High-Efficiency System for the Construction of Adenovirus Vectors and Its Application to the Generation of Representative Adenovirus-Based cDNA Expression Libraries

Moritz Hillgenberg,^{1*} Christian Hofmann,^{1†} Herbert Stadler,² and Peter Löser^{1‡}

DeveloGen AG NL Berlin, Robert-Rössle-Strasse 10, D-13125 Berlin, Germany,¹ and Affectis Pharmaceuticals AG, Kraepelinstrasse 2, D-80804 Munich, Germany²

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We here describe a convenient system for the production of recombinant adenovirus vectors and its use for the construction of a representative adenovirus-based cDNA expression library. The system is based on direct site-specific insertion of transgene cassettes into a replicating donor virus. The transgene is inserted into a donor plasmid containing the viral 5' inverted terminal repeat, the complete viral packaging signal, and a single *loxP* site. The plasmid is then transfected into a Cre recombinase-expressing packaging cell line that has been infected with a donor virus containing a partially deleted packaging signal flanked by *loxP* sites. Cre recombinase, by two steps of action, sequentially catalyzes the generation of a nonpackageable donor virus acceptor substrate and the generation of the desired recombinant adenovirus vector. Due to its growth impairment, residual donor virus can efficiently be counterselected during amplification of the recombinant adenovirus vector. By using this adenovirus construction system, a plasmid-based human liver cDNA library was converted by a single step into an adenovirus-based cDNA expression library with about 10^6 independent adenovirus clones. The high-titer purified library was shown to contain about 44% of full-length cDNAs with an average insert size of 1.3 kb. cDNAs of a gene expressed at a high level (human α_1 -antitrypsin) and a gene expressed at a relatively low level (human coagulation factor IX) in human liver were isolated from the adenovirus-based library using an enzyme-linked immunosorbent assay-based screening procedure.

In the postsequencing era of genome projects, the biological function of the majority of the human genes is still unknown (42). The identification of gene function is an increasingly relevant issue, especially in the search for new targets for improved therapy of human disease. Functional genomics approaches, which aim at the identification of genes via phenotypes induced in biological systems, require measurement of gene function on the genomic scale in cell-based assays. cDNA expression libraries representing the population of expressed genes in a given cell/tissue type have classically been cloned into plasmids, which can be introduced into cells by transient transfection using high-throughput automatization. However, transfection efficiency varies widely between cell types, and automatization costs and efforts are considerable. Therefore, a variety of virus-derived vector systems have been developed for improved cDNA transduction, expression, and screening in mammalian cells, including simian virus 40 replicons in COS cells (9, 21, 35, 43) and cDNA cloning and expression vectors derived from retrovirus (18, 32, 40, 41), baculovirus (11), alphavirus (19), human immunodeficiency virus (38), vaccinia virus (36), and adenovirus (8, 13, 24, 25).

Recombinant E1-deleted vectors derived from human adenovirus serotype 5 (Ad5) are highly efficient for *in vitro* and *in*

vivo gene transfer into a variety of mammalian cells and tissues and have been used in functional and gene therapy studies (17, 20), vaccination (6), and, lately, the introduction of cDNA libraries into cell-based assay systems for gene discovery (8, 13, 24, 25). Numerous methods for their construction have been described (for an overview, see reference 7). These vectors also offer the advantage of high levels of transient transgene expression and relative ease of construction, propagation, and purification to high-titer stable virus (17). Therefore, they are considered to be particularly suited as vector systems for functional genomics and cDNA expression cloning (31, 39).

It is assumed that there are up to $\sim 10^5$ different mRNA species present in mammalian cells and that an adequate representation in a cDNA library requires at least 10^6 independent clones (32). Construction of complex populations of recombinant adenoviruses with classical methods for adenovirus construction is hampered by inefficient rescue of infectious virus from cloned DNA. Using virus genomes cloned in a plasmid or cosmid generates only around 20 to 70 plaques per 60-mm dish after transfection into 293 cells (2, 4). This is mainly caused by the low infectivity of cloned viral DNA, which is recognized as $\sim 10^3$ -fold less efficient than infectious virus due to the lack of terminal protein (TP), which is covalently attached to both 3' and 5' termini of natural viral genomes and plays an important role in initiation of adenovirus replication (14). Michiels et al. (25) combined a classical system of recombinant adenovirus generation with high-level automatization of rescue of individual virus in a 96-well format. They were thus able to generate an arrayed adenoviral library containing human placental cDNA with around 1.2×10^5 independent clones and, by applying functional cell-based assays, to isolate

* Corresponding author. Present address: BiRDS Pharma GmbH, Prenzlauer Promenade 190, 13189 Berlin, Germany. Phone: 49 30 479075820. Fax: 49 30 479075812. E-mail: mhillgenberg@birdspharma.com.

† Present address: Apit Laboratories GmbH, Hermanswerder 16, D-14473 Potsdam, Germany.

‡ Present address: Robert-Koch-Institut, Seestr. 10, D-13353 Berlin, Germany.

known as well as yet unknown potential regulators of osteogenesis, metastasis, and angiogenesis. However, these procedures as well as currently existing approaches for one-step rescue of adenovirus-based cDNA expression libraries (8, 13) are technically demanding and time-consuming. Thus, there is a requirement for a fast, efficient, and generally applicable system for adenovirus-based cDNA library construction, which should allow for efficient one-step rescue of around 10^6 independent clones.

In this report we describe a new and efficient system for the generation of adenovirus vectors that is based on rescue by site-specific Cre/*loxP*-mediated insertion of foreign DNA into replicating donor virus, followed by an amplification procedure that counterselects residual donor virus. It fulfills the criterion of combining convenient procedures with high-efficiency virus rescue. The beneficial properties of the system result from a combination of principles for adenovirus vector construction, which individually have also been employed by others in the past but have not been combined before. We demonstrate our system to be well suited for the fast and efficient generation of clonal and complex populations of recombinant adenovirus vectors. Furthermore, in a proof-of-concept experiment, we applied our system to the construction of an adenovirus-based human liver cDNA library. cDNAs of genes expressed at either very high or low levels were successfully isolated from this library using enzyme-linked immunosorbent assay (ELISA)-based screening procedures, demonstrating the usefulness of our system for the construction of adenovirus-based cDNA libraries for gene discovery.

MATERIALS AND METHODS

Cell lines. E1-transformed human embryonic kidney cell line 293 (10) and human hepatoma cell line Huh7 (27) were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Eggenstein, Germany) supplemented with 2 mM glutamine (Sigma, Deisenhofen, Germany), antibiotics (penicillin and streptomycin), and 10% fetal calf serum (Biochrom, Berlin, Germany). Cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂. Previously described packaging cell line CIN1004, which had been derived from 293 cells by stable transfection with a bicistronic human cytomegalovirus (hCMV) immediate-early promoter-driven expression cassette coding for the neomycin resistance gene and nuclear-localized Cre recombinase (15), was cultivated under the same conditions plus 1 mg/ml G418.

