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Adenoviral preterminal protein stabilizes mini-adenoviral genomes in vitro and in vivo

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In the absence of host immunity, nonintegrating, first-generation adenoviral vectors remain stable in the nucleus of quiescent transduced cells in mice. A mini-adenoviral genome (9 kb) deleted for viral E1, E2, E3, and late genes, but containing the viral inverted terminal repeats (ITRs), transgene expression cassette (human α_1-antitrypsin), and the viral E4 genes was equally efficient at transducing cells in vitro or in vivo as first generation, E1-deleted vectors. In contrast to a first generation vector, gene expression as well as vector DNA was short-lived in cells transduced with the deleted adenoviral genome. We demonstrate that coexpression of the adenoviral E2-preterminal protein from the vector or in trans stabilizes the mini-genome in vitro and in vivo without evidence of cellular toxicity.

Keywords: gene therapy, episomal vectors

In vivo gene transfer by recombinant, E1-deficient adenoviral vectors results in early and late gene expression that induces vector-related toxicity and a host immune response limiting the duration of transgene expression[1,2]. We generated adenoviruses lacking the genes for the immunogenic viral proteins encoded in the E1, E2, E3, and corresponding late region[3]. The mini-vector deleted for 33 kb of viral DNA and constituting a final size of 9 kb can be produced at high titers using a technique based on cre-lox recombination. The deleted virus transduced hepatocytes in vivo efficiently after intravenous infusion and without associated toxicity. Transgene expression was transient due to instability of the vector genome in the nucleus of transduced cells. The deleted vector can be stabilized in trans by coinfection of an equal amount of first-generation adenovirus, suggesting that proteins encoded in the deleted region are needed for genome stabilization.

The deleted region includes the pIX and E2 expression units[4,5]. Protein IX is a minor structural protein while the E2 proteins are involved in viral DNA replication. Three of the major E2 mRNAs are generated by posttranscriptional processing of a common precursor producing the E2a gene product—DNA binding protein (DBP) (57 kDa)—and the two E2b products, pTP (precursor terminal protein) (77 kDa) and the viral DNA polymerase (pol) (140 kDa). The region corresponding to E2 on the other DNA strand contains the major late promoter and the L1–L4 RNA family including penton (protein III/IIIa), hexon (II), and core proteins (VII, VI, VIII).

As potential candidates for factors stabilizing the deleted genome, we focused on the nonstructural, nuclear-localized E2 proteins pTP, pol, and DBP. The E2 promoter in E1a-deleted, first-generation adenoviruses is active in transduced hepatocytes in vivo[2,3]. Our hypothesis is that hepatocyte-specific transcription factors can functionally substitute E1a in its transactivator function for the E2 promoter, and that a low level of E2 protein expression contributes to genome stability of first generation vectors in hepatocytes[3]. The respective functions of the E2 proteins during the life cycle of wild-type adenovirus are well studied[4–6]. DBP binds cooperatively ssDNA synthesized during replication by forming a protein chain that is wound around the DNA. DBP influences all stages of replication by facilitating duplex unwinding and inhibition of intramolecular renaturation between complementary ends of ssDNA. After forming the final double-stranded replication product, DBP rapidly dissociates from the viral genome.

The pTP expressed early from the transduced parental viral DNA binds as heterodimer with pol within the origin found on each end of the linear genome within the ITRs[5]. pTP is then covalently linked to dCTP, providing a free 3′-hydroxyl group to begin the synthesis of a daughter DNA catalyzed by the adenoviral polymerase. pTP serves as the site of primary attachment of the viral DNA to specific protein(s) in the nuclear matrix forming replicative complexes[7,8]. Late in infection, pTP is proteolytically cleaved by the viral protease generating the 55 kDa terminal protein.

