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Genetics

The U3 promoter region of the acutely lethal simian immunodeficiency virus clone smmPBj1.9 confers related biological activity on the apathogenic clone agm3mc

(acute disease/apathogenicity/virulence/genetic determinants of pathogenicity/replication competence in nonstimulated peripheral blood mononuclear cells)

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ABSTRACT **Infection with the acutely pathogenic molecular virus clone $SIV_{smmPBj1.9}$, cloned from isolate PBj14 of simian immunodeficiency virus (SIV) from sooty mangabey monkeys (*Cercocebus atys*), leads to acute viral and often lethal disease within days or weeks. $SIV_{smmPBj1.9}$ has the unique property of replicating in nonstimulated peripheral blood mononuclear cells from pig-tailed macaques. In contrast, molecular virus clone SIV_{agm3mc} of SIV from African green monkeys (*Cercopithecus aethiops*), which is apathogenic in its natural host and in pig-tailed macaques, is unable to grow in nonstimulated peripheral blood cells. Chimeric proviruses were constructed by exchanging defined regions of SIV_{agm3mc} against comparable regions of $SIV_{smmPBj1.9}$. Four of five hybrid viruses generated by transfection into the CD4-positive T-cell line C8166 replicated in T-cell lines permissive for SIV_{agm3mc} replication and in stimulated peripheral blood cells from pig-tailed macaques and from African green monkeys. Three hybrid viruses displayed the distinct biological property of $SIV_{smmPBj14}$ to replicate in nonstimulated peripheral blood cells from pig-tailed macaques and from African green monkeys. Replication in nonstimulated peripheral blood cells was dependent on the presence of the U3 promoter region of $SIV_{smmPBj1.9}$ within the viral long terminal repeat.**

Simian immunodeficiency virus (SIV) isolated from African green monkeys (AGMs; *Cercopithecus aethiops*) belongs to an evolutionary old and distinct group of lentiviruses (1–4). Provirus $pSIV_{agm3}$ (SIV_{agm3mc}) was molecularly cloned from isolate SIV_{agm3} obtained from a long-term infected healthy AGM and replicates in CD4-positive human T-cell lines such as Molt 4 clone 8 or C8166 (5). SIV_{agm3mc} used for infections of AGMs and pig-tailed macaques (*Macaca nemestrina*) did not cause acquired immunodeficiency during an observation period of at least 4 years (5, 6). In contrast, an isolate of SIV_{smm} from sooty mangabey monkeys (*Cercocebus atys*) termed $SIV_{smmPBj14}$ induces an acute disease in macaques. $SIV_{smmPBj14}$ was isolated from an experimentally infected, moribund pig-tailed macaque suffering from severe immunodeficiency (7, 8). Full-length provirus 1.9 ($pSIV_{smmPBj1.9}$; ref. 9) was subsequently cloned and the molecular virus clone $SIV_{smmPBj1.9}$ was demonstrated to also induce an acute disease in pig-tailed macaques (9, 10). Both cloned proviruses, $pSIV_{agm3}$ and $pSIV_{smmPBj1.9}$, comprise cistronic *gag, pol, env*, as well as *tat, rev, vif, vpx*, and *nef* genes, whereas only $pSIV_{smmPBj1.9}$ includes the *vpr* gene. The viral proteins encoded by the two proviruses display amino acid homologies between 20% and 60%. This genetic similarity was presumed to allow the exchange of comparable subgenomic regions between both viruses without loss of replication competence.

The acute disease induced by experimental infection of pig-tailed macaques with $SIV_{smmPBj1.9}$ was shown to correlate with the unique capability of this virus variant to replicate to high titers in nonstimulated peripheral blood mononuclear cells (PBMCs) from pig-tailed macaques *in vitro* (9–13). In contrast, apathogenic SIV_{agm3mc} replicates in cultured PBMCs from AGMs and pig-tailed macaques only after mitogenic stimulation of the cells. To define genomic regions of $SIV_{smmPBj1.9}$ that would empower SIV_{agm3mc} to also replicate in nonstimulated monkey PBMCs and to cause disease *in vivo* we generated replication competent hybrid viruses by exchanging defined subgenomic regions of $pSIV_{agm3}$ against respective regions of $pSIV_{smmPBj1.9}$. We were able to demonstrate that a single genomic region of $SIV_{smmPBj1.9}$ confers upon SIV_{agm3mc} the capacity to grow in nonstimulated PBMCs, the hallmark of $SIV_{smmPBj14}$ acute virulence and pathogenicity.

