An Adenovirus Type 5 (Ad5) Amplicon-Based Packaging Cell Line for Production of High-Capacity Helper-Independent Δ E1-E2-E3-E4 Ad5 Vectors

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Production of multiply deleted adenoviral (Ad) vectors with increased cloning capacity and reduced immunogenicity to adenovirus gene products requires the concomitant generation of efficient packaging cell lines. High expression levels of the complementing genes must be achieved in a coordinated fashion with viral replication. This is a particularly difficult task in light of the significant cytotoxicity displayed by adenoviral proteins. To this end, we developed a novel adenovirus-based amplicon with an Epstein-Barr virus origin of replication, Ad type 5 (Ad5) inverted terminal repeats, all Ad5 early region 2 (E2) genes, and the early region 4 (E4) open reading frame 6 (ORF6) under the control of a tetracycline-dependent promoter. The amplicon (pE2) was stably maintained in multiple copies in the nuclei of 293 cells stably expressing the Epstein-Barr virus nuclear antigen 1 (EBNA1) and allowed replication as a linear DNA upon induction of E2 and ORF6 gene expression. A stable cell line (2E2) was generated by introducing pE2 into 293EBNA1 cells expressing the tetracycline-dependent transcriptional silencer and the reverse Tet transactivator (rtTA2). Upon induction with doxycycline, 2E2 cells produced higher levels of polymerase, precursor terminal protein (pTP), and DNA binding protein than noninduced 2E2 cells infected with first-generation Ad5 vector and supported efficient amplification of a multiply deleted Ad5 vector lacking E1, E2, E3, and E4 genes (Ad5 Δ E₁₋₄). The high cloning capacity of Ad5 Δ E₁₋₄ (up to 12.6 kb) was exploited to construct a vector encoding the entire hepatitis C virus (HCV) polyprotein. Infection of HeLa cells by the resulting vector showed high levels of correctly processed HCV proteins.

Adenoviruses (Ads) are characterized by broad tropism in that they are able to infect both quiescent and proliferating cells of a wide variety of tissues. This characteristic, together with ease of manipulation of the viral genome and propagation to high titers, makes Ad-based vectors particularly attractive for in vitro protein expression, as well as for in vivo applications of gene therapy and vaccination. Early region 1 (E1)-deleted first-generation (FG) Ad vectors are ideal recombinant viral vaccines due to their ability to elicit strong cell-mediated and humoral immunities (7, 23, 42, 43, 45–47). In contrast, use of FG Ad for gene therapy has been hampered by its short-term transgene expression as a result of leaky adenoviral structural-gene expression, leading to immune clearance of transduced cells (11, 33, 38, 45). To improve longevity of expression, new generations of Ad type 5 (Ad5)-based vectors have been constructed by introducing additional deletions of viral genes, resulting in further attenuation of the adenoviral gene expression in vivo. Vectors with E1, E2a/b, E3, and E4 deletions in different combinations displayed lower in vitro cytotoxicity and higher stability in vivo than classic FG Ad5 (4, 5, 21, 29). However, conclusive evidence that these second- and third-generation Ad vectors are capable of significantly prolonging gene expression in vivo is still missing. The systems presently available have low production efficiencies, presumably due to

suboptimal expression levels and timing of the complementing genes stably introduced in the packaging cell lines (5, 17, 29, 49).

Helper-dependent (HD) Ad vectors, which contain only the *cis*-acting DNA elements necessary for replication and packaging but lack all adenovirus genes, represent the most efficient and safe gene transfer vectors (14, 32, 35, 36, 37, 39). The current system for HD Ad vector production is based on three components: an E1 complementing cell line, the HD backbone, and a helper virus that provides in *trans* the whole repertoire of viral proteins required for replication and assembly of the HD progeny. This method inevitably leads to contamination of HD vector preparations with variable amounts of helper virus. Additionally, due to difficulties in optimizing the helper/HD ratio during the amplification cycles, production levels seldom reach those of FG vectors. A number of different approaches have been reported in the attempt to solve this problem, including use of a baculovirus-adenovirus hybrid to deliver the Ad helper functions (8), but in this case also, the system needs improvement to prevent the generation of replication-competent adenovirus.

An ideal solution to these problems would be to develop a helper cell line that would simplify production of high-titer HD vectors. Despite several attempts, efforts to construct such helper cell lines have failed so far. A major obstacle is the strong cytotoxic effect of adenoviral proteins resulting from the leaky gene expression observed with native viral promoters, such as the major late promoter. Additionally, it has proved

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extremely difficult to reproduce the right sequence of events to precisely coordinate viral DNA replication with expression of the structural proteins that lead to massive production of viral particles during the late phase of natural infection (12). Accordingly, the viral cycle cannot be mimicked by adopting strategies to express Ad early genes based on integration into the host cell chromosome of multiple Ad transcription units.

