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Replication-Competent Chimeric Lenti-Oncovirus with Expanded Host Cell Tropism

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Baboon bone marrow was grafted into human immunodeficiency virus type 1-infected patients in the course of recent trials for AIDS treatment. Since the baboon genome harbors multiple copies of an endogenous oncovirus, chimeric lenti-oncoviruses could emerge in the xenotransplant recipient. To analyze the potential replication competence of hybrid viruses between different genera of retroviruses, we replaced most of the *env* gene of simian immunodeficiency virus with the *env* gene of an amphotropic murine leukemia virus. The hybrid virus could be propagated in human T-cell lines, in peripheral blood mononuclear cells of rhesus macaques, and in CD4$^-$ B-cell lines. Because of the expanded cell tropism, the hybrid virus might have a selective advantage in comparison to parental viruses. Therefore, emerging chimeric viruses may be considered a serious risk of xenotransplantation. A note of caution is also suggested for the use of pseudotyped lentiviral vectors for human gene therapy.

The transplantation of baboon bone marrow into a human immunodeficiency virus type 1 (HIV-1)-infected patient has fueled controversy. Criticism has been raised that the procedure of xenotransplantation might lead to a species transfer of baboon viruses to humans. In addition to a high mutation rate, retroviruses recombine with high frequency. Recombinations can occur between exogenous viruses, between exogenous and endogenous viruses, and between retroviruses and unrelated cellular genes (2). Since baboons harbor endogenous retroviruses (19), HIV-1-infected patients receiving baboon bone marrow might provide a favorable environment for the generation of hybrid viruses between the endogenous baboon retrovirus (BaEV) and HIV-1. The BaEV belongs to the mammalian type C retroviruses, formerly classified as oncoviruses. To investigate if hybrid viruses between a mammalian type C virus and a lentivirus can be replication competent, chimera were constructed between simian immunodeficiency virus (SIV) and an amphotropic murine leukemia virus (MLV), a well-characterized mammalian type C virus. Since previous experiments had already indicated that immunodeficiency viruses could be pseudotyped with the Env protein of MLV (10, 18), we tried to replace the *env* gene of SIV by the MLV *env* gene.

With a PCR approach, a deletion was introduced into the *env* gene of SIVmac239 (14) from nucleotides 6604 to 7757 (numbering according to reference 17). The second exons of *tat* and *rev*, which overlap the *env* reading frame, and the Rev-responsive element (RRE) were not removed. The full-length *env* reading frame derived from the 4070A isolate (6) of amphotropic MLV was amplified by PCR from the plasmid SV-A-MLV (10) and inserted in place of the deleted SIV *env* region (Fig. 1). To reduce the overall length of the hybrid viral genome, a fragment spanning deletions of 513 nucleotides in *nef* and the U3 region (nef/U3 deletion mutant) was cloned from SIVΔNU (5) into the SIV/MLV hybrid virus plasmid, resulting in pMuSIV. These deletions do not impair SIV replication in cell culture, since SIVΔNU replicated with similar kinetics as wild-type SIVmac239 in CEMx174 cells and peripheral blood mononuclear cells (PBMCs) of rhesus macaques (own unpublished observations and reference 9) as has been previously observed with another nef/U3 deletion mutant of SIV (4). In macaques, replication of SIVΔNU (5) was similar to that of another well-characterized *nef* deletion mutant (8). After transfection of two different plasmid clones of pMuSIV into CEMx174 cells, an increased proportion of small pycnotic

