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DIVERSITY IN RELATION TO VIRAL PATHOGENESIS AND  
TREATMENT

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13. Abstract (continued)

(iv) the identification by PCR amplification of HIV-2 viruses in humans that are phylogenetically indistinguishable from SIV<sub>sm</sub> in sooty mangabeys, thereby providing direct evidence for HIV-2 as a zoonotic infection of man. The implications of these findings to HIV pathogenesis, treatment, and prevention are discussed.



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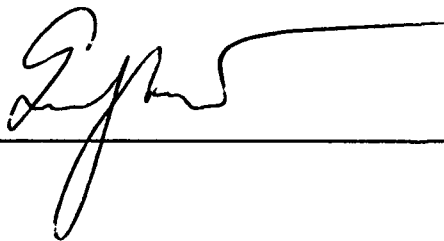
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"Genetic Variation of HIV: Viral Load and  
Genotypic Diversity in Relation to  
Viral Pathogenesis and Treatment"

Introduction

Elucidation of HIV-1 and HIV-2 pathogenesis and development of effective antiviral treatments and vaccines will require a thorough understanding of viral replication patterns, viral load, and the molecular details of viral variation occurring during natural infection. The specific objectives of our project (contract DAMD17-90-C-0064) address each of these important aspects of HIV pathogenesis, and in the first 18 months of the contract period, we have made substantial progress. In this mid-term report, we describe our studies that have led to five published manuscripts, one submitted, and two in preparation. The results of these studies reveal for the first time the changes in replication patterns for HIV-1 throughout the entire spectrum of infection from acute (CDC stage I) to endstage disease (CDC stage IV) (1,2); the genotypic characteristics of HIV-1 transmitted sexually from donor to recipient and its subsequent evolution over time (2, 3, in preparation); the genetic and biologic characteristics of HIV-1 in *uncultured* brain tissue (4,5); and finally, the identification of a strain of HIV-2 in West African humans that is so similar to SIV<sub>SM</sub> from sooty mangabeys as to be phylogenetically indistinguishable (6). In addition to this work, we have also performed PCR studies in an attempt to amplify and clone the envelope gene from uncultured tissues for genetic and biologic analysis and as a quantitative measure of virus replication and burden.

Infection with human immunodeficiency virus type 1 (HIV-1) causes a chronic progressive illness characterized by deterioration in immune and neurologic function and frequent abnormalities in other organ systems including hematologic, pulmonary, cardiac, renal, and gastrointestinal. Direct and indirect roles for HIV-1 have been postulated to explain the diverse clinical sequelae resulting from viral infection, but even the progressive loss of CD4<sup>+</sup> lymphocytes leading to the acquired immunodeficiency syndrome (AIDS) has not been explained mechanistically (7,8). HIV-1 is classified as a lentivirus because of its characteristic genomic organization, nontransforming biological properties, and slowly (lenti-) developing clinical sequelae that are analogous to other lentiviruses such as visna virus, equine infectious anemia virus (EIAV), and simian immunodeficiency virus (SIV) which infect different animal species (9,10). For these other lentiviruses, there is compelling evidence for the importance of viral replication patterns being critical determinants of viral pathogenicity and natural history. For EIAV and SIV in particular, high level viral replication leading to high titer plasma viremia has been directly associated with efficient transmission and disease induction (11,12).

Relatively little is known about replication patterns and viral burden of HIV-1 in vivo and their relation to disease pathogenesis. Recent studies have shown that both CD4<sup>+</sup> lymphocytes in blood and monocyte-derived cell types in brain serve as primary reservoirs for HIV-1 and that the proportion of virally infected cells and the abundance of viral DNA increases with disease progression (13-16). Circulating HIV p24 antigen, a marker of HIV-1 replication, has been shown to occur transiently during acute HIV-1 infection and to reappear in later stages of infection coincident

with a decline in CD4<sup>+</sup> lymphocytes (17-19). Cell-free infectious virus in plasma, another indicator of HIV-1 replication, was first demonstrated by Zagury and co-workers (20) followed by other reports of viremia in association with p24 antigenemia in acute and chronic infection (21-26). A major objective of our studies was to obtain systematic and quantitative data for HIV-1 plasma viremia and its relation to p24 antigenemia, anti-p24 antibody, clinical stage, and likelihood of disease progression in both children and adults. We thus examined HIV-1 plasma virus titers in 81 consecutively enrolled HIV-1 infected adults and children representing all stages of viral infection from acute (CDC stage I) to chronic (CDC stages II-IV). Once this data was obtained, we planned to characterize the genetic properties of virus as it exists *in vivo* and the changes in genetic complexity and burden that ensue with the establishment of persistent infection.

