

# SIMIAN ADENOVIRUS NUCLEIC ACID AND AMINO ACID SEQUENCES, VECTORS CONTAINING SAME, AND METHODS OF USE

## 5 BACKGROUND OF THE INVENTION

Adenovirus is a double-stranded DNA virus with a genome size of about 36 kilobases (kb), which has been widely used for gene transfer applications due to its ability to achieve highly efficient gene transfer in a variety of target tissues and large transgene capacity. Conventionally, E1 genes of adenovirus are deleted and replaced with a  
10 transgene cassette consisting of the promoter of choice, cDNA sequence of the gene of interest and a poly A signal, resulting in a replication defective recombinant virus.

Adenoviruses have a characteristic morphology with an icosahedral capsid consisting of three major proteins, hexon (II), penton base (III) and a knobbed fibre (IV), along with a number of other minor proteins, VI, VIII, IX, IIIa and IVa2 [W.C. Russell, *J. Gen Virol.*, 81:2573-2604 (Nov 2000)]. The virus genome is a linear, double-stranded  
15 DNA with a terminal protein attached covalently to the 5' termini, which have inverted terminal repeats (ITRs). The virus DNA is intimately associated with the highly basic protein VII and a small peptide termed mu. Another protein, V, is packaged with this DNA-protein complex and provides a structural link to the capsid via protein VI. The  
20 virus also contains a virus-encoded protease, which is necessary for processing of some of the structural proteins to produce mature infectious virus.

Recombinant adenoviruses have been described for delivery of molecules to host cells. See, US Patent 6,083,716, which describes the genome of two chimpanzee adenoviruses.

25 What is needed in the art are more effective vectors which avoid the effect of pre-existing immunity to selected adenovirus serotypes in the population and/or which are useful for repeat administration and for titer boosting by second vaccination, if required.

## Summary of the Invention

30 The present invention provides the isolated nucleic acid sequences and amino acid sequences of six simian adenoviruses, vectors containing these sequences, and cell lines expressing simian adenovirus genes. Also provided are a number of methods for using the vectors and cells of the invention.

The methods of the invention involve delivering one or more selected heterologous gene(s) to a mammalian patient by administering a vector of the invention. Because the various vector constructs are derived from simian rather than from human adenoviruses, the immune system of the non-simian human or veterinary patient is not primed to respond immediately to the vector as a foreign antigen. Use of the compositions of this invention thus permits a more stable expression of the selected transgene when administered to a non-simian patient. Use of the compositions of this invention for vaccination permits presentation of a selected antigen for the elicitation of protective immune responses. Without wishing to be bound by theory, the ability of the adenoviruses of the invention to transduce human dendritic cells is at least partially responsible for the ability of the recombinant constructs of the invention to induce an immune response. The recombinant simian adenoviruses of this invention may also be used for producing heterologous gene products *in vitro*. Such gene products are themselves useful in a variety for a variety of purposes such as are described herein.

These and other embodiments and advantages of the invention are described in more detail below.

#### Brief Description of the Drawings

Fig. 1 provides an alignment of the amino acid sequences of the L1 and a portion of the L2 loops of the capsid protein hexon of the chimpanzee adenovirus C1 [SEQ ID NO:13], chimpanzee adenovirus C68 (Pan-9) [SEQ ID NO:14], and the novel Pan5 [SEQ ID NO:15], Pan6 [SEQ ID NO: 16] and Pan7 [SEQ ID NO: 17] chimpanzee adenovirus sequences of the invention. The intervening conserved region is part of the pedestal domain conserved between adenovirus serotypes.

Fig. 2 provides an alignment of the amino acid sequences of the fiber knob domains of chimpanzee C68 (Pan-9) [SEQ ID NO:18], Pan-6 [SEQ ID NO:19], Pan-7 [SEQ ID NO:20], and Pan-5 [SEQ ID NO:21] and the human adenoviruses serotypes 2 [SEQ ID NO:22] and 5 [SEQ ID NO:23].

#### DETAILED DESCRIPTION OF THE INVENTION

The invention provides novel nucleic acid and amino acid sequences from Ad Pan5 [SEQ ID NO:1-4, 15 and 21], Ad Pan6 [SEQ ID NO: 5-8, 16, 19], and Ad serotype

Pan7 [SEQ ID NO: 9-12, 17, 20], which were originally isolated from chimpanzee lymph nodes. In several instances throughout the specification, these adenoviruses are alternatively termed herein C5, C6 and C7, respectively. Also provided are sequences from adenovirus SV1 [SEQ ID NO: 24-28], which was originally isolated from the kidney cells of cynomolgus monkey. The invention also provides sequences of adenoviruses SV-25 [SEQ ID NO:29-33] and SV-39 [SEQ ID NO: 34-37], which were originally isolated from rhesus monkey kidney cells.

The present invention provides novel adenovirus vectors and packaging cell lines to produce those vectors for use in the *in vitro* production of recombinant proteins or fragments or other reagents. The invention further provides compositions for use in delivering a heterologous molecule for therapeutic or vaccine purposes. Such therapeutic or vaccine compositions contain the adenoviral vectors carrying an inserted heterologous molecule. In addition, novel sequences of the invention are useful in providing the essential helper functions required for production of recombinant adeno-associated viral (AAV) vectors. Thus, the invention provides helper constructs, methods and cell lines which use these sequences in such production methods.

The term "substantial homology" or "substantial similarity," when referring to a nucleic acid or fragment thereof, indicates that, when optimally aligned with appropriate nucleotide insertions or deletions with another nucleic acid (or its complementary strand), there is nucleotide sequence identity in at least about 95 to 99% of the aligned sequences.

The term "substantial homology" or "substantial similarity," when referring to amino acids or fragments thereof, indicates that, when optimally aligned with appropriate amino acid insertions or deletions with another amino acid (or its complementary strand), there is amino acid sequence identity in at least about 95 to 99% of the aligned sequences. Preferably, the homology is over full-length sequence, or a protein thereof, or a fragment thereof which is at least 8 amino acids, or more desirably, at least 15 amino acids in length. Examples of suitable fragments are described herein.

The term "percent sequence identity" or "identical" in the context of nucleic acid sequences refers to the residues in the two sequences that are the same when aligned for maximum correspondence. The length of sequence identity comparison may be over the full-length of the genome (e.g., about 36 kbp), the full-length of an open reading frame of a gene, protein, subunit, or enzyme [see, e.g., the tables providing the adenoviral coding

regions], or a fragment of at least about 500 to 5000 nucleotides, is desired. However, identity among smaller fragments, e.g. of at least about nine nucleotides, usually at least about 20 to 24 nucleotides, at least about 28 to 32 nucleotides, at least about 36 or more nucleotides, may also be desired. Similarly, "percent sequence identity" may be readily  
5 determined for amino acid sequences, over the full-length of a protein, or a fragment thereof. Suitably, a fragment is at least about 8 amino acids in length, and may be up to about 700 amino acids. Examples of suitable fragments are described herein.

Identity is readily determined using such algorithms and computer programs as are defined herein at default settings. Preferably, such identity is over the full length of  
10 the protein, enzyme, subunit, or over a fragment of at least about 8 amino acids in length. However, identity may be based upon shorter regions, where suited to the use to which the identical gene product is being put.

As described herein, alignments are performed using any of a variety of publicly or commercially available Multiple Sequence Alignment Programs, such as "Clustal W",  
15 accessible through Web Servers on the internet. Alternatively, Vector NTI utilities are also used. There are also a number of algorithms known in the art that can be used to measure nucleotide sequence identity, including those contained in the programs described above. As another example, polynucleotide sequences can be compared using Fasta, a program in GCG Version 6.1. Fasta provides alignments and percent sequence  
20 identity of the regions of the best overlap between the query and search sequences. For instance, percent sequence identity between nucleic acid sequences can be determined using Fasta with its default parameters (a word size of 6 and the NOPAM factor for the scoring matrix) as provided in GCG Version 6.1, herein incorporated by reference. Similarly programs are available for performing amino acid alignments. Generally, these  
25 programs are used at default settings, although one of skill in the art can alter these settings as needed. Alternatively, one of skill in the art can utilize another algorithm or computer program that provides at least the level of identity or alignment as that provided by the referenced algorithms and programs.

As used throughout this specification and the claims, the term "comprise" and its  
30 variants including, "comprises", "comprising", among other variants, is inclusive of other components, elements, integers, steps and the like. The term "consists of" or "consisting of" are exclusive of other components, elements, integers, steps and the like.

I. The Simian Adenovirus Sequences

The invention provides nucleic acid sequences and amino acid sequences of Pan5, Pan6, Pan7, SV1, SV25 and SV39, which are isolated from the other viral material with which they are associated in nature.

A. Nucleic Acid Sequences

The Pan5 nucleic acid sequences of the invention include nucleotides 1 to 36462 of SEQ ID NO:1. The Pan6 nucleic acid sequences of the invention include nucleotides 1 to 36604 of SEQ ID NO: 5. The Pan7 nucleic acid sequences of the invention include nucleotides 1 to 36535 of SEQ ID NO: 9. The SV1 nucleic acid sequences of the invention include nucleotides 1 to 34264 of SEQ ID NO: 24. The SV25 nucleic acid sequences of the invention include nucleotides 1 to 31044 of SEQ ID NO: 29. The SV39 nucleic acid sequences of the invention include nucleotides 1 to 34115 of SEQ ID NO: 34. See, Sequence Listing, which is incorporated by reference herein.

The nucleic acid sequences of the invention further encompass the strand which is complementary to the sequences of SEQ ID NO: 5, 9, 24, 29 and 34, as well as the RNA and cDNA sequences corresponding to the sequences of these sequences figures and their complementary strands. Further included in this invention are nucleic acid sequences which are greater than 95 to 98%, and more preferably about 99 to 99.9% homologous or identical to the Sequence Listing. Also included in the nucleic acid sequences of the invention are natural variants and engineered modifications of the sequences provided in SEQ ID NO: 5, 9, 24, 29 and 34 and their complementary strands. Such modifications include, for example, labels that are known in the art, methylation, and substitution of one or more of the naturally occurring nucleotides with a degenerate nucleotide.

The invention further encompasses fragments of the sequences of Pan5, Pan6, Pan7, SV1, SV25 and SV39, their complementary strand, cDNA and RNA complementary thereto. Suitable fragments are at least 15 nucleotides in length, and encompass functional fragments, i.e., fragments which are of biological interest. For example, a functional fragment can express a desired adenoviral product or may

be useful in production of recombinant viral vectors. Such fragments include the gene sequences and fragments listed in the tables below.

The following tables provide the transcript regions and open reading frames in the simian adenovirus sequences of the invention. For certain genes, the transcripts and open reading frames (ORFs) are located on the strand complementary to that presented in SEQ ID NO: 5, 9, 24, 29 and 34. See, e.g., E2b, E4 and E2a. The calculated molecular weights of the encoded proteins are also shown. Note that the E1a open reading frame Pan5 [nt 576-1436 of SEQ ID NO:1], Pan6 [nt 576 to 1437 of SEQ ID NO: 5] and Pan7 [nt 576 to 1437 of SEQ ID NO: 9] contain internal splice sites. These splice sites are noted in the following tables.

| Ad Pan-5 [SEQ ID NO:1] |              |                     |             |                   |
|------------------------|--------------|---------------------|-------------|-------------------|
| Regions                |              | Start<br>(nt)       | End<br>(nt) | M.W.<br>(Daltons) |
| ITR                    |              | 1                   | 120         | -                 |
| E1a                    | Transcript   | 478                 |             | -                 |
|                        | 13S          | 576-664,1233-1436   |             | 28120             |
|                        | 12S          | 576-1046, 1233-1436 |             | 24389             |
|                        | 9S           | 576-644,1233-1436   |             | 9962              |
|                        | Transcript   |                     | 1516        | -                 |
| E1b                    | Transcript   | 1552                |             | -                 |
|                        | Small T      | 1599                | 2171        | 22317             |
|                        | Large T      | 1904                | 3412        | 55595             |
|                        | LX           | 3492                | 3920        | 14427             |
|                        | Transcript   |                     | 3959        | -                 |
| E2b                    | Transcript   | 10349               |             | -                 |
|                        | PTP          | 10349               | 8451        | 72930             |
|                        | Polymerase   | 8448                | 5083        | 127237            |
|                        | IVa2         | 5604                | 3980        | 50466             |
|                        | Transcript   |                     | 3960        |                   |
| 28.1 kD                |              | 5155                | 5979        | 28141             |
| Agnoprotein            |              | 7864                | 8580        | 25755             |
| L1                     | Transcript   | 10849               |             | -                 |
|                        | 52/55D       | 10851               | 12025       |                   |
|                        | IIIa         | 12050               | 13819       | 65669             |
|                        | Transcript   |                     | 13832       | -                 |
|                        | Transcript   | 13894               |             | -                 |
| L2                     | Penton       | 13898               | 15490       | 59292             |
|                        | VII          | 15494               | 16078       | 21478             |
|                        | V            | 16123               | 17166       | 39568             |
|                        | Mu           | 17189               | 17422       | 8524              |
|                        | transcript   |                     | 17442       | -                 |
|                        | Transcript   | 17488               |             | -                 |
| L3                     | VI           | 17491               | 18222       | 26192             |
|                        | Hexon        | 18315               | 21116       | 104874            |
|                        | Endoprotease | 20989               | 21783       | 28304             |
|                        | transcript   |                     | 21811       | -                 |

| Ad Pan-5 (cont'd) [SEQ ID NO:1] |               |               |             |                   |
|---------------------------------|---------------|---------------|-------------|-------------------|
| Regions                         |               | Start<br>(nt) | End<br>(nt) | M.W.<br>(Daltons) |
| E2a                             | Transcript    | 26782         |             | -                 |
|                                 | DBP           | 23386         | 21845       | 57358             |
|                                 | transcript    |               | 21788       | -                 |
| L4                              | Transcript    | 23406         |             | -                 |
|                                 | 100kD         | 23412         | 25805       | 88223             |
|                                 | 33 kD homolog | 25525         | 26356       | 24538             |
|                                 | VIII          | 26428         | 27111       | 24768             |
|                                 | transcript    |               | 27421       | -                 |
| E3                              | Transcript    | 26788         |             | -                 |
|                                 | Orf #1        | 27112         | 27432       | 12098             |
|                                 | Orf #2        | 27386         | 28012       | 23040             |
|                                 | Orf #3        | 27994         | 28527       | 19525             |
|                                 | Orf #4        | 28557         | 29156       | 22567             |
|                                 | Orf #5        | 29169         | 29783       | 22267             |
|                                 | Orf #6        | 29798         | 30673       | 31458             |
|                                 | Orf #7        | 30681         | 30956       | 10477             |
|                                 | Orf #8        | 30962         | 31396       | 16523             |
|                                 | Orf #9        | 31389         | 31796       | 15236             |
|                                 | transcript    |               | 31837       | -                 |
| L5                              | Transcript    | 32032         |             | -                 |
|                                 | Fiber         | 32035         | 33372       | 47670             |
|                                 | transcript    |               | 33443       | -                 |
| E4                              | Transcript    | 36135         |             | -                 |
|                                 | Orf 7         | 33710         | 33462       | 9191              |
|                                 | Orf 6         | 34615         | 33710       | 35005             |
|                                 | Orf 4         | 34886         | 34521       | 13878             |
|                                 | Orf 3         | 35249         | 34896       | 13641             |
|                                 | Orf 2         | 35635         | 35246       | 14584             |
|                                 | Orf 1         | 36050         | 35676       | 13772             |
|                                 | Transcript    |               | 33437       | -                 |
| ITR                             |               | 36343         | 36462       | -                 |

| Ad Pan-6 [SEQ ID NO: 5] |              |                     |          |                   |
|-------------------------|--------------|---------------------|----------|-------------------|
| Regions                 |              | Start (nt)          | End (nt) | M.W.<br>(Daltons) |
| ITR                     |              | 1                   | 123      | -                 |
| E1a                     | transcript   | 478                 |          | -                 |
|                         | 13S          | 576-1143, 1229-1437 |          | 28291             |
|                         | 12S          | 576-1050, 1229-1437 |          | 24634             |
|                         | 9S           | 576-645, 1229-1437  |          | 10102             |
|                         | transcript   |                     | 1516     | -                 |
| E1b                     | transcript   | 1553                |          | -                 |
|                         | Small T      | 1600                | 2172     | 22315             |
|                         | LargeT       | 1905                | 3413     | 55594             |
|                         | IX           | 3498                | 3926     | 14427             |
|                         | transcript   |                     | 3965     | -                 |
| E2b                     | transcript   | 10341               |          | -                 |
|                         | PTP          | 10340               | 8451     | 72570             |
|                         | Polymerase   | 8445                | 5089     | 126907            |
|                         | IVa2         | 5610                | 3986     | 50452             |
|                         | transcript   |                     | 3966     | -                 |
| L1                      | transcript   | 10838               |          | -                 |
|                         | 52/55 kD     | 10840               | 12012    | 44205             |
|                         | IIIa         | 12036               | 13799    | 65460             |
|                         | Transcript   |                     | 13812    | -                 |
| 28.1 kd                 |              | 5161                | 5985     | 28012             |
| Agnoprotein             |              | 7870                | 8580     | 25382             |
| L2                      | transcript   | 13874               |          | -                 |
|                         | Penton       | 13878               | 15467    | 59314             |
|                         | VII          | 15471               | 16055    | 21508             |
|                         | V            | 16100               | 17137    | 39388             |
|                         | Mu           | 17160               | 17393    | 8506              |
|                         | transcript   |                     | 17415    | -                 |
| L3                      | transcript   | 17466               |          | -                 |
|                         | VI           | 17469               | 18188    | 25860             |
|                         | Hexon        | 18284               | 21112    | 106132            |
|                         | Endoprotease | 21134               | 21754    | 23445             |
|                         | transcript   |                     | 21803    | -                 |
| E2a                     | transcript   | 26780               |          | -                 |
|                         | DBP          | 23375               | 21837    | 57299             |
|                         | transcript   |                     | 21780    | -                 |

| Ad Pan-6 (cont'd) [SEQ ID NO:5] |               |            |          |                |
|---------------------------------|---------------|------------|----------|----------------|
| Regions                         |               | Start (nt) | End (nt) | M.W. (Daltons) |
| L4                              | Transcript    | 23398      |          | -              |
|                                 | 100kD         | 23404      | 25806    | 88577          |
|                                 | 33 kD homolog | 25523      | 26357    | 24609          |
|                                 | VIII          | 26426      | 27109    | 24749          |
|                                 | transcript    |            | 27419    | -              |
| E3                              | transcript    | 26786      |          | -              |
|                                 | Orf #1        | 27110      | 27430    | 12098          |
|                                 | Orf #2        | 27384      | 28007    | 22880          |
|                                 | Orf #3        | 27989      | 28519    | 19460          |
|                                 | Orf #4        | 28553      | 29236    | 25403          |
|                                 | Orf #5        | 29249      | 29860    | 22350          |
|                                 | Orf #6        | 29875      | 30741    | 31028          |
|                                 | Orf #7        | 30749      | 31024    | 10469          |
|                                 | Orf #8        | 31030      | 31464    | 16540          |
|                                 | Orf #9        | 31457      | 31864    | 15264          |
|                                 | transcript    |            | 31907    | -              |
|                                 |               |            |          |                |
| L5                              | transcript    | 32159      |          |                |
|                                 | Fiber         | 32162      | 33493    | 47364          |
|                                 | transcript    |            | 33574    | -              |
| E4                              | transcript    | 36276      |          | -              |
|                                 | Orf 7         | 33841      | 33593    | 9177           |
|                                 | Orf 6         | 34746      | 33841    | 35094          |
|                                 | Orf 4         | 35017      | 34652    | 13937          |
|                                 | Orf 3         | 35380      | 35027    | 13627          |
|                                 | Orf 2         | 35766      | 35377    | 14727          |
|                                 | Orf 1         | 36181      | 35807    | 13739          |
|                                 | transcript    |            | 33558    | -              |
| ITR                             |               | 36482      | 36604    | -              |

| Ad Pan-7 [SEQ ID NO:9] |              |             |             |                |
|------------------------|--------------|-------------|-------------|----------------|
| Regions                |              | Start (nt)  | End (nt)    | M.W. (Daltons) |
| ITR                    |              | 1           | 132         | -              |
| E1a                    | transcript   | 478         |             | -              |
|                        | 13S          | 576 – 1143, | 1229-1437   | 28218          |
|                        | 12S          | 576 – 1050, | 1229- 1437  | 24561          |
|                        | 9S           | 576 – 645,  | 1229 – 1437 | 10102          |
|                        | transcript   |             | 1516        | -              |
| E1b                    | transcript   | 1553        |             | -              |
|                        | Small T      | 1600        | 2178        | 22559          |
|                        | LargeT       | 1905        | 3419        | 55698          |
|                        | IVa2         | 3992        | 5616        | 50210          |
|                        | transcript   |             | 3971        | -              |
| E2b                    | transcript   | 10341       |             | -              |
|                        | PTP          | 10340       | 8457        | 72297          |
|                        | Polymerase   | 8451        | 5095        | 126994         |
|                        | IX           | 3504        | 3932        | 14441          |
|                        | transcript   |             | 3972        | -              |
| 28.1kD                 |              | 5167        | 5991        | 28028          |
| Agnoprotein            |              | 7876        | 8586        | 25424          |
| L1                     | transcript   | 10834       |             |                |
|                        | 52/55 kD     | 10836       | 12011       | 44302          |
|                        | IIIa         | 12035       | 13795       | 65339          |
|                        | transcript   |             | 13808       | -              |
| L2                     | transcript   | 13870       |             | -              |
|                        | Penton       | 13874       | 15469       | 59494          |
|                        | VII          | 15473       | 16057       | 21339          |
|                        | V            | 16102       | 17139       | 39414          |
|                        | Mu           | 17167       | 17400       | 8506           |
|                        | transcript   |             | 17420       | -              |
| L3                     | transcript   | 17467       |             | -              |
|                        | VI           | 17470       | 18198       | 26105          |
|                        | Hexon        | 18288       | 21086       | 104763         |
|                        | Endoprotease | 21106       | 21732       | 23620          |
|                        | transcript   |             | 21781       | -              |
| E2a                    | transcript   | 26764       |             | -              |
|                        | DBP          | 23353       | 21815       | 57199          |
|                        | transcript   |             | 21755       | -              |