MATERIALS AND METHODS

Plasmid Constructions To Generate Recombinant Viral Genomes. The construction and the complete sequences of proviral plasmid clones $pSIV_{agm3}$ [SIV_{agm3mc}; GenBank accession no. M30931; numbering started at nucleotide 1 (A1) of the R region of the long terminal repeat (LTR)] and clones 1 and 9 of $pSIV_{smmPBj1.9}$ [$SIV_{smmPBj1.9}$; GenBank accession no. M31325; numbering started at nucleotide 1 (P1) as in ref. 9] have been described (4, 9). Briefly, $pSIV_{agm3}$ was obtained by insertion of the *Eco*RI fragment of pSIVMB1 (5) into plasmid pEX2.4, which contains the *Eco*RI (A6990)–*Xho* I (A334) 3′ half fragment of pSIVMB1 inserted into *Eco*RI and *Sal* I of pUC8 (Pharmacia). Plasmid pSN3.9 was constructed by inserting the subgenomic region from *Sac* I (A5386) to *Nar* I (A222) containing the 3′ half of provirus $pSIV_{agm3}$ including the 3′ LTR into plasmid pBluescript (Stratagene). Plasmid pES7.5 containing the 5′ genomic half of $pSIV_{agm3}$ (LTR, *gag, pol*) was obtained by insertion of the *Eco*RI (A6990)–*Sac* I (A5386) fragment of $pSIV_{agm3}$ into the respective sites of pBluescript. To generate subclone pTZ-HCR containing a recombinant central region (CR), three subgenomic fragments were amplified according to Horton *et al.* (14) using primers A4479(+) (CATCGTCGACACTTATGGCATGCCA-CATC) and A5554(−) (CCCTCCTTTCTTCGTATGGTTC) (5′ fragment), A5778(+) (ATGAAGCTGACATTACTG-ATAGGG) and A6029(−) (TCAGGTCGACTACTA-AGGGGTTTCTGTCAG) (3′ fragment), and AP-vpr(+)