We have taken a different approach by generating an episomal plasmid capable of inducible adenovirus-like replication in the nucleus of the host cell. This amplicon (pE2) contains an Ad inverted terminal repeat (ITR) junction and all the genes necessary for adenoviral DNA replication (polymerase, preterminal protein [pTP], and DNA binding protein [DBP]) under tight transcription control of a tetracycline-dependent promoter. The cytotoxic effect of E2-E4 open reading frame 6 (ORF6) gene expression (19, 22, 24) was blocked by reducing the basal level of transcription of *tetO* with the constitutive expression of the Tet-dependent transcriptional silencer (tTS) in the cell line (15). An Epstein-Barr virus (EBV) origin of replication allowed stable pE2 maintenance in Epstein-Barr virus nuclear antigen 1 (EBNA1)-expressing 293 cells by virtue of the nuclear retention features of the EBV latent replication system (44). Upon induction of E2 gene expression, pE2 was replicated in the form of linear DNA using the Ad ITR junction as the replication origin, and as a result, large amounts of E2 viral proteins were expressed. This cellular system was capable of supporting the propagation of a novel adenoviral vector deleted of E1, E2, E3, and E4 (Ad5ΔE₁₋₄) to levels comparable to those of FG Ad. Ad5ΔE₁₋₄ was characterized by a cloning capacity of up to 12.5 kb and by a reduced leakiness of viral gene expression. An Ad5ΔE₁₋₄ vector expressing the entire hepatitis C virus (HCV) polyprotein was constructed and shown to direct efficient expression of correctly processed HCV proteins in vitro.

MATERIALS AND METHODS

Plasmid construction. The structure of pIREStet containing a Tet silencer and a reverse Tet transactivator expression cassette is described in Fig. 1a. The Tet system was combined in a single expression vector as follows. An EcoRI-ClaI DNA fragment containing the tTS was isolated from the plasmid pUHS6-1 (15) (kindly provided by H. Bujard) and inserted downstream of the internal ribosome entry site (IRES) sequence into the vector pIRES-Neo (Clontech), replacing the Neo gene. The new vector, pIRES-tTS, was modified with the insertion of a reverse Tet transactivator 2 (rtTA2) gene (from pUHD 52-1; kindly provided by H. Bujard) into the unique EcoRV restriction site downstream of the human cytomegalovirus (HCMV) immediate-early promoter, generating pIREStTS/rtTA. In order to introduce a selection marker to isolate cell clones stably expressing Tet proteins, a puromycin resistance expression cassette obtained from the pPUR vector (Clontech) was inserted in the XhoI site of pIREStTS/rtTA, generating pIREStTS/rtTApuro.

The structure of pE2 is described in Fig. 1c. More details of the plasmid constructions will be provided upon request. In brief, a dicistronic cassette expressing Ad5 polymerase and preterminal protein was constructed by inserting in the vector pBI (Clontech), under the control of *tetO*, the ClaI/SphI fragment obtained from plasmid pVAC-Pol, including Ad5 polymerase cDNA, and the Acc65/EcoRV fragment from pVAC-pTP containing Ad5-pTP cDNA (pVAC-Pol and pVAC-pTP were kindly provided by P. C. van der Vliet). A second bidirectional inducible cassette was constructed by inserting into the same vector, pBI, the Ad5 E4 ORF6 (Ad5 nucleotides [nt] 33190 to 34084) obtained by PCR with the oligonucleotides 5'-TTATACGCGTGCCACCATGACTACGTCG G-3' and 5'-TTATGCTAGCGCGAAGGAGAAGTCCACG-3', as well as the Ad5 DBP gene (Ad5 nt 22490 to 24079) obtained from pFG140 (18). The EBV OriP (EBV nt 7421 to 8042) region derived from pCEP4 (Invitrogen) flanked by HS4 insulators was obtained by cloning it into the BamHI site of pJC13-1 (9).

FIG. 1. (a and c) Structure of pIREStTS/rtTApuro and pE2 vectors and (b) control of luciferase expression in 293EBNA clones. (a) Schematic representation of plasmid pIREStTS/rtTApuro. The reverse Tet transactivator (rtTA), Tet transcriptional silencer (tTS), and puromycin resistance (Puro^R) elements are indicated by solid arrows. The HCMV promoter, ECMV IRES, and intron sequence (intS) are shown as grey, open, and stippled boxes, respectively. (b) Control of luciferase expression in 293EBNA clones infected with AdTetLuc. 293EBNA cells and different 293EBNA clones were infected with AdTetLuc (MOI, 10) in the presence (solid bars) or absence (empty bars) of 1 μ g/ml of doxycycline. Luciferase activity was evaluated 48 h postinfection in cell lysates. (c) Schematic representation of pE2 plasmid. A head-to-tail junction of Ad5 inverted terminal repeats derived from pFG140 was cloned in the plasmid (ITRs, grey arrowheads). Ad5 early genes are indicated by black arrows. Polymerase (Pol), preterminal protein (pTP), DBP, and E4orf6 were inserted into two bicistronic expression cassettes driven by Tet-responsive elements (TRE; white boxes); EBV latent origin of replication (OriP) and flanking chicken β -globin insulator sequences (HS4) are indicated by the dotted box and grey boxes. Expression of the hygromycin B phosphotransferase gene (Hyg^R; white arrow) is regulated by the thymidine kinase promoter and polyadenylation signal.

The Ad5 ITR junction was amplified by PCR from pFG140 using the oligonucleotides 5'-AACTACAATTCACACATAC-3' and 5'-CACATCCGTCGC TTACATG-3'. Finally, the thymidine kinase (tk)-hygromycin-B phosphotransferase cassette was derived from pCEP4 (Invitrogen). All the elements composing pE2 were sequentially transferred into the pBI-pol/pTP vector, finally generating pE2.