| Ad Pan-7 (cont'd) [SEQ ID NO: 9] |               |            |          |                |
|----------------------------------|---------------|------------|----------|----------------|
| Regions                          |               | Start (nt) | End (nt) | M.W. (Daltons) |
| L4                               | transcript    | 23370      |          | -              |
|                                  | 100kD         | 23376      | 25781    | 88520          |
|                                  | 33 kD homolog | 25489      | 26338    | 25155          |
|                                  | VIII          | 26410      | 27093    | 24749          |
|                                  | transcript    |            | 27403    | -              |
| E3                               | transcript    | 26770      |          | -              |
|                                  | Orf #1        | 27094      | 27414    | 12056          |
|                                  | Orf #2        | 27368      | 27988    | 22667          |
|                                  | Orf #3        | 27970      | 28500    | 19462          |
|                                  | Orf #4        | 28530      | 29150    | 22999          |
|                                  | Orf #5        | 29163      | 29777    | 22224          |
|                                  | Orf #6        | 29792      | 30679    | 32153          |
|                                  | Orf #7        | 30687      | 30962    | 10511          |
|                                  | Orf #8        | 30968      | 31399    | 16388          |
|                                  | Orf #9        | 31392      | 31799    | 15205          |
|                                  | transcript    |            | 31842    | -              |
| L5                               | transcript    | 32091      |          | -              |
|                                  | Fiber         | 32094      | 33425    | 47344          |
|                                  | transcript    |            | 33517    | -              |
| E4                               | transcript    | 36208      |          | -              |
|                                  | Orf 7         | 33784      | 33536    | 9191           |
|                                  | Orf 6         | 34689      | 33784    | 35063          |
|                                  | Orf 4         | 34960      | 34595    | 13879          |
|                                  | Orf 3         | 35323      | 34970    | 13641          |
|                                  | Orf 2         | 35709      | 35320    | 14644          |
|                                  | Orf 1         | 36123      | 35749    | 13746          |
|                                  | transcript    |            | 33501    | -              |
| ITR                              |               | 36404      | 36535    | -              |

|        | Ad SV-1<br>[SEQ ID NO:24] |       | Ad SV-25<br>[SEQ ID NO:29] |       | Ad SV-39<br>[SEQ ID NO: 34] |       |
|--------|---------------------------|-------|----------------------------|-------|-----------------------------|-------|
| Region | Start                     | End   | Start                      | End   | Start                       | End   |
| ITR    | 1                         | 106   | 1                          | 133   | 1                           | 150   |
| E1a    | 352                       | 1120  | -                          | -     | 404                         | 1409  |
| E1b    | 1301                      | 2891  | 359                        | 2273  | 1518                        | 3877  |
| E2b    | 9257                      | 2882  | 9087                       | 2754  | 10143                       | 3868  |
| E2a    | 24415                     | 20281 | 24034                      | 20086 | 25381                       | 21228 |
| E3     | 24974                     | 27886 | 24791                      | 25792 | 25790                       | 29335 |
| E4     | 33498                     | 30881 | 30696                      | 28163 | 33896                       | 31157 |
| ITR    | 34145                     | 34264 | 30912                      | 31044 | 33966                       | 34115 |

|        | Ad SV-1<br>[SEQ ID NO:24] |       | Ad SV-25<br>[SEQ ID NO:29] |       | Ad SV-39<br>[SEQ ID NO: 34] |       |
|--------|---------------------------|-------|----------------------------|-------|-----------------------------|-------|
| Region | Start                     | End   | Start                      | End   | Start                       | End   |
| ITR    | 1                         | 106   | 1                          | 133   | 1                           | 150   |
| L1     | 9513                      | 12376 | 9343                       | 12206 | 10416                       | 13383 |
| L2     | 12453                     | 15858 | 12283                      | 15696 | 13444                       | 16877 |
| L3     | 15910                     | 20270 | 15748                      | 20080 | 17783                       | 21192 |
| L4     | 21715                     | 25603 | 21526                      | 25420 | 22659                       | 26427 |
| L5     | 28059                     | 30899 | 25320                      | 28172 | 29513                       | 31170 |
| ITR    | 34145                     | 34264 | 30912                      | 31044 | 33966                       | 34115 |

|     | protein      | Ad SV-1, SEQ ID NO: 24 |       |        |
|-----|--------------|------------------------|-------|--------|
|     |              | Start                  | End   | M.W.   |
| ITR |              | 1                      | 106   | -      |
| E1a | 13S          | 459                    | 953   | 18039  |
|     | 12S          |                        |       |        |
| E1b | Small T      |                        |       |        |
|     | LargeT       | 1301                   | 2413  | 42293  |
|     | IX           | 2391                   | 2885  | 16882  |
| E2b | IVa2         | 4354                   | 2924  | 54087  |
|     | Polymerase   | 6750                   | 4027  | 102883 |
|     | PTP          | 9257                   | 7371  | 72413  |
|     | Agno-protein | 6850                   | 7455  | 20984  |
| L1  | 52/55 kD     | 9515                   | 10642 | 42675  |
|     | IIIa         | 10663                  | 12372 | 636568 |
| L2  | Penton       | 12454                  | 13965 | 56725  |
|     | VII          | 13968                  | 14531 | 20397  |
|     | V            | 14588                  | 15625 | 39374  |
|     | Mu           | 15645                  | 15857 | 7568   |
| L3  | VI           | 15911                  | 16753 | 30418  |
|     | Hexon        | 16841                  | 19636 | 104494 |
|     | Endoprotease | 19645                  | 20262 | 23407  |
| 2a  | DBP          | 21700                  | 20312 | 52107  |
| L4  | 100kD        | 21721                  | 24009 | 85508  |
|     | VIII         | 24591                  | 25292 | 25390  |

|     | protein  | Ad SV-1 (cont'd) |       |              |
|-----|----------|------------------|-------|--------------|
|     |          | SEQ ID NO: 24    |       |              |
|     |          | Start            | End   | <i>M.W.</i>  |
| E3  | Orf #1   | 25292            | 25609 | <i>11950</i> |
|     | Orf #2   | 25563            | 26081 | <i>18940</i> |
|     | Orf #3   | 26084            | 26893 | <i>30452</i> |
|     | Orf #4   | 26908            | 27180 | <i>10232</i> |
|     | Orf #5   | 27177            | 17512 | <i>12640</i> |
|     | Orf #6   | 27505            | 27873 | <i>13639</i> |
| L5  | Fiber #2 | 28059            | 29150 | <i>39472</i> |
|     | Fiber #1 | 29183            | 30867 | <i>61128</i> |
| E4  | Orf 7    | 31098            | 30892 | <i>7837</i>  |
|     | Orf 6    | 31982            | 31122 | <i>33921</i> |
|     | Orf 4    | 32277            | 31915 | <i>14338</i> |
|     | Orf 3    | 32629            | 32279 | <i>13386</i> |
|     | Orf 2    | 33018            | 32626 | <i>14753</i> |
|     | Orf 1    | 33423            | 33043 | <i>14301</i> |
| ITR |          | 34145            | 34264 |              |

|     | protein           | Ad SV-25, SEQ ID NO:29 |       |             | Ad SV-39, SEQ ID NO:34 |       |             |
|-----|-------------------|------------------------|-------|-------------|------------------------|-------|-------------|
|     |                   | Start                  | End   | <i>M.W.</i> | Start                  | End   | <i>M.W.</i> |
| ITR |                   | 1                      | 133   | -           | 1                      | 150   | -           |
| E1a | 13S               |                        |       |             | 492                    | 1355  | 28585       |
|     | 12S               |                        |       |             | 492                    | 1355  | 25003       |
| E1b | Small T           | 478                    | 1030  | 20274       | 1518                   | 2075  | 21652       |
|     | Large T           | 829                    | 2244  | 52310       | 1823                   | 3349  | 55534       |
|     | IX                | 2306                   | 2716  | 13854       | 3434                   | 3844  | 14075       |
| E2b | IVa2              | 4208                   | 2755  | 54675       | 3912                   | 5141  | 46164       |
|     | Poly-<br>merase   | 6581                   | 3858  | 102839      | 7753                   | 5033  | 103988      |
|     | PTP               | 9087                   | 7207  | 71326       | 10143                  | 8335  | 69274       |
|     | Agno-<br>protein  | 6681                   | 7139  | 16025       | -                      | -     | -           |
| L1  | 52/55 kD          | 9345                   | 10472 | 42703       | 10418                  | 11608 | 44232       |
|     | IIIa              | 10493                  | 12202 | 63598       | 11574                  | 13364 | 66078       |
| L2  | Penton            | 12284                  | 13801 | 56949       | 13448                  | 14959 | 56292       |
|     | VII               | 13806                  | 14369 | 20369       | 14960                  | 15517 | 20374       |
|     | V                 | 14426                  | 15463 | 39289       | 15567                  | 16628 | 39676       |
|     | Mu                | 15483                  | 15695 | 7598        | 16650                  | 16871 | 7497        |
| L3  | VI                | 15749                  | 16591 | 30347       | 16925                  | 17695 | 28043       |
|     | Hexon             | 16681                  | 19446 | 104035      | 17785                  | 20538 | 102579      |
|     | Endo-<br>protease | 19455                  | 20072 | 23338       | 20573                  | 21181 | 22716       |
| 2a  | DBP               | 21511                  | 20123 | 52189       | 22631                  | 21231 | 53160       |
| L4  | 100kD             | 21532                  | 23829 | 85970       | 22659                  | 25355 | 100362      |
|     | VIII              | 24408                  | 25109 | 25347       | 25410                  | 26108 | 25229       |

|     | protein  | Ad SV-25, SEQ ID NO:29<br>(cont'd) |       |              | Ad SV-39, SEQ ID NO:34,<br>(cont'd) |       |              |
|-----|----------|------------------------------------|-------|--------------|-------------------------------------|-------|--------------|
|     |          | Start                              | End   | <i>M.W.</i>  | Start                               | End   | <i>M.W.</i>  |
| E3  | Orf #1   | 25109                              | 25426 | <i>11890</i> | 26375                               | 27484 | <i>42257</i> |
|     | Orf #2   |                                    |       |              | 27580                               | 28357 | <i>29785</i> |
|     | Orf #3   |                                    |       |              | 28370                               | 28645 | <i>10514</i> |
|     | Orf #4   |                                    |       |              | 28863                               | 29333 | <i>18835</i> |
|     | Orf #5   |                                    |       |              |                                     |       |              |
|     | Orf #6   |                                    |       |              |                                     |       |              |
| L5  | Fiber #2 | 25380                              | 26423 | <i>37529</i> |                                     |       |              |
|     | Fiber #1 | 26457                              | 28136 | <i>60707</i> | 29515                               | 31116 | <i>56382</i> |
| E4  | Orf 7    |                                    |       |              | 31441                               | 31118 | <i>11856</i> |
|     | Orf 6    | 29255                              | 28395 | <i>33905</i> | 32292                               | 31438 | <i>33437</i> |
|     | Orf 4    | 29550                              | 29188 | <i>14399</i> | 32587                               | 32222 | <i>13997</i> |
|     | Orf 3    | 29902                              | 29552 | <i>13284</i> | 32954                               | 32607 | <i>13353</i> |
|     | Orf 2    | 30291                              | 29899 | <i>14853</i> | 33348                               | 32959 | <i>14821</i> |
|     | Orf 1    | 30316                              | 30696 | <i>14301</i> | 33764                               | 33378 | <i>14235</i> |
| ITR |          | 30912                              | 31044 |              | 33966                               | 34115 |              |

The Pan5, Pan6, Pan7, SV1, SV25 and SV39 adenoviral nucleic acid  
 5 sequences are useful as therapeutic agents and in construction of a variety of vector  
 systems and host cells. As used herein, a vector includes any suitable nucleic acid  
 molecule including, naked DNA, a plasmid, a virus, a cosmid, or an episome. These  
 sequences and products may be used alone or in combination with other adenoviral  
 sequences or fragments, or in combination with elements from other adenoviral or non-  
 10 adenoviral sequences. The adenoviral sequences of the invention are also useful as  
 antisense delivery vectors, gene therapy vectors, or vaccine vectors. Thus, the invention  
 further provides nucleic acid molecules, gene delivery vectors, and host cells which  
 contain the Ad sequences of the invention.

For example, the invention encompasses a nucleic acid molecule containing simian Ad ITR sequences of the invention. In another example, the invention provides a nucleic acid molecule containing simian Ad sequences of the invention encoding a desired Ad gene product. Still other nucleic acid molecule constructed using the sequences of the invention will be readily apparent to one of skill in the art, in view of the information provided herein.

In one embodiment, the simian Ad gene regions identified herein may be used in a variety of vectors for delivery of a heterologous molecule to a cell. For example, vectors are generated for expression of an adenoviral capsid protein (or fragment thereof) for purposes of generating a viral vector in a packaging host cell. Such vectors may be designed for expression in trans. Alternatively, such vectors are designed to provide cells which stably contain sequences which express desired adenoviral functions, e.g., one or more of E1a, E1b, the terminal repeat sequences, E2a, E2b, E4, E4ORF6 region.

In addition, the adenoviral gene sequences and fragments thereof are useful for providing the helper functions necessary for production of helper-dependent viruses (e.g., adenoviral vectors deleted of essential functions or adeno-associated viruses (AAV)). For such production methods, the simian adenoviral sequences of the invention are utilized in such a method in a manner similar to those described for the human Ad. However, due to the differences in sequences between the simian adenoviral sequences of the invention and those of human Ad, the use of the sequences of the invention essentially eliminate the possibility of homologous recombination with helper functions in a host cell carrying human Ad E1 functions, e.g., 293 cells, which may produce infectious adenoviral contaminants during rAAV production.

Methods of producing rAAV using adenoviral helper functions have been described at length in the literature with human adenoviral serotypes. See, e.g., US Patent 6,258,595 and the references cited therein. See, also, US Patent 5,871,982; WO 99/14354; WO 99/15685; WO 99/47691. These methods may also be used in production of non-human serotype AAV, including non-human primate AAV serotypes. The simian adenoviral gene sequences of the invention which provide the necessary helper functions (e.g., E1a, E1b, E2a and/or E4 ORF6) can be particularly useful in providing the necessary adenoviral function while minimizing or eliminating the possibility of

recombination with any other adenoviruses present in the rAAV-packaging cell which are typically of human origin. Thus, selected genes or open reading frames of the adenoviral sequences of the invention may be utilized in these rAAV production methods.

Alternatively, recombinant adenoviral simian vectors of the invention may  
5 be utilized in these methods. Such recombinant adenoviral simian vectors may include, e.g., a hybrid chimp Ad/AAV in which chimp Ad sequences flank a rAAV expression cassette composed of, e.g., AAV 3' and/or 5' ITRs and a transgene under the control of regulatory sequences which control its expression. One of skill in the art will recognize that still other simian adenoviral vectors and/or gene sequences of the invention will be  
10 useful for production of rAAV and other viruses dependent upon adenoviral helper.

In still another embodiment, nucleic acid molecules are designed for delivery and expression of selected adenoviral gene products in a host cell to achieve a desired physiologic effect. For example, a nucleic acid molecule containing sequences encoding an adenovirus E1a protein of the invention may be delivered to a subject for use  
15 as a cancer therapeutic. Optionally, such a molecule is formulated in a lipid-based carrier and preferentially targets cancer cells. Such a formulation may be combined with other cancer therapeutics (e.g., cisplatin, taxol, or the like). Still other uses for the adenoviral sequences provided herein will be readily apparent to one of skill in the art.

In addition, one of skill in the art will readily understand that the Ad  
20 sequences of the invention can be readily adapted for use for a variety of viral and non-viral vector systems for in vitro, ex vivo or in vivo delivery of therapeutic and immunogenic molecules. For example, the Pan5, Pan6, Pan7, SV1, SV25 and/or SV39 simian Ad genomes of the invention can be utilized in a variety of rAd and non-rAd vector systems. Such vectors systems may include, e.g., plasmids, lentiviruses,  
25 retroviruses, poxviruses, vaccinia viruses, and adeno-associated viral systems, among others. Selection of these vector systems is not a limitation of the present invention.

The invention further provides molecules useful for production of the simian and simian-derived proteins of the invention. Such molecules which carry polynucleotides including the simian Ad DNA sequences of the invention can be in the  
30 form of naked DNA, a plasmid, a virus or any other genetic element.

## B. Simian Adenoviral Proteins of the Invention

The invention further provides gene products of the above adenoviruses, such as proteins, enzymes, and fragments thereof, which are encoded by the adenoviral nucleic acids of the invention. The invention further encompasses Pan5, Pan6 and Pan7, 5 SV1, SV25 and SV39 proteins, enzymes, and fragments thereof, having the amino acid sequences encoded by these nucleic acid sequences which are generated by other methods. Such proteins include those encoded by the open reading frames identified in the tables above, in Figs. 1 and 2, and fragments thereof.

Thus, in one aspect, the invention provides unique simian adenoviral 10 proteins which are substantially pure, i.e., are free of other viral and proteinaceous proteins. Preferably, these proteins are at least 10% homogeneous, more preferably 60% homogeneous, and most preferably 95% homogeneous.

In one embodiment, the invention provides unique simian-derived capsid 15 proteins. As used herein, a simian-derived capsid protein includes any adenoviral capsid protein that contains a Pan5, Pan6, Pan7, SV1, SV25 or SV39 capsid protein or a fragment thereof, as defined above, including, without limitation, chimeric capsid proteins, fusion proteins, artificial capsid proteins, synthetic capsid proteins, and recombinantly capsid proteins, without limitation to means of generating these proteins.

Suitably, these simian-derived capsid proteins contain one or more Pan5, 20 Pan6, Pan7, SV1, SV25 or SV39 regions or fragments thereof (e.g., a hexon, penton, fiber or fragment thereof) in combination with capsid regions or fragments thereof of different adenoviral serotypes, or modified simian capsid proteins or fragments, as described herein. A "modification of a capsid protein associated with altered tropism" as used herein includes an altered capsid protein, i.e., a penton, hexon or fiber protein region, or 25 fragment thereof, such as the knob domain of the fiber region, or a polynucleotide encoding same, such that specificity is altered. The simian-derived capsid may be constructed with one or more of the simian Ad of the invention or another Ad serotypes which may be of human or non-human origin. Such Ad may be obtained from a variety of sources including the ATCC, commercial and academic sources, or the sequences of the 30 Ad may be obtained from GenBank or other suitable sources.

The amino acid sequences of the simian adenoviruses penton proteins of the invention are provided herein. The AdPan5 penton protein is provided in SEQ ID

NO:2. The AdPan7 penton is provided in SEQ ID NO:6. The AdPan6 penton is provided in SEQ ID NO:10. The SV1 penton is provided in SEQ ID NO:25. The SV25 penton protein is provided in SEQ ID NO:30. The SV39 penton is provided in SEQ ID NO:35. Suitably, any of these penton proteins, or unique fragments thereof, may be utilized for a variety of purposes. Examples of suitable fragments include the penton having N-terminal and/or C-terminal truncations of about 50, 100, 150, or 200 amino acids, based upon the amino acid numbering provided above and in SEQ ID NO:2; SEQ ID NO:6; SEQ ID NO:25; SEQ ID NO:30, or SEQ ID NO:35. Other suitable fragments include shorter internal, C-terminal, or N-terminal fragments. Further, the penton protein may be modified for a variety of purposes known to those of skill in the art.

The invention further provides the amino acid sequences of the hexon protein of Pan5 [SEQ ID NO:3], Pan6 [SEQ ID NO:7], Pan 7 [SEQ ID NO:11], SV1 [SEQ ID NO:26], SV25 [SEQ ID NO:31], and/or SV39 [SEQ ID NO:36]. Suitably, this hexon protein, or unique fragments thereof, may be utilized for a variety of purposes. Examples of suitable fragments include the hexon having N-terminal and/or C-terminal truncations of about 50, 100, 150, 200, 300, 400, or 500 amino acids, based upon the amino acid numbering provided above and in SEQ ID NO: 3, 7, 11, 26, 31 and 36. Other suitable fragments include shorter internal, C-terminal, or N-terminal fragments. For example, one suitable fragment the loop region (domain) of the hexon protein, designated DEI and FG1, or a hypervariable region thereof. Such fragments include the regions spanning amino acid residues about 125 to 443; about 138 to 441, or smaller fragments, such as those spanning about residue 138 to residue 163; about 170 to about 176; about 195 to about 203; about 233 to about 246; about 253 to about 264; about 287 to about 297; and about 404 to about 430 of the simian hexon proteins, with reference to SEQ ID NO: 3, 7, 11, 26, 31 or 36. Other suitable fragments may be readily identified by one of skill in the art. Further, the hexon protein may be modified for a variety of purposes known to those of skill in the art. Because the hexon protein is the determinant for serotype of an adenovirus, such artificial hexon proteins would result in adenoviruses having artificial serotypes. Other artificial capsid proteins can also be constructed using the chimp Ad penton sequences and/or fiber sequences of the invention and/or fragments thereof.

