Genetic Identity and Biological Phenotype of a Transmitted/Founder Virus Representative of Non-pathogenic Simian Immunodeficiency Virus Infection in African Green Monkeys

Running head: Infectious transmitted/founder clone of SIVagm

Clement Wesley Gnanadurai\(^1\)*, Ivona Pandrea\(^2,3\)*, Nicholas F. Parrish\(^4\), Matthias H. Kraus\(^4\), Gerald H. Learn\(^4\), Maria G. Salazar\(^4\), Ulrike Sauermann\(^5\), Katharina Töpfer\(^5\), Rajeev Gautam\(^2\), Jan Münch\(^1\), Christiane Stahl-Hennig\(^5\), Christian Apetrei\(^2,3\), Beatrice H. Hahn\(^4\)§, and Frank Kirchhoff\(^1\)§

\(^1\) Institute of Molecular Virology
\(^2\) University Clinic of Ulm
\(^3\) Tulane National Primate Research Center
\(^4\) Tulane University
\(^5\) Covington, LA 70433, USA
\(^6\) University of Pittsburgh
\(^7\) Pittsburgh, PA 15261-9045, USA
\(^8\) Departments of Medicine and Microbiology
\(^9\) University of Alabama at Birmingham
\(^10\) Birmingham, Alabama 35294, USA
\(^11\) Unit of Infection Models
\(^12\) German Primate Centre
\(^13\) 37077 Goettingen, Germany
\(^14\) * both contributed equally
\(^15\) § Corresponding authors

Beatrice H. Hahn
Phone: 205-934-0412
bhahn@uab.edu

Frank Kirchhoff
Phone: 49-731-50065150
frank.kirchhoff@uni-ulm.de
Understanding the lack of disease progression in non-pathogenic simian immuno-deficiency virus (SIV) infections is essential for deciphering the immunopathogenesis of human AIDS. Yet, *in vivo* studies have been hampered by a paucity of infectious molecular clones (IMCs) of SIV suitable to dissect the viral and host factors responsible for the non-pathogenic phenotype. Here, we describe the identification, cloning and biological analysis of the first transmitted/founder (T/F) virus representing a non-pathogenic SIV infection. Blood was collected at peak viremia from an acutely infected sabaeus monkey (*Chlorocebus sabaeus*) inoculated intravenously with an SIVagm strain (Sab92018) that had never been propagated *in vitro*. To generate IMCs, we first used conventional (bulk) polymerase chain reaction (PCR) to amplify full-length viral genomes from peripheral blood mononuclear cell (PBMC) DNA. Although this yielded two intact SIVagmSab genomes, biological characterization revealed that both were replication-defective. We then performed single genome amplification (SGA) to generate partially overlapping 5’ (n=10) and 3’ (n=13) half genomes from plasma viral RNA. Analysis of these amplicons revealed clusters of nearly identical viral sequences representing the progeny of T/F viruses. Synthesis of the consensus sequence of one of these generated an IMC (Sab92018ivTF) that produced infectious CCR5-tropic virions, and replicated to high titers in Molt-4 clone 8 cells and African green monkey PBMCs. Sab92018ivTF also initiated productive infection in sabaeus monkeys, and faithfully recapitulated the replication kinetics and non-pathogenic phenotype of the parental Sab92018 strain. These results thus extend the T/F virus concept to non-pathogenic SIV infections and provide an important new tool to define viral determinants of disease non-progression.
INTRODUCTION

Simian immunodeficiency viruses (SIVs) have been identified in over 40 African non-human primate (NHP) species (reviewed in 48, 73), although to date only sooty mangabeys (SIVsmm), African green monkeys (SIVagm) and mandrills (SIVmnd), have been studied in detail (reviewed in 47, 48, 70, 73). Natural SIV infections share many similarities with human immunodeficiency virus (HIV) infections, including high levels of viral replication and marked depletion of mucosal CD4+ T cells during acute infection (reviewed in 47, 48, 70, 71). However, fundamental differences have also been identified. Most importantly, AGM and SM hosts of SIV show only an early transient increase in immune activation and do not develop AIDS (7, 10, 23, 30, 44, 49, 52, 67, 68).

Comparisons of non-pathogenic and pathogenic primate lentiviral infections have become an important area of AIDS research (reviewed in 29, 47, 50, 70). It is now clear that host factors can play an important role in the clinical outcome of infection. This is perhaps best illustrated by the fact that SIV from SMs is usually non-pathogenic in its natural mangabey host, but highly virulent in experimentally infected macaques (reviewed in 29, 47, 50, 70).