Construction of multiply deleted Ad backbone pAd5ΔE₁₋₄. An Ad5ΔE1-E3 backbone deleted of E2b genes was obtained by transferring the partial deletion of Ad5 polymerase (Ad5 nt 7274 to 7883) and preterminal protein (Ad5 nt 8919 to 9462) from the pAdCMV/LacZ/ΔPol vector (kindly provided by A. Amalfitano) and Ad5Δ308Δ_{pTP}-gα (kindly provided by J. Schaack) (41), respectively, into MRKpAd5E3- (48). Additionally, a site-specific mutagenesis of the polymerase start codon (ATG to CTG) was also performed, finally obtaining a pAd5 ΔE1, E3, E2b vector. pBluescript KSII(+) (Stratagene), which contains the BamHI/XhoI fragment of Ad5 (nt 21563 to 24797) deleted of the Drai-MscI fragment (Ad5 nt 22445 to 24029) comprising the DBP gene, was kindly provided by Rocco Savino.

The pAd-ΔE₁₋₂ vector was obtained by homologous recombination cotransforming the ΔDBP fragment and the AdΔE1, E3, E2b vector into *Escherichia coli* BJ5183. Deletion of all E4 units but ORF3 (nt 32931 to 34343 and nt 34895 to 35462) was performed as described below. The ORF3 region with AvrII and MfeI restriction sites at the termini was amplified by PCR (ΔE4orf3_fw_AvrII [5'-GCCTAGGGA TGCGTGTCTAATCAGTGTGGGTTC-3'] and ΔE4orf3_rew_MfeI [5'-CAAT TGAAAAGTGAGCGGGAAGAGCTGGAAAGACCATG-3']) and cloned in an E4 shuttle vector digested with the same enzymes. E4 ORF3 maintains E4 promoter and poly(A) signals. The pAd5ΔE₁₋₄orf3⁺ vector was obtained by cotransforming such DNA with the pAd5ΔE₁₋₂ vector in *E. coli* BJ5183.

Human and mouse CMV (MCMV) in expression cassettes were constructed in the context of an Ad5 shuttle vector that contains, in addition to CMV promoters and BGH poly(A) signal for transgene expression, the Ad5 sequences nt 1 to 450 (left) and nt 3511 to 5792 (right) to allow the insertion in the E1 region of pAd5ΔE₁₋₄orf3⁺ by homologous recombination in *E. coli* BJ5183 as described previously (48). Enhanced green fluorescent protein (EGFP) cDNA was obtained from the pEGFP plasmid (Clontech, BD Bioscience, San Jose, CA) and then cloned in an Ad shuttle plasmid, producing pShAd5 EGFP. The HCV-BK virus cDNA (HCV_BK nt 342 to 9374) deleted of 5' and 3' untranslated terminal repeats was derived from plasmid pCMV(1-9.4) (13).

The NS5B ORF was mutated at three amino acid positions corresponding to the catalytic triad of the viral RNA-dependent RNA polymerase (G-2737 to A, D-2738 to A, and D-2739 to G) to abolish enzymatic activity. The HCV cDNA fused to an optimized Kozak sequence was cloned in a modified version of the pAd5 shuttle obtained by substituting the HCMV promoter with the MCMV promoter, finally constructing pShAd5HCV. Insertion of all expression cassettes in the E1 region of pAd5ΔE₁₋₄orf3⁺ was obtained by homologous recombination in *E. coli* as described previously (39).

Cells. The 293EBNA cell line (Invitrogen) was cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), 2 mM glutamine, and 250 µg/ml G-418 (GIBCO BRL). 293EBNATet cells were selected by using the same medium with 0.5 µg/ml puromycin. To select 2E2 cells, 90 µg/ml of hygromycin B were added to the previously described medium. Plasmid DNA transfections were performed with Lipofectamine 2000 (Invitrogen) as described by the manufacturer. To obtain a 293EBNA clone expressing reverse Tet transactivator and Tet silencer proteins, 1 day prior to transfection, 1 × 10⁶ 293EBNA cells were seeded into 6-cm plates and transfected with 5 µg of SapI-linearized pIRES/tTA/puro; 48 h posttransfection, the cells were trypsinized and seeded into 15-cm plates in puromycin-containing DMEM. Resistant clones were isolated and subsequently screened with a recombinant Ad5 carrying a Tet-luciferase cassette. Cells (5 × 10⁵) of each clone were seeded in triplicate into 24-well plates and infected with Ad5 Tet-luc with a multiplicity of infection (MOI) of 10 with and without doxycycline. Twenty-four hours postinfection, cells were harvested and the luciferase activity was measured in the cell lysate (luciferase assay system; Promega). Both induction and silencing of gene expression were scored for each clone as a ratio with relative light unit values obtained in control experiments with parental 293EBNA cells.

To obtain a 2E2 packaging cell line, 293EBNATet cells were transfected with pE2 vector following the protocol described above. Stable transfectants were selected using DMEM containing 90 µg/ml of hygromycin B. Resistant clones were expanded and screened by transfection of an Ad5ΔE₁₋₂EGFP DNA. Positive clones were identified by cytopathic effect (CPE) appearance and confirmed by serial passaging of the Ad5ΔE₁₋₂EGFP vector.