In one example, it may be desirable to generate an adenovirus having an altered hexon protein utilizing the sequences of a hexon protein of the invention. One suitable method for altering hexon proteins is described in US Patent 5,922,315, which is incorporated by reference. In this method, at least one loop region of the adenovirus hexon is changed with at least one loop region of another adenovirus serotype. Thus, at least one loop region of such an altered adenovirus hexon protein is a simian Ad hexon loop region of the invention (e.g. Pan7). In one embodiment, a loop region of the Pan7 hexon protein is replaced by a loop region from another adenovirus serotype. In another embodiment, the loop region of the Pan7 hexon is used to replace a loop region from another adenovirus serotype. Suitable adenovirus serotypes may be readily selected from among human and non-human serotypes, as described herein. Pan7 is selected for purposes of illustration only; the other simian Ad hexon proteins of the invention may be similarly altered, or used to alter another Ad hexon. The selection of a suitable serotype is not a limitation of the present invention. Still other uses for the hexon protein sequences of the invention will be readily apparent to those of skill in the art.

The invention further encompasses the fiber proteins of the simian adenoviruses of the invention. The fiber protein of AdPan 5 has the amino acid sequence of SEQ ID NO:4. The fiber protein AdPan6 has the amino acid sequence of SEQ ID NO: 8. The fiber protein of AdPan7 has the amino acid sequence of SEQ ID NO: 12. SV-1 has two fiber proteins; fiber 2 has the amino acid sequence of SEQ ID NO:27 and fiber 1 has the amino acid sequence of SEQ ID NO:28. SV-25 also has two fiber proteins; fiber 2 has the amino acid sequence of SEQ ID NO:32 and fiber 1 has the amino acid sequence of SEQ ID NO:33. The fiber protein of SV-39 has the amino acid sequence of SEQ ID NO:37.

Suitably, this fiber protein, or unique fragments thereof, may be utilized for a variety of purposes. One suitable fragment is the fiber knob, which spans about amino acids 247 to 425 of SEQ ID NO: 4, 8, 12, 28, 32, 33 and 37. See Fig. 2. Examples of other suitable fragments include the fiber having N-terminal and/or C-terminal truncations of about 50, 100, 150, or 200 amino acids, based upon the amino acid numbering provided above and in SEQ ID NO: 4, 8, 12, 28, 32, 33 and 37. Still other suitable fragments include internal fragments. Further, the fiber protein may be modified using a variety of techniques known to those of skill in the art.

The invention further encompasses unique fragments of the proteins of the invention which are at least 8 amino acids in length. However, fragments of other desired lengths can be readily utilized. In addition, the invention encompasses such modifications as may be introduced to enhance yield and/or expression of a Pan5, Pan6, Pan7, SV1, SV25 or SV39 gene product, e.g., construction of a fusion molecule in which all or a fragment of the Pan5, Pan6, Pan7, SV1, SV25 or SV39 gene product is fused (either directly or via a linker) with a fusion partner to enhance. Other suitable modifications include, without limitation, truncation of a coding region (e.g., a protein or enzyme) to eliminate a pre- or pro-protein ordinarily cleaved and to provide the mature protein or enzyme and/or mutation of a coding region to provide a secretable gene product. Still other modifications will be readily apparent to one of skill in the art. The invention further encompasses proteins having at least about 95% to 99% identity to the Pan5, Pan6, Pan7, SV1, SV25 or SV39 proteins provided herein.

As described herein, vectors of the invention containing the adenoviral capsid proteins of the invention are particularly well suited for use in applications in which the neutralizing antibodies diminish the effectiveness of other Ad serotype based vectors, as well as other viral vectors. The rAd vectors of the invention are particularly advantageous in readministration for repeat gene therapy or for boosting immune response (vaccine titers).

Under certain circumstances, it may be desirable to use one or more of the Pan5, Pan6, Pan7, SV1, SV25 and/or SV39 gene products (e.g., a capsid protein or a fragment thereof) to generate an antibody. The term "an antibody," as used herein, refers to an immunoglobulin molecule which is able to specifically bind to an epitope. Thus, the antibodies of the invention bind, preferably specifically and without cross-reactivity, to a Pan5, Pan6, Pan7, SV1, SV25 or SV39 epitope. The antibodies in the present invention exist in a variety of forms including, for example, high affinity polyclonal antibodies, monoclonal antibodies, synthetic antibodies, chimeric antibodies, recombinant antibodies and humanized antibodies. Such antibodies originate from immunoglobulin classes IgG, IgM, IgA, IgD and IgE.

Such antibodies may be generated using any of a number of methods know in the art. Suitable antibodies may be generated by well-known conventional techniques, e.g. Kohler and Milstein and the many known modifications thereof.

Similarly desirable high titer antibodies are generated by applying known recombinant techniques to the monoclonal or polyclonal antibodies developed to these antigens [see, e.g., PCT Patent Application No. PCT/GB85/00392; British Patent Application Publication No. GB2188638A; Amit *et al.*, 1986 *Science*, 233:747-753; Queen *et al.*, 5 1989 *Proc. Nat'l. Acad. Sci. USA*, 86:10029-10033; PCT Patent Application No. PCT/WO9007861; and Riechmann *et al.*, *Nature*, 332:323-327 (1988); Huse *et al.*, 1988a *Science*, 246:1275-1281]. Alternatively, antibodies can be produced by manipulating the complementarity determining regions of animal or human antibodies to the antigen of this invention. See, e.g., E. Mark and Padlin, "Humanization of Monoclonal Antibodies", 10 Chapter 4, *The Handbook of Experimental Pharmacology*, Vol. 113, *The Pharmacology of Monoclonal Antibodies*, Springer-Verlag (June, 1994); Harlow *et al.*, 1999, *Using Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, NY; Harlow *et al.*, 1989, *Antibodies: A Laboratory Manual*, Cold Spring Harbor, New York; Houston *et al.*, 1988, *Proc. Natl. Acad. Sci. USA* 85:5879-5883; and Bird *et al.*, 1988, *Science* 15 242:423-426. Further provided by the present invention are anti-idiotypic antibodies (Ab2) and anti-anti-idiotypic antibodies (Ab3). See, e.g., M. Wettendorff *et al.*, "Modulation of anti-tumor immunity by anti-idiotypic antibodies." In *Idiotypic Network and Diseases*, ed. by J. Cerny and J. Hiernaux, 1990 *J. Am. Soc. Microbiol.*, Washington DC: pp. 203-229]. These anti-idiotypic and anti-anti-idiotypic antibodies are produced 20 using techniques well known to those of skill in the art. These antibodies may be used for a variety of purposes, including diagnostic and clinical methods and kits.

Under certain circumstances, it may be desirable to introduce a detectable label or a tag onto a Pan5, Pan6, Pan7, SV1, SV25 or SV39 gene product, antibody or other construct of the invention. As used herein, a detectable label is a molecule which is 25 capable, alone or upon interaction with another molecule, of providing a detectable signal. Most desirably, the label is detectable visually, e.g. by fluorescence, for ready use in immunohistochemical analyses or immunofluorescent microscopy. For example, suitable labels include fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), coriphosphine-O (CPO) or tandem dyes, PE-cyanin-5 (PC5), and PE-Texas Red 30 (ECD). All of these fluorescent dyes are commercially available, and their uses known to the art. Other useful labels include a colloidal gold label. Still other useful labels include radioactive compounds or elements. Additionally, labels include a variety of enzyme

systems that operate to reveal a colorimetric signal in an assay, e.g., glucose oxidase (which uses glucose as a substrate) releases peroxide as a product which in the presence of peroxidase and a hydrogen donor such as tetramethyl benzidine (TMB) produces an oxidized TMB that is seen as a blue color. Other examples include horseradish  
5 peroxidase (HRP) or alkaline phosphatase (AP), and hexokinase in conjunction with glucose-6-phosphate dehydrogenase which reacts with ATP, glucose, and NAD<sup>+</sup> to yield, among other products, NADH that is detected as increased absorbance at 340 nm wavelength.

Other label systems that are utilized in the methods of this invention are  
10 detectable by other means, e.g., colored latex microparticles [Bangs Laboratories, Indiana] in which a dye is embedded are used in place of enzymes to form conjugates with the target sequences provide a visual signal indicative of the presence of the resulting complex in applicable assays.

Methods for coupling or associating the label with a desired molecule are  
15 similarly conventional and known to those of skill in the art. Known methods of label attachment are described [see, for example, Handbook of Fluorescent probes and Research Chemicals, 6th Ed., R. P. M. Haugland, Molecular Probes, Inc., Eugene, OR, 1996; Pierce Catalog and Handbook, Life Science and Analytical Research Products, Pierce Chemical Company, Rockford, IL, 1994/1995]. Thus, selection of the label and  
20 coupling methods do not limit this invention.

The sequences, proteins, and fragments of the invention may be produced by any suitable means, including recombinant production, chemical synthesis, or other synthetic means. Suitable production techniques are well known to those of skill in the art. See, e.g., Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring  
25 Harbor Press (Cold Spring Harbor, NY). Alternatively, peptides can also be synthesized by the well known solid phase peptide synthesis methods (Merrifield, *J. Am. Chem. Soc.*, 85:2149 (1962); Stewart and Young, Solid Phase Peptide Synthesis (Freeman, San Francisco, 1969) pp. 27-62). These and other suitable production methods are within the knowledge of those of skill in the art and are not a limitation of the present invention.

30 In addition, one of skill in the art will readily understand that the Ad sequences of the invention can be readily adapted for use for a variety of viral and non-viral vector systems for *in vitro*, *ex vivo* or *in vivo* delivery of therapeutic and

immunogenic molecules. For example, in one embodiment, the simian Ad capsid proteins and other simian adenovirus proteins described herein are used for non-viral, protein-based delivery of genes, proteins, and other desirable diagnostic, therapeutic and immunogenic molecules. In one such embodiment, a protein of the invention is linked, directly or indirectly, to a molecule for targeting to cells with a receptor for adenoviruses. Preferably, a capsid protein such as a hexon, penton, fiber or a fragment thereof having a ligand for a cell surface receptor is selected for such targeting. Suitable molecules for delivery are selected from among the therapeutic molecules described herein and their gene products. A variety of linkers including, lipids, polyLys, and the like may be utilized as linkers. For example, the simian penton protein may be readily utilized for such a purpose by production of a fusion protein using the simian penton sequences in a manner analogous to that described in Medina-Kauwe LK, et al, *Gene Ther.* 2001 May; 8(10):795-803 and Medina-Kauwe LK, et al, *Gene Ther.* 2001 Dec; 8(23): 1753-1761. Alternatively, the amino acid sequences of simian Ad protein IX may be utilized for targeting vectors to a cell surface receptor, as described in US Patent Appln 20010047081. Suitable ligands include a CD40 antigen, an RGD-containing or polylysine-containing sequence, and the like. Still other simian Ad proteins, including, e.g., the hexon protein and/or the fiber protein, may be used for these and similar purposes.

Still other adenoviral proteins of the invention may be used as alone, or in combination with other adenoviral protein, for a variety of purposes which will be readily apparent to one of skill in the art. In addition, still other uses for the adenoviral proteins of the invention will be readily apparent to one of skill in the art.

## II. Recombinant Adenoviral Vectors

The compositions of this invention include vectors that deliver a heterologous molecule to cells, either for therapeutic or vaccine purposes. As used herein, a vector may include any genetic element including, without limitation, naked DNA, a phage, transposon, cosmid, episome, plasmid, or a virus. Such vectors contain simian adenovirus DNA of Pan5, Pan6, Pan7, SV1, SV25 and/or SV39 and a minigene. By "minigene" is meant the combination of a selected heterologous gene and the other

regulatory elements necessary to drive translation, transcription and/or expression of the gene product in a host cell.

Typically, an adenoviral vector of the invention is designed such that the minigene is located in a nucleic acid molecule which contains other adenoviral sequences in the region native to a selected adenoviral gene. The minigene may be inserted into an existing gene region to disrupt the function of that region, if desired. Alternatively, the minigene may be inserted into the site of a partially or fully deleted adenoviral gene. For example, the minigene may be located in the site of such as the site of a functional E1 deletion or functional E3 deletion, among others that may be selected. The term "functionally deleted" or "functional deletion" means that a sufficient amount of the gene region is removed or otherwise damaged, e.g., by mutation or modification, so that the gene region is no longer capable of producing functional products of gene expression. If desired, the entire gene region may be removed. Other suitable sites for gene disruption or deletion are discussed elsewhere in the application.

For example, for a production vector useful for generation of a recombinant virus, the vector may contain the minigene and either the 5' end of the adenoviral genome or the 3' end of the adenoviral genome, or both the 5' and 3' ends of the adenoviral genome. The 5' end of the adenoviral genome contains the 5' cis-elements necessary for packaging and replication; i.e., the 5' inverted terminal repeat (ITR) sequences (which functions as origins of replication) and the native 5' packaging enhancer domains (that contain sequences necessary for packaging linear Ad genomes and enhancer elements for the E1 promoter). The 3' end of the adenoviral genome includes the 3' cis-elements (including the ITRs) necessary for packaging and encapsidation. Suitably, a recombinant adenovirus contains both 5' and 3' adenoviral cis-elements and the minigene is located between the 5' and 3' adenoviral sequences. Any adenoviral vector of the invention may also contain additional adenoviral sequences.

Suitably, these adenoviral vectors of the invention contain one or more adenoviral elements derived from an adenoviral genome of the invention. In one embodiment, the vectors contain adenoviral ITRs from Pan5, Pan6, Pan7, SV1, SV25 or SV39 and additional adenoviral sequences from the same adenoviral serotype. In another embodiment, the vectors contain adenoviral sequences that are derived from a different

adenoviral serotype than that which provides the ITRs. As defined herein, a pseudotyped adenovirus refers to an adenovirus in which the capsid protein of the adenovirus is from a different serotype than the serotype which provides the ITRs. The selection of the serotype of the ITRs and the serotype of any other adenoviral sequences present in vector is not a limitation of the present invention. A variety of adenovirus strains are available from the American Type Culture Collection, Manassas, Virginia, or available by request from a variety of commercial and institutional sources. Further, the sequences of many such strains are available from a variety of databases including, e.g., PubMed and GenBank. Homologous adenovirus vectors prepared from other simian or from human adenoviruses are described in the published literature [see, for example, US Patent No. 5,240,846]. The DNA sequences of a number of adenovirus types are available from GenBank, including type Ad5 [GenBank Accession No. M73260]. The adenovirus sequences may be obtained from any known adenovirus serotype, such as serotypes 2, 3, 4, 7, 12 and 40, and further including any of the presently identified human types. Similarly adenoviruses known to infect non-human animals (e.g., simians) may also be employed in the vector constructs of this invention. See, e.g., US Patent No. 6,083,716.

The viral sequences, helper viruses, if needed, and recombinant viral particles, and other vector components and sequences employed in the construction of the vectors described herein are obtained as described above. The DNA sequences of the Pan5, Pan6, Pan7, SV1, SV25 and/or SV39 simian adenovirus sequences of the invention are employed to construct vectors and cell lines useful in the preparation of such vectors.

Modifications of the nucleic acid sequences forming the vectors of this invention, including sequence deletions, insertions, and other mutations may be generated using standard molecular biological techniques and are within the scope of this invention.

#### A. *The "Minigene"*

The methods employed for the selection of the transgene, the cloning and construction of the "minigene" and its insertion into the viral vector are within the skill in the art given the teachings provided herein.

##### 1. The transgene

The transgene is a nucleic acid sequence, heterologous to the vector sequences flanking the transgene, which encodes a polypeptide, protein, or other product, of interest. The nucleic acid coding sequence is operatively linked to regulatory

components in a manner which permits transgene transcription, translation, and/or expression in a host cell.

The composition of the transgene sequence will depend upon the use to which the resulting vector will be put. For example, one type of transgene sequence includes a reporter sequence, which upon expression produces a detectable signal. Such reporter sequences include, without limitation, DNA sequences encoding  $\beta$ -lactamase,  $\beta$ -galactosidase (LacZ), alkaline phosphatase, thymidine kinase, green fluorescent protein (GFP), chloramphenicol acetyltransferase (CAT), luciferase, membrane bound proteins including, for example, CD2, CD4, CD8, the influenza hemagglutinin protein, and others well known in the art, to which high affinity antibodies directed thereto exist or can be produced by conventional means, and fusion proteins comprising a membrane bound protein appropriately fused to an antigen tag domain from, among others, hemagglutinin or Myc. These coding sequences, when associated with regulatory elements which drive their expression, provide signals detectable by conventional means, including enzymatic, radiographic, colorimetric, fluorescence or other spectrographic assays, fluorescent activating cell sorting assays and immunological assays, including enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and immunohistochemistry. For example, where the marker sequence is the LacZ gene, the presence of the vector carrying the signal is detected by assays for beta-galactosidase activity. Where the transgene is GFP or luciferase, the vector carrying the signal may be measured visually by color or light production in a luminometer.

However, desirably, the transgene is a non-marker sequence encoding a product which is useful in biology and medicine, such as proteins, peptides, RNA, enzymes, or catalytic RNAs. Desirable RNA molecules include tRNA, dsRNA, ribosomal RNA, catalytic RNAs, and antisense RNAs. One example of a useful RNA sequence is a sequence which extinguishes expression of a targeted nucleic acid sequence in the treated animal.

The transgene may be used for treatment, e.g., of genetic deficiencies, as a cancer therapeutic or vaccine, for induction of an immune response, and/or for prophylactic vaccine purposes. As used herein, induction of an immune response refers to the ability of a molecule (e.g., a gene product) to induce a T cell and/or a humoral immune response to the molecule. The invention further includes using multiple

transgenes, e.g., to correct or ameliorate a condition caused by a multi-subunit protein. In certain situations, a different transgene may be used to encode each subunit of a protein, or to encode different peptides or proteins. This is desirable when the size of the DNA encoding the protein subunit is large, e.g., for an immunoglobulin, the platelet-derived growth factor, or a dystrophin protein. In order for the cell to produce the multi-subunit protein, a cell is infected with the recombinant virus containing each of the different subunits. Alternatively, different subunits of a protein may be encoded by the same transgene. In this case, a single transgene includes the DNA encoding each of the subunits, with the DNA for each subunit separated by an internal ribozyme entry site (IRES). This is desirable when the size of the DNA encoding each of the subunits is small, e.g., the total size of the DNA encoding the subunits and the IRES is less than five kilobases. As an alternative to an IRES, the DNA may be separated by sequences encoding a 2A peptide, which self-cleaves in a post-translational event. See, e.g., M.L. Donnelly, *et al*, *J. Gen. Virol.*, 78(Pt 1):13-21 (Jan 1997); Furler, S., *et al*, *Gene Ther.*, 8(11):864-873 (June 2001); Klump H., *et al.*, *Gene Ther.*, 8(10):811-817 (May 2001). This 2A peptide is significantly smaller than an IRES, making it well suited for use when space is a limiting factor. However, the selected transgene may encode any biologically active product or other product, e.g., a product desirable for study.

Suitable transgenes may be readily selected by one of skill in the art. The selection of the transgene is not considered to be a limitation of this invention.

## 2. Regulatory Elements

In addition to the major elements identified above for the minigene, the vector also includes conventional control elements necessary which are operably linked to the transgene in a manner that permits its transcription, translation and/or expression in a cell transfected with the plasmid vector or infected with the virus produced by the invention. As used herein, "operably linked" sequences include both expression control sequences that are contiguous with the gene of interest and expression control sequences that act in *trans* or at a distance to control the gene of interest.

Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (i.e., Kozak consensus

sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. A great number of expression control sequences, including promoters which are native, constitutive, inducible and/or tissue-specific, are known in the art and may be utilized.

5                   Examples of constitutive promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, e.g., Boshart *et al*, *Cell*, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the  $\beta$ -actin promoter, the phosphoglycerol kinase (PGK) promoter, 10                   and the EF1 $\alpha$  promoter [Invitrogen].

                  Inducible promoters allow regulation of gene expression and can be regulated by exogenously supplied compounds, environmental factors such as temperature, or the presence of a specific physiological state, e.g., acute phase, a particular differentiation state of the cell, or in replicating cells only. Inducible 15                   promoters and inducible systems are available from a variety of commercial sources, including, without limitation, Invitrogen, Clontech and Ariad. Many other systems have been described and can be readily selected by one of skill in the art. For example, inducible promoters include the zinc-inducible sheep metallothioneine (MT) promoter and the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter. 20                   Other inducible systems include the T7 polymerase promoter system [WO 98/10088]; the ecdysone insect promoter [No *et al*, *Proc. Natl. Acad. Sci. USA*, 93:3346-3351 (1996)], the tetracycline-repressible system [Gossen *et al*, *Proc. Natl. Acad. Sci. USA*, 89:5547-5551 (1992)], the tetracycline-inducible system [Gossen *et al*, *Science*, 268:1766-1769 (1995), see also Harvey *et al*, *Curr. Opin. Chem. Biol.*, 2:512-518 (1998)]. Other systems 25                   include the FK506 dimer, VP16 or p65 using castradiol, diphenol murislerone, the RU486-inducible system [Wang *et al*, *Nat. Biotech.*, 15:239-243 (1997) and Wang *et al*, *Gene Ther.*, 4:432-441 (1997)] and the rapamycin-inducible system [Magari *et al*, *J. Clin. Invest.*, 100:2865-2872 (1997)]. The effectiveness of some inducible promoters increases over time. In such cases one can enhance the effectiveness of such systems by 30                   inserting multiple repressors in tandem, e.g., TetR linked to a TetR by an IRES. Alternatively, one can wait at least 3 days before screening for the desired function. One can enhance expression of desired proteins by known means to enhance the effectiveness

of this system. For example, using the Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE).

