INTRODUCTION

While replication-deficient adeno-virus (Ad) vectors are used extensively as gene delivery vehicles in the research environment to analyze gene function, they are increasingly being adopted in large scale studies of gene function (1), drug discovery (www5.gelifesciences.com/aptrix/upp00919.nsf/Content/viralvectors_homepage), and as a delivery platform for RNAi (2). Clinically, Ad type 5 is the most commonly used vector in human gene therapy protocols. The human adenovirus 5 (Ad5) genome (∼36 kb) comprises a linear dsDNA, bracketed by inverted repeats. Genes are grouped into four early transcription units, numbered E1, E2, E3, and E4, as well as delayed early units and a major late unit (3). Ad vectors are rendered replication-deficient by deleting the essential E1 gene region, and must therefore be propagated on helper cells expressing E1 functions (4,5). The E3 region is nonessential for replication in vitro, and its additional excision allows the insertion of transgenes up to ∼8 kb. Additional sequences can be deleted from the Ad genomic backbone; however, this renders the vector substantially more difficult to produce and use in routine in vitro applications.

The Ad genome can readily be cloned and manipulated in Escherichia coli. A wide range of popular vector systems have been developed that are based on recombination between prokaryotic transfer vectors and the Ad genomic backbone in E. coli, or following plasmid co-transfection into E1-expressing cell lines (e.g., 293 or 911 cells) (6–9). Nevertheless, the procedure for generating Ad recombinants still remains relatively labor-intensive and poorly suited to cloning multiple genes simultaneously: sequential sub-cloning steps are required to configure transgenes into the transfer vector, while the recombination step required to insert the transgene into the Ad genomic backbone can be problematic. Finally, the recombinant virus will not be generated if transgene expression is not compatible with vector replication. We were motivated to radically redesign the Ad vector to overcome these shortcomings. To this end we have developed a vector compatible with recombineering technology.

The novel vector system, herein designated AdZ (Ad with zero cloning steps), utilizes recombination-mediated genetic engineering (recombineering) to allow for direct insertion into the Ad vector of PCR products, synthesized sequences, or oligonucleotides encoding shRNAs without requirement for a transfer vector. Vectors were optimized for high-throughput applications by making them “self-excising” through incorporating the I-SceI homing endonuclease into the vector, removing the need to linearize vectors prior to transfection into packaging cells. AdZ vectors allow genes to be expressed in their native form or with strep, V5, or GFP tags. Insertion of tetracycline operators downstream of the human cytomegalovirus major immediate early (HCMV MIE) promoter permits silencing of transgenes in helper cells expressing the tet repressor, thus making the vector compatible with the cloning of toxic gene products. The AdZ vector system is robust, straightforward, and suited to both sporadic and high-throughput applications.

With the enhanced capacity of bioinformatics to interrogate extensive banks of sequence data, more efficient technologies are needed to test gene function predictions. Replication-deficient recombinant adenovirus (Ad) vectors are widely used in expression analysis since they provide for extremely efficient expression of transgenes in a wide range of cell types. To facilitate rapid, high-throughput generation of recombinant viruses, we have re-engineered an adenovirus vector (designated AdZ) to allow single-step, directional gene insertion using recombineering technology. Recombineering allows for direct insertion into the Ad vector of PCR products, synthesized sequences, or oligonucleotides encoding shRNAs without requirement for a transfer vector. Vectors were optimized for high-throughput applications by making them “self-excising” through incorporating the I-SceI homing endonuclease into the vector, removing the need to linearize vectors prior to transfection into packaging cells. AdZ vectors allow genes to be expressed in their native form or with strep, V5, or GFP tags. Insertion of tetracycline operators downstream of the human cytomegalovirus major immediate early (HCMV MIE) promoter permits silencing of transgenes in helper cells expressing the tet repressor, thus making the vector compatible with the cloning of toxic gene products. The AdZ vector system is robust, straightforward, and suited to both sporadic and high-throughput applications.
double-stranded oligonucleotides or custom synthesized genes such that the transgene is inserted directly and in the desired orientation into the vector. The DNA element encoding the transgene is introduced by electroporation into recombineering bacteria that already carrying a bacterial artificial chromosome (BAC) containing the AdZ vector. The AdZ vector was further optimized to provide an extremely robust technology suited to the simultaneous construction of large numbers of recombinant Ads (RAds).

