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Adenovirus vector library: an approach to the discovery of gene and protein function

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A method was developed to generate a complex cDNA expression library within an adenovirus type 5 (Ad5)-based vector backbone, termed AdLibrary. Construction of the AdLibrary entailed the conversion of an Ad5 genome-containing cosmid to infectious virus particles. The Ad5 genome was modified by replacing the E1A and E1B genes with a Rous sarcoma virus-driven expression cassette. Conversion was accomplished by liberating the viral genome by restriction enzyme digestion and transfection in HEK 293 cells, which support the growth of E1A/E1B-deficient virus. A test AdLibrary demonstrated the possibility of converting and identifying a marker gene present at a frequency of 1/10^5 in the cosmid library. To demonstrate the utility of this technology, an AdLibrary was used to isolate a viral gene by its biological function. Virus growth was selected for with an AdLibrary on A549 cells, which do not complement for E1A/E1B function. The AdLibrary was generated with cDNAs derived from HeLa cells productively infected with Ad5. A cDNA corresponding to Ad5 E1A 13S was selected and isolated from the AdLibrary using this strategy. Since multiple genes are assayed simultaneously, this technology should expedite the discovery of genes affecting defined biological activities. This AdLibrary approach provides an opportunity to exploit the efficient gene delivery capabilities of adenovirus vectors for the rapid discovery of gene and protein function.

INTRODUCTION

Following the completed sequencing of the human genome, and indeed the genomes of other organisms, a continuing challenge is to link individual genes with the control of biological phenotypes. Improvements in methods that facilitate genetic analysis in cells from higher organisms are likely to speed the understanding of gene function. Screening methods have been developed to provide a means to select a gene capable of inducing a particular phenotype from among a population of candidates. These methods include the transferal of a library of genes to test cells growing in culture and induction of gene

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expression. Selection is applied to this population of cells and the desired phenotype is isolated, amplified and studied. Such genetic selection techniques have been useful for the identification of protein–protein interactions (Toby & Golemis, 2001) or specific cell surface molecules with ligand- (Walter *et al.*, 2001) or antibody-binding (Griffiths & Duncan, 1998) characteristics. In certain situations, plasmid libraries and libraries of recombinant retroviruses have been used in genetic screens to identify genes conferring specific functions (Kitamura, 1998; Mahlmann *et al.*, 1998). While powerful, these methods can be limited, either by the inability to transduce some cells in culture or by insufficient gene expression to induce the phenotype of interest. The construction of adenovirus expression libraries as an array of single vectors (Michiels *et al.*, 2002) or as a population of vectors (Elahi *et al.*, 2002) has been described recently. In both cases, an *in vivo* recombination event was required to generate the vectors. Here, a cDNA expression library was cloned from the viral genome and converted directly into an adenovirus expression library.

Adenovirus gene transfer methods provide advantages for the assay of gene function. Adenovirus vectors can efficiently transduce both quiescent and cycling cells, which, along with their broad tropism, make adenoviruses well suited as gene transfer vectors. The adenovirus genome can be readily modified and propagated in bacteria. The genomes produced can then be used in complementing cell lines to create virus (Berkner & Sharp, 1983; Hanahan & Gluzman, 1984). In this study, we adapted the adenovirus genome construction and transfer methods to the challenge of generating gene libraries for the identification of gene function.

Human adenovirus type 5 (Ad5) is a 36 kb, double-stranded DNA virus which is widely used to express heterologous genes replacing the Ad5 E1 region. Deletion of the E1 region renders the virus replication deficient. The E1 region comprises the E1A and E1B transcription units (Berk & Sharp, 1977, 1978; Chow *et al.*, 1979; Kitchingman *et al.*, 1977). E1A is the first region transcribed after virus infection to yield the alternatively spliced 12S and 13S transcripts (Berk *et al.*, 1979; Jones & Shenk, 1979; Nevins, 1981; Nevins *et al.*, 1979). Analysis of various mutant viruses revealed the 13S gene product is required and sufficient to complement all E1A functions necessary for productive virus infection in cell culture (Berk *et al.*, 1979; Jones & Shenk, 1979; Montell *et al.*, 1982; Nevins, 1981; Nevins *et al.*, 1979). In this study, we demonstrated that adenovirus libraries can be generated with a complexity of $>1\times10^5$. Using a library screening strategy, this level of complexity is sufficient to confirm the complementing function of E1A 13S. This 'proof of principle' experiment demonstrates that adenovirus vector libraries can be generated for the identification of genes with specific biological function.