In another embodiment, the native promoter for the transgene will be used. The native promoter may be preferred when it is desired that expression of the transgene should mimic the native expression. The native promoter may be used when expression of the transgene must be regulated temporally or developmentally, or in a tissue-specific manner, or in response to specific transcriptional stimuli. In a further embodiment, other native expression control elements, such as enhancer elements, polyadenylation sites or Kozak consensus sequences may also be used to mimic the native expression.

Another embodiment of the transgene includes a transgene operably linked to a tissue-specific promoter. For instance, if expression in skeletal muscle is desired, a promoter active in muscle should be used. These include the promoters from genes encoding skeletal  $\beta$ -actin, myosin light chain 2A, dystrophin, muscle creatine kinase, as well as synthetic muscle promoters with activities higher than naturally occurring promoters (see Li *et al.*, *Nat. Biotech.*, **17**:241-245 (1999)). Examples of promoters that are tissue-specific are known for liver (albumin, Miyatake *et al.*, *J. Virol.*, **71**:5124-32 (1997); hepatitis B virus core promoter, Sandig *et al.*, *Gene Ther.*, **3**:1002-9 (1996); alpha-fetoprotein (AFP), Arbuthnot *et al.*, *Hum. Gene Ther.*, **7**:1503-14 (1996)), bone osteocalcin (Stein *et al.*, *Mol. Biol. Rep.*, **24**:185-96 (1997)); bone sialoprotein (Chen *et al.*, *J. Bone Miner. Res.*, **11**:654-64 (1996)), lymphocytes (CD2, Hansal *et al.*, *J. Immunol.*, **161**:1063-8 (1998); immunoglobulin heavy chain; T cell receptor chain), neuronal such as neuron-specific enolase (NSE) promoter (Andersen *et al.*, *Cell. Mol. Neurobiol.*, **13**:503-15 (1993)), neurofilament light-chain gene (Piccioli *et al.*, *Proc. Natl. Acad. Sci. USA*, **88**:5611-5 (1991)), and the neuron-specific vgf gene (Piccioli *et al.*, *Neuron*, **15**:373-84 (1995)), among others.

Optionally, vectors carrying transgenes encoding therapeutically useful or immunogenic products may also include selectable markers or reporter genes may include sequences encoding geneticin, hygromycin or purimycin resistance, among others. Such selectable reporters or marker genes (preferably located outside the viral genome to be packaged into a viral particle) can be used to signal the presence of the plasmids in bacterial cells, such as ampicillin resistance. Other components of the vector may include

an origin of replication. Selection of these and other promoters and vector elements are conventional and many such sequences are available [see, e.g., Sambrook et al, and references cited therein].

These vectors are generated using the techniques and sequences provided  
5 herein, in conjunction with techniques known to those of skill in the art.  
Such techniques include conventional cloning techniques of cDNA such as those described in texts [Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY], use of overlapping oligonucleotide sequences of the adenovirus genomes, polymerase chain reaction, and any suitable  
10 method which provides the desired nucleotide sequence.

### III. Production of the Recombinant Viral Particle

In one embodiment, the simian adenoviral plasmids (or other vectors) are used to produce recombinant adenoviral particles. In one embodiment, the recombinant  
15 adenoviruses are functionally deleted in the E1a or E1b genes, and optionally bearing other mutations, e.g., temperature-sensitive mutations or deletions in other genes. In other embodiments, it is desirable to retain an intact E1a and/or E1b region in the recombinant adenoviruses. Such an intact E1 region may be located in its native location in the adenoviral genome or placed in the site of a deletion in the native adenoviral  
20 genome (e.g., in the E3 region).

In the construction of useful simian adenovirus vectors for delivery of a gene to the human (or other mammalian) cell, a range of adenovirus nucleic acid sequences can be employed in the vectors. For example, all or a portion of the adenovirus delayed early gene E3 may be eliminated from the simian adenovirus sequence which forms a part of  
25 the recombinant virus. The function of simian E3 is believed to be irrelevant to the function and production of the recombinant virus particle. Simian adenovirus vectors may also be constructed having a deletion of at least the ORF6 region of the E4 gene, and more desirably because of the redundancy in the function of this region, the entire E4 region. Still another vector of this invention contains a deletion in the delayed early gene  
30 E2a. Deletions may also be made in any of the late genes L1 through L5 of the simian adenovirus genome. Similarly, deletions in the intermediate genes IX and IVa<sub>2</sub> may be useful for some purposes. Other deletions may be made in the other structural or non-

structural adenovirus genes. The above discussed deletions may be used individually, i.e., an adenovirus sequence for use in the present invention may contain deletions in only a single region. Alternatively, deletions of entire genes or portions thereof effective to destroy their biological activity may be used in any combination. For example, in one  
5 exemplary vector, the adenovirus sequence may have deletions of the E1 genes and the E4 gene, or of the E1, E2a and E3 genes, or of the E1 and E3 genes, or of E1, E2a and E4 genes, with or without deletion of E3, and so on. As discussed above, such deletions may be used in combination with other mutations, such as temperature-sensitive mutations, to achieve a desired result.

10 An adenoviral vector lacking any essential adenoviral sequences (e.g., E1a, E1b, E2a, E2b, E4 ORF6, L1, L2, L3, L4 and L5) may be cultured in the presence of the missing adenoviral gene products which are required for viral infectivity and propagation of an adenoviral particle. These helper functions may be provided by culturing the adenoviral vector in the presence of one or more helper constructs (e.g., a plasmid or  
15 virus) or a packaging host cell. See, for example, the techniques described for preparation of a "minimal" human Ad vector in International Patent Application WO96/13597, published May 9, 1996, and incorporated herein by reference.

#### 1. Helper Viruses

Thus, depending upon the simian adenovirus gene content of the  
20 viral vectors employed to carry the minigene, a helper adenovirus or non-replicating virus fragment may be necessary to provide sufficient simian adenovirus gene sequences necessary to produce an infective recombinant viral particle containing the minigene. Useful helper viruses contain selected adenovirus gene sequences not present in the adenovirus vector construct and/or not expressed by the packaging cell line in which the  
25 vector is transfected. In one embodiment, the helper virus is replication-defective and contains a variety of adenovirus genes in addition to the sequences described above. Such a helper virus is desirably used in combination with an E1-expressing cell line.

Helper viruses may also be formed into poly-cation conjugates as described in Wu *et al*, *J. Biol. Chem.*, 264:16985-16987 (1989); K. J. Fisher and J. M.  
30 Wilson, *Biochem. J.*, 299:49 (April 1, 1994). Helper virus may optionally contain a second reporter minigene. A number of such reporter genes are known to the art. The presence of a reporter gene on the helper virus which is different from the transgene on

the adenovirus vector allows both the Ad vector and the helper virus to be independently monitored. This second reporter is used to enable separation between the resulting recombinant virus and the helper virus upon purification.

## 2. Complementation Cell Lines

5 To generate recombinant simian adenoviruses (Ad) deleted in any of the genes described above, the function of the deleted gene region, if essential to the replication and infectivity of the virus, must be supplied to the recombinant virus by a helper virus or cell line, i.e., a complementation or packaging cell line. In many circumstances, a cell line expressing the human E1 can be used to transcomplement the  
10 chimp Ad vector. This is particularly advantageous because, due to the diversity between the chimp Ad sequences of the invention and the human AdE1 sequences found in currently available packaging cells, the use of the current human E1-containing cells prevents the generation of replication-competent adenoviruses during the replication and production process. However, in certain circumstances, it will be desirable to utilize a  
15 cell line which expresses the E1 gene products can be utilized for production of an E1-deleted simian adenovirus. Such cell lines have been described. See, e.g., US Patent 6,083,716.

If desired, one may utilize the sequences provided herein to generate a packaging cell or cell line that expresses, at a minimum, the adenovirus E1  
20 gene from Pan5, Pan6, Pan7, SV1, SV25 or SV39 under the transcriptional control of a promoter for expression in a selected parent cell line. Inducible or constitutive promoters may be employed for this purpose. Examples of such promoters are described in detail elsewhere in this specification. A parent cell is selected for the generation of a novel cell line expressing any desired AdPan5, Pan6, Pan7, SV1, SV25 or SV39 gene. Without  
25 limitation, such a parent cell line may be HeLa [ATCC Accession No. CCL 2], A549 [ATCC Accession No. CCL 185], HEK 293, KB [CCL 17], Detroit [e.g., Detroit 510, CCL 72] and WI-38 [CCL 75] cells, among others. These cell lines are all available from the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209. Other suitable parent cell lines may be obtained from other sources.

30 Such E1-expressing cell lines are useful in the generation of recombinant simian adenovirus E1 deleted vectors. Additionally, or alternatively, the invention provides cell lines that express one or more simian adenoviral gene products,

e.g., E1a, E1b, E2a, and/or E4 ORF6, can be constructed using essentially the same procedures for use in the generation of recombinant simian viral vectors. Such cell lines can be utilized to transcomplement adenovirus vectors deleted in the essential genes that encode those products, or to provide helper functions necessary for packaging of a helper-dependent virus (e.g., adeno-associated virus). The preparation of a host cell according to this invention involves techniques such as assembly of selected DNA sequences. This assembly may be accomplished utilizing conventional techniques. Such techniques include cDNA and genomic cloning, which are well known and are described in Sambrook et al., cited above, use of overlapping oligonucleotide sequences of the adenovirus genomes, combined with polymerase chain reaction, synthetic methods, and any other suitable methods which provide the desired nucleotide sequence.

In still another alternative, the essential adenoviral gene products are provided in *trans* by the adenoviral vector and/or helper virus. In such an instance, a suitable host cell can be selected from any biological organism, including prokaryotic (e.g., bacterial) cells, and eukaryotic cells, including, insect cells, yeast cells and mammalian cells. Particularly desirable host cells are selected from among any mammalian species, including, without limitation, cells such as A549, WEHI, 3T3, 10T1/2, HEK 293 cells or PERC6 (both of which express functional adenoviral E1) [Fallaux, FJ *et al.*, (1998), *Hum Gene Ther*, 9:1909-1917], Saos, C2C12, L cells, HT1080, HepG2 and primary fibroblast, hepatocyte and myoblast cells derived from mammals including human, monkey, mouse, rat, rabbit, and hamster. The selection of the mammalian species providing the cells is not a limitation of this invention; nor is the type of mammalian cell, i.e., fibroblast, hepatocyte, tumor cell, etc.

### 3. Assembly of Viral Particle and Transfection of a Cell Line

Generally, when delivering the vector comprising the minigene by transfection, the vector is delivered in an amount from about 5  $\mu$ g to about 100  $\mu$ g DNA, and preferably about 10 to about 50  $\mu$ g DNA to about  $1 \times 10^4$  cells to about  $1 \times 10^{13}$  cells, and preferably about  $10^5$  cells. However, the relative amounts of vector DNA to host cells may be adjusted, taking into consideration such factors as the selected vector, the delivery method and the host cells selected.

The vector may be any vector known in the art or disclosed above, including naked DNA, a plasmid, phage, transposon, cosmids, episomes, viruses, etc.

Introduction into the host cell of the vector may be achieved by any means known in the art or as disclosed above, including transfection, and infection. One or more of the adenoviral genes may be stably integrated into the genome of the host cell, stably expressed as episomes, or expressed transiently. The gene products may all be expressed transiently, on an episome or stably integrated, or some of the gene products may be expressed stably while others are expressed transiently. Furthermore, the promoters for each of the adenoviral genes may be selected independently from a constitutive promoter, an inducible promoter or a native adenoviral promoter. The promoters may be regulated by a specific physiological state of the organism or cell (i.e., by the differentiation state or in replicating or quiescent cells) or by exogenously-added factors, for example.

Introduction of the molecules (as plasmids or viruses) into the host cell may also be accomplished using techniques known to the skilled artisan and as discussed throughout the specification. In preferred embodiment, standard transfection techniques are used, e.g.,  $\text{CaPO}_4$  transfection or electroporation.

Assembly of the selected DNA sequences of the adenovirus (as well as the transgene and other vector elements into various intermediate plasmids, and the use of the plasmids and vectors to produce a recombinant viral particle are all achieved using conventional techniques. Such techniques include conventional cloning techniques of cDNA such as those described in texts [Sambrook et al, cited above], use of overlapping oligonucleotide sequences of the adenovirus genomes, polymerase chain reaction, and any suitable method which provides the desired nucleotide sequence. Standard transfection and co-transfection techniques are employed, e.g.,  $\text{CaPO}_4$  precipitation techniques. Other conventional methods employed include homologous recombination of the viral genomes, plaquing of viruses in agar overlay, methods of measuring signal generation, and the like.

For example, following the construction and assembly of the desired minigene-containing viral vector, the vector is transfected *in vitro* in the presence of a helper virus into the packaging cell line. Homologous recombination occurs between the helper and the vector sequences, which permits the adenovirus-transgene sequences in the vector to be replicated and packaged into virion capsids, resulting in the recombinant viral vector particles. The current method for producing such virus particles is transfection-based. However, the invention is not limited to such methods.

The resulting recombinant simian adenoviruses are useful in transferring a selected transgene to a selected cell. In *in vivo* experiments with the recombinant virus grown in the packaging cell lines, the E1-deleted recombinant simian adenoviral vectors of the invention demonstrate utility in transferring a transgene to a non-simian, preferably a human, cell.

#### IV. Use of the Recombinant Adenovirus Vectors

The recombinant simian adenovirus vectors of the invention are useful for gene transfer to a human or non-simian veterinary patient *in vitro*, *ex vivo*, and *in vivo*.

The recombinant adenovirus vectors described herein can be used as expression vectors for the production of the products encoded by the heterologous genes *in vitro*. For example, the recombinant adenoviruses containing a gene inserted into the location of an E1 deletion may be transfected into an E1-expressing cell line as described above. Alternatively, replication-competent adenoviruses may be used in another selected cell line. The transfected cells are then cultured in the conventional manner, allowing the recombinant adenovirus to express the gene product from the promoter. The gene product may then be recovered from the culture medium by known conventional methods of protein isolation and recovery from culture.

A Pan5, Pan6, Pan7, SV1, SV25 or SV39-derived recombinant simian adenoviral vector of the invention provides an efficient gene transfer vehicle that can deliver a selected transgene to a selected host cell *in vivo* or *ex vivo* even where the organism has neutralizing antibodies to one or more AAV serotypes. In one embodiment, the rAAV and the cells are mixed *ex vivo*; the infected cells are cultured using conventional methodologies; and the transduced cells are re-infused into the patient. These compositions are particularly well suited to gene delivery for therapeutic purposes and for immunization, including inducing protective immunity.

More commonly, the Pan 5, Pan6, Pan7, SV1, SV25, or SV39 recombinant adenoviral vectors of the invention will be utilized for delivery of therapeutic or immunogenic molecules, as described below. It will be readily understood for both applications, that the recombinant adenoviral vectors of the invention are particularly well suited for use in regimens involving repeat delivery of recombinant adenoviral vectors. Such regimens typically involve delivery of a series of viral vectors in which the viral

capsids are alternated. The viral capsids may be changed for each subsequent administration, or after a pre-selected number of administrations of a particular serotype capsid (e.g., one, two, three, four or more). Thus, a regimen may involve delivery of a rAd with a first simian capsid, delivery with a rAd with a second simian capsid, and  
5 delivery with a third simian capsid. A variety of other regimens which use the Ad capsids of the invention alone, in combination with one another, or in combination with other Ad serotypes will be apparent to those of skill in the art. Optionally, such a regimen may involve administration of rAd with capsids of other non-human primate adenoviruses, human adenoviruses, or artificial serotypes such as are described herein. Each phase of  
10 the regimen may involve administration of a series of injections (or other delivery routes) with a single Ad serotype capsid followed by a series with another Ad serotype capsid. Alternatively, the recombinant Ad vectors of the invention may be utilized in regimens involving other non-adenoviral-mediated delivery systems, including other viral systems, non-viral delivery systems, protein, peptides, and other biologically active molecules.

15 The following sections will focus on exemplary molecules which may be delivered via the adenoviral vectors of the invention.

#### A. Ad-Mediated Delivery of Therapeutic Molecules

In one embodiment, the above-described recombinant vectors are administered to humans according to published methods for gene therapy. A simian viral  
20 vector bearing the selected transgene may be administered to a patient, preferably suspended in a biologically compatible solution or pharmaceutically acceptable delivery vehicle. A suitable vehicle includes sterile saline. Other aqueous and non-aqueous isotonic sterile injection solutions and aqueous and non-aqueous sterile suspensions known to be pharmaceutically acceptable carriers and well known to those of skill in the  
25 art may be employed for this purpose.

The simian adenoviral vectors are administered in sufficient amounts to transduce the target cells and to provide sufficient levels of gene transfer and expression to provide a therapeutic benefit without undue adverse or with medically acceptable physiological effects, which can be determined by those skilled in the medical arts.  
30 Conventional and pharmaceutically acceptable routes of administration include, but are not limited to, direct delivery to the retina and other intraocular delivery methods, direct delivery to the liver, inhalation, intranasal, intravenous, intramuscular, intratracheal,

subcutaneous, intradermal, rectal, oral and other parenteral routes of administration. Routes of administration may be combined, if desired, or adjusted depending upon the transgene or the condition. The route of administration primarily will depend on the nature of the condition being treated.

5                    Dosages of the viral vector will depend primarily on factors such as the condition being treated, the age, weight and health of the patient, and may thus vary among patients. For example, a therapeutically effective adult human or veterinary dosage of the viral vector is generally in the range of from about 100  $\mu$ L to about 100 mL of a carrier containing concentrations of from about  $1 \times 10^6$  to about  $1 \times 10^{15}$  particles, 10    about  $1 \times 10^{11}$  to  $1 \times 10^{13}$  particles, or about  $1 \times 10^9$  to  $1 \times 10^{12}$  particles virus. Dosages will range depending upon the size of the animal and the route of administration. For example, a suitable human or veterinary dosage (for about an 80 kg animal) for intramuscular injection is in the range of about  $1 \times 10^9$  to about  $5 \times 10^{12}$  particles per mL, for a single site. Optionally, multiple sites of administration may be delivered. In another 15    example, a suitable human or veterinary dosage may be in the range of about  $1 \times 10^{11}$  to about  $1 \times 10^{15}$  particles for an oral formulation. One of skill in the art may adjust these doses, depending the route of administration, and the therapeutic or vaccinal application for which the recombinant vector is employed. The levels of expression of the transgene, or for an immunogen, the level of circulating antibody, can be monitored to determine the 20    frequency of dosage administration. Yet other methods for determining the timing of frequency of administration will be readily apparent to one of skill in the art.

                    An optional method step involves the co-administration to the patient, either concurrently with, or before or after administration of the viral vector, of a suitable amount of a short acting immune modulator. The selected immune modulator is defined 25    herein as an agent capable of inhibiting the formation of neutralizing antibodies directed against the recombinant vector of this invention or capable of inhibiting cytolytic T lymphocyte (CTL) elimination of the vector. The immune modulator may interfere with the interactions between the T helper subsets ( $T_{H1}$  or  $T_{H2}$ ) and B cells to inhibit neutralizing antibody formation. Alternatively, the immune modulator may inhibit the 30    interaction between  $T_{H1}$  cells and CTLs to reduce the occurrence of CTL elimination of the vector. A variety of useful immune modulators and dosages for use of same are disclosed, for example, in Yang *et al.*, *J. Virol.*, 70(9) (Sept., 1996); International Patent

Application No. WO96/12406, published May 2, 1996; and International Patent Application No. PCT/US96/03035, all incorporated herein by reference.

#### 1. Therapeutic Transgenes

Useful therapeutic products encoded by the transgene include

5 hormones and growth and differentiation factors including, without limitation, insulin, glucagon, growth hormone (GH), parathyroid hormone (PTH), growth hormone releasing factor (GRF), follicle stimulating hormone (FSH), luteinizing hormone (LH), human chorionic gonadotropin (hCG), vascular endothelial growth factor (VEGF), angiopoietins, angiostatin, granulocyte colony stimulating factor (GCSF), erythropoietin (EPO),

10 connective tissue growth factor (CTGF), basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), epidermal growth factor (EGF), transforming growth factor (TGF), platelet-derived growth factor (PDGF), insulin growth factors I and II (IGF-I and IGF-II), any one of the transforming growth factor superfamily, including TGF, activins, inhibins, or any of the bone morphogenic proteins (BMP) BMPs 1-15, any

15 one of the heregulin/neuregulin/ARIA/neu differentiation factor (NDF) family of growth factors, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins NT-3 and NT-4/5, ciliary neurotrophic factor (CNTF), glial cell line derived neurotrophic factor (GDNF), neurturin, agrin, any one of the family of semaphorins/collapsins, netrin-1 and netrin-2, hepatocyte growth factor (HGF), ephrins,

20 noggin, sonic hedgehog and tyrosine hydroxylase.