Features that distinguish SMs and AGMs from macaques that may allow them to avoid chronic immune activation and disease progression include effective regulatory T cell responses that establish an anti-inflammatory milieu, reduced levels of CCR5 on the surface of CD4+ T cells, and the maintenance of an intact mucosal barrier that prevents microbial translocation (reviewed in 29, 47, 50, 70). However, viral properties that differentiate SIVagm and SIVsmm from HIV-1 and its chimpanzee precursor SIVcpz, such as the lack of a vpu gene and the expression of Nef proteins that block the activation of virally infected T cells by TCR-CD3 down-modulation (3, 64, 65), may also contribute to an anti-inflammatory environment (reviewed in 29). The recent finding that SIVcpz is pathogenic in naturally infected chimpanzees in the wild (28) is consistent with this possibility.
One approach to explore viral determinants of \textit{in vivo} pathogenicity is to generate infectious proviral clones and mutants that differ in virus-specific properties, such as coreceptor tropism or accessory gene functions. Site-specific mutants of SIV have been used extensively in the pathogenic SIVmac/macaque model (reviewed in 2). However, this has not been done for non-pathogenic SIV infections, such as SIVagm infection of AGMs and SIVsmm infection of SMs, which represent the best studied model systems (reviewed in 47, 48, 70, 73). Although some infectious SIVsmm clones exist (19, 72), SMs are classified as endangered and can thus not be used for invasive studies. In contrast, AGMs are available in large numbers in captivity and there is no restriction concerning \textit{in vivo} experimentation. To date a number of infectious molecular clones from vervet (TYO-1, 155, 3, 9063), grivet (GRI-1), tantalus (TAN-1) and sabaeus (SAB-1) monkeys have been described (1, 4, 13, 20, 24, 25, 69). However, all of these were obtained after extensive passage in human cell lines and many exhibit features selected by \textit{in vitro} propagation, such as a truncated transmembrane envelope glycoprotein (gp41) or nonsense mutations in accessory genes (4, 13, 24, 69). Since it is unknown to what extent \textit{in vitro} culture alters \textit{in vivo} viral properties, existing SIVagm clones may not be suitable for \textit{in vivo} pathogenesis experiments.

To generate a physiologically relevant SIVagm proviral clone, we thus selected a virus stock (Sab92018) that had never been propagated \textit{in vitro}. This strain was originally identified in a wild-caught sabaeus monkey from Senegal and thus represents a viral lineage that circulated in the wild (10). Plasma from this chronically infected animal was used to infect two captive sabaeus monkeys to generate high titer viral stocks without \textit{in vitro} propagation. In each case, this was done by intravenous inoculation of plasma, followed by the harvest of plasma from the experimentally infected animal at peak viral replication (10, 49). The plasma stocks of Sab92018 have already been studied extensively \textit{in vitro} and \textit{in vivo} and the biological properties of this strain are thus well known (10, 14, 15, 17, 49-53).
To clone Sab92018, we first generated two full-length SIVagmSab proviruses (A5, E2), using conventional PCR and cloning techniques. Since this approach yielded replication defective clones, we next used single genome amplification (SGA) and direct sequencing of plasma viral RNA to infer the sequence of a transmitted/founder (T/F) virus that had established a productive infection. We have used this approach in the past to generate replication competent clones of HIV-1 and SIVmac (34, 62; Lopker, Hahn and Shaw, unpublished data). Chemical synthesis of a Sab92018 T/F genome generated a biologically active molecular clone that replicated to high titers both \textit{in vitro} and \textit{in vivo}. Our results thus show that the same principles of T/F virus inference and analysis previously established for HIV-1 and SIVmac also apply to non-pathogenic SIV infections, and that this strategy can thus be used to generate molecular clones that recapitulate the \textit{in vivo} replication properties of the parental virus strains.

MATERIALS AND METHODS

\textbf{Passage history of Sab92018.} The original Sab92018 strain was identified in a wild-caught adult sabaeus monkey from Senegal (10). Plasma from this index animal was used to infect a captive sabaeus monkey (92018) originating from Senegal by intravenous inoculation to generate a high titer virus stock without \textit{in vitro} propagation (10). This plasma stock was used for various \textit{in vitro} and \textit{in vivo} experiments (10, 30) and subsequently replenished by infecting a second sabaeus monkey originating from St. Kitts. This Caribbean AGM, termed EI43, was also infected by intravenous inoculation with uncultured plasma using 300 50\% tissue culture infectious doses (49). Sab92018 thus represents a naturally occurring SIVagm strain that was never adapted in tissue culture. Sab92018 was passaged only twice, and then only in its natural host. Moreover, \textit{in vivo} passage was performed using sufficiently large inocula to maintain the quasispecies complexity of the original viral strain. Importantly, there
was no evidence of increased viral loads or altered pathogenicity following the two passages
(10, 30, 49). Animal EI43 was euthanized 9 days post-inoculation and maximum volumes of
blood were collected. Plasma and PBMCs were separated and frozen in aliquots at -80°C.
The new plasma virus stock was named SIVagmSab92018 (EI43), quantified by real-time
PCR, and titered on SupT1 as described previously (10, 49).

Generation of near-full length SIVagmSab92018 genomes by conventional PCR.
High molecular weight DNA was isolated by phenol/chloroform extraction from the PBMCs
of animal EI43 9 days post-infection (p.i.). Near-full length genomes were amplified by
nested PCR using the expand long-template PCR system (Roche) according to
manufacturer’s instructions. Long terminal repeat (LTR) regions were amplified separately in
a single round PCR The resulting 768 bp amplicon was cloned into pCR-Script SK+ and used
to generate a complete provirus.