Virus amplification and characterization. The production of the multiply deleted virus was carried out in a 2E2 packaging cell line. Adenovirus genomes

were released from the respective plasmids by PacI digestion and transfected in 2E2 cells in the presence of 1 µg/ml doxycycline. Four to 6 days posttransfection, the cells were lysed by three freeze-thaw cycles, and one-fifth of the lysate was used to amplify the virus by serial passaging. Large-scale amplification was performed by infecting 2E2 cells seeded into two-layer cell factories (NUNC). Adenoviral vectors were purified by CsCl gradients, dialyzed, and quantified by real-time PCR. The infectivity of the CsCl-purified vector was evaluated on 2E2 cells as the 50% tissue culture infectious dose (39).

Southern blot analysis. pE2 replication was evaluated by Southern blot analysis. 293EBNATet cells were seeded in 6-cm dishes and transfected by Lipofectamine 2000 (Invitrogen) with 5 µg of pE2 vector with or without doxycycline (1 µg/ml). Extrachromosomal DNA was isolated after 48 h by the Hirt method (20). Then, the DNA was digested with NotI and DpnI and subjected to Southern analysis according to standard procedures using a ³²P-labeled DNA probe. Signals were detected by autoradiography with the PhosphorImager system (Molecular Dynamics).

Episomal DNA from stable pE2 clones was extracted following the Hirt protocol, digested with BamHI, and analyzed by Southern blotting using ³²P-labeled pE2 DNA as a probe.

The genetic stability of the multiply deleted virus was evaluated by Southern blot analysis upon serial passages in 2E2 cells. Ad5ΔE₁₋₄HCV vector was propagated up to passage 14. The vector was amplified on 10⁸ 2E2 cells at passages 10, 12, and 14 and purified on a CsCl gradient, and the viral DNA was purified by proteinase K digestion and phenol-chloroform extraction. The purified DNA was digested with HindIII and subjected to Southern blot analysis according to the standard procedure by using as probes pAd5ΔE₁₋₄orf3⁺HCV and an XbaI-HindIII DNA fragment containing the E1 region obtained from pXC1 (kindly provided by F. Graham) labeled by using the Nick Translation kit (Promega).

Western blot analysis. Analysis of protein expression was performed 48 h posttransfection as follows. 2E2 cells were washed twice with phosphate-buffered saline and lysed by adding 0.5 ml of RIPA buffer (1× phosphate-buffered saline, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.05 mM phenylmethylsulfonyl fluoride) per 6-cm plate. The plates were incubated for 1 h on ice, and then the soluble proteins were collected from the cell lysates after centrifugation at 10,000 × g at 4°C. Western blot analysis was performed on 30 µg of proteins. Samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto Protan nitrocellulose membranes (Schleicher and Schuell). The membranes were incubated with rabbit antisera directed against polymerase or pTP and with anti-DBP monoclonal antibody (MAb) (clone H2-19, kindly provided by F. Graham, McMaster University, Hamilton, Canada). After incubation with horseradish peroxidase-conjugated secondary antibodies, proteins were detected by Supersignal West Pico chemiluminescent substrate (Pierce). HCV protein expression was detected by using the following reagents: anti-core MAb B12.F8 (kindly provided by M. Mondelli, University of Pavia), anti-E2 MAb 185.C7, anti-NS3 MAb 10E5/24, anti-NS5a rabbit polyclonal antiserum, and anti-NS5B MAb 20B6/13.

RESULTS

Development of a 293 cell line coexpressing the Tet-based transcriptional silencer and reverse transactivator factors. We aimed at generating a packaging cell line capable of complementing a multiply deleted adenovirus vector lacking all early genes. To this end, we used the Tet regulatory system to achieve tight control over expression of the complementing adenovirus E2 and E4 ORF6 genes. The combination of the Tet transcriptional silencer tTS (15) and of the improved version of the reverse Tet transactivator (26) was chosen to obtain very low basal expression while maintaining high levels of inducible transcription. The tTS was constructed by fusing the KRAB domain of the kidney protein Kid-1, which is a known transcriptional silencer, with the Tet repressor DNA binding domain. In the absence of the effector drug, tTS binds to the Tet operator sequence (*tetO*) and reduces the basal level of transcription (40), while rtTA does not bind. When tetracycline (or its analog, doxycycline) is added to the medium, the situation is reversed, leading to strong induction of the Tet-dependent promoter.

FIG. 2. Tet control of E2 protein expression and pE2 DNA replication. (a) Schematic structure of pE2 in circular and linear forms. The DNA segment used as a probe and NotI-digested fragments generated from circular and linear forms of pE2 are also indicated. (b) Southern blot analysis of pE2 plasmid and DNA extracted from 293EBNATet cells transfected with pE2. A total of 10^8 copies of NotI-digested pE2 were loaded in the first lane. Episomal DNA extracted from 293EBNATet cells 48 h after transfection with pE2 without (-) or with (+) doxycycline (dox) and digested with NotI and DpnI was loaded in lanes 2 and 3. The 12.6- and 4.4-kb bands, indicative of circular and linear monomeric forms, are indicated with black arrows. The sizes of DNA markers are indicated on the right. (c) Expression of DBP, pTP, and polymerase (Pol) was monitored by Western blotting on whole-cell extracts from 293EBNATet cells transfected with the pE2 amplicon with (+) or without (-) doxycycline (dox). E2 proteins were detected with specific antisera. Migration of molecular mass markers (kDa) is indicated on the left of the figure.