Other useful transgene products include proteins that regulate the immune system including, without limitation, cytokines and lymphokines such as thrombopoietin (TPO), interleukins (IL) IL-1 through IL-25 (including, e.g., IL-2, IL-4, IL-12 and IL-18), monocyte chemoattractant protein, leukemia inhibitory factor,

25 granulocyte-macrophage colony stimulating factor, Fas ligand, tumor necrosis factors and, interferons, and, stem cell factor, flk-2/flt3 ligand. Gene products produced by the immune system are also useful in the invention. These include, without limitation, immunoglobulins IgG, IgM, IgA, IgD and IgE, chimeric immunoglobulins, humanized antibodies, single chain antibodies, T cell receptors, chimeric T cell receptors, single

30 chain T cell receptors, class I and class II MHC molecules, as well as engineered immunoglobulins and MHC molecules. Useful gene products also include complement

regulatory proteins such as complement regulatory proteins, membrane cofactor protein (MCP), decay accelerating factor (DAF), CR1, CF2 and CD59.

Still other useful gene products include any one of the receptors for the hormones, growth factors, cytokines, lymphokines, regulatory proteins and immune system proteins. The invention encompasses receptors for cholesterol regulation, including the low density lipoprotein (LDL) receptor, high density lipoprotein (HDL) receptor, the very low density lipoprotein (VLDL) receptor, and the scavenger receptor. The invention also encompasses gene products such as members of the steroid hormone receptor superfamily including glucocorticoid receptors and estrogen receptors, Vitamin D receptors and other nuclear receptors. In addition, useful gene products include transcription factors such as *jun*, *fos*, max, mad, serum response factor (SRF), AP-1, AP2, *myb*, MyoD and myogenin, ETS-box containing proteins, TFE3, E2F, ATF1, ATF2, ATF3, ATF4, ZF5, NFAT, CREB, HNF-4, C/EBP, SP1, CCAAT-box binding proteins, interferon regulation factor (IRF-1), Wilms tumor protein, ETS-binding protein, STAT, GATA-box binding proteins, e.g., GATA-3, and the forkhead family of winged helix proteins.

Other useful gene products include, carbamoyl synthetase I, ornithine transcarbamylase, arginosuccinate synthetase, arginosuccinate lyase, arginase, fumarylacetylacetate hydrolase, phenylalanine hydroxylase, alpha-1 antitrypsin, glucose-6-phosphatase, porphobilinogen deaminase, factor VIII, factor IX, cystathione beta-synthase, branched chain ketoacid decarboxylase, albumin, isovaleryl-coA dehydrogenase, propionyl CoA carboxylase, methyl malonyl CoA mutase, glutaryl CoA dehydrogenase, insulin, beta-glucosidase, pyruvate carboxylate, hepatic phosphorylase, phosphorylase kinase, glycine decarboxylase, H-protein, T-protein, a cystic fibrosis transmembrane regulator (CFTR) sequence, and a dystrophin cDNA sequence.

Other useful gene products include non-naturally occurring polypeptides, such as chimeric or hybrid polypeptides having a non-naturally occurring amino acid sequence containing insertions, deletions or amino acid substitutions. For example, single-chain engineered immunoglobulins could be useful in certain immunocompromised patients. Other types of non-naturally occurring gene sequences include antisense molecules and catalytic nucleic acids, such as ribozymes, which could be used to reduce overexpression of a target.

Reduction and/or modulation of expression of a gene are particularly desirable for treatment of hyperproliferative conditions characterized by hyperproliferating cells, as are cancers and psoriasis. Target polypeptides include those polypeptides which are produced exclusively or at higher levels in hyperproliferative cells  
5 as compared to normal cells. Target antigens include polypeptides encoded by oncogenes such as myb, myc, fyn, and the translocation gene bcr/abl, ras, src, P53, neu, trk and EGRF. In addition to oncogene products as target antigens, target polypeptides for anti-cancer treatments and protective regimens include variable regions of antibodies made by B cell lymphomas and variable regions of T cell receptors of T cell lymphomas  
10 which, in some embodiments, are also used as target antigens for autoimmune disease. Other tumor-associated polypeptides can be used as target polypeptides such as polypeptides which are found at higher levels in tumor cells including the polypeptide recognized by monoclonal antibody 17-1A and folate binding polypeptides.

Other suitable therapeutic polypeptides and proteins include those  
15 which may be useful for treating individuals suffering from autoimmune diseases and disorders by conferring a broad based protective immune response against targets that are associated with autoimmunity including cell receptors and cells which produce self-directed antibodies. T cell mediated autoimmune diseases include Rheumatoid arthritis (RA), multiple sclerosis (MS), Sjögren's syndrome, sarcoidosis, insulin dependent  
20 diabetes mellitus (IDDM), autoimmune thyroiditis, reactive arthritis, ankylosing spondylitis, scleroderma, polymyositis, dermatomyositis, psoriasis, vasculitis, Wegener's granulomatosis, Crohn's disease and ulcerative colitis. Each of these diseases is characterized by T cell receptors (TCRs) that bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases.

25 The simian adenoviral vectors of the invention are particularly well suited for therapeutic regimens in which multiple adenoviral-mediated deliveries of transgenes is desired, e.g., in regimens involving redelivery of the same transgene or in combination regimens involving delivery of other transgenes. Such regimens may involve administration of a Pan5, Pan6, Pan7, SV1, SV25 or SV39 simian adenoviral vector,  
30 followed by re-administration with a vector from the same serotype adenovirus. Particularly desirable regimens involve administration of a Pan5, Pan6, Pan7, SV1, SV25 or SV39 simian adenoviral vector of the invention, in which the serotype of the viral

vector delivered in the first administration differs from the serotype of the viral vector utilized in one or more of the subsequent administrations. For example, a therapeutic regimen involves administration of a Pan5, Pan6, Pan7, SV1, SV25 or SV39 vector and repeat administration with one or more adenoviral vectors of the same or different serotypes. In another example, a therapeutic regimen involves administration of an adenoviral vector followed by repeat administration with a Pan5, Pan6, Pan7, SV1, SV25 or SV39 vector of the invention which differs from the serotype of the first delivered adenoviral vector, and optionally further administration with another vector which is the same or, preferably, differs from the serotype of the vector in the prior administration steps. These regimens are not limited to delivery of adenoviral vectors constructed using the Pan5, Pan6, Pan7, SV1, SV25 or SV39 simian serotypes of the invention. Rather, these regimens can readily utilize vectors other adenoviral serotypes, including, without limitation, other simian adenoviral serotypes (e.g., Pan9 or C68, C1, etc), other non-human primate adenoviral serotypes, or human adenoviral serotypes, in combination with one or more of the Pan5, Pan6, Pan7, SV1, SV25 or SV39 vectors of the invention. Examples of such simian, other non-human primate and human adenoviral serotypes are discussed elsewhere in this document. Further, these therapeutic regimens may involve either simultaneous or sequential delivery of Pan 5, Pan6, Pan7, SV1, SV25, and/or SV39 adenoviral vectors of the invention in combination with non-adenoviral vectors, non-viral vectors, and/or a variety of other therapeutically useful compounds or molecules. The present invention is not limited to these therapeutic regimens, a variety of which will be readily apparent to one of skill in the art.

#### B. Ad-Mediated Delivery of Immunogenic Transgenes

The recombinant simian adenoviruses may also be employed as immunogenic compositions. As used herein, an immunogenic composition is a composition to which a humoral (e.g., antibody) or cellular (e.g., a cytotoxic T cell) response is mounted to a transgene product delivered by the immunogenic composition following delivery to a mammal, and preferably a primate. The present invention provides a recombinant simian Ad that can contain in any of its adenovirus sequence deletions a gene encoding a desired immunogen. The simian adenovirus is likely to be better suited for use as a live recombinant virus vaccine in different animal species compared to an adenovirus of human origin, but is not limited to such a use. The

recombinant adenoviruses can be used as prophylactic or therapeutic vaccines against any pathogen for which the antigen(s) crucial for induction of an immune response and able to limit the spread of the pathogen has been identified and for which the cDNA is available.

Such vaccinal (or other immunogenic) compositions are formulated in a suitable delivery vehicle, as described above. Generally, doses for the immunogenic compositions are in the range defined above for therapeutic compositions. The levels of immunity of the selected gene can be monitored to determine the need, if any, for boosters. Following an assessment of antibody titers in the serum, optional booster immunizations may be desired.

Optionally, a vaccinal composition of the invention may be formulated to contain other components, including, e.g. adjuvants, stabilizers, pH adjusters, preservatives and the like. Such components are well known to those of skill in the vaccine art. Examples of suitable adjuvants include, without limitation, liposomes, alum, monophosphoryl lipid A, and any biologically active factor, such as cytokine, an interleukin, a chemokine, a ligands, and optionally combinations thereof. Certain of these biologically active factors can be expressed *in vivo*, e.g., via a plasmid or viral vector. For example, such an adjuvant can be administered with a priming DNA vaccine encoding an antigen to enhance the antigen-specific immune response compared with the immune response generated upon priming with a DNA vaccine encoding the antigen only.

The recombinant adenoviruses are administered in a "an immunogenic amount", that is, an amount of recombinant adenovirus that is effective in a route of administration to transfect the desired cells and provide sufficient levels of expression of the selected gene to induce an immune response. Where protective immunity is provided, the recombinant adenoviruses are considered to be vaccine compositions useful in preventing infection and/or recurrent disease.

Alternatively, or in addition, the vectors of the invention may contain a transgene encoding a peptide, polypeptide or protein which induces an immune response to a selected immunogen. The recombinant adenoviruses of this invention are expected to be highly efficacious at inducing cytolytic T cells and antibodies to the inserted heterologous antigenic protein expressed by the vector.

For example, immunogens may be selected from a variety of viral families. Example of desirable viral families against which an immune response would be desirable include, the picornavirus family, which includes the genera rhinoviruses, which are responsible for about 50% of cases of the common cold; the genera

5 enteroviruses, which include polioviruses, coxsackieviruses, echoviruses, and human enteroviruses such as hepatitis A virus; and the genera apthoviruses, which are responsible for foot and mouth diseases, primarily in non-human animals. Within the picornavirus family of viruses, target antigens include the VP1, VP2, VP3, VP4, and VPG. Another viral family includes the calcivirus family, which encompasses the

10 Norwalk group of viruses, which are an important causative agent of epidemic gastroenteritis. Still another viral family desirable for use in targeting antigens for inducing immune responses in humans and non-human animals is the togavirus family, which includes the genera alphavirus, which include Sindbis viruses, Ross River virus, and Venezuelan, Eastern & Western Equine encephalitis, and rubivirus, including Rubella

15 virus. The flaviviridae family includes dengue, yellow fever, Japanese encephalitis, St. Louis encephalitis and tick borne encephalitis viruses. Other target antigens may be generated from the Hepatitis C or the coronavirus family, which includes a number of non-human viruses such as infectious bronchitis virus (poultry), porcine transmissible gastroenteric virus (pig), porcine hemagglutinating encephalomyelitis virus (pig), feline

20 infectious peritonitis virus (cats), feline enteric coronavirus (cat), canine coronavirus (dog), and human respiratory coronaviruses, which may cause the common cold and/or non-A, B or C hepatitis. Within the coronavirus family, target antigens include the E1 (also called M or matrix protein), E2 (also called S or Spike protein), E3 (also called HE or hemagglutinin-esterase) glycoprotein (not present in all coronaviruses), or N

25 (nucleocapsid). Still other antigens may be targeted against the rhabdovirus family, which includes the genera vesiculovirus (e.g., Vesicular Stomatitis Virus), and the general lyssavirus (e.g., rabies). Within the rhabdovirus family, suitable antigens may be derived from the G protein or the N protein. The family filoviridae, which includes hemorrhagic fever viruses such as Marburg and Ebola virus, may be a suitable source of antigens. The

30 paramyxovirus family includes parainfluenza Virus Type 1, parainfluenza Virus Type 3, bovine parainfluenza Virus Type 3, rubulavirus (mumps virus), parainfluenza Virus Type 2, parainfluenza virus Type 4, Newcastle disease virus (chickens), rinderpest,

morbillivirus, which includes measles and canine distemper, and pneumovirus, which includes respiratory syncytial virus. The influenza virus is classified within the family orthomyxovirus and is a suitable source of antigen (e.g., the HA protein, the N1 protein). The bunyavirus family includes the genera bunyavirus (California encephalitis, La  
5 Crosse), phlebovirus (Rift Valley Fever), hantavirus (puremala is a hemahagin fever virus), nairovirus (Nairobi sheep disease) and various unassigned bungaviruses. The arenavirus family provides a source of antigens against LCM and Lassa fever virus. The reovirus family includes the genera reovirus, rotavirus (which causes acute gastroenteritis in children), orbiviruses, and cultivirus (Colorado Tick fever, Lebombo (humans), equine  
10 encephalosis, blue tongue).

The retrovirus family includes the sub-family oncorivirinal which encompasses such human and veterinary diseases as feline leukemia virus, HTLVI and HTLVII, lentivirinal (which includes human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), equine infectious  
15 anemia virus, and spumavirinal). Among the lentiviruses, many suitable antigens have been described and can readily be selected. Examples of suitable HIV and SIV antigens include, without limitation the gag, pol, Vif, Vpx, VPR, Env, Tat, Nef, and Rev proteins, as well as various fragments thereof. For example, suitable fragments of the Env protein may include any of its subunits such as the gp120, gp160, gp41, or smaller fragments  
20 thereof, e.g., of at least about 8 amino acids in length. Similarly, fragments of the tat protein may be selected. [See, US Patent 5,891,994 and US Patent 6,193,981.] See, also, the HIV and SIV proteins described in D.H. Barouch et al, J. Virol., 75(5):2462-2467 (March 2001), and R.R. Amara, et al, *Science*, 292:69-74 (6 April 2001). In another example, the HIV and/or SIV immunogenic proteins or peptides may be used to form  
25 fusion proteins or other immunogenic molecules. See, e.g., the HIV-1 Tat and/or Nef fusion proteins and immunization regimens described in WO 01/54719, published August 2, 2001, and WO 99/16884, published April 8, 1999. The invention is not limited to the HIV and/or SIV immunogenic proteins or peptides described herein. In addition, a variety of modifications to these proteins have been described or could readily be made  
30 by one of skill in the art. See, e.g., the modified gag protein that is described in US Patent 5,972,596. Further, any desired HIV and/or SIV immunogens may be delivered alone or in combination. Such combinations may include expression from a single vector or from

multiple vectors. Optionally, another combination may involve delivery of one or more expressed immunogens with delivery of one or more of the immunogens in protein form. Such combinations are discussed in more detail below.

The papovavirus family includes the sub-family polyomaviruses (BKU  
5 and JCU viruses) and the sub-family papillomavirus (associated with cancers or malignant progression of papilloma). The adenovirus family includes viruses (EX, AD7, ARD, O.B.) which cause respiratory disease and/or enteritis. The parvovirus family feline parvovirus (feline enteritis), feline panleucopeniavirus, canine parvovirus, and porcine parvovirus. The herpesvirus family includes the sub-family alpha herpesvirinae,  
10 which encompasses the genera simplexvirus (HSV I, HSV II), varicellovirus (pseudorabies, varicella zoster) and the sub-family beta herpesvirinae, which includes the genera cytomegalovirus (HCMV, muromegalovirus) and the sub-family gamma herpesvirinae, which includes the genera lymphocryptovirus, EBV (Burkitt's lymphoma), infectious rhinotracheitis, Marek's disease virus, and rhadinovirus. The  
15 poxvirus family includes the sub-family chordopoxvirinae, which encompasses the genera orthopoxvirus (Variola (Smallpox) and Vaccinia (Cowpox)), parapoxvirus, avipoxvirus, capripoxvirus, leporipoxvirus, suipoxvirus, and the sub-family entomopoxvirinae. The hepadnavirus family includes the Hepatitis B virus. One unclassified virus which may be suitable source of antigens is the Hepatitis delta virus. Still other viral sources  
20 may include avian infectious bursal disease virus and porcine respiratory and reproductive syndrome virus. The alphavirus family includes equine arteritis virus and various Encephalitis viruses.

The present invention may also encompass immunogens which are useful to immunize a human or non-human animal against other pathogens including  
25 bacteria, fungi, parasitic microorganisms or multicellular parasites which infect human and non-human vertebrates, or from a cancer cell or tumor cell. Examples of bacterial pathogens include pathogenic gram-positive cocci include pneumococci; staphylococci; and streptococci. Pathogenic gram-negative cocci include meningococcus; gonococcus. Pathogenic enteric gram-negative bacilli include enterobacteriaceae; pseudomonas,  
30 acinetobacteria and eikenella; melioidosis; salmonella; shigella; haemophilus; moraxella; *H. ducreyi* (which causes chancroid); brucella; *Francisella tularensis* (which causes tularemia); yersinia (pasteurella); streptobacillus moniliformis and spirillum; Gram-

positive bacilli include *Listeria monocytogenes*; *Erysipelothrix rhusiopathiae*; *Corynebacterium diphtheria* (diphtheria); cholera; *B. anthracis* (anthrax); donovanosis (granuloma inguinale); and bartonellosis. Diseases caused by pathogenic anaerobic bacteria include tetanus; botulism; other clostridia; tuberculosis; leprosy; and other mycobacteria. Pathogenic spirochetal diseases include syphilis; treponematoses: yaws, pinta and endemic syphilis; and leptospirosis. Other infections caused by higher pathogen bacteria and pathogenic fungi include actinomycosis; nocardiosis; cryptococcosis, blastomycosis, histoplasmosis and coccidioidomycosis; candidiasis, aspergillosis, and mucormycosis; sporotrichosis; paracoccidioidomycosis, petriellidiosis, torulopsosis, mycetoma and chromomycosis; and dermatophytosis. Rickettsial infections include Typhus fever, Rocky Mountain spotted fever, Q fever, and Rickettsialpox. Examples of mycoplasma and chlamydial infections include: *Mycoplasma pneumoniae*; lymphogranuloma venereum; psittacosis; and perinatal chlamydial infections. Pathogenic eukaryotes encompass pathogenic protozoans and helminths and infections produced thereby include: amebiasis; malaria; leishmaniasis; trypanosomiasis; toxoplasmosis; *Pneumocystis carinii*; *Trichans*; *Toxoplasma gondii*; babesiosis; giardiasis; trichinosis; filariasis; schistosomiasis; nematodes; trematodes or flukes; and cestode (tapeworm) infections.

Many of these organisms and/or toxins produced thereby have been identified by the Centers for Disease Control [(CDC), Department of Health and Human Services, USA], as agents which have potential for use in biological attacks. For example, some of these biological agents, include, *Bacillus anthracis* (anthrax), *Clostridium botulinum* and its toxin (botulism), *Yersinia pestis* (plague), variola major (smallpox), *Francisella tularensis* (tularemia), and viral hemorrhagic fevers [filoviruses (e.g., Ebola, Marburg), and arenaviruses [e.g., Lassa, Machupo]], all of which are currently classified as Category A agents; *Coxiella burnetii* (Q fever); *Brucella* species (brucellosis), *Burkholderia mallei* (glanders), *Burkholderia pseudomallei* (meloidosis), *Ricinus communis* and its toxin (ricin toxin), *Clostridium perfringens* and its toxin (epsilon toxin), *Staphylococcus* species and their toxins (enterotoxin B), *Chlamydia psittaci* (psittacosis), water safety threats (e.g., *Vibrio cholerae*, *Cryptosporidium parvum*), Typhus fever (*Rickettsia powazeki*), and viral encephalitis (alphaviruses, e.g., Venezuelan equine encephalitis; eastern equine encephalitis; western equine

encephalitis); all of which are currently classified as Category B agents; and Nipah virus and hantaviruses, which are currently classified as Category C agents. In addition, other organisms, which are so classified or differently classified, may be identified and/or used for such a purpose in the future. It will be readily understood that the viral vectors and  
5 other constructs described herein are useful to deliver antigens from these organisms, viruses, their toxins or other by-products, which will prevent and/or treat infection or other adverse reactions with these biological agents.

Administration of the vectors of the invention to deliver immunogens against the variable region of the T cells elicit an immune response  
10 including CTLs to eliminate those T cells. In RA, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include V-3, V-14, V-17 and V $\alpha$ -17. Thus, delivery of a nucleic acid sequence that encodes at least one of these polypeptides will elicit an immune response that will target T cells involved in RA. In MS, several specific variable regions of TCRs which are involved in  
15 the disease have been characterized. These TCRs include V-7 and V $\alpha$ -10. Thus, delivery of a nucleic acid sequence that encodes at least one of these polypeptides will elicit an immune response that will target T cells involved in MS. In scleroderma, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include V-6, V-8, V-14 and V $\alpha$ -16, V $\alpha$ -3C, V $\alpha$ -7, V $\alpha$ -14,  
20 V $\alpha$ -15, V $\alpha$ -16, V $\alpha$ -28 and V $\alpha$ -12. Thus, delivery of a recombinant simian adenovirus that encodes at least one of these polypeptides will elicit an immune response that will target T cells involved in scleroderma.

