USE OF NON-SUBTYPE B GAG PROTEINS FOR LENTIVIRAL PACKAGING

Background of the Invention

Recombinant vaccines have been developed with the progress of recombinant DNA technology, allowing the modification of viral genomes to produce modified viruses. In this manner, it has been possible to introduce genetic sequences into non-pathogenic viruses, so that they encode immunogenic proteins to be expressed in target cells upon infection, in order to develop a specific immune response in their host.

Such vaccines constitute a major advance in vaccine technology (Kutzler et al., *Nat Rev Genet,* 9(10) : 776-788, 2008). In particular, they have the advantage over traditional vaccines of avoiding live (attenuated) virus and eliminating risks associated with the manufacture of inactivated vaccines.

Gene delivery using modified retroviruses (retroviral vectors) was introduced in the early 1980s by Mann et al. (*Cell,* 33(1):153-9, 1983). The most commonly used oncogenic retroviral vectors are based on the Moloney murine leukemia virus (MLV). They have a simple genome from which the polyproteins Gag, Pol and Env are produced and are required in *trans* for viral replication (Breckpot *et al*., 2007, *Gene Ther,* 14(11):847-62; He *et al*. 2007, *Expert Rev vaccines,* 6(6):913-24). Sequences generally required in *cis* are the long terminal repeats (LTRs) and its vicinity: the inverted repeats (IR or att sites) required for integration, the packaging sequence Ψ, the transport RNA-binding site (primer binding site, PBS), and some additional sequences involved in reverse transcription (the repeat R within the LTRs, and the polypurine tracts, PPT, necessary for plus strand initiation). To generate replication-defective retroviral vectors, the *gag*, *pol*, and *env* genes are generally entirely deleted and replaced with an expression cassette.

Retroviral vectors deriving from lentivirus genomes (i.e. lentiviral vectors) have emerged as promising tools for both gene therapy and immunotherapy purposes, because they exhibit several advantages over other viral systems. In particular, lentiviral vectors themselves are not toxic and, unlike other retroviruses, lentiviruses are capable of transducing non-dividing cells, in particular dendritic cells (He *et al*. 2007, *Expert Rev vaccines,* 6(6):913-24), allowing antigen presentation through the endogenous pathway.

Lentiviruses represent a genus of slow viruses of the Retroviridae family, which includes the human immunodeficiency viruses (HIV), the simian immunodeficiency virus (SIV), the equine infectious encephalitis virus (EIAV), the caprine arthritis encephalitis

virus (CAEV), the bovine immunodeficiency virus (BIV) and the feline immunodeficiency virus (FIV). Lentiviruses can persist indefinitely in their hosts and replicate continuously at variable rates during the course of the lifelong infection. Persistent replication of the viruses in their hosts depends on their ability to circumvent host defenses.

The design of recombinant lentiviral vectors is based on the separation of the *cis*- and *trans*-acting sequences of the lentivirus. Efficient integration and replication in non-dividing cells requires the presence of two *cis*-acting sequences in the lentiviral genome, the central polypurine tract (cPPT) and the central terminal sequence (CTS). These lead to the formation of a triple-stranded DNA structure called the central DNA "flap", which maximizes the efficiency of gene import into the nuclei of non-dividing cells, including dendritic cells (DCs) (Zennou *et al.,* 2000, Cell, 101(2) 173-85; Arhel *et al.,* 2007, *EMBO J,* 26(12):3025-37).

HIV-1 lentiviral vectors have been generated based on providing the subtype B Gag, Pol, Tat and Rev proteins for packaging vectors *in trans* from a packaging construct (Naldini *et al*, PNAS 15: 11382-8 (1996); Zufferey *et al*, Nature Biotechnology 15:871-875, 1997); Dull *et al*, Journal of Virology (1997)). These studies were performed with subtype B Gag and Pol proteins. The effect of non-subtype B *gag* and *pol* sequences in a HIV-1 packaging construct was not assessed.

There are many different subtypes of HIV-1 other than subtype B. Some subtypes of HIV-1, such as C, E, and A, appear to be transmitted more efficiently than HIV-1 subtype B, which is the major subtype in the United States and Europe. Essex et al., Adv Virus Res. 1999; 53:71-88. The predominant subtype of HIV-1 that is found in the developed Western World, clade B, differs considerably from those subtypes and recombinants that exist in Africa and Asia, where the vast majority of HIV-infected persons reside. Spira et al., J. Antimicrobial Chemotherapy (2003) 51, 229-240. Thus, serious discrepancies may exist between the subtype B retrovirus encountered in North America and Europe and those viral subtypes that plague humanity on a global scale. *Id.* Subtype diversity may impact on modes of HIV transmission. Homosexual and intravenous drug abuse are the primary modes of transmission observed for clade B strains in Europe and the Americas. *Id.* In contrast, clades A, C, D and E predominate in Africa and Asia where heterosexual transmission predominates. *Id.* In addition, some studies suggest that AIDS progression differs as a function of infecting subtype. *Id.* Thus, it appears that HIV-1 subtype B is quite different than the other HIV-1 subtypes.

HIV phylogenic classifications are normally based either on nucleotide sequences derived from multiple sub genomic regions (*gag, pol* and *env*) of the same isolates, or on full-length genome sequence analysis. A phylogenic analysis of HIV-1 near-full length sequences revealed that HIV-1 subtype B was most closely related genetically to HIV subtype D (Figure 1). Phylogenic analyses of HIV-1 Gag and Pol protein sequences also showed that HIV-1 subtype B was most closely related genetically to HIV subtype D (Figure 2).

Nevertheless, HIV-1-NDK, a subtype D virus, is significantly more cytopathic for CD4+ lymphocytes than the HIV-1-BRU prototype, a subtype B virus. This may be due to enhanced fusogenicity and infectivity of subtype D viruses. De Mareuil et al., J. Virol. 66: 6797 (1992). Phenotypic analysis of recombinant viruses indicated that 75 amino acids from the N-terminal part of HIV-1-NDK matrix (MA) protein, together with the HIV-1-NDK envelope glycoprotein, are responsible for enhanced fusogenicity of HIV-1-NDK in CD4+ lymphocytes as well as for enhanced infectivity of HIV-1-NDK in some CD4- cell lines. *Id*.