### Body (Results)

Seventy-two HIV-1 infected adults, 14 HIV-1 infected or exposed children, and 20 uninfected normal donors were enrolled in our studies of plasma viremia and viral pathogenesis. The clinical protocol was approved by the Human Use Committee of the University of Alabama at Birmingham (UAB) Institutional Review Board. Patients at all stages of clinical disease who attended the UAB AIDS Outpatient Center and The Children's Hospital of Alabama were identified and recruited to participate in the study. All volunteers underwent detailed clinical and laboratory evaluations including lymphocyte subset analyses and were staged according to the Centers for Disease Control (CDC) classification system (27,28). Among the adults, 4 had acute infection



(CDC stage I), 19 were asymptomatic (CDC stage II), 34 were classified as early AIDS-related complex (ARC; CDC stage III/IVC2), and 15 patients had AIDS (CDC stage IVA/B/C1/D).

Among the 14 children, 5 were exposed to HIV-1 in utero but exhibited no clinical or laboratory evidence of infection (CDC stage P0) based on negative peripheral blood lymphocyte cultures, undetectable serum p24 Ag, decreasing intensity of reactivity of enzyme linked immunoabsorbent assay (ELISA) and Western blot for HIV-1 antibodies over time, and absence of polymerase chain reaction (PCR) amplification of HIV-1 DNA. The other 9 children, 5 of whom were exposed to virus in utero and the remainder by transfusion of blood or clotting factors, had established HIV-1 infection as determined by positive HIV-1 lymphocyte cultures and presence of anti-HIV-1 antibody in children  $\geq 15$  months of age. Three of the HIV-1 infected children had AIDS (P2B, C, D1, D2, or E1), five had clinical findings indicative of symptomatic HIV-1 infection (P2A, D3, E2, F, or IVC2), and one had asymptomatic infection with normal immune function (P1A).

For the entire group of adults and children, the CD4 counts ranged from 3 cells/mm<sup>3</sup> to 2848 cells/mm<sup>3</sup> and the CD4:CD8 ratios from 0.01 to 4.30. All but 7 patients were studied before the institution of antiviral therapy, and 22 patients were studied both before and after zidovudine treatment. One patient had taken dithiocarb (DTC) and another patient had taken hypericin. On the day of clinical evaluation, 60 ml of peripheral blood from adults and 3 ml from infants and children were obtained in sterile acetate citrate dextran (ACD) vacutainers via antecubital venipuncture. The blood was transported to the laboratory at ambient temperatures

and within two hours was centrifuged at 675 g x 15 minutes to separate plasma and cellular fractions. The collected plasma fraction was centrifuged at 1800 g x 10 minutes to remove residual cellular and platelet components. It was then passed through a sterile 1.2  $\mu$ m nonabsorbing filter that had been prewetted with RPMI containing 15% heat inactivated fetal calf serum (FCS) to insure the removal of any residual cells or platelets. Filtered plasma was then serially diluted from  $10^0$  to  $10^8$  with RPMI-1640 containing 15% FCS. One milliliter aliquots of each plasma dilution ( $10^0$  to  $10^8$ ) were then combined in 12 well plates with  $2 \times 10^6$  type O normal donor peripheral blood mononuclear cells (PBMC) (total volume 2 ml) which had been stimulated for 48-72 hours with phytohemagglutinin (PHA; 2  $\mu$ g/ml), washed, and resuspended in RPMI-1640 containing IL-2 (30 units/ml), gentamicin (0.1 mg/ml), L-glutamine (2 mM), and 15% FCS. Cultures were maintained for five weeks with 1:3 cell splits performed weekly. Supernatants were tested weekly for HIV-1 p24 antigen in a solid phase sandwich-type enzyme linked immunosorbent assay (Abbott Laboratories, Chicago, IL) beginning on days 3 to 7. Positive cultures were defined as those which achieved absorbance values greater than 2 standard deviations above normal control samples (greater than 30 pg p24 antigen per ml) and had rising levels of HIV-1 p24 antigen reflecting active viral replication. Selected cultures were further characterized by reverse transcriptase assay and PCR analysis and were amplified and frozen for subsequent analyses. The final endpoint plasma titer represents the highest plasma dilution during the five week culture period yielding a positive result. Uncultured and unfiltered plasma samples from each study subject were frozen at -70°C and analyzed for quantitative p24 antigen levels as described elsewhere (26).

