Genetic engineering of onco/lentivirus hybrids results in formation of infectious but not of replication-competent viruses

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To achieve specific gene transfer into human CD4+ cells, murine leukaemia virus (MLV)-based pseudotype vector particles were generated employing Env variants derived from human or simian immunodeficiency virus (HIV-1 or SIVagm). Here, we describe the generation of full-length onco/lentivirus hybrid genomes comprising components of MLV and HIV-1 or SIVagm, respectively, to assess the possibility of replication-competent hybrid virus formation. The env reading frame of an infectious molecular clone of MLV was replaced with the analogous coding regions of HIV-1 or SIVagm encompassing the env gene and accessory genes. Resulting MLV/HIV-1 or MLV/SIVagm hybrid genomes were transfected into 293T cells. Expression of viral proteins and budding of retroviral particles was shown by specific immunostaining and electron microscopy. The viral particles mediated CD4- and co-receptor-specific infection of human cells as demonstrated by PCR and immunostaining in the respective target cells. However, no productive infection resulting in the generation of infectious virus was detected in these cells. Thus, these onco/lentivirus hybrids, although able to initiate single-round infection, were not replication competent. Thus, MLV-based pseudotype vectors carrying Env variants of HIV-1 or SIVagm are not prone to form replication-competent retroviruses, suggesting a favourable safety profile for MLV-based CD4-specific pseudotype vectors.

INTRODUCTION

Retroviral vectors are powerful tools for gene transfer into mammalian cells. Murine leukaemia virus (MLV)-based vectors have been extensively used as gene delivery systems in clinical gene therapy trials. The inadvertent production of replication-competent retroviruses (RCRs) constitutes one of the major safety issues concerning the use of retroviral vectors. Such viruses may lead to viraemia and subsequent formation of malignant tumours as a result of insertional mutagenesis (Cornetta et al., 1991; Purcell et al., 1996), as demonstrated in monkeys experimentally infected with replication-competent MLVs (Donahue et al., 1992). Thus, the risk of infecting recipient patients with RCRs arising from viral packaging cell lines has to be assessed and minimized. RCRs can be generated by recombination events between the vector components or between vector components and endogenous retroviral sequences (Chong & Vile, 1996; Chong et al., 1998). Since recombination events are more frequent at regions of high sequence homology (Zhang & Temin, 1993), this risk may be reduced by using MLV-derived vectors pseudotyped with glycoproteins from distantly related viruses such as lentiviruses.

We and others have previously reported the generation of MLV-based vectors pseudotyped with C-terminally truncated variants of the envelope (Env) glycoproteins of human and simian immunodeficiency virus (HIV-1 and SIVagm). Packaging cells expressing an MLV-derived, psi-positive transfer vector, a psi-negative MLV gag–pol gene and a psi-negative variant lentiviral env gene release [MLV(HIV-1)] or [MLV(SIVagm)] pseudotype vectors, respectively. These vectors have been shown to allow efficient and selective transduction of human CD4+ cells expressing appropriate co-receptors (Schnierle et al., 1997; Mammano et al., 1997; Lodge et al., 1998; Stitz et al., 2000; Steidl et al., 2002). In the respective high-titre packaging cell lines, as well as in transduced target cells, formation of RCRs has not been observed using PCR, RT assays or rescue tests, challenging the so far theoretical possibility of replication-competent MLV/lentivirus hybrid formation. No naturally occurring recombination between different genera of retroviruses leading to replication-competent hybrid viruses has yet been reported. However, recently generation of a hybrid virus consisting of an MLV genome carrying envelope regions of a foamy virus by molecular cloning has been
described (Shikova-Lekova et al., 2003). The only case of a replication-competent chimeric virus composed of elements from lentiviruses and MLV has been described by Reiprich et al. (1997). In that report, the env gene of SIVmac239 was replaced by the env gene of amphotropic MLV 4070A leading to a replication-competent SIVmac/MLV hybrid virus, which showed an expanded host tropism. Other MLV/lentivirus chimeras where the matrix genes had been exchanged revealed only restricted replication capacity (Deminin & Emerman, 1994; Chen et al., 2001; Reed et al., 2002).

To assess the possibility of replication-competent MLV/lentivirus hybrid formation from genome components present in packaging cells releasing [MLV(HIV-1)] and [MLV(SIVagm)] pseudotype vectors, we constructed hybrid genomes encompassing a lentiviral env gene in the context of an otherwise MLV-derived genome. The possible replication competency of the resulting viruses was analysed.