Donor viruses and donor plasmids. The genomes of donor viruses *AdlantisI* and *AdlantisII* were constructed using homologous recombination of plasmids in *Escherichia coli* similar to the method described by Chartier et al. (4). Both genomes contain the 5' inverted terminal repeat (ITR) of Ad5, a *loxP* site, a partially deleted packaging signal, a 929-bp noncoding spacer fragment, a second *loxP* site in parallel orientation, and the sequence from nucleotide [nt] 3524 to the 3' end of Ad5 with a 2.7-kb deletion in the E3 region (the details on the complex cloning procedure are available on request). The packaging signal present in *AdlantisI* contains A repeats I through V (corresponding to nt 190 to 341 of the Ad5 genome), while the packaging signal of *AdlantisII* contains A repeats I and II and VI and VII (corresponding to nt 190 to 272 and nt 354 to 542). Viruses were produced by transfection of linear viral genomes onto 293 cells, and resulting infectious viruses were amplified on 293 cells. For *AdlantisI* a purified stock (10^9 infectious particles/ml [IP/ml]) was obtained by CsCl density gradient centrifugation. For *AdlantisII* a freeze-thaw lysate from 2×10^8 infected cells with a titer of 10^7 IP/ml was used as a stock for the experiments. Construction of donor plasmids pCBI-3 and pCBII-3 was performed using standard methods and starting from cosmid vector pMV (15) and pBSKS- (Stratagene). In pCBI-3, two *lox* sites flank the Ad5 packaging signal and a polylinker for convenient insertion of a transgene expression cassette. pCBII-3 contains nt 1 to 542 of the Ad5 genome (5' ITR and complete packaging signal), a polylinker, and a *loxP* site, and all three elements are framed by two sites for the rare cutting endonuclease I-SceI. For construction of DsRed (red fluorescent protein from *Discosoma coral*) and *lacZ* reporter plasmids (pCBI-DsRed, pCBII-DsRed,

pCBI-*lacZ*, and pCBII-*lacZ*), expression cassettes containing DsRed or *lacZ* genes, respectively, driven by the Rous sarcoma virus 3' LTR, were inserted into the polylinkers of pCBI-3 or pCBII-3, respectively. Plasmid pCBII-CMVII was derived from pCBII-3 by insertion of the hCMV immediate-early promoter, a short polylinker, and the hCMV early polyadenylation signal into the pCB3-II polylinker (the details of the complex cloning procedures are available upon request).

Production and characterization of recombinant adenoviruses. For recombinant virus rescue, 10^6 CIN1004 cells in 60-mm cell culture dishes were infected overnight with 1.2 ml of donor virus suspension in complete cell culture medium at a multiplicity of infection (MOI) of 1 or 5, respectively. Cells were replenished with fresh medium and subsequently transfected with 12 µg of I-SceI-digested donor plasmid using the calcium phosphate coprecipitation method. After cytopathic effect (CPE) became complete, virus was harvested by three freeze-thaw cycles. This rescue procedure was designated as amplification round 0 (passage A0), and the respective cell lysate was called the A0 lysate. One milliliter of this lysate was used to infect 293 or CIN1004 cells in a 60-mm culture dish of subconfluent cells (amplification round 1, or A1). After CPE was complete, cells were lysed to obtain the A1 lysate, all of which was applied to a further amplification round following the same scheme and resulting in the A2 lysate. All of this lysate was then used to infect cells in 10 150-ml dishes of subconfluent cells, which resulted in amplification round 3 (A3). Virus produced in this final amplification round was purified by CsCl banding as previously described (33). The adenovirus band was collected, and CsCl was removed by purification on NAP25 columns (Amersham Pharmacia Biotech, Uppsala, Sweden). Infectious virus titer (IP/ml) was determined by dilution endpoint analysis on 293 cells. Total number of viral particles per milliliter was determined by optical absorption as described by Maizel et al. (23). For restriction analysis, viral DNA was extracted from purified viral particles following standard procedures. Isolation of viral DNA from infected cells was done by the Hirt extraction procedure (16). Restriction analysis of viral DNA was done by digestion with PshAI, followed by separation of fragments in 0.4% agarose gels.

Reporter gene assays. For detection of reporter gene transduction, 293 or CIN1004 cells (10^6 cells/dish in 60-mm cell culture dishes or 10^5 cells/well in 24-well plates, respectively, seeded 24 h prior to infection) or Huh7 cells (4×10^4 cells/well in 24-well plates, seeded 24 h prior to infection) were infected with freeze-thaw lysates of intermediate amplifications rounds or with purified virus and analyzed at 36 h postinfection. DsRed expression was visualized by fluorescence microscopy. *lacZ* expression was visualized by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining using standard procedures. For titration of DsRed transducing units (DTU/ml) or *lacZ* transducing units (LTU/ml), a series of dilutions of the sample was used.

Construction and characterization of liver cDNA library in donor plasmid. Poly(A)⁺ RNA from human liver (5 µg; Stratagene) was converted to cDNA using a Stratagene cDNA cloning kit according to the instructions of the manufacturer, resulting in cDNA with EcoRI/XhoI ends. Total cDNA was size-fractionated by gel filtration. Selected fractions were pooled and ligated to plasmid pCBII-CMVII previously digested with restriction endonucleases MfeI/XhoI and dephosphorylated with calf intestine phosphatase. A total of 12 ligations were carried out, each with 20 ng of cDNA and 30 ng of pCBII-CMVII. After transformation of *E. coli* (XL10 GOLD; Stratagene), bacteria were plated onto a total of 24 15-mm ampicillin-containing (100 µg/ml) agar plates. Clones were counted, pooled, and amplified overnight in the total of 2 liters of LB supplemented with 100 µg of ampicillin/ml. For isolation and purification of the resulting plasmid library pCBII-CMV-LIVERcDNA, a QIAGEN Maxi Kit was used. For characterization, *E. coli* (XL10 GOLD) cells were transformed with pCBII-CMV-LIVERcDNA, and individual clones were characterized with respect to the size of the inserted cDNA by restriction analysis using SnaBI. The presence of human α1-antitrypsin (hATT) and human factor IX (hFIX) cDNAs in the library was verified by PCR using hAAT-specific PCR primers (KhAAT-s, 5'-ATGCCGTCTCTGTCTCGTG-3', and KhAAT-as, 5'-CATGAAGAGG GGAGACTTG-3'; 1,228-bp product including translation initiation site) or hFIX-specific PCR primers (KhFIX-s, 5'-ATGCAGCGCGTGAACATGAT-3', and hFIXcDNA-as, 5'-CTTCATGGAAGCCAGCACAG-3'; 1,206-bp product including translation initiation site).

Generation and characterization of adenovirus-based cDNA library. A total of 26 60-mm cell culture dishes with subconfluent CIN1004 cells (10^6 cells/dish seeded on the previous day) were infected overnight with *AdlantisI* at an MOI of 5. Fresh medium was added, and 20 dishes were transfected with 12 µg of I-SceI-digested pCBII-CMV-LIVERcDNA per dish. As controls, three 60-mm dishes were transfected with a total of 12 µg of a 1:50,000 mixture of I-SceI-digested plasmid pCBII-*lacZ* and plasmid library pCBII-CMVII-LIVERcDNA (control for complexity of virus rescue) and three 60-mm dishes were transfected

with 12 μ g of I-SceI-digested pCBII-*lacZ* alone (control for overall efficiency of virus rescue). After onset of CPE, cells were lysed by the freeze-thaw method, and the A0 lysates were pooled and passaged through 293 or CIN1004 cells to generate crude virus stocks of A1 and A2. Virus from the final amplification round (A3, obtained as described above) was purified as described above to obtain the purified adenovirus-based library *Adlantis*LIVERcDNA stock, which was stored in aliquots at -80°C . A1 lysates from the control experiments were used to infect HuH7 cells overnight (1 ml of lysate per 60-mm dish containing 5×10^5 cells), medium was added to 5 ml, and the number of LTU was determined by X-Gal staining 36 h later. Titer determination of the adenovirus-based library *Adlantis*LIVERcDNA was done as mentioned above. For characterization of the insert size range, percentage of full-length cDNAs, and insert sequences, individual adenovirus clones were isolated and characterized as described below. For verification of the presence of cDNAs for hAAT and hFIX, viral DNA was extracted from *Adlantis*LIVERcDNA and subjected to PCR analyses using a sense primer binding to the 5' end of the hCMV promoter sequence (primer CEK-s, TGGTACCGGAGCTTAAGGTG-3') and antisense primers khAAT-as and hFIX-cDNA-as, respectively.

