsCl adenovirus particles in dialysis buffer were digested with 100  $\mu\text{l}$  of 0.1% conc in 10 mM Tris-Cl (pH 7.5)-1% sodium dodecyl sulfate (SDS)-10 mM EDTA for 3 h at  $37^\circ\text{C}$ . After phenol-chloroform extraction, DNA was ethanol precipitated and dissolved in 100  $\mu\text{l}$  of TE (10 mM Tris [pH 7.5], 1 mM EDTA). DNA quantification was performed either with ethidium bromide-stained agarose gels loaded with concentration markers or spectrophotometrically. Generally, 10  $\mu\text{l}$  of DNA was used for *HindIII* digestion.

**Animal studies.** Animal studies were performed in accordance with the institutional guidelines set forth by the University of Washington. Female C57BL/6 and C57BL/6-*scid* mice (Jackson Laboratories, Bar Harbor, Maine) aged 5 to 6 weeks were used in the experiments described here. All animals were housed in specific-pathogen-free facilities. Adenovirus injection was performed by tail vein infusion with 200  $\mu\text{l}$  of adenovirus diluted in adenovirus dialysis buffer. Generally,  $10^{10}$  PFU or transducing particles were injected per mouse, a dose that transduces  $\sim 100\%$  of hepatocytes (35). [*methyl*-<sup>3</sup>H]thymidine (1  $\mu\text{Ci/g}$  of body weight) diluted in pyrogen-free physiologic saline or bromodeoxyuridine (BrdU) labelling agent (Amersham) was injected intraperitoneally in 200  $\mu\text{l}$ . Blood samples for hAAT or serum glutamic pyruvic transaminase (SGPT) analysis were obtained by retroorbital or tail bleeding, respectively. A two-thirds partial hepatectomy was performed as described earlier (32).

**Histological analysis.** For histological analysis, liver samples (left half of the large upper lobe) were fixed in 10% neutral formalin, embedded in paraffin, sectioned (6  $\mu\text{m}$ ), and stained with hematoxylin and eosin.

Autoradiography after [*methyl*-<sup>3</sup>H]thymidine labelling was performed on paraffin sections that were dip coated with Kodak NTB-2 emulsion diluted 1:1 (vol/vol) with water and developed after a 2-week exposure. All slides were

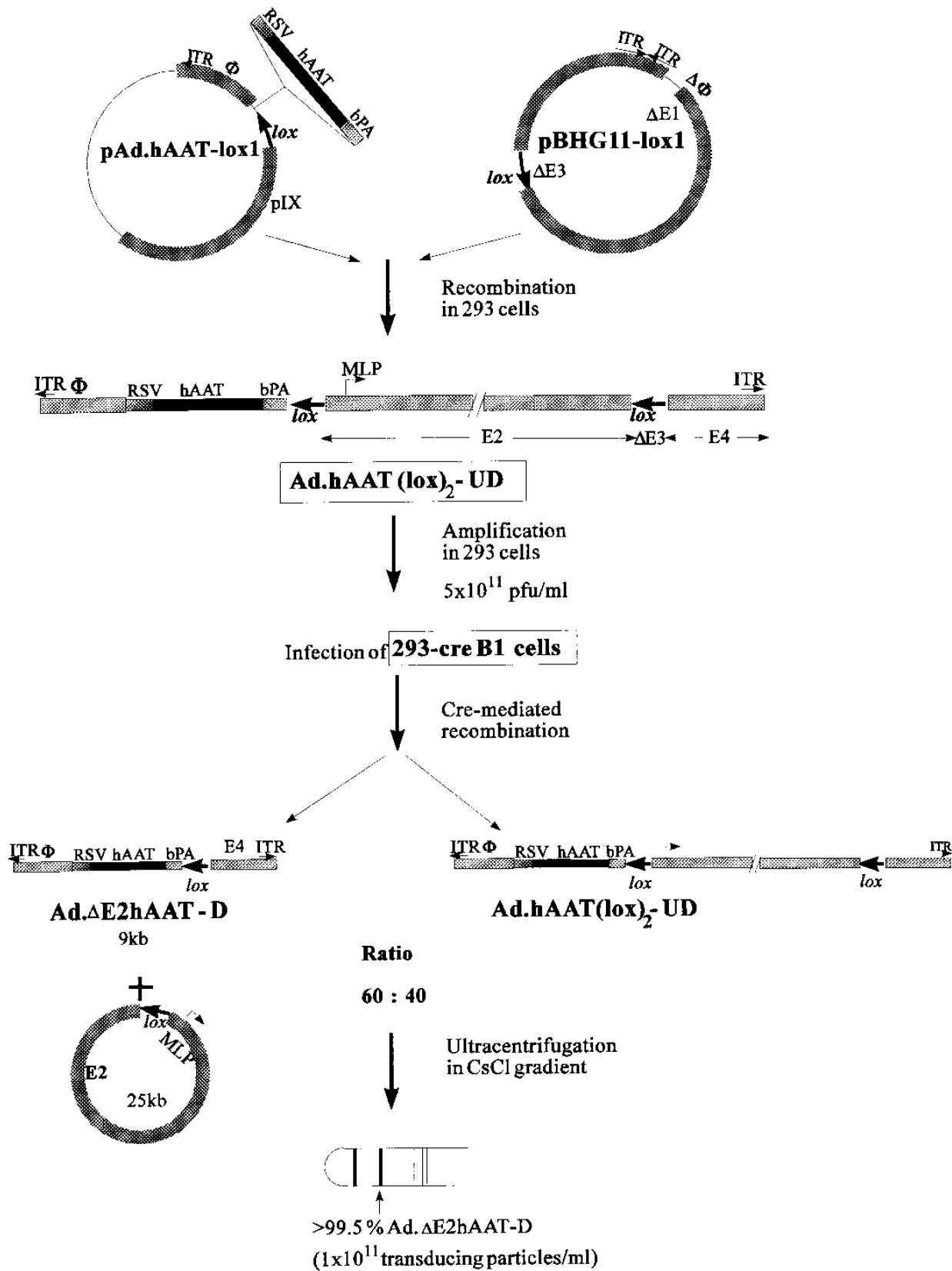


FIG. 1. Strategy for producing deleted adenoviruses by using the Cre-lox system. Φ, packaging signal; hAAT, hAAT cDNA, bPA, bovine growth hormone polyadenylation signal; MLP, major late promoter; RSV, RSV-LTR.

counterstained with hematoxylin and eosin, and at least 1,000 cells from random fields were counted. Immunohistochemical staining was carried out on liver tissues that were frozen in OCT compound (Miles, Inc., Elkhart, Ind.) and sectioned (10  $\mu$ m) or on cells cultured on chambers slides (Nunc) after fixation with 1:1 (vol/vol) acetone-methanol. The following antibodies were used: goat anti-hAAT (MN55082; Atlantic Antibodies) (diluted 1:50), rabbit anti-ORF3 and anti-ORF4 (1:1 mixture) (personal gift from G. Ketner) (1:15), rabbit anti-hexon (fluorescein isothiocyanate conjugated) (AB 1056F; Chemicon) (1:20), mouse monoclonal antifiber (4D25) (personal gift from J. Engler) (1:20), and mouse monoclonal anti-DNA-binding protein (anti-DBP) (Mab 37-3; GenVec) (1:50). The corresponding species-specific antibodies conjugated to fluorescein isothiocyanate were obtained from Sigma. All antibody dilutions were performed with Dulbecco modified Eagle medium with 10% fetal calf serum.

For examination of viral particles in the transmission electron microscopy studies, CsCl-purified virions were fixed with glutaraldehyde and stained with uranyl acetate as described previously (39).

**Cell culture.** 293 (Microbix Biosystems Inc.), HeLa (American Type Culture Collection) (CCL2), and Hepa 1a (American Type Culture Collection) (HB8065) cells were grown in high-glucose Dulbecco modified Eagle medium with 10% fetal calf serum (Hyclone). Primary mouse hepatocytes were obtained by collagenase perfusion as described before (32). Hepatocytes with >90% viability were plated at a density of  $2 \times 10^6$  on collagen I-coated 6-cm-diameter dishes in Williams E medium with 10% fetal calf serum. After 2 h, the Williams E-fetal calf serum medium was replaced by a hormonally defined medium (37); these conditions are known to maintain differentiated hepatocytes.

**Biochemical analysis of serum samples.** Serum hAAT concentrations were determined by enzyme-linked immunosorbent assay as previously described (30). A Sigma diagnostic kit was used for colorimetric determination of the activity of SGPT with 10  $\mu$ l of serum (Sigma procedure no. 505).

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of viral proteins.** Viral proteins were reduced in 0.25 M Tris-Cl (pH 6.8)–8% SDS–1%  $\beta$ -mercaptoethanol–10% glycerol with boiling for 5 min and then loaded on a Tris-glycine-SDS–10% polyacrylamide gel, run at 35 mA for 3 hours, and silver stained.

**Southern analysis.** For genomic DNA preparation, cultured cells were washed three times with PBS before analysis. Mouse livers were flushed with 5 ml of PBS via the portal vein. After removal, the livers were homogenized, and a 100-mg portion was used for DNA extraction as described previously (55). DNA concentrations were determined spectrophotometrically. Ten micrograms of genomic DNA was digested with *Bam*HI, run on an 0.8% agarose gel, and electrophoretically transferred to a Hybond nylon filter (Amersham). The blots were hybridized in rapid hybridization buffer (Amersham) with [ $\alpha$ - $^{32}$ P]dCTP-labelled DNA probes, using a random priming kit (Gibco BRL). As a control, DNA from noninfected animals or cells was spiked with 10 pg of undeleted viral genomic DNA and was loaded on each gel. The relative amount of adenovirus DNA was determined by Phosphorimager analysis as a ratio between the sample signal and the control signal. Small variations in DNA loading or transfer between lanes were adjusted by rehybridization of the blots with probes for the mouse metallothionein I gene (for mouse DNA) or for the genomic hAAT gene (for human DNA). The following DNA fragments were used for labelled probes: 1.4 kb of the hAAT cDNA (*Eco*RI fragment of pAd.RSVhAAT) (30), 6 kb of the Ad5 E4 region (*Eco*RI fragment of pBGH11), 0.65 kb of the E1a gene (PCR product [nucleotides 571 to 1220 of pFG140] [Microbix]), and 2 kb of the mouse metallothionein I gene (*Hind*III-*Eco*RI fragment of pmMMT-1) (55).