Both plasma and tissue culture supernatant samples were treated with 0.5% Triton X-100 in order to disrupt virions prior to incubation with the primary solid phase anti-HIV-1 antibody. In addition, patient samples were also analyzed for p24 antibody levels using a new enzyme immunoassay developed at Abbott Laboratories. In this assay, recombinant p24 antigen is used on the solid phase as well as conjugated to horseradish peroxidase to specifically capture p24 antibodies from plasma or serum. Serial five-fold dilutions of the specimen permits determination of the endpoint titer which is the greatest dilution at which p24 antibodies are still reproducibly detected. The endpoint titer is a function of the amount of antibody present in the specimen and the avidity of the p24 antibody population, this being a measure of the overall p24 antigen binding strength. Statistical analyses were performed by chi square analysis.

A total of 72 infected adults and 14 children were evaluated 161 times during the course of this study. Tables 1 and 2 summarize the initial plasma culture results for each subject with CDC stage II/P1 disease or greater in relation to other clinical characteristics including disease stage, CD4<sup>+</sup> lymphocyte counts, plasma HIV-1 p24 antigen and antibody levels, and concurrent antiretroviral treatment, if any. In the adult group, 14/15 patients with AIDS (CDC stage IVA/B/C1/D) were viremic compared with 4/53 patients with less advanced illness (CDC stage II/III/IVC2;  $P < 0.001$ ). In the pediatric group, 5 of 9 children with established HIV-1 infection were plasma viremic. Interestingly, this included all 5 children who had been infected with HIV-1 in the perinatal period and none of the 4 children infected after the age of 3 months as a result of blood product transfusion. None of five children (stage P0)

who were exposed to HIV-1 in utero but who remained virus negative based on repeated lymphocyte cultures and absence of viral specific PCR amplification of viral DNA nor any of 20 additional HIV-1 seronegative control subjects included as blinded controls.

Levels of plasma viremia were determined for all subjects and ranged from  $10^1$  to  $10^8$  TCID (tissue culture infectious doses) per milliliter of plasma. The mean geometric endpoint titer for HIV-1 viremia in adults with CD4<sup>+</sup> lymphocyte counts of  $>400/\text{mm}^3$ ,  $200\text{--}400/\text{mm}^3$ , and  $<200/\text{mm}^3$  were 0,  $10^{0.5}$ , and  $10^{2.8}$ , respectively. Of 15 patients with CDC defined AIDS, 7 had virus levels between  $10^3$  and  $10^8$  TCID/ml. For perinatally infected children, the geometric mean titer was  $10^{2.6}$  TCID/ml plasma with a range of  $10^1$  to  $10^3$  TCID/ml. For the 24 viremic patients studied, plasma cultures became detectably positive at one or more dilutions by day 6 on average (range 3 to 14 days) while the time required for the final endpoint plasma culture to become positive averaged 12 days (range of 4 to 39 days). In 21 out of 23 viremic subjects, culture endpoint was reached by day 21. In all instances but one, all cultures corresponding to plasma dilutions less than the endpoint titer were also positive for virus (e.g., plasma dilutions  $10^0$  to  $10^{-4}$  and  $10^0$  to  $10^{-7}$  were positive along with the  $10^{-5}$  and  $10^{-8}$  endpoint cultures of patients DODO-0116 and JOJI-0070, Table 1).