There is a need in the art for lentiviral packaging constructs producing higher titers of packaged lentiviral vectors, in order to reduce injection volumes, increase dosages, reduce the cost of vaccination, and increase the number of patients that could be treated with one batch. The current invention fulfills this need.

Brief Summary of the Invention

The *gag-pol* gene of subtype B of HIV-1 in a lentiviral packaging plasmid (construct p8.74) was replaced by the *gag-pol* gene of a subtype D HIV-1 to generate construct pThV-GP-N. The constructs were used for lentiviral vector production. Approximately 2-fold higher titers were obtained using the pThV-GP-N plasmid as compared to construct p8.74. Thus, the Gag-Pol of a subtype D virus increases the titer of lentiviral vector particles relative to a Gag-Pol of a subtype B virus.

The invention encompasses a lentiviral packaging vector comprising a subtype D *gag-pol* sequence, particularly from HIV-1 NDK. In a preferred embodiment, the lentiviral packaging vector comprises the nucleotide sequence of SEQ ID NO:1. In a preferred embodiment, the lentiviral packaging vector encodes the amino acid sequence of SEQ ID NO:2.

The nucleotide sequence of SEQ ID NO 1 is:

atgggtgcgagagcgtcagtattaagcgggggaaaattagatacatgggaaagaattcggttac
ggccaggaggaaagaaaaaatatgcactaaaacatttgatatgggcaagcagggagctagaacg

atttacacttaatcctggccttttagagacatcagaaggctgtaaacaaataataggacagcta
caaccatctattcaaacaggatcagaagaaattagatcattatataatacagtagcaaccctct
attgtgtacatgaaaggatagaggtaaaagacaccaaagaagctgtagaaaagatggaggaaga
acaaaacaaaagtaagaaaaagacacagcaagcagcagctgatagcagccaggtcagccaaaat
taccctatagtgcagaacctacaggggcaaatggtacatcaggccatatcacctagaactttga
acgcatgggtaaaagtaatagaagaaaaggccttcagcccggaagtaatacccatgttttcagc
attatcagaaggagccaccccacaagatttaaacaccatgctaaacacagtgggggggacatcaa
gcagctatgcaaatgctaaaagagaccatcaatgacgaagctgcagaatgggacagattacatc
cagtgcatgcagggcctgttgcaccaggccaaatgagagaaccaaggggaagtgatatagcagg
aactactagtacccttcaggaacaaatagcatggatgacaagcaacccacctatcccagtagga
gaaatctataaaagatggataatcctgggattaaataaaatagtaagaatgtatagccctgtca
gcattttggacataagacagggaccaaaggaacctttagagactatgtagaccggttctataa
aactctaagagccgagcaagcttcacaggatgtaaaaaactggatgacagaaaccttgttggtc
caaaatgcaaacccagattgtaaaactatcttaaaagcattgggaccacaggctacactagaag
aaatgatgacagcatgccagggagtgggggggcccggccataaagcaagagttttggctgaggc
aatgagccaagtaacaggttcagctactgcagtaatgatgcagagaggcaattttaagggccca
agaaaaagtattaagtgtttcaactgtggcaaggaagggcacacagcaaaaaattgcagggccc
ctagaaaaaagggctgttggaaatgcggaagggaaggacaccaaatgaaagattgcactgaaag
acaggctaatttttagggaagatttggccttcccacaagggaaggccggggaattttcttcag
agcagaccagagccaacagccccaccagcagagagcttcgggtttggggaggagataacccccct
ctcagaaacaggagcagaaagacaaggaactgtatcctttagcttccctcaaatcactctttgg
caacgacccctcgtcacaataaagataggggacagctaaaggaagctctattagatacaggag
cagatgatacagtattagaagaaataaatttgccaggaaaatggaagccaaaaatgatagggggg
aattggaggttttatcaaagtaagacagtatgatcaaatactcatagaaatctgtggatataaa
gctatgggtacagtattagtaggacctacacctgtcaacataattggaagaaatttgttgaccc
agattggctgcactttaaattttccaattagtcctattgaaactgtaccagtaaaattaaagcc
aggaatggatggcccaaaagttaaacaatggccattgacgaagaaaaaataaaagcattaacag
aaatttgtacagaaatggaaaaggaaggaaaaatttcaagaattgggcctgaaaatccatataa
tactccaatatttgccataaagaaaaaagacagtaccaagtggagaaaattagtagatttcaga
gaacttaataagagaactcaagatttctgggaggttcaattaggaataccgcatcctgcagggc
tgaaaaagaaaaaatcagtaacagtactggatgtgggtgatgcatatttctcagttcccttaga
tgaagattttaggaaatataccgcatttaccatacctagtataaacaatgagacaccagggatt
agatatcagtacaatgtgctcccacagggatggaaaggatcaccggcaatattccaaagtagca
tgacaaaaatcttagagccctttagaaaacaaaatccagaaatagttatctatcaatacatgga