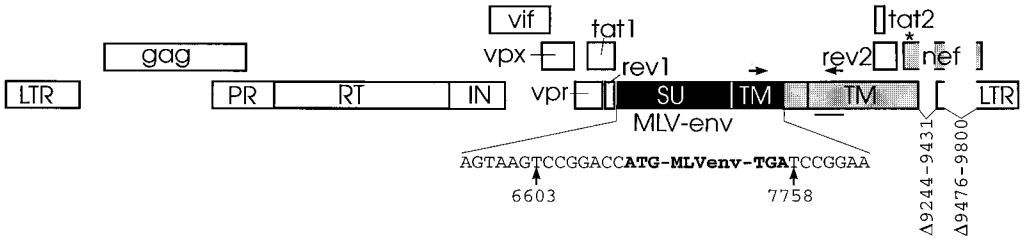

FIG. 1. Structure of the SIV/MLV hybrid virus MuSIV. SIV-derived open reading frames and regulatory regions are marked by a white box, the MLV *env* gene is marked by a black box, and nonfunctional reading frames are shaded. The position of the RRE element (11) is underlined. The nucleotide sequence at the SIV/MLV borders and the deletions in the *nef* and U3 region (5) are given below the drawing. The numbering is that of Regier and Desrosiers (17). The first and second ATG codons of *nef* were mutated to ACG as indicated by an asterisk. The position of the primers used to amplify the junction between MLV *env* and the truncated SIV *env* is marked by horizontal arrows. LTR, long terminal repeat; PR, protease; RT, reverse transcriptase; IN, integrase; TM, transmembrane protein; SU, surface protein.

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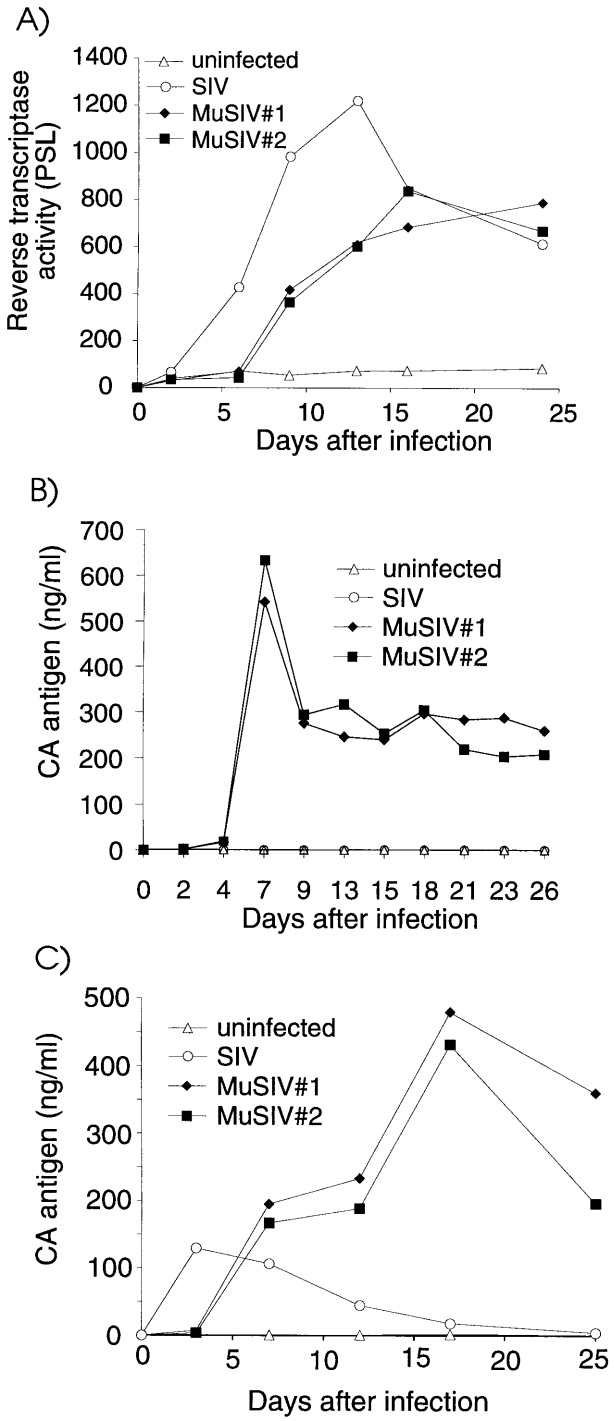