Single genome amplifications of 5’ and 3’ genomic halves. Viral RNA was extracted
from plasma of animal EI43 collected 9 days p. i. using the QIAamp viral RNA mini Kit
(Qiagen). cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen)
and strain specific primers a829r (5’-CCAAAGAGGTCTGACTATCC-AAGCTTTTC-3’)
for the 5` half, and a796r (5’-CTCCTCCCTGGAAAGTCCCGCT-3’) for the 3` half,
respectively. Single genome amplification was performed as described (5, 34, 61). Briefly,
cDNA was serially diluted and distributed in replicates of 8 PCR reactions in 96-well plates.
Dilutions where positive wells constituted less than 30% of PCR reactions were identified
and used for additional amplifications. At these dilutions, most wells contained amplicons
from single cDNA molecules. PCR amplifications were carried out using 1x High Fidelity
Platinum Taq PCR buffer, 2 mM MgSO$_4$, 0.2 mM of each deoxynucleoside triphosphate, 0.2
μM of each primer, and 0.02 U/μl of Platinum High Fidelity polymerase in 20 μl reactions
for the first round and 50 μl reactions for the second round. The nested primers for
generating 5' half genome amplicons (covering U5, gag and pol) included 798f (5’-CAAGTGTGTGCCCATTTATCCTCAG-3’) and a829r (5’-CCCAAGAGGTCTGAATCCTATCCAAAGCCTTTTC-3’) in the first round, a799f (5’-GTTAACCCTGGTTTACTAACGGATCCCTG-3’) and a828r (5’-TTCTCTGTATCACACTGCTCCTCTCTCTCTTTCCA-3’) in the second round, respectively. The nested primers for generating 3' half genome amplicons (covering vif, vpr, rev, tat, env, nef and U3) included a787f (5’-TGYTGGTGGGAGAAATAGAGCACAC-3’) and a796r (5’-CTCTCTCTGGAAGTCCCGCT-3’) for the first round, and a788f (5’-CACAATTTTAAAAGAAARGGRGGRATGGGG-3’) and a797r (5’-GGATGTGGTTTGTGGTTAGGCAGA-3’) for the second round, respectively. PCR conditions were as follows: 94°C for 1 minute, then 35 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 5 minutes and 30 seconds, followed by a final extension of 15 minutes at 68°C. One µl of the first round reaction was transferred as template to the second round. Amplicons were gel purified and sequenced directly using an ABI 3730 DNA analyzer.

**Molecular cloning a Sab92018 transmitted/founder virus.** To clone a full-length SIVagmSab T/F virus, we first inferred its sequence from 5' and 3' half genome SGA amplicons and 426 bp from the LTR sequence obtained following conventional PCR. This sequence was then synthesized (Blue Heron Biotechnology, Bothell, WA) as two fragments, 4,707 bp and 5,322 bp in lengths, which were joined at a unique BglI site (Fig. 1). *NotI* and *MluI* cloning sites attached during synthesis at the 5' and 3' ends of the half genome fragments, respectively, facilitated cloning into the pCR-XL-TOPO vector. The clone, designated pSab92018ivTF, was grown in XL2-MRF cells at 30°C (Stratagene).

**Phylogenetic analyses.** The Sab92018 T/F virus was compared to representative SIVagm strains and other primate lentiviruses in the viral Env region. Deduced Env amino acid sequences were used from the following SIV strains (GenBank accession numbers are in parentheses) (31): SIVagmSab (SAB1, U04005), SIVagmSab (P1, U59187), SIVagmGri...
(GRI1, M66437), SIVagmVer (155, M29975; 9063, L40990), SIVagmTan (Tan1, U58991),
SIVsmm (SL92B, AF334679), SIVrcm (GAB1, AF382829), SIVcpz (TAN1, AF447763),
SIVmus-1 (CM1085, AY340700), SIVtal (00CM266, AY655744), SIVsyk (KE51,
AY523867), SIVdeb (CM40, AY523865), SIVden (CD1, AJ580407), SIVmnd-2 (CM16,
AF367411), and SIVcol (CGU1, AF301156). Sequences were aligned using CLUSTAL W
(32) and the resulting alignments were visually adjusted. Sites that could not be aligned
unambiguously, or sites with a gap in any sequence, were excluded. Phylogenetic trees, along
with support using 1000 bootstrap replicates (12), were inferred by the neighbor-joining
method (60), as implemented in CLUSTAL W.

**Cell culture and virus stocks.** TZM-bl cells, which express CD4, CXCR4, and CCR5
and contain Tat-responsive reporter genes for β-galactosidase and the firefly luciferase under
the control of an HIV-1 long terminal repeat (LTR) sequence (56, 74), were obtained from the
NIH ARRRP, as contributed by John Kappes and Xiaoyun Wu. These cells and 293T cells
were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum.
For the generation of virus stocks, 293T cells were transfected with proviral constructs by the
calcium phosphate method as described previously (38). The medium was changed after
overnight incubation, and virus was harvested 24 hrs later. Residual cells in the supernatants
were pelleted, and the supernatants were stored at –80°C. The content of viral p27 capsid
antigen was quantified by SIV core p27 antigen capture assay kit (Zeptometrix Corp.,
Buffalo, NY). Quantification was done according to the manufacturer’s instructions.