**DNA replication studies.** C57BL/6 or C57BL/6-*scid* mice were injected intravenously with  $10^{10}$  transducing particles of undeleted Ad.hAAT(*lox*)<sub>2</sub>-UD or deleted Ad. $\Delta$ E2hAAT-D vector. At 4 and 2 h before sacrifice, 200  $\mu$ l of BrdU labelling reagent (RPN 201; Amersham) was injected intraperitoneally. Fifty micrograms of genomic liver DNA was digested with *Bam*HI and separated on a 0.8% agarose gel. After depurination in HCl, DNA was transferred in  $10\times$  SSC ( $1\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate) by capillary transfer to a nitrocellulose filter (ECL; Amersham). DNA was immobilized by baking at 80°C for 2 h under vacuum. The filter was first blocked for 3 h at room temperature in 50 mM Tris-Cl (pH 7.5)–150 mM NaCl–5% dry milk–0.1% Tween 20 and then incubated overnight at 4°C with a monoclonal mouse antibody against BrdU (M0744; DAKO) diluted 1:50 in blocking buffer. After washing, biotinylated anti-mouse immunoglobulin G antibody (BA-2001; Vector Laboratories) diluted 1:100 in blocking buffer was added and left for 2 h at room temperature. Filters were then incubated for 1 h at room temperature in blocking buffer with avidin D-horseradish peroxidase (A-2004; Vector Laboratories) diluted 1:2,000. Specific binding was detected by using the ECL system (Amersham). At 40 and 42 h after a two-thirds partial hepatectomy, C57BL/6 mice were injected with BrdU. Genomic DNA was isolated from livers at 44 h after partial hepatectomy and analyzed as described above. Cell cycle arrest in confluent cultured cells was assessed by [*methyl*- $^3$ H]thymidine incorporation as described previously (23).

## RESULTS

**Production of deleted vectors.** Recombinant adenoviruses with two parallel *lox* sites flanking the pIX and E2 region were generated after recombination of pAd.hAAT-*lox*1 and

pBGH11-*lox*1 in 293 cells and purified to titers of  $5 \times 10^{11}$  PFU/ml (Fig. 1). A vector with antiparallel *lox* sites was constructed and served as a control for the Cre-mediated excision. No spontaneous homologous recombination events between the *lox* sites in Ad.hAAT(*lox*)<sub>2</sub>-UD were detectable after viral DNA analysis. The plasmid pCMV-*nlsc*re was cotransfected with pSV2neo into 293 cells, and G418-resistant cells were selected. Fifty single colonies were screened for *cre* mRNA expression by RNA in-solution hybridization. Clones with different *cre* mRNA levels were infected with Ad.hAAT(*lox*)<sub>2</sub>-UD, and the released virus was analyzed by ultracentrifugation in a CsCl step gradient.

After the first CsCl density step gradient, three clearly separated bands (separated by a distance of  $\sim 1$  cm) were visible. DNA was extracted from banded virus, and the genome size was analyzed by gel electrophoresis. The band with the highest density (1.35 g/cm<sup>3</sup>) contained undeleted virus [Ad.hAAT(*lox*)<sub>2</sub>-UD]. The middle band (1.32 g/cm<sup>3</sup>) contained the deleted virus (Ad. $\Delta$ E2hAAT-D), and the lightest band ( $\sim 1.25$  g/cm<sup>3</sup>) had mostly empty particles. Deleted virus obtained at this stage of purification contained about 10% contamination with undeleted virus as judged by quantitative DNA analysis of viruses collected from different bands in the CsCl gradient (Fig. 2A). After ultracentrifugation in a CsCl equilibrium gradient, the deleted virus could be purified to 99.5% purity as estimated by DNA analysis (Fig. 2A) and plaque assay on 293 cells, which revealed fewer than five to eight plaques per  $10^6$  total particles. A second equilibrium gradient did not significantly increase the purity of the deleted virus (Fig. 2A). The ratio of total particles to transducing (or infectious) particles was determined by hAAT and E4 gene expression in HeLa cells and was similar, at about 100:1, in all preparations for deleted and undeleted virus. The output yield of particles (based on optical density) per cell ( $\sim 200$  particles [deleted plus undeleted] per 293-*cre* cell) was comparable to amounts obtained after infection of 293 cells with E1-deficient adenovirus, indicating that viral protein synthesis in 293-*cre* cells was not limiting and virions were efficiently released.

The critical variables in maximizing the output of deleted vectors were the multiplicity of infection of Ad.hAAT(*lox*)<sub>2</sub>-UD in 293-*cre* cells, the time of adenovirus harvest, and the appropriate Cre-producing cell line as listed in Materials and Methods. The most efficient cell line for production of deleted vector (293-*cre*B1) contained about 150 *cre* mRNA molecules per cell over 60 passages. Interestingly, the clones with the highest *cre* mRNA concentrations did not necessarily give the best output yield of deleted virus. It is possible that a certain amount ( $\sim 40\%$ ) of virus DNA must remain undeleted to serve as a template for the production of proteins necessary for the efficient replication of deleted and undeleted virus DNA and for assembly of the corresponding virions.

Under optimal conditions, the ratio of deleted to undeleted particles after the first CsCl gradient was 60:40, with  $\sim 10^{11}$  transducing particles containing the deleted genome obtained from  $10^9$  cells. Deleted vector could not be generated with adenoviruses containing antiparallel *lox* sites.

**Characterization of the deleted vector.** The restriction analysis of deleted DNA isolated from purified virions (Fig. 2B) and sequence analysis of the region around the *lox* site showed that Cre-mediated recombination occurred accurately only between the two *lox* sites of Ad.hAAT(*lox*)<sub>2</sub>-UD joining the left genome end, with the ITR, packaging signal, and hAAT expression cassette, and the right end, with the 6-kb E4 region and right ITR.

The concentrations of viral DNA extracted from the same number of particles (based on optical density) were similar for

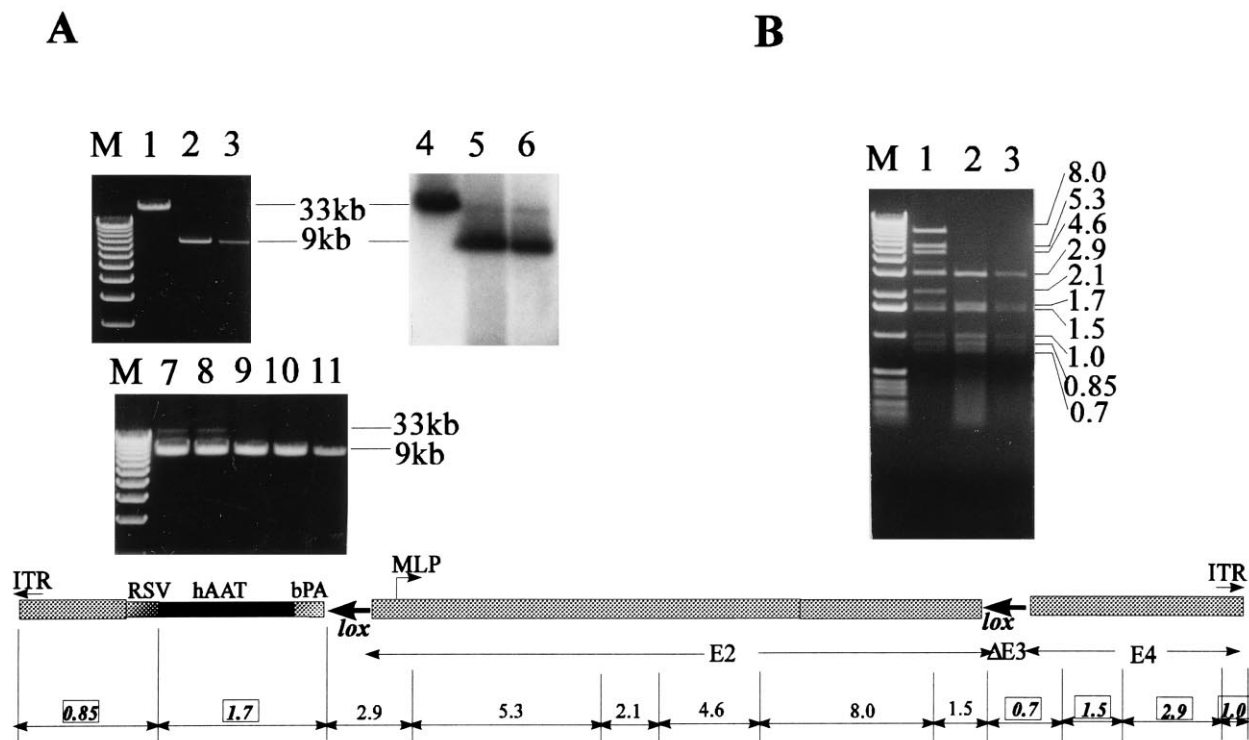


FIG. 2. Analysis of Ad.hAAT(lox)<sub>2</sub>-UD and Ad.ΔE2hAAT viral DNAs. (A) Quantitation of Ad.ΔE2hAAT genomes. Two hundred nanograms of viral DNA isolated from vectors purified by CsCl gradients were separated in a 0.8% agarose gel and ethidium bromide stained (lanes 1 to 3 and 7 to 11) or transferred to a nylon filter for hybridization with a 6.2-kb E4-specific, <sup>32</sup>P-labelled probe (lanes 4 to 6). DNA was isolated from Ad.hAAT(lox)<sub>2</sub>-UD virus (lanes 1 and 4) or Ad.ΔE2hAAT virus (lanes 2, 3, and 5 to 9) after the first CsCl step gradient from two independent preparations. Lanes 9 to 11 show Ad.ΔE2hAAT viral DNA after one (lanes 9 and 10) or two (lane 11) additional CsCl equilibrium gradients. 33kb, undeleted genome; 9kb, deleted genome; M, 1-kb DNA ladder. (B) Five hundred nanograms of viral DNA isolated from undeleted adenovirus (lane 1) or two different preparations of deleted adenovirus (lane 2 and 3) after purification by one CsCl step gradient were digested with *Hind*III and separated on a 0.8% agarose gel stained with ethidium bromide. The *Hind*III restriction map of Ad.hAAT(lox)<sub>2</sub>-UD DNA is shown in the lower part of the figure. All *Hind*III fragments (not in boxed italics) within the intervening region between the two *lox* sites are absent in DNA from deleted virus. The restriction fragments present in the deleted vector are indicated in italics in boxes. The Cre-mediated excision was confirmed by sequencing of the region around the remaining *lox* site within the deleted DNA with a bovine growth hormone polyadenylation signal (bPA)-specific primer. MLP, major late promoter; RSV, RSV-LTR.

deleted and undeleted viruses, indicating that the 9-kb genome was efficiently packaged. To characterize the protein composition of the deleted vector, the same amounts of purified particles of deleted and undeleted vectors were analyzed by denaturing SDS-10% PAGE after silver staining (Fig. 3). Capsid proteins were present in similar amounts in deleted and undeleted virions. Core proteins (VII and V) were reduced in virions containing the deleted DNA, possibly because of the reduced number of core binding sites. There were additional bands found in the deleted vector preparations. The origins of these bands are not known, but perhaps they represent non-specific degradation products.

Virions containing deleted viral DNA retained their icosahedral shape as seen in electron microscopy (Fig. 4). Staining with uranyl acetate caused the central viral cores to appear electron dense. Virions with deleted DNA had only a spotted luminal dark staining. This may have resulted from the smaller DNA genomes packaged within the deleted vectors compared with the undeleted vector.