FIG. 2. Replication kinetics of MuSIV and SIV in CEMx174 cells (A), Raji cells (B) or rhesus monkey PBMCs (C). MuSIV 1 and 2 are derived from two independent plasmid clones. The relative reverse transcriptase activity is given as photostimulated luminescence (PSL). Capsid (CA) antigen levels were determined with the HIV-1/HIV-2 antigen test from Innogenetics (Zwijnaarde, Belgium) (B) or Coulter (Luton, United Kingdom) (C).

cells was observed, and cell growth was reduced. Reverse transcriptase activity could be detected in the culture supernatant (data not shown). In contrast to SIV, no syncytia could be observed in cultures transfected with pMuSIV.

To compare the replication kinetics of the hybrid virus MuSIV and SIV, CEMx174 cells were infected with cell-free stocks of both MuSIV clones and SIVmac239 after normalization for reverse transcriptase activity as described previously (5, 16). Replication of MuSIV was slightly retarded compared to that of SIV (Fig. 2A). A similar delay was observed in the human $CD4^+$ T-cell line C8166 (data not shown). MuSIV also replicated to high titers in the $CD4^-$ human B-cell line Raji. This clearly demonstrated an expanded cell tropism, since SIV did not replicate in these cells (Fig. 2B). Replication of MuSIV but not SIV was also observed in a herpesvirus papio-transformed B-cell line from rhesus macaques (data not shown). SIV replicated slightly faster than MuSIV in the PBMCs of rhesus macaques, but after the peak in the antigen levels, titers declined to hardly detectable levels (Fig. 2C). Both MuSIV-infected parallel cultures reached peak antigen levels later, but the amounts of antigen produced were higher, and high antigen levels persisted for the duration of the culture (Fig. 2C).

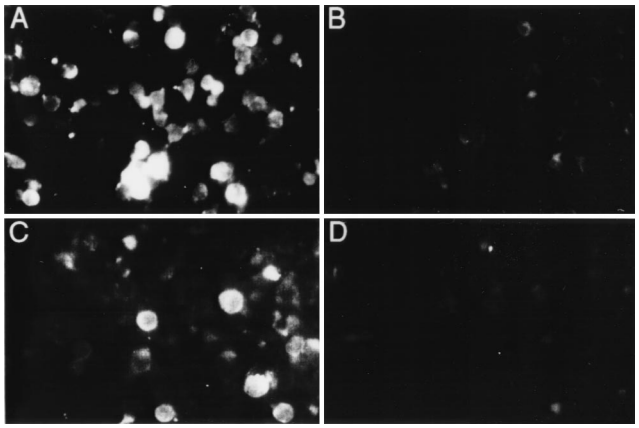

FIG. 3. Detection of MLV Env expression by immunofluorescence analysis. MuSIV-infected (A) or SIV-exposed (B) Raji cells and MuSIV- or SIV-infected PBMCs (C and D, respectively) of a rhesus macaque were stained for MLV Env expression with the monoclonal antibody 83A25 (3).

Expression of the MLV Env protein was analyzed by immunofluorescence staining to further demonstrate replication of MuSIV in Raji cells and PBMCs. MuSIV-infected or SIV-exposed Raji cells were fixed in 100% ethanol and stained with antibody 83A25 (3) directed against MLV Env followed by a fluorescein isothiocyanate-labelled rabbit anti-rat immunoglobulin G (Dianova, Hamburg, Germany). SIV-exposed Raji cells were negative for MLV Env (Fig. 3B), while a high percentage of MLV Env-positive MuSIV-infected Raji cells were detected (Fig. 3A). MLV Env-positive cells could also be detected in MuSIV-infected PBMCs (Fig. 3C), but not in uninfected or SIV-infected cultures (Fig. 3D).