Full protocols for the use of the AdZ system are provided on our web site (AdZ.cf.ac.uk).

MATERIALS AND METHODS

Cells

Cells were grown in DMEM (Gibco, Paisley, UK) containing 10% FCS. Ad recombinants were propagated in 911, 293, or 293TREx cells (Invitrogen, Paisley, UK) that express the tet repressor (4,13). HFFF-htert-hCar are human fetal foreskin fibroblasts (HFFs) that have been immortalized using htert (14), and engineered to express the human Coxsackie-adenovirus receptor (hCAR) using retrovirus derived from LX5N-hCAR (15), provided by J. DeGregori (University of Colorado Health Sciences Center, Denver, USA).

PCR

PCR products were amplified using the Expand Hi-Fi PCR system (Roche Applied Science, East Sussex, UK) according to manufacturer’s instructions with 100 pmol of each primer (desalted purity) (Invitrogen). PCR products were gel purified with GFX DNA purification kit (GE Healthcare, Bucks, UK) and eluted in 30 μl ddH2O.

Plasmids

Plasmid pGalK (11) was supplied by N. Copeland (National Cancer Institute, Frederick, MD, USA) and expresses GalK. Plasmid pBeloBAC11 (New England Biolabs, Ipswitch, MA, USA) is a single copy vector expressing chloramphenicol resistance. The LoxP and cos sites were removed by digesting with HpaI and ApaLI, overhanging sequences filled in with the Klenow polymerase and religated to generate pAL767. pMV100 contains the strain AD169 HCMV MIE promoter (-299 to +69) and polyadenylation sequence from the same gene (+2757 to +3053) (16). pCAG- l-SceI was a gift from M. Jasin (Memorial Sloan-Kettering Cancer Center, New York, NY, USA) and expresses the l-SceI ORF with NLS and HA tag (17). pShuttle and pAdEasy-1 are components of the AdEasy vector construction system and were obtained from B. Vogelstein (Johns Hopkins University, Baltimore, MD, USA) (18).

Recombineering

Recombineering was performed essentially as described in Reference 11. Unless otherwise stated, E. coli was grown at 32°C. Briefly, E. coli SW102 (11) cultures were grown overnight in LB with appropriate antibiotics. 1 mL of culture was inoculated into 50 mL LB and grown to OD600 = 0.6. Expression of λ phage Red genes was induced by incubating for 15 min at 42°C. Cells were cooled on ice (15 min), and then washed twice in 25 mL of ice-cold ddH2O and resuspended in 400 μL of ice-cold ddH2O. DNA (PCR product or synthesized sequence) was added, and cells were electroporated in 0.2 cm cuvettes (BioRad, Herts, UK) at 2.5 kV. For positive selection, 1 mL LB was added and cells recovered for 1 h before being plated on selective media. For negative selection, 5 mL LB was added and cells recovered for 4.5 h before being plated on selective media.

For positive selection using the sacBlacZamp cassette (see section “Generating the recombineering targeting cassette”) cells were plated into LB agar (Melford, Ipswich, UK) containing 12.5 μg/mL chloramphenicol (Sigma, Dorset, UK), 50 μg/mL ampicillin (Sigma), 80 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Melford), and 200 μM isopropyl-
β-D-thiogalactopyranoside (IPTG) (Melford). For negative selection against the same cassette, cells were plated on LB agar containing 12.5 μg/mL chloramphenicol, 80 μg/mL X-Gal, 200 μM IPTG and 5% (w/v) sucrose (Sigma). E. coli expressing sacB are efficiently inhibited by sucrose in media lacking salt (19); LB for negative selection was therefore made with 10 g/L tryptone (Sigma) and 5 g/L yeast extract (Sigma) only.