**Western blot.** To assess viral gene expression, 293T cells were transfected with 5 µg of
dNA of the SIVagmSab constructs A5, E2, Sab92018ivTF and SAB-1. Two days post-
transfection, cells were lysed in 500 µl RIPA buffer. To analyze viral particles produced from
the molecular clones, 500 µl of the culture supernatants were centrifuged at 14,000 rpm for
30’ and the resulting pellets resuspended in 25 µl RIPA buffer. Proteins were separated on a
4-12% NuPAGE Novex Bis-Tris precast gel (Invitrogen) and transferred to poly-vinylidene
(PVDF) microporous membranes (Millipor, Bedford, MA) by electroblotting. Viral proteins
were detected using serum from an AGM infected with SIVagmsab92018 and probed with
horseradish peroxidase-conjugated goat anti-human immunoglobulin G (γ-chain specific)
secondary antibody (Sigma). Antibody complexes were detected by DAB (3, 3’-
diaminobenzidine, Vector lab).

**Viral infectivity and coreceptor usage.** To determine the infectivity of virions produced
by the various SIVagmSab clones, TZM-bl cells were seeded in 96-well plates at a density of
5000 cells/well and infected after overnight incubation with virus stocks containing 10 ng of
p27 capsid antigen produced by transiently transfected 293T cells. Two days post-infection
infectivity was detected using a galactosidase screen kit from Tropix as recommended by the
manufacturer. β-Galactosidase activities were quantified as relative light units per second
using an Orion Microplate Luminometer. TZM-bl cells were also used to determine the
coreceptor preference of the SIVagmSab92018ivTF clone. Cells were seeded at 5000
cells/well in 96-well plates overnight and then treated with Maraviroc (10 nM) and/or
AMD3100 (10 µM) for 1 hr. Infections were performed using virus stocks containing
normalized quantities of p24 or p27 antigen (10 ng). After 48 h of incubation at 37°C,
supernatant was removed, and cells were processed for detection of luciferase activity as
described above. To further analyze the coreceptor usage of the SIVagmSab92018ivTF clone,
GHOST cells were used as previously described (36). The six GHOST cell lines used express
CD4 alone or together with the viral coreceptors CCR5, CXCR4, BOB/GPR15 or
Bonzo/STRL33 and contain the gene encoding the green fluorescent protein (GFP) under the
control of the HIV LTR (36). A total of 5 x 10^4 cells were exposed to virus stocks containing
10 ng of p24 or p27 antigen produced by transient transfection of 293T cells. Three days after
infection the percentage of virally infected GFP-positive cells was analyzed by fluorescence-activated cell sorting (FACS). Uninfected cells were used as a negative control.

**Viral replication in African green monkey PBMCs.** PBMCs from SIV uninfected AGMs (n=4), were isolated by density gradient centrifugation over lymphocyte separation media (MP Biomedical, Irvine, CA). Briefly, blood was layered over density gradient media in a ratio of 2:1 and centrifuged at 18 °C at 400×g for 25 min. The monolayer containing PBMCs was resuspended with RPMI1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin–streptomycin (1 mg/ml, Invitrogen, CA) for subsequent studies. Freshly isolated PBMCs were stimulated with 10 μg PHA per ml of medium for 2 days followed by overnight incubation in IL-2 media. Activated PBMCs (5×10^6) were infected with virus stocks containing 4 ng of p27 capsid antigen of the SIVagmSab92018ivTF clone produced by transiently transfected 293T cells or the parental SIVagmSab92018 (EI43) plasma stock at 37 °C for 4 h; cells were then washed extensively to remove any cell-free virus. Cells were maintained in IL-2 media for 4 weeks. Virus production in culture supernatants was monitored weekly by SIV P27 antigen capture assay.

An aliquot of PBMCs were also depleted of CD8^+ cells employing a positive selection procedure (CD8 microbead kit, Miltenyi Biotech, Auburn, CA). This was done to improve the levels of viral replication since the CD4/CD8 T cell ratio is relatively low (about 1/4) in uninfected AGMs. The CD8^+-depleted cells were then stimulated with 10 μg/ml PHA for 2 days, followed by overnight incubation in IL-2 media. 5×10^5 CD8^+-depleted cells were infected as described above for the PBMCs. For 4 weeks, one half of the supernatant was collected every third day and replaced with fresh IL-2-containing media. Virus production in culture supernatants was monitored by SIV p27 antigen capture assay.

**Infection of AGMs.** One Caribbean AGM housed at the Tulane National Primate Research Center (TNPRC), an Association for Assessment and Accreditation of Laboratory
Animal Care (AAALAC) International facility, was infected with a virus stock of Sab92018ivTF derived from the supernatant of transiently transfected 293T cells containing 4 ng of p27 capsid antigen. Additionally, viral replication was compared to that recorded in 5 AGMs infected with 300 TCID50 of the SIVagmSab92018 (El43) plasma stock. The animals were fed and housed according to regulations set forth by the Guide for the Care and Use of Laboratory Animals (40) and the Animal Welfare Act. This study was approved by the Tulane University Institutional Animal Care and Use Committee (IACUC). Blood was collected from the animals at two preinfection times (days -15 and -7) at the time of virus inoculation, biweekly for the first two weeks, weekly for the next four weeks and monthly thereafter, up to 100 days p.i. Another three Caribbean AGMs imported from Barbados were infected intravenously with 500 ng of p27 capsid antigen of the SIVagmSab92018ivTF viral clone at the German Primate Centre. This animal experiment was licenced by the ethical board enacted through the German Animal Welfare Act under the number 33.11.42502-04-094/08 issued by the Lower Saxony State Office for Consumer protection and food safety. Blood was collected from all animals twice to three times before and at regular intervals after infection. Plasma viral loads were determined as previously described (49).