To confirm replication of the hybrid virus in the absence of SIV Env, immunoprecipitation experiments were performed with uninfected, SIV- or MuSIV-infected CEMx174 cells with serum from a SIV-infected macaque as described previously (20). SIV Env could be detected in SIV-infected cells but not in MuSIV-infected cells (Fig. 4). Because of the *nef* deletion of MuSIV, the SIV Nef protein was only detectable in the SIV-infected cells. However, the p27CA protein was present in MuSIV- and SIV-infected cells. Precipitation of the same cell lysates with the 83A25 antibody (3) revealed a band similar in size to the gp70 MLV Env protein. To analyze whether replication of MuSIV depends on MLV Env, CEMx174 cells containing the secreted alkaline phosphatase (SEAP) gene under the control of the SIV long terminal repeat (kindly provided by R. Means and R. C. Desrosiers) were infected with MuSIV or

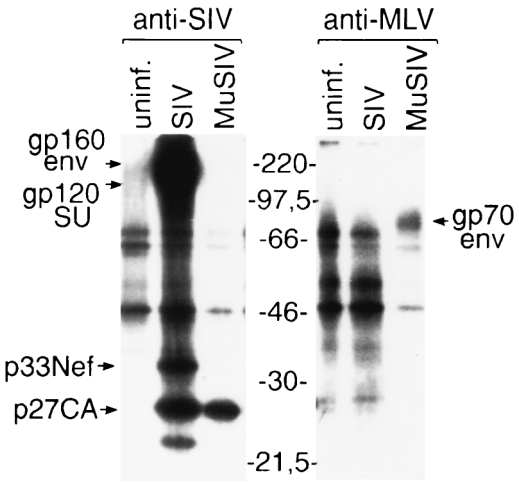

FIG. 4. Viral protein expression in MuSIV- or SIV-infected CEMx174 cells. Immunoprecipitation assays of metabolically labelled uninfected (uninf.) and infected cells were performed with a serum from an SIVmac-infected rhesus monkey (left panel) or the monoclonal antibody 83A25 (3) (right panel). The positions of the molecular mass markers (in kilodaltons) are shown between the panels.

SIV. Because of Tat transactivation, virus replication can be monitored by measuring SEAP activity. In the presence of the 83A25 antibody, replication of MuSIV but not of SIV could be inhibited in a dose-dependent manner (Fig. 5). By using the primers indicated in Fig. 1, a fragment spanning the junction between the MLV *env* gene and the truncated SIV *env* gene could be amplified from MuSIV-infected cells but not from uninfected or SIV-infected cells. The nucleotide sequence of the MLV/SIV border region of the amplified fragment was identical to that of the plasmid clone of MuSIV. Since the MLV *env* gene is separated by a stop codon and a frameshift

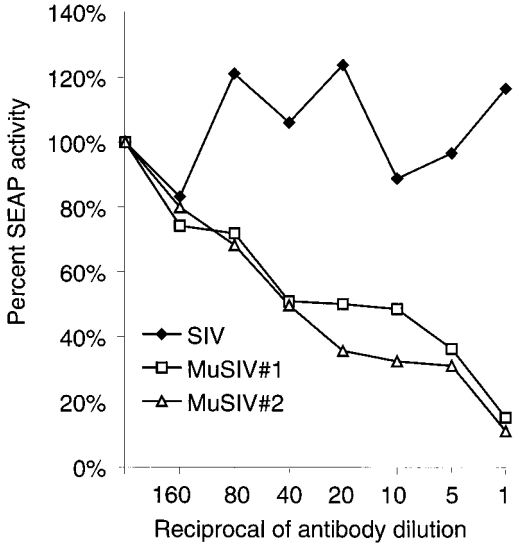

FIG. 5. Neutralization assay with an anti-MLV antibody. SIV-SEAP-CEMx174 cells were infected in triplicates with the viruses indicated after preincubation of the viruses with serial dilutions of the 83A25 antibody (3). SEAP activity was determined 3 days after infection with the Phospha-Light reporter gene assay (Serva, Heidelberg, Germany) as described by the manufacturer. The mean values of the triplicates are expressed as the percentage of SEAP activity of cultures infected in the absence of antibody.

from the truncated SIV *env* gene, MLV Env seems to be the functional envelope protein rather than a fusion protein of MLV Env and the truncated SIV Env.