**METHODS**

**Generation of hybrid virus genomes.** The plasmid pKA1558 (encompassing a molecular clone of Moloney murine leukaemia virus (MoMuLV); Skov & Andersen, 1993) was used for molecular cloning of hybrid viruses. First, a 30 nt linker containing an SfiI site was introduced at nt 5389 (all positions according to GenBank accession no. J02255), resulting in pKA1558L. An additional SfiI site at nt 5389 was subsequently mutated, forming the construct pKA1558LDS. Prior to insertion of lentiviral env genes, the start codon of the MLV env (nt 5777) was deleted to avoid generation of a chimeric Rev (pKA1558LDS.ATGm; details on primers are available on request). HIV-1 env and flanking accessory genes rev and vpu were amplified from plasmid pL128, which is derived from SIVagm3mc (Baier et al., 1990) and into the second exon of BH10. To insert 5′ SfiI- and 3′ ClaI sites, plL128 sequences were amplified using the following primers: SfiIBH10Rev(+), SfiIGA-3′, and ClaIBH10Rev(−), ClaIGA-3′. A 30 nt linker containing an SfiI site was introduced into plRep293T cells seeded in six-well dishes (Nunc) was performed using 10% of one of the molecular clones of hybrid or wt viruses and the transfection reagent Lipofectamin Plus (Gibco, Life Technologies) according to the manufacturer’s instructions. For generation of replication-incompetent pseudotype vectors by double transfection of 293T cells, 2.5 μg pKA1558LDS.ATMg and 2.5 μg of one of the env expression plasmids plL128, pRepA10 or pRepA10SA were used. Supernatants were harvested 48 h after transfection and filtered through a 0.45 μm pore size filter (Sartorius). One ml of the supernatant was then used for infection of 2 × 10⁵ GHOST cells or 1 × 10⁶ T cells, respectively. After 4 h of incubation, virus-containing supernatant was removed.

**Transfection and infection of cells.** Transfection of 6 × 10⁵ 293T cells seeded in six-wells dishes (Nunc) was performed using 3 μg of plasmid DNA of one of the molecular clones of hybrid or wt viruses and the transfection reagent Lipofectamin Plus (Gibco, Life Technologies) according to the manufacturer’s instructions. For generation of replication-incompetent pseudotype vectors by double transfection of 293T cells, 2.5 μg pKA1558LDS.ATMg and 2.5 μg of one of the env expression plasmids plL128, pRepA10 or pRepA10SA were used. Supernatants were harvested 48 h after transfection and filtered through a 0.45 μm pore size filter (Sartorius). One ml of the supernatant was then used for infection of 2 × 10⁵ GHOST cells or 1 × 10⁶ T cells, respectively. After 4 h of incubation, virus-containing supernatant was removed.

**Determination of infectivity.** To determine infectivity of hybrid viruses and the respective pseudotyped MLV-derived vectors, filtered virus- or vector-containing supernatants were titrated by serial dilution on GHOST cells. In parallel, RT activity of supernatants was measured. Four hours after infection of GHOST cells, supernatants were replaced with fresh medium. Two hours later, 10 μg AZT ml⁻¹ was added to prevent spreading of replicative virus. Seventy-two hours after infection, cells were analysed by in situ immunostaining for MLV Gag and titres were defined as the number of gag-expressing cells per ml inoculum. Infectivity was determined as virus or vector titre per ml RT activity.

**Membrane fusion.** 293T cells were transfected with plasmids encoding the hybrid virus variants. On the following day, transfected cells were overlaid with Molot/8 T cells, co-cultivated for 2 days and monitored for the appearance of syncytia as previously described (Steidl et al., 2002).

**In situ immunostaining.** Transfected or infected cells were analysed for expression of viral genes by in situ immunostaining as described by Schnierle et al. (1997) and Stitz et al. (2000). For detection of MLV Gag, a goat anti-RLV p30-CA serum was used (Viromed; cross-reactive with MLV p30-CA) diluted 1 : 1000 in PBS/1% BSA. For detection of HIV-1 or SIVagm Env, sera from an HIV-1-infected donor or an SIVagm-infected pig-tailed macaque (Macaca nemestrina, Nem 170) diluted 1 : 1000 and 1 : 400, respectively, were used as described previously (Stitz et al., 2000). Antibodies were stained using 1:1000-diluted peroxidase-coupled protein G (Sigma).

**Electron microscopy.** For electron microscopy, cells were fixed for 45 min in 2.5% glutaraldehyde in warm medium. After postfixation in OsO₄ for 1 h, cells were tannin-treated to improve the visibility of viral spikes (Gelderblom et al., 1987). After dehydration in a series of graded ethanol, cells were embedded in Epon 812...
according to standard protocols and finally cut as 80 nm ultrathin sections. Staining was performed using 2 % uranyl acetate for 5 min and 1 % lead citrate for 2 min at room temperature.

**Provirus detection in genomic DNA by PCR.** Genomic DNA was isolated using the QIAamp DNA mini kit (Qiagen) according to manufacturer’s instructions. Detection of integrated proviruses was performed by standard PCR using a primer from the MLV component and one from the lentiviral env gene as follows: MLV5753(+) 5'-GGGTGGACCATCCTCTAGAC-3' and HIV6173(-), 5'-CCTTCACCTCATTGGCCTTG-3', or SIV6054(-), 5'-CTGTGACCTATTGTTGCCTGG-3', respectively. This led to 420, 301 or 310 bp fragments for MLV/HIV-1, MLV/SIVagm and MLV/SIVagmSA, respectively. For the detection of MLV/HIV-1 in T cells, the minus primer HIV6041(-), 5'-GCTACTACTAATGCTACTATTGC-3', was used, resulting in a 288 bp fragment.