The tTS and rtTA genes were combined in a bicistronic transcription unit using the encephalomyocarditis virus (ECMV) IRES (Fig. 1a). The resulting plasmid (pIREStTS/rtTApuro) was transfected into 293EBNA cells, and stable clones were obtained by puromycin selection. To identify the cells with the lowest basal level of Tet-dependent transcription, several independent clones were expanded and screened for the ability to tightly regulate luciferase expression from a Tet-

inducible expression cassette delivered by infection of a first-generation Ad vector (Ad Tet-luc). A number of clones showed reduced basal levels of luciferase with respect to parental 293EBNA cells and >20-fold induction in the presence of doxycycline (Fig. 1b). Clone 1.1 (named 293EBNATet), which displayed 25-fold reduction in basal expression and the best induction ratio (>100-fold), was chosen for further studies.

FIG. 3. ΔE_{1-2} vector propagation in pE2 stable clones. pE2 stable clones were established in the 293EBNATet cell line by hygromycin B selection. The clones were screened by transfection of an Ad ΔE_{1-2} EGFP vector. Rescue of the vector (P₀) and amplification passages (P₁ to P₃) were performed in the presence of doxycycline (1 μ g/ml). Virus production was evaluated by real-time PCR in crude lysates. Results from clones that support vector propagation (continuous lines), as well as from negative clones (dashed lines), are shown.

Construction of an E2-E4 ORF6 amplicon. To functionally complement an Ad vector deleted of all early genes, we constructed an Ad5-based amplicon containing the following elements: (i) the latent origin of replication of EBV (OriP) for stable maintenance in the nuclei of dividing cells expressing the EBNA1 protein (44), (ii) the tk-hygromycin B selection marker, (iii) an Ad5 ITR junction derived from pFG140 (18) to allow plasmid replication in an Ad-based fashion, and (iv) the Ad5 E2 (polymerase, preterminal protein, and DNA binding protein) and E4 ORF6 genes arranged in two divergent transcriptional units under the control of bidirectional tetracycline-inducible promoters. Two chicken β -globin HS4 insulator dimers (9) flanking the OriP element were also introduced to reduce the enhancer effect of the OriP on the E2 and E4 ORF6 distal promoters (16). The structure of the resulting plasmid (pE2) is shown in Fig. 1c.

Since both *cis*- and *trans*-acting elements necessary for Ad replication are present in the above-described system, we tested whether induction of E2 gene expression would also trigger pE2 DNA replication in 293EBNATet-transfected cells. Plasmid replication was detected by Southern blotting 48 h posttransfection on total DNA. Since the restriction endonuclease DpnI cleaves only when its recognition site is *dam* methylated, in order to discriminate between *E. coli*-replicated input plasmid and DNA replicated in transfected cells, samples were digested first with DpnI to get rid of the input plasmid DNA. Samples were then digested with NotI to further differentiate between residual input circular plasmid or a circular form replicated via EBNA1-OriP and linear forms replicated via Ad ITRs (Fig. 2a). Blots were hybridized to a DNA probe derived from pE2, as indicated in Fig. 2a, and showed a plasmid-derived band of 12.6 kb for the circular form of pE2 (Fig.

2b, lane 1). When total DNA from pE2-transfected cells was analyzed, a band of the expected size for the replicated amplicon was visible only when the cells were induced with doxycycline (Fig. 2b, lanes 2 and 3). No evidence of DNA replication was detected from transfection of a pE2 plasmid derivative deleted of ITR junctions (data not shown).

Induction of E2 gene expression upon addition of doxycycline in the medium of pE2-transfected 293EBNATet cells was measured by Western blotting. As shown in Fig. 2c (lanes 2, 5, and 8), no protein expression was detected 48 h posttransfection in the absence of effector drug, while strong expression of all E2 proteins was evident when transcription was induced by adding 1 μ g/ml of doxycycline (lanes 3, 6, and 9). Similar results were obtained 4 and 6 days after transfection (data not shown).

These data indicated that the tTS-rtTA silencing/activation system allows the complete shutoff of pE2 functions even when the plasmid is present in high copy numbers after transient transfection but can support efficient replication of the amplicon in an adenovirus-specific fashion upon doxycycline induction of early gene expression. They also provided solid evidence in favor of the pE2 amplicon in combination with 293EBNATet cells as a suitable system for rescue and growth of adenovirus vectors deleted of the early genes.