Screening of adenovirus-based cDNA library. In the first screening round, 293 cells seeded in 96-well plates were infected with either 50 IP/well of *Adlantis* LIVERcDNA (for hAAT screening; three plates with 72 wells per plate and a total of 10,800 IP) or with 500 IP/well (for hFIX screening; nine plates with 72 wells per plate and a total of 324,000 IP). After onset of CPE, cells were lysed by freeze-thawing, and lysates were kept in the 96-well plates to generate master plates designated S1A1. Lysates from S1A1 were used for amplification through 293 cells in 96-well plates, which gave rise to master plates designated S1A2. Supernatants from S1A2 were then applied to ELISAs specific for hAAT or hFIX. In the event that a well of S1A2 master plates gave positive ELISA results, virus titer in this well was determined. Subsequently, selected virus-containing supernatants were subjected to a second screening round in which one 96-well plate seeded with 293 cells was used for each lysate selected from S1A2 plates. Seventy-two wells per plate were infected with 1 IP/well (in the case of screening for hAAT expression) or 10 IP/well (in the case of screening for hFIX expression), resulting in master plates designated S2A1. Supernatants were used for one (hFIX) or two (hAAT) amplification rounds through 293 cells in 96-well plates, resulting in master plates S2A2 (hFIX) and S2A2/S2A3 (hAAT). Supernatants from S2A2 (hFIX screening) or S2A3 (hAAT screening) were then used to measure concentrations of hAAT and hFIX by ELISA. Again, virus titers in selected cell lysates from wells containing hAAT or hFIX were determined. For hFIX, a third screening round was performed using virus from S2A2 at an MOI of 1 for infection of 293 cells, which resulted in master plates S3A1 and was followed by two amplification rounds which gave rise to master plates S3A2 and S3A3, respectively. Analysis of supernatants by ELISA and determination of virus titers in positive wells were done as before. Selected wells from master plates S2A3 (hAAT) or S3A3 (hFIX) were then used to isolate individual adenovirus clones by plaque assay for the characterization of inserts. For all infections performed with a defined IP number, 293 cells were seeded at a density of 3×10^3 cells/well 24 h before infections. For amplification steps, 293 cells were seeded at a density of 3×10^4 cells/well. For ELISA or amplification, one-third of the supernatant was used, and supernatants applied to ELISAs were generally collected prior to freeze-thawing. Cross-contamination of wells was prevented by applying plastic sealing prior to freeze-thawing, and master plates were stored at -80°C . For hAAT- and hFIX-specific ELISAs, a 1:4 dilution of supernatants from master plates generated during hAAT and hFIX screening was used. A hAAT-specific ELISA was done according to the method described by Cichon and Strauss (5); hFIX levels were measured by an ELISA according to the method described by Baru et al. (3), with the exception that a horseradish peroxidase-linked antibody (Pierce, Rockford, Ill.) was used for visualization.

Isolation and characterization of individual adenovirus clones. Individual adenovirus clones were obtained by plaque assay on 293 cells using standard protocols. Plaque isolates were amplified by passage through 293 cells, and viral DNA was isolated from infected cells by the Hirt extraction procedure (16). Restriction analysis of viral DNA was done by digestion with PshAI, followed by the separation of fragments in 0.4% agarose gels. For sequencing of unknown cDNA inserts, fragments containing the end of the CMV promoter, the cDNA insert, and the beginning of the CMV polyadenylation signal were amplified from isolated viral DNA by PCR using the primer pair CMVPr-s (5'-ACCGTCAGATCGCCTGGAGA-3') and CMVpA-as (5'-CGCTGCTAACGCTGCAAGAG-3'). For sequencing of hAAT and hFIX cDNA inserts, fragments containing the CMV promoter and the respective open reading frames of hAAT and hFIX were amplified from isolated viral DNA by PCR using sense primer CEK-s and antisense primers khAAT-as and hFIX-cDNA-as, respectively. PCR products were cloned into pBSKS- (Stratagene) and sequenced with primers binding to

T3 and T7 promoter sequences located upstream and downstream of the insertion site in pBSKS-, respectively. Sequences were identified by a BLASTN search at default settings (www.ncbi.gov).

RESULTS

Principles of the system and construction of donor viruses and donor plasmids. The principles underlying our system for adenovirus generation are outlined in Fig. 1. A donor virus contains a partially deleted packaging signal framed by two parallel oriented *loxP* sites. In an E1-complementing cell line expressing the Cre recombinase gene (CIN1004 [15]), the packaging signal is excised. The resulting donor virus $\Delta\Psi$ acceptor substrate can replicate in the usual manner but cannot be packaged due to the lack of a packaging signal. The remaining single *loxP* site can serve as a site-specific insertion site (acceptor site) for insertion of foreign DNA by Cre/*loxP*-mediated recombination (Fig. 1A). To insert foreign DNA into the viral acceptor site, two types of plasmids were constructed that make use of different reaction types catalyzed by Cre recombinase (Fig. 1B): type I donor plasmids (pCBI) contain the complete viral packaging signal, a gene of interest (GOI), and two parallel oriented *loxP* sites flanking the bacterial backbone. The bacterial backbone is excised by Cre recombinase, and the resulting construct that contains a single *loxP* site, the complete packaging signal and the GOI is recombined into the acceptor site of the donor virus $\Delta\Psi$ acceptor substrate by Cre recombinase via an insertion/excision type reaction. Type II donor plasmids (pCBII) contain a cassette that is flanked by I-SceI sites and that harbors the viral 5' ITR and the complete packaging signal, followed by the GOI and a single *loxP* site. After digestion with I-SceI and transfection onto CIN1004 cells, this cassette is inserted into the donor virus $\Delta\Psi$ acceptor substrate via a terminal exchange type reaction. The resulting recombinant adenoviruses can be packaged efficiently into viral capsids because they contain a complete adenoviral packaging signal and, as a prerequisite for efficient packaging, the TP attached to the 3' ITR (type II donor plasmid) or to both ITRs (type I donor plasmid). Since the novel recombinant viruses differ from the original donor viruses in that they contain a complete packaging signal, residual donor virus will be lost during amplification due to impaired packaging. Additionally, recombinant adenovirus made from type II donor plasmids is no longer a substrate for Cre-mediated excision of the packaging signal, whereby selection against residual donor virus can be achieved by propagation on the CIN1004 cell line.