### C. Ad-Mediated Delivery Methods

The therapeutic levels, or levels of immunity, of the selected gene can be  
25 monitored to determine the need, if any, for boosters. Following an assessment of CD8+ T cell response, or optionally, antibody titers, in the serum, optional booster immunizations may be desired. Optionally, the recombinant simian adenoviral vectors of the invention may be delivered in a single administration or in various combination regimens, e.g., in combination with a regimen or course of treatment involving other  
30 active ingredients or in a prime-boost regimen. A variety of such regimens have been described in the art and may be readily selected.

For example, prime-boost regimens may involve the administration of a DNA (e.g., plasmid) based vector to prime the immune system to second, booster, administration with a traditional antigen, such as a protein or a recombinant virus carrying the sequences encoding such an antigen. See, e.g., WO 00/11140, published March 2, 2000, incorporated by reference. Alternatively, an immunization regimen may involve the administration of a recombinant simian adenoviral vector of the invention to boost the immune response to a vector (either viral or DNA-based) carrying an antigen, or a protein. In still another alternative, an immunization regimen involves administration of a protein followed by booster with a vector encoding the antigen.

In one embodiment, the invention provides a method of priming and boosting an immune response to a selected antigen by delivering a plasmid DNA vector carrying said antigen, followed by boosting with a recombinant simian adenoviral vector of the invention. In one embodiment, the prime-boost regimen involves the expression of multiproteins from the prime and/or the boost vehicle. See, e.g., R.R. Amara, *Science*, 292:69-74 (6 April 2001) which describes a multiprotein regimen for expression of protein subunits useful for generating an immune response against HIV and SIV. For example, a DNA prime may deliver the Gag, Pol, Vif, VPX and Vpr and Env, Tat, and Rev from a single transcript. Alternatively, the SIV Gag, Pol and HIV-1 Env is delivered in a recombinant adenovirus construct of the invention. Still other regimens are described in WO 99/16884 and WO 01/54719.

However, the prime-boost regimens are not limited to immunization for HIV or to delivery of these antigens. For example, priming may involve delivering with a first chimp vector of the invention followed by boosting with a second chimp vector, or with a composition containing the antigen itself in protein form. In one example, the prime-boost regimen can provide a protective immune response to the virus, bacteria or other organism from which the antigen is derived. In another desired embodiment, the prime-boost regimen provides a therapeutic effect that can be measured using convention assays for detection of the presence of the condition for which therapy is being administered.

The priming composition may be administered at various sites in the body in a dose dependent manner, which depends on the antigen to which the desired immune response is being targeted. The invention is not limited to the amount or situs of

injection(s) or to the pharmaceutical carrier. Rather, the regimen may involve a priming and/or boosting step, each of which may include a single dose or dosage that is administered hourly, daily, weekly or monthly, or yearly. As an example, the mammals may receive one or two doses containing between about 10  $\mu$ g to about 50  $\mu$ g of plasmid in carrier. A desirable amount of a DNA composition ranges between about 1  $\mu$ g to about 10,000  $\mu$ g of the DNA vector. Dosages may vary from about 1  $\mu$ g to 1000  $\mu$ g DNA per kg of subject body weight. The amount or site of delivery is desirably selected based upon the identity and condition of the mammal.

The dosage unit of the vector suitable for delivery of the antigen to the mammal is described herein. The vector is prepared for administration by being suspended or dissolved in a pharmaceutically or physiologically acceptable carrier such as isotonic saline; isotonic salts solution or other formulations that will be apparent to those skilled in such administration. The appropriate carrier will be evident to those skilled in the art and will depend in large part upon the route of administration. The compositions of the invention may be administered to a mammal according to the routes described above, in a sustained release formulation using a biodegradable biocompatible polymer, or by on-site delivery using micelles, gels and liposomes. Optionally, the priming step of this invention also includes administering with the priming composition, a suitable amount of an adjuvant, such as are defined herein.

Preferably, a boosting composition is administered about 2 to about 27 weeks after administering the priming composition to the mammalian subject. The administration of the boosting composition is accomplished using an effective amount of a boosting composition containing or capable of delivering the same antigen as administered by the priming DNA vaccine. The boosting composition may be composed of a recombinant viral vector derived from the same viral source (e.g., adenoviral sequences of the invention) or from another source. Alternatively, the "boosting composition" can be a composition containing the same antigen as encoded in the priming DNA vaccine, but in the form of a protein or peptide, which composition induces an immune response in the host. In another embodiment, the boosting composition contains a DNA sequence encoding the antigen under the control of a regulatory sequence directing its expression in a mammalian cell, e.g., vectors such as well-known bacterial or viral vectors. The primary requirements of the boosting composition are that the antigen

of the composition is the same antigen, or a cross-reactive antigen, as that encoded by the priming composition.

In another embodiment, the simian adenoviral vectors of the invention are also well suited for use in a variety of other immunization and therapeutic regimens.

5 Such regimens may involve delivery of simian adenoviral vectors of the invention simultaneously or sequentially with Ad vectors of different serotype capsids, regimens in which adenoviral vectors of the invention are delivered simultaneously or sequentially with non-Ad vectors, regimens in which the adenoviral vectors of the invention are delivered simultaneously or sequentially with proteins, peptides, and/or other biologically  
10 useful therapeutic or immunogenic compounds. Such uses will be readily apparent to one of skill in the art.

The following examples illustrate the cloning of the simian adenoviruses and the construction of exemplary recombinant adenovirus vectors of the present invention. These examples are illustrative only, and do not limit the scope of the present  
15 invention.

#### Example 1 - Viral Propagation

The Pan5 [ATCC Accession No. VR-591], Pan6 [ATCC Accession No. VR-592], and Pan7 [ATCC Accession No. VR-593] viruses, originally isolated from  
20 lymph nodes from chimpanzees, were propagated in 293 cells [ATCC CRL1573]. Typically, these cells are cultured in Dulbecco's Modified Eagles Medium (DMEM; Sigma, St. Louis, MO.) supplemented with 10% fetal calf serum (FCS) [Sigma or Hyclone, Logan, UT] and 1 % Penicillin-Streptomycin (Sigma). Infection of 293 cells is carried out in DMEM supplemented with 2% FCS for the first 24 hours, after which FCS  
25 is added to bring the final concentration to 10%. Infected cells are harvested when 100% of the cells exhibit virus-induced cytopathic effect (CPE), and are then collected, and concentrated by centrifugation. Cell pellets are resuspended in 10 mM Tris (pH 8.0), and lysed by 3 cycles of freezing and thawing. Virus preparations are obtained following two ultra centrifugation steps on cesium chloride density gradients and stocks of virus are  
30 diluted to 1 to  $5 \times 10^{12}$  particles/ml in 10 mM Tris/100 mM NaCl/50% glycerol and stored at -70°C.

The ability of 293 cells to propagate these adenoviruses exceeded expectations which were based on knowledge of other non-human adenovirus serotypes.

|   | <u>Virus</u> | <u>Yield (virus particles produced in <math>8 \times 10^8</math> cells)</u> |
|---|--------------|-----------------------------------------------------------------------------|
|   | Pan5         | $8.8 \times 10^{13}$                                                        |
| 5 | Pan6         | $1.6 \times 10^{14}$                                                        |
|   | Pan7         | $8.8 \times 10^{13}$                                                        |

#### Example 2 – Characterization of Viral Genomic DNA

10                    Genomic DNA was isolated from the purified virus preparations of Example 1 and digested with HindIII or BamHI restriction enzymes following the manufacturers' recommendations. The results (not shown) revealed that that the Pan5, Pan6, Pan7 genomes of the invention and the published Pan 9 (C68) genome show different restriction patterns, and thus, are distinct from each other.

15                    The nucleotide sequences of Pan5, Pan6 and Pan7 were determined. The nucleotide sequence of the top strand of Pan5 DNA is reported in SEQ ID NO: 1. The nucleotide sequence of the top strand of Pan6 DNA is reported in SEQ ID NO: 5. The nucleotide sequence of the top strand of Pan7 DNA is reported in SEQ ID NO: 9.

20                    Regulatory and coding regions in the viral DNA sequences were identified by homology to known adenoviral sequences using the "Clustal W" program described above at conventional settings. See the tables above providing the adenoviral sequences. Open reading frames were translated and the predicted amino acid sequences examined for homology to previously described adenoviral protein sequences, Ad4, Ad5, Ad7, Ad12, and Ad40.

25                    Analysis of the sequence revealed a genome organization that is similar to that present in human adenoviruses, with the greatest similarity to human Ad4. However, substantial differences in the hexon hypervariable regions were noted between the chimpanzee adenoviruses and other known adenoviruses, including AdHu4. These differences fit well with the serological cross-reactivity data that has been obtained (see  
30 below).

                      An alignment of a portion of the hexon sequences is shown in Fig. 1. The portion shown is from the region of the hexon that corresponds to the outwardly disposed extended loops DE1 and FG1 where the most variability between serotypes is observed.

An intervening portion that contributes to the base of the hexon (corresponding to residues 308-368 of the published AdC68 sequence; US Patent 6,083,716), and is highly conserved between serotypes, is also present. The following table summarizes the pair-wise comparisons of the amino acids in the hexon proteins.

5

| Comparison |       | Hexon amino-acid<br>Similarity (%) |
|------------|-------|------------------------------------|
| #1         | #2    |                                    |
| AdC5       | AdC7  | 99.0                               |
| AdC5       | AdC68 | 98.3                               |
| AdC5       | AdC6  | 88.0                               |
| AdC5       | AdC1  | 84.9                               |
| AdC6       | AdC7  | 87.7                               |
| AdC6       | AdC68 | 87.3                               |
| AdC6       | AdC1  | 84.9                               |
| AdC7       | AdC68 | 97.5                               |
| AdC7       | AdC1  | 84.8                               |
| AdC68      | AdC1  | 84.9                               |

Analysis of the fiber knob domain (which is responsible for receptor binding) of the chimpanzee adenoviruses shows an overall similarity in structure (Fig. 2).

10

The degree of sequence similarity between the E1 proteins of huAd5 and C68 (see Tables below) is similar to that between huAd5 and Pan-5, Pan-6, and Pan-7.

| Comparison |       | E1a (13S) amino-acid identity (%) |
|------------|-------|-----------------------------------|
| #1         | #2    |                                   |
| AdHu5      | AdC5  | 36.6                              |
| AdHu5      | AdC6  | 28.5                              |
| AdHu5      | AdC7  | 34.9                              |
| AdHu5      | AdC68 | 35.6                              |
| AdHu5      | AdC1  | 35.6                              |
| AdC5       | AdC6  | 68.3                              |
| AdC5       | AdC7  | 96.9                              |
| AdC5       | AdC68 | 80.4                              |
| AdC5       | AdC1  | 51.3                              |
| AdC6       | AdC7  | 69.3                              |
| AdC6       | AdC68 | 59.4                              |
| AdC6       | AdC1  | 37.7                              |
| AdC7       | AdC68 | 81.5                              |
| AdC7       | AdC1  | 51.0                              |
| AdC68      | AdC1  | 54.9                              |

|       | Sequence Identity with human Ad5 |                     |
|-------|----------------------------------|---------------------|
|       | E1b Small T Protein              | E1b Large T Protein |
| C68   | 47.3%                            | 55.8%               |
| Pan-5 | 43.2%                            | 54.5%               |
| Pan-6 | 45.3%                            | 54.5%               |
| Pan-7 | 46.4%                            | 53.8%               |

Replication-defective versions of AdC5, AdC6 and AdC7 were created by molecular cloning methods described in the following examples in which minigene cassettes were inserted into the place of the E1a and E1b genes. The molecular clones of the recombinant viruses were rescued and grown up in 293 cells for large-scale purification using the published CsCl sedimentation method [K. Fisher *et al.*, J. Virol., 70:520 (1996)]. Vector yields were based on 50 plate (150 mm) preps in which approximately  $1 \times 10^9$  293 cells were infected with the corresponding viruses. Yields were determined by measuring viral particle concentrations spectrophotometrically. After having constructed E1-deleted vectors, it was determined that HEK 293 cells (which express human adenovirus serotype 5 E1 functions) trans-complement the E1 deletions of the novel viral vectors and allow for the production of high titer stocks. Examples of virus yields for a few of these recombinant viruses are shown in the table below.

The transgenes for these vectors,  $\beta$ -galactosidase (LacZ), green fluorescent protein (GFP), alpha-1-anti-trypsin (A1AT), ebola glycoprotein (ebo), a soluble ebola glycoprotein variant lacking the transmembrane and cytoplasmic domains (sEbo), and three deletion mutants of the ebola glycoprotein (Ebo $\Delta$ 2, Ebo $\Delta$ 3, and Ebo $\Delta$ 4), were expressed by the cytomegalovirus promoter (CMV). In the following table, ND indicates that the study has not yet been done.

| Transgene         | Viral backbone/Vector yield (Viral particles $\times 10^{13}$ ) |  |      |       |      |
|-------------------|-----------------------------------------------------------------|--|------|-------|------|
|                   | AdHu5                                                           |  | AdC7 | AdC68 | AdC6 |
| CMVLacZ           | 1.5                                                             |  | 1.4  | 3.3   | 6.1  |
| CMVGFP            | 2.5                                                             |  | 3.6  | 8     | 10   |
| CMVA1AT           | 3.7                                                             |  | 6    | 10    | ND   |
| CMVEbo            | 1.1                                                             |  | 4.3  | ND    | ND   |
| CMVsEbo           | 4.9                                                             |  | 5.4  | ND    | ND   |
| CMVEbo $\Delta$ 2 | 1                                                               |  | 9.3  | ND    | ND   |
| CMVEbo $\Delta$ 3 | 0.8                                                             |  | 9.5  | ND    | ND   |
| CMVEbo $\Delta$ 4 | 1.4                                                             |  | 6.2  | ND    | ND   |

The ability of human adenovirus E1 to trans-complement the E1-deleted chimpanzee viruses of the invention is highly advantageous, as it permits the production of E1-deleted chimpanzee adenoviral vectors of the invention, while reducing or eliminating the risk of homologous recombination due to the differences in sequences  
5 between human Ad and the chimpanzee adenoviruses described herein.

### Example 3 – Serological Studies of Pan 5, 6, and 7 Viruses

Because of the differences in the hexon hypervariable region, it was anticipated that the C5, C6, and C7 viruses would be serologically distinct from human adenoviruses,  
10 including AdHu4.

#### 1. *Cross-Reactivity of Wild-type Viruses*

For screening of wild-type viruses in order to make a determination of antibody cross-reactivity, the replication competent viruses were used and inhibition of cytopathic effects (CPE) was measured. Briefly, preparations of adenoviruses (Adhu5,  
15 Pan-5, Pan-6, Pan-7 and AdC68) stored at  $5 \times 10^{12}$  particles/ml were diluted 1/600 for the assays. This concentration of virus was selected since it results in 100% CPE within 48 hours in the absence of neutralization. Prior to adding the virus to 293 cells ( $4 \times 10^4$  cells/well in a 96 well dish), 1:20 dilutions of sera were added. The assay is read as the presence or absence of CPE; full neutralization would read as no cytopathic effect. The  
20 results are summarized in the Table below. The fact that 9/36 human sera neutralized Adhu5 induced CPE is consistent with previous estimates of neutralizing antibodies in the human population. The numbers indicate the total individuals who showed neutralization (numerator) versus the total number screened (denominator). ND = not determined.

|       | Neutralization by 1/20 diln of serum |                  |                      |
|-------|--------------------------------------|------------------|----------------------|
|       | Human<br>(N=36)                      | Rhesus<br>(N=52) | Chimpanzee<br>(N=20) |
| Adhu5 | 9/36                                 | ND               | ND                   |
| AdC68 | 1/36                                 | 0/52             | 12/20                |
| Pan 5 | 0/36                                 | 0/52             | 10/20                |
| Pan 6 | 0/36                                 | 0/52             | 9/20                 |
| Pan 7 | 0/36                                 | 0/52             | 12/20                |

Of all human sera screened, 35/36 were negative for neutralization to AdC68 while 36/36 were negative for neutralization to Pan-5, Pan-6 and Pan-7. Of 52 rhesus monkeys screened, none showed neutralization to any chimpanzee adenovirus; rhesus monkey is the preferred pre-clinical model for evaluating HIV vaccines. Between 9 to 12  
5 out of 20 chimpanzees had substantial neutralization to one or another of the chimpanzee adenoviruses consistent with the fact these are indeed endemic chimpanzee-specific pathogens. Interestingly, there are chimpanzees with neutralizing antibodies only to Pan-5, Pan-6 or AdC68 supporting the hypothesis that several of these chimpanzee adenoviral vectors will not cross neutralize each other and are distinct serotypes.

10 The same assay was carried out for 20 chimpanzee serum samples. Fifty percent (50%) of the samples reacted serologically, in different degrees to Pan5; 40% to Pan6; 55% to Pan7 and 60% to C68. Among the positive serum samples, one of them had strong neutralizing activity to all four chimp viruses.

## 2. *Cross-neutralization with Recombinant Viruses*

15 High-titer polyclonal antibodies were obtained to each of the simian adenoviruses in order to more precisely gauge the degree of cross-neutralization among the different serotypes. This was done by intramuscular immunization of rabbits using a recombinant virus containing GFP based on previously the described C68 chimpanzee adenovirus as an adjuvant. The serum was then used to assay for neutralizing activity  
20 against each of the three chimpanzee adenoviruses of the invention, AdC5, AdC6 and AdC7. A rabbit was injected with  $5 \times 10^{12}$  viral particle per kg of C68CMVGFP vector intramuscularly and boosted 5 weeks later using the same dose. A bleed collected at the 9 week time point revealed extremely potent neutralizing activity against C68 as well as Pan-5 and Pan-7 but not against Pan-6 (see Table below), indicating that the  
25 administration of a C68 (or Pan-5 and Pan-7) based vaccine could be effectively followed by a boost using a vector based on Pan-6. However, it has been found that this level of inter-relatedness does not necessarily prevent with re-administration in a setting where antiviral antibody titers were not as high as was achieved in this rabbit. In the following table, + indicates 33% CPE; ++ indicates 66% CPE; +++ indicates 100% CPE.

30

| Infection on 293 cells with virus: |      |      |           |            |                   |
|------------------------------------|------|------|-----------|------------|-------------------|
| Pan5                               | Pan6 | Pan7 | Pan9(C68) | C68<br>GFP | Serum<br>Dilution |
| -                                  | +++  | -    | -         | -          | 1/20              |
| -                                  | +++  | -    | -         | -          | 1/40              |
| -                                  | +++  | -    | -         | -          | 1/80              |
| -                                  | +++  | -    | -         | -          | 1/160             |
| -                                  | +++  | -    | -         | -          | 1/320             |
| -                                  | +++  | -    | -         | -          | 1/640             |
| -                                  | +++  | -    | -         | -          | 1/1,280           |
| -                                  | +++  | -    | -         | -          | 1/2,560           |
| -                                  | +++  | -    | -         | -          | 1/5,120           |
| +                                  | +++  | -    | -         | -          | 1/10,240          |
| +                                  | +++  | ++   | -         | -          | 1/20,480          |
| ++                                 | +++  | +++  | -         | -          | 1/40,960          |
| ++                                 | +++  | +++  | +         | +          | 1/81,920          |
| +++                                | +++  | +++  | ++        | ++         | 1/163,840         |
| +++                                | +++  | +++  | +++       | +++        | 1/327,680         |
| +++                                | +++  | +++  | +++       | +++        | 1/665,360         |
| +++                                | +++  | +++  | +++       | +++        | 1/1,310,720       |
| +++                                | +++  | +++  | +++       | +++        | 1/2,621,440       |

### 3. *Quantitative Assay for Detection of Neutralizing Antibody*

The result was validated by a more quantitative-based assay for detecting neutralizing antibody, which is based on transduction of a GFP vector. Briefly, groups of C57BL/6 mice were immunized intramuscularly or intravenously with  $5.0 \times 10^{10}$  particles/ml Pan5, Pan6, Pan7 or C68. Sera from day 28 bleeds were tested for cross-neutralizing activity against C68CMVEGFP at dilutions of 1/20 and 1/80. In summary, when a pharmaceutical preparation of human immunoglobulin was tested for serological reactions to Pan 5, 6, and 7, and C68, some low levels of neutralizing activities against Pan 7 and C68 were detected. No neutralizing activity against Pan5 or Pan6 was detected. Serum samples from 36 human subjects were run for the same assay. Serum samples were tested at a 1/20 dilution. The results indicated that only one individual has clear neutralizing activity to C68. No neutralizing activity to Pan5, Pan6 or Pan7 was detected.

### 4. *In Vitro Cross-Neutralization*

Cross-neutralization of the simian adenoviruses by high-titer rabbit polyclonal antibodies raised against each of the adenoviruses Pan-5, Pan-6, Pan-7, and C68 was tested.

Rabbits were immunized with intra-muscular injections of  $10^{13}$  particles of each of the chimpanzee adenoviruses and boosted 40 days later with the same dose with incomplete Freund's adjuvant. Sera were analyzed for the presence of neutralizing antibodies by incubating serial two-fold dilutions with  $10^9$  genome copies of each of the appropriate chimpanzee adenovirus vector expressing GFP and testing for the attenuation of GFP expression when applied to 293 cells. The serum dilution which produced a 50% reduction of GFP expression was scored as the neutralizing antibody titer against that particular virus.