Our results demonstrate that chimeras formed between retroviruses from different genera can be replication competent. The SIV/MLV hybrid virus MuSIV replicated in PBMCs to higher levels than did SIV. The increased virus production observed in MuSIV-infected PBMCs could be a consequence of the expanded cell tropism, reduced cytopathogenicity, or combination of both. At least under these experimental conditions, the chimeric virus seems to have a selective advantage over the parental SIV. MuSIV might be a useful tool with which to better understand differences in the pathogenesis of lentiviruses and oncoviruses. Because of the expanded tropism, such hybrid viruses might also allow establishment of new animal models. However, MuSIV did not replicate in mouse NIH 3T3 cells (data not shown), confirming that the blocking of viral entry is not the only restriction of SIV replication in mouse cells (13).

The question of whether recombinants between different genera of retroviruses can arise spontaneously remains to be addressed. Sequence comparison of different endogenous and exogenous retroviruses suggests that the acquisition of heterologous *env* genes can lead to new viral species (2). Experimentally, recombinants formed between HIV-1 and MLV were not observed in SCID mice harboring an HIV-1-infected human cell line or in cell cultures coinfected with HIV-1 and MLV (12). However, recombinants presumably do not have a selective advantage in these assay systems and, therefore, may have escaped detection. Numerous retroviruses have independently captured unrelated cellular genes. The recombinant retroviruses were detected by means of their transforming potential. Evidence suggests that recombination between cellular genes and retroviruses occurs during reverse transcription after copackaging of the viral genome and cellular RNA into the same virus particle (2). Therefore, coinfection of the same cell with two retroviruses must be postulated for recombination. This could occur in the case of an HIV-1-infected baboon transplant recipient. The baboon genome harbors multiple full-length copies of BaEV (21). Coculture of various baboon tissues including bone marrow and spleen with human cell lines repeatedly led to the isolation of BaEV (19). In the HIV-1-infected xenotransplant recipient, BaEV could therefore enter human cells that are also susceptible to HIV-1 infection. Multiple crossover events can be observed during reverse transcription because of frequent template switching (7). Therefore, a more complex recombination event required for the generation of replication-competent hybrid viruses might occur also. The emergence and detection of such a recombinant virus depends on the selective advantage of the recombinant over the parental viruses. Conditions in HIV-1-infected xenotransplant recipients may favor the outgrowth of a recombinant. If, for example, baboon retroviruses replicate poorly in human cells, an HIV-1 virus in which the *env* gene is replaced by the *env* gene of BaEV, similar to the construct described here, might have an expanded host cell tropism leading to a selective advantage. The immunosuppression or immunodeficiency in this patient might further contribute to an uncontrolled replication of the virus resulting in further adaptation. Too little is known about the recombination frequency of nonhomologous retroviruses, the replication competence of recombinants, and the pathogenicity of hybrid viruses to dismiss such a scenario. Since a hybrid formed between Rous sarcoma virus and MLV is replication competent (1), *env* genes might be more generally exchangeable between heterologous retroviruses without loss of replication competence. In addi-

tion to the implications of MuSIV for xenotransplantation, the SIV/MLV hybrid virus mimics a potential recombinant of a recently described lentiviral vector-packaging system for human gene therapy (15). Since the vector and the *gag-pol* genes are derived from HIV-1, while the *env* gene is of MLV origin, a replication-competent recombinant could emerge. As an alternative to the MLV Env protein, the G glycoprotein of vesicular stomatitis virus (VSV-G) was used. VSV-G confers increased stability and a wide host cell tropism. Therefore, the mode of transmission of a potential HIV-1–VSV recombinant could also be altered, suggesting a note of caution. Since emerging chimeric retroviruses have to be considered a serious risk of several recent experimental approaches, the study of recombinant viruses under appropriate biosafety conditions is important.

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