Two donor viruses (*Adlantis*I and *Adlantis*II) that contain different partially deleted packaging signals flanked by *loxP* sites were engineered (Fig. 2A). In *Adlantis*I, A repeats VI and VII are deleted (" $\Psi\Delta$ I-V") whereas *Adlantis*II has deletions in A repeats III through V (" $\Psi\Delta$ II, II, VI, and VII") (Fig. 2B). After infection of CIN1004 cells with the donor viruses, the expected donor virus $\Delta\Psi$ acceptor substrate was produced as verified by the size of NheI restriction fragments of extracted viral DNA, whereas no donor virus $\Delta\Psi$ acceptor substrate was produced in infected 293 cells (data not shown). As expected, packaging of both viruses was severely reduced in CIN1004 cells compared to 293 cells (55-fold for *Adlantis*I and 115-fold for *Adlantis*II) (Fig. 2C). However, compared to a control virus

FIG. 1. Principle of recombinant adenovirus generation by site-specific insertion into replicating donor virus. The donor virus is converted into nonpackageable donor virus $\Delta\Psi$ acceptor substrate by Cre-mediated excision of the packaging signal (A). Foreign DNA is inserted into the resulting single *loxP* site via alternative reactions catalyzed by Cre recombinase depending on the type of donor plasmid (B). See text for details. Black boxes, 5' and 3' ITRs of Ad5; hatched boxes, packaging signal of Ad5, either partially deleted ($\Psi\Delta$) or complete (Ψ); $\Delta E1\Delta E3$, coding region of Ad5 with a deletion of early region 1 and 3; P, promoter; pA, polyadenylation signal; *amp*, bacterial ampicillin resistance gene; ori, bacterial origin of replication; *loxP*, recognition site for Cre recombinase; I-SceI, recognition site for I-SceI.

with a wild-type (wt) packaging signal, the number of infectious virus particles produced in 293 cells was only slightly reduced with *AdlantisI* but decreased by almost two orders of magnitude with *AdlantisII*, indicating that the deletion within the packaging signal of the latter leads to a severe reduction of packaging efficiency.

Establishment of a method for generating recombinant adenoviruses. We wished to determine the efficiency of type I or type II donor plasmids in generating recombinant adenovirus.

Two basic donor plasmids (pCBI-3 and pCBII-3) were constructed that correspond to type I or type II donor plasmids, respectively, but additionally contain a large polylinker sequence 3' to the complete packaging signal of Ad5. Insertion of a Rous sarcoma virus promoter-driven DsRed reporter gene into the polylinker resulted in donor plasmids pCBI-DsRed and pCBII-DsRed. CIN1004 cells were infected with *AdlantisI* and transfected 8 h later with either pCBI-DsRed or pCBII-DsRed to rescue recombinant adenoviruses AdCBI-DsRed

FIG. 2. Construction and characterization of donor viruses. (A) Genome structures of donor viruses AdlantisI and AdlantisII. The emergence of the donor virus $\Delta\Psi$ acceptor substrate generated by Cre processing of these viruses can be identified by the size of the N-terminal *NheI* fragment. Hatched boxes (Ψ^* or Ψ^{**}), partially deleted packaging signals; S, 0.9-kb noncoding spacer. (B) Detailed structures of the partially deleted packaging signals of donor viruses AdlantisI and AdlantisII and the complete packaging signal in pCBI and pCBII donor plasmids. The organization of the wt packaging signal of Ad5 (wt Ψ) with A repeats I to VII is shown on top. Numbers represent the nucleotide positions of the A repeats in the Ad5 genome. Restriction sites within the wt Ad5 genome used to generate the deletions are indicated. (C) Output of infectious virus progeny after infection of 293 or CIN1004 cells with AdlantisI, AdlantisII, or AdC, a control virus containing the wt Ad5 packaging signal that was not flanked by *loxP* sites. Infections were done at an MOI of 5 (AdlantisI and AdC) or 1 (AdlantisII). IP/cell. Virus titer in cell lysates was determined by endpoint dilution analysis on 293 cells after the appearance of complete CPE. Numbers above the bars represent the mean values from three independent experiments.

and AdCBII-DsRed, respectively (Fig. 3A). Rescue of recombinant adenovirus was detected by the emergence of DTU. With both donor plasmids, the emergence of DTU could reproducibly be detected in CIN1004 cells, indicating the emergence of recombinant viruses expressing the DsRed gene. Passaging of the virus through 293 cells resulted in a significant increase in DTU number (Fig. 3B). However, the number of DTU differed significantly between viruses produced from pCBI- and pCBII-derived plasmids (10^4 DTU versus 10^8 DTU in 10^6 cells after a single amplification round on 293 cells) (Fig. 3B), although the number of total IP was comparable in both

situations. Thus, whereas use of pCBI-DsRed resulted in a high degree of donor virus contamination within the virus population (only 1 DTU in about 10,000 IP), in the case of pCBII-DsRed the number of DTU was in the same range as the total IP number, indicating that the majority of the viruses contain the DsRed gene in the latter case, which was confirmed by restriction analysis of low-molecular-weight DNA isolated from infected cells (Fig. 3C). PCR analysis also confirmed the presence of AdCBI-DsRed and AdCBII-DsRed in the respective infected cells (data not shown).

Next, we wished to reduce donor virus contamination by

FIG. 3. (A) Structure of donor plasmids and resulting viruses expressing the DsRed gene. The sizes of fragments produced by digestion of viral DNA with PshAI are indicated. (B) Number of DTU (black bars) and IP (white bars) in virus populations at A0 and A1, respectively, using either pCBI or pCBII as donor plasmids. CIN1004 cells were infected and transfected as described in Materials and Methods; cells were lysed after the appearance of total CPE, and 20% of the lysates (passage A0) was used to reinfect 293 cells to produce passage A1. DTU and IP titers were determined by titration of lysates on 293 cells. Shown are results from three independent experiments with standard deviations. (C) Restriction analysis of low-molecular-weight DNA isolated from 293 cells at passage A1. The position of the 5' terminal PshAI fragments characteristic for *AdlantisI* (3,906 bp) and DsRed viruses (4,796 and 4,581 bp) are indicated. Whereas no band specific for AdCBI-DsRed is visible, the AdCBII-DsRed-specific 5'-PshAI fragment of 4,581 bp is clearly detectable.

making use of the growth advantages of recombinant DsRed virus produced from pCBII-DsRed over the donor virus *AdlantisI* or *AdlantisII*. To this end, 12 separate populations of AdCBII-DsRed were rescued independently on CIN1004 cells and grown over three passages to counterselect against donor virus. To distinguish between counterselection by reduced virus packaging (due to the deletions in the *Adlantis* packaging

signals) and counterselection caused by Cre-mediated excision of the packaging signal from the donor virus genome, passaging was done on 293 and CIN1004 cells. At passage three (A3), CsCl-banded virus was prepared, and viral DNA was analyzed following digestion with PshAI. In the A1 passage, DNA of virus derived from *AdlantisI* and passaged on 293 cells (but not on CIN1004 cells) shows bands typical for both viruses

FIG. 4. Analyses of pCBII-derived viruses after repeated passaging. (A) Viruses rescued from pCBII-DsRed and *AdlantisI* or *AdlantisII* were passaged three times on either 293 or CIN1004 cells. Viral DNA was isolated from cell lysates by Hirt extraction (passage A1) or from CsCl-banded virus (passage A3) and analyzed after digestion with PshAI. Viral DNA isolated from 293 cells infected with *AdlantisI* or *AdlantisII* alone (left panels) or cloned DNA of *AdlantisI* and *AdlantisII* (right panels) was also digested with PshAI and used for controls. The positions of the characteristic 5' terminal PshAI fragments are indicated. Lane M, 1-kb DNA ladder. (B) Southern blot analysis for quantification of minor contamination by donor virus. One microgram of viral DNA isolated from CsCl-banded viruses was digested with PshAI and applied to Southern blot analysis using a radiolabeled probe specific for the spacer fragment located between the packaging signal and the second *loxP* site in both donor viruses. This spacer fragment is not present in the recombinant viruses (Fig. 2). As controls, cloned *AdlantisI* or *AdlantisII* DNAs were digested with PshAI, and 10 ng (corresponding to a 10^2 -fold dilution of 1 μ g of recombinant adenovirus DNA), 1 ng (10^3 -fold dilution), 0.1 ng (10^4 -fold dilution), or 0.01 ng (10^5 -fold dilution) of digested DNA was loaded onto the gel. Contamination with donor virus was determined by quantification of signal intensities in sample and control lanes. Each sample in panels A and B represents an individual adenovirus virus population which was independently rescued and passaged.