**FACS analysis for CD4+ T cells.** CD4+ T cell proportions were determined by staining whole blood leucocytes with a pre-titrated antibody cocktail comprising anti-CD11a-APC (clone HI 111), CD3-Alexa700 (clone SP34-2) and CD4-Horizon V450 (clone L200), all obtained from Becton Dickinson (BD). Following staining with the antibody mixture red blood cells were lysed with BD FACS lysing solution and labelled lymphocytes analyzed for their expression of cell surface markers by flow cytometry on a LSR II flow cytometer (Becton Dickinson, Heidelberg, Germany). Lymphocyte populations were gated based on forward and side scatter characteristics, then on exclusion of doublets and expression of
CD11a followed by CD3. Data were generated with BD FACS Diva 6.1.3 Software before analysis with FlowJo 8.8 Software (Treestar).

**GenBank accession numbers.** The sequence of the molecular SIVagmSab92018ivTF clone generated in this study will be submitted to GenBank and an accession number will be provided upon acceptance for publication.

### RESULTS

**Generation of two replication defective SIVagmSab proviral clones by conventional PCR.** Our first attempt to generate infectious molecular clones of Sab92018 involved conventional bulk amplification and cloning of near full-length genome fragments from PBMC DNA. Two such clones, termed E2 and A5, were selected for proviral construction because sequence analysis revealed that both encoded uninterrupted gag, pol, vif, vpr, tat, rev, env and nef genes, as well as intact cis-regulatory sequence elements, such as the TATA box, the primer-binding site, the ribosomal frame shift region at the gag-pol junction, and the poly-purine tract. Both clones were thus reconstructed to contain full-length LTRs. However, biological analysis showed that the two clones produced virions that were only poorly infectious and failed to replicate in CD4+ target cells **in vitro** (data not shown). Thus, conventional PCR and cloning approaches failed to generate biologically active clones of SIVagmSab92018, despite yielding fragments with a seemingly intact genomic organization.

**SGA analysis identifies a transmitted/founder SIVagmSab genome.** Since plasma was collected from animal EI43 during the acute phase of infection, we reasoned that SGA of plasma viral RNA would allow us to infer the sequence of viruses that had established the infection 9 days earlier (26, 27). We thus used SGA to generate 5' (n=10) and 3' (n=13) genome halves and sequenced them directly (Fig. 1A). As shown in Fig. 2B, the resulting amplicons exhibited a substantial amount of sequence diversity, consistent with the fact that
monkey EI43 was infected with virus from a chronically infected animal. However, phylogenetic analysis also revealed one low diversity lineage among both the 5’ and 3’ amplicons, which comprised sequences that differed by only few nucleotides (indicated by brackets in Fig. 1B). Such discrete low diversity lineages represent the progeny of T/F viruses, whose sequences can be inferred by determining their consensus sequences (26, 27, 33, 62). Since both 5’ and 3’ consensus sequences were identical in the 298 bp region of sequence overlap, we reasoned that they represented the same T/F virus. Missing LTR sequences were derived by conventional PCR (using a high fidelity polymerase) and direct sequencing, and the entire T/F virus genome was synthesized as two fragments, which were joined at a unique BglII site (Fig. 1C).

The full-length Sab92018 T/F virus was 10,004 bp in length and encoded intact gag, pol, vif, vpr, tat, rev, env and nef genes. Like the previously reported SAB-1 molecular clone and the genomes of SIVsmm and HIV-2 (69), the Sab92018 T/F virus had a single NF-kb binding site in its LTR, three NF-AT interaction sites and duplicated TAR sequences. Known cis-regulatory sequence elements were also preserved. Unlike other molecular clones of SIV obtained after extensive in vitro propagation, the Sab92018ivTF env gene encoded a full-length gp41 with a cytoplasmic tail of 157 amino acids (aa). The predicted length of the Sab92018ivTF Vpr protein is 138 aa, which is similar to that of SAB-1 (140 aa) but longer than those of SIVagmVer and SIVagmGri (120 aa) (24). As expected, in phylogenetic trees of Env amino acid sequences, Sab92018ivTF clustered closely with SAB-1 and P1, forming a species-specific clade within the SIVagm radiation (Fig. 2).

The SIVagmSab transmitted/founder virus is replication competent. To determine whether the Sab92018ivTF virus produces infectious virus, we infected TZM-bl indicator cells with 293T cell-derived virus stocks containing 10 ng of p27 capsid antigen and determined the β-galactosidase activities 2 days later. Previously described SAB-1 and TAN-
1 clones (24, 69) were used as positive controls. In contrast to the SIVagmSab A5 and E2 proviruses derived by conventional PCR, the Sab92018ivTF clone yielded highly infectious viral particles (Fig. 3A) that replicated efficiently in Molt-4 clone 8 cells (Fig. 3B). To determine whether this was due altered protein expression or particle assembly, we performed western blot analyses. The results showed that A5, E2 and Sab92018ivTF proviral constructs expressed all major viral proteins at levels similar to the SAB-1 positive control (Fig. 3C). Moreover, transfection derived supernatants contained viral particles that could be pelleted (Fig. 3C). Thus, the replication block in the A5 and E2 proviruses must be due to defects following protein expression and virion assembly.