Abbreviations: CR, central region; HY, hybrid virus; IL-2, interleukin 2; LTR, long terminal repeat; PBMC, peripheral blood mononuclear cell; SIV, simian immunodeficiency virus; AGM, African green monkey; PHA, phytohemagglutinin; moi, multiplicity of infection.
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(A5531–A5553/*P5919–P5939*) (GAACCATACGAAGA-AAGGAGGG-*ATGACAGAAAGACCTCCAGAAGATG*) and AP-rev(−) (A5778–A5802/*P6341–P6365*) (CCCTAT-CAGTAATGTCAGCTTCAT-*ACTTACTTGTCTGATGCA-GAAGATG*) (central fragment). The chimeric subgenomic 1.7-kbp fragment was subcloned into plasmid pTZ18U (Bio-Rad) after digestion by *Bcl* I and *Sph* I. The chimeric subgenomic *Sph* I (A4490)–*Xba* I (P6207) fragment was inserted into the respective sites of $pSIV_{smmPBj1.9}$, resulting in the chimeric 3′ half plasmid. After digestion by *Sph* I and ligation with a separately digested 5′ half genome of pES7.5 and transfection of the ligation mixture into C8166 cells, hybrid virus HY-vpr/env/U3 was obtained. The central fragment from *Sac* I (P6100) to *Bcl* I (A5937) of plasmid pTZ-HCR was inserted into pSN3.9 instead of the comparable region from *Sac* I (A5386) to *Bcl* I (A5937). The resulting plasmid containing the chimeric 3′ half genome was digested with *Xba* I and ligated with the 5′ half genome of $SIV_{smmPBj1.9}$ (clone 1) digested by *Xba* I. After transfection of the ligation mixture, chimeric virus HY-gag/pol/CR was generated. For the construction of hybrid virus HY-vif, a 1.5-kbp *Sph* I (A4490)–*Bcl* I (A5937) fragment encompassing the *vif* gene of $pSIV_{smmPBj1.9}$ in place of the *vif* gene of pSIVagm3 was obtained after PCR fusion of the three fragments amplified using primer pairs A4479(+) and A4762(−) (AATCCTTTATAATTTTTGC) (5′ fragment), A5461(+) (ATATTATAAATTGGTGC) and A6029(−) (3′ fragment), and AP-vif(+) (A4744–A4762/*P5107–P5127*) (GCAAAAAATTATAAAGGATT-*ATGGAG-GAGGAAAAGAATTGG*) and AP-vif(−) (A5461–A5478/*P5737–P5751*) (GCACCAATTTATAATAT-*TCATCATGC-CAGTATTCC*) (central fragment). The resulting chimeric fragment was inserted into *Sph* I (A4490)–*Bcl* I (A5937)-digested plasmid pSIVMB1 (5). After digestion by *Eco*RI, purification of the hybrid proviral fragment, ligation, and transfection, hybrid virus HY-vif was obtained. For the generation of hybrid virus HY-TM/nef/U3, a fused PCR product was synthesized by PCR from three fragments amplified with primer pairs A7494(+) (ATTCGTCGACCAGCATTT-ACTTGCTGGG) and A8244(−) (ATTGTATGTAGTG-GAAAGCGCTCC) (5′ fragment), A9096(+) (CAGTCTCT-TACTAGGAGACCAGC) and pUC18(−) (GATTGTC-GACGCCAAGCTTGGCTGCAG) (3′ fragment), and AP-nef(+) (A8220–A8244/*P8868–P8892*) (GGAGCGCTTTC-CACTACATACAAT-*ATGGGTGGCGTTACCTCCAAGA-AG*) and AP-U3(−) (A9069–A9092/*P9764–P9786*) (GCTGG-TCTCCTAGTAAGAGACTG-*AATACAGAGCGAAATGC-AGTTG*) (central fragment). The resulting 2.1-kbp amplification product was inserted into the *Eco*RI (A6990)–*Nar* I (A222) vector fragment of plasmid pSN3.9, resulting in a chimeric 3′ half clone used for ligation with the 5′ half genome of pES7.5 after digestion of both plasmids by *Sac* I. For construction of the hybrid virus HY-U3Δnef, three fragments were obtained by PCR using primer pairs A7494(+) and A8588(−) (CCCCCCTTTTCTTTTAAA) (5′ fragment), A9096(+) and pUC18(−) (3′ fragment), and AP-U3(+) (A8590–A8588/*P9248–P9269*) (TTTAAAAGAAAAGGG-GGG-*TGGAAGGGATTTATTACAGTG*) and AP-U3(−) (central fragment). The PCR fusion product of those fragments was inserted into the *Eco*RI (A6990)–*Nar* I (A222) vector fragment of plasmid pSN3.9 and of pES7.5, resulting in a chimeric 3′ half plasmid and a chimeric 5′ half plasmid. These two plasmids were digested by *Sac* I, ligated, and transfected. For construction of $pSIV_{agm3}\Delta$nef, a polynucleotide linker triplet (5′-CTAGCTAGCTAG-3′) was inserted into the *Stu* I (A8389) site of plasmid pEX2.4. The *Eco*RI fragment of pSIVMB1 was then inserted in correct orientation into the single *Eco*RI site of the linker-containing subgenomic plasmid.

Transfection and Reverse Transcriptase Activity. For transfection, 10 μg of each of two plasmids containing the respective 5′ or 3′ genomic half of the full-length hybrid provirus to be formed was digested by treatment with the appropriate restriction enzymes (see above) and ligated in a total volume of 100 μl using T4 DNA ligase (New England Biolabs). C8166 cells (1×10^6) were transfected using the DOTAP transfection reagent {*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate; Boehringer Mannheim}. Reverse transcriptase activity was measured as described (15).

Direct Detection of Genomic RNA of Cell-Free Virions. To verify the chimeric structure of the hybrid viruses, viral RNA was purified from virus concentrates (10× of cell culture supernatants) according to the procedure described by Bagnarelli *et al.* (16). After preparation of genomic RNA from viruses HY-gag/pol/CR and HY-vif, cDNA synthesis was performed using random hexamers (GIBCO/BRL) and the CRs were subsequently amplified by PCR using primer pairs APvif(+)/A6029(−) and A4479(+)/A6029(−), respectively. To clone subgenomic regions comprising the U3 region of hybrid viruses HY-TM/nef/U3, HY-U3Δnef, and HY-gag/pol/CR, cDNA synthesis was performed using an oligo$(dT)_{16}$ primer. PCR amplification was carried out using primers