Generation of E1-, E2-, E4-complementing cell line. 293EBNATet cells transfected with pE2 were selected in the presence of hygromycin B as described in Materials and Methods. Individual clones were expanded and screened for the ability to support rescue and propagation of an Ad5 vector carrying a deletion of E2 genes and an expression cassette for the enhanced green fluorescent protein (Ad5 ΔE_{1-2} EGFP). Positive clones were scored by direct observation of CPE at

FIG. 4. Structure of pE2 extracted from 2E2 clone and expression of E2 proteins. (a) DNA extracted following the Hirt method was digested with BamHI, separated on a 1% agarose gel, transferred onto a nylon membrane, and hybridized with pE2 DNA labeled with ^{32}P . pE2 vector was loaded in the first lane as a reference; DNAs extracted from 293EBNATet cells (negative control) and from a 2E2 clone were loaded in the second and third lanes, respectively. (b) E2a and E2b protein expression by a 2E2 clone was evaluated by Western blotting in the presence (+) and in the absence (–) of doxycycline (dox; 1 $\mu\text{g}/\text{ml}$) (lanes 2 and 3) and compared with the expression levels of E2 proteins from noninduced 2E2 cells infected with an MOI of 500 of FG Ad5 vector (lane 1). Migration of molecular mass markers (kDa) is indicated on the left. Pol, polymerase.

results indicated that no rearrangement occurred and the amplicon is stably maintained in episomal form in the cell lines in the presence of hygromycin B (data not shown).

Clone 2E2 was selected for further characterization and for amplification of multiply deleted adenovirus vectors. The structure of pE2 was evaluated by Southern blotting using the entire plasmid as a probe on extrachromosomal DNA extracted from 2E2 cells. Our experiment demonstrated that pE2 is stably maintained without detectable rearrangements in 2E2 cells cultivated in the presence of hygromycin B for over 15 passages (Fig. 4a). Efficient expression of E2 proteins upon doxycycline induction was confirmed after 15 passages of the 2E2 cell line and compared to E2 protein expression detected in 2E2 cells infected with an FG Ad5 vector in the absence of doxycycline.

As shown in Fig. 4b, upon induction, 2E2 cells express considerably higher levels of E2 proteins (lanes 3) than the same cells infected with 500 particles per cell of an FG vector (lanes 1), whereas no protein expression was detected in the absence of induction (lanes 2).

Construction of a multiply deleted Ad5 backbone. A large-capacity Ad5-based vector was generated by deleting the early genes. A schematic representation of this multiply deleted vector backbone is shown in Fig. 5. Besides the classical deletion of the E1 and E3 regions (reviewed in reference 10), we removed the entire coding sequence of the DNA binding protein (nt 22445 to 24029; 1,584-bp deletion) without affecting the other functions encoded in the r-strand, which encompasses the L4 intron. Partial deletion of polymerase (nt 7274 to 7883; 609-bp deletion) and preterminal protein (nt 8919 to 9462; 543-bp deletion) genes corresponding to the introns of the tripartite leader sequence and major late units were introduced to knock out E2b gene expression. Furthermore, to prevent a truncated nonactive form of polymerase being produced, the ATG start codon was mutated to CTG. The E4 region was totally deleted (nt 32931 to 34343 and 34895 to 35462; 1,979-bp deletion) with the exception of ORF3, which was directly fused to the E4 promoter. Retention of open reading frame 3 is required for persistent expression in vivo and in vitro of transgenes regulated by an internal CMV promoter (17, 30). Thus, in addition to the 5,700-bp deletion of a standard Δ E1E3 FG vector, we deleted 4,715 bp, for a total deletion of 10,415 bp. Thus, given the estimated packaging capacity for genomes corresponding to 105% of the wild type (wt) (6), the new Ad5 Δ E₁₋₄orf3⁺ viral vector should be able to accommodate transgenes up to 12.4 kb.

A shuttle vector containing the left ITR and the packaging signal (Ad5 nt 1 to 452), as well as a pIX fragment (Ad5 nt 3511 to 5792) flanking the expression cassette, was created in order to facilitate the vector construction.

To evaluate the growth properties and transduction efficiency of the new vector system, pAd5 Δ E₁₋₄orf3⁺EGFP was constructed, transfected into 2E2 cells, and incubated with or without doxycycline. EGFP-transducing viral particles (vp), as well as CPE, were produced only when E2 and E4 ORF6 genes were induced by doxycycline. No viral particles were generated in 293EBNATet cells due to the lack of complementing genes. Vector production reached a plateau after two serial passages, when 100% of the cells were EGFP positive (data not shown). 2E2 cells were able to support growth and amplification of the

FIG. 5. Schematic representation of multiply deleted adenoviral vector and structural characterization of Ad5ΔE₁₋₄orf3⁺HCV vector. (a) Schematic map of Ad5. Deleted regions are indicated in the diagram by grey boxes. E1, E2a, E3, and six of the seven E4 ORFs, with the exception of ORF3, were completely deleted from the vector backbone. Partial deletions of polymerase and preterminal protein were also introduced. Genes close to the deleted regions are represented by white arrows. The positions of HindIII restriction sites are indicated by vertical bars. (b) Schematic representation of the Ad5ΔE₁₋₄orf3⁺HCV vector showing the HCV polyprotein expression cassette introduced in the E1 region of the vector and the coordinates of the HindIII sites. (c and d) Southern blot analysis of Ad5ΔE₁₋₄orf3⁺HCV. Viral DNA extracted from CsCl-purified viral particles and plasmid DNAs were digested with HindIII, separated by electrophoresis on 1% agarose gels, transferred to nylon membranes, and hybridized either with ³²P-labeled Ad5ΔE₁₋₄orf3⁺HCV preAd plasmid (c) or with a ³²P-labeled XbaI-HindIII (nt 1340 to 2805) Ad5 fragment derived from the E1 region (d). HindIII-digested Ad5 wt (500, 50, 5, and 0.5 pg) was loaded in lanes 1 to 4; 1 μg of HindIII-digested Ad5ΔE₁₋₄orf3⁺HCV vector purified at passages 10, 12, and 14 was loaded in lanes 5 to 7; 1 μg of Ad5ΔE₁₋₄orf3⁺HCV was loaded in lanes 8 (c and d). (c) The 2-kb plasmid band is indicated by *. (d) A 2.8-kb HindIII fragment containing the left end of the viral genome is indicated by the arrow. No structural rearrangements or E1-specific signals were detected in the lanes loaded with the multiply deleted vector DNA.