Generating the recombineering targeting cassette

All restriction enzymes were purchased from New England Biolabs. To generate a targeting construct for recombineering, a cassette encoding sacB, lacZα and ampicillin resistance was constructed. Plasmid pEL04 encoding sacB and chloramphenicol resistance was provided by N. Copeland (10). lacZα was amplified using primers LacZaF (GGCC AGATCT AGCGCCCAA TACGCAAACCG, BglII site underlined), and LacZaR (GGCC CCCGGGTTAATGCGCCGCTA CAGGCC, XmaI site underlined) cut with XmaI and BglII and cloned into XmaI/BamHI digested pEL04, generating pAL814 expressing sacB, lacZα and chloramphenicol resistance. Finally chloramphenicol resistance was switched for ampicillin resistance using recombineering. The ampicillin resistance cassette was amplified using primers AmpF (ATGCGACTCCTGCAC CTTCTCGTCTTCGAAAT AAATACCTGTGACGG AACGATACTTCG AGTACTGAGAGTCGAC CATAG, arms of homology underlined) and AmpR (ATCACTTATTCAGGCGTAGCAACC AGGCGTTTAAGGGCCA CCAATAACTGCCTTA AAAAGCTCAGTGG AACGAAAACTC, arms of homology underlined). The ampicillin cassette was recombineered into pAL814 and cells selected on ampicillin, generating pAL820. Plasmid DNA was isolated (Qiagen Spin miniprep kit; Qiagen, West Sussex, UK), retransformed into SW102 bacteria and reselected on ampicillin to generate pure plasmid stocks.

Generating an Ad5 BAC vector

Recombineering works most effectively on single copy vectors. In order to generate the Ad5 vector, the complete Ad5 genome from the AdEasy cloning system (18) was moved from its original vector into pAL767. In addition, the CMV promoter and polyadenylation sequence from pMV100 were inserted in place of the E1 region.

![Diagram of Ad5 BAC vector](image.png)

**Figure 2. The range of AdZ vectors generated.** (A) Plasmid numbers, characteristics of the vector (self-excising and/or whether expression of transgenes can be repressed by expression of tet operators) are given along with the arrangement of the expression cassette in each vector. Layout of the various tags that can be attached to cloned genes are depicted. (B) Diagram of a complete AdZ plasmid. Scale diagram of pAL1112 showing features common to all AdZ vectors, including the self-excision system (I-SceI expression cassette and restriction sites; see section “A self-excising system”), tet-restricted CMV promoter (see section “Conditional expression of genes”) and polyadenylation sequence with the sacB/lacZ selectable marker and epitope tag. Adenovirus genome backbone numbering is taken from GenBank sequence AC_000008.
In order to do this the CMV promoter and polyadenylation site were first amplified from pMV100 using primers pMV100F (TAGCGCCGCCGCAAG GTTGGACCTGCGGCTGCTG, NotI site undelined) and pMV100R (TAGCGATCTAAAGCTGGGGCT GCAGGTCGGG, BglII site underlined) and cloned into pShuttle on a BglII/NotI fragment. pShuttle was recombined with pAdEasy-1 in E. coli BJ5183 as previously described (18), generating pAL787 containing an E1/E3-deleted Ad5 genome with the HCMV promoter and polyadenylation signal from pMV100 inserted in place of the E1 region. The viral genome was then transferred into pAL767 by homologous recombination as previously described (20). The ends of the viral genome were first amplified using primers AdTerm (GGGCGGATCCCTTGGAAAT TAGAATTAATAGACATTCA AATAATACCTTATGG, restriction sites for BamHI, BstBI, SwaI, and PacI underlined) and Ad5Left (GGGCGCGATCCCTTGGAAAT TAGAATTAATAGACATTCA AATAATACCTTATGG, restriction sites for BamHI, BstBI, SwaI, and PacI underlined) and either Ad5Right (GGGCGGATCCCTTGGAAAT TAGAATTAATAGACATTCA AATAATACCTTATGG, restriction sites for BamHI, BstBI, SwaI, and PacI underlined) or Ad5Left (GGGCGGATCCCTTGGAAAT TAGAATTAATAGACATTCA AATAATACCTTATGG, restriction sites for BamHI, BstBI, SwaI, and PacI underlined) to generate two arms of homology. The two PCR products were digested with NheI and ligated, and then the purified ligated product was excised with BamHI and cloned into pAL767, generating pAL838 containing the two ends of the genome (to act as regions of homology) with a unique NheI site between them.