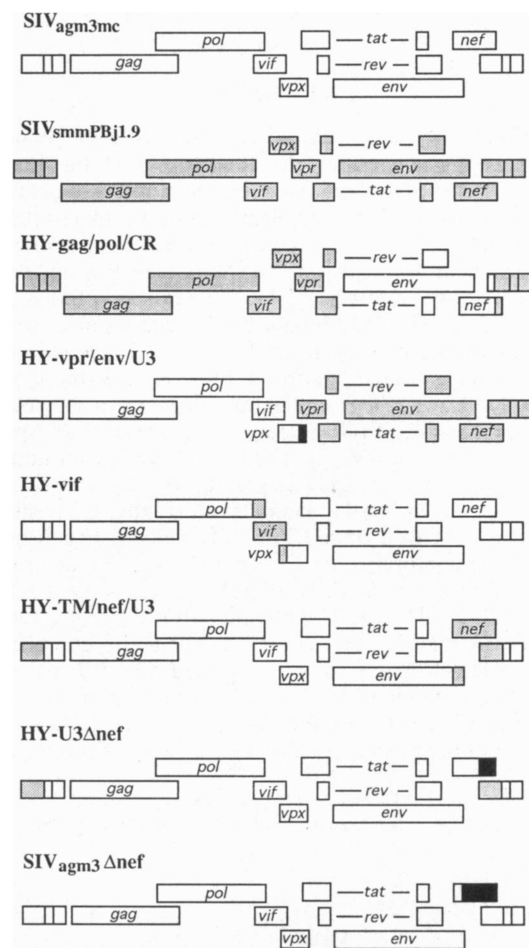

FIG. 1. Proviral structures of chimeric $SIV_{agm3mc}/SIV_{smmPBj1.9}$, of the parental viruses, and of deletion mutant $SIV_{agm3}\Delta$nef. Shown are the viral genomes generated after transfection of the respective proviruses into C8166 cells. All hybrid viruses displayed the genomic structures expected from their construction. Only hybrid virus HY-gag/pol/CR underwent an early recombination leading to the linkage of the viral promotor fragment of $pSIV_{smmPBj1.9}$ (nucleotides P9367–P9787) to proviral fragment A8587 to A8709 of $pSIV_{agm3}$. The respective genomic region comprising the viral promoter of $pSIV_{agm3}$ was apparently deleted. The two parental proviruses are shown at the top. White boxes represent genomic regions derived from $pSIV_{agm3}$; shaded boxes represent those of $pSIV_{smmPBj1.9}$; black boxes represent truncated genes due to the presence of premature stop codons introduced during the construction of respective plasmids.

A8194(+) (ACAGGTCGACCATCTGGCTATACAACAG) and A9099(−) (CTGGTCGACTCTCCTAGTAAGAGAC-TG). The resulting subgenomic DNA fragments were inserted into plasmid pDK101 (17) and at least two plasmid clones from each virus were subsequently sequenced.

Infection of PBMCs. Virus stocks were diluted in 3-fold steps using RPMI 1640 medium and eight 50-μl aliquots of each sample were used for the titration of viruses infectious for C8166 cells. Immunoperoxidase assay was used for the detection of SIV antigen-expressing cells using sera from an SIV_{agm3} infected pig-tailed macaque and a human immunodeficiency virus 2 (HIV-2) infected human, respectively (18). PBMCs were isolated from SIV-seronegative AGMs and pig-tailed macaques by Ficoll/Hypaque density gradient centrifugation and either infected immediately—i.e., nonstimulated—or incubated for 2 days in the presence of 5 μg of phytohemagglutinin (PHA) per ml (Wellcome) and 100 units of recombinant interleukin 2 (IL-2) per ml (Eurocetus, Frankfurt am Main, Germany) before infection [multiplicity of infection (moi) = 0.01]. After 2 days the cells were washed and cell supernatants were removed for titration after an incubation period of 15 days (see above).