tgatttgtatgtaggatctgacttagaaatagggcagcatagaacaaaaatagaggaattaaga
gaacatctattgaggtggggatttaccacaccagataaaaaacatcagaaagaacctccatttc
tttggatgggttatgaactccatcctgataaatggacagtacagcctataaacctgccagaaaa
agaaagctggactgtcaatgatatacagaagttagtggggaaattaaactgggcaagccagatt
tatgcaggaattaaagtaaagcaattatgtaaactccttaggggaaccaaagcactaacagaag
tagtaccactaacagaagaagcagaattagaactggcagaaaacagggaaattctaaaagaacc
agtacatggagtgtattatgacccatcaaaagacttaatagcagaactacagaaacaaggggac
ggccaatggacataccaaatttatcaagaaccatttaaaaatctaaaaacaggaaagtatgcaa
gaacgaggggtgcccacactaatgatgtaaaacaattaacagaggcagtgcaaaaaatagccac
agaaagcatagtgatatggggaaagactcctaaatttaaactacccatacaaaaggaaacatgg
gaaacatggtggatagagtattggcaagccacctggattcctgagtgggaatttgtcaataccc
ctcctttagtaaaattatggtaccagttagagaaggaacccataataggagcagaaactttcta
tgtagatggggcagctaatagagagactaaattaggaaaagcaggatatgttactgacagagga
agacagaaagttgtccctttcactgacacgacaaatcagaagactgagttacaagcaattaatc
tagctttacaggattcgggattagaagtaaacatagtaacagattcacaatatgcactaggaat
cattcaagcacaaccagataagagtgaatcagagttagtcagtcaaataatagagcagctaata
aaaaaggaaaaggtttacctggcatgggtaccagcacacaaaggaattggaggaaatgaacaag
tagataaattagtcagtcagggaatcaggaaagtactatttttggatggaatagataaggctca
ggaagaacatgagaaatatcacaacaattggagagcaatggctagtgattttaacctaccacct
gtggtagcgaaagaaatagtagctagctgtgataaatgtcagctaaaaggagaagccatgcatg
gacaagtagactgtagtccaggaatatggcaattagattgtacacatctggaaggaaaagttat
cctggtagcagttcatgtagccagtggctatatagaagcagaagttattccagcagaaacgggg
caagaaacagcatactttctcttaaaattagcaggaagatggccagtaaaagtagtacatacag
ataatggcagcaatttcaccagtgctacagttaaggccgcctgttggtgggcagggatcaaaca
ggaatttggaattccctacaatccccaaagtcaaggagtagtagaatctatgaataaagaatta
aagaaaattataggacaggtaagagatcaagctgaacatcttaagacagcagtacaaatggcag
tatttatccacaattttaaaagaaaaggggggattgggggatacagtgcaggggaaagaataat
agacataatagcaacagacatacaaactagagaattacaaaaacaaatcataaaaattcaaaat
tttcgggtttattacagggacagcagagatccaatttggaaaggaccagcaaagcttctctgga
aaggtgaaggggcagtagtaatacaagacaatagtgacataaaggtagtaccaagaagaaaagt
aaagatcattagggattatggaaaacagatggcaggtgatgattgtgtggcaagtagacaggat
gaggattaac (SEQ ID NO 1).

The amino acid sequence of SEQ ID NO 2 is:

MGARASVLSGGKLDAWERIRLRPGGKKKYALKHLIWASRELERIALNPGLLETSEGCK
QIIGQLQPSIQTGSEELRSLYNTIATLYCVHERIEVKDTKEAVEKMEEEQNKSKKKTQQ
AAADSSQVSQNYPIVQNLQGQMVHQAISPRTLNAWVKVIEEKAFSPEVIPMFSALSEG
ATPQDLNTMLNTVGGHQAAMQMLKETINDEAAEWDRLHPVHAGPVAPGQMREPRG
SDIAGTTSTLQEQIAWMTSNPPIPVGEIYKRWIILGLNKIVRMYSPVSILDIRQGPKEPFR
DYVDRFYKTLRAEQASQDVKNWMTETLLVQNANPDCKTILKALGPQATLEEMMTACQ
GVGGPGHKARVLAEAMSQVTGSVTAVMMQRGNFKGPRKSIKCFNCGKEGHTAKNC
RAPRKKGCWKCGREGHQMKDCSERQANFLGKIWPSHKGRPGNFLQSRPEPTAPPA
ESFGFGEEITPSQKQEQKDKELYPLASLKSLFGNDPSSQFFREDLAFPQGKAGEFSSE
QTRANSPTSRELRVWGGDNPLSETGAEGQGTVSFSFPQITLWQRPLVTIKIGGQLKEA
LLDTGADDTVLEEMNLPGKWKPKMIGGIGGFIKVRQYDQILIEICGYKAMGTVLVGPTP
VNIIGRNLLTQIGCTLNFPISPIETVPVKLKPGMDGPKVKQWPLTEEKIKALTEICTEMEK
EGKISRIGPENPYNTPIFAIKKKDSTKWRKLVDFRELNKRTQDFWEVQLGIPHPAGLKK
KKSVTVLDVGDAYFSVPLDEDFRKYTAFTIPSINNETPGIRYQYNVLPQGWKGSPAIFQ
SSMTKILEPFRKQNPEIVIYQYMDDLYVGSDLEIGQHRTKIEELREHLLRWGFTTPDKK
HQKEPPFLWMGYELHPDKWTVQPIKLPEKESWTVNDIQKLVGKLNWASQIYAGIKVK
QLCKLLRGTKALTEVVPLTEEAELELAENREILKEPVHGVYYDPSKDLIAELQKQGDGQ
WTYQIYQEPFKNLKTGKYARTRGAHTNDVKQLTEAVQKIATESIVIWGKTPKFKLPIQK
ETWETWWIEYWQATWIPEWEFVNTPPLVKLWYQLEKEPIIGAETFYVDGAANRETKL
GKAGYVTDRGRQKVVPFTDTTNQKTELQAINLALQDSGLEVNIVTDSQYALGIIQAQPD
KSESELVSQIIEQLIKKEKVYLAWVPAHKGIGGNEQVDKLVSQGIRKVLFLDGIDKAQEE
HEKYHNNWRAMASDFNLPPVVAKEIVASCDKCQLKGEAMHGQVDCSPGIWQLDCTH
LEGKVILVAVHVASGYIEAEVIPAETGQETAYFLLKLAGRWPVKVVHTDNGSNFTSATV
KAACWWAGIKQEFGIPYNPQSQGVVESMNKELKKIIGQVRDQAEHLKTAVQMAVFIHN
FKRKGGIGGYSAGERIIDIIATDIQTRELQKQIIKIQNFRVYYRDSRDPIWKGPAKLLWKG
EGAVVIQDNSDIKVVPRRKVKIIRDYGKQMAGDDCVASRQDED (SEQ ID NO:2).

The invention encompasses a lentiviral packaging vector comprising a subtype D MA sequence, particularly from HIV-1 NDK. In a preferred embodiment, the lentiviral packaging vector encodes an MA protein comprising the amino acid sequence of SEQ ID NO:3.

The amino acid sequence of SEQ ID NO 3 is:

MGARASVLSGGKLDTWERIRLRPGGKKKYALKHLIWASRELERFTLNPGLLETSEGCK
QIIGQLQPSIQTGSEEIRSLYNTVATLYCVHERIEVKDTKEAVEKMEEEQNKSKKKTQQ
AAADSSQVSQNY (SEQ ID NO 3).