These results taken together demonstrate that a small, 9-kb deleted genome can be packaged into capsids that appear to be structurally similar to those of undeleted adenovirus; however, the results do not directly demonstrate that the structural proteins were correctly processed into a particle that can transduce its genetic material into cells.

**Transduction in vitro.** To determine the ability of the deleted vector to transduce cells, different cell lines (293, HeLa,

and Hepa 1a) were infected with deleted or undeleted vectors at an MOI of 100 and analyzed for hAAT, E4 (ORF3/4), fiber, hexon, and DBP expression 40 h later. The data for all cell lines were similar, and an example of the results with HeLa cells is shown in Fig. 5. The immunofluorescence signals for the transgene hAAT were comparable for undeleted and deleted vectors, while the E4 gene products were found in a similar number of cells but at a slightly reduced intensity. The L2, L5, and E2a region products (hexon and fiber [Fig. 5] and DBP [not shown], respectively) were absent in Ad.ΔE2hAAT-D but not in Ad.hAAT(lox)<sub>2</sub>-UD vector-transduced cells. Although the fiber-coding sequences located downstream of the E3 region were still present in the deleted genome, because of the deletion of the major late promoter, no fiber expression was detected in cells transduced with this vector (Fig. 5).

hAAT and E4 expression proved that the deleted genome was efficiently transduced into target cells and transported to the nucleus, where it was transcribed by cellular RNA polymerases. The relative transducing abilities of the deleted and undeleted vectors were more accurately assessed in HeLa cells (Fig. 6A), mouse hepatoma cells (Fig. 6B), and primary hepatocytes (Fig. 6C) (the *in vivo* target cells) grown to confluency and arrested in the cell cycle (to preclude loss of adenovirus DNA from rapid division of cells) as determined by [<sup>3</sup>H]thymidine labelling. Gene expression was quantified by periodic measurements of secreted hAAT concentrations. In all three cell culture studies, the hAAT expression from both vectors

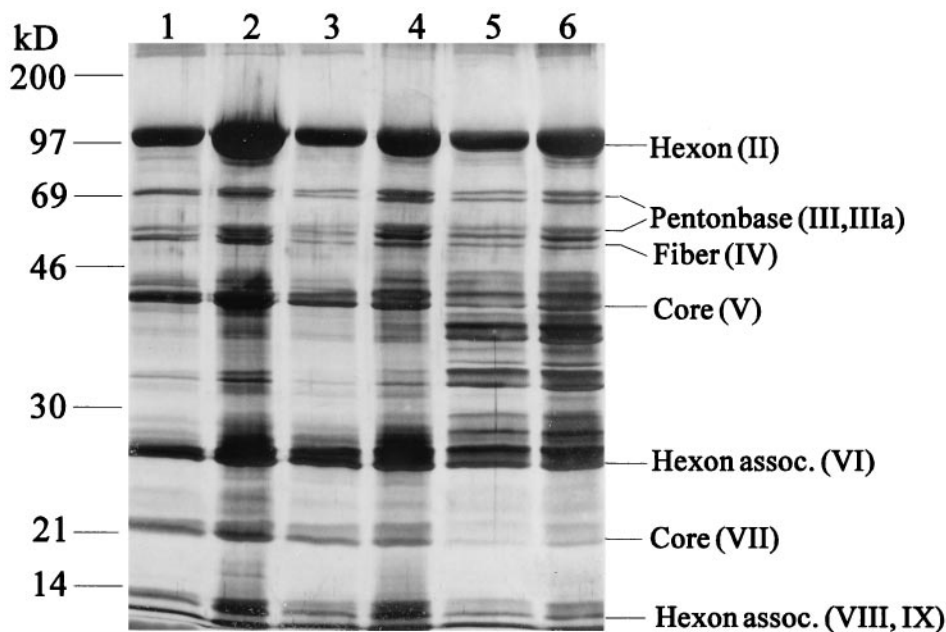


FIG. 3. Protein analysis of virions. Proteins from  $0.3 \times 10^{10}$  (lanes 1, 3, and 5) or  $1.0 \times 10^{10}$  (lanes 2, 4, and 6) viral particles were separated on an SDS-PAGE gel and then silver stained. Lanes 1 and 2, Ad/RSVhAAT(30); lanes 3 and 4, Ad.hAAT(lox)<sub>2</sub>-UD; lanes 5 and 6, Ad.ΔE2hAAT-D. The localizations of main viral proteins are indicated.

was initially similar, but that from the deleted vector declined (Fig. 6). To investigate the reasons for this, undeleted and deleted viral DNAs were analyzed by quantitative Southern blots of genomic DNAs from transduced cells with hAAT- or E4- specific probes (Fig. 6).

Deleted viral DNA declined to undetectable levels by days 2 to 3 after infection, whereas viral DNA from undeleted (E1-minus) virus accumulated by severalfold over time. In order to attempt to induce cellular transcription factors that may substitute for E1a (50), primary mouse hepatocytes were incubated with interleukin-6 (IL-6), a cytokine that is produced *in vivo* after adenovirus administration. Undeleted DNA initially accumulated at a greater rate but then declined in hepatocytes in the presence of IL-6 (Fig. 6C). Ad.hAAT(lox)<sub>2</sub>-UD DNA replication was also dependent on the virus titer (Fig. 6C); the greater the MOI used, the higher the relative amount of vector DNA that accumulated. This suggests that a critical number of genome copies per cell was required for vector stabilization and/or accumulation.

Ad.hAAT(lox)<sub>2</sub>-UD vector DNA accumulation was blocked by hydroxyurea (Fig. 6A), an inhibitor of viral DNA replication that allows the synthesis of viral proteins (53), demonstrating that replication of the vector DNA was responsible for vector DNA accumulation. Interestingly, when DNA replication was prevented, Ad.hAAT(lox)<sub>2</sub>-UD viral DNA concentrations fell in a manner similar to that for deleted DNA. There was no effect of hydroxyurea on the stability of Ad.ΔE2hAAT vector DNA. hAAT gene expression lagged behind but correlated well with the changes in DNA concentrations (Fig. 6A), which is probably related to hAAT mRNA stability. These results imply that both the stability and accumulation of the undeleted vector are properties related to vector DNA replication, while the loss of deleted genomes is most likely the result of their inability to replicate.

**In vivo properties of deleted and undeleted viruses.** To compare the efficiencies of deleted and undeleted vectors in transducing hepatocytes *in vivo*,  $10^{10}$  transducing particles of each

vector were injected intravenously into C57BL/6 and C57BL/6-*scid* mice. Serum samples were analyzed for hAAT expression, and the viral DNA in genomic liver DNA at different time points after infection was quantified (Fig. 7). The concentration of undeleted vector DNA in the liver decreased within the first 36 h by 77 and 51% in C57BL/6 and C57BL/6-*scid* mice, respectively, with the major decline occurring within the first 30 min. Subsequently, viral DNA levels were relatively stable over a time period of 7 days. During the next 12 weeks, a further gradual decline was observed. Serum hAAT concentrations were higher (30%) in *scid* than in non-*scid* mice. Following the rapid decline within the first 30 min, the deleted virus genome was cleared from the liver at 7 h postinfusion. Serum hAAT was detectable only at days 1 and 2 (peak, 80 ng/ml) (Fig. 7). Most likely, the deleted viral DNA template was eliminated before efficient transcription from the RSV-LTR promoter could be initiated. Expressed E4 proteins were detected 16 h after infusion of Ad.ΔE2hAAT-D and Ad.hAAT(lox)<sub>2</sub>-UD viruses in livers by immunofluorescence in similar percentages of hepatocytes; however, the signal intensity was lower in livers infected with the deleted vector (Fig. 8). Notably, in mice infused with the Ad.hAAT(lox)<sub>2</sub>-UD vector, about 25% of hepatocytes still expressed E4 proteins at 12 weeks. DBP immunofluorescence at 16 h postinfusion was undetectable in livers infected with the deleted vector and clearly positive in livers infected with the undeleted virus (not shown).

**In vivo hepatocellular toxicity.** The deleted vector was shown to transduce cells with efficiencies similar to those of the undeleted vector. A major unanswered question that remains is whether the relative toxicity of first-generation adenovirus is related to the input virus particle itself or to *de novo*-synthesized viral proteins. To study toxic effects of adenoviruses, serum pyruvic glutamic transaminase (SGPT) concentration, an early and sensitive marker for hepatocellular injury, was measured. Intravenous administration of the E1a-deficient adenovirus Ad.hAAT(lox)<sub>2</sub>-UD resulted in biphasic elevations in SGPT concentrations (Fig. 9). Early hepatocellular damage

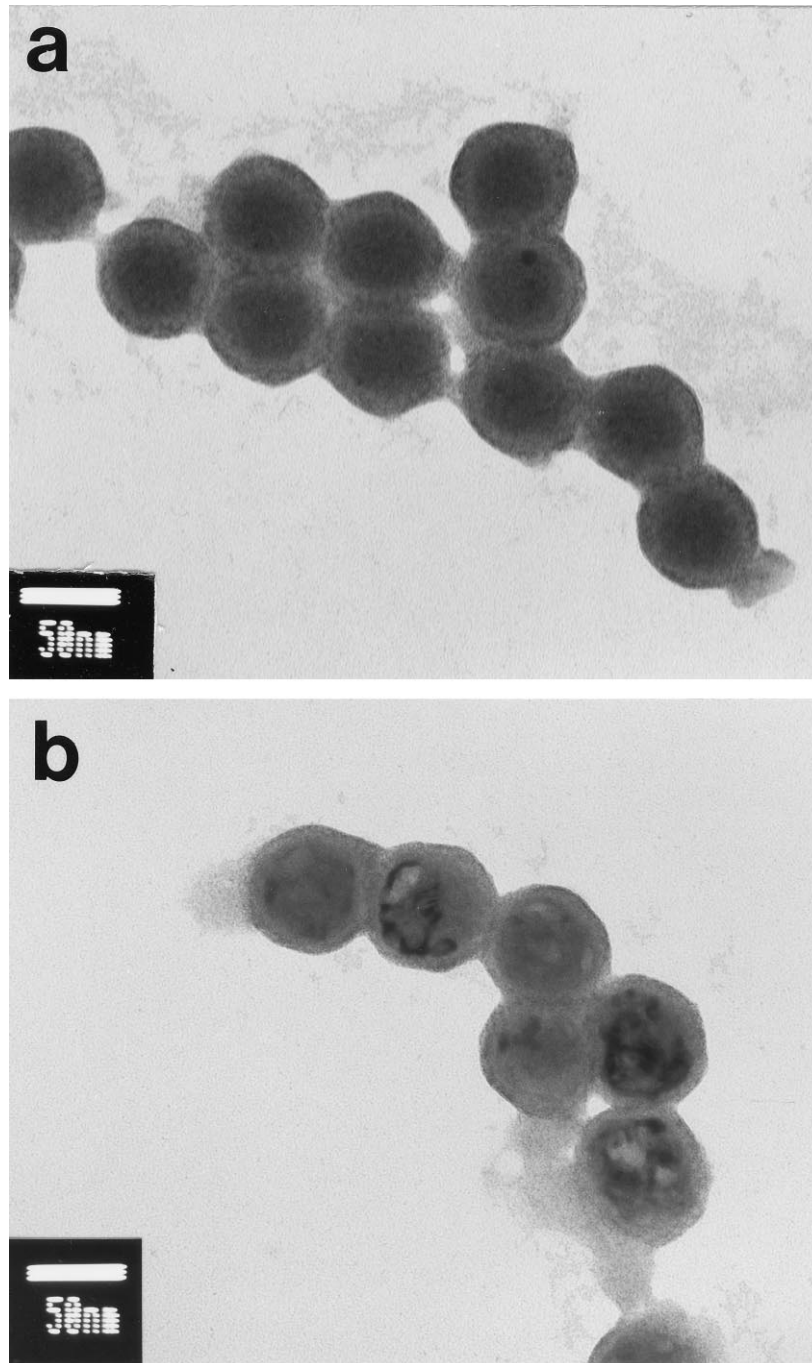


FIG. 4. Electron photomicrographs of adenovirus particles. (a) Particles with Ad.hAAT(lox)<sub>2</sub>-UD genomes; (b) particles with Ad.ΔE2hAAT-D genomes. Magnification, ×100,000. Bar, 50 nm.