RESULTS

Chimeric $SIV_{agm3mc}/SIV_{smmPBj1.9}$ Replicate in Human T-Cell Lines Permissive for the Replication of the Parental Viruses. To identify subgenomic regions capable of conferring on SIV_{agm3mc} the ability to replicate in nonstimulated PBMCs, chimeric $SIV_{agm3mc}/SIV_{smmPBj1.9}$ proviruses were constructed as described in *Materials and Methods* (see Fig. 1). Briefly, provirus HY-vif comprises the *vif* gene of $pSIV_{smmPBj1.9}$ (nucleotides 5107–5754) in place of a comparable genomic region of $pSIV_{agm3}$ (nucleotides 4762–5459). Provirus HY-gag/pol/CR contained the proviral region from the R region of the 5′ LTR up to the 3′ end of the first *tat* exon, which marks the end of the CR of $pSIV_{smmPBj1.9}$ in place of the respective proviral region of $pSIV_{agm3}$. The hybrid virus generated after transfection of provirus HY-gag/pol/CR was later shown to contain, in addition, the major part of the U3 region of $pSIV_{smmPBj1.9}$ within both LTRs (see below). Provirus HY-TM/nef/U3 comprised the C-terminal 55 amino acids of the transmembrane protein (TM) of $pSIV_{smmPBj1.9}$ linked in frame to the TM of $pSIV_{agm3}$. The complete $pSIV_{smmPBj1.9}$ *nef* gene and the U3 region were also included in the construction. Provirus HY-U3Δnef only comprised the U3 region of $pSIV_{smmPBj1.9}$ (nucleotides 9248–9786) instead of the comparable genomic region (nucleotides 8588–9096) comprising the U3 region of the $pSIV_{agm3}$ LTR. The *nef* gene of $pSIV_{agm3}$ was therefore truncated after codon 106. Provirus HY-vpr/env/U3 was constructed by insertion of nucleotides 5919–9996 of $pSIV_{smmPBj1.9}$ instead of nucleotides 5554–9214 of $pSIV_{agm3}$. As a control, a stretch of nine stop codons was inserted at position 8404 of $pSIV_{agm3}$, thereby truncating the *nef* gene of the resulting provirus $pSIV_{agm3}Δnef$ after codon 52 of 229 amino acids encoded by the wild-type *nef* gene of $pSIV_{agm3}$. Except for proviruses HY-vif and $pSIV_{agm3}Δnef$, which were stable in bacteria as full-length clones with one and two LTRs, respectively, all other hybrid proviruses were obtained by ligation of suitable 5′ and 3′ genomic halves and subsequent transfection into the T-cell line C8166.

After transfection into C8166 cells, reverse transcriptase activity released into the cell supernatant was followed for 25 days. As shown in Table 1, except for HY-vpr/env/U3, infectious hybrid viruses were generated from all proviruses constructed. In addition, the parental viruses and deletion mutant $SIV_{agm3}Δnef$ were generated as controls. Provirus HY-vpr/env/U3 was repeatedly transfected and expression of viral antigen was demonstrated, but infection of fresh cells could not be achieved. In contrast, all other chimeric viruses were infectious and replicated in C8166 cells with kinetics similar to those of SIV_{agm3mc}, $SIV_{agm3}Δnef$, or $SIV_{smmPBj1.9}$ (Table 1). Molt 4 clone 8 and CEMx174 cells were subsequently infected at the low moi of 0.01 (data not shown). Viruses HY-gag/pol/CR, HY-vif, and HY-U3Δnef replicated in Molt 4 clone 8 cells with kinetics similar to those of SIV_{agm3mc}, whereas chimeric virus HY-TM/nef/U3 and parental virus $SIV_{smmPBj1.9}$ were unable to replicate in these cells. In contrast, only $SIV_{smmPBj1.9}$, but none of the chimeric viruses nor SIV_{agm3mc} nor $SIV_{agm3}Δnef$, infected CEMx174 cells. In conclusion, SIV_{agm}-based hybrid viruses expressing various subgenomic regions of $pSIV_{smmPBj1.9}$ in place of the respective regions of $pSIV_{agm3}$ were demonstrated to replicate in human CD4-positive T-cell lines permissive for SIV_{agm} infection.

Chimeric $SIV_{agm3mc}/SIV_{smmPBj1.9}$ Replicate in Stimulated PBMCs from AGMs and Pig-Tailed Macaques. PBMCs from pig-tailed macaques stimulated for 2 days in the presence of PHA and IL-2 were infected at a moi of 0.01 with SIV_{agm3mc}, $SIV_{agm3}Δnef$, $SIV_{smmPBj1.9}$, and the replication competent hybrid viruses. As shown in Fig. 2 *A* and *B*, $SIV_{smmPBj1.9}$ replicated with kinetics similar to those of SIV_{agm3mc} and $SIV_{agm3}Δnef$ but released about 10-fold more infectious particles at the replication maximum. Interestingly, two of the hybrid viruses—namely, HY-gag/pol/CR and HY-TM/nef/U3—replicated each to a maximum titer comparable with that of $SIV_{smmPBj1.9}$. Although virus HY-U3Δnef replicated with kinetics similar to those of these two chimeras, the maximum amount of virus released was slightly lower. In contrast, chi-