For amplification of whole env genes, the following primers flanking env were used: for MLV/SIVagm or MLV/SIVagmSA: (+) 5'-ATGGGTCACAGTATTTATG-3' and (-) 5'-CCTTCCTGTGCTCTCTGGTCCTCTC-3' (amplification product 1973 bp); for MLV/HIV-1: (+) 5'-CTGTGAGAGATTGGGCTTGAG-3' and (-) 5'-TACACAGCIAAAACTCATTCTA-3' (2078 bp); and for MLV-A: (+) 5'-CTGTGAGAGATTGGGCTTGAG-3' and (-) 5'-CATGTGAGTAAGGATCC-3' (1866 bp). For restriction analysis, PCR products were extracted from agarose gels using the QIAex II agarose gel extraction kit (Qiagen) and digested with EcoRI (MLV/SIVagm, MLV/SIVagmSA or MLV-A) or AseI (MLV/HIV-1).

As an internal control, a 570 bp fragment of the human gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified. As a positive control, the respective hybrid virus-encoding plasmids were used.

**Detection of viral RNA by RT-PCR.** For detection of env mRNA, total cellular RNA was extracted from transfected 293T cells by standard procedures using Trizol (Invitrogen) and reverse transcribed using SuperScript III reverse transcriptase following manufacturer’s instructions (Invitrogen). Specific reverse primers were located within the respective env genes: 5'-CTGTGAGAGATTGGGCTTGAG-3' for MLV/SIVagm or MLV/SIVagmSA, 5'-CTGTGAGAGATTGGGCTTGAG-3' for MLV/HIV-1, and 5'-TAAGCTCTACCCGGTGTCTCAC-3' for MLV-A. PCR amplification of cDNA from spliced env mRNA was performed using one primer complementary to sequences located upstream of the MLV splice donor site and one primer from the env gene: 5'-GGGTCTTTCATTTGGGGCTCGTC-3' and 5'-CTGTGAGAGATTGGGCTTGAG-3' for MLV/HIV-1; 5'-GGGTCTTTCATTTGGGGCTCGTC-3' and 5'-GAGCTGAGTAAGGATCC-3' for MLV/SIVagm or MLV/SIVagmSA; and 5'-TAAGCTCTACCCGGTGTCTCAC-3' and 5'-GAGCTGAGTAAGGATCC-3' for MLV-A.
For detection of particle-associated RNA, particles were purified and concentrated by ultracentrifugation (35 000 r.p.m. for 2 h at 4°C in a Sorval SW41 rotor) of 10 ml of cell culture supernatant through 20% sucrose in STE. RNA was isolated using a QIAamp Viral RNA preparation kit (Qiagen) as following the manufacturer’s instructions and digested with RNase-free DNase (Promega). cDNA was synthesized using MvULV RT (Applied Biosystems) and a specific MLV gag reverse primer (5'-GGCCCGGCGCCCGGCCCCCTTTCTA-3'). Detection of viral gag sequences was done by standard PCR (35 cycles) using primers binding to MLV gag (+) 5'-ACCCGCCGCTGGACCTCTCCCTTTAT-3' and (-) 5'-TCCGGTCAGCAGAGTCCCAACAG-3', resulting in a 471 bp fragment.

Reverse transcriptase activity. RT activity in supernatants was measured using a C-type RT activity assay for MLV wt or onco/ lentivirus hybrids (Malmösten et al., 1998) and a Lentiviral RT assay for HIV-1 or SIV Agm viruses (Cavidi Tech).

RESULTS

Generation of MLV/HIV-1 and MLV/SIV Agm hybrid viruses

To investigate the properties of MLV/HIV-1 and MLV/ SIV Agm hybrid viruses, various chimeric genomes were constructed. The infectious molecular clone of ecotropic MLV strain MoMuLV pKA1558 (Skov & Andersen, 1993) served as the source of MLV genome components. After modification of S/F restriction sites to facilitate exchange of env genes, the resulting clone pKA1558LDS was still able to generate replication-competent MLV after transfection of 293T cells (data not shown). The start codon of env was then removed and the MLV env gene of the now replication-incompetent clone designated pKA1558LDS.ATGm was replaced with the respective variant lentiviral env genes. Genome structures of MLV and the resulting hybrid viruses are given in Fig. 1. The MLV env coding region was replaced by complete open reading frames encoding a C-terminally truncated Env variant of HIV-1 (Wilk et al., 1992) and the accessory HIV-1 proteins Rev and Vpu, resulting in plasmid pKAHIV encompassing an MLV/HIV-1 hybrid genome (Fig. 1A). Similarly, a hybrid MLV/SIV Agm genome designated pKASIV was constructed, which harboured a C-terminally truncated variant of the SIV Agm3 env gene (Stitz et al., 2000) as well as the SIV Agm3mc rev gene (Fig. 1C). A third construct, designated pKASIVSA, comprised an additional MLV-derived splice acceptor site between the first exon of rev and the env gene variant of KASIV (Fig. 1D). In all cases, the accurate insertion of the lentiviral genes was confirmed by nucleotide sequencing of the respective plasmids (data not shown).