Experiments were also performed to determine the consistency of plasma viral titers in untreated patients over time, the effect of normal lymphocyte donor selection on viremia titers, and the intra-experiment reproducibility of the assay. Twelve subjects who did not receive antiviral therapy were studied on more than one

occasion over a 1-7 month period. All 5 individuals who were initially viremic remained so, and similarly, all 7 individuals who were initially not viremic remained without detectable virus in their plasma. Of the 5 viremic subjects who did not receive antiviral therapy (BIJA-0205, DODO-0116, SMDO-0157, DEDA-0006, and WHRO-C002), virus titers varied by 2 log dilutions or less (Table 3). Plasma cultures for two subjects (BIJA-0205 and NAPH-0073) were each established using cells from three different normal donors (D2,D3, D4 and D15, 16, 17, respectively) and from single normal donors in triplicate (D4a, b, c and D17 a, b, c, respectively), and the titers of HIV-1 measured varied by 1 log dilution or less (Table 3).

A striking inverse correlation was found between the presence of viremia and CD4<sup>+</sup> lymphocyte counts in adult patients (Figure 1). Fifteen of 16 subjects with <200 CD4<sup>+</sup> cells/mm<sup>3</sup> and 3 of 17 subjects with 200-400 CD4<sup>+</sup> cells/mm<sup>3</sup> were viremic as opposed to 0 of 35 subjects with CD4<sup>+</sup> lymphocyte counts > 400 cells/mm<sup>3</sup>. In contrast to these results in adults, all 5 children who acquired HIV-1 infection perinatally were viremic regardless of their CD4<sup>+</sup> lymphocyte counts which ranged from 42 to 2848 cells/mm<sup>3</sup> (Table 2). Children infected by transfusion of blood products at older ages were similar to adult patients with none of them having plasma viremia.

Of 77 HIV-1 infected subjects, 30 had detectable HIV-1 p24 antigen in plasma. There was a positive correlation between level of p24 antigenemia and HIV-1 viremia ( $r = 0.60$ ;  $P < 0.001$ ; Figure 2), although in both adults and children there was substantial discordance between the two measures of viral replication. For example, of 18 adults and 5 children who were viremic, 4 (22%) and 2 (40%), respectively,

lacked detectable p24 antigen in their plasma. Conversely, among 50 adults and 4 children who were not viremic, 10 (22%) and 3 (75%), respectively, were p24 antigenemic. Of 16 adults with CD4<sup>+</sup> counts less than 200/mm<sup>3</sup>, 15 (94%) were viremic and 12 (75%) were antigenemic. Of 17 adults with CD4<sup>+</sup> counts between 200 and 400/mm<sup>3</sup>, 3 (18%) were viremic and 6 (35%) were antigenemic. Of 35 adults with CD4<sup>+</sup> counts greater than 400/mm<sup>3</sup>, none were viremic and 6 (17%) were antigenemic. Of 15 patients with CDC defined AIDS, 14 had HIV-1 plasma viremia and 11 HIV-1 p24 antigenemia. The mean antigen levels in viremic and nonviremic groups of patients were 130 pg/ml and 7 pg/ml, respectively ( $P < 0.001$ ).

Anti-p24 antibody assays were performed on all but four HIV-1 infected study subjects. Fifty-four out of 73 individuals studied had detectable anti-p24 antibodies. Twenty-nine of 36 (81%) subjects with  $> 400$  CD4<sup>+</sup> cells/mm<sup>3</sup>, 17 of 19 (89%) subjects with 200-400 CD4<sup>+</sup> cells/mm<sup>3</sup>, and 8 of 18 (44%) subjects with  $<200$  CD4<sup>+</sup> cells/mm<sup>3</sup> had anti-p24 antibodies. Of 15 subjects with CDC defined AIDS, 14 were viremic and 8 had anti-p24 antibodies. Forty-three of 52 (83%) nonviremic subjects had anti-p24 antibodies as opposed to 11 of 21 (52%) viremic subjects. The mean titers of anti-p24 antibodies in nonviremic versus viremic subjects were 17,756 and 62, respectively ( $P < 0.001$ ).