Results

Trans-complementation with first-generation adenovirus confers stability of mini-vector. Coinfection studies strongly suggest that trans-acting, viral factor(s) missing in the deleted vector play a role in viral genome persistence[3]. To further elucidate this in animals, different amounts of E1-deleted Ad.RSVβgal[9] were added as helper to 5×10^9 transducing particles of ΔAd.hAAT (deleted vector) prior to intravenous infusion into C57Bl/6 mice (Fig. 1). In mice receiving ΔAd.hAAT alone or with 5×10^6 (0.1%) transducing particles of Ad.RSVβgal, serum hAAT were only detectable for less than 5 days. In contrast, mice that received as little as 1% helper (5×10^7 transducing particles) expressed hAAT for more than 2 months, similar to the length of β-galactosidase expression in these mice[10]. At a dose of 5×10^9 transducing particles (moi estimated at 50 with 1×10^8 hepatocytes per liver) about 15 to 30 adenoviral genomes were found in hepatocytes transduced in vivo[11,12]. Thus with 1% of this amount added as helper, we estimate about one helper vector genome for every two to three hepatocytes.

Transient pTP coexpression stabilizes ΔAd.hAAT genomes in vitro. Elements in the helper virus that provide stabilization of the

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deleted genome could represent the helper genome itself or early/late proteins expressed from this genome. We investigated here whether the nuclear-localized, DNA-binding proteins encoded in the E2 region (pTP, DBP, pol) would provide the stabilization of the deleted genome when transiently expressed (separately or in combination) prior to transduction with ΔAd.hAAT. To do this, expression plasmids with the individual E2 genes, pTP, DBP, or pol individually or in combination were cotransfected onto 5×10^5 HeLa cells, and 36 hours later cells were transduced with ΔAd.hAAT. Expression studies with specific antibodies (immunofluorescence or Western blot analysis) demonstrated detectable pTP or DBP expression between 24 hours and 7 days after plasmid transfection (data not shown). Transduced cells were analyzed for vector DNA at 5 hours (input) and 7 days (Fig. 2). Cells receiving either pTP or pTP in combination with pol had stable concentrations of ΔAd.hAAT vector DNA over a 1-week period. pTP-expressing cells had even a slightly higher concentration of vector DNA at 7 days compared with the input measured at 5 hours. In contrast, cells receiving the other E2-derived plasmids or the control plasmid lost most of the ΔAd.hAAT DNA within the week. DBP expression resulted in decreased cell viability (data not shown).

The pTP mini-vector confers self-stability and stability of another coinfected deleted vector. A second deleted vector, ΔAd.pTP (9 kb) containing the pTP gene in place of the hAAT cDNA was constructed in order to determine whether pTP expression can self-stabilize ΔAd.pTP or ΔAd.hAAT when coinfected. Western blot analysis on liver extracts (Fig. 3) or cultured cells (data not shown) transduced with ΔAd.pTP revealed the 77 kDa pTP-protein. The 77 kDa pTP-specific band was also detected in livers transduced with a first-generation adenovirus (Ad/RSVhAAT), demonstrating that pTP is expressed from first generation vectors in hepatocytes in vivo.

The ΔAd.pTP vector genome was stable after transduction into cultured HeLa and baby hamster kidney (BHK) cells for at least a week whereas by 24 hours ΔAd.hAAT vector DNA transduced in parallel experiments declined to very low levels (Fig. 4A). Southern

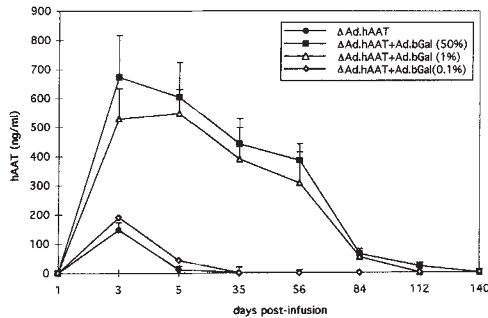

Figure 1. Complementation of ΔAd.hAAT with first generation adenovirus in C57Bl/6-mice. 5×10^9 transducing particles of ΔAd.hAAT, alone or in combination with 50% (2.5×10^9), 1% (5×10^7) or 0.1% (5×10^6) Ad.RSVβGal (Ad.bGal), a first generation (E1 deleted) adenovirus, were administered to C57 Bl/6 mice by tail vein infection. n=3 per group.