Table 1. Tropism and replication competence in monkey PBMCs of chimeric $SIV_{agm3mc}/SIV_{smmPBj1.9}$ viruses, the parental viruses, and variant $SIV_{agm3}Δnef$

				PBMCs			
	Cell lines			Stimulated		Nonstimulated	
Virus	C8166	Molt 4/8	CEMx174	NEM	AGM	NEM	AGM
$SIV_{smmPBj1.9}$	+	−	+	+	+	+	+
SIV_{agm3mc}	+	+	−	+	+	−	−
$SIV_{agm3}Δnef$	+	+	−	+	+	−	−
HY-gag/pol/CR	+	+	−	+	+	+	+
HY-vpr/env/U3	−						
HY-TM/nef/U3	+	−	−	+	+	+	+
HY-vif	+	+	−	+	+	−	−
HY-U3Δnef	+	+	−	+	+	+	+

The outcome of infections of CD4-positive cell lines and PBMCs isolated from pig-tailed macaques (*M. nemestrina*; NEM) and AGMs with respective viruses generated by transfection into C8166 cells (see Fig. 1 for proviral structures) is shown. Nonstimulated PBMCs were infected directly after isolation, whereas stimulation of PBMCs prior to infection was performed for 2 days in the presence of PHA/IL-2. All infections were done at a moi of 0.01.

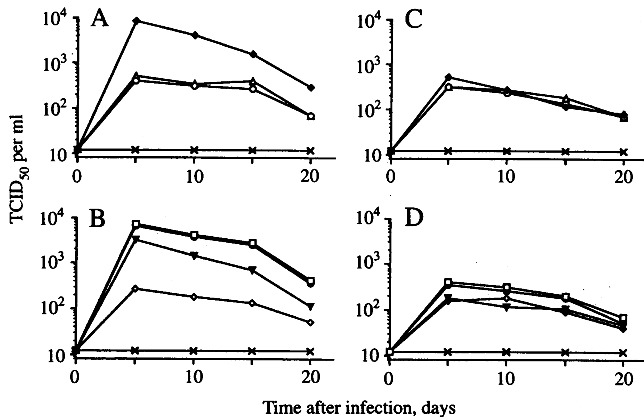

FIG. 2. Time course of viral replication of parental and hybrid viruses in stimulated PBMCs. Replication was followed after infection of 10^7 PBMC from pig-tailed macaques (*A* and *B*) and AGM (*C* and *D*) growth stimulated by PHA/IL-2 [infection: 10^5 tissue culture 50% infective dose ($TCID_{50}$) titrated in C8166 cells]. In *A* and *C* the time courses of replication of the parental viruses [SIV_{agm3mc} (○); $SIV_{smmPBj1.9}$ (◆)] and of the *nef*-negative variant $SIV_{agm3}\Delta nef$ (△) are shown. In *B* and *D* the replication curves of hybrid viruses HY-gag/pol/CR (□), HY-TM/nef/U3 (●), HY-U3Δnef (▼), and HY-vif (◇) are depicted. Supernatants were collected on days 5, 10, 15, and 20 and aliquots were titrated in C8166 cells. In *B* and *D*, replication curves of HY-gag/pol/CR (□) and HY-TM/nef/U3 (●) coincided. ×, Mock infection.

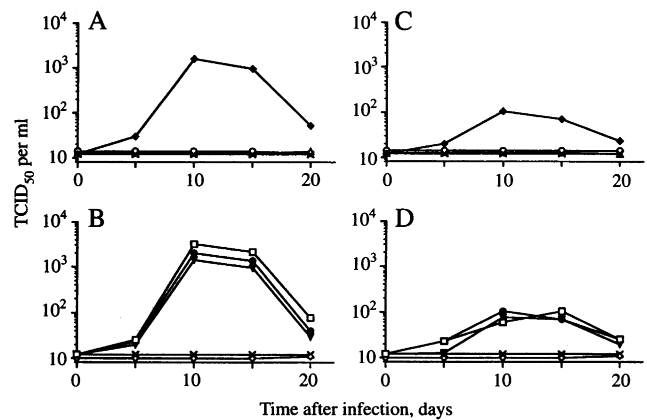

FIG. 3. Time course of viral replication of parental and hybrid viruses in nonstimulated PBMCs. Replication was followed after infection of 10^7 PBMCs from pig-tailed macaques (*A* and *B*) or from AGMs (*C* and *D*) directly after isolation of PBMCs (infection: 10^5 $TCID_{50}$ titrated in C8166 cells). In *A* and *C*, replication curves of SIV_{agm3mc} (○) and $SIV_{agm3}\Delta nef$ (△) coincided. See the legend to Fig. 2 for further details.

meric virus HY-vif replicated to a 10-fold lower maximum and with a kinetic very similar to that of SIV_{agm3mc} and $SIV_{agm3}\Delta nef$. Thus, genes other than *vif* conferred upon SIV_{agm3mc} a replication behavior comparable with that of $SIV_{smmPBj1.9}$.