The three constructs were separately transfected into 293T cells. Infectious molecular clones of the parental wt viruses were transfected as positive controls. Two days after transfection, expression of the MLV capsid protein p30-CA and the lentiviral Env proteins was investigated by immunostaining of the transfected cells in situ using appropriate antisera (Fig. 2A). In all hybrid virus genome-transfected cell cultures, MLV p30-CA and the lentiviral env genes were shown to be expressed. Typically, the proportion of cells expressing MLV p30-CA was about 30–40%, whereas HIV-1 Env was detected in a maximum of 1% and SIV Agm Env in about 3% of the cells. Remarkably, pKASIVSA revealed higher expression levels of SIV Agm Env compared with pKASIV, as indicated by stronger signals and a slightly higher proportion of about 5% positive cells. This is most likely due to more efficient splicing of subgenomic env mRNA utilizing the additional MLV splice acceptor site located directly upstream of the env gene. Remarkably, the presence of spliced env mRNA attributable to the use of the MLV splice acceptor was proven for all hybrid viruses (Fig. 2B). For pKASIVSA, an additional faint band of about 400 bp reflecting the use of the inserted splice acceptor site was detected.

To test the functionality of the expressed Env proteins, transfected 293T cells were co-cultivated with the CD4+ and co-receptor-positive human T-cell line Molt4/8. After 2 days of co-cultivation, formation of syncytia was observed in all hybrid virus-transfected cultures, indicating expression of lentiviral Env proteins at the cell surface and maintenance of their fusogenic capacity (data not shown). Since efficient lentiviral env expression is Rev-dependent, this result also pointed to the functional expression of rev from respective genomes.

Supernatants of transfected cultures were tested for RT activity. Detected RT levels from pKAHIV-, pKASIV- and pKASIVSA-transfected cultures were between 363 and 625 mU RT ml⁻¹, which was only slightly lower than that of a culture transfected with an infectious molecular clone of amphotropic MLV (pM91) used as positive control (750 mU RT ml⁻¹). Therefore, functional expression of pol in addition to gag and env was also proven for the hybrid virus genomes.

Finally, production of virus particles was investigated by electron microscopic examination of the 293T cells 3 days after transfection (Fig. 3). Ultrathin sections of cells transfected with hybrid virus plasmids as well as with MLV-A revealed high numbers of budding and mature retroviral
particles, again confirming functional expression of all viral proteins required for particle formation. Some of the viral particles revealed envelope spikes, pointing to incorporation of the foreign lentiviral Env protein or of amphotropic MLV Env, respectively. In mock-transfected cells, no viral particles were detected.

Fig. 3. Retroviral particles observed in ultrathin sections of 293T cells transfected with proviral constructs of MLV-A (a, b), MLV/HIV-1 (c, d), MLV/SIVagm (e, f) and MLV/SIVagmSA (g, h). Immature budding particles are shown on the left and free mature particles are shown on the right. Note the visibility of prominent spikes on the particles in (e), (f) and (h). Bar, 100 nm.
MLV/HIV-1 and MLV/SIVagm hybrid viruses are capable of co-receptor-dependent single-round infection of human osteosarcoma cells expressing HIV receptors

To assess the infectivity of the hybrid viruses, GHOST cells expressing human CD4 and either the HIV-1 co-receptor CXCR4 or CCR5 (GHOST/CXCR4 or GHOST/CCR5 cells; Cecilia et al., 1998), respectively, were employed. T-cell-tropic HIV-1 virus is able to infect GHOST/CXCR4 cells, while SIVagm3 infects GHOST/CCR5 cells and, to a lesser extent, GHOST/CXCR4 cells, both of which additionally express the co-receptor Bonzo (Edinger et al., 1998) also amenable for use during SIVagm infection (König et al., 2002). Moreover, GHOST/CXCR4 or GHOST/CCR5 cells were previously shown to also be permissive for [MLV(HIV-1)] and [MLV(SIVagm)] pseudotype vectors carrying the T-cell-tropic HIV-1 envelope or the CCR5- and Bonzotrophic SIVagm Env (Stitz et al., 2000; Steidl et al., 2002).