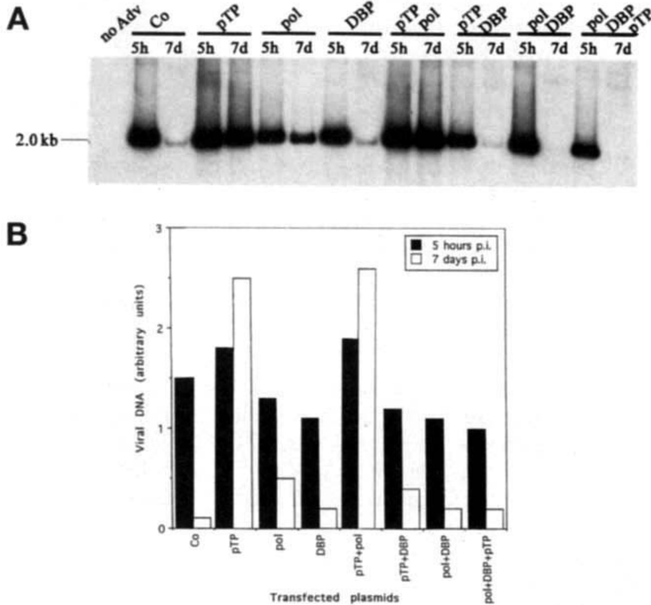

Figure 2. Effect of transient expression of the E2 proteins pTP, polymerase (pol), and DBP on the stability of ΔAd.hAAT DNA in vitro. 5×10^5 HeLa cells were transfected with pCMV pTP (pTP), pCMVpol (pol), or pBZ20 (DBP) DNA. Thirty six hours after transfection, ΔAd.hAAT at a multiplicity of infection (moi) of 100 was added to the cells. Co: all transfected DNA was pCDNA3. (A) Southern blot analysis using a radiolabelled hAAT probe. (B) Quantification by phosphoimager-analysis after adjusting for loading differences. The data are expressed as the mean arbitrary units relative to a concentration standard loaded on the gel from three separate experiments. The standard deviation between experiments was less than 10% for each sample.

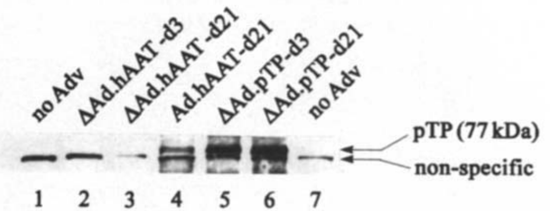

Figure 3. Western blot analysis of pTP expression in mouse livers after infusion of 5×10^9 vector transducing particles. Liver homogenates were immunoprecipitated with a monoclonal anti-pTP antibody that crossreacted with another protein (nonspecific). The signal in the 77-kDa band may not reflect the total amount of pTP expressed from transduced vectors, as the protein extraction protocol may not release all pTP from the nuclear matrix.

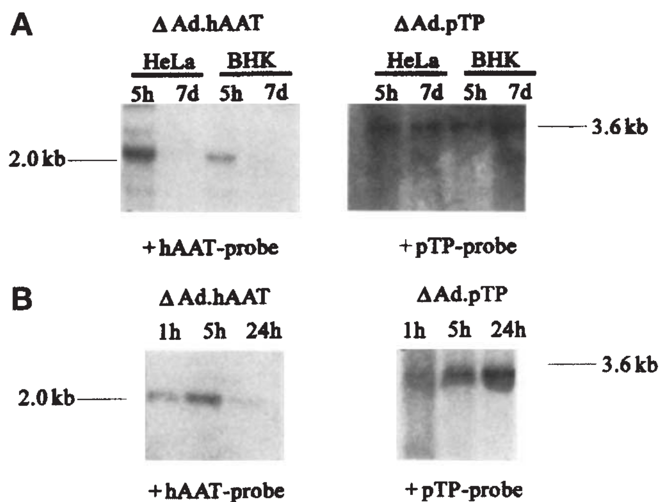

Figure 4. Southern blot analysis of viral DNA after transduction in vitro with ΔAd.hAAT and ΔAd.pTP (moi 100). (A) Five hours or 7 days after infection of the cells, genomic DNA was analyzed with a radiolabelled hAAT- or pTP probe. (B) Confluent HeLa cells were transduced with deleted vectors and specific viral DNA was analyzed from nuclear DNA isolated from cells collected at 1, 5, or 24 h postinfection.