was detected between 12 and 48 h postinfusion and was more pronounced in *scid* mice. In the immunocompetent animals, this early phase was followed by chronic hepatocyte destruction that lasted as long as transgene and viral expression was detectable, suggesting that it was the result of antigen-specific immunity.

In contrast to Ad.hAAT(lox)<sub>2</sub>-UD, the same number of particles of Ad.ΔE2hAAT-D adenovirus did not lead to early or late hepatocellular injury. This indicated that the early hepatocellular toxicity after systemic adenovirus injection was not

related to capsid or other proteins of the input virus but was possibly due to the direct cytotoxicity of de novo-synthesized vector proteins and/or was mediated by innate, nonspecific immune mechanisms involving cytokines, complement, and NK cell activity. The fact that SGPT levels at 24 h postinfusion were higher in *scid* mice with elevated cytokine and NK cell activities (41) supports the latter hypothesis. In earlier studies by Duncan et al. (12), it was demonstrated that UV-inactivated wild-type virus injected intravenously in mice had no influence on SGPT levels. The SGPT levels were normal after intramus-

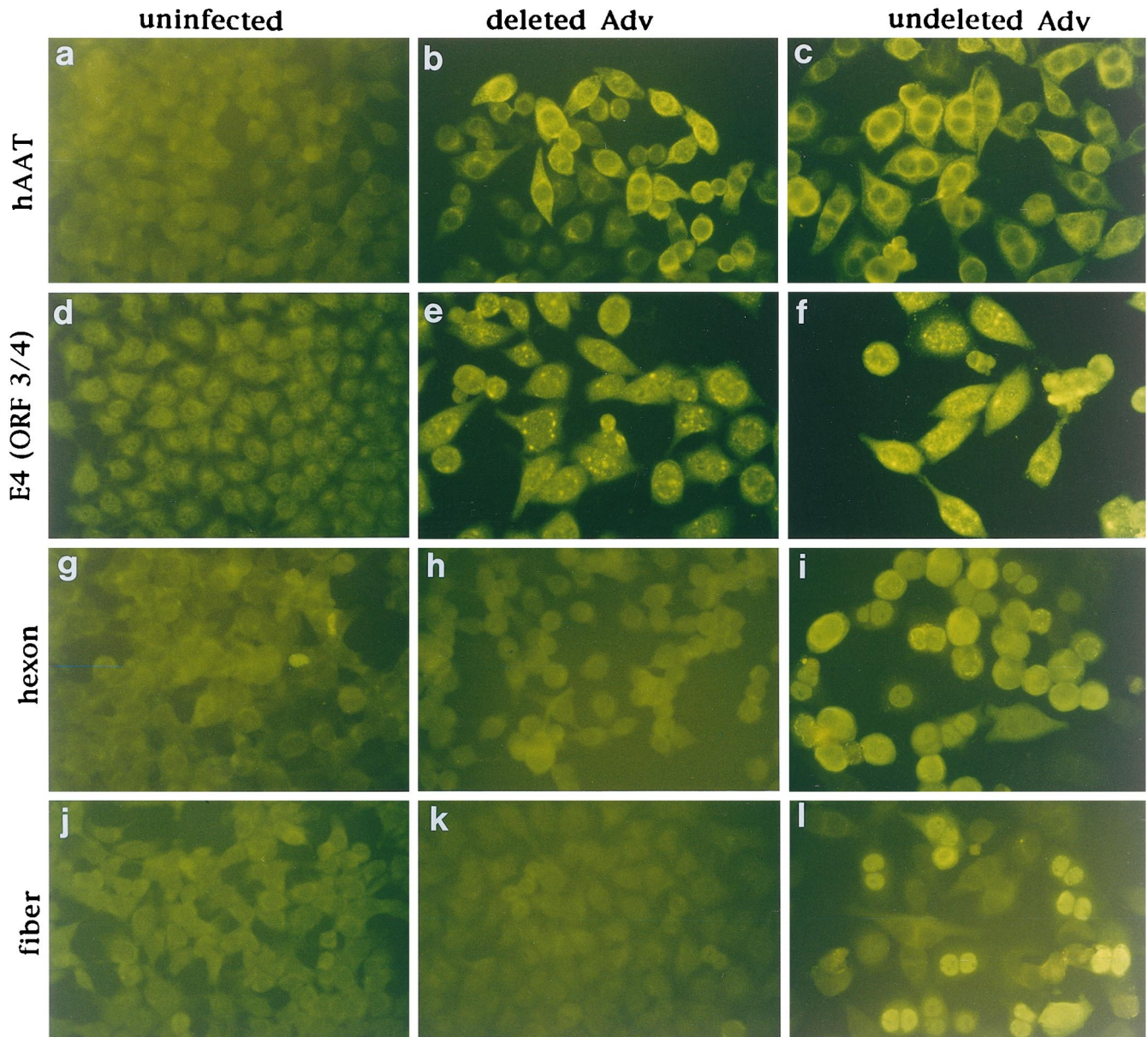


FIG. 5. Immunofluorescence analysis with HeLa cells. HeLa cells were infected with Ad.hAAT(lox)<sub>2</sub>-UD or Ad.ΔE2hAAT-D adenoviruses at an MOI of 100. At 40 h postinfection, the cells were washed with PBS, fixed in methanol-acetone, and incubated with primary antibodies for 1 h. After washing, hAAT, E4, and fiber detection was completed by 30 min of incubation with the corresponding species-specific antibody conjugated to fluorescein isothiocyanate. Adenovirus assembly takes place in the host cell nucleus; thus, hexon and fiber signals in cells infected with undeleted adenovirus are localized in the nucleus.

cular or intranasal administration of  $10^{10}$  transducing particles of undeleted virus or after infusion of virus buffer in mice (not shown).

The patterns of liver injury determined by the SGPT elevations were supported by histological studies which showed a sparse neutrophil infiltration on days 1 and 2 postinfection, lymphocytic infiltration, and signs of hepatocellular degeneration beginning on day 4 in animals receiving undeleted but not deleted vector (not shown). The effects of hepatic injury are manifested by a compensatory proliferation of hepatocytes (16). The percentage of regenerating hepatocytes was quantitated by use of [<sup>3</sup>H]thymidine, whose incorporation into DNA during the S phase can be detected in liver sections. After systemic administration of E1-minus adenovirus, DNA synthe-

sis in hepatocyte nuclei with a peak at day 4 postinfusion was observed (Fig. 10). About 75 and 45% of the hepatocyte nuclei were labelled in the livers of *scid* and congenic non-*scid* mice, respectively. In contrast, rarely labelled (less than 1%) hepatocytes were detected in mice receiving an equivalent amount of deleted vector. While hepatocellular replication disappeared after 9 days in *scid* mice, about 5 to 10% of hepatocyte nuclei were still replicating over a period of 70 days in C57BL/6 livers, an observation consistent with chronic inflammation.

**Replication of E1-deficient adenovirus in mouse livers.** There was no significant viral DNA accumulation in mouse livers after injection with E1-deleted virus (Fig. 7). Since an equilibrium state between viral replication and antiviral host immune mechanisms leading to viral DNA or hepatocyte deg-