Preferably, the lentiviral packaging vector encoding the HIV Gag MA protein generates at least a 1.5 fold increase, or at least a 2-fold increase, in the titer of a packaged lentiviral vector as compared to the lentiviral packaging vector encoding an HIV Gag MA protein of HIV-1 BRU, e.g., p8.74. Preferably, the lentiviral packaging vector is replication-defective and lacks a Ψ site.

In a preferred embodiment, the lentiviral packaging vector encodes an HIV Gag MA protein having an amino acid at position 12 that is not a glutamic acid and an amino acid at position 15 that is not an arginine. Preferably, the lentiviral packaging vector does not have both a valine at position 46 and a leucine at position 61.

In a preferred embodiment, the amino acid at position 12 of the MA protein is a lysine. In a preferred embodiment, the amino acid at position 15 is a threonine. In a preferred embodiment, the amino acid at position 15 is an alanine. In a preferred embodiment, the amino acid at position 46 is a leucine. In a preferred embodiment, the amino acid at position 61 is an isoleucine. In a preferred embodiment, the amino acid at position 61 is a methionine.

In one embodiment, the vector does not encode a functional Env protein.

The invention also encompasses methods for making the above lentiviral packaging vectors and methods for using these lentiviral packaging vectors.

Brief Description of the Drawings

The invention is more fully understood through reference to the drawings.

Fig. 1 depicts phylogenic trees of HIV viruses.

Fig. 2A and B depict phylogenic trees of HIV GAG (A) and POL (B) proteins.

GAG and POL protein sequences were obtained from: http://www.hiv.lanl.gov/content/sequence/NEWALIGN/align.html. For each known HIV clade, one patient's (for clade B) or two patients' virus sequences were randomly chosen and GAG and POL protein sequences were compared to the reference clade B proteins (B.FR.83.HXB2_LAI_IIIB_BRU.KO3455). Alignments were performed using Vector NTI advance 11 (Invitrogen).

Fig. 3 depicts titers obtained using the p8.74 and the pThV-GP-N plasmids for vector production. Lentiviral particles were produced using the proviral plasmid (pFLAP-CMV-GFP), the pseudotyping plasmid (pTHV-VSV.G) and either the commonly used packaging plasmid (derived from the BRU strain, p8,74) or an NDK-derived packaging plasmid (pTHV-GP-N). With each packaging plasmid, 18 independent transfections were performed and the particles titers were measured by FACS analysis. Similar

results were also obtained using a vector employing a proviral plasmid containing the β2 microglobulin promoter driving HIV antigen expression.

Fig. 4 depicts production of wild type BRU and NDK viruses and evaluation of their respective early phase efficiency. 293T cells were transfected either with plasmid encoding for the wild type BRU (pBRU) or NDK (pNDK-N) virus. Viral supernatants were collected after 48 hours and diluted to infect P4-CCR5 cells (encompassing a stable luciferase reporting gene which expression is driven by the HIV LTR, allowing a luciferase production in presence of the TAT protein (brought by the virus). Serial dilution of either BRU or NDK viruses were used to infect P4-CCR5 cells and the luciferase expression (A) or the luciferase/P24 ratio (B) were measured.

Fig. 5 depicts vector production using different ratios of BRU (p8,74) and NDK (pThV GP-N) derived packaging plasmids. For each conditions (from 0µg NDK + 10µg BRU to 10µg NDK + 0µg BRU), the titer (gray bars) and the P24 level (black squares) were measured.

Fig. 6 depicts a Western blot of vector supernatants produced using either BRU (8,74) or NDK (pThV GP-N) derived packaging plasmids. The P24 protein and precursors detection was performed using the NIH anti-P24 MAB (183-H12-5C).

Fig. 7 depicts a sequence alignment of the N-terminal MA sequences of a clade B virus (BRU; SEQ ID NO:3) with a clade D virus (NDK; SEQ ID NO:4).

Fig. 8 depicts a sequence alignment of the N-terminal MA sequences of clade B viruses.

Fig. 9 depicts a sequence alignment of the N-terminal MA sequences of clade D viruses.

Fig. 10 depicts a sequence alignment of the N-terminal MA sequences of a clade B virus (BRU) with viruses of other clades.

Detailed Description of the Invention

Subtype B HIV-1 viruses differ from other HIV-1 subtypes in that subtype B viruses appear to be transmitted less efficiently and have a different mode of transmission. Nevertheless, subtype B viruses have been used extensively in the generation of HIV-1 lentiviral vectors and lentiviral packaging vectors. To determine whether the Gag and Pol proteins of non-subtype B viruses could be used for the generation of lentiviral packaging vectors, the *gag-pol* gene of HIV-1 subtype B in a lentiviral packaging plasmid (construct p8.74) was replaced by the *gag-pol* gene of a subtype D HIV-1 to generate construct pThV-GP-N. The constructs were used for

lentiviral vector production. Approximately 2-fold higher titers were obtained using the pThV-GP-N plasmid as compared to construct p8.74 (Figure 3). Thus, the subtype D HIV-1 Gag-Pol in the packaging vector increased the titer of lentiviral vector over the titer seen with a subtype B HIV-1 Gag-Pol.

Increased titer of the lentiviral vector is beneficial in allowing the reduction in contaminants in a given dose of lentiviral vector. This facilitates a reduction in injection volumes, and an increase in possible dosages. It further facilitates reducing the cost of vaccination by reducing the quantity of materials and labor necessary to achieve a particular dose, and by increasing the number of patients that could be treated with a single batch of lentival vector.

Serial dilutions of HIV-1 BRU and HIV-1 NDK viruses indicated that the HIV-1 NDK virus was more efficient in the early phases of the virus life cycle (Figure 4). By mixing different amounts of the subtype B and subtype D packaging vectors, it was demonstrated that the subtype D packaging vector increased both the titer and the level of p24 in the lentiviral vector preparations (Figure 5). The p24 in the lentiviral vector preparations using the subtype D packaging vector was also observed to be less completely processed, showing higher levels of p24 precursors (Figure 6). Thus, the Gag protein in the subtype D packaging vector was exhibiting various differences from the subtype B packaging vectors.