Cell-free supernatants were harvested from 293T cells 2 days after transfection with plasmids encompassing hybrid virus genomes or controls, respectively, and subsequently used to infect GHOST/CCR5 or GHOST/CXCR4 cells. Four days after infection, cells were analysed for the expression of viral genes by *in situ* immunostaining and 7 days after infection for the presence of proviral DNA by PCR. PCR primers are indicated in the genome structures presented in Fig. 1(B and C). RT activity in the supernatant was determined at different time points over 20 days (Fig. 4A, GHOST I). The RT activity assay used has been shown to detect as little as 0.3 µU MLV RT, corresponding to less than 1000 viral particles (Malmsten et al., 1998). Furthermore, occurrence of viral particles was assessed by RT-PCR of gag sequences present in particles after purification and concentration by ultracentrifugation of supernatant from day 7 through a sucrose cushion (Fig. 4B, GHOST I).

After infection with MLV/HIV-1 hybrid virus, a faint provirus-specific band of 420 bp could be detected in GHOST/CXCR4 cells but not in GHOST/CCR5 cells by PCR using primers complementary to the pol gene of MLV and the env gene of HIV-1 (Fig. 5B). The presence of proviral DNA solely in GHOST/CXCR4 cells indicated the restricted tropism of the utilized HIV-1 envelope protein, which was derived from a CXCR4-tropic strain (Stitz et al., 2000). However, expression of neither MLV p30-CA nor HIV-1 Env could be detected in GHOST/CCR5 or GHOST/ CXCR4 cells by *in situ* immunostaining (Fig. 5A; a–d) and no RT activity or particle fraction-associated RNA could be detected in supernatant from infected cells (Fig. 4, GHOST I).

After infection with MLV/SIVagm hybrid virus, provirus-specific bands were detected in both GHOST cell lines by PCR using primers complementary to the pol gene of MLV and the env gene of SIVagm (Fig. 5C), reflecting usage of both co-receptors, CCR5 and Bonzo. In contrast, only GHOST/CCR5 cells, but not GHOST/CXCR4 cells, were demonstrated to express viral genes by *in situ* immunostaining (Fig. 5A; e–h). About 5% of GHOST/CCR5 cells expressed MLV p30-CA, whereas only a few cells, i.e. less than 1% of the infected GHOST/CCR5 cells, expressed SIVagm Env. Accordingly, RT activity could be detected only in the supernatant of infected GHOST/CCR5 cells (Fig. 4A, GHOST I). Furthermore, only low amounts of viral RNA were detected by RT-PCR in the particle fraction in supernatant from infected GHOST/CCR5 cells (Fig. 4B, GHOST I).

After infection of GHOST/CCR5 cells with MLV/SIVagmSA, about 20% of the cells were found to express MLV p30-CA. However, again less than 1% were found to be positive for SIVagm Env. Again, the usage of Bonzo by SIVagm was confirmed by expression of MLV p30-CA in a few GHOST/CXCR4 cells infected with MLV/SIVagmSA, although no expression of SIV Env could be detected (Fig. 5A, i–l). For MLV/SIVagm, RT activity or RT-PCR signals could be detected exclusively in the supernatant of MLV/SIVagmSA-infected GHOST/CCR5 cells (Fig. 4, GHOST I).

To check the genomic integrity of the hybrid proviral sequences, the complete env reading frames were amplified by PCR from genomic DNA extracted from GHOST/CXCR4 or GHOST/CCR5 cells 4 days after infection with the respective hybrid viruses or wt MLV. Complete and virtually unmodified env sequences were obtained from all infected cells expressing the respective co-receptors as shown by unaltered molecular mass and restriction patterns compared with those of sequences amplified from the parental plasmids (Fig. 5D). Remarkably, for amplification of the complete env of MLV/HIV-1 two rounds of PCR were required, pointing again to the low amount of proviral DNA present in cells infected with this virus.

Overall, all hybrid viruses were able to infect target cells in a co-receptor-dependent manner as shown by detection of proviral DNA by PCR. This indicates that gene transfer occurred by receptor-mediated retroviral infection, which implies efficient incorporation of the truncated lentiviral Env proteins into hybrid virus particles. However, expression of viral genes could be detected exclusively in MLV/SIVagm- or MLV/SIVagmSA-infected but not in MLV/HIV-1-infected cells.