Next, we analyzed the coreceptor tropism of SIVagm90218ivTF using two specific small molecule coreceptor antagonists, i.e. Maraviroc, which specifically blocks CCR5(R5)-tropic HIV-1 infection, and AMD3100, a specific inhibitor of CXCR4(X4)-mediated HIV-1 entry (reviewed in Ref. 58). The X4-tropic wild-type HIV-1 NL4-3 strain, an R5-tropic derivative thereof containing the gp120 V3 loop of HIV-1 TH014 (55), SIVmac239, and SIVagm SAB-1 were analyzed in the same experiment for control. As expected, AMD3100 blocked wild-type NL4-3 infection, whereas Maraviroc specifically inhibited the R5-tropic HIV-1 derivative (Fig. 3D). Infection of SIVmac239, SIVagmSAB-1 and SIVagm90218ivTF was inhibited by Maraviroc but not affected by AMD3100 (Fig. 3D) demonstrating that all three molecular clones of SIV utilize R5 but not X4 for entry into TZM-bl cells. However, infection by SIVagm92018ivTF and SIVmac239 was not blocked entirely suggesting that these viruses may utilize alternative coreceptors to gain entry into TZM-bl cells. To further investigate the coreceptor tropism of SIVagm92018ivTF, we infected GHOST cells, which stably express CD4 alone or together with different coreceptors and contain the GFP reporter gene under the control of the viral LTR promoter (36), with virus stocks containing normalized amounts of p24 or p27 antigen. In agreement with published data (9, 11),
SIVmac239 infected cells expressing CCR5 or the orphan receptors BOB/GPR15 and BONZO/STRL33 (Fig. 3E). Unexpectedly, Sab92018ivTF utilized BONZO/STRL33 about as efficiently as R5 for entry into GHOST target cells (Fig. 3E). Sab92018ivTF also infected GHOST cells expressing X4 and BOB/GPR15, albeit with lower efficacy. Thus, like most primary SIV and HIV-1 strains, Sab92018ivTF utilizes R5 for infection but is also able to enter cells via alternative coreceptors, particularly BONZO/STRL33.

We next compared the replicative capacities of the Sab92018ivTF clone and its parental SIVagmSab92018 strain in AGM PBMCs. In the presence of CD8\(^+\) T cells, Sab92018ivTF replicated efficiently only in the PBMCs from two of four animals. Replication in PBMCs from the remaining two AGMs was markedly reduced, but titers were higher than those of the parental SIVagmSab92018 isolate, which did not replicate to detectable levels (Fig. 4A). In contrast, both cloned and uncloned SIVagm strains were capable of establishing a productive infection in AGM PBMCs in the absence of CD8\(^+\) T cells (Fig. 4B). Again, the Sab92018ivTF clone consistently replicated with higher efficiency than the parental SIVagmSab92018 isolate in cells derived from all four animals examined. These results demonstrate that the Sab92018ivTF clone shows high replication fitness in primary cells derived from its natural AGM host.

To evaluate whether the T/F clone was also able to establish a productive infection in vivo, we initially infected one Caribbean AGM (Chlorocebus sabaeus) intravenously with a virus stock produced by transfection of 293T cells. For comparison, five other animals received the parental SIVagmSab92018 strain. We found that the Sab92018ivTF clone replicated about as efficiently in vivo as the parental SIVagmSab92018 strain (Fig. 5A). Peak levels of viral RNA were observed at 10 days p. i. for the molecular Sab92018ivTF clone (7.91\(\times\)10\(^6\) copies/ml) and at 8 days p. i. for the uncloned Sab92018 virus (2.81\(\pm\)1.91\(\times\)10\(^7\) copies/ml). Most importantly, Sab92018ivTF established a set-point viral load by day 42 p.i.,...
ranging from $10^5$ to $10^6$ copies per ml and which was maintained up to day 100 p.i., (Fig. 5A). Thus, the levels of Sab92018ivTF replication during the chronic phase of infection were as high or even higher as those detected in the wild-type SIVagmSab92018-infected animals.

To examine further the replication fitness of Sab92018ivTF in vivo three additional AGMs were infected at the German Primate Research Center. The results showed that the SIVagm Sab92018ivTF molecular clone replicated persistently at high levels for almost one year of follow-up (Fig. 5B). Furthermore, the three infected AGMs maintained stable CD4+ T cell counts (Fig. 5C) and did not show signs of disease progression (data not shown).

**DISCUSSION**

In the present study, we generated and functionally characterized three full-length molecular clones of a strain of SIVagmSab that has only been propagated in its natural host. We found that two clones (A5, E2) derived by conventional PCR produced poorly infectious virions that were replication-defective. In contrast, a proviral clone (Sab92018ivTF) representing a T/F virus was highly infectious and replicated efficiently both in vitro and in vivo. Virus generated from the T/F IMC exhibited biological properties that were very similar to those of the parental SIVagmSab92018 strain, including CCR5 tropism and the ability to establish a productive and persistent infection in AGMs in vivo. Thus, we have generated the first IMC that exhibits the properties of non-pathogenic SIV infection but is not compromised by interim propagation in tissue culture.