pAL838 was digested with NheI, linearizing it in between the arms of homology, and co-transfected with PacI-digested pAL787 into induced E. coli SW102, with colonies being selected on media containing chloramphenicol. pAL840 contains the AdEasy (E1/E3-) Ad5 genome with HCMV promoter and polyadenylation sequence in a single copy vector. Plasmids were named with a pAL prefix followed by a number, and RAd5 (see section “Generating RAds”) were named with an RAd prefix followed by the cognate number.

Inserting tags into the Ad vectors

The sacB/lacZ/amp\(^\prime\) cassette was inserted in between the HCMV promoter and polyadenylation site of pAL840 along with various tags as follows. Primers SacBF-Strep-CMV and SacBR-pA (Supplementary Table 1, available at www.BioTechniques.com) amplified the sacB/lacZ/amp\(^\prime\) cassette with a ribosomal entry site and strep-2 tag at the 5’ end (21,22) generating pAL848. Primers SacBF-CMV and SacBR-Strep-pA (Supplementary Table 1) amplified the sacB/lacZ/amp\(^\prime\) cassette with a strep-2 tag at the 3’ end generating pAL841. The GFP ORF was then inserted into the HCMV promoter between the PacI sites in order to recover virus, plasmids were cut with PacI and linearized using primers SacBF-SacBR-eGFP (Supplementary Table 1) and recombined into pAL841, generating pAL854. The sacB/lacZ/amp\(^\prime\) cassette was then re-inserted at either the 5’ (primers SacBF and SacBR-eGFP) or 3’ (primers SacBF-eGFP and SacBR-pA) of the GFP ORF generating pAL862 and 863, respectively. To generate a vector containing a strep-3 tag (21) oligonucleotides Strep3F and Strep3R were used as template in a PCR, using primers Strep3F-CMV and Strep3R-pA (Supplementary Table 1), and the product was recombined into pAL841. The sacB/lacZ/amp\(^\prime\) cassette was then amplified using primers SacBF-CMV and SacBR-Strep-pA (Supplementary Table 1) and reinserted upstream of the strep-3 tag to generate pAL878.

RAds encoding HCMV UL120 or human herpes virus 7 (HHV-7) IE1 were generated by amplifying the respective ORFs (primers in Supplementary Table 2, available at www.BioTechniques.com) and recombineering into pAL878 to generate pAL925 and pAL943, respectively. RA941 was made using the AdEasy system (18) by cloning the eGFP ORF from pEGFP-N1 (Clontech) on a KpnI/NotI fragment into pShuttle-CMV to generate pAL931, which was then recombined with pAdEasy-1 to generate pAL941.

Inserting tet operators into the HCMV MIE promoter

For several constructs, tet operators were inserted into the HCMV promoter by recombineering. The sacB/lacZ/amp\(^\prime\) cassette was amplified using primers SacBF-tet-CMV and SacBR-tet-pA (Supplementary Table 1) and recombined into the HCMV promoter. The cassette was then replaced by recombineering using annealed oligos TetF and TetR (Supplementary Table 1), leaving behind two tet operators.

Rendering AdZ vectors self-excising

In order to make the vector self-excising, I-SceI sites were inserted in between the PacI sites and the termini of the Ad5 genome in pAL840 by recombineering, following which an expression cassette expressing I-SceI from the RSV promoter and SV40 polyadenylation signal was inserted into the prokaryotic vector backbone, again by recombineering (see Supplementary Table 3, available at www.BioTechniques.com).