To compare the infectivity of all hybrid viruses with that of replication-competent vectors, corresponding MLV-derived pseudotype vectors were generated. Therefore, the env-deleted molecular MLV clone pKA1558LDS.ATGm, which comprises the MLV components of the hybrid viruses, was used as the transfer vector. In so doing, determination of infectious titres of both hybrid viruses and pseudotype vectors by the same method was possible, by *in situ* staining of MLV Gag. Likewise, the same expression constructs from which the lentiviral envelope genes had been derived for generation of hybrid viruses, namely pL128, pRepΔA10 or pRepΔA10SA for MLV/HIV-1, MLV/SIVagm or MLV/SIVagmSA, respectively, were used for
Fig. 4. RT activity and particle-associated gag RNA in cell culture supernatant. (A) Kinetics of RT activity in GHOST/CXCR4 or GHOST/CCR5 cells after infection with supernatant of transfected 293T cells (GHOST I) or after infection with supernatant of infected GHOST I cells (GHOST II) using hybrid or control viruses as indicated. (B) Gag RT-PCR of particle fractions purified by ultracentrifugation through 20% sucrose from supernatant of 293T cells transfected with the plasmids indicated or from supernatant of GHOST I or GHOST II cells infected with the viruses indicated. Amplification products were separated on a 2% agarose gel. Gag-specific amplification products are 471 bp. SIV, MLV/SIVagm; SIV/SA, MLV/SIVagmSA; HIV, MLV/HIV-1; --, uninfected; M, molecular mass marker; +RT/−RT, generation of cDNA with/without RT.
pseudotyping. To generate pseudotype vector particles, 293T cells were co-transfected with pKA1558LDS.ATGm and one of the enve expression constructs. In parallel, 293T cells were transfected separately with the hybrid virus genomes. Supernatants of transfected cells were analysed for RT activity and used for infection of GHOST/CXCR4 or GHOST/CCR5 cells, respectively. Viral and vector titres were determined by in situ immunostaining of MLV Gag. The resulting mean values of two independent experiments and the calculated relative infection rates per RT unit are given in Table 1. Using SIVagm or SIVagmSA envelopes, replication-incompetent vectors were about two- to three-fold more infectious than the respective hybrid viruses, whereas the vector carrying HIV-1 env was more than 1000-fold more infectious than the MLV/HIV-1 hybrid virus.

**MLV/HIV-1 and MLV/SIVagm hybrid viruses are replication incompetent in GHOST cells**

Next, we addressed the capacity of hybrid virus-infected cells to produce infectious virus particles. As described, positive RT-PCR signals detected in purified fractions from supernatants of MLV/SIVagm or MLV/SIVagmSA-infected GHOST/CCR5 cells pointed to the presence of minimal amounts of viral particles. However, by electron microscopy, no particles could be detected in ultrathin sections of GHOST/CXCR4 or GHOST/CCR5 cells 3 days after infection with any of the hybrid viruses, whereas MLV-A-infected cells revealed retroviral particles as expected (data not shown). To demonstrate hybrid virus particles in an infection assay, infected GHOST cells were expanded for 7 days, followed by preparation of cell-free supernatants, which were subsequently used to infect fresh GHOST/ CXCR4 or GHOST/CCR5 cell cultures designated GHOST II, which were analysed as described for the first round of infection. However, neither the presence of proviral DNA nor expression of MLV, HIV-1 or SIVagm genes could be detected in any of the GHOST II cell cultures by PCR or in situ immunostaining (data not shown) or RT assays (Fig. 4A, GHOST II). No viral particles could be detected, even by RT-PCR in supernatant from GHOST II cells (Fig. 4B, GHOST II). In contrast, amphotropic MLV used as a positive control was readily detected. These results revealed that the generated MLV/HIV-1, MLV/SIVagm and MLV/SIVagmSA hybrid viruses could perform single-round but not productive infection of GHOST cells.

**MLV/HIV-1 and MLV/SIVagm hybrid viruses are replication incompetent in human T cells**

Amphotropic MLV, HIV-1 and SIVagm wt viruses have been demonstrated to productively infect GHOST cells that express suitable receptors (Edinger et al., 1998; König et al., 2002). However, HIV-1 and SIVagm are adapted for

### Table 1. Infectivity of hybrid viruses and corresponding pseudotype vectors

Mean values of two independent experiments are given.

<table>
<thead>
<tr>
<th>Genome</th>
<th>Particle type</th>
<th>Titre (IU ml⁻¹)</th>
<th>RT activity (mU ml⁻¹)</th>
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replication in T cells. Thus, we investigated the replication competence of hybrid viruses in the permissive human lymphoid T-cell line Molt4/8, known to facilitate replication of all three parental viruses. As a control, HIV wt virus strain NL4-3, which has been shown to exhibit equal replication capacity to the NL4-3/BH10 chimeric virus (Wilk et al., 1992), used as the source of env of MLV/HIV-1 hybrid virus, was employed. In addition, SIVagm3 wt virus (Baier et al., 1990), the origin of env for the MLV/SIVagm or MLV/SIVagmSA hybrid viruses, was used. Infectious particles of MLV-A, HIV-1 and SIVagm wt viruses, as well as MLV/HIV-1, MLV/SIVagm and MLV/SIVagmSA hybrid viruses, were generated by transient transfection of 293T cells, and cell-free supernatants were used to infect Molt4/8 cells. Supernatants of untransfected cells served as negative controls. Infected human T cells were expanded for 21 days and virus replication was detected using standard lentivirus or C-type retrovirus RT assays, respectively. As illustrated in Fig. 6(A), replication of wt viruses increased from day 2 post-infection to a maximum of 355 mU RT ml⁻¹ supernatant for MLV-A, 511 ng RT ml⁻¹ for HIV-1 and 560 ng RT ml⁻¹ for SIVagm on day 12. In contrast, no MLV RT activity was observed in MLV/HIV-1, MLV/SIVagm and MLV/SIVagmSA hybrid virus-infected T cells over the observed time period of 21 days. However, using PCR with respective primers, proviral DNA of MLV/HIV-1, MLV/SIVagm and MLV/SIVagmSA was detected in genomic DNA isolated 17 days post-infection, suggesting infection of Molt4/8 cells (Fig. 6B and C).