Replication kinetics in stimulated PBMCs isolated from negative AGMs were subsequently tested for all infectious viruses described above (Fig. 2 *C* and *D*). $SIV_{smmPBj1.9}$ and all the replication competent chimeric viruses displayed replication kinetics comparable with those of wild-type SIV_{agm3mc} and of $SIV_{agm3}\Delta nef$. However, maximum infectious viruses released into the cell supernatants by all tested viruses were about 10-fold lower than that of $SIV_{smmPBj1.9}$ in stimulated PBMCs isolated from pig-tailed macaques. Again, the replication phenotype of hybrid viruses HY-gag/pol/CR and HY-TM/nef/U3 was very similar to that of parental virus $SIV_{smmPBj1.9}$. Hybrid viruses HY-vif and HY-U3Δnef yielded slightly lower titers than the parental viruses, despite growing with similar kinetics. In conclusion, HY-gag/pol/CR and HY-TM/nef/U3 displayed a replication behavior reminiscent of $SIV_{smmPBj1.9}$ when tested in stimulated PBMCs from pig-tailed macaques and from AGMs. In contrast, hybrid viruses HY-U3Δnef and HY-vif replicated to slightly lower titers than $SIV_{smmPBj1.9}$ in AGM PBMCs. SIV_{agm3mc}, $SIV_{agm3}\Delta nef$, and HY-vif replicated to much lower titers than the other viruses in stimulated PBMCs from pig-tailed macaques, where HY-U3Δnef was only slightly less replicative than hybrid viruses HY-gag/pol/CR and HY-TM/nef/U3 and parental virus $SIV_{smmPBj1.9}$.

Chimeric Viruses Comprising the Putative Promoter Region in U3 of $SIV_{smmPBj1.9}$ Replicate in Nonstimulated PBMCs from AGMs and Pig-Tailed Macaques. As an *in vitro* correlate of its capacity to induce acute disease in infected pig-tailed macaques, $SIV_{smmPBj14}$ was shown to replicate in nonstimulated PBMCs from pig-tailed macaques (11–13). The infectious chimeric viruses generated were therefore used for infections of nonstimulated PBMCs from pig-tailed macaques and from AGMs. As illustrated in Fig. 3, parental virus SIV_{agm3mc}, mutant $SIV_{agm3}\Delta nef$, and hybrid virus HY-vif were unable to grow in nonstimulated primary cells, whereas $SIV_{smmPBj1.9}$ and chimeras HY-gag/pol/CR, HY-TM/nef/U3, and HY-U3Δnef replicated to high titers in PBMCs from pig-tailed macaques. To our surprise, $SIV_{smmPBj1.9}$ and hybrid viruses HY-gag/pol/CR, HY-TM/nef/U3, and HY-U3Δnef were also capable of replicating in nonstimulated AGM PBMCs, although to about 15-fold lower titers than those achieved in nonstimulated pig-tailed macaque PBMCs. Again, hybrid viruses HY-gag/pol/CR, HY-TM/nef/U3, and HY-U3Δnef replicated to maximum titers and with kinetics similar to those of $SIV_{smmPBj1.9}$.

Sequence analyses of all viral regions encompassing junctions between the genome fragments of the two parental viruses were performed prior to and after infection of stimulated PBMCs. All chimeric viruses except HY-gag/pol/CR showed the expected genome structures of the respective proviral constructs. Subgenomic chimeric regions of virus HY-gag/pol/CR showed a critical alteration of the viral LTR, which now comprised nucleotides 8588–8708 of SIV_{agm3} linked to nucleotides 9367–9787 of $pSIV_{smmPBj1.9}$. The putative viral promoter (9, 19) of this chimeric virus was thus derived from $SIV_{smmPBj1.9}$ and not from $pSIV_{agm3}$. Therefore, one genomic region of $SIV_{smmPBj1.9}$—namely, the viral promoter within the U3 region of the viral LTR—was common to all infectious chimeric viruses generated except for HY-vif. In conclusion, the viral promoter region of U3 of $SIV_{smmPBj1.9}$ inserted in place of the respective genome region of SIV_{agm3mc} elicited in all chimeric viruses a replication behavior comparable with that of parental $SIV_{smmPBj1.9}$ when tested in PBMCs from AGMs and pig-tailed macaques.