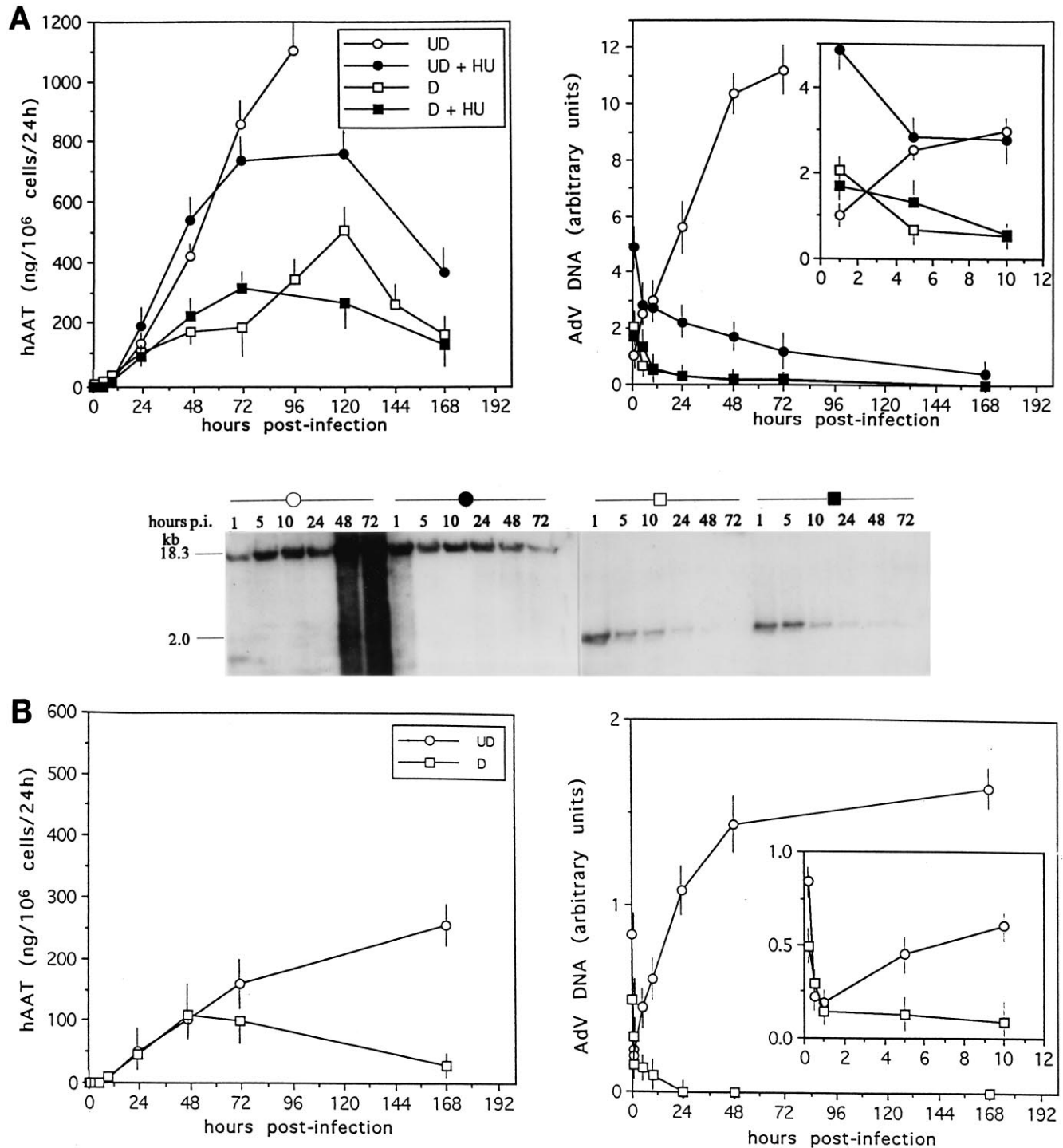


FIG. 6. Transgene expression and quantitative DNA blot analysis in vitro. Confluent cultured cells were infected with the Ad.hAAT(lox)<sub>2</sub>-UD (UD) or Ad.ΔE2hAAT-D (D) vector. The medium was changed 1 h after infection and then daily for hAAT determinations. At different times postinfection (p.i.), genomic DNA was extracted. Ten-microgram samples were digested with *Bam*HI and analyzed by Southern blotting with an hAAT-specific probe. Ad.hAAT(lox)<sub>2</sub>-UD and Ad.ΔE2hAAT-D genomes can be distinguished by specific *Bam*HI fragments containing the hAAT gene (UD, 18.3 kb; D, 2.0 kb). The DNA was quantified by Phosphorimager analysis. The data are expressed as arbitrary units relative to a concentration standard loaded on each gel; 1.0 arbitrary unit is 10 pg ( $5 \times 10^{10}$  PFU) of undeleted viral genomic DNA mixed with 10 μg of DNA from the corresponding uninfected cells that were used. Each value represents the mean and standard deviation from at least three independent experiments. One example of a Southern blot is shown. The symbols above the blot are the same as used for hAAT and DNA quantification. AdV, adenovirus. (A) HeLa cells transduced with Ad.hAAT(lox)<sub>2</sub>-UD and Ad.ΔE2hAAT-D at an MOI of 100. Hydroxyurea (HU) was added to the culture medium at a final concentration of 10 mM 1 h postinfection and later with each medium change. Ad.hAAT(lox)<sub>2</sub>-UD-infected HeLa cells in the absence of hydroxyurea developed toxicity by 72 h. (B) Hep1a (mouse hepatocarcinoma) cells transduced with Ad.hAAT(lox)<sub>2</sub>-UD and Ad.ΔE2hAAT-D at an MOI of 100. (C) Primary mouse hepatocytes transduced with Ad.ΔE2hAAT-D (MOI, 100) and Ad.hAAT(lox)<sub>2</sub>-UD (MOIs, 100, 200, and 500). Five hundred units of recombinant murine IL-6 (Quality Controlled Biochemicals, Inc.) per ml was added to hepatocytes 6 h before adenovirus infection (MOI, 100) and with each medium change.

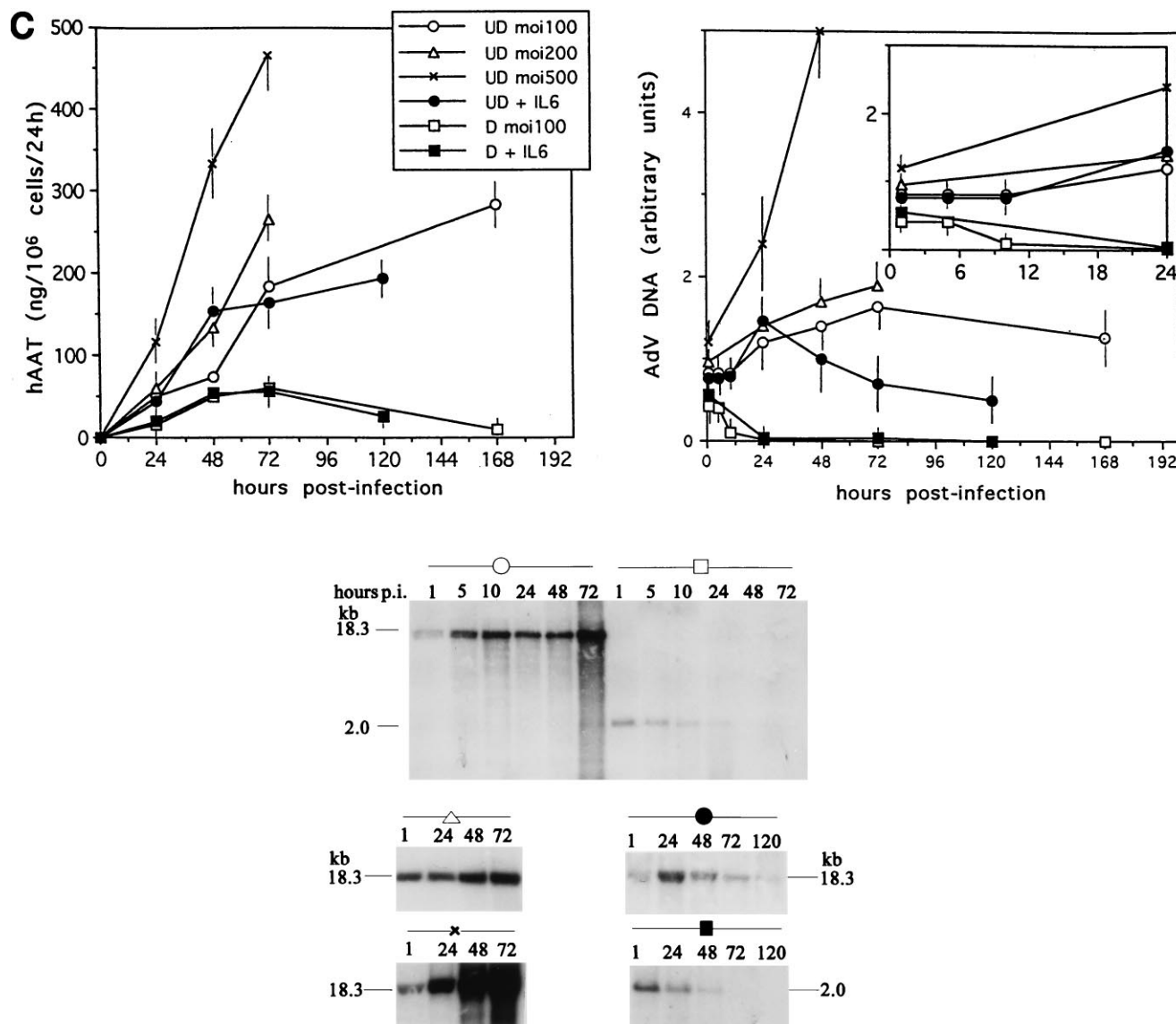


FIG. 6—Continued.

radiation could not be excluded, de novo DNA synthesis studies with BrdU incorporation were carried out. After BrdU injection, total DNA extracted from mouse livers at different time points after adenovirus infusion was digested with *Bam*HI and separated on an agarose gel. After transfer, the nitrocellulose filter was incubated with anti-BrdU antibodies. Specific antibody binding was enhanced and then made visible by using the ECL system (Amersham) (Fig. 11).

Cellular DNA replication after adenovirus injection appears as a background smear of DNA with BrdU incorporated that becomes most intense 4 days postinfection, similar to that seen for DNA isolated from hepatocytes 48 h after partial hepatectomy. Specific *Bam*HI fragments of adenovirus DNA (18.3, 6.2, 4.8, and 2.0 kb) with incorporated BrdU appear in DNA isolated from mice after injection of undeleted virus but not in partially hepatectomized mice. The larger, 18-kb *Bam*HI fragment was better visualized when less genomic DNA was loaded on the gel (not shown). Undeleted virus DNA replication in mouse livers was first detectable between 8 and 12 h postin-

fection. At later time points (32 to 36 h and 92 to 96 h), BrdU-labelled bands became more intense. At day 4 postinfection, the background BrdU signal was stronger than the signal from the adenovirus DNA fragments, hindering viral replication studies. Replication was detectable for at least 10 days. The relative amounts of viral DNA replication in the livers of C57BL/6-*scid* and C57BL/6 mice were comparable. Neither cellular or viral DNA replication occurred after injection with Ad.ΔE2hAAT-D adenovirus or in naive animals.

The relative amounts of undeleted adenovirus DNA found in the spleen, lung, and liver were within 50% of each other 1 day after intravascular infusion (Fig. 11B), yet adenovirus DNA declined more rapidly, and to very low levels, in the spleen and lung compared with the liver. Furthermore, no replication was detected during the first 24 h in the nonhepatic tissues (not shown), suggesting that the relative instability of Ad.hAAT(lox)<sub>2</sub>-UD in nonhepatic tissues may be linked to vector DNA replication.