The results are shown in the Table. The data are consistent with the expectation from sequence analysis of the hexon amino-acid sequences, which indicated that Ad Pan-6 was likely to be the most serologically distinct compared to the other chimpanzee adenoviruses.

|                                   | Infection of 293 cells with $10^9$ genome copies of |          |           |        |
|-----------------------------------|-----------------------------------------------------|----------|-----------|--------|
| Serum from rabbit immunized with: | Ad Pan-5                                            | Ad Pan-6 | Ad Pan-7  | Ad C68 |
| Ad Pan-5                          | 1/5120                                              | <1/20    | 1/2560    | 1/2560 |
| Ad Pan-6                          | No neutralization                                   | 1/20,480 | <1/20     | <1/20  |
| Ad Pan-7                          | 1/2560                                              | 1/160    | 1/163,840 | 1/2560 |
| Ad C68                            | No neutralization                                   | <1/20    | <1/20     | 1/5120 |

In order to determine whether antibodies cross-reacting with the simian adenoviruses were likely to be of low prevalence in humans, simian adenoviruses SV1, SV39, and SV25 were tested for their ability to withstand neutralization when incubated with commercially available pooled human immunoglobulins (Ig). The same assay was also performed with Adhu5 and the chimpanzee adenoviruses Pan-5, Pan-6, Pan-7, and C68. In a further study, sera from mice has been immunized with one of the chimpanzee adenoviruses C5, C6, C7, and C68 and their ability to cross-neutralize the simian adenoviruses SV-15, SV-23, SA-17, and Baboon Adenovirus has been tested. No cross-reactivity was observed in any case.

#### Example 4 – Generation of Recombinant E1-Deleted Pan5 Vector

A modified pX plasmid was prepared by destroying the FspI site in the bla gene region of pX (Clontech) by site-directed mutagenesis. The resulting modified plasmid, termed pX', is a circular plasmid of 3000 bp which contains an fl ori and an ampicillin resistance gene (AmpR-cds).

##### A. Production of Pan-5 Adenovirus Plasmid

A polylinker for sequential cloning of the Pan5 DNA fragments into pX' is created. The polylinker is substituted for the existing pX' polylinker following digestion with *MluI* and *EcoRI*. The blunt-*FseI* fragment of the Pan 5 is inserted into the *SmaI* and *FseI* sites of the polylinker. This fragment contains the 5' end

of the adenoviral genome (bp 1 to 3606, SEQ ID NO:1). The *SnaBI-FspI* fragment of Pan 5 (bp 455 to 3484, SEQ ID NO:1) is replaced with a short sequence flanked by *I-CeuI* and *PI-SceI* sites from pShuttle (Clontech), to eliminate the E1 region of the adenoviral genome. The *EcoRI*-blunt fragment of Pan5 (bp 28658 to 36462, SEQ ID NO:1) is inserted into the *EcoRI* and *EcoRV* sites of the polylinker (to provide the 3' end of the adenoviral genome); the *FseI-MluI* fragment (bp 3606 to 15135, SEQ ID NO:1) is inserted into the polylinker; and the *MluI-EcoRI* fragment is inserted into the polylinker (bp 15135 to 28658, SEQ ID NO:1). Optionally, a desired transgene is inserted into *I-CeuI* and *PI-SceI* sites of the newly created pX'Pan5ΔE1 vector.

10                   B.       *Alternative Method of Generating pX'Pan5ΔE1.*

The initial plasmid pX is derived from pAdX adenovirus plasmid available from Clontech, as described above. Thereafter, a *PacI-XhoI* region of pX' was deleted and the blunt-ended Pan5 polylinker was inserted into the *FspI* site to generate pX'PLNK (2994 bp). The 5'-end-*FseI* region of Pan 5 (bp 1-3607, SEQ ID NO:1) was inserted into *SmaI* and *FseI* sites of pX'LNK to generate the pX'Pan5-5' plasmid (6591 bp). The *SnaBI-NdeI* region of pX'Pan5-5' was excised and replaced with the *Ceu/Sce* cassette, which had been PCR amplified from pRCS to create pX'Pan5-5'ΔE1 (4374 bp). Briefly, a sequence containing *I-CeuI* and *PI-SceI* rare cutter sites was PCR amplified from pRCS (3113bp). The 3' PCR primer was introduced an *NdeI* site into the PCR product.

20                   To extend the Pan5 DNA in pX'Pan5-5'ΔE1 (4374 bp), the *FseI-MluI* region of Pan 5 (bp 3607-15135, SEQ ID NO:1) is added, to create pX'Pan5-5'Mlu (15900 bp). The remaining *MluI*-3' end of the Pan5 sequence (bp 15135-36462, SEQ ID NO:1) is added to the vector between the *MluI* and *EcoRV* sites of the vector polylinker to form pX'Pan5ΔE1 which contains the full-length Pan5 sequence containing a deletion in the E1 region.

C.       Generation of Recombinant Viruses

To generate the recombinant adenoviruses from pX'Pan5ΔE1, the plasmid is co-transfected with a helper expressing E1, or from an E1-expressing packaging cell line, such as 293 cell line or a cell line prepared as described herein. The expression of E1 in the packaging cell permits the replication and packaging of

the Pan5ΔE1 into a virion capsid. In another embodiment, the packaging cell transfected with pX'Pan5ΔE1 is transfected with an adenovirus vector as described above bearing the transgene of interest. Homologous recombination occurs between the helper and the plasmid, which permits the adenovirus-transgene sequences in the vector to be replicated and packaged into virion capsids, resulting in the recombinant adenovirus.

Transfection is followed by an agar overlay for 2 weeks, after which the viruses are plaqued, expanded and screened for expression of the transgene. Several additional rounds of plaque purification are followed by another expansion of the cultures. Finally the cells are harvested, a virus extract prepared and the recombinant chimpanzee adenovirus containing the desired transgene is purified by buoyant density ultracentrifugation in a CsCl gradient or by alternative means known to those of skill in the art.

#### 15 Example 5 – Generation of Recombinant E1-Deleted Pan6 Vector

##### A. Strategy for Construction of Pan-6 Adenoviral Plasmid

###### 1. *Cloning of terminal fragments*

Pan 6 virus is deproteinated by pronase and proteanase K treatment and phenol extraction. Synthetic 12 bp Pme I linkers are ligated onto the viral DNA as described by Berkner and Sharp, *Nucleic Acids Research*, 11: 6003 (1983). The viral DNA is then digested with Xba I to isolate a 5' terminal fragment (6043 bp). The Ad6 XbaI 5' fragment is then ligated into pX link at Sma I and Xba I sites to form pX-AdPan6-0-16.5. The viral DNA with Pme I linkers is also digested with Pac I to isolate the 6475 bp 3' terminal fragment and cloned into pX link at Pac I and Sma I sites, resulting in pXAdPan6-82-100.

###### 2. *Deletion of E1 from the 5' clone*

To delete E1 (m.u.1.2-9), the BsiWi-Xba I fragment in pX-AdPan6-0-16.5 is replaced with a PCR fragment spanning m.u.9-16.7 fragment treated with BsiWi and Xba I, leading to pX-Ad-Pan6 m.u.0-1, 9-16.5 .

3. *Fusion of 5' and 3' clones and to create an anchor site to accept the middle Hind III fragment*

First, the 5' clone, pX-Ad-Pan6 m.u.0-1, 9-16.5, is further  
5 expanded by inserting the 2<sup>nd</sup> Xba I fragment (4350 bp, m.u.16.5 – 28) from Pan 6 genome into the Xba I site in the pX-Ad-Pan6 m.u.0-1, 9-16.5. This construct is named pXAd-Pan6-mu 0-1, 9-28.

Second, the 3' clone is also expanded by inserting the 15026 bp Mlu I/Pac I fragment covering m.u.41-82 from Pan 6 genome into the Mlu I/Pac I sites of  
10 pXAdPan6-82-100, generating pXAdPan6-m.u.41-100.

Then, a 8167 bp Hind III/Eco 47III Pan 6 fragment is isolated from pXAd-Pan6-mu 0-1, 9-28 and subcloned into pXAdPan6-m.u.41-100 at Hind III and Xba I blunt sites. This 5' and 3' fusion clone is called pXAdPan6mu0-1, 9-19.5, 64-100.

4. *Drop of the middle fragment of the genome into the fusion*  
15 *clone*

A 16335 bp Hind III fragment (m.u.19.5 – 64) from Pan 6 is inserted into Hind III site of pXAdPan6mu0-1, 9-19.5, 64-100 to form pXAdPan6-0-1, 9-100.

5. *Introduction of a PKGFP selective marker into the final*  
20 *construct for direct cloning the gene of interest and green/white selection of recombinant transformants.*

A minigene cassette that expresses GFP under a lac promoter and is flanked with recognition sites of rare intron encoding restriction enzymes, PI-Sce I and I-Ceu I, was isolated from pShuttle-pkGFP (bare) by Sap I and Dra III digestions  
25 followed by filling-in reaction. The pShuttle-pkGFP (bare) plasmid is 4126 bp in length, and contains a ColE1-Ori, a kanamycin resistance gene, plac, a LacZ promoter-GFPmut3-1 cds (Clontech), and a GFPmut3-1 cds (Clontech). This cassette is subcloned into Srf I cut and blunted pXAdPan6-0-1, 9-100. This final construct is called pX-Pan6-pkGFP mu.0-1, 9-100, which is useful for generating recombinant E1-deleted Pan 6 molecular  
30 clones carrying genes of interest by direct ligation and green/white selection in combination with the generic pShuttlepkGFP vectors.

## B. Alternative Strategy for Generation of Pan-6 Plasmid

### 1. Cloning of 5' terminal fragment

The Pan 6 virus is deproteinated by pronase and proteanase K treatment and phenol extraction as described above and synthetic 12 bp  
5 Pme I linkers are ligated onto the viral DNA as described. The AdPan6 5' XbaI fragment is isolated and ligated into pX to form pX-AdPan6-0-16.5 (9022 bp) as described in Part A above.

### 2. Deletion of E1 from the 5' clone

To delete E1 (m.u. 1.2-9), pX-AdPan6-0-16.5 is digested  
10 with SnaBI and NdeI to remove the regions encoding the E1a and E1b proteins (3442-6310 bp). This vector is subsequently digested with BsiWI in preparation for blunting with the minigene cassette carrying a selective marker.

### 3. Introduction of a selective marker

A minigene cassette that expressed GFP under a lac  
15 promoter and which is flanked with recognition sites of rare intron encoding restriction enzymes, PI-XceI and I-CeuI, was isolated from pShuttle-pkGFP as described above. The DraIII-SapI fragment is then ligated with the digested pX-AdPan6-0-16.5 to form pX-AdPan6 MU 0-16.5ΔE1 (7749 bp).

### 4. Extension of Pan-6 Adenoviral Sequences

pX-AdPan6 MU 0-16.5ΔE1 was subjected to XbaI  
20 digestion to permit insertion of an XbaI-RsrII linker. An XbaI/RsrII digestion fragment from the AdPan6 genome was isolated (mu 28-100, 26240 bp) and ligated into the Xba/RsrII-digested pX-AdPan6 MU 0-16.5ΔE1 to provide pX-AdPan6 MU 0-1, 9-16.5, 28-100. A second XbaI fragment from the Pan6 genome (mu 16.5-28, 4350 bp) is then  
25 ligated into this plasmid to form pX-AdPan6 MU 0-1, 9-100 (38551 bp).

## C. Generation of Recombinant Adenoviruses

To generate the recombinant adenoviruses from a E1-deleted Pan6 plasmid prepared as described in Parts A or b, the plasmid is co-transfected with a helper expressing E1, or from an E1-expressing packaging cell line, such as 293 cell line or a  
30 cell line prepared as described herein. The expression of E1 in the packaging cell permits the replication and packaging of the Pan6-pkGFP mu.0-1, 9-100 into a virion capsid. Alternatively, the packaging cell transfected with pX-Pan6-pkGFP mu.0-1, 9-100 is

transfected with an adenovirus vector as described above bearing another transgene of interest.

#### Example 6 – Generation of Recombinant E1-Deleted Pan7 Vector

##### 5                   A.       *Generation of Pan7 Plasmids*

A synthetic linker containing the restriction sites PacI-SmaI-FseI-MluI-EcoRV-PacI was cloned into pBR322 that was cut with EcoRI and NdeI. The left end (bp1 to 3618) of Ad Pan7 was cloned into the linker between the SmaI and FseI sites. The adenovirus E1 was then excised from the cloned left end by cutting with SnaBI and NdeI and inserting an I-CeuI-GFP-PI-SceI cassette from pShuttle (Clontech) in its place. The resulting plasmid was cut with FseI and MluI and Ad Pan7 fragment FseI (bp 3618) to MluI (bp 155114) was inserted to extend the left end. The construct (pPan7pGFP) was completed by inserting the 21421 bp Ad Pan7 right end fragment from the MluI site (bp 15114) into the above plasmid between MluI and EcoRV to generate a complete molecular clone of E1 deleted adenovirus Pan7 suitable for the generation of recombinant adenoviruses. Optionally, a desired transgene is inserted into the I-CeuI and PI-SceI sites of the newly created pPan7 vector plasmid.

##### B.       *Construction of E1-Deleted Pan7 Viral Vectors*

To generate the recombinant adenoviruses from pPan7ΔE1, the plasmid is co-transfected with a helper expressing E1, or from an E1-expressing packaging cell line, such as 293 cell line or a cell line prepared as described herein. The expression of E1 in the packaging cell permits the replication and packaging of the Pan7ΔE1 into a virion capsid. In another embodiment, the packaging cell transfected with pX'Pan7 ΔE1 is transfected with an adenovirus vector as described above bearing the transgene of interest. Homologous recombination occurs between the helper and the plasmid, which permits the adenovirus-transgene sequences in the vector to be replicated and packaged into virion capsids, resulting in the recombinant adenovirus. Transfection and purification is as described above.

##### 30       Example 7 - Generation of Plasmid Vectors Expressing the E1 Genes

Plasmid vectors are constructed which encode the Pan5 E1 region gene, and these plasmids are used to generate stable cell lines expressing viral E1 proteins.

The E1 region of Pan5 is cloned into pX', essentially as described in Example 4 above, prior to replacement of this region with the fragment from pShuttle (Clontech). The expression plasmid contains the Pan5 adenoviral genome sequence spanning at least bp 1 to 3959 in the Pan5 genomic sequence. Thus, the expression plasmid contains the sequence encoding E1a and E1b of chimpanzee Ad Pan5 under the control of a heterologous promoter. Similar expression plasmids can be generated using the Ad Pan6 and AdPan 7 E1 regions, identified in the tables above.

#### Example 8 - Generation of Cell Lines Expressing Chimpanzee Adenovirus E1 Proteins

Cell lines expressing viral E1 proteins are generated by transfecting HeLa (ATCC Acc. No. CCL2) with the plasmid of Example 6. These cell lines are useful for the production of E1-deleted recombinant chimpanzee adenoviruses by co-transfection of genomic viral DNA and the expression plasmids described above. Transfection of these cell lines, as well as isolation and purification of recombinant chimpanzee adenoviruses therefrom are performed by methods conventional for other adenoviruses, i.e., human adenoviruses [see, e.g., Horwitz, cited above and other standard texts].

##### *A. Cell lines expressing Pan5 E1 proteins*

HeLa cells in 10cm dishes are transfected with 10 µg of pX-Pan51-E1 DNA using a Cellfect™ kit (Pharmacia, Uppsala, Sweden) and following the manufacturer's protocol. 22 hours post-transfection, the cells are subjected to a three minute glycerol shock (15% glycerol in Hepes Buffered Saline, pH 7.5) washed once in DMEM (HeLa) or F12K (A549; Life Technologies, Inc., Grand Island, NY) media supplemented with 10% FCS, 1% Pen-Strep, then incubated for six hours at 37°C in the above described media. The transfected cells are then split into duplicate 15cm plates at ratios of 1:20, 1:40, 1:80, 1:160, and 1:320. Following incubation at 37°C overnight, the media is supplemented with G418 (Life Technologies, Inc.) at a concentration of 1 µg/ml. The media is replaced every 5 days and clones are isolated 20 days post-transfection.

HeLa E1 cell clones are isolated and assayed for their ability to augment adeno-associated virus (AAV) infection and expression of recombinant LacZ protein as described below.

## B. AAV Augmentation Assay for Screening E1 Expressing Cell Lines

AAV requires adenovirus-encoded proteins in order to complete its life cycle. The adenoviral E1 proteins as well as the E4 region-encoded ORF6 protein are necessary for the augmentation of AAV infection. An assay for E1 expression based on AAV augmentation is used. Briefly, the method for identifying adenoviral E1-expressing cells comprises the steps of infecting in separate cultures a putative adenovirus E1-expressing cell and a cell containing no adenovirus sequence, with both an adeno-associated virus (AAV) expressing a marker gene and an AAV expressing the ORF6 of the E4 gene of human adenovirus, for a suitable time. The marker gene activity in the resulting cells is measured and those cells with significantly greater measurable marker activity than the control cells are selected as confirmed E1-expressing cells. In the following experiment, the marker gene is a lacZ gene and the marker activity is the appearance of blue stain.

For example, the cell lines described above, as well as untransfected control cells (HeLa) are infected with 100 genomes per cell of an AAV vector bearing a marker gene, e.g., AV.LacZ [K. Fisher *et al.*, J. Virol., 70:520 (1996)] and an AAV vector expressing the ORF6 region of human 5 (AV.orf6). The DNA sequence of the plasmid generates a novel recombinant adeno-associated virus (rAAV) containing the LacZ transgene and the Ad E4 ORF 6, which is an open reading frame whose expression product facilitates single-stranded (ss) to double-stranded (ds) conversion of rAAV genomic DNA. These vectors are incubated in medium containing 2% FCS and 1% Pen-Strep at 37°C for 4 hours, at which point an equal volume of medium containing 10% FCS is added. It should be understood by one of skill in the art that any marker gene (or reporter gene) may be employed in the first AAV vector of this assay, e.g., alkaline phosphatase, luciferase, and others. An antibody-enzyme assay can also be used to quantitate levels of antigen, where the marker expresses an antigen. The assay is not limited by the identity of the marker gene. Twenty to twenty-four hours post-infection, the cells are stained for LacZ activity using standard methods. After 4 hours the cells are observed microscopically and cell lines with significantly more blue cells than the A549 or HeLa cell controls are scored as positive.

### Example 9 - Delivery of Transgene to Host Cell

The resulting recombinant chimpanzee adenovirus described in Example 4, 5 or 6 above is then employed to deliver the transgene to a mammalian, preferably human, cell. For example, following purification of the recombinant virus, human embryonic kidney  
5 293 cells are infected at an MOI of 50 particles per cell. GFP expression was documented 24 hours post-infection.

#### A. Gene Transfer in Mouse Models via Pan-6, Pan-7, and Pan-9 vectors

Gene transfer efficiencies and toxicological profile of recombinant chimpanzee adenoviruses were compared in mouse liver directed gene transfer, mouse  
10 lung directed gene transfer, and mouse muscle directed gene transfer.

E1-deleted adenoviral vectors containing LacZ under the control of the CMV promoter were constructed using the techniques herein for human Ad5, chimpanzee Pan 6, chimpanzee Pan 7 and chimpanzee Pan 9 (C68). The vectors were delivered to immune-deficient NCR nude mice (80 for each study) as follows. For the liver study, 100  
15  $\mu$ l ( $1 \times 10^{11}$  particles) were injected into the tail vein. For the lung study, 50  $\mu$ l ( $5 \times 10^{10}$  particles) were delivered intratracheally. For the muscle study, 25  $\mu$ l ( $5 \times 10^{10}$  particles) were injected into tibialis anterior. The mice were sacrificed on days 3, 7, 14 and 28 post-vector injection (5 animals per group at each time point). At each necropsy, the liver/lung/muscle tissue was harvested and prepared for cryoblocks and paraffin  
20 embedding. The cryoblocks were sectioned for X-gal staining and the paraffin sections are H&E stained for histopathic analysis. At each time point, terminal bleeding was performed. Serum samples were subjected to liver function tests.

It was observed in this experiment the chimpanzee adenoviruses Pan-6, Pan-7, and Pan-9 were less efficient than huAd5 in gene transfer to the liver and to the lung.  
25 However, this may be desirable in certain circumstances, to reduce liver toxicity observed for huAd5. The gene transfer efficiency in muscle varied less between serotypes.

#### B. *Mouse study to feasibility of re-administration of adenovirus vectors by serotype switching between Adhu5, Pan-6, Pan-7, and Pan-9 vectors*

Mice were administered (C57/Bl6; 4/group) LacZ vectors based on  
30 huAd5, Pan-6, Pan-7, and Pan-9 (H5.040CMVLacZ, Pan6.000CMVLacZ, Pan7.000CMVLacZ, Pan9.000CMVLacZ;  $10^{11}$  particles/injection) by tail vein. Thirty days later the mice were re-administered adenovirus vectors expressing  $\alpha$ 1-antitrypsin

(H5.040CMVhA1AT, Pan6.000CMVhA1AT,  $1 \times 10^{11}$  particles, Pan7.000CMVhA1AT, Pan9.000CMVhA1AT,  $10^{11}$  particles/injection). Successful transduction by the re-administered vector is monitored by measuring serum  $\alpha$ 1-antitrypsin on days 3 and 7, following re-administration.