Generating RAd5s

In order to recover virus, plasmids were purified from 250 mL overnight culture (BacMax 100 kit; Macherey-Nagel, Duren, Germany). This allowed the isolation of ∼25 μg DNA. Where necessary plasmids were cut with PacI (New England Biolabs) and then precipitated before being transfected into 293 or 293TREx cells in a 6-well plate using Effectene (Qiagen), requiring only 1 μg DNA. Virus was extracted from infected
cells using an equal volume of Arklone-P (Sigma) and titered in 293TREx cells. Where plaques were counted, cells were overlayed with 1% AgarPlaque Plus (BD Biosciences, Oxford, UK) 72 h post transfection.

**Re-deriving the Ad5 genome**

In order to generate a vector system with a fully-defined Ad5 sequence, a wild-type Ad5 genome (donated by Vivien Mautner, University of Birmingham, Birmingham, UK) was also cloned into the self-excising BAC vector by homologous recombination, sequenced, the E3 region deleted by homologous recombination, and identical expression cassettes inserted, as described in “Inserting tags into the Ad vectors.”

**Sequencing**

All cloned PCR products were sequenced in an ABI Prism 3130 genetic analyzer (Applied Biosystems, Warrington, UK). Cycle sequencing was performed according to manufacturer’s instructions, with the exception that 100 cycles were used instead of 25. Sequencing reactions were purified using Performa DTR columns (Edge Biosystems, Gaithersburg, MD, USA) and sequence verification was performed with Vector NTI software (Invitrogen).

**Immunocchemistry**

SDS-PAGE was performed as previously described (23) using anti-GFP antibody (clone B-2; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-mouse HRP (GE Healthcare). For flow cytometry, cells were fixed with 2% paraformaldehyde and imaged using FACS Caliber (Becton Dickinson, Oxford, UK).

**RESULTS**

**Vector construction**

The prototype AdZ BAC plasmid (pAL840) contains an E1/E3-deleted Ad5 viral genome, with an expression cassette in place of the E1 region. The expression cassette consists of a truncated HCMV MIE promoter in combination with the polyadenylation signal from the same gene (24). PacI, SwaI, and BsrBI sites (each absent from the Ad5 genome) at the junction of the viral genome and the plasmid backbone allow the vector genome to be excised by restriction endonuclease digestion prior to transfection into helper 293 cells (25,26).

To allow transgenes to be inserted directly into the vector it was necessary...
to incorporate a selectable marker. A prokaryotic expression cassette comprising \textit{ sacB}, \textit{lacZ}a, and \textit{amp}r and a strep-2 tag was recombineered in between the eukaryotic HCMV promoter and polyadenylation sequence to generate pAL841. Genes to be cloned into the vector were either synthesized de novo or PCR amplified with 50–80 bases of homology to the HCMV MIE promoter at the start of the forward primer and to the polyadenylation sequence at the end of the reverse primer. Thus, transgenes are always inserted in the desired orientation. Linear dsDNAs were transformed into \textit{ E. coli} SW102 containing pAL841, the $\lambda$ Red genes were transiently induced by temperature shift, and following homologous recombination the transgene replaced the selectable markers (\textit{sacB}/\textit{lacZ}/\textit{lacZ}/\textit{lacZ}/\textit{amp}/\textit{amp}). In the presence of 5% sucrose, \textit{sacB} expression is lethal to gram-negative bacteria and thus the marker allows selection against the parental vector. The second marker (\textit{lacZ}a) could differentiate recombinant colonies from a background of false positives arising from spontaneous mutations in \textit{sacB} (19). Thus, when the desired recombination event occurs, the \textit{sacB}/\textit{lacZ}a/\textit{amp} cassette is replaced by the transgene resulting in readily identifiable white colonies (Figure 1A). Using this system to construct >100 viruses, every construct analyzed had the desired structure. Through the use of a proof-reading Taq DNA polymerase mix (see Materials and Methods), yields of PCR products are high and errors are rare, even with the very long (100 bp) primers needed to amplify genes with the arms of homology. It was seldom necessary to sequence more than one clone.