Nineteen adults and 3 children were evaluated before and after initiation of therapy with zidovudine (dosages ranging from 300 mg/d to 1200 mg/d). Among the adults, 13/19 had negative plasma cultures pretreatment and all 13 remained without plasma viremia while on zidovudine (mean follow-up 18.5 weeks; range 8-32 weeks). Among the 6 adults and 2 children who were viremic on initial

evaluation (pretreatment), all 8 exhibited a decrease in plasma viremia titers by 10 to  $10^6$  fold (Figure 3) with a geometric mean pretreatment titer of  $10^{2.9}$  which decreased to  $10^{0.6}$  while on zidovudine (mean follow-up 18.5 weeks, range 15 to 40 weeks). Five of the eight patients became plasma culture negative while receiving zidovudine therapy. In contrast, four adults who were viremic on initial evaluation and did not receive antiviral therapy remained viremic on subsequent assessments with an initial geometric mean titer of  $10^{4.0}$  versus a mean follow-up titer of  $10^{3.5}$  TCID (mean duration of follow-up 18 weeks; range 1 to 32 weeks). HIV-1 p24 antigen was detectable in 10 patients (8 adults and 2 children) before initiation of zidovudine therapy and decreased in 8 of them during therapy with mean values falling from 166 pg/ml to 117 pg/ml (mean follow-up 21 weeks, range 6-40 weeks).

We also determined the level of cell-free infectious virus in plasma samples in four subjects with acute HIV-1 infection. Acute HIV-1 infection (CDC stage I) represents a dynamic period during which high level viral replication and widespread viral dissemination occur. These virologic events frequently occur in association with severe clinical symptoms (Table 4) and signs of immune activation and are followed by prompt resolution of plasma viremia, antigenemia, and clinical symptoms. In our studies, we determined the titers and biological properties of infectious HIV-1 in plasma during primary, symptomatic infection in three patients and studied the relation of these factors to p24 antigenemia, seroconversion, and clinical course. We also performed molecular analyses of HIV-1 proviruses from an acutely infected person and his sexual partner, and we documented that sexual transmission and the resulting acute, symptomatic infection are associated with high-level expression of

replication-competent, cytopathic virus. Finally, we employed polymerase chain reaction (PCR) amplification of HIV-1 DNA sequences from uncultured peripheral blood mononuclear cells (PBMC) to show that acute HIV-1 infection is associated with high level replication of a relatively homogeneous population of virus which then evolves over time into the quasispecies mixture of viruses characteristic of established HIV-1 infection.

Figure 4 demonstrates the seroconversion profiles of four acutely infected individuals. Patients #1-#3 correspond to the same patients whose antigenemia and viremia titers are profiled in Figure 5. In all patients, the peak plasma virus titers were observed during the earliest, most symptomatic phase of acute illness, approximately 6 to 15 days after the onset of symptoms and at a time when all serological assays for HIV-1 antibodies were negative. HIV-1 specific seroreactivity as determined by ELISA first became positive (ratio of sample/cutoff for normal control > 1.0) between 11 and 20 days after the onset of symptoms and specific reactivity to HIV-1 *gag* (p24) and *env* (gp160) proteins on Western immunoblot was first detectable at 9 to 24 days. Broader reactivity to additional viral proteins followed, along with an increase in total antibody response reflected in rising absorption titers on ELISA over the course of the study.

HIV-1 TCID titers in plasma ranged from  $10^1$  to  $10^3$  TCID per milliliter initially. In a fourth patient (SUMA), peak titers were  $10^4$  TCID/ml. In three of the patients, plasma virus titers decreased rapidly within 8 to 14 days at the same time that a strong HIV-1 antibody response appeared. These patients had no detectable plasma viremia 27 and 14 days after the onset of symptoms. Patient 2, (INME), on the other



hand, did not have complete resolution of plasma viremia until 75 days after onset of symptoms. Of the three patients studied, this patient had the latest appearance of serologic reactivity to *gag* and *env* proteins. Once plasma viremia resolved, it was not again detected in any of the four patients in cultures performed over the next one to six months. In all four patients, levels of HIV-1 p24 antigen closely paralleled the level of virus in plasma ( $r = 0.73$ ,  $p < 0.0001$ ), and both were inversely correlated with anti-HIV-1 ELISA reactivity ( $r = -0.72$  p24,  $r = -0.73$  viremia,  $p < 0.001$ ).