- 5                      The ability of adenovirus vectors based on huAd5, Pan-6, Pan-7, and Pan-9 respectively to transduce the livers of mice in the presence of neutralizing antibodies to the other serotypes was determined. The results are tabulated here.

| 1 <sup>st</sup> injection | 2 <sup>nd</sup> injection | Cross-neutralization |
|---------------------------|---------------------------|----------------------|
| Adhu5                     | Adhu5                     | Yes (+ve control)    |
|                           | Pan-6                     | No                   |
|                           | Pan-7                     | No                   |
|                           | Pan-9 (C68)               | No                   |
| Pan-6                     | Adhu5                     | No                   |
|                           | Pan-6                     | Yes (+ve control)    |
|                           | Pan-7                     | Yes                  |
|                           | Pan-9 (C68)               | No                   |
| Pan-7                     | Adhu5                     | No                   |
|                           | Pan-6                     | Yes                  |
|                           | Pan-7                     | Yes (+ve control)    |
|                           | Pan-9 (C68)               | Yes                  |

| 1 <sup>st</sup> injection | 2 <sup>nd</sup> injection | Cross-neutralization |
|---------------------------|---------------------------|----------------------|
| Pan-9 (C68)               | Adhu5                     | No                   |
|                           | Pan-6                     | No                   |
|                           | Pan-7                     | Yes                  |
|                           | Pan-9 (C68)               | Yes (+ve control)    |

Ability of vectors to transduce murine liver in the presence of neutralizing antibodies to other serotypes.

5 Thus, immunization with huAd5 does not prevent re-administration with either of the chimpanzee adenovirus vectors Pan-6, Pan-7, or Pan-9 (C68). This experiment also appears to indicate that Pan-7 is between Pan-6 and Pan-9 in the spectrum of antigenic relatedness and cross-reacts with both; however Pan-6 and Pan-9 do not neutralize each other. This is a surprising result based on homology comparisons, which indicates that  
10 Pan-6 is quite distinct from Pan-7 and Pan-9. Evaluation of antisera generated against Pan-9 indicated no cross-neutralization against Pan-6 but some neutralization against Pan-7, arguing that Pan-6 is distinct from the others.

#### 15 Example 10 - Generation of Recombinant E1-Deleted SV-25 Vector

A plasmid was constructed containing the complete SV-25 genome except for an engineered E1 deletion. At the site of the E1 deletion recognition sites for the restriction enzymes I-CeuI and PI-SceI which would allow insertion of transgene from a shuttle plasmid where the transgene expression cassette is flanked by these two enzyme  
20 recognition sites were inserted.

A synthetic linker containing the restriction sites SwaI-SnaBI-SpeI-AflIII-EcoRV-SwaI was cloned into pBR322 that was cut with EcoRI and NdeI. This was done by annealing together two synthetic oligomers SV25T (5'-AAT TTA AAT ACG TAG CGC ACT AGT CGC GCT AAG CGC GGA TAT CAT TTA AA-3', SEQ ID NO: 38) and  
25 SV25B (5'-TAT TTA AAT GAT ATC CGC GCT TAA GCG CGA CTA GTG CGC

TAC GTA TTT A-3', SEQ ID NO:39) and inserting it into pBR322 digested with EcoRI and NdeI. The left end (bp1 to 1057, SEQ ID NO:29) of Ad SV25 was cloned into the above linker between the SnaBI and SpeI sites. The right end (bp28059 to 31042, SEQ ID NO: 29) of Ad SV25 was cloned into the linker between the AflIII and EcoRV sites.

5 The adenovirus E1 was then excised between the EcoRI site (bp 547) to XhoI (bp 2031) from the cloned left end as follows. A PCR generated I-CeuI-PI-SceI cassette from pShuttle (Clontech) was inserted between the EcoRI and SpeI sites. The 10154 bp XhoI fragment of Ad SV-25 (bp2031 to 12185, SEQ ID NO:29) was then inserted into the SpeI site. The resulting plasmid was digested with HindIII and the construct (pSV25) was

10 completed by inserting the 18344 bp Ad SV-25 HindIII fragment (bp11984 to 30328, SEQ ID NO:29) to generate a complete molecular clone of E1 deleted adenovirus SV25 suitable for the generation of recombinant adenoviruses. Optionally, a desired transgene is inserted into the I-CeuI and PI-SceI sites of the newly created pSV25 vector plasmid.

To generate an AdSV25 carrying a marker gene, a GFP (green fluorescent

15 protein) expression cassette previously cloned in the plasmid pShuttle (Clontech) was excised with the restriction enzymes I-CeuI and PI-SceI and ligated into pSV25 (or another of the Ad chimp plasmids described herein) digested with the same enzymes. The resulting plasmid (pSV25GFP) was digested with SmaI to separate the bacterial plasmid backbone and transfected into the E1 complementing cell line HEK 293. About

20 10 days later, a cytopathic effect was observed indicating the presence of replicative virus. The successful generation of an Ad SV25 based adenoviral vector expressing GFP was confirmed by applying the supernatant from the transfected culture on to fresh cell cultures. The presence of secondarily infected cells was determined by observation of green fluorescence in a population of the cells.

25

#### Example 11 - Construction of E3 deleted Pan-5, Pan-6, Pan-7 and C68 vectors

In order to enhance the cloning capacity of the adenoviral vectors, the E3 region can be deleted because this region encodes genes that are not required for the propagation of the virus in culture. Towards this end, E3-deleted versions of Pan-5, Pan-6, Pan-7, and

30 C68 have been made (a 3.5 kb Nru-AvrII fragment containing E31-9 is deleted).

A. *E3 deleted Pan5 based vector*

E1-deleted pPan5-pkGFP plasmid was treated with Avr II endonuclease to isolate a 5.8 kb fragment containing the E3 region and re-circulate pPan5-pkGFP with Avr II deletion to form construct pPan5-pkGFP-E3-Avr II. Subsequently, the 5.8 kb Avr II fragment was subcloned into pSL-Pan5-E3-Avr II for a further deletion of E3 region by Nru I digestion. This led to a plasmid pSL-Pan5-E3-deletion. The final construct pPan5-E3-pkGFP was produced by removing a 4.3 kb Avr II/Spe I fragment from pSL-Pan5-E3-deletion plasmid and inserting into pPan5-pkGFP-E3-Avr II at Avr II site. In this final construct, a 3.1 kb deletion in E3 region was accomplished.

B. *E3 deletion in Pan6 based vector*

E1-deleted pPan6- pkGFP molecular clone was digested with Sbf I and Not I to isolate 19.3 kb fragment and ligated back at Sbf I site. The resulting construct pPan6-Sbf I-E3 was treated with Eco 47 III and Swa I, generating pPan6-E3. Finally, 21 kb Sbf I fragment from Sbf I digestion of pPan6- pkGFP was subcloned into pPan6-E3 to create pPan6-E3-pkGFP with a 4 kb deletion in E3.

C. *E3 deleted Pan7 and Pan9 vectors*

The same strategy was used to achieve E3 deletions in both vectors. First, a 5.8 kb Avr II fragment spanning the E3 region was subcloned pSL-1180, followed by deletion of E3 by Nru I digestion. The resulting plasmids were treated with Spe I and Avr II to obtain 4.4 kb fragments and clone into pPan7- pkGFP and pPan9-pkGFP at Avr II sites to replace the original E3 containing Avr II fragments, respectively. The final pPan7-E3- pkGFP and pPan9-E3- pkGFP constructs have 3.5 kb E3-deletions.

Example 12 - Construction of E3- and E4-deleted Pan-7 vector

Although the deletion of the E1 region of adenoviruses (first generation adenovirus vectors) renders them replication-incompetent, expression of the adenoviral vector backbone genes is not fully abolished. Deletion of the E4 region considerably attenuates this residual gene expression and may confer a safety advantage. An E4-deleted Pan-7 vector containing a 2.5 kb deletion (a PvuII-AgeI fragment containing E4ORF1-ORF7 is deleted) has been constructed. High titer stocks of this virus were generated using a HEK 293-based cell line, which in addition to E1, expresses an essential E4 gene (orf 6).

1. *E4 deletion in the molecular clone of Pan7*

A 19 kb Xba I fragment was deleted from pPan7- pkGFP to create pPan7-Xba I from which a 2.5 kb E4 fragment was deleted by Age I and Pvu II partial digestion, resulting in pPan7-Xba I-E4. pPan7-E4- pkGFP plasmid was generated from pPan7-Xba I-E4 in two sequential cloning steps, adding 19 kb Xba I and 15 kb I-Ceu I/Mlu I fragments, both of which came from pPan7- pkGFP construct.

2. *Introduction of E3 and E4 deletions in Pan9 vector*

A 11 kb plasmid, pPan9-EcoRI, containing E4 region was created by retrieving 11 kb EcoRI fragment from pPan9 pkGFP after EcoRI digestion and self-ligation. E4 region was deleted from this construct by Age I digestion/filled in and Pvu II partial digestion and self-ligation to generate pPan9-EcoR I-E4. A 23 kb EcoR I fragment was isolated from pPan9-pkGFP and inserted into pPan9-EcoR I-E4 at EcoR I site, followed by adding 5.8 kb Avr II fragment from pPan9-pkGFP, to form the final product pPan9-E3-E4- pkGF. Compared to the genome size of wild type Pan9, this E1-E3-E4-deleted vector could have a transgene capacity up to 8 kb.

3. *Introduction of minigene cassettes with genes of interest including reporter genes, glyco- and nuclear proteins of Ebo into molecular clones of Pan vectors*

A highly efficient direct cloning and green/white selection procedure was employed for creating molecular clones of recombinant viruses. Briefly, genes of interest were cloned into pShuttlepkGFP by screening white colonies for recombinants. Subsequently, the minigene cassettes were transferred into chimpanzee adenovirus backbone plasmids, pPanX-pkGFP with various deletions, easily by swapping with pkGFP cassette at I-Ceu I and PI-Sce I sites and screening a few white colonies for correct recombinants.

4. *Rescue of molecular clones of Pan vectors with multiple deletions in early regions and virus propagation*

For rescue of E1-E3-deleted molecular clones of chimpanzee adenovirus vectors, the clones were linearized with appropriate restriction enzymes and transfected into regular 293 cells. Once a full cytopathic effect (CPE) observed in the transfected

cells, crude lysate was harvested and expanded in 293 cells to large-scale infections. The viruses were purified by CsCl sedimentation method.

For E1-E4 and E1-E3-E4-deleted Pan vectors, 10-3 cells, a 293-based E1-E4-complementing cell line, were used for rescue and propagation of vectors. E4  
5 ORF6 gene expression in 10-3 cells was induced by addition of 150  $\mu$ M ZnSO<sub>4</sub> to the culture medium.

Example 13 - Vaccination with adenovirus vectors expressing wild type and variant EboZ GP.

10 AdHu5 or AdC7 vectors expressing Ebola envelope chimeras were produced for *in vivo* immunization experiments in C57BL/6 mice. Recombinant viruses with different viral backbones were created by molecular cloning method in which the minigene cassettes were inserted into the place of E1-deletions. The molecular clones of all recombinant viruses were rescued and grown up in 293  
15 cells for large-scale purification using CsCl sedimentation method.

Five EboZ variants encoded by AdHu5 or AdPan7 (C7) were selected and produced to evaluate their relative immunogenicity following an intramuscular Ad injection. The wt Ebo, a soluble Ebo variant, Ebo $\Delta$ 1, Ebo $\Delta$ 2, Ebo $\Delta$ 3, Ebo $\Delta$ 4, Ebo $\Delta$ 5S, Ebo $\Delta$ 6S, Ebo $\Delta$ 7S and Ebo $\Delta$ 8S were evaluated in the initial vaccine  
20 studies. For the data summarized in the following table, the number of viral particles (per ml or total) produced and amplified from infected 293 cells was established by spectrophotometry reading.

Table: Production of AdHu5 or AdC7 Adenoviral vector encoding EboZ variant.

| Gene   | HuAd5                                   |                                               | AdC7                                    |                                               |
|--------|-----------------------------------------|-----------------------------------------------|-----------------------------------------|-----------------------------------------------|
|        | Titer<br>(VP x<br>10 <sup>12</sup> /ml) | Total<br>yield<br>(VP x<br>10 <sup>12</sup> ) | Titer<br>(VP x<br>10 <sup>12</sup> /ml) | Total<br>yield<br>(VP x<br>10 <sup>12</sup> ) |
| Ebo wt | 2.6                                     | 12                                            | 4.3                                     | 43                                            |
| EboS   | 4.9                                     | 49                                            | 4.6                                     | 55                                            |
| EboΔ2  | 2.1                                     | 9                                             | 5.8                                     | 93                                            |
| EboΔ3  | 1.7                                     | 8                                             | 5.3                                     | 95                                            |
| EboΔ4  | 3                                       | 12                                            | 4.1                                     | 62                                            |

Vector was administered intramuscularly (10<sup>11</sup> genome copies/cell) in C57BL/6 mice and the presence of virus neutralizing antibody (VNA) was evaluated 28 days later as a first measure of an immune response generated against the Ebola envelope glycoprotein. VNA is defined here as serum antibody able to inhibit transduction of HeLa cells mediated by HIV-based vector pseudotyped with the wild-type Ebola envelope.

VNA to the EboZ pseudotypes was detected with AdPan7 (C7) yielding higher titers than AdHu5. The EboZΔ3 elicited the highest VNA in terms of the transgene targets. For the data summarized in the following table, neutralizing antibody titers to HIV-EboZ-GFP pseudotypes (reciprocal dilution) are provided (N=5 animals/group).

|       | VNA Titers    |       |        |
|-------|---------------|-------|--------|
|       | EboZ wildtype | EboZs | EboZΔ3 |
| AdHu5 | 12            | 16    | 12     |
| AdC7  | 44            | 12    | 140    |

#### Example 14 – Pan7-mediated Expression of Ebola Proteins

Mouse studies to evaluate Pan-7 vectors expressing Ebola envelope proteins and the Ebola nuclear antigen have been initiated. These are directed towards evaluation of neutralizing antibodies in C57Bl/6 mice injected intramuscularly (IM) with Adhu5 or Pan-7 expressing each of 4 Ebola env constructs.

##### A. *Evaluation of CTL from C57Bl/6 mice injected IM with Adhu5 or Pan-7 expressing the Ebola env constructs.*

##### 1. *Challenge experiment in mice with Ebola virus.*

Neutralizing antibody (NAB) responses to the Ebola envelope were analyzed by looking at immunized mouse sera mediated neutralization of a lentiviral (HIV) vector pseudotyped with the several constructs (eEbo, NTD2, NTD3, NTD4) of the Ebola envelope glycoprotein. C57BL/6 or BALB/c mice received a single intramuscular injection of  $5 \times 10^{10}$  particles per mouse of C7 (Ad Pan-7) encoding Ebola envelope variant. Neutralizing antibody was evaluated 30 days post-vaccination. Briefly, Ebola Zaire pseudotyped HIV vector encoding for  $\beta$ -galactosidase (EboZ-HIV-LacZ) was incubated for 2 hr at 37°C with different dilution of heat inactivated mouse serum. Following the incubation with serum, EboZ-HIV-LacZ was then used to infect HeLa cells for 16 hr at 37°C. Infectivity was revealed by X-gal staining of transduced HeLa cells positive for  $\beta$ -galactosidase. Neutralizing titer represent the serum reciprocal dilution where a 50% decrease in the number of  $\beta$ -galactosidase positive blue cells was observed. Sera were collected 30 days post-immunization, which consisted in a single intramuscular (I.M.) administration of  $5 \times 10^{10}$  particles/animal. Neutralizing antibody to Ebola pseudotyped HIV could be detected from all groups with antibody titers ranging from 20 for Ad-EboZ (Adhu5 expressing EboZ), Ad-NTD3 (Adhu5 expressing NTD3) and C7-sEbo (Ad Pan-7 expressing soluble EboZ) to over 130 for C7-NTD3 (Ad Pan-7 expressing soluble NTD3) and C7-NTD4 (Ad Pan-7 expressing soluble NTD3). The same immunization strategy in BALB/c mice resulted in lower neutralizing antibody titers for Ad- and C7-NTD2, and NTD4.

##### B. *Cellular Immune Response*

The cellular immune response to the Ebola envelope in C57BL/6 mice was evaluated 8 days after a single I.M. administration of  $5 \times 10^{10}$  particles of C7-LacZ or C7-Ebola envelope variant per animal. Mice were vaccinated I.M. with  $5 \times 10^{10}$  particles

of C7 encoding LacZ or Ebola envelope variant. Splenic lymphocytes from immunized mice were collected 8 days post vaccination and stimulated in vitro with feeder cells (splenic lymphocytes from untreated mice infected with human Adenovirus serotype 5 encoding for the wild-type Ebola envelope and irradiated). Standard 5-hr CTL assays  
5 were performed using <sup>51</sup>Cr-labeled syngenic C57 cells transfected with an expressor of EboZ.

A positive MHC-restricted cytotoxic T lymphocyte (CTL) response was observed from all AdPan-7 encoding for Ebola envelope variants with a higher response from NTD2, NTD3 or NTD4 immunized mice. Indeed, effector cells from C7 encoding Ebola  
10 envelope variant immunized mice recognized EboZ transfected target cells and gave recall CTL responses up to 30% specific lysis. Less than 5% lysis was seen with effector cells from naïve or LacZ immunized control mice confirming that lysis was specific for Ebola envelope antigens.

### C. *Protection Studies*

15 The most direct means of evaluating C7 (Ad Pan-7) encoding for the EboZ variants as a successful vaccine in mice was to assess protection against weight loss and death following lethal challenge with mouse adapted Ebola Zaire virus. BALB/c mice were immunized with a single dose of  $5 \times 10^{10}$  particles per animal as performed previously and vaccinated animals were challenged with 200 LD<sub>50</sub> of mouse adapted  
20 Ebola Zaire 21 days later. All control mice (vehicle and C7-LacZ) died between day 5 to day 9 post-challenge. In contrast, all vaccinated mice but one, (from the C7-sEbo group), survived the challenge with Ebola Zaire.

Weight loss was observed from mice vaccinated with C7-sEbo from day 4 to day 7. Signs of illness such as pilo-erection and from light to severe lethargy were also noted  
25 from mice vaccinated with C7-sEbo, NTD2 and NTD3 between day 4 to day 7. Mice immunized with C7-EboZ and C7-NTD4 did not show sign of illness. Overall, a single dose of C7-EboZ and C7-NTD4 completely protected immunized mice from illness and death possibly due to a significant T cell mediated immunity.

All documents recited above are incorporated herein by reference. Numerous modifications and variations of the present invention are included in the scope of the above-identified specification and are expected to be obvious to one of skill in the art.

- 5 Such modifications and alterations to the compositions and processes of the present invention, such as selections of different minigenes or selection or dosage of the vectors or immune modulators are believed to be within the scope of the claims appended hereto.

## CLAIMS:

1. A recombinant adenovirus having a capsid comprising an AdPan7 hexon protein having the amino acid sequence of SEQ ID NO:11, a fiber protein, and a penton protein, the adenovirus further comprising adenovirus sequences functionally deleted in the E1a and/or E1b genes, 5' and 3' adenovirus cis-elements necessary for replication and encapsidation, and a transgene heterologous to the adenovirus and operatively linked to sequences which direct expression of said gene in a host cell.
2. The recombinant adenovirus according to claim 1, wherein the fiber protein is the AdPan7 fiber protein of SEQ ID NO:20.
3. The recombinant adenovirus according to claim 1, wherein the fiber protein is the AdPan7 fiber protein of SEQ ID NO: 12.
4. The recombinant adenovirus according to any one of claims 1 to 3, wherein the penton protein is an AdPan7 penton protein of SEQ ID NO: 10.
5. The recombinant adenovirus according to any one of claims 1 to 4, wherein said recombinant adenovirus is a pseudotyped adenovirus, wherein said genome comprises 5' and 3' adenovirus cis-elements necessary for replication and encapsidation, said cis-elements comprising an adenovirus 5' inverted terminal repeat and an adenovirus 3' inverted terminal repeat from an adenovirus different from that supplying the hexon protein.
6. A recombinant adenovirus having a capsid comprising a hexon containing a fragment of the AdPan7 hexon protein, a fiber protein, and a penton protein, the adenovirus further comprising an adenovirus genome functionally deleted in the E1a and/or E1b genes, 5' and 3' adenovirus cis-elements necessary for replication and encapsidation, and a transgene heterologous to the adenovirus, wherein the fragment of the AdPan7 hexon protein is an N-terminal or C-terminal truncation of about 50 amino acids in length of the AdPan7 hexon protein sequence of SEQ ID NO:11.

7. The recombinant adenovirus according to any one of claims 1 to 4 or 6, wherein the adenovirus cis elements comprise 5' inverted terminal repeat (ITR) sequences and the 3' ITR, of AdPan7 of SEQ ID NO: 9 or a sequence complementary thereto.

8. The adenovirus according to claim 6 comprising at least one simian capsid protein selected from:

the penton protein of AdPan7 SEQ ID NO:10; and  
the fiber protein of AdPan7 SEQ ID NO:12.

9. An isolated host cell comprising a recombinant adenovirus according to any one of claims 1 to 8.

10. A composition comprising a recombinant adenovirus according to any one of claims 1 to 8 and a pharmaceutically acceptable carrier.

11. Use of a recombinant adenovirus of any one of claims 1 to 8 for the manufacture of a medicament formulated for eliciting an immune response against an infective agent in a mammalian host.

12. A recombinant adenovirus according to any one of claims 1 to 8 for use in eliciting an immune response against an infective agent in a mammalian host.