(AdCBII-DsRed, 4,581 bp; *AdlantisI*, 3,906 bp). In contrast, DNA of virus derived from *AdlantisII* contains only the fragment specific for the DsRed virus and no visible band typical for *AdlantisII* at this stage. After two additional passages

through either 293 or CIN1004 cells, no major donor virus contamination was detected by this method in any virus preparation, indicating a reproducible and reliable counterselection against contaminating donor virus (Fig. 4A). To verify these

data, identical experiments were performed using a virus containing the *E. coli lacZ* gene instead of the DsRed gene with similar results (data not shown). The titers of the large-scale preparations were 10^{10} to 10^{11} IP/ml at a particle-to-infectious particle ratio of 15 to 30. These titers are comparable to those obtained with classical methods for recombinant adenovirus production (7). Gene transfer efficiency into cultured cells was measured by expression of the reporter genes *lacZ* and DsRed and was comparable to the respective adenovirus vectors constructed with conventional methods (data not shown).

In order to detect possible minor donor virus contamination, Southern blot analyses were performed using a probe that specifically binds to the 5' terminus of the donor viruses (Fig. 4B). When 293 cells were used for virus amplification, donor virus contamination was about 1% with *AdlantisI* as donor virus and about 0.025% with *AdlantisII* as donor virus. This is consistent with the observation that growth of *AdlantisII* on 293 cells is far less efficient than that of *AdlantisI*. When CIN1004 cells were used for virus amplification, residual donor virus contamination was below the sensitivity of the assay with either donor virus ($<0.001\%$), which is consistent with the observation that Cre-mediated excision of the donor virus packaging signal is highly efficient in these cells. Similar data on residual contamination with donor virus were obtained with the viruses expressing the *E. coli lacZ* gene (data not shown).

Rescue of adenoviruses from plasmid mixtures. A prerequisite for using our system for construction of adenovirus-based cDNA libraries from plasmid-based cDNA libraries is the rescue of complex populations of recombinant adenovirus which includes the conversion of low-abundance donor plasmids into virus. To test for this, pCBII-*lacZ* was diluted into pCBII-DsRed in a range from 1:50 to 1:500,000, and plasmid mixtures were used for virus rescue from 10^6 CIN1004 cells previously infected with *AdlantisI* or *AdlantisII*. At passage A1, LTU (indicative for generation of AdCBII-*lacZ* from pCBII-*lacZ*) were titrated. When *AdlantisI* was used for virus rescue, LTU were present in nearly all preparations up to a dilution of 1:50,000. At higher dilutions, only a minority of preparations contained LTU. With *AdlantisII* as donor virus, LTU was present only up to a dilution of 1:5,000 (Fig. 5). Thus, assuming that both pCBII-DsRed and pCBII-*lacZ* act as equally efficient templates for virus rescue, the number of independent recombinant adenovirus clones generated during rescue is about 50,000 from 10^6 cells when *AdlantisI* is used as the donor virus and less than 5,000 when *AdlantisII* is used.

Generation and characterization of an adenovirus-based human liver cDNA expression library. The general scheme of cDNA library generation and screening is outlined in Fig. 6. A plasmid-based cDNA library was created starting with plasmid pCBII-CMV, which was derived from plasmid pCBII and contains the hCMV immediate-early promoter, a polylinker for cDNA insertion, and a polyadenylation signal derived from hCMV. cDNA generated from human liver poly(A)⁺ RNA (fractions of 0.3 to 4 kb in size) was unidirectionally inserted into pCBII-CMV to create a plasmid-based cDNA library, termed pCBII-CMV-LIVERcDNA, that contained a total of 0.82×10^6 independent clones. Seventeen retransformed clones were analyzed with respect to the size of the cDNA insert by restriction analysis with SnaBI (Fig. 7A). In 16 of 17 clones the insert sizes were between 0.4 and 2.1 kb, with an estimated average

FIG. 5. Determination of numbers of independent recombinant adenovirus clones generated when *AdlantisI* and *AdlantisII* are used as donor viruses for rescue. For rescue, CIN1004 cells infected with *AdlantisI* (top) or *AdlantisII* (bottom) were transfected with a total of 12 μ g of mixtures of I-SceI-digested pCBII-*lacZ* and pCBII-DsRed at ratios between 1:50 and 1:500,000. After rescue, virus mixtures were amplified by one passage through 293 cells (A1). The total number of LTU present in the lysates was titrated by X-Gal staining of Huh7 cells infected with a dilution series of A1 lysates. As a positive control (PC), 12 μ g of I-SceI-digested pCBII-*lacZ* was used for rescue; as a negative control 12 μ g of I-SceI-digested pCBII-DsRed was used. Bars represent average titers of LTU in A1 with standard deviations; numbers above the bars indicate the total number of experiments versus the number of experiments in which the emergence of LTU could be detected.

of 1.3 kb. Only one clone contained a significantly larger insert of about 3.2 kb. The presence of cDNAs derived from an mRNA species with a high copy number in human liver hAAT and from a low-abundance mRNA (hFIX) was verified by PCR (Fig. 7B).

In the attempt to construct an adenovirus cDNA library, we aimed at the generation of 2×10^6 independent clones. As shown in Fig. 5, about 5×10^4 clones were reproducibly generated from 10^6 cells with donor virus *AdlantisI*. Therefore,

FIG. 6. Principle of construction of adenovirus-based cDNA libraries. cDNA reversely transcribed from cellular mRNA is directionally inserted into the polylinker of a pCBII-derived donor plasmid, thereby generating a donor plasmid-based cDNA library. The latter can be digested with I-SceI and is used to transfect packaging cell line CIN1004 previously infected with donor virus *Adlantis*I. Site-specific insertion of cDNAs into replicating donor virus results in an adenovirus-based cDNA library that contains about 10⁶ independent adenovirus clones. This library is then titrated, and the diluted library can be used for infection of 293 producer cells in 96-well plates. After replication of the subpopulations, master plates are generated by freeze-thawing and contain infectious supernatants that represent subpopulations of the adenovirus library. Supernatants can be used to infect a functional cell-based assay in a 96-well format. Recombinant adenoviruses from positive wells containing candidate clones are isolated by plaque assay on 293 cells and characterized by sequencing of the cDNA insert. CMV, hCMV immediate-early promoter; pA, hCMV early polyadenylation signal. Other symbols and abbreviations are as described in the legend of Fig. 1.