E1a-containing replication-competent helper virus gener-

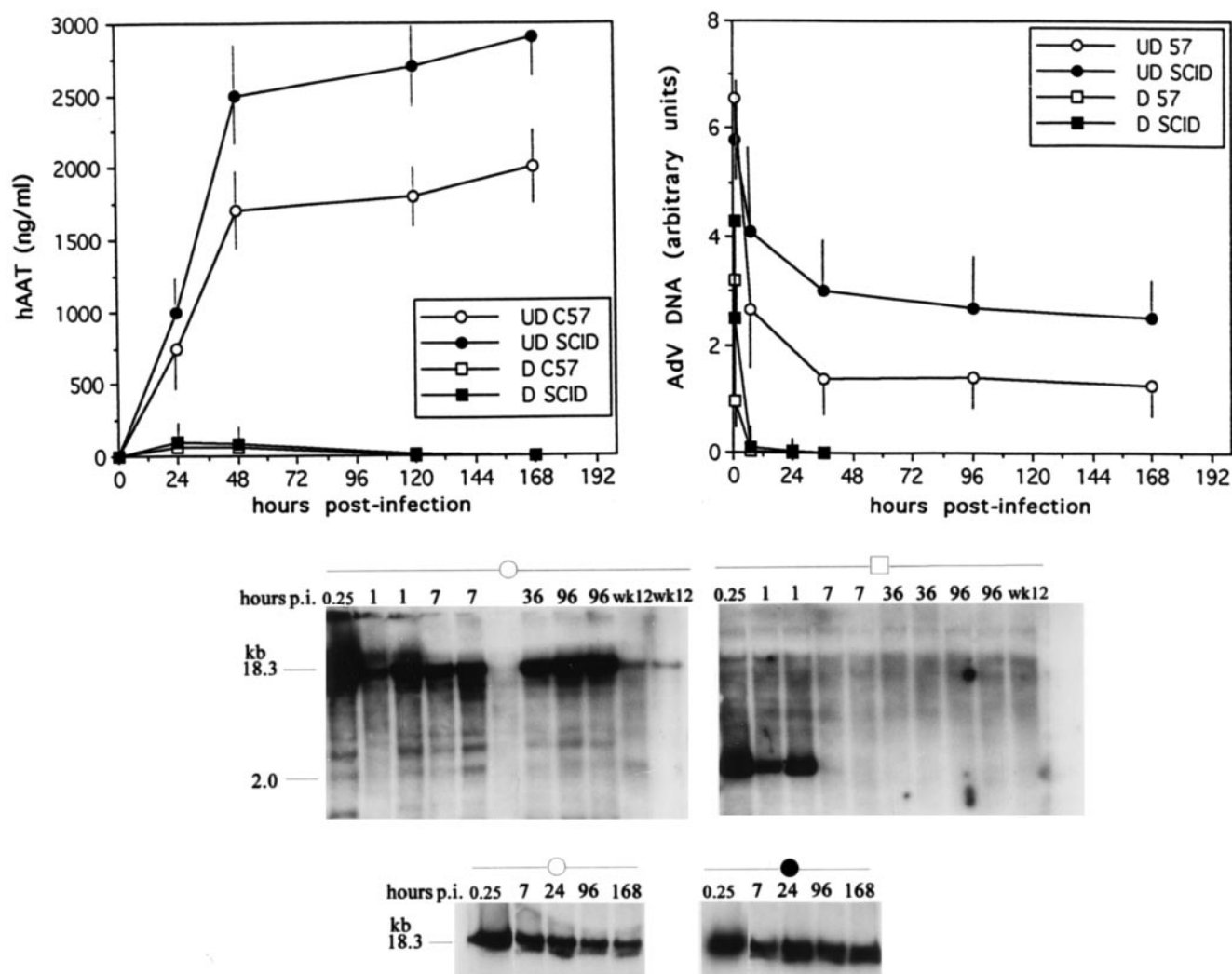


FIG. 7. In vivo gene transfer with deleted and undeleted adenovirus (AdV). C57BL/6 or C57BL/6-*scid* mice (5 to 6 weeks old) were injected with  $10^{10}$  infectious particles of deleted (D) and undeleted (UD) viruses by tail vein infusion. At different times postinfection (p.i.) serum samples were collected for hAAT quantitation. Genomic DNA was extracted from livers and flushed with PBS via the portal vein prior to sacrifice. Ten-microgram genomic DNA samples were analyzed as described in the legend to Fig. 6. The concentration standard was mixed with DNA from untreated mice ( $n =$  three mice per point).

ated by recombination with E1a sequences in 293 cells may be present in low concentrations in highly purified first-generation vector stocks (27). Additional studies were performed to exclude low levels of replication-competent virus as an explanation for the accumulation and replication of adenovirus genomes. First, no plaquable virus was recovered from 0.5 ml of serum and whole liver homogenates from Ad.hAAT(lox)<sub>2</sub>-UD vector-infected animals (not shown). Second, the BrdU-labelled band pattern was specific for undeleted virus and differed from *Bam*HI fragments obtained from E1a plus (wild-type) Ad5 (not shown). This excludes the possibility that de novo-synthesized DNA originated from contaminating non-vector-derived sequences. Third, no E1a-containing sequences were detectable by Southern DNA analysis in mice injected with undeleted vector (Fig. 12). Finally, the limit of detection of helper replication-competent adenovirus (RCA) was at least 1 in  $1 \times 10^6$  PFU (3) with research-grade vectors and less than 1 in  $2.5 \times 10^9$  PFU with clinical-grade vector (Fig. 11A, AdA and AdB). Assuming the worst-case scenario, there may be at most 10,000 and 4 RCA particles in research- and clinical-

grade preparations infused in a mouse (of  $10^{10}$  PFU), respectively. Even if each particle infected a liver cell (mouse liver contains  $10^8$  hepatocytes), only 1 in  $10^4$  or 2 in  $10^7$  hepatocytes would contain the RCA genome.

**Stabilization of deleted vector DNA in trans.** To begin to understand whether the original DNA structure of the input virus or the absence of E2 gene expression was responsible for the instability of deleted genomes, E2 complementation studies were performed (Fig. 13). An E1a-deficient adenovirus with  $\beta$ -galactosidase as a transgene (Ad. $\beta$ Gal) was infected onto HeLa cells 5 h after transduction with Ad. $\Delta$ E2hAAT-D adenovirus (MOI, 100) (Fig. 13A). E2-DBP expression was detected by immunofluorescence after 16 h in 100 and 40% of cells coinfecting with Ad. $\beta$ Gal at MOIs of 100 and 5, respectively (not shown). Cellular DNA was analyzed for hAAT-specific DNA present within the deleted virus genome at different times postinfection. While deleted DNA was completely cleared from HeLa cells at 7 days after infection with deleted vector alone, about 50 and 95% of the deleted genome was still

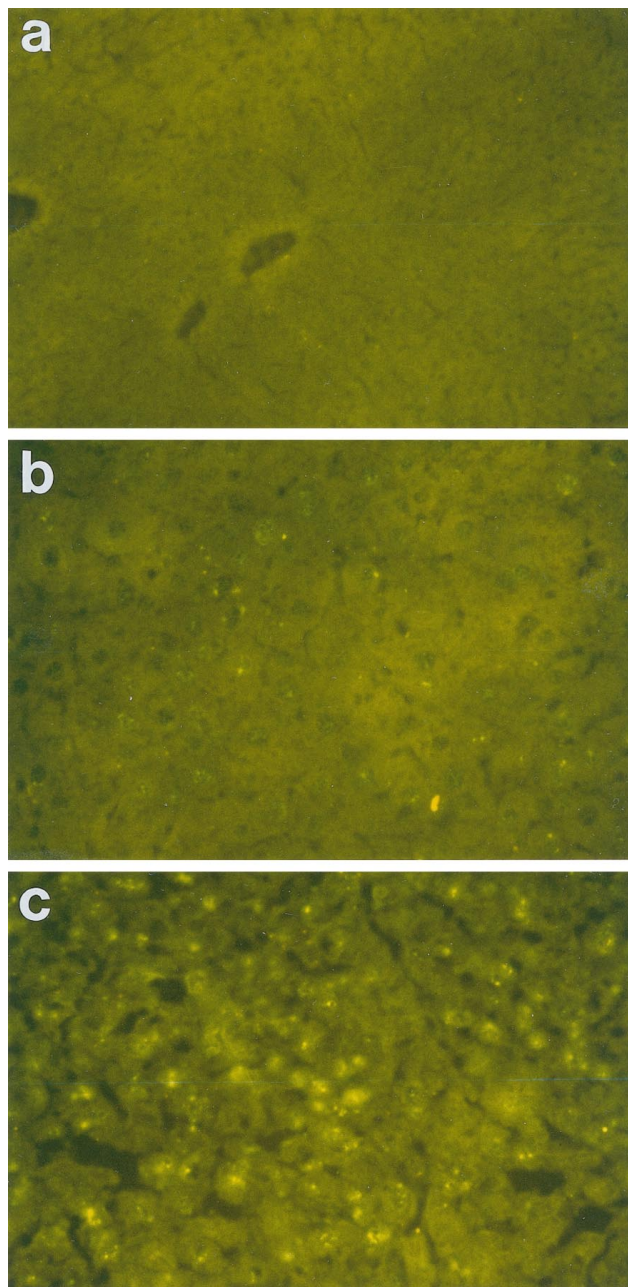


FIG. 8. Immunofluorescence analysis for E4 protein expression in liver. Liver cryosections from C57BL/6 mice were stained with antibodies directed against E4-ORF3/4 16 h after infusion. (a) Control liver; (b and c) after infusion of  $10^{10}$  transducing particles of Ad.ΔE2hAAT-D (b) or Ad.hAAT(lox)<sub>2</sub>-UD (c).

present at 7 days postinfection in cells infected with Ad.βGal at MOIs of 5 and 100, respectively.

To confirm the *trans* complementation in vivo, mice were infused with Ad.ΔE2hAAT-D or equal amounts of Ad.ΔE2hAAT-D and Ad.βgal. Animals receiving both vectors had sustained serum hAAT concentrations for at least 16 days, compared with 2 days in animals receiving Ad.ΔE2hAAT-D (Fig. 13B). Thus, in both cultured cells and primary mouse hepatocytes in vivo, deleted vectors can be made to persist in the presence of first-generation vectors, presumably because they can supply the appropriate stabilizing adenovirus proteins.

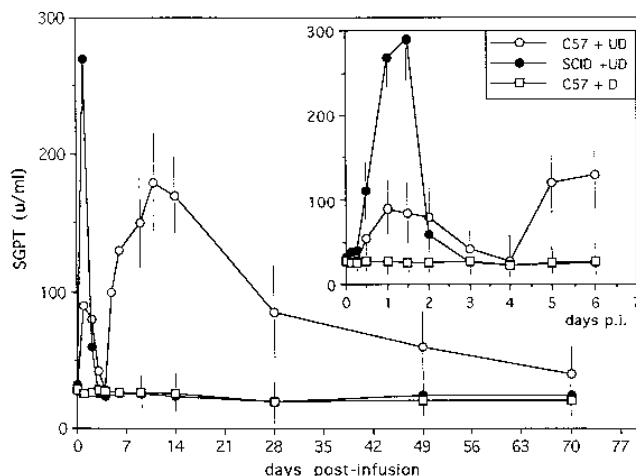


FIG. 9. SGPT in mice infused with adenovirus. SGPT levels in 5- to 6-week-old C57BL/6 and C57BL/6-*scid* mice were determined periodically after intravenous infusion of  $10^{10}$  infectious units of Ad.hAAT(lox)<sub>2</sub>-UD (UD) and Ad.ΔE2hAAT-D (D) vector ( $n =$  three or more per point). p.i., postinfection.