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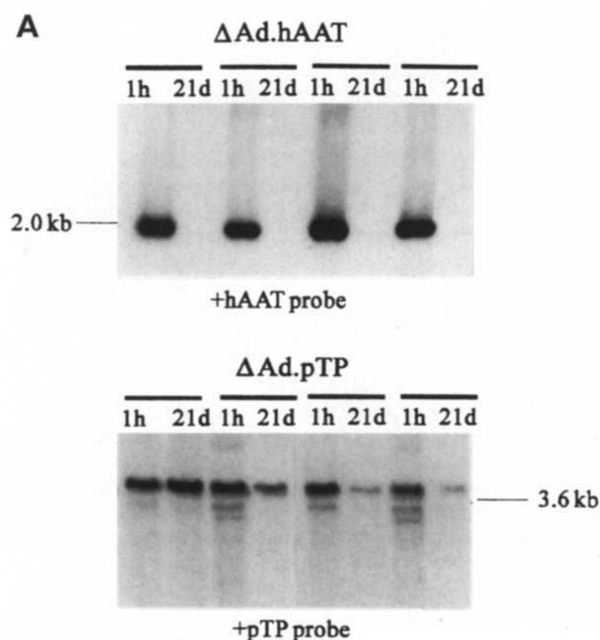
blot analysis performed on nuclear DNA isolated from transduced cells at the early time points indicated that both ΔAd.hAAT and ΔAd.pTP vector DNA could enter the nucleus (Fig. 4B). This is consistent with previous studies showing similar expression of hAAT after transduction with the ΔAd.hAAT and Ad/RSVhAAT (first generation equivalent) vector[3]. Taken together, a deleted adenoviral vector genome can be stabilized in the nucleus of cells in culture when pTP is expressed from the vector.

Similar results to those obtained in cell culture were achieved in animals infused with deleted vector expressing pTP (Figure 5). ΔAd.pTP-transduced mouse livers had only slightly reduced amounts of vector DNA at 21 days (the length of the experiment) compared with 1 hour postinfection, while similar to previous results[3], the ΔAd.hAAT vector was undetectable by 7 days. The ΔAd.hAAT and ΔAd.pTP viruses were mixed prior to infusion into mice to determine if trans-complementation would stabilize the genome and transgene expression (Fig. 6). Mice receiving both vectors had stable serum hAAT expression (Fig. 6A) and persistence of both vector genomes (Fig. 6B) for at least 3 weeks, whereas mice receiving ΔAd.hAAT alone had transient serum hAAT levels as well as vector DNA.

pTP expression is nontoxic in vivo. Attempts to establish stable, pTP-expressing cell lines indicated that high-level pTP production may induce cell-cycle arrest and cell death[13,14]. To determine if pTP expression in vivo was associated with liver toxicity, serum levels of



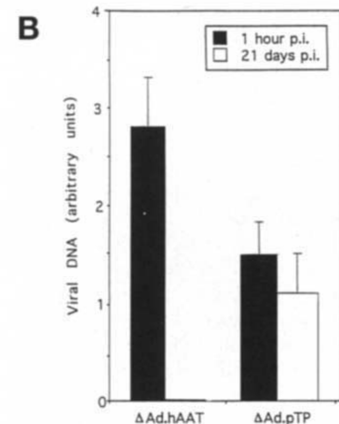
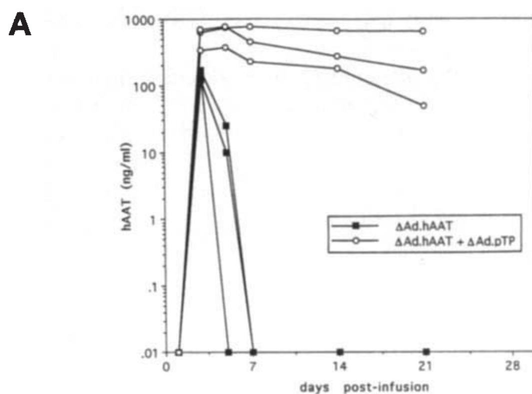
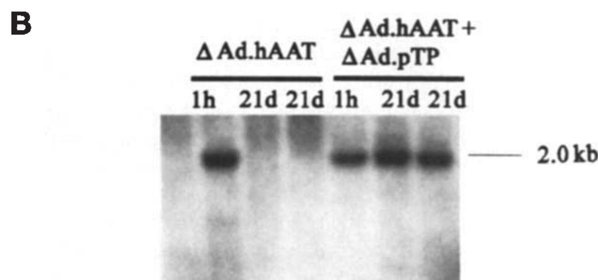