Conventional PCR can generate in vitro artefacts, especially when used to amplify genetically diverse sequence mixtures such as viral quasispecies (61). Single genome amplification precludes Taq polymerase induced recombination (template switching) and nucleotide misincorporation, thereby ensuring an accurate representation of viral variants as they exist in vivo. Given that animal EI43 was infected with SIVagm from a chronically
infected monkey, it was not surprising that the two proviruses derived by conventional PCR
were replication defective. Notably, most biologically active SIVagm clones available thus
far (3, 155, 9063, TYO, GRI-1, SAB-1, TAN-1) were generated by lambda phage cloning of
proviral DNA (1, 4, 13, 24, 25, 69), which is not prone to these same artefacts. To avoid bulk
PCR shortcomings, we opted to clone a T/F provirus inferred from SGA amplicons, a
strategy that has yielded replication competent molecular clones in the past (34, 62). T/F
viruses are responsible for initiating productive clinical infections. It is thus not surprising
that the T/F IMC of SIVagmSab replicated to high titers both in vitro and in vivo.

Sabaeus monkey EI43 was infected by intravenous inoculation with a plasma stock
originally derived from a chronically infected sabaeus monkey. Although in this study we
focused on cloning only one T/F IMC, it is clear that additional transmitted founder viruses
would have been identified had we generate additional SGA amplicons. Recent studies in
SIVmac infected rhesus macaques have shown that intravenous infection is more than 2,000-
fold more efficient than intrarectal infection, and frequently associated with multivariant
transmissions (27). Similarly, humans infected with HIV-1 by intravenous routes are more
likely to acquire multiple variants than humans exposed by sexual routes (5). Thus, the T/F
virus that we identified in the plasma of animal EI43 is likely one of many. Since we cloned a
T/F virus that was not subjected to a mucosal bottleneck, we also examined AGMs that were
infected intra-rectally with the same plasma stock used to derive SAB92018ivTF.

Characterizing the transmitted founder viruses in four such animals, we found one that
differed from SAB92018ivT/F by only a single synonymous substitution (Pandrea, Apetrei
and Hahn, unpublished observations). These results suggest that SAB92018ivTF will also be
capable of infecting AGMs by mucosal transmission routes.

In vivo analyses suggest that Sab92018ivTF faithfully reproduced the properties of its
parental strain, which has been extensively characterized (10, 14, 15, 17, 49-53). All four
Sabaeus monkeys infected with the Sab92018ivTF clone maintained high viral loads without developing immunodeficiency during follow-up. Thus, the newly generated Sab92018ivTF will be useful to characterize viral determinants of non-progression in the natural NHP hosts. For example, it has been suggested that the lack of a *vpu* gene and the ability of Nef to block T cell activation by down-modulation of TCR-CD3 may contribute to the low levels of immune activation (29). To examine these hypotheses, we have already created derivatives of the Sab92018ivTF clone expressing the SIVgsn Vpu, which antagonizes AGM tetherin (63, 74) and the HIV-1 Nef protein that is unable to down-modulate CD3 (64). Preliminary results show that this chimeric SIVagmSab clone is capable of establishing a persistent infection in sabaeus monkeys (Sauermann, Stahl-Hennig and Kirchhoff, unpublished data). Future studies will thus be able to address whether this "HIV-1-like" SIVagmSab derivative causes higher levels of immune activation and is more pathogenic than the original Sab92018ivTF clone. The Sab92018ivTF clone will also be useful to evaluate other determinants of virus transmission and pathogenicity, such as the role of viral properties in the frequency of vertical transmissions (46, 51) or the importance of coreceptor tropism for the maintenance of stable CD4+ T cell counts (35).

Like previously reported for the parental SIVagmSab92018 strain (52), the Sab92018ivTF molecular clone mainly uses CCR5 for viral entry but is capable of infecting GHOST cells engineered to express high levels of CXCR4. Unexpectedly, Sab92018ivTF entered GHOST cells expressing Bonzo/STRL33 as efficiently as those expressing CCR5. It has been reported that STRL33 is used by a variety of SIVs and by some HIV-1 strains and is expressed on specific subsets of CD4+ naive T cells, B lymphocytes, and natural killer cells (57, 66). It has been suggested that STRL33 may be a relevant coreceptor for HIV-1 *in vivo* (66), but experimental evidence is missing. Studies using derivatives of Sab92018ivTF that maintain the capability to use CCR5 as entry cofactor but are unable to utilize
Bonzo/STRL33 will provide novel insights into the role of this orphan receptor for viral replication in vivo.

In summary, we have generated the first replication-competent molecular clone of SIVagm that has not been adapted to growth in cell culture. Our results suggest that Sab92018ivTF recapitulates the biological properties of the naturally occurring parental SIVagmSab90218 strain and will thus be an important resource to identify those viral properties that contribute to the lack of disease progression in the natural AGM hosts.
REFERENCES


Murayama, Y., A. Amano, R. Mukai, H. Shibata, S. Matsunaga, H. Takahashi, Y. Yoshikawa, M. Hayami, and A. Noguchi. 1997. CD4 and CD8 expressions in African...


64. Schindler, M., J. Munch, O. Kutsch, H. Li, M. L. Santiago, F. Bibollet-Ruche, M. C.
   mediated suppression of T cell activation was lost in a lentiviral lineage that gave rise to

downmodulation of CD3 and MHC-I correlates with loss of CD4+ T cells in natural SIV infection. PLoS Pathog. 4:e1000107.


primary simian immunodeficiency virus SIVsm infection of natural sooty mangabey and nonnatural rhesus macaque hosts. J. Virol. 79:4043-4054.

mangabeys is characterized by limited bystander immunopathology despite chronic high-
level viremia. Immunity 18:441-452.


natural simian immunodeficiency virus infections of African nonhuman primate hosts.
Nat. Med. 15:861-865.