HIV-1 was also cultured from PBMCs obtained from patients at the time of initial presentation and subsequently. Virus isolated from the plasma and PBMCs of all three patients with primary infection replicated to high levels and formed syncytia in PBMC cultures, and isolates established productive infections in H9 and Hut 78 after cell-free transmission. Sequential virus isolates from Patient 1 obtained on days 15, 51, and 86, as well as genetically defined virus strains derived by molecular cloning and transfection of HIV-1 proviruses from Patient 1 obtained on day 15, were all highly cytopathic and exhibited replicative and fusogenic properties ( $>10^4$  TCID<sub>50</sub>/ml;  $>10^4$  cpm/ml RT activity;  $>10^4$  pg/ml p24 antigen in culture supernatants) equivalent to the most cytopathic strains of HIV-1 previously described.

The genetic composition of HIV-1 virus that is transmitted sexually is unknown. Therefore, we used PCR amplification of viral DNA from *uncultured* PBMC to characterize envelope V3 loop sequences from an individual with AIDS (patient RIER) who transmitted virus to patient # 1 (WEAU; shown in Figure 4 and 5). A summary of that analysis is depicted in Figure 6. In patient RIER, sequence diversity characteristic of established HIV-1 infection was found. The predominant viral species

present in RIER lymphocytes was the only viral form transmitted to the recipient WEAU. As shown, sequence analysis of 24 viral clones from 5/30/91, 20 clones from 6/21/90, and 22 clones from 1/18/91 demonstrated that acute viral infection is associated with high level replication of a relatively homogeneous population of viruses and that over time virus complexity broadens. Interestingly, few changes evolved and persisted within the V3 loop of HIV-1 in this patient over an eight month period.

Analysis of the genetic and biologic characteristics of the viral strains present in patients RIER and WEAU provided certain insights into the pathogenesis of HIV-1 disease. The restriction-enzyme cleavage patterns of the predominant viruses from both patients were identical, but they differed from those of isolates from unrelated subjects in 48 to 68 percent of the sites mapped. Proof of transmission between these patients was obtained by sequence analysis of the viral envelope (Figure 6). Proviruses molecularly cloned from the initial viral culture of Patient 1 (WEAU) were shown, after transfection into Cos cells and cell-free passage onto H9 cells, to be fully replication-competent and highly cytopathic. These data, along with the finding of high titer plasma viremia in most individuals with symptomatic primary infection, suggest that virus with high replicative potential may be commonly be transmitted by sexual routes in such individuals and accounts for their clinical symptoms and rapid seroconversion usually within one to three months. Other persons may be exposed to smaller amounts of virus, to less virulent viral strains, or even to defective virus, which could explain the reports of prolonged virus-positive, antibody-negative periods in persons with subclinical primary infection.

This study, and two other reports (25,26), were the first to examine in a systematic and quantitative fashion the role of plasma viremia in HIV-1 natural history and pathogenesis. Our report differs from the other reports by inclusion of children exposed to HIV-1 in utero and postpartum, the methodology for plasma virus cultivation, and the resultant data concerning the magnitude of viremia and its relation to CD4 lymphocyte levels and clinical stage in adult and pediatric groups. The principal findings were that HIV-1 plasma titers reached levels as high as  $10^8$  TCID/ml, that viremia was closely associated with advanced disease and low CD4<sup>+</sup> lymphocyte counts in adults and older children but not in perinatally infected infants, and that treatment with zidovudine led to a decrease in plasma virus titers in most patients.