pAd5ΔE₁₋₄orf3⁺EGFP vector to high titers (2×10^{12} vp/ml) with a productivity of 5,000 vp/cell, corresponding to about half the production efficiency of FG Ad vectors (Table 1).

Construction and characterization of a ΔE₁₋₄Ad vector expressing the entire HCV polyprotein. The large capacity of the new vector system was exploited to insert an expression cas-

sette for the entire HCV polyprotein gene fused to the MCMV promoter. The HCV polyprotein expression cassette was constructed by eliminating the 5' and 3' untranslated regions and by inserting an optimal Kozak sequence upstream of the start codon. Mutation of the catalytic domain of NS5B replicase was introduced to eliminate its enzymatic activity (28). In order to

TABLE 1. Productivity of Ad5 vectors carrying different deletions^a

Deletion	Transgene	Insert size (kb)	Yield (vp/cell)
E1-E3	EGFP	1.8	1.0×10^4
E1-E2-E3	EGFP	1.8	5.9×10^3
E1-E2-E3-E4orf3 ⁺	EGFP	1.8	5.0×10^3
E1-E2-E3-E4orf3 ⁺	HCV	10.5	4.9×10^3

^a Titers were evaluated by real-time PCR on CsCl-purified vector stocks.

increase the efficiency of transgene expression, we substituted the human CMV promoter with a mouse CMV promoter that was reported to be 4- to 30-fold more potent in FG adenoviral vectors (1).

The Ad5ΔE₁₋₄orf3⁺HCV vector was successfully rescued by transfection and propagated by serial passages in 2E2 cells. Amplification was monitored by evaluating Ad genome concentrations in crude cell lysates by real-time PCR as described in Materials and Methods. To obtain a large-scale preparation, 2.8×10^9 2E2 cells were infected with an MOI of about 100 genomes/cell using a crude lysate obtained after four amplification cycles. Cells were harvested 48 h postinfection when a full CPE was clearly evident. The final yield of purified virus was 6.2×10^{12} physical particles/ml. Also in this case, productivity was comparable to that of FG Ad vectors (5,000 particles per cell) (Table 1).

Since the deletions introduced in the polymerase and pre-terminal protein involved only portions of both genes, the homology between the Ad vector and pE2 episome are theoretically sufficient to rescue the wild-type genes back into the viral genome. In addition, the large regions of homology between Ad5 DNA fragments integrated into 293 cells and the vector DNA flanking the E1 deletion can lead to E1 region reversion and transgene loss upon amplification in the 293-derived 2E2 cells. In Fig. 5c is shown the Southern blot analysis of the HindIII restriction pattern of Ad5 wt DNA (lanes 1 to 4) compared to the vector DNA extracted at passages 10, 12, and 14 and digested with the same restriction enzyme (lanes 5 to 7). The pre-Ad5ΔE₁₋₄orf3⁺HCV plasmid DNA was also included in the experiment (lane 8). As indicated by Fig. 5c (lanes 1 to 4), our detection limit is on the order of 5 to 50 pg of Ad5 wt, corresponding to 10^5 to 10^6 viral genomes; however, the restriction pattern of Ad5ΔE₁₋₄orf3⁺HCV viral DNA evaluated up to passage 14 is correct and identical to that of the original pre-Ad plasmid, indicating that the concentration of a possible mutant virus generated by homologous recombination remains below our threshold. More specifically, we assessed the genetic stability of the E1 region by Southern blotting using as a probe the XbaI-HindIII Ad5 fragment (nt 1340 to 2805) that was deleted in the Ad5ΔE₁₋₄orf3⁺ vector. As shown in Fig. 5d, we can clearly detect as little as 5 pg of Ad5 wt DNA, corresponding to 1×10^5 copies of the viral genome. However, no signals were observed in lanes 5 to 7 containing Ad5ΔE₁₋₄orf3⁺HCV vector DNA, indicating that, at least up to passage 14, an E1-positive species was not amplified by serial passage of the HCV vector.

The efficiency of expression and correct processing of HCV proteins were evaluated in vitro by infecting 293 and HeLa cell lines using different vector MOIs. Western blot analysis with specific monoclonal antibodies or polyclonal antisera against

HCV core, E1, E2, NS3, NS4, NS5a, and NS5B demonstrated the presence of HCV proteins in the infected cells, indicating correct processing of the HCV polyprotein (Fig. 6).