It is known that, with HIV-1 Env, the 75 amino acids from the N-terminal part of HIV-1-NDK matrix (MA) protein is responsible for enhanced fusogenicity of HIV-1-NDK in CD4+ lymphocytes as well as for enhanced infectivity of HIV-1-NDK in some CD4- cell lines. De Mareuil et al., J. Virol. 66: 6797 (1992). Since the Env protein used for generation of lentiviral vectors with the subtype B and subtype D packaging vectors is the same (i.e., VSV), only the differences in HIV-1 MA are present in the subtype B and subtype D packaging vectors.

The conservation and divergence of amino acids in the N-terminal 75 amino acids of M from various clades of HIV-1 viruses was examined. HIV-1 NDK showed 10 differences in amino acids from HIV-1 BRU (Figure 7). However, only eight of these differences were seen when HIV-1 NDK was compared to an assortment of 20 different HIV-1 subtype B viruses (Figure 8). When other subtype D viruses were included in the comparison, only 4 of these differences remained (Figure 9). These differences are the absence of a glutamic acid at amino acid position 12, the absence of an arginine at

amino acid position 15, the absence of a valine at amino acid position 46, and the absence of a leucine at amino acid position 61 in subtype D viruses.

When other subtype viruses were included in the comparison, the other subtypes appeared to align with subtype D, preserving nearly all of these differences (Figure 10). Nearly all of the non-subtype B viruses exhibited a lysine at position 12. Many of the non-subtype B viruses exhibited an alanine at position 15. Nearly all of the non-subtype B viruses exhibited a leucine at position 46. Nearly all of the non-subtype B viruses exhibited a methionine or isoleucine at position 61. The non-subtype B viruses exhibited the consensus of a lysine at position 12, an amino acid other than arginine at position 15, a leucine at position 46, and an isoleucine or methionine at position 61.

The invention encompasses packaging vectors encoding non-subtype B Gag and/or Pol proteins and host cells comprising these vectors. The invention further encompasses methods for making packaging vectors encoding non-subtype B Gag and/or Pol proteins. The invention also encompasses methods for using these packaging vectors to generate lentiviral vectors, and lentiviral vectors comprising non-subtype B Gag and/or Pol proteins.

Packaging Vectors

The invention encompasses packaging vectors encoding non-subtype B Gag and/or Pol proteins. A lentiviral "packaging vector" is defined herein as a nucleic acid sequence not encoding functional HIV-1 Env and lacking a Ψ site, but capable of expressing lentiviral Gag and/or Pol proteins that can be incorporated into viral particles when cotransfected with a vector containing appropriate lentiviral cis-acting signals for packaging. The lentiviral packaging vector of the invention is unable to replicate itself by packaging and reverse transcribing its own sequence.

The packaging vector can be an RNA or DNA vector. The non-subtype B Gag and Pol proteins can be selected from subtype A, subtype C, subtype D, subtype E, subtype F1, subtype F2, subtype G, subtype H, and subtype J proteins, and recombinants thereof. A preferred packaging vector comprises SEQ ID NO:1 or encodes SEQ ID NO:2.

The invention encompasses packaging vectors encoding non-subtype B Gag proteins and host cells comprising these vectors. The non-subtype B Gag proteins can be selected from subtype A, subtype C, subtype D, subtype E, subtype F1, subtype F2, subtype G, subtype H, and subtype J proteins, and recombinants thereof. A preferred packaging vector encodes the Gag protein portion of SEQ ID NO:2.

The invention encompasses packaging vectors encoding non-subtype B MA proteins and host cells comprising these vectors. The non-subtype B MA proteins can be selected from subtype A, subtype C, subtype D, subtype E, subtype F1, subtype F2, subtype G, subtype H, and subtype J proteins, and recombinants thereof. A preferred packaging vector encodes SEQ ID NO:3 or the MA protein portion of SEQ ID NO:2.

In various embodiments, the packaging vector comprises SEQ ID NO:1 or a nucleic acid sequence that is at least 95%, 96%, 97%, 98%, 99% identical with SEQ ID NO:1. In various embodiments, the packaging vector encodes SEQ ID NO:2, SEQ ID NO:3, or an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99% identical with SEQ ID NO:2 or SEQ ID NO:3.

As used herein, the percent identity of two nucleic acid sequences can be determined by comparing sequence information using the GAP computer program, version 6.0 described by Devereux et al. (Nucl. Acids Res. 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG), using the default parameters for the GAP program including: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess, Nucl. Acids Res. 14:6745, 1986, as described by Schwartz and Dayhoff, eds., Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

In various embodiments, the vector comprises a sequence encoding an HIV-1 MA protein having one or more of the following features:

the absence of a glutamic acid at amino acid position 12;

the absence of an arginine at amino acid position 15;

the absence of a valine at amino acid position 46; and

the absence of a leucine at amino acid position 61.

In various embodiments, the vector comprises a sequence encoding an HIV-1 MA protein having one or more of the following features:

the amino acid at position 12 is a lysine;

the amino acid at position 15 is a threonine;

the amino acid at position 15 is an alanine;

the amino acid at position 46 is a leucine;

the amino acid at position 61 is an isoleucine; and/or

the amino acid at position 61 is a methionine.

The packaging vector preferably encodes HIV-1 Gag and Pol. Most preferably, the packaging vector encodes an HIV-1 Gag MA protein.

The packaging vector can contain viral or non-viral sequences for expression of Gag and Pol. The packaging vector can contain an HIV-1 LTR or the U3 region of an HIV-1 LTR. In other embodiments, the packaging vector does not contain HIV-1 LTRs. Any promoter can be used to drive expression of Gag and Pol. Preferably, the promoter is a strong promoter in human cells. Most preferably, the packaging vector contains a Cytomegalovirus (CMV) promoter, a CMV early enhancer/chicken β actin (CAG) promoter, a Rous Sarcoma Virus (RSV) promoter, a human phosphoglycerate kinase (hPGK) promoter, or a U3 from an LTR (e.g., myeloproliferative sarcoma virus (MPSV) U3) promoter driving expression of the encoded genes, e.g. *gag* and *pol*.

Preferably, the packaging vector contains a polyadenylation signal. Any polyadenylation signal can be. Preferably, polyadenylation signal is a strong signal in human cells. Most preferably, the polyadenylation signal is a human α2 globin or a Bovine Growth hormone (BGH) polyadenylation signal.