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METHODS

Description of pAdRSV.XS. The cosmid pAdRSV.XS (Fig. 1) was generated by ACE phage homologous recombination in *Escherichia coli* between pACE1(BN)E3(10) (McVey *et al.*, 2002) and pAdRSVTKXS. pAdRSVTKXS contains the Rous sarcoma virus (RSV) promoter and a thymidine kinase polyadenylation signal separated by the unique *Xba*I and *Swa*I restriction sites. The expression cassette is flanked by Ad5 sequences (nt 1–355 and 3328–5788) and is orientated to direct transcription towards the left inverted terminal repeat (ITR). The Ad5 genome used in constructing pACE1(BN)E3(10) from a derivative of isolate dl309 was cloned into pACYC177 (New England BioLabs), which had been cut to completion with *Drd*I/*Dra*I in order to retain the p15 origin of replication and kanamycin-resistance gene. The Ad5 left ITR resides nearest to the *Dra*I site. The lambda phage cos packaging site is 32 bp from the left ITR, containing nt 48460–190 of lambda. The Lac I^Q expression cassette contains 69 bp of upstream regulatory sequences and is oriented to direct transcription towards the cos packaging site. An E3 deletion was generated by cutting the Ad5 genome with *Xba*I, nt 28592–30470, treating the DNA with the Klenow fragment of DNA polymerase I and subsequently allowing the genome to self ligate. *Pac*I restriction sites reside next to the right ITR and the Lac I^Q promoter.

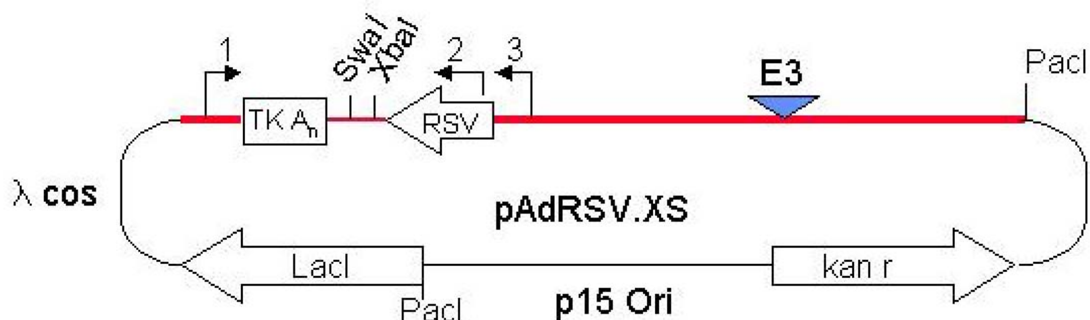

Fig. 1. Schematic representation of pAdRSV.XS. The plasmid backbone comprises the lambda phage packaging signal (cos), the LacI^Q repressor transcription unit (LacI), a low copy bacterial origin of replication (p15 Ori), a gene that confers kanamycin resistance (Kan^r) and *Pac*I restriction sites. The Ad5 genome, in the standard left-to-right orientation, with modifications. The E1 region was replaced with an expression cassette containing an RSV promoter, shown as an open arrow, thymidine kinase polyadenylation signal (TK A_n) and *Xba*I and *Swa*I restriction sites, into which the cDNA library is cloned. The E3 deletion is shown as a triangle. The thin, closed arrows numbered 1, 2 and 3 represent the direction (5′→3′) and location of oligonucleotides used in PCR.

Lambda cDNA library. A total of 20 10 cm plates containing HeLa cells (50 % confluent) were infected with wild-type Ad5 at an m.o.i. of 10. At 6 h post-infection (p.i.), cells were washed with 1× PBS (Life Technologies). Total RNA was isolated using an RNAgent kit (Promega) and poly(A) mRNA was subsequently isolated using the Oligotex mRNA Isolation kit (Qiagen), following the manufacturers' instructions. mRNA was converted to cDNA using the UniZAP XR cDNA Construction kit (Stratagene),