2×10^7 CIN1004 cells (20 60-mm dishes) were used for infection with *Adlantis*I and subsequent transfection with I-SceI-digested pCBII-CMVII-LIVERcDNA. As controls for complexity and overall efficiency of adenovirus generation, each of three dishes was infected in parallel under the same conditions and transfected with a mixture of pCBII-*lacZ* and pCBII-CMVII-LIVERcDNA at a ratio of $1:5 \times 10^4$. Virus generated in the controls was amplified by passing once through 293 cells and used for infection of HuH7 cells. X-Gal staining of

HuH7 cells revealed the presence of LTU, indicative for generation of recombinant adenovirus AdCBII-*lacZ* from plasmid pCBII-*lacZ* (data not shown). Thus, conditions used for virus rescue were adequate for the generation of at least 5×10^4 independent clones per 10^6 CIN1004 cells. Virus from the cells transfected with pCBII-CMVII-LIVERcDNA alone was pooled to generate round A0 of the adenovirus-based cDNA library and was amplified by passing through CIN1004 cells, resulting in A1. After a second amplification round (A2) by

FIG. 7. Characterization of pCBII-CMVII-LIVERcDNA plasmid-based cDNA library. (A) Determination of cDNA insert sizes in the donor plasmid-based cDNA library (pCBII-CMVII-LIVERcDNA). One microgram of plasmid DNA from individual clones (1 to 17, isolated from the cDNA library by retransformation) was digested with *Sna*BI and analyzed on a 0.4% agarose gel. *Sna*BI restriction sites are located at the 5' end of the hCMV promoter and at the 3' end of the hCMV polyadenylation signal of pCBII-CMVII. As a control, pCBII-CMVII without insert was digested in parallel, resulting in a fragment of 632 bp. Sizes of DNA marker fragments are indicated. (B) Verification of the presence of hAAT cDNA and hFIX cDNA in pCBII-CMVII-LIVERcDNA. Indicated amounts of plasmid library were used in the PCRs with hAAT- or hFIX-specific primers. Specific products (1,228 bp for hAAT cDNA and 1,206 bp for hFIX cDNA) are indicated. H₂O and 10 ng of empty pCBII-CMV were used as negative controls; plasmids with cloned full-length hAAT and hFIX cDNAs served as positive controls. Lanes M, 1-kb DNA ladder.

passage through 293 cells, the adenovirus-based library was purified by CsCl banding. The total virus particle titer of the preparation was 1.9×10^{12} particles per ml, and the infectious virus titer was 2.3×10^{11} IP/ml, indicating a total viral particle-to-infectious particle ratio of ~ 12 .

For characterization of the insert size range and percentage of full-length cDNAs, individual adenovirus clones were isolated from *Adlantis*LIVERcDNA by plaque assay. Sixteen plaque isolates were amplified, followed by isolation and restriction analysis of viral DNA by digestion with *Psh*AI, which

generates 5' ends with a size of 3,667 bp plus the cDNA insert size (Fig. 8A). Insert sizes were between 0.8 and 2.4 kb, with an average of about 1.3 kb (Fig. 8B). Although only a limited number of clones was analyzed, these insert sizes were comparable to those found in the donor plasmid library, indicating that probably no significant shift of insert sizes had occurred during conversion to the virus-based library. cDNA inserts were then amplified from viral DNA and sequenced after cloning into pBSSK. Sequences were identified by alignment with public sequence databases (BLASTN search at www.ncbi.gov). The results are summarized in Table 1. A total of 12 of 16 inserts could be identified as either full-length (7/12, or $\sim 58\%$) or 5'-truncated (5/12, or $\sim 42\%$) cDNAs of genes known to be expressed in human liver. Of these, seven were serum proteins synthesized in liver (apolipoprotein A1, histidine-rich glycoprotein [two], vitronectin, haptoglobin α S1 [two], and complement component 4 binding protein α) and five were intracellular or membrane-located proteins (proteasomal modulator subunit p27, deoxiguanosine kinase, melanoma-associated antigen, and adipose differentiation-associated antigen). For 4 of 16 inserts no other matches than homologies to human chromosomal DNA sequences were found. Finally, the presence of cDNAs for hAAT and hFIX in the adenovirus library was verified by PCR (Fig. 8C).

Screening of the adenovirus-based human liver cDNA expression library for specific genes. Screening for adenovirus clones expressing hAAT and hFIX genes (the products of which are secreted into the supernatant) was done by hAAT- and hFIX-specific ELISA with supernatants of 293 cells infected with subpopulations of *Adlantis*LIVERcDNA in 96-well plates. The results of the ELISAs are summarized in Fig. 9A (hAAT screening) and Fig. 9B (hFIX screening). Briefly, screening for hAAT started with three 96-well plates infected with 50 IP of *Adlantis*LIVERcDNA/well (total 10,800 IP), and screening for hFIX started with nine 96-well plates infected with 500 IP/well (total 324,000 IP). In screening round 1, a total of 52 of 216 subpopulations were positive for hAAT (Fig. 9A), but only 2 of 648 subpopulations were positive for hFIX (Fig. 9B), confirming the high abundance of hAAT and low abundance of hFIX cDNAs, respectively, in the adenovirus library. Selected subpopulations of *Adlantis*LIVERcDNA expressing the hAAT or hFIX cDNA were titrated and used for a second screening round on one (each) 96-well plate which was infected with 1 IP/well (hAAT screening) or 10 IP/well (hFIX screening). Three positive subpopulations from a second screening round for hAAT were used for isolation and characterization of virus clones. In hFIX screening, two selected positive subpopulations from the second screening round were titrated and used for a third screening round on one 96-well plate which was infected with 1 IP/well. Two positive subpopulations from the third screening round for hFIX were used for isolation and characterization of viral clones.

Six individual adenovirus clones were isolated from each of the three or two positive subpopulations of the final hAAT and hFIX screening rounds, respectively. Plaque isolates were amplified through 293 cells, followed by isolation and restriction analysis of viral DNA. In all cases, the six clones isolated from the same subpopulation had identical fragment patterns, verifying the clonality of the subpopulation (data not shown). One isolate from each subpopulation was then used for PCR am-

A.

B.

C.

FIG. 8. Characterization of adenovirus-based cDNA library *Adantis* LIVERcDNA. (A) Structure of adenovirus backbone with unidirectionally inserted cDNA and recognition sites for *PshAI* used for restriction analysis. The positions of the PCR primers used for insert amplification (CMVPr-s and CMVpA-as) and detection of hAAT and hFIX cDNAs in the library (CEK-s, hFIXcDNA-as, and khAAT-as) are indicated. (B) Determination of the average cDNA insert size by *PshAI* digestion of DNA from individual adenovirus clones. Clones were isolated from *Adantis*LIVERcDNA by plaque assay and amplified by passage through 293 cells. One microgram of viral DNA isolated from infected 293 cells by Hirt extraction was used in the experiment. (C) Verification of the presence of hAAT and hFIX cDNAs in

plification of the insert with primers, as shown in Fig. 10. PCR products were cloned, sequenced, and identified by alignment with public sequence databases (BLASTN search at www.ncbi.gov). All three clones isolated from hAAT-positive subpopulations were full-length cDNAs for hAAT, with a perfect match to GenBank accession number X01663. The number of nucleotides between the 5' cDNA insertion site and the beginning of the hAAT gene open reading frame (ORF) varied (27, 131, and 168 bp), indicating that the viruses present in the three subpopulations were derived from different reverse transcripts (Fig. 10A). Similarly, both clones isolated from hFIX-positive subpopulations contained at least the 5'-terminal 1,233 bp of the total 1,385 bp of the hFIX cDNA ORF, with a perfect match to GenBank accession number NM_000133. Although not completely verified by the sequencing, it can be concluded from the functional assay that both clones contained the full-length cDNA. The numbers of nucleotides between the 5' cDNA insertion site and the beginning of the hFIX ORF were identical (13 bp), indicating that both clones might originate from the same reverse transcript (Fig. 10B).

DISCUSSION

In the present study we have established a system for the generation of adenovirus vectors that is based on rescue by site-specific insertion of foreign DNA into replicating donor virus, followed by an amplification procedure that counter-selects residual donor virus. While this system might also be well suited for the fast and efficient generation of single recombinant adenovirus vectors, it can be better used for construction of adenovirus-based cDNA libraries. The beneficial properties of the present system result from a combination of principles for adenovirus vector construction that individually have also been employed by others in the past but have not previously been combined.