DISCUSSION

Despite the great potential of Ad vectors for gene therapy and vaccination purposes, their possible applications have been hampered as a consequence of several problems limiting their overall in vivo efficacy (11, 31, 33, 38, 45). To date, several vector systems based on Ad5 have been proposed with the aim of improving the safety profile of FG vectors and increasing their cloning capacities. Ad5 helper-dependent vectors devoid of all viral genes are considered one of the most efficient and safe vectors for in vivo gene transfer (14, 25, 32, 35, 37, 39). However, propagation of HD Ad vectors is based on coinfection of 293 cells with the vector plus a helper virus and is not readily amenable to scaling up. In addition, HD Ad vector preparations invariably display some level of contamination with helper virus. Helper-independent second- and third-generation Ad vectors have been constructed by deleting some of the E2 genes and/or the E4 region or by combining deletions of different early genes (2-4, 17, 21, 27, 29, 34, 41).

Development of new vector systems based on the deletion of adenoviral genes from the vector backbone requires the concomitant generation of suitable packaging cell lines. This has been attempted by stable introduction of the complementing genes into the host cell chromosomes. However, chromosomal integration of the viral genes in low copy numbers proved inefficient when multiple deletions had to be complemented. Andrews et al. (5) found that such an approach failed to support production of high titers of a vector deleted of E1, E2a, E3, and E4 regions. Zhou and Beaudet (49) demonstrated that multiple integrated copies of the DBP gene are necessary to achieve efficient amplification of an E1-E2a-deleted vector with titers approaching those usually reached with FG vectors.

Here, we propose a novel multiply deleted adenovirus vector and packaging cell line system that incorporates new features

FIG. 6. Expression of HCV proteins in Ad5ΔE₁₋₄HCV-infected cells. HeLa cells were infected with Ad5ΔE₁₋₄HCV at an MOI of 10. HCV proteins were detected in cell extracts by Western blot analysis with HCV-specific antibodies. Lysates from infected HeLa cells, prepared 48 h postinfection, were loaded in lanes 3. Lanes 1, lysate from uninfected control cells; lanes 2, lysate from HeLa cells transfected with MCMV-HCV vector DNA. Specific bands are indicated by arrowheads.

and established technologies to produce a large-capacity Ad5 vector. In the design of an efficient production cell line capable of complementing an Ad5 vector carrying multiple deletions, we introduced the complementing Ad5 viral genes in an EBV-based episome, pE2, containing the EBV latent origin of replication, as well as the adenovirus origins of replication. The presence of EBNA1 in combination with the OriP allowed autonomous replication and nuclear retention of pE2 as a stable episome replicating only once every cell cycle (44). Coding sequences for Ad5 polymerase, pTP, and DBP factors, which are required for adenovirus DNA replication, and for E4 ORF6 were arranged into two dicistronic transcription units under the dual control of the Tet transcriptional silencer and reverse transactivator. Thus, when transcriptionally silenced, the Ad-EBV episome was stably maintained as a latent genetic element. However, pE2 stability in the 2E2 cell line was assessed only in the presence of hygromycin B, and we cannot exclude the possibility that a progressive loss of the episome can occur in the absence of selection. We were able to induce the replicative phase of the episome, as demonstrated by accumulation of linear DNA upon doxycycline induction of E2 gene expression. We observed that the 2E2 cell line expresses levels of E2a and E2b proteins higher than those in noninduced 2E2 cells infected by FG vectors (Fig. 4b). Consistently, high yields of multiply deleted vector particles per cell were produced, to a level comparable to those observed with FG vectors.

It seems likely that the selection for nondefective variants which can replicate more efficiently than the main vector population is very strong during serial propagation. However, in spite of the theoretical possibility that Pol and pTP genes could be rescued in the Ad5 Δ E₁₋₄orf3⁺HCV backbone by homologous recombination, no variants containing the E2 wt genes were detected to passage 14. The observed high expression levels of complementing proteins possibly reduced the selective advantage of an E2b wild-type virus over the multiply deleted vector. In addition, since the structure of the E1 region present in the multiply deleted vector is identical to that of FG vectors, it seems likely that E1 reversion would occur at the same rate as with FG vectors upon vector amplification in 293 cells. Nevertheless, while E1 reversion with FG vectors leads to increasing titers of replication-competent adenovirus during multiple passages, we did not observe a strong positive selection for E1⁺ variants generated in the context of a viral backbone carrying multiple deletions of the essential E2 genes.

The high cloning capacity of the Ad5 Δ E₁₋₄orf3⁺ backbone (about 12.4 kb) was exploited to construct an Ad5 vector expressing the entire HCV cDNA. The resulting vector was stable over several amplification cycles and directed expression of high levels of polyprotein precursor, eventually leading to mature products through correct processing by host and polyprotein-encoded proteases, as demonstrated by Western blot analysis of infected cells. Experiments with mice and nonhuman primates are in progress to characterize the immune response elicited by the Ad5 Δ E₁₋₄HCV vector administration.

In conclusion, the system described in this work offers the potential for developing a new strategy to produce efficient packaging cell lines for helper-independent adenovirus vectors. The Ad-EBV amplicon provides an important contribution to the production of helper cell lines able to propagate at

high-titer adenovirus vectors incorporating deletions of multiple genes. We believe that the strategy described above represents a step toward the construction of packaging helper cell lines for fully deleted Ad vectors.

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