following the manufacturer's protocol, with modifications of the oligonucleotides used for cDNA synthesis. The primer for first-strand cDNA synthesis was 5′-GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGATTTAAATAAAAAAAAAAAAAAAAAA-3′, which contains a *Swa*I restriction site. Oligonucleotides containing a *Spe*I restriction site (5′-AATTCGGACTAGTGG-3′ and 5′-CCACTAGTCCG-3′) were annealed and ligated to the cDNA. The cDNA (100 ng) was then restricted with *Xho*I, ligated with 1 μg lambda UniZAP XR vector (Stratagene), packaged *in vitro* with GIGA Pack Gold extract (Stratagene) and transduced in *E. coli* XL-1 Blue, following the manufacturer's instructions. The library expressed a total of $2{\cdot}5\times10^5$ p.f.u. of virus. An inoculum from a fresh overnight culture of XL-1 Blue cells was started in LB broth supplemented with 0·2 % maltose and 10 mM $MgSO_4$. XL-1 Blue cells transduced with the packaged phage was plaqued in top agar (LB with 10 mM $MgSO_4$ and 0·6 % Bacto-agar) at a titre of ~50 000 plaques per 150 mm agarose plate for 5–6 h, following standard protocols (Silhavy *et al.*, 1984). Then, 10 ml ice-cold SM buffer (10 mM Tris, pH 7·4, 5 mM $MgSO_4$ and 0·01 % gelatin) was added to each plate and phage were eluted overnight at 4 °C. The titre of the eluted phage was 4.5×10^9 p.f.u. ml^{-1}, which constitutes the first expansion of the lambda cDNA library.

Cosmid cDNA library construction. A total of 20 150 mm plates with 50 000 plaques from the first expansion of the lambda cDNA library were eluted in SM buffer and DNA was isolated with a lambda genomic purification kit from Promega, following the manufacturer's instructions. Phage DNA was restricted with *Swa*I/*Spe*I, passed through a plasmid purification cartridge from Qiagen and ligated into *Xba*I/*Swa*I-restricted pAdRSV.XS, which had been pretreated with shrimp alkaline phosphatase (Boehringer Mannheim). The ligation mixture was packaged into phage heads using GIGA Pack Gold extract. XL-1 Blue cells were then transduced and plated on 150 mm agarose plates. Colonies were harvested after overnight incubation with LB and brought to 30 % glycerol before being snap–frozen on dry ice and stored at –80 °C. After freezing, the cosmid library contained 4×10^7 kanamycin-resistant c.f.u. ml^{-1}. Next, 200 μg cosmid library DNA was isolated from an overnight culture grown in the presence of kanamycin at 30 °C after being inoculated with 1 ml of the cosmid library and purified using a Qiagen endotoxin-free DNA column.

Creation of the AdLibrary. Cosmid library DNA was restricted with *Pac*I, extracted with phenol and phenol/chloroform/isoamyl alcohol (25:25:1) before being precipitated with ethanol, dried and resuspended in pyrogen-free H_2O. The DNA (5 μg) was resuspended in H_2O containing 100 mM $CaCl_2$ to bring the final volume to 250 μl. Next, 250 μl 2× HBS buffer (250 mM NaCl, 50 mM HEPES, pH 7·4, and 1·5 mM $NaHPO_4$) was quickly added, mixed and incubated at room temperature for 1–2 min before being added to a 60 mm plate containing 1×10^6 HEK 293 cells that had been incubated in DMEM with 5

% FCS 2 h before. At 4 h post-transfection, the cells were washed once with 1 ml 1 mM EGTA in HBS buffer (125 mM NaCl, 25 mM HEPES, pH 7·4, and 0·75 mM $NaHPO_4$) and twice with 2ml DMEM containing 2 % FCS. Cells were then incubated in 5 ml DMEM containing 5 % FCS. After 5 days, the cells were freeze–thawed three times and 1 ml of lysate was used to infect a 10 cm plate containing HEK 293 cells (70 % confluent) for 1 h before being incubated with 5 ml DMEM supplemented with 5 % FCS. Four days later, CPE was observed and the cells were freeze–thawed three times to create the AdLibrary.

Selection of AdLibrary. A 10 cm plate of A549 cells (70 % confluent) was infected with 1 ml AdLibrary lysate. At 5 days p.i., cells were harvested and freeze–thawed three times to create passage 1. Two additional passages were carried out to create passage 2 and 3 lysates, except that passage 3 was harvested at 4 days p.i.

Detection of active virus particles. Six-well plates were seeded with $1{\cdot}2\times10^6$ HEK 293 cells per well in DMEM supplemented with 5 % FCS. At 20–26 h later, the cells were washed with 1× PBS, infected with 200 µl of serial dilutions from the virus lysates made with DMEM containing 5 % FCS. Virus was removed 1 h later and the cells were fed with 3 ml DMEM containing 5 % FCS. At 22–24 h p.i., cells were washed with PBS and fixed with 2 ml ice–cold methanol for 15 min at room temperature and washed once more with PBS. A mouse antibody specific for adenovirus DNA-binding protein in 500 µl PBS was incubated with the cells for 1 h before being removed. Cells were subsequently washed with PBS before the addition of an FITC-conjugated anti-mouse antibody and incubation for 1 h in the dark. The number of UV-induced green fluorescent cells was determined under a microscope.