Recombination mediated by Cre recombinase has previously been shown to significantly enhance the efficiency of adenovirus construction compared to homology-mediated recombination (28, 37). In the present study, on the basis of different Cre/*lox*-mediated mechanisms, two types of donor plasmids were investigated with respect to their efficiency in recombinant adenovirus rescue. Although recombinant viruses were obtained with both donor plasmid types, the virus rescue was much more efficient with type II donor plasmids. Two reasons might account for this observation. (i) For thermodynamic reasons, the equilibrium of the excision/insertion reaction involved in the insertion of transgene sequences is shifted toward excision when type I donor plasmids are used, thereby leading to a reduced overall insertion rate, whereas the terminal exchange reaction has a balanced equilibrium (1). (ii) The recombinants resulting from type I donor plasmids, like the do-

*Adantis*LIVERcDNA. The indicated amounts of DNA isolated from purified *Adantis*LIVERcDNA stocks were used as templates for PCR with hAAT- or hFIX-specific primers as described in Materials and Methods. H₂O and 10 ng of cloned *Adantis*I donor virus DNA served as negative controls. The sizes of DNA marker fragments are indicated to the left. Lanes M, 1-kb DNA ladder.

TABLE 1. Identification of cDNA inserts present in 16 adenovirus clones isolated from *Adlantis*LIVERcDNA

Plaque isolate no.	Insert size (bp)	Match to GenBank accession no. ^a	Insert
1	896	NM_000039, nt 15-896	Apolipoprotein AI (ApoAI); full-length (803-bp ORF)
2	1,075	NM_002813.2, nt 71-1099	Proteasomal modulator subunit p27 (PSMD9); full-length (695-bp ORF)
3	2,275	BC022312.1, nt 1-2232	Complement component 4 binding protein a; full-length (1,793-bp ORF)
4	1,263	AL160276, nt 17317-18582	ND; match to region on chromosome 9
5	1,663	NM_000412.2, nt 18-1639	Histidine-rich glycoprotein (HRG); full-length (1,577-bp ORF)
6	973	XM_002341.6, nt 188-1128	Deoxyguanosine kinase (DGUOK); 5'-truncated (744/833-bp ORF)
7	1,306	AC008781, nt 134809-135922	ND, match to region on chromosome 5
8	1,303	XM_031492.1, nt 334-1584	Vitronectin (VTN); 5'-truncated (1168/1438-bp ORF)
9	1,120	XM_042621, nt 327-1411	Haptoglobin a S1 (Hpa S1); 5'-truncated (920/1,220-bp ORF)
10	1,254	XM_042621, nt 3-1222	Haptoglobin a S1 (Hpa S1); full-length (1,220-bp ORF)
11	1,070	AC090376.6, nt 1151714-150677	ND; match to region on chromosome 18
12	1,698	AK057754.1, nt 536-1594	Rearranged IgG heavy chain; 5'-truncated
13	1,981	NM_000412.2, nt 18-1636	Histidine-rich glycoprotein (HRG); full length (1,577-bp ORF)
14	1,955	AF200348.1, nt 3605-5510	Melanoma-associated antigen (MG50); 5'-truncated (886/4,439-bp ORF)
15	1,584	AC104958.6, nt 148626-150169	ND; match to region on chromosome 8
16	1,900	BC005127.1, nt 9-1877	Adipose differentiation associated antigen (ADRP); full-length (1,313-bp ORF)

^a Alignment was performed with public sequence databases (BLASTN search at www.ncbi.gov). ND, gene not defined.

nor viruses, have a packaging signal framed by two *loxP* sequences and are, therefore, growth impaired in the Cre recombinase-expressing packaging cell line CIN1004 used for rescue. In contrast, type II donor plasmids lead to recombinants that retain only one single *loxP* site and therefore can grow normally in CIN1004 cells.

TP, which is covalently attached to both the 3' and 5' termini of natural adenoviral genomes, plays an important role in the initiation of adenovirus replication, and cloned linear adenovirus DNA devoid of TP is recognized $\sim 10^3$ -fold less efficiently by the viral replication machinery than natural virus genomes (14). Using virus genomes cloned in a plasmid or cosmid generated only about 20 to 70 plaques per 60-mm dish after transfection into 293 cells (2, 4). In contrast, McVey et al. (24) reported the generation of an adenovirus cDNA library with high complexity by simply using transfection of cloned viral DNA into 293 cells, but the reasons for this surprising finding remain to be elucidated. However, in accordance with the accepted importance of TP for substrate recognition by the adenovirus replication machinery, a high efficiency of recombinant adenovirus rescue was reported when virus genome termini linked to TP were used as a source of the right-end viral sequences (13, 29). Unfortunately, transfection of left-end-truncated genomes requires technically demanding and time-consuming preparation of large amounts of virus DNA-TP complexes from purified virus. Therefore, use of an infectious donor virus with TP as a substrate for foreign DNA insertion is more convenient (8, 12, 26). In general, however, the use of a donor virus for rescue of recombinants results in the problem of coreplication of donor virus (8, 12, 26). To solve

this problem, Elahi et al. (8) used a donor virus with the viral protease gene (PS) deleted, which can only perform one replication cycle in noncomplementing cells (30). In this system, the recombination event leads to incorporation of both the cDNA cassette and an ectopic PS expression cassette into the E1 region of the recombinant, which permits a positive selection. However, as the recombinants can complement the PS deficiency of the donor viruses in *trans*, laborious plaque assays are essential in order to obtain donor virus-free libraries, which is time-consuming and not efficient for generating complex mixed adenovirus populations.

In contrast, our dual-principle counterselection method, which is based on the Cre/*lox*-mediated excision of the donor virus packaging signal in the cell line CIN1004 and the presence of partially deleted packaging signals in the donor viruses, proved to be highly efficient. It resulted in recombinant adenovirus preparations with less than 0.001% residual donor virus contamination after only three rounds of amplification of clonal populations of recombinant adenoviruses in CIN1004 cells with either donor virus. Although we did not examine residual donor virus contamination in the adenovirus-based human liver cDNA library, results from restriction analysis of viral DNA isolated from the library stocks did not indicate contamination with donor virus (data not shown). This was also confirmed by the finding that none of the virus clones isolated for insert characterization was the original donor virus. Notably, others were not able to efficiently reduce donor virus contamination by a Cre/*loxP*-mediated excision of donor virus packaging signal alone (12). This difference might be due to the presence of partially deleted packaging signals in our

FIG. 9. Screening of *Adlantis*LIVERcDNA for adenovirus clones expressing the hAAT (A) or hFIX (B) genes. hAAT and hFIX concentrations were measured by ELISA in supernatants from master plates obtained after the first, second, or third screening round. Shown are the optical density values at 490 nm, minus blank values, according to the gray scale code under each plate. In each 96-well plate, the designation of wells refers to the row number (A to H, from top to bottom) and column number (1 to 12, from right to left). Wells A1 to F12 are supernatants of infected cells from master plates, wells G1 to G12 are negative controls (supernatants of 293 cells infected with donor virus *Adlantis*I lacking transgenes), wells H1 to H9 are a 1:2 dilution series of recombinant protein starting with 250 ng/ μ l (hAAT) or 100 ng/ μ l (hFIX) in H1, and wells H10 to H12 are blanks. Positive wells selected for subsequent screening rounds or for plaque purification of individual adenoviruses are highlighted with white frames. In each screening round, infection of 96-well plates was performed with decreasing IP numbers per well, as indicated to the left.

A.

B.