To assess the efficiency of the cloning system, 100 ng or 10 ng of a short HCMV ORF (UL120, 0.6 kb) and equimolar amounts of a longer ORF (U90/IE-1, 3.7 kb) from human herpes virus-7 (HHV-7) were recombineered into pAL841. While the efficiency of transgene insertion decreases with insert size, even small amounts of insert DNA (10 ng) yielded a sizeable proportion of positive colonies (Figure 1B). Operationally, the robust dual-selection system ensures reliable identification of positive colonies even when using larger inserts.

Having established that recombinant viruses could readily be generated using pAL841, the versatility of the system was enhanced by generating vector variants that enabled proteins to be expressed with various tags at the N or C terminus. This was accomplished by introducing the strep-2, strep-3, V5, or GFP tags into the vectors by recombineering (Figure 2A). Genes are amplified using primers with homology to the required tag and recombined with the vector in a single step. The final complete configuration of the AdZ vectors (including features discussed in “Conditional expression of genes” and “A self-excising vector” sections) is shown in Figure 2B.

High level expression with the AdZ vector

It is generally desirable to express transgenes from the AdZ vectors both at high levels and for extended periods. To maximize expression, a truncated version of the HCMV promoter (-299 to +69) (16) was used in the AdZ vectors. Elements located primarily in upstream HCMV enhancer sequences (-580 to -300) have been associated with the binding of factors that suppress levels, host cell range, and longevity of expression (27,28). We sought to evaluate the capacity of the AdZ vector to promote efficient transgene expression and directly compare the efficacy of the different HCMV MIE promoters used in the AdZ and AdEasy-1 vectors. Recombinant viruses were constructed encoding \textit{lacZ} using the AdZ vector (truncated promoter; RAd866) and AdEasy vector (extended promoter (-572 to +7); RAd871). HFFF-htert-hCAR was infected with either RAd866 or RAd871 at a multiplicity of infection (moi) of either 10 or 100. 72 h post infection (p.i.), direct staining of total cell extracts demonstrated that the AdZ (RAd866) recombinant showed a modest yet consistent
was further enhanced by making the vector self-excising. Recognition sites for the I-SceI homing endonuclease are 18 bp long, and consequently, they are extremely rare and I-SceI expression is compatible with eukaryotic cell growth (31). I-SceI sites were therefore recombinated between the PacI sites and the genome termini of the Ad genome and an expression cassette for I-SceI (under an RSV promoter) cloned into the plasmid backbone. Thus, transfection of circular, self-excisable vectors into a eukaryotic cell line results in expression of I-SceI, which releases the viral genome from the plasmid to render it infectious. A greater number of plaques formed when the self-excising pAL947 BAC (expressing GFP) was transfected in circular form than when cut with I-SceI, precipitated, and transfected as linear DNA (Figure 5), presumably due to the greater efficiency of transfection of circular DNA compared with linear. Self-excision thus represented a significant enhancement in efficiency, and the strategy was applied to all expression constructs as in Figure 2, A and B.

Direct insertion of a synthetic gene

The AdZ system allows for high-throughput, direct cloning of inserts into the Ad genome by recombineering. Clones containing inserts were identified with 100% accuracy using dual selectable markers, and Ad recombinants can be generated by direct transfection of packaging cells with circular DNA. PCR inherently needs a DNA/RNA template and requires all inserts be sequenced to detect errors introduced during amplification. However, even these requirements could be bypassed by using synthesized gene sequences. To test this, the 1.7-kb gene UL122 (IE2) from HCMV strain Merlin (32) was used. IE2 is translated from the major immediate early region of HCMV along with UL123 (IE1) by alternative splicing. IE2 is incompatible with RAd vector growth, and it has not been possible to recover viruses expressing IE2 using non-repressible vectors (data not shown). Thus, IE2 was used as a test of both synthesized genes and the tet repression system. The IE2 gene was synthesized by Genscript (Piscataway, NJ, USA), including 50 bp of homology to the target insertion site and PacI restriction sites at either end of the insert outside of the arms of homology. Within 14 days of supplying sequence information, 4 μg of the cloned synthesized fragment were delivered, digested with PacI to release the insert, gel-purified, and cloned directly into pAL944. Recombineering generated 70% white colonies, of which two were picked and confirmed as being correct. Following transfection of 293TREx cells virus made on the first attempt. The use of in vitro synthesized gene sequence eliminates the need to perform PCR and the absolute requirement to sequence cloned insert, requiring only a single, simple and efficient recombineering step to insert the transgene into the AdZ vector.