DISCUSSION

With the future goal to understand the mechanisms underlying the apathogenicity of SIV_{agm} in its natural host and in pig-tailed macaques we tested the ability of hybrid viruses based on the genome of apathogenic SIV_{agm3mc} to acquire replicative properties of the pathogenic $SIV_{smmPBj1.9}$. Unique among SIVs, it was previously shown that $SIV_{smmPBj14}$ replication in nonstimulated monkey PBMCs correlated with its acute virulence and pathogenicity *in vivo* (11–13). Three of the biologically active chimeras generated here replicated in nonstimulated PBMCs and comprised the putative viral promoter of $SIV_{smmPBj1.9}$ within the U3 region of the LTR, replacing the viral promoter of SIV_{agm3mc}.

Virus HY-TM/nef/U3 expressed the $SIV_{smmPBj1.9}$ *nef* gene and virus HY-U3Δnef coded for a truncated form of the SIV_{agm3mc} *nef* gene. A *nef*-negative mutant of SIV_{agm3mc} was

generated as a control and shown to be unable to replicate in nonstimulated PBMCs. Thus, it was ruled out that the deletion of the SIV_{agm3mc} *nef* gene or the presence of the $SIV_{smmPBj1.9}$ *nef* gene contributed to the replication competence of the respective hybrid viruses in nonstimulated PBMCs. Whereas the chimeric viruses HY-TM/nef/U3 and HY-U3Δnef encompassed the complete U3 region, virus HY-gag/pol/CR did not comprise the complete LTR but only the viral promoter region of $SIV_{smmPBj1.9}$ (9, 19). Therefore, it was shown that the presence and the transcriptional activity of the $SIV_{smmPBj1.9}$ promoter are necessary and sufficient to endow replication competence in nonstimulated monkey PBMCs of hybrid viruses expressing all structural and regulatory proteins of the apathogenic SIV_{agm3mc}.

Viral stimulation of proliferation of pig-tailed macaque PBMCs was shown to correlate with the capability of $SIV_{smmPBj14}$ to replicate in nonstimulated PBMCs. The growth stimulatory activity was correlated by Novembre *et al.* (13, 20) with the acute virulence and lethality *in vivo* of another acutely pathogenic molecular virus clone of $SIV_{smmPBj14}$—namely, $SIV_{smmPBj6.6}$. Using virus chimeras between $SIV_{smmPBj6.6}$ and its less virulent relative SIV_{smmH4} (21), the growth stimulatory activity of $SIV_{smmPBj6.6}$ was shown to depend on the presence of its promoter region in the viral genome. This is in agreement with the results presented here. The genomic regions of SIV_{agm3mc} expressed by the viral promoter of $SIV_{smmPBj1.9}$ included all cistronic genes except *nef.* Thus it can also be concluded that so far undefined regions of structural or regulatory viral proteins of SIV_{agm3mc} are competent to induce proliferation of nonstimulated PBMCs.

In light of these results it will be crucial to test *in vivo* the acute pathogenicity of the hybrid viruses in pig-tailed macaques. $SIV_{smmPBj6.6}$ induced death of two of two experimentally infected monkeys (13). Surprisingly, hybrid viruses expressing all cistronic genes of SIV_{smmH4} from the viral promoter of $SIV_{smmPBj6.6}$ were not acutely pathogenic *in vivo* and were unable to stimulate the growth of resting monkey PBMCs. This indicates the absence of pathogenic and growth stimulatory determinants from cistronic genes of SIV_{smmH4}. In contrast, the cistronic genes of apathogenic SIV_{agm3mc} were shown here to comprise growth stimulatory determinants *in vitro.* This is surprising in light of the absence of acute or chronic immunosuppressive effects of SIV_{agm3mc} *in vivo* (18, 21–23) and its relatively low expression in peripheral blood following natural and experimental infections of AGMs and pig-tailed macaques (24). However, it seems clear that the growth stimulatory properties of $SIV_{smmPBj6.6}$ are a necessary component of its acute pathogenicity and virulence *in vivo.* Hybrid viruses expressing growth stimulatory genes of SIV_{agm3mc} under transcriptional control of the $SIV_{smmPBj1.9}$ promoter may therefore be immunosuppressive *in vivo.*

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