PCR analysis. A 5 µl sample of lysate in a 100 µl reaction mixture was heated at 94 °C for 5 min. PCR was carried out using *Taq* polymerase, buffers and conditions (30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min) as suggested by the manufacturer (Boehringer Mannheim). The sequences for oligonucleotides 1 (Ad5 nt 278–297), 2 (RSV promoter) and 3 (Ad5 nt 3349–3368) are 5′-CGCGGGAAAACTGAATAAGA-3′, 5′-CGATTGGTGGAAGTAAGG-3′ and 5′-CCTGGTGCGGGTCTCATCGTA-3′, respectively.

Southern blot analysis for E1 sequences. DNA was resolved on a 0·8 % agarose gel in 1× TBE buffer, transferred to a nylon membrane (Boehringer Mannheim) and UV crossed-linked in a Stratagene Stratalinker 2400. Membranes were prehybridized with DIG Easy Hybridization buffer (Boehringer Mannheim) at 55 °C for 1 h. The membrane was then probed with a PCR product of Ad5 (nt 559–798) labelled using the DIG Luminescent Detection kit (Boehringer Mannheim) (E1 probe) overnight at 55 °C. The membrane was washed three times at 55 °C in 0·1× SSC and 0·1 % SDS before being exposed to film and developed.

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RESULTS

AdLibrary detection limits

The use of expression libraries relies on the ability to select or screen for the presence of a gene(s) encoding a desired biological activity. The more complex the library is, the greater its utility will be. The method used to create the present AdLibrary requires the conversion of the viral genome residing within a plasmid to a functional virus. This is accomplished by liberating the viral genome from the plasmid by restriction enzyme digestion and transfecting HEK 293 cells (Graham *et al.*, 1977), which support the conversion process. To estimate the potential complexity generated in the AdLibrary, a mixing experiment was carried out. Two plasmids, which were identical except for the transgene expressed, were mixed and converted to virus simultaneously. A dilution series was made in which plasmids bearing an adenovirus genome expressing either green fluorescent protein (pAdGFP) or β-galactosidase (pAdZ) were mixed in ratios ranging from 10^{-1} to 10^{-7}. The genomes were converted to virus and passaged once on HEK 293 cells before being plated on A549 cells to detect the presence of AdGFP virus. The total number of GFP-positive cells is found in Table 1. The data from Table 1 shows that one pAdGFP plasmid in a background of 10^5 pAdZ plasmids can be converted and detected as virus. Hence, this system has the ability to detect a gene product present at a frequency of $1/10^5$.

Table 1. Estimation of AdLibrary detection limits

pAdGFP representation*	GFP-positive cells†
1/10	>1500
$1/10^2$	1500
$1/10^3$	250
$1/10^4$	25
$1/10^5$	5
$1/10^6$	0
$1/10^7$	0

*Ratio of pAdGFP to pAdZ, comprising the Ad5 genome used to convert to virus.

†Number of GFP-positive cells per plate for each dilution point.

AdLibrary strategy

To investigate the utility of this technology, a cDNA expression AdLibrary was created to select for Ad5 E1 complementing activity. Therefore, the Ad5 genome in the cosmid used to create the cDNA AdLibrary (Fig. 1, pAdRSV.XS) had the entire E1 region replaced with an RSV expression cassette. cDNA was cloned into the expression cassette to create a cosmid library, the precursor to the AdLibrary. The cosmid library was subsequently converted to an AdLibrary in HEK 293 cells (Graham *et al.*, 1977),

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since these cells can complement productive growth of E1-deficient virus. Virus growth was selected on A549 cells with the AdLibrary. A549 cells are human lung carcinoma cells that support full virus infection and lytic growth of wild-type, but not of E1-deficient, virus. They do, however, support the growth of E1B-deficient virus in the presence of E1A (Hay *et al.*, 1999). Therefore, in order for virus to propagate on these cells, they must express a cDNA capable of complementing the E1A activities of the virus.