Figure 5. Southern blot analysis of viral DNA after transduction in vivo with ΔAd.hAAT and ΔAd.pTP. C57Bl/6 mice were injected with 5×10^9 infectious particles of ΔAd.hAAT or ΔAd.pTP. (A) At 1 h or 21 days after virus infusion genomic liver DNA was analyzed for specific viral DNA using a radiolabelled hAAT- or pTP probe. (B) The results from 4 different mice per time point are averaged after phosphoimager quantitation.





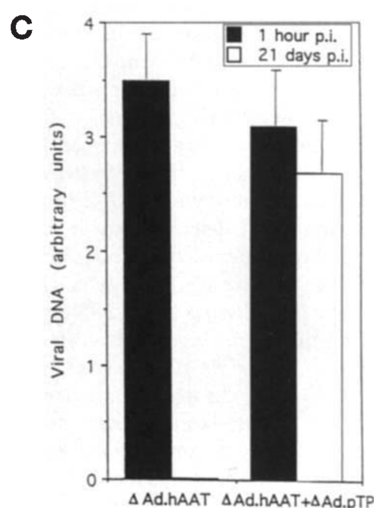


Figure 6. Combined infusion of ΔAd.hAAT and ΔAd.pTP. 5×10^9 transducing particles of ΔAd.hAAT alone or ΔAd.hAAT in combination with 5×10^9 transducing particles of ΔAd.pTP were infused in C57Bl/6 mice. (A) Serum hAAT levels. (B) Genomic liver DNA at day 1 and day 21 was isolated for Southern blot analysis using a hAAT specific probe. (C) Quantitation of Southern blot analysis. Mean value with the standard errors from at least 3 mice per group.

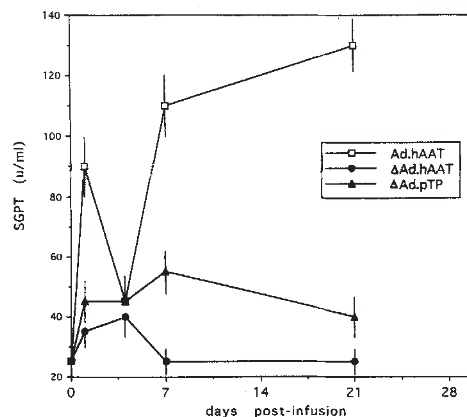


Figure 7. SGPT levels in mice infused with first-generation adenovirus hAAT, ΔAd.hAAT, or ΔAd.pTP. SGPT levels were determined periodically after intravenous infusion of 5×10^9 transducing particles. n = 3 per time point.

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glutamic pyruvic transaminase (SGPT), an early and sensitive marker for hepatocellular injury, were measured in animals transduced with ΔAd.pTP and found to be only slightly higher than those from mice receiving ΔAd.hAAT (Fig. 7). Based on these studies, the level of pTP expression from ΔAd.pTP does not appear to be toxic in vivo. Taken together, expression of pTP-protein in trans or cis results in stabilization of E2-deleted adenoviral genomes in vivo without obvious toxic side effects.

Discussion

A 9-kb adenoviral vector deleted for the entire E1, E2, E3, and corresponding late gene region transduced hepatocytes efficiently in vitro and in vivo; however, the viral DNA, was rapidly lost from the nucleus[3]. The fact that this mini-virus is efficiently packaged appears to be in conflict with a recent report demonstrating a lower limit to packaging of adenovirus to about 27 kb[15]. Our vector, however, starts out full length and is deleted in the producer cell perhaps after a critical event required for packaging has occurred. The complementation data demonstrate that a certain low level of E2 proteins produced from first-generation vectors is necessary for stabilization of the mini-vector. Another practical consequence of our coinfection experiments is that expression/persistence studies with deleted or minimal (so-called "gutless") vectors can be influenced by a small percentage of contaminating helper virus copurified during vector preparation.