Preferably, the packaging vector contains a Rev-responsive element (RRE). In a preferred embodiment, the packaging vector expresses an HIV-1 Rev protein. In a preferred embodiment, the packaging vector contains at least one splice donor site and at least one splice acceptor site. In one embodiment, the packaging vector expresses an HIV-1 Tat protein.

In preferred embodiments, the packaging vector lacks sequences encoding HIV-1 Vif, Vpr, Vpu, and/or Nef. The packaging vector can comprise a sequence encoding an HIV-1 MA protein having one or more of the features discussed herein.

In one embodiment, the packaging vector encodes only 1 HIV-1 protein, Gag. In one embodiment, the packaging vector encodes only 2 HIV-1 proteins, Gag and Pol. In one embodiment, the packaging vector encodes only 3 HIV-1 proteins, selected from Gag, Pol, Rev, and Tat. In one embodiment, the packaging vector encodes only 4 HIV-1 proteins, Gag, Pol, Rev, and Tat.

In one embodiment, the vector comprises (from 5' to 3') a CMV promoter, a nucleic acid sequence encoding HIV-1 Gag-Pol, an exon encoding part of Tat and Rev, a splice donor site, an intron containing an RRE, a splice acceptor site, an exon encoding part of Tat and Rev, and a polyadenylation signal. Preferably, the HIV-1 Gag-Pol is a subtype D HIV-1 Gag-Pol.

The packaging vector may further contain an origin for replication in bacteria or eukaryotic cells. The packaging vector may contain a selectable marker gene for selection in bacteria or eukaryotic cells.

The invention encompasses host cells comprising the packaging vectors of the invention. The host cells can be transiently transfected with the packaging vectors of the invention. The host cells can be cell lines with the packaging vectors of the invention integrated into the genome of the host cell. Many different cells are suitable host cells Preferably, the cells are human cells, most preferably an immortalized human cell line. In one embodiment, the cells are HEK 293T cells. In one embodiment, the cells are HeLA, HT1080 or PER C6 cells (Delenda *et al,* Cells for Gene Therapy and vector Production, from Methods in Biotechnology, Vol 24 : Animal Cell Biotechnology : Methods and Protocols, 2nd Ed. Edited by R. Pörtner, Humana Press Inc., Totowa, NJ).

Packaging Systems

The invention encompasses lentiviral packaging systems comprising cells expressing non-subtype B Gag and/or Pol proteins. A lentiviral "packaging system" is defined herein as a cell-based system comprising cells expressing at least lentiviral Gag and Pol proteins in the absence of a Ψ site, and capable of packaging and reverse transcribing an exogenous nucleic acid containing a Ψ site. The cells of the lentiviral packaging system can also express other viral proteins. Preferably, the lentiviral packaging system expresses an envelope protein. The envelope protein can be a lentiviral (e.g., HIV-1 Env) or non-lentiviral (e.g., VSV, Sindbis virus, Rabies virus) envelope protein. In various embodiments, the lentiviral packaging system expresses an HIV-1 Tat and/or Rev protein.

In various embodiments, the cells of the lentiviral packaging system contain sequences encoding HIV-1 Gag and/or Pol stably integrated into their genome. In various embodiments, the cells of the lentiviral packaging system contain sequences encoding an envelope protein stably integrated into their genome. In various embodiments, the cells of the lentiviral packaging system contain sequences encoding HIV-1 Tat and/or Rev stably integrated into their genome.

In various embodiments, the cells of the lentiviral packaging system transiently express HIV-1 Gag and/or Pol proteins. In various embodiments, the cells of the lentiviral packaging system transiently express an envelope protein. In various embodiments, the cells of the lentiviral packaging system transiently express HIV-1 Tat and/or Rev proteins.

The cells of the lentiviral packaging system can express non-subtype B Gag and Pol proteins selected from subtype A, subtype C, subtype D, subtype E, subtype F1, subtype F2, subtype G, subtype H, and subtype J proteins, and recombinants thereof. In one embodiment, the cells of the lentiviral packaging system comprise SEQ ID NO:1 or express SEQ ID NO:2.

In various embodiments, the cells of the lentiviral packaging system express non-subtype B Gag proteins. The non-subtype B Gag proteins can be selected from subtype A, subtype C, subtype D, subtype E, subtype F1, subtype F2, subtype G, subtype H, and subtype J proteins, and recombinants thereof.

In various embodiments, the cells of the lentiviral packaging system express non-subtype B MA proteins. The non-subtype B MA proteins can be selected from subtype A, subtype C, subtype D, subtype E, subtype F1, subtype F2, subtype G, subtype H, and subtype J proteins, and recombinants thereof. A preferred packaging vector encodes SEQ ID NO:3 or the MA protein portion of SEQ ID NO:2.

In various embodiments, the cells of the lentiviral packaging system can contain any of the lentiviral vectors of the invention.

In various embodiments, the cells of the lentiviral packaging system contain SEQ ID NO:1 or a nucleic acid sequence that is at least 95%, 96%, 97%, 98%, 99% identical with SEQ ID NO:1. In various embodiments, the cells of the lentiviral packaging system express a protein with the amino acid sequence of SEQ ID NO:2, SEQ ID NO:3, or an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99% identical with SEQ ID NO:2 or SEQ ID NO:3.

Methods of Producing Packaging Vectors

The invention encompasses methods for making packaging vectors encoding non-subtype B Gag and/or Pol proteins. The packaging vector can comprise any of the features discussed herein.

In one embodiment, the method comprises inserting a Gag and/or Pol sequence from a non-subtype B HIV-1 virus into a plasmid under the control of a non-HIV promoter (e.g., CMV promoter) to generate a packaging vector. In one embodiment, the packaging vector encodes only 1 HIV-1 protein. In one embodiment, the packaging vector encodes only 2 HIV-1 proteins. In one embodiment, the packaging vector encodes only 3 HIV-1 proteins, selected from Gag, Pol, Rev, and Tat. In one embodiment, the packaging vector encodes only 4 HIV-1 proteins, Gag, Pol, Rev, and Tat.

In various embodiments, the plasmid comprises one or more of a CMV promoter, an exon encoding part of Tat and Rev, a splice donor site, an intron containing an RRE, a splice acceptor site, an exon encoding part of Tat and Rev, and a polyadenylation signal.