AdLibrary construction

Construction of the cDNA AdLibrary proceeded through a three-step process. Initially, the cDNA generated from HeLa cells infected with wild-type Ad5 was cloned as a lambda library with a complexity of $2{\cdot}5\times10^5$ p.f.u. Of these plaques, 0·4 % contained E1A sequences, as assessed by plaque-lift analysis. The cDNA used in creating the lambda cDNA library had a median size of 1·6 kb when resolved on an agarose gel (data not shown). The second step was to move the cDNA from the lambda library into the RSV expression cassette found in pAdRSV.XS to create the cosmid library. The resulting cosmid library had a total complexity of 4×10^5 c.f.u. Restriction analysis of cosmid DNA isolated from 18 random colonies showed them to all contain an insert. Finally, the cosmid library was converted to an AdLibrary in HEK 293 cells. This was accomplished by standard $CaPO_4$ transfection techniques with Ad5 genomic DNA liberated from the cosmid backbone by *Pac*I restriction. Analysis of the AdLibrary lysate revealed it to contain 4×10^7 active particles ml^{-1}, as measured by a f.f.u. assay (Table 2).

Table 2. Active particle assessment of virus lysates

Lysates, isolated from the cell lines indicated, were assayed: AdLibrary, starting lysate; passage 1 (P1); and passage 3 (P3).

Lysate	Cell line	No. of active virus particles (f.f.u. ml^{-1})
AdLibrary	293	4.0×10^7
P1	A549	3.8×10^6
P3	A549	2.5×10^8

Selection for E1 function

Virus growth was selected on A549 cells with the AdLibrary. A549 cells were infected with the AdLibrary at an m.o.i. of approximately 8–10 f.f.u. per cell. Virus growth on the first two passages (P1 and P2) was allowed to proceed for 5 days before the cells were frozen and the lysate used for the next round of infection. In passage 3 (P3), full virus infection was observed at 2 days p.i., whereupon the cells were frozen. Quantification of the process, as assessed by f.f.u. assay, is shown in Table 2. With the initiation of selection, the relative abundance of adenovirus decreased by 10-fold (from 4×10^7 to $3{\cdot}8\times10^6$

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f.f.u. ml^{-1}) between the AdLibrary and P1. Outgrowth from P1 to P3 was nearly 100-fold ($3{\cdot}8\times10^6$ to $2{\cdot}5\times10^8$ f.f.u. ml^{-1}), consistent with the cellular cytopathology observed.

Characterization of E1 complementing activity

The DNA sequences with E1 complementing activity were identified by PCR isolation. Lysates from P1 and P3 were used as template with oligonucleotides designed to amplify all sequences present within the expression cassette. The primers used annealed to the Ad5 genome (Fig. 1, 1) and RSV promoter (Fig. 1, 2). In the absence of a transgene, these two primers are 744 bp apart in the vector. As can be seen in Fig. 2(A), a single prominent band of approximately 1·8 kb was generated from the P3 lysate, while no bands were observed from the P1 lysate. The insert is predicted to be near 1 kb in size. Although not quantitative, these data are consistent with the selection and expansion of Ad5 observed both phenotypically and by f.f.u. analysis. The PCR product was subjected to Southern blot analysis employing an E1A probe. The result identified the major band to have identity with the viral E1A region (Fig. 2B) and was of a size predicted for the 13S splice variant. A second primer set using oligonucleotides 1 and 3 (positions shown in Fig. 1) confirmed these results (data not shown). Direct sequencing of the PCR product identified it to be derived from the E1A 13S transcript (data not shown). The 5′ and 3′ junction of the cDNA mapped to Ad5 nt 522 and 1632, which compares favourably to previously mapped sites of transcription initiation and termination at positions 499 and 1633, respectively.

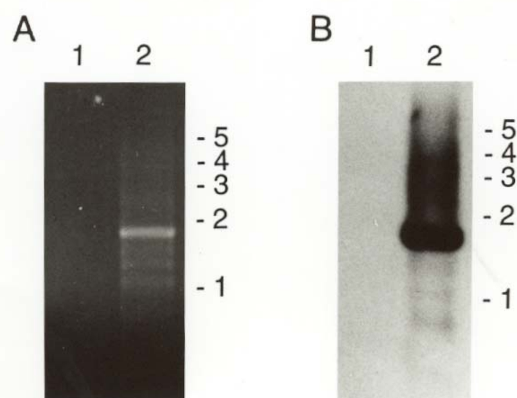

Fig. 2. Complementing genome analysis. After 30 cycles of PCR amplification with oligonucleotides 1 and 2 (see Fig. 1) using P1 (lane 1) and P3 (lane 2) lysate as template, the reactants were resolved on a 0·8 % ethidium bromide-stained agarose gel (A). The DNA was transferred to a nylon membrane, probed with E1 sequences and exposed to film (B). Size markers (kb) are indicated to the right.