Preterminal protein coexpression clearly prolongs persistence of E2-deleted genomes in the nucleus of transduced cells in vitro and in vivo. The sizes of the deleted vectors (ΔAd.hAAT and ΔAd.pTP) compared in this study are similar (approximately 9 kb). Because pTP production from ΔAd.pTP stabilizes hAAT expression from ΔAd.hAAT in trans when coinfected, the newly introduced pTP sequence can not be responsible for stabilization in cis. We hypothesize that pTP-mediated matrix attachment of viral DNA is probably a crucial element in stabilization of the deleted vector in trans by pTP coexpression. Although in the absence of viral DNA replication, pTP may not covalently bind to the virus genome, association with the nuclear matrix may still be provided via the pTP C-terminus. The mechanisms underlying the stabilizing effect or attachment of viral DNA to the nuclear matrix via pTP may include: structural support or changes in the viral chromatin, circularization of the viral genome, protection against exonucleases, or contact with enzymes localized in the matrix (e.g., topoisomerases)[16–18]. Cellular genomic DNA is organized into supercoiled loops that are attached to the nuclear matrix via matrix attachment regions (MARs)[19,20]. If matrix attachment of deleted genomes is indeed crucial for stability, then specific MARs should provide a stabilizing effect in cis, when cloned into the deleted vector. Finally, if the near full-length "gutless" vectors[21–24] do result in persistence of gene expression in the absence of pTP it is likely that other factors related to DNA size/structure or specific sequences, like MARs, confer stability. Thus, it is possible that there are more than one mechanism to stabilize episomal DNA molecules in quiescent cells in vivo.

Based on the amount of pTP produced in the adenovirus life cycle, only a low expression level of pTP may be needed for stabilization of deleted genomes, reducing the risk of potential toxic or immunologic side effects. In our coexpression studies, the expression level and/or ratio of pTP:pol and DBP after plasmid transfection, may not reflect the situation during infection with wild-type virus, and at this time we cannot predict the minimal amount of pTP that will be required for stable gene expression. Little is known about the half-life of pTP expressed in hepatocytes from first-generation vectors, and our study cannot definitively determine whether or not continuous pTP synthesis will be required for vector persistence.

pTP alone does not induce DNA replication of vectors deleted for all other E2 genes. Unlike first-generation vectors in which vector DNA accumulates in cell culture, no accumulation of ΔAd.pTP vector DNA was observed in this study even though the genomes and gene expression remained stable. This is in contrast to our earlier hypothesis that viral replication observed after transduction with first generation vectors of hepatocytes in vitro and in vivo at low levels is necessary for genome persistence[3]. Moreover, our recent demonstration that first-generation vector DNA molecules that enter the nucleus of mouse hepatocytes in vivo are stable for at least 3 weeks without replication[25] is also consistent with our new conclusion that the production of a certain level of pTP protein is sufficient to maintain E1a-deleted genomes in hepatocytes over an extended period of time.

Unlike first-generation vectors, in vivo pTP expression from ΔAd.pTP had no detectable toxic effects on transduced hepatocytes. It remains to be determined whether exogenous pTP expression in vivo has any long-term subtle effects on the host, or whether ΔAd.pTP will be cleared by the immune system. This information together with long-term expression studies using a deleted virus containing both transgene and the pTP gene are necessary to evaluate the usefulness of these vectors. With its properties to transduce efficiently all hepatocytes in vivo[3] and to provide prolonged transgene expression without associated toxic or immunologic side effects, deleted vectors stabilized by pTP expression whose production is technically straightforward may be a valuable tool for human gene therapy. Finally, the principles learned from the interaction of pTP with the adenoviral episome may also be useful for developing nonviral vectors in which some level of persistent gene expression will be required.