Further experiments using the human T-cell lines SupT-1 and C8166 as target cells led to similar results. Both cell lines could be infected with MLV/HIV-1, MLV/SIVagm and MLV/SIVagmSA as shown by provirus detection, but no further propagation was observed (data not shown).

In accordance with the observations of hybrid virus-infected GHOST cells, these results underline the ability of MLV/SIVagm and MLV/HIV-1 hybrid viruses to form infectious virions allowing receptor binding, cell entry and integration into the host cell’s genome. However, hybrid viruses facilitated only single-round infection, but were not able to initiate productive infection.

**DISCUSSION**

To assess the possibility of replication-competent onco/lentivirus hybrid formation, various hybrid genomes were constructed carrying a lentiviral env gene within an MLV genome. All constructs were capable of generating infectious
retroviral particles. However, these particles were found to enable only abortive single-round infection with no productive virus replication. The reason for this inability remains elusive. As shown by Reiprich et al. (1997), generation of replication-competent hybrid viruses consisting of components of onco- and lentiviruses is achievable. In that study, an amphotropic MLV env was introduced into a lentiviral genome. To our knowledge, reciprocal attempts at generation of replication-competent hybrid viruses carrying lentiviral envelopes have not yet been described. However, it has been shown that MLV capsids can efficiently be pseudotyped with C-terminally truncated HIV-1 or SIVagm envelope proteins resulting in infectious replication-incompetent vectors (Schnierle et al., 1997; Stitz et al., 2000). Therefore, we used the same modified HIV-1 and SIVagm envelope genes for generation of hybrid virus genomes, which allowed the generation of functional pseudotype vectors. Accordingly, the inability of these hybrid virus constructs to generate replication-competent viruses is probably not due to an incompatibility of MLV capsids with the lentiviral envelope proteins used.

The MLV/HIV-1 and MLV/SIVagm hybrid virus constructs were designed to enable expression of MLV gag and pol genes as well as the lentiviral env genes, including the flanking rev genes, by retaining rev splicing signals (Fig. 1). Indeed, virtually all viral genes required for formation of particles were expressed, as shown by immunostaining of MLV Gag and lentiviral envelope proteins in 293T cells transfected with hybrid virus genomes and by RT assays of supernatants. Expression of lentiviral envelope proteins also points to the expression of functional Rev proteins, since these are required for efficient expression of env (Nasioulas et al., 1994). However, expression of detectable levels of HIV-1 or SIVagm envelope proteins was only found in about 1–5% of the transfected cells, in contrast to expression of MLV Gag, which revealed a transfection efficiency of about 40%. Insertion of an additional MLV-derived splice acceptor site between SIVagm rev and env resulting in MLV/SIVagmSA resulted in a moderately enhanced expression of SIVagm env, probably due to low amounts of the additional env mRNA, detectable by RT-PCR. Nevertheless, functionality of Env proteins was shown for all hybrid virus genomes using a fusion assay. Moreover, the detection of significant numbers of spiked virus particles by electron microscopy unequivocally proved functional expression of all viral proteins required for particle formation for all hybrid viruses and pointed to incorporation of the foreign lentiviral Env proteins into the hybrid virus particles. Lack of detectable envelope spikes in most of the particles visualized by electron microscopy may be explained by shedding of viral envelope proteins or by preparation difficulties due to the labelling procedure (Gelderblom et al., 1987). Env incorporation was also demonstrated by Western blot analysis of supernatant from transfected 293T cells (data not shown).

Using particle-containing supernatant from transfected cells for infection of target cells, successful infection was shown for MLV/HIV-1 hybrid virus by detection of proviral DNA by PCR resulting in a 420 or 288 bp fragment for infected GHOST cells or human T-cell lines, respectively, but not by viral gene expression. However, MLV/HIV-1 DNA was detected by amplification of the 420 bp fragment exclusively in GHOST cells expressing the HIV-1 co-receptor CXCR4, which is required for infection via the HIV-1 envelope used. Therefore, it can be concluded that this DNA actually originated from receptor-mediated infection by infectious particles rather than from carry-over of input DNA of the transfected cells or PCR contamination.