FIG. 10. Characterization of cDNA inserts obtained from individual adenovirus clones after plaque isolation of virus present in wells staining positive for hAAT (A) or hFIX (B). Viruses from final screening rounds for hAAT (clones a-B9-E10, b-D10-E4, and c-B6-C4) and hFIX (clones a-A11-F1-B1 and b-C5-B12-C11) were plaque purified and amplified by passing through 293 cells. Viral DNA was isolated by Hirt extraction and used for PCR analysis. The general structure of the adenoviral vectors with the cDNA inserted between the MnlI/EcoRI and XhoI sites is shown, and the positions of PCR primers used for amplification of cDNA inserts are indicated. Sequences of cloned PCR products were used for a BLASTN search at www.ncbi.gov. Regions of perfect match of cDNA inserts of hAAT adenovirus clones and of hFIX adenovirus clones to the respective reference sequences (GenBank accession number X01683 for hAAT cDNA and NM_000133 for hFIX cDNA) are shown. White boxes represent 5' nontranslated sequences; black boxes represent coding sequences. SP, signal peptide; MP, mature protein. N_n indicates the number of nucleotides between the MnlI/EcoRI insertion site of the cDNA and the first nucleotide matching the reference sequence. Other abbreviations are as described in the legend of Fig. 1.

donor viruses and the very high Cre recombinase expression levels in the CIN1004 cell line (15). Interestingly, while contamination with donor virus was about 50% directly after rescue of recombinants when *AdlantisI* was used, contamination

was much lower with *AdlantisII* as donor virus. This is in agreement with the earlier finding that deletion of A repeats VI and VII leads to a less severe packaging inhibition than deletion of the central A repeats III to V (34). The residual donor virus contamination obtained with the present system (less than 0.001%) is probably not critical for most research-oriented applications of clonal or complex preparations of recombinant adenovirus vectors.

Donor viruses *AdlantisI* and *AdlantisII* have complementary favorable features. Whereas in preliminary experiments *AdlantisII* turned out to be unsuitable as a donor virus for the construction of highly complex populations of recombinant adenovirus vectors, 5×10^4 independent adenovirus clones per 10^6 cells were achieved in rescue experiments when *AdlantisI* was used as the donor virus. This corresponds to 1 recombinant virus generated per 20 cells. This is, to our knowledge, the highest rescue efficiency for recombinant adenovirus vectors reported so far. Consequently, *AdlantisI* was used for construction of a high-titer adenoviral cDNA library from human liver cDNA. However, testing for contamination with replication-competent adenovirus (RCA) revealed that use of *AdlantisI* as a donor virus for the construction of clonal populations of recombinant adenovirus correlated with the presence of RCA in all preparations tested (data not shown). In contrast, when *AdlantisII* was used as the donor virus for the construction of clonal populations of recombinant adenovirus vectors, only 1 of 12 preparations tested was positive for RCA (data not shown). This is in the range also observed with classical methods of adenovirus construction. The emergence of RCA by recombination of the E1-deleted vectors with the left-end sequences of Ad5 inserted into the genome of 293 cells is a well-known problem when these cells or derivatives thereof are used for adenovirus rescue and amplification (22) and was also observed with other systems for adenovirus-based cDNA library construction (24). This problem remains unsolved so far, since the reasons for the RCA contaminations of *AdlantisI*-derived virus preparations are not clear, especially in face of the fact that the *AdlantisI* stock used for the experiments was RCA free (below $1/10^8$ IP) (data not shown). Thus, whereas the use of *AdlantisII* as a donor virus seems favorable for the generation of RCA-free high-titer preparations of clonal recombinant adenovirus populations, only the use of *AdlantisI* as the donor virus leads to a rescue complexity suitable for the generation of highly complex populations of recombinant adenovirus vectors, e.g., adenovirus-based cDNA expression libraries. Here, however, as shown by our results, limited contamination with RCA (about 0.005% of viral particles) does not seem to be critical for screening procedures. However, if the objective is to screen for cDNAs inducing highly complex biological processes that might need more time in order to induce a detectable phenotype, RCA contamination even at low levels would, in fact, impose a problem.

Despite this potential need for future optimization, the present study unequivocally demonstrates the usefulness of our system in its present state for adenovirus-based cDNA expression library construction and screening. In our proof-of-concept experiment, we have shown that our novel system can be applied to the construction of representative adenovirus-based cDNA libraries for gene discovery, as illustrated by the generation of a high-titer adenoviral cDNA library from human liver

cDNA that contained about 10^6 independent viral clones and about 44% full-length cDNAs. Furthermore, the suitability of adenoviral cDNA libraries generated with our system for the isolation of genes in cell-based assay systems is illustrated by the successful isolation of cDNAs for hAAT and hFIX. The number of positive subpopulations identified in screening round 1 is in accordance with the level of expression of these genes in liver and, thus, the abundance of the respective cDNAs in the cDNA library. Screening for hAAT revealed, that 52 wells were positive for hAAT when a total of 10,800 adenovirus clones were used, indicating a frequency of 1/207 and corresponding to 0.5% frequency of hAAT cDNA. When screening for hFIX was performed, only two wells turned out to be positive. Since screening was started with a total of 324,000 adenovirus clones, the frequency of hFIX in the library was 1/164,000, which corresponds to a frequency of hFIX cDNA of 0.0006% of total cDNAs. Taken together, these results show that low-abundance cDNA can be isolated from adenovirus-based cDNA libraries generated with our system with relative ease, and they confirm that the complexity of the liver mRNA pool was maintained in the adenovirus-based cDNA library. Analysis of the library for the presence of other genes expressed at low levels in human liver has not been performed yet but will be done in the future to verify these results. In addition, our results confirm the usefulness of pooled subpopulation approaches in adenovirus library screening. Although screening round 1 started with 50 and 500 clones/well for hAAT and hFIX screening, respectively, positive subpopulations could unambiguously be identified. Furthermore, by this approach, only a total of 6 or 13 96-well ELISAs was needed for the whole screening procedure for hAAT or hFIX, respectively.

In contrast to the pooled adenovirus cDNA expression library generated in the present study and by others (8, 13, 24), Michiels et al. (25) generated an arrayed library by automated rescue of individual viruses in a 96-well format. The arrayed libraries clearly offer the advantage of defined clones with little redundancy. On the other hand, pooled libraries generated by our system are more versatile, can be generated faster, are less costly, and can be more easily adapted for individual needs. Thus, both pooled and arrayed adenovirus cDNA libraries offer advantages of their own and can act as complementary tools for various gene discovery applications. Similar to the present study, other studies have also shown that cDNAs can be successfully isolated from adenoviral cDNA libraries, mainly in the context of cell-based assay systems: Michiels et al. (25) applied three different functional cell-based assays to isolate known as well as yet unknown regulators of osteogenesis, metastasis, and angiogenesis from their arrayed adenovirus-based placental cDNA library; Hatanaka et al. (13), by applying a screening procedure based on histochemistry, isolated the CD2 cDNA which was present at a frequency of less than 0.003% of cDNAs in their adenoviral T-cell cDNA library; and McVey et al. (24) were able to isolate the cDNA for Ad5 E1A 13S, which was present in their cDNA library at a frequency of 0.4%. Thus, one can conclude that adenoviral cDNA libraries today are a proven tool for gene discovery in cell-based assays.

In summary, the system described in this report, with the complementary favorable features of the donor virus *Adlantis*I for the construction of highly complex populations of recom-

binant adenoviruses and *Adlantis*II for the construction of clonal populations of recombinant adenovirus vectors, represents significant progress in recombinant adenovirus construction. Most importantly, the system facilitates the construction of adenovirus cDNA libraries for gene discovery in cell-based assays and might significantly contribute to the identification of genes based on specific biological functions for a variety of purposes. Furthermore, the validated adenovirus-based human liver cDNA library generated in the present study could be useful for the future discovery of liver genes involved in various biological processes.

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