DISCUSSION

The AdZ system was developed by an iterative process in which the technology was systematically developed and optimized to eliminate steps and to facilitate simultaneous processing of multiple transgenes. To achieve these objectives (i) all conventional DNA cloning steps were eliminated by utilizing recombineering for directional insertion, (ii) inclusion of dual selectable markers eliminated the requirement to screen bacterial colonies for transgene insertion, (iii) incorporation of epitope/fluorescence tags into vectors enabled routine validation and tracking of transgene expression, (iv) the Ad vector was rendered self-excising to overcome limitations associated with low copy number BAC-based plasmids, and (v) suppression of transgene expression in the helper cell line enabled the generation of RAd encoding gene products incompatible with vector replication. High-throughput applications demand rapid, reliable, and robust technology, and the AdZ system meets these criteria. More than 100 recombinant viruses have already been produced using this system. Recombineering allows DNA sequences to be inserted directly and directionally into the Ad vector genome in a single step, and with all manipulations being carried out in a single E. coli strain. Growth of Ad recom-
binants in helper cells is normally associated with high level transgene expression as transcription from the HCMV promoter is stimulated by the E1A helper functions and replication increases the copy number of the transgene. Selective pressure may be exerted against a toxic or incompatible transgene during viral propagation (33). A further proportion of Ad recombinants cannot be grown to high titer; this is normally attributed to cytotoxicity associated with the transgene. By inserting the tet operator downstream of the HCMV promoter [step (+), above], transgene expression is efficiently suppressed in 293TREx helpers cells, but not in conventional 293 helper or target cells lacking the tet repressor. This feature has been exploited to produce a series of Ad recombinants that did not grow, or grew inefficiently, using conventional vectors. During the cloning of a contiguous panel of 36 genes from the HCMV genome, it proved impossible to generate virus for 7 of the genes using non-repressible vectors. Virus for all 7 was generated on the first attempt using the AdZ vector and 293TREx cells, and grown to titers comparable to the other 29 viruses (data not shown). A great amount of time can be saved by eliminating the snagging process associated with such problematic constructs. Crucially, the process of generating a large, defined bank of recombinants can be approached with confidence.

The AdZ vectors were initially designed to provide for direct insertion of PCR-generated sequences containing an appropriate tag to track expression. However, the technology is simplified even further when custom gene synthesis is used; gene synthesis can be deployed most beneficially when an appropriate PCR template is not available or complex manipulations of the transgene are required. RAd vectors are increasingly being exploited for the delivery of RNAi. The coding sequence for a shRNA together with sufficient flanking vector homology required for recombineering can readily be contained in a synthetic single-stranded oligonucleotide (~150 bases) and inserted directly into AdZ vectors (unpublished result).

Most existing commercial Ad vector systems are derived from strains that have been extensively passaged in vitro and subjected to ill-defined experimental genetic manipulations. We therefore generated an additional vector based on a defined wild-type Ad5 strain. This vector has been fully sequenced and may be considered preferable for certain applications. Recombineering technology can be utilized to rapidly modify the viral genome (34), promoters, and epitope tags in order to suit any cloning project. Having constructed a vector system using this method, it would also be relatively straightforward to clone other Ad genomes into the same self-excising vector, enabling the rapid production of cloning systems utilizing other Ad species.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Address correspondence to Richard J. Stanton, Department of Medical Microbiology, Tcovanus Building, Cardiff University, Heath Park, Cardiff, CF14 4XN, UK. email: stantonrj@cf.ac.uk

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