For MLV/SIVagm and MLV/SIVagmSA hybrid viruses, successful infection of target cells was shown by detection of viral gene expression in addition to proviral DNA. In addition, co-receptor-dependent infection was found for these viruses, since 5 or 20%, respectively, of the infected GHOST/CCR5 cells expressed MLV gag, whereas only a few infected GHOST/CXCR4 cells were positive due to the less efficient utilization of co-receptor Bonzo (Edinger et al., 1998). Expression of SIVagm envelope from both hybrid viruses was found in less than 1% of infected GHOST/CCR5 cells and not at all in GHOST/CXCR4 cells. Moreover, RT activity, as well as viral RNA detected by RT-PCR in sucrose gradient-purified particle fractions, was also detected exclusively in supernatant from GHOST/CCR5 cells.

From these infection experiments it can be concluded that all the hybrid virus constructs enabled formation of particles able to infect target cells carrying the required receptors and co-receptors. The efficiencies of infection were different and reflected the extent of env expression in transfected 293T cells: for MLV/HIV-1 hybrid virus, proviral DNA but not gene expression could be detected in infected cells, whereas for MLV/SIVagm and MLV/SIVagmSA, viral gene expression was found to an increasing extent.

Comparing the efficiency of infection of the hybrid viruses with that of corresponding replication-incompetent pseudotype vectors resulted in only approximately two- to three-fold differences for MLV/SIVagm or MLV/SIVagmSA, whereas the infectivity of MLV/HIV-1 was more than three orders of magnitude less than that of the corresponding [MLV(HIV-1)] pseudotype vector when the virus or vector inocula were adjusted to give equal RT activity. Although the replication-incompetent vectors were designed to exhibit high similarity to the corresponding hybrid viruses, the significance of this comparison may be questionable due to major differences in vector or virus production, e.g. generation by single in contrast to double transfection. Nevertheless, this result indicates that the infectivity of MLV/SIVagm or MLV/SIVagmSA virus particles is not significantly impaired, whereas that of the MLV/HIV-1 hybrid virus is highly reduced when compared with that of the [MLV(HIV-1)] pseudotype vector. The reason for this remains unknown.
Using electron microscopy, viral particles were not detected in infected cells. However, in the supernatant of infected GHOST cells, the presence of viral gag RNA could be demonstrated by RT-PCR in particle fractions after purification by ultracentrifugation through a sucrose cushion, which may indicate the formation of small amounts of infectious particles. Otherwise, the presence of this viral RNA could also be explained by contamination with co-sedimented RNA or by formation of defective viral particles. However, more importantly, attempts to infect further GHOST cell cultures, designated GHOST II, using supernatant from infected cells failed. Neither proviral DNA nor expression of viral proteins could be detected and no RT activity or viral RNA could be detected using sensitive RT assays or RT-PCR, respectively. Replication incompetency of all chimeric viruses was also confirmed when supernatants from transfected 293T cells were used to infect human T-cell lines. Besides proviral DNA, no marker of infection was observed. Moreover, preliminary experiments of infection of anti-CD3/anti-CD28-stimulated human PBMC did not lead to detectable hybrid virus replication (data not shown). Remarkably, in contrast to infection of cell lines, no provirus integration could be detected in the genomic DNA of the primary cells. Thus, although all hybrid viruses generated by transfection of 293T cells were able to infect target cells and although in some cases few viral particles may be released from infected cells, none of them could induce productive virus replication in infected cells.

The most probable explanation for this incompetency of the hybrid viruses generated is an impaired env expression as observed, which is sufficient for particle formation following efficient transfection of 293T cells with molecular clones, but may not be adequate after infection of target cells. This poor env expression is probably not due to modifications of the hybrid virus genomes, since analysis of proviral DNA from infected cells by PCR and restriction analysis did not reveal major alterations. It seems more probable that the impaired env gene expression results from inefficient generation of spliced env mRNA or from impaired Rev function, although the design of the constructs should allow satisfactory expression of env and rev. Actually, generation of env mRNA could be demonstrated by RT-PCR in transfected cells. Other possible explanations include impaired incorporation of the truncated lentiviral Env into particles, e.g. due to the lack of palmitoylation sites present in the C-tail of unmodified lentiviral envelope proteins and necessary for targeting membrane proteins to lipid rafts (Rousso et al., 2000). Furthermore, infection may be impaired by lack of complex and often unknown regulation mechanisms necessary for lentivirus infection, e.g. downregulation of CD4 as determined for HIV-1 and SIV. Thus, generation of replication-competent hybrid viruses consisting of MLV capsids and lentiviral envelope proteins will require a number of further modifications, mainly to enhance env expression and to compensate for the lack of the Env C-tail.

Regardless of the mechanisms underlying our observations, the incompetency of formation of replication-competent chimeric viruses by relatively simple genome substitutions strengthens the safety of MLV-derived vectors pseudotyped with lentiviral envelope proteins, since the formation of replication-competent retroviruses is considered a major risk when using retroviral vectors for human gene therapy. Accordingly, [MLV/HIV-1] and [MLV/SIVagm] pseudotype vectors selective for CD4+ target cells have possibilities for future ex vivo and in vivo applications in various gene therapy approaches.

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