In various embodiments, the packaging vector comprises a CMV promoter, an exon encoding part of Tat and Rev, a splice donor site, an intron containing an RRE, a splice acceptor site, an exon encoding part of Tat and Rev, and a polyadenylation signal.

The non-subtype B HIV-1 virus can be selected from subtype A, subtype C, subtype D, subtype E, subtype F1, subtype F2, subtype G, subtype H, and subtype J viruses, and recombinants thereof.

In one embodiment, the method comprises providing a packaging vector comprising a Gag sequence of an HIV-1 subtype B virus and replacing Gag sequences in the vector with sequences from an HIV-1 non-subtype B virus.

The non-subtype B HIV-1 virus can be selected from subtype A, subtype C, subtype D, subtype E, subtype F1, subtype F2, subtype G, subtype H, and subtype J viruses, and recombinants thereof. In a preferred embodiment, non-subtype B HIV-1 virus is HIV-1 NDK.

Methods of Producing Lentiviral Vectors

The invention also encompasses methods for using packaging vectors encoding HIV-1 non-subtype B Gag and/or Pol proteins to generate lentiviral vectors. In one embodiment, the invention encompasses administering a packaging vector encoding an HIV-1 non-subtype B Gag or Pol protein to a cell with a lentiviral vector. The packaging vector can comprise any of the features discussed herein.

The lentiviral vector comprises cis-acting sequences for packaging and reverse transcription, including a Ψ site and primer binding site. Preferably, the lentiviral vector comprises two HIV-1 LTR sequences. In one embodiment, one of the LTRs is deleted for U3 and R sequences. Preferably, the lentiviral vector comprises a central polypurine tract (cPPT) and a central terminal sequence (CTS). The lentiviral vector preferably encodes a lentiviral or non-lentiviral protein, such as a selectable marker or tumor antigen.

In one embodiment, the lentiviral vector comprises one or more HIV antigen, preferably an HIV-1 antigen. Most preferably, the antigen is a Gag, Pol, Env, Vif, Vpr, Vpu, Nef, Tat, or Rev antigen. The antigen can be a single antigen, a mix of antigens,

an antigenic polypeptide, or a mix of antigenic polypeptides from these proteins. In a preferred embodiment, the lentiviral vector comprises an HIV-1 p24 Gag antigen.

In one embodiment, the invention encompasses a lentiviral vector comprising an NIS-containing promoter. An "NIS-containing promoter" comprises an NF-Kb binding site, an interferon stimulated response element (ISRE), and an SXY module (SXY). Examples of naturally occurring NIS-containing promoters are the β2m promoter and the MHC class I gene promoters. These naturally occurring NIS-containing promoters are generally cloned or reproduced from the promoter region of a gene encoding a protein β2m or a MHC class I protein, or referred to as putatively encoding such proteins in genome databases (ex: NCBI polynucleotide database http://www.ncbi.nlm.nih.gov/guide/dna-rna). Both β2m and class I MHC proteins enter the Major Histocompatibility Complex (MHC). β2m and class I MHC promoter sequences are also usually referred to as such in genome databases - i.e. annotated as being β2m and class I MHC promoter sequences.

MHC class I and β2-microglobulin promoters contain the shared structural homologies of NIS-containing promoters. These promoters also share the ability to be strongly activated in dendritic cells, as well as, to lower intensity, in the majority of the other human body tissues.

In one embodiment, the packaging vector and the lentiviral vector are introduced together into a cell to allow the formation of lentiviral vector particles containing the Gag protein produced by the packaging vector and the nucleic acid produced by the lentiviral vector. Preferably, this is achieved by cotransfection of the cells with the packaging vector and the lentiviral vector. The cells can also be transfected with a nucleic acid encoding an Env protein, preferably a VSV Glycoprotein G. Preferably, the lentiviral vector particles are capable of entry, reverse transcription, and expression in an appropriate host cell.

In one embodiment, the packaging vector or the lentiviral vector is stably integrated into cells, and the non-integrated vector is transfected into the cells to allow the formation of lentiviral vector particles.

In one embodiment, the method further comprises collecting the lentiviral vector produced by the cells.

In one embodiment, the method further comprises selecting for a packaging vector that packages a higher titer of the lentiviral vector than a same packaging vector

encoding a subtype B Gag or Pol protein. Preferably, the titer is increased at least 1.5 or 2-fold relative to the packaging vector encoding a subtype B Gag or Pol protein.

Lentiviral Vector Particles

The invention also encompasses lentiviral vector particles comprising HIV-1 non-subtype B Gag and/or Pol proteins. The non-subtype B Gag and Pol proteins can comprise any of the features discussed herein.

The lentiviral vector particle comprises a nucleic acid comprising cis-acting sequences for packaging and reverse transcription, including a Ψ site and primer binding site in association with Gag, Pol and Env proteins. Preferably, the nucleic acid comprises two HIV-1 LTR sequences. In one embodiment, one of the LTRs is deleted for U3 and R sequences. Preferably, the nucleic acid of the lentiviral vector particle comprises a central polypurine tract (cPPT) and a central terminal sequence (CTS). The nucleic acid preferably encodes a lentiviral or non-lentiviral protein, such as a selectable marker or tumor antigen. Preferably, the lentiviral vector particle comprises a VSV Glycoprotein.

Preferably, the lentiviral vector comprises an NIS-containing promoter. In one embodiment, the promoter is a β2m promoter.

In one embodiment, the lentiviral vector comprises one or more HIV antigen, preferably an HIV-1 antigen. Most preferably, the antigen is a Gag, Pol, Env, Vif, Vpr, Vpu, Nef, Tat, or Rev antigen.

The lentiviral vectors of the invention can be administered to a host cell, including a human host.

The lentiviral vector particle can contain a targeting mechanism for specific cell types. *See, e.g.,* Yang et al., Targeting lentiviral vectors to specific cell types in vivo, PNAS 113(31):11479-11484 (2006), which is hereby incorporated by reference. Targeting can be achieved through an antibody that binds to a cell surface protein on a cell. The targeted cell type is preferably a dendritic cell, a T cell, a B cell. Targeting to dendritic cell type is preferred and can be accomplished through envelope proteins that specifically bind to a DC surface protein. *See, e.g.,* Yang et al., Engineered Lentivector Targeting of Dendritic Cells for In Vivo, Nat Biotechnol. 2008 March ; 26(3): 326–334, which is hereby incorporated by reference.