ACKNOWLEDGMENTS

We thank Daniela Krnavek for expert technical assistance, and Dre van der Merwe and Kristina Wohllaib for critical reading of the manuscript. This work was supported by grants from NIH (R01 AI064066, R37 AI 050529) and the Deutsche Forschungsgemeinschaft. Nicholas Parrish is supported by the UAB Medical Scientist Training Program (T32 GM008361)
FIGURE LEGENDS

FIG. 1. Molecular cloning of a SIVagmSab transmitted/founder virus. (A) Single genome amplification was used to generate 5’ (n=10) and 3’ (n=13) half genome sequences (drawn to scale) from the plasma of a sabaeus monkey acutely infected with a high titer stock of isolate SIVagmSab92018. (B) Phylogenetic trees and Highlighter plots of 5’ (top row) and 3’ (bottom row) half genome sequences. Tick marks indicate differences compared to the top sequence, which also represents the inferred transmitted/founder sequence (red, T; green, A; blue, C; and orange, G). Trees were inferred by the neighbour joining method and are midpoint rooted. The scale bar represents 0.002 substitutions per site. Discrete low diversity lineages representing the progeny of a transmitted founder virus are indicated by brackets. (C) The consensus sequence of low diversity lineages was used to synthesize the Sab92018ivTF genome as proviral halves. Flanking NotI and MluI restriction sites and an internal BglII site allowed cloning into the pCR-XL TOPO vector.

FIG. 2. Phylogenetic relationship of the SIVagmSab transmitted/founder virus to other primate lentiviruses. The tree was inferred from Env amino acid sequences. Numbers at nodes are percent bootstrap support (only values of 70% or greater are shown). The scale represents 0.2 substitutions per site.

FIG. 3. Functional characterization of the transmitted/founder Sab92018ivTF clone. (A) TZM-bl indicator cells were infected with the indicated SIVagm molecular clones. Infections were performed with virus stocks containing 10 ng of p27 antigen. Panels A and D show average values of triplicate infections ± SD. Abbreviation: ivTF, SIVagmSab92018ivTF. (B) Replication of SIVagmSab92018ivTF in Molt-4 clone 8 cells. (C) Western blot analysis of
cellular extracts of 293T cells transfected with the indicated molecular clones of SIVagmSab (left) and virions pelleted from the culture supernatant (right). (D) TZM-bl cells were left untreated (Control) or pretreated with specific inhibitors of CCR5 (Maraviroc), CXCR4 (AMD3100) or a combination thereof prior to infection with the indicated molecular clones of HIV-1 or SIV. (E) Coreceptor usage by SIVagmSab92018ivTF and the indicated control viruses tested in GHOST cells expressing CD4 alone (parental) or together with and CCR5, CXCR4, or the orphan receptors BOB/GPR15 or Bonzo/STRL33. All infectivity data were confirmed in one or two independent experiments.

**FIG. 4. Replication of SIVagmSab molecular clones in AGM PBMCs.** PBMCs (left) or PBMCs depleted of CD8+ T cells (right) derived from four AGMs were infected with the parental SIVagmSab92018 strain or the 92018ivTF molecular clone. Virus production was monitored by p27 antigen ELISA.

**FIG. 5. Replication of the SIVagmSab92018ivTF clone in African green monkeys.** (A) Viral RNA loads in one AGM infected with SIVagmSab92018ivTF and five animals that received the uncloned parental SIVagmSab92018 strain at the TNPRC. (B) Levels of plasma viremia and (C) CD4+ T-cell counts in three sabaeus monkeys infected with SIVagmSab92018ivTF at the German Primate Center. Individual animal numbers are indicated by five-digit numbers. The vertical line marks the time point of infection. The limit of viral RNA detection is approximately 100 copies/ml of plasma. Values were determined as described in Materials and Methods.
Gnanadurai et al. Fig. 1

A

\[ \text{SIVagm.sab} \]

5' LTR

gag

pol

vif

vpr

tat

rev

enr

3' LTR

5' half SGA amplicons

3' half SGA amplicons

B

5' half

5_F5

5_H3

5_E7

5_A5

5_B2

5_E11

5_E8

5_H1

5_A7

0.002

3' half

3_A6

3_A2

3_A8

3_D5

3_C4

3_F10

3_B12

3_E2

3_D8

3_G9

3_E3

3_B8

0.002

Base number

0

1000

2000

3000

4000

T

A

C

G

Gaps

C

5' half consensus (A5, E7, F5, H3)

3' half consensus (A2, A6, A8)

Chemical synthesis of 5' and 3' halves

Not I

5' half (4,707 bp)

Bgl I

3' half (5,322 bp)

Mlu I

Assembly of 5' and 3' halves

Not I

Mlu I

Full-length SIVagmSab90218lvTF proviral clone
Gnanadurai et al. Fig. 3
Gnanadurai et al., Fig. 5

A

Viral RNA load (copies/ml)

Days post-infection

B

Viral RNA load (copies/ml)

Weeks post-infection

C

CD4+ T cells (%)

Weeks post-infection