Experimental protocol

Plasmids and adenoviruses. pCMVpTP and pCMV pol were provided by Jerry Schaack (University of Colorado, Denver). The pTP gene contained the pTP coding sequence (Ad5 bp: 8533–10589) including the N-terminal amino acids (encoding a small exon around Ad map unit 39 containing the initiation codon). The pol cDNA (Ad5 bp: 5187-8357) contains a mutation at the C-terminus to create a SphI site that does not impair enzymatic activity. The pTP or pol genes were cloned into pcDNA3 (Invitrogene, Carlsbad, CA) between the CMV promoter and the bovine growth hormone polyadenylation signal (bPA). The plasmid expressing DBP (pBZ20) was obtained from Arthur Beaudet (Baylor College of Medicine, Houston, TX)[26]. The construct contains the E2a gene with its own promoter. pAd.hAAT.lox used for generation of ΔAd.hAAT was described earlier[3]. It includes the RSV-promoter–human α_1-antitrypsin (hAAT) cDNA and the bPA signal followed by a lox site. To construct pAd.pTPlox, the 1.68-kb Hind III/Hind III fragment from Ad.hAATlox (with hAAT-bPA) was removed and replaced by the 2.1-kb Hind III/Eco RI fragment (with pTP) from pCMVpTP linked to a 0.28-kb bovine growth hormone polyadenylation signal. The generation of the deleted adenovirus containing the hAAT expression cassette (ΔAd.hAAT) was described earlier[3]. To produce ΔAd.pTP, first, vectors with two lox sites flanking the pIX and E2 regions were generated by recombination of pAd.pTPlox with pBHGlox1 with the lox site cloned into the E3 region. Then these vectors were infected into 293-cre cells[3] to delete the intervening region between the lox sites. Recombinant vector with the two lox sites from single plaques was amplified in 293 cells and analyzed for pTP expression by Western blot analysis with monoclonal antibodies against pTP (obtained from Sarah Jones, University of St. Andrews, UK). The intensity of the specific 77-kDa pTP-band from vectors with exogenous pTP expression was approximately 20-fold stronger than the pTP band derived from endogenous pTP expression in first generation adenoviruses (data not shown). Virus from positive plaques was amplified in large scale in 293 cells infected with a moi of >200. Considerable effort was required to amplify the vector with the two lox sites in 293 cells because of observed recombination between the endogenous pTP sequence located within the E2 region[5,6] and the exogenous pTP gene downstream of the RSV promoter. To generate deleted virus expressing pTP (ΔAd.pTP), the vector containing the two lox sites was infected onto 293 cells expressing cre recombinase (293-cre) at an moi of 200. (Cre mediated the efficient excision of the intervening 25 kb region, joining the left genome end with ITR and the transgene cassette, and the right genome end with the E4 region and the right ITR together.) Infected 293-cre cells were harvested at 48 h postinjection and deleted virus was banded by multiple rounds of CsCl ultracentrifugation as described[3]. Titers given in

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transducing particles of ΔAd.hAAT or ΔAd.pTP were determined on HeLa cells by immunofluorescence using antibodies specific to E4 proteins (rabbit anti-ORF-3 and anti-ORF4, 1:1 mixture; provided by Gary Ketner, [Johns Hopkins University, Baltimore, MD]). All preparation of deleted viruses were tested for contaminating first generation adenovirus (with two lox sites) by plaque assay on 293 cells[27]. Only preparations with fewer than 5 plaques per 10^6 transforming units (less than 0.0005% contamination) were used in the described experiments. Viruses were stored at a titer of 3×10^{10} transducing particles/ml at -80°C in 10 mM Tris-Cl, pH 8.0. 1 mM $MgCl_2$, 10% glycerol.

Cell lines/plasmid transfection. 293 (Microbix Biosystems, Toronto, CA) and HeLa (CCL2; American Type Culture Collection, Rockville, MD) cells were grown in high glucose-DMEM with 10% FCS. 5×10^5 HeLa cells in 6-cm dishes were transfected by calcium-phosphate coprecipitation. Generally, the transfection efficiency was approximately 50% as determined by X-Gal staining after transfection with 10 μg pCMV β-Gal[28]. The plasmids pCMVpTP, pCMVpol or pBZ20 (DBP) were transfected separately (3.3 μg each) or in combination in two sets of dishes. To maintain the total concentration of plasmid DNA at 10 μg/dish, control plasmid DNA with the CMV-promoter, but without the pTP gene (pCDNA3) was added. The pCDNA3 plasmid was used as the control in transfection experiments. ΔAd.hAAT vector (moi 100) was added 36 h after transfection. At this time, cells had reached confluence.