### DISCUSSION

The results from this study demonstrate that by using the *Cre-lox* recombination system, an adenovirus deficient in E1, E2, E3, and late gene expression can be produced at a high titer. The genome with 25 kb deleted is efficiently packaged into virions that can transduce cells in vitro and in vivo at high efficiency. The deleted vector appears to be nontoxic. However, after gene transfer, gene expression is transient because of instability of the vector genome. The vector can be stabilized in *trans*, suggesting that virus-encoded proteins are needed for genome stabilization. Further studies established that the persistence of first-generation vectors, at least in part, resulted from DNA replication of vector genome in both cultured cells and primary mouse hepatocytes, in vitro and in vivo. Plaqueable virus was not recovered from the liver. The small amount of

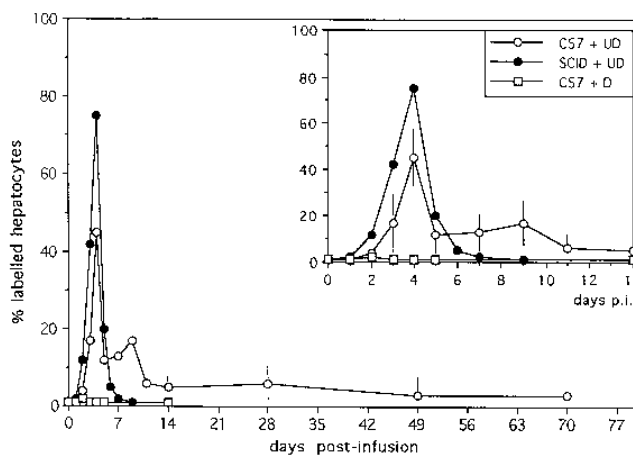


FIG. 10. Analysis of hepatocellular DNA synthesis in mouse livers after injection with Ad.hAAT(lox)<sub>2</sub>-UD (UD) and Ad.ΔE2hAAT-D (D) vectors. A total of  $10^{10}$  transducing particles of Ad.hAAT(lox)<sub>2</sub>-UD or Ad.ΔE2hAAT-D were injected in 5- to 6-week-old C57BL/6 or C57BL/6-*scid* mice. At 12 and 2 h before sacrifice, the animals were infused with [*methyl*-<sup>3</sup>H]thymidine. Liver sections were exposed to film emulsion, and the proportion of positive hepatocyte nuclei was scored for at least 1,000 cells per slide. Symbols without standard deviations,  $n = 2$ ; other samples,  $n = 3$ . p.i., postinfection.

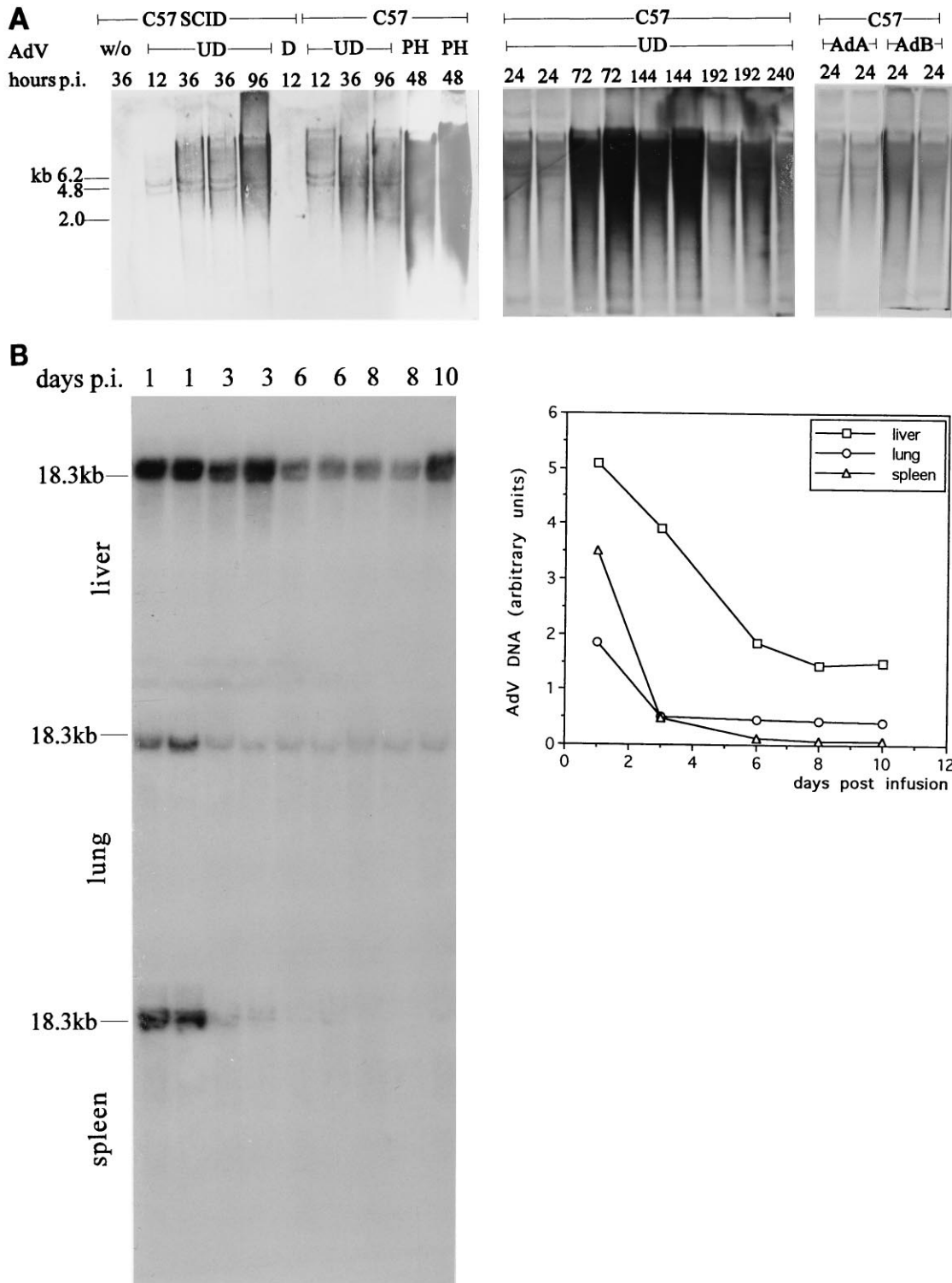


FIG. 11. Adenovirus (AdV) DNA synthesis in vivo. (A) Replication of adenovirus DNA in mouse liver. C57BL/6 or C57BL/6-*scid* mice were injected intravenously with  $10^{10}$  transducing particles of Ad.hAAT(lox)<sub>2</sub>-UD (UD), Ad.ΔE2hAAT-D (D), Ad2/CMVβgal-2 (AdA), or Ad2/CMVβgal-4 (AdB). At 4 and 2 h before sacrifice, 200 μl of BrdU labelling reagent was injected intraperitoneally. Fifty micrograms of genomic liver DNA was digested with *Bam*HI, separated on a 0.8% agarose gel, and transferred to a nitrocellulose filter, and immobilized. After blocking, the filter was incubated with a monoclonal mouse antibody against BrdU. After washing, the blot was exposed to biotinylated anti-mouse immunoglobulin G antibody and then to avidin D-horseradish peroxidase. Specific binding was detected by using the ECL system (Amersham). Control animals were subjected to a partial hepatectomy (PH), and BrdU was administered 40 and 42 h later. Each time point represents an individual animal. w/o, no treatment; p.i., postinfection. (B) Quantitation of adenovirus DNA in different tissues. Adenovirus DNA in the spleen, liver, and lung was quantitated as described in the legend to Fig. 6.

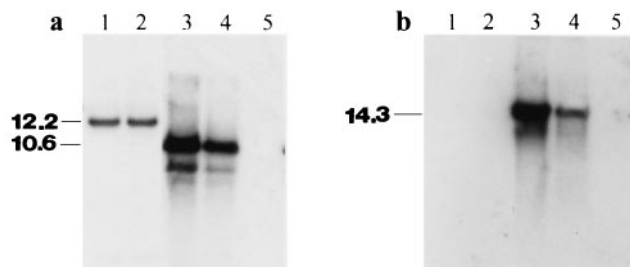


FIG. 12. Southern blot analysis of liver DNA for E1a sequences. C57BL/6 mice were injected intravenously with  $10^{10}$  Ad.hAAT(lox)<sub>2</sub>-UD transducing particles (lanes 1 and 2),  $10^9$  PFU of wild-type Ad5 (lanes 3 and 4), or virus dialysis buffer (lanes 5). Seven days later, 10  $\mu$ g of genomic DNA was digested with *Bam*HI and probed with an E4-specific probe (a) or an E1a-specific probe (b). 12.2 and 10.6 kb, E4-specific fragments for Ad.hAAT(lox)<sub>2</sub>-UD and wild-type Ad5, respectively; 14.3 kb, E1a-specific *Bam*HI fragment of wild-type adenovirus DNA.

late gene products made in the liver was probably not sufficient to package replicating genomes.

Cellular proteins can functionally substitute for E1a in its role as a transcriptional transactivator for early and late promoters. Proteins with E1a-like activities have been found in mouse oocytes, in preimplantation embryos (11), in mouse embryonal carcinoma F9 cells (34), in human hepatoblastoma HepG2 cells (50, 51), in cervix carcinoma HeLa cells (28, 29), and in other cell lines derived from human malignant tumors. E1a activity in transformed cells may result from expressed cellular oncogenes or tumorigenic viruses. For example, HeLa cells contain an integrated human papillomavirus type 18 genome expressing the E7 gene, which is known to have E1a features (42, 48).

The expression of viral early and late genes after *in vivo* gene transfer with E1-deficient adenovirus suggests that E1a-like products exist in primary cells as well. The E1a-independent activation of the E2a promoter can lead to expression of E2 proteins at levels which can trigger virus DNA replication. The E2a early promoter at 76.0 map units is regulated by E1a via interaction with cellular transcription factors like E2F, ATF2, and OTF1. At later time points, transcription is switched to a new E2a late promoter (E1a independent) at 72.0 map units in order to maintain the high-level E2 expression that is necessary for DNA replication. Three E2 messages are generated by posttranscriptional processing of a common precursor that is transcribed from the E2a promoter(s). The E2a gene product is the 72-kDa DBP. In addition to its role in the initiation and elongation of DNA synthesis, DBP activates the transcription from the major late promoter, represses E4 transcription, and is involved in assembly of virus particles. The E2b region contains the genes for the 140-kDa viral DNA polymerase and the 80-kDa preterminal protein (pTP). The pTP acts as protein primer for DNA replication. At late times postinfection, pTP is processed by the viral protease to the 55-kDa TP, which covalently binds to the 5' virus DNA termini and which is packaged with the DNA. After infection and virus uncoating, DNA-bound TP has functions in directing the core particle to the nucleus, in protecting the viral DNA from exonucleases, and in attachment to the nuclear matrix (54).