E1 complementing activity by E1A 13S was confirmed by plaque analysis. Virus from the P3 lysate was plaque-purified twice on A549 cells. Nineteen plaques were expanded and the E1 region was investigated. Sequence analysis of PCR products generated with oligonucleotides 1 and 3 confirmed 16 of the viruses to contain the 13S transcript. The remaining three plaques contained the wild-type E1 region; these do not represent wild-type contaminating virus since they contain the E3 deletion present in the cosmid genome. The presence of the wild-type sequences replacing the entire RSV expression cassette most likely arose by homologous recombination between vector and the Ad5 genome present in HEK 293

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cells (Lochmuller *et al.*, 1994), on which the AdLibrary was created. As expected, the biological selection for virus growth also encouraged the growth of this minority population. These results validate the use of an AdLibrary strategy to isolate gene(s) that complement virus growth.

DISCUSSION

This work describes the construction of a cDNA adenovirus library and selection of a gene by its function. AdLibraries provide a new genetic tool to elucidate gene function in mammalian cells. Efficient virus transduction capabilities coupled to the ability to generate libraries with sufficient complexity and representation allow for gene discovery by function. Genetic complementation of a virus growth defect was used in this study to select for a gene of interest. This feature is particularly well suited to AdLibrary technology since the biological assay of virus growth results in both selection and amplification of the complementing gene. The reiterative process of selection, often required in library analysis, is easily accomplished by passaging of the virus lysate when selecting for virus growth.

Complementation of E1 function was the basis for selection in this study. A library in which the E1A and E1B regions of the virus were replaced with an expression cassette was assayed for virus growth on A549 cells. This cell line is known to contain an E1B complementing activity (Hay *et al.*, 1999). The majority of isolates contained E1A 13S cDNA and a minority contained wild-type genomic E1 regions, presumably the result of recombination with the complementing cell during construction of the library. Plaque purification of the isolates confirmed E1A 13S to be sufficient to complement for all E1 functions in A549 cells.

When using screening methods, the probability of finding a gene that encodes a desired trait is directly related to the number of different genes or mutations available to test. The methods described in this study are applicable to the generation of highly complex libraries. Cosmid cloning was chosen as the method to generate the AdLibrary genomes before their conversion to virus because of its proven ability for use in library generation. The cosmid library used in this study had a complexity of 4×10^5 c.f.u. The cDNA used in constructing the cosmid library had E1A sequences with a representation of approximately $1–4\times10^{-3}$. These results suggest that AdLibrary technology may be applicable to the isolation of genes that are represented at relatively low levels in a cDNA library.

Alternate genome configurations will be required for different AdLibrary gene discovery projects. To facilitate such constructions, we have generated two systems: a plasmid (D. E. Brough and others, unpublished data) and an ACE phage system (McVey *et al.*, 2002) to modify the vector genome. In these systems and in those of other investigators (Chartier *et al.*, 1996; Crouzet *et al.*, 1997; Ketner *et al.*, 1994;

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Miyake *et al.*, 1996), the viral genome is modified and propagated in bacteria or yeast and genomes converted to virus when transduced into complementing cells. These construction technologies allow any region of the genome to be modified for library purposes. It is, therefore, possible to directly address in the vector design the particular attributes required for the biological assay.

AdLibraries may be applicable to many types of genetic selection, employing both gain and loss of function. In the field of virology and virus vector systems, it is anticipated that additional genes will be identified that will complement other virus growth defects, which will assist in cell line construction. Alteration of the virus coat for a novel tropism could be used to direct a therapeutic vector to a defined cell type. This would greatly enhance vector utility and safety for gene therapy. AdLibraries can also be applied to mammalian cell lines with well-defined loss of function mutations (Hollstein *et al.*, 1991; Shapiro *et al.*, 1995). Such cell lines provide the opportunity to search for genes capable of complementing these defects. AdLibraries also have the potential for use in gain of function studies in which the generation of a biological phenotype is caused by gene expression. Examples of assays readily adaptable to AdLibrary screening are promoter activation assays, employing a marker gene such as β-lactamase, or a flow cytometry assay based on the detection of an extracellular receptor. All of these applications will result in the identification of genes by their function with no prior gene characterization required.

NOTE ADDED IN PROOF

An additional method of creating an adenovirus library has been published since the submission of this manuscript (Hatanaka *et al.*, 2003).

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