Western blot analysis. At different time points after plasmid transfection, cell pellets were lysed on ice for 30 min in 20 mM HEPES pH 7.5, 2 mM EGTA, 10% glycerol, 1% Triton X-100, 0.1 M DTT, and protease inhibitors. After 5 min boiling, 100 μg total protein in 1× Laemmli buffer with 4% β-mercaptoethanol, proteins were separated on a 10% SDS-PA-gel. After electrotransfer and blocking, filters were incubated with monoclonal anti-pTP antibodies (1:40 diluted) or anti-DBP antibodies (1:50 diluted, mab 37-3, GenVec, Rockville, MD), followed by an incubation with peroxidase-labeled anti-mouse Ig antibodies (1:1000). Filters were developed using the ECL detection kit (Amersham, Burlington, MA). For pTP detection after in vivo transduction with ΔAd.pTP, or Ad/RSVhAAT livers were first flushed with 5 ml PBS via the portal vein, then 300 mg tissue was homogenized in 1 ml RIPA (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors) and incubated for 30 min on ice. After adding $MgCl_2$ to a final concentration of 5 mM, cell lysates were incubated with 20 μg/ml DNaseI for 30 min at room temperature. Lysates with a total of 12 mg protein were incubated with anti-pTP antibodies (1:40) overnight at 4°C, followed by an incubation with protein-A-sepharose (Sigma, St. Louis, MO) for 1 h at 4°C. After 4 washes with RIPA, sepharose bead pellets were resuspended in an equal volume of 2× Laemmli buffer with 8% β-mercaptoethanol and boiled for 5 min before loading on a 10% SDS-PA gel. The gel was analyzed by Western blot analysis as described above.

Animal studies. Animal studies were performed in accordance with the institutional guidelines of the University of Washington. Female C57Bl/6 mice (Jackson Labs, Bar Harbor, ME) aged 5 to 6 weeks were used in the described experiments. All animals were housed in SPF facilities. Adenovirus injections were performed by tail vein infusions with 200 μl of virus diluted in adenovirus storage buffer. Generally, 5×10^9 transducing particles were injected per mouse, a dose that transduces approximately 95% of hepatocytes[29]. Blood samples for hAAT or SGPT analysis were obtained by retroorbital or tail vein bleeding, respectively. hAAT concentrations in serum or tissue culture samples were measured by ELISA[27]; a Sigma diagnostic kit was used for colorimetric determination of the activity of SGPT with 10 μl serum[29].

Southern blot analysis. Cultured cells were washed three times with PBS before harvesting; mouse livers were flushed with 5 ml PBS via the portal vein. Genomic DNA was extracted from 100 mg liver[3]. For preparations of nuclear DNA, nuclei were isolated and purified[30]. For analysis, 10 μg of genomic DNA were digested with BamHI, run on a 0.8% agarose gel, and electrotransferred to Hybond nylon filters (Amersham). The blots were hybridized in rapid hybridization buffer (Amersham) with [α-^{32}P]dCTP-labeled DNA probes. As reference standard, DNA from noninfected cells or livers was spiked with 5 pg of deleted viral genomic DNA and loaded on each gel. The relative amount of adenovirus DNA was determined by Phosphorimager analysis as a ratio between the sample signal and the standard signal, and expressed as arbitrary units. All blots were rehybridized with probes for the mouse metallothionein gene (mouse liver DNA) or for the genomic hAAT gene (HeLa DNA) to adjust loading differences. The following DNA fragments were used as labeled probes: 1.4 kb of the hAAT cDNA (EcoRI fragment of pAd.RSVhAAT[27]), 3.6 kb probe of pTP DNA (HindIII/EcoRI fragment of pCMVpTP) or 2 kb fragment of the mouse MT gene (HindIII/EcoRI fragment of pmMMT[11]).

Acknowledgments

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