**Does adenovirus DNA have a special fate in transduced livers compared with other organs?** Among the cytokines induced after vascular adenovirus administration, IL-6 may play a special role in the fate of adenovirus DNA in the liver. A specific IL-6 receptor on hepatocytes is implicated in acute-phase reactions in the liver and in the regulation of liver re-

generation after partial hepatectomy (24). This cytokine is rapidly induced after adenovirus administration to the pulmonary (8, 19, 20) and vascular (60) systems. Human NF-IL6 (1, 43) and the rodent analog, C/EBP-b (LAP) (57), are transcription factors of the C/EBP family present in differentiated hepatocytes and induced by IL-6. IL-6 induction of NF-IL6 stimulated adenovirus replication in the absence of E1a in HepG2 (50, 51) and embryonal carcinoma (26) cells, via OTF I (NFIII), one of at least three host cell factors required for DNA replication of Ad2 and Ad5. Spergel and Chen-Kiang (50) demonstrated that NF-IL6 transactivates the E2a early promoter at low concentrations and represses it at high concentrations by binding to a different site in the E2a early promoter. Our data for primary mouse hepatocytes support this observation. After an acute increase in DNA replication in the presence of IL-6, replication was suppressed by IL-6, possibly because of reduced E2 protein production. On the basis of these observations, the question that arises is whether the phenomenon of adenovirus genome replication will occur in other tissues. Notably, after intravenous injection of wild-type Ad5, the level of viral replication was 100 to 1,000 times higher in the liver than in other organs (12). The rapid loss of undeleted vector DNA and the inability to detect viral replication in nonhepatic tissues is also consistent with the idea that vector replication may be more pronounced in the liver. Although the absence of cellular transcription factors that can substitute for adenovirus in nonhepatic tissues is an attractive hypothesis to explain the results, the possibility that the vector DNA concentration after transduction is not high enough in these cells to produce enough adenovirus proteins to replicate their genomes cannot be excluded.

**Why is deleted DNA unstable in transduced cells?** The loss of deleted adenovirus DNA in cell culture and mouse liver was rapid. There was no evidence of hepatocyte degeneration in histological or biochemical analysis at 5, 12, and 24 h after infusion of the deleted vector, making it unlikely that the rapid loss of transduced DNA is due to hepatocyte destruction by innate immune mechanisms.

The levels of hAAT expression in tissue culture were similar for both the deleted virus and undeleted vector during the first 24 to 48 h after infection. Furthermore, expressed E4 proteins could be found *in vitro* and *in vivo*, albeit at lower concentrations in similar numbers of cells. Taking these data into account, inefficient delivery of deleted genomes into the nucleus cannot explain the relatively short persistence compared with first-generation vectors. Thus, the processes of virus adsorption, penetration, uncoating, and nuclear transport seem to be similarly efficient for the deleted and undeleted viral vectors.

We speculate that, among several others, there are the following possible explanations for the rapid degradation of deleted virus DNA inside the nucleus: (i) the input virus DNA does not have the appropriate chromatin structure; (ii) because of the absence of E2 protein expression, the deleted genome is unable to maintain a stable structure; and/or (iii) viral replication is temporarily or persistently required for stabilization.

(i) **Chromatin structure.** Packaged Ad5 DNA exists inside the capsid in a supercoiled structure associated with core proteins VII, V, and IV $\alpha_2$ , forming the virus chromatin. In contrast to polyomavirus and papillomavirus, adenovirus is not packaged with cellular histones. According to a model proposed by Wong and Hsu (59), adenovirus core proteins (VII and V) bind to seven defined,  $\sim 200$ -bp regions on the Ad5 DNA. The DNA regions between the core binding sites form characteristic supercoiled loop domains. About eight loops can be accommodated in an Ad5 virion as demonstrated by elec-

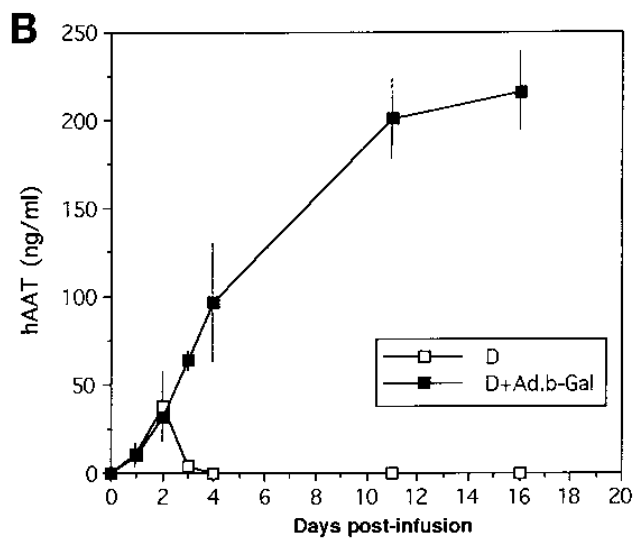
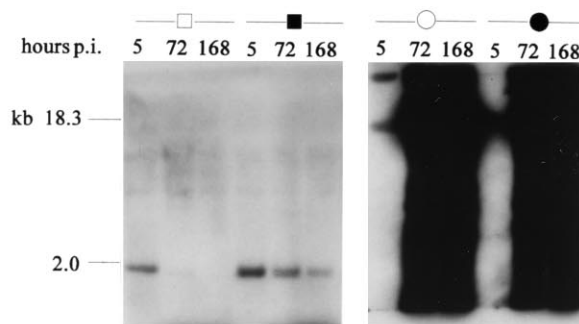
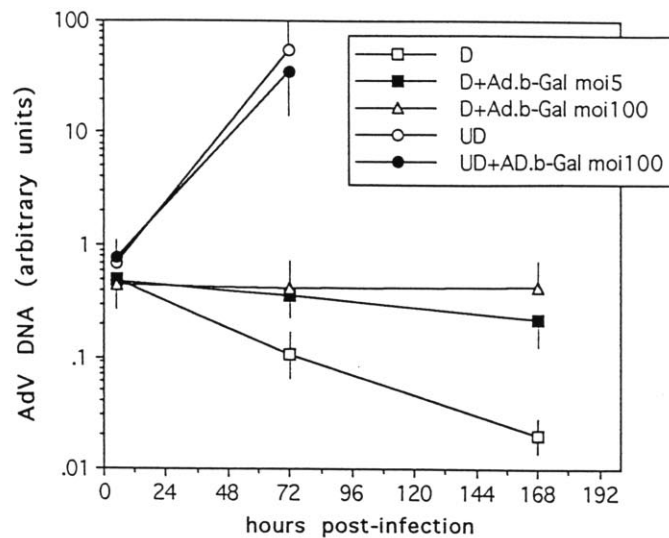
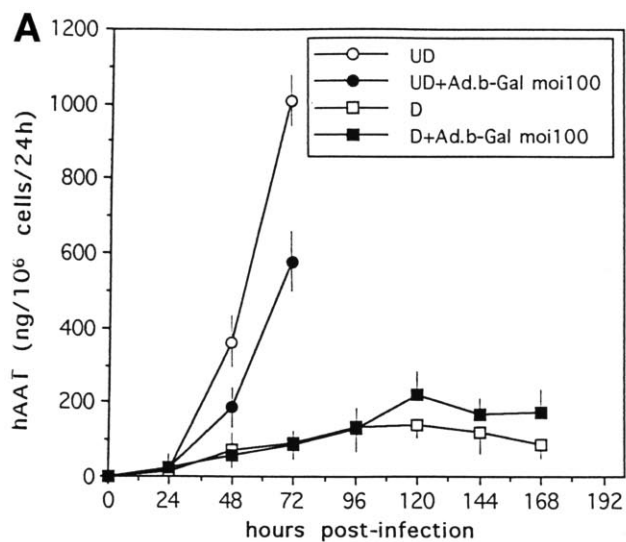


FIG. 13. Complementation of deleted vectors. E1a-deficient adenovirus (Ad.RSVβgal) (MOIs, 5 and 100) was infected onto HeLa cells 5 h after transduction with deleted or undeleted adenovirus (AdV) (MOI, 100). hAAT quantitation and Southern blot analysis with an hAAT specific probe were carried out as described for Fig. 6. p.i., postinfection. (B) A total of  $5 \times 10^9$  transducing particles of Ad.ΔE2hAAT-D or  $5 \times 10^9$  transducing particles each of Ad.βgal and Ad.ΔE2hAAT-D were administered to C57BL/6 by tail vein injection. Each value represents the mean and standard deviation from at least three independent experiments.

tron microscopy. In the deleted, 9-kb genome, five of the potential core binding sites are deleted. Theoretically, the ratio of the number of core proteins to the DNA length unit is unchanged. The TP that protects the DNA termini from exonucleases and is implicated in nuclear transport and in nuclear matrix attachments (18) is theoretically bound to deleted DNA

inside the nucleus. However, the total amount of core proteins is reduced, and it is unclear whether a compact structure similar to that of the undeleted genome can be formed. On the other hand, the viral chromatin structure of the deleted DNA taken up by the nucleus seems not to be critical for genome stability. If the absent E2 proteins are provided by coinfection of the deleted virus with an undeleted virus, then the deleted genome becomes stabilized.

(ii) **E2 protein expression.** Little is known about the half-life and turnover of TP and DNA-bound core proteins in hepatocytes in vivo after transduction with E1-deficient viruses. It is possible that de novo synthesis of E2 (and late proteins like viral protease for pre-TP processing) is essential to renew the chromatin packaging of viral DNA in order to protect it from nucleases.

(iii) **Viral replication.** De novo DNA synthesis could be necessary to counterbalance DNA degradation that is stimulated or enhanced by nonspecific or specific host immune mechanisms. In the present study, significant viral DNA replication in mouse livers began at 8 to 12 h postinfection, the

time when wild-type adenovirus replication begins in mouse hepatocytes *in vivo* (12). Notably, this time point corresponds to the moment when replication-deficient, deleted virus DNA disappears from transduced cells. Dynamic changes in the viral chromatin during wild-type virus infection have been reported (9, 10, 38, 58). Several models support the theory that viral replication contributes to the replacement of the virion core structure by a nucleosome-like structure involving cellular histones. Furthermore, the activation of viral promoters seems to be dependent on viral DNA replication. The observation that the concentration of viral DNA begins to decline if DNA replication is inhibited by hydroxyurea supports the latter hypothesis.

Clearly, the mechanisms described above are not mutually exclusive. Taking the facts together, we speculate that under nonlytic infection conditions, a low-level, (early) viral DNA synthesis is needed to maintain adenovirus vector genomes in hepatocytes. Furthermore, this can be accomplished only with the production of a threshold level of early gene products. Thus, a certain number of adenovirus genomes in transduced cells, in a dose-dependent manner, may need to be present in order to obtain persistence.

The next phase of study will be to focus on determining the minimal combination of E2 or E2 and E4 genes that stabilize deleted genomes. The importance of E2a is uncertain, because vectors with E1a and E2a deleted and expressing the same hAAT transgene have periods of gene persistence similar to those of the E1a-deleted vector (66). Other factors may play a role in the persistence of the vector, including the size of the packaged genome. Once these issues are resolved, the genes required for genome stability can be placed back into the deleted vector to analyze whether the vector toxicity returns and whether the expression of these genes will elicit an immune response. These experiments may be critical for the future of recombinant adenovirus vectors for human gene therapy.

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