The present invention further relates to the use of the lentiviral vectors according to the invention, especially in the form of lentiviral vector particles, for the preparation of therapeutic compositions or vaccines which are capable of inducing or contributing to

the occurrence or improvement of an immunogical reaction against epitopes, more particularly those encoded by the transgene present in the vectors.

Examples

Example 1. Plasmid construction

The *gag-pol* gene was amplified by PCR, using two primers and pNDK-N, a clone of HIV-1 NDK, as template. In order to obtain pThV-GP-N plasmid, the PCR product was digested with EagI /SalI and inserted in packaging construct p8.74, also digested by EagI /SalI.

Example 2. Production of lentiviral vector by transfection

Lentiviral vector stock was produced using pFLAP CMV GFP bis and pTHV-VSV.G (INDI-CO)bis in combination with p8.74 or pThV-GP-N. 36 transfections were done, 18 with p8.74 and 18 with pThV-GP-N. All the supernatant were stored at -80°C.

The plasmid pFLAP-CMV GFP bis encoded for the Green Fluorescence protein (GFP), which expression can be detected by flow cytometry.

Example 3. Titration of lentiviral vector production

Vector titer was determined by the frequency of GFP expression in 293T cells. Cells were cultivated in 24-well plates, in DMEM containing 10% FBS, until they reached a density of 1×10^5 cells per well. The cells were then transduced with different volume of vector supernatant in a final volume of 300 µL. After 2 hours, 700 µl of fresh medium containing 10% FBS was added in each well. 72 hours after transduction, the medium was then removed and the cells washed in Dulbecco's phosphate-buffered saline (DPBS; Gibco). Cells were removed with 0.05% Trypsin-EDTA (Gibco). Trypsinization was stopped by the addition of 300 µl complete DMEM, and the cells were transferred to a tube for FACS, after which the number of GFP-expressing cells was counted with a FACSCalibur (BD Biosciences) using an excitation wavelength of 509 nm. Only the percent of GFP positive cells under 30% was considered.

The results are shown in Figure 3. A significant difference between the pThV-GP-N and the p8.74 vectors productions was seen, with higher titers obtained using the pThV-GP-N plasmid. Indeed, the vector titer obtained with the packaging plasmid pThV-GP-N was 2 fold higher than the vector titer obtained with the classically used plasmid p8.74 ($p<0.001$ according to Student test).

Example 4. Increased titer of lentiviral vector with pThV-GP-N

HIV-1 BRU and HIV-1 NDK viruses were made on 293T cells and used to transduced P4 CCR5 cells. These cells encompass a stable luciferase gene under the

control of the HIV LTR: If they are transduced with TAT protein (which is the case when they are infected with a WT HIV), the LacZ gene is expressed and a luciferase expression can be measured. HIV-1 Gag p24 and luciferase expression were measured. The results are shown in Figure 4, and confirm that wild type NDK virus has a higher transduction rate than the wild type BRU one.

Example 5. Increased p24 and titer with pThV-GP-N

Different ratios of p8,74 and pSD GP NDK packaging vectors were used to produce lentiviral vector particles. For each ratio, the titer and the P24 level were measured. The results are shown in Figure 5 and demonstrate that it is the presence of the NDK packaging plasmid that is responsible for the enhancement of the production titers and of the P24 level .

Example 6. Decreased p24 processing with pThV-GP-N

Packaging vectors p8.74 and pTHV-GP-N were used to produce supernatants containing lentiviral vector particles. Western blots were performed on the supernatants using the NIH anti-P24 MAB (183-H12-5C). The results are shown in Figure 6, and confirm that the difference between the NDK and BRU packaging plasmids relies on the production of P24 protein and precursor, as the NDK seems to generate a higher P24 synthesis (presence of P24 precursor in the viral supernatant) when the BRU shows only P24 in viral supernatant .

Claims

We claim:

1. A replication-defective lentiviral packaging vector lacking a Ψ site and encoding an HIV-1 Gag MA protein;

wherein the HIV-1 Gag MA protein:

a) has an amino acid at position 12 that is not a glutamic acid,

b) has an amino acid at position 15 that is not an arginine,

c) does not have both a valine at position 46 and a leucine at position 61; and

wherein the lentiviral packaging vector encoding the HIV-1 Gag MA protein generates at least a 1.5 fold increase in the titer of a packaged lentiviral vector as compared to the lentiviral packaging vector encoding an HIV-1 Gag MA protein of HIV-1 BRU.

2. The vector of claim 1, wherein the amino acid at position 12 is a lysine.

3. The vector of claim 1, wherein the amino acid at position 12 is an arginine.

4. The vector of claim 1, wherein the amino acid at position 15 is a threonine.

5. The vector of claim 1, wherein the amino acid at position 15 is an alanine.

6. The vector of claim 1, wherein the amino acid at position 46 is a leucine.

7. The vector of claim 1, wherein the amino acid at position 61 is an isoleucine.

8. The vector of claim 1, wherein the amino acid at position 61 is a methionine.

9. The vector of claim 2, wherein the amino acid at position 15 is a threonine.

11. The vector of claim 2, wherein the amino acid at position 46 is a leucine.

12. The vector of claim 2, wherein the amino acid at position 61 is an isoleucine.

13. The vector of claim 9, wherein the amino acid at position 46 is a leucine.

14. The vector of claim 9, wherein the amino acid at position 61 is an isoleucine.

15. The vector of claim 13, wherein the amino acid at position 61 is an isoleucine.

16. The vector of claim 15, wherein the HIV-1 Gag MA protein is the HIV-1 Gag MA protein of HIV-1 NDK.

17. The vector of claim 16, wherein the vector encodes the amino acid sequence of SEQ ID NO:4.

18. A method for generating a packaging vector comprising inserting a Gag and/or Pol sequence from a non-subtype B HIV-1 virus into a plasmid under the control of a non-HIV promoter to generate the packaging vector.

19. A method for generating a lentiviral vector comprising administering a packaging vector encoding a non-subtype B HIV-1 Gag or Pol protein to a cell with a lentiviral vector.

20. The method of claim 19, further comprising selecting for a packaging vector that packages a higher titer of the lentiviral vector than a same packaging vector encoding a subtype B HIV-1 Gag or Pol protein.