Whereas the overall geometric mean titers of plasma HIV-1 in AIDS patients in all three studies (25, 26) were similar ( $10^{2.5}$  to  $10^{2.8}$  TCID/ml), maximum titers in our study reached  $10^8$  TCID/ml with 4 of 14 viremic patients with AIDS having  $\geq 10^4$  TCID/ml. In studies by Ho (25) and Coombs (26), maximum virus titers in plasma were between  $2 \times 10^4$  and  $5 \times 10^4$ /ml with only 1 of 20 AIDS patients in one study having titers reaching  $10^4$ /ml. Thus, our data indicate that a greater range of quantitative plasma viremia exists in HIV-1 infection and that a substantial proportion of AIDS patients has HIV-1 plasma titers equalling or exceeding  $10^4$ /ml. Another difference between the results of this study and others is the proportion of HIV-1 infected individuals at earlier stages of infection who were found to be plasma viremic. All 54 HIV-1 infected individuals studied by Ho et al. (20) had cell free infectious virus in their plasma regardless of clinical stage or CD4<sup>+</sup> lymphocyte

counts. Coombs, et al. (26) detected plasma viremia was detected in 75 of 92 patients with AIDS, in 32 of 71 patients with advanced ARC, and in 11 of 48 patients with asymptomatic infection . In our study, 15 of 16 AIDS patients were viremic whereas only 4 of 34 ARC patients and none of 19 asymptomatic individuals had detectable viremia. We also identified four adult subjects with acute HIV-1 infection (CDC stage I) and determined each of their cell-free plasma infectious HIV-1 titers to be  $10^1$  -  $10^4$  TCID/ml.

Methodological differences in procedures for establishing plasma cultures may explain the differences in results of between our study and others. Ho et al. (25) centrifuged the blood of HIV-1 infected subjects at high gravitational forces (3000 g) as opposed to our procedure (675 g) to separate plasma from cells and they did not subsequently filter the plasma before cultivation. Lymphocyte-plasma cultures were then washed after 24 hours to remove patient plasma from the cultures so that no plasma was present for the duration of the 4 week culture period. Coombs et al (26) initially diluted the blood of HIV-1 infected subjects 1:1 with saline prior to underlayering with lymphocyte separation medium and centrifugation at 800 g. The saline diluted plasma was then filtered prior to co-culture with PHA-stimulated normal donor lymphocytes in medium containing DEAE dextran. In our study, patient blood was centrifuged at low gravitational forces initially (675 g), and plasma was collected and centrifuged again at 1800 g prior to passage through a  $1.2\ \mu\text{m}$  filter to remove any residual cellular or platelet elements. Plasma dilutions were then added to PHA-stimulated PBMCs and cultured for 5 weeks without washing plasma from the cultures. It is thus possible that in the previously reported studies virus

could be released from damaged cells in the preparation phase or be actually transmitted in platelet- or cell-associated form, whereas, in our procedure, low titer virus could be either lost by filtration or neutralized by the patients' minimally diluted plasma.

In contrast to the close correlations observed between plasma viremia, advanced disease, and low CD4<sup>+</sup> lymphocyte counts in adults, we found all 5 perinatally infected children to be viremic regardless of CD4<sup>+</sup> count, duration of infection, or clinical stage. For example, three of five children (HUJO-C005, MOBG-C004, GRAL-C015) with perinatal HIV-1 infection and plasma viremia had CD4-lymphocytes counts within the normal range for age-matched control subjects (see Table 2 legend) as opposed to eighteen viremic adults all of whom had CD4-lymphocyte counts less than 400/mm<sup>3</sup>. The five viremic children also had much shorter durations of infection ranging from 6 weeks to 3 years and clinical stages ranging from asymptomatic (P1) to AIDS (P2B, C, D1, and D2). In contrast, viremic adults were uniformly symptomatic and infected for more than 3 years. These findings suggest that either adults are immunologically more capable than neonates of controlling HIV-1 replication for prolonged periods or the developing immune system of neonates supports greater viral replication than that of older children and adults. The ability of adults to clear the acute viremic phase of initial HIV-1 infection (2), the demonstration of protection from SIV induced disease in macaques and mangabeys by immunization or prior infection with attenuated viral strains (12), and the more rapid progression of HIV-1 disease in children as compared to adults (29) argues for an important role for the host immune system in suppressing viral replication.

We also examined the relationship between plasma p24 antigen and antibody levels, plasma virus titers, and clinical stage. Plasma viremia was highly predictive of AIDS (14 of 15 adults) and CD4<sup>+</sup> lymphocyte counts less than 200/mm<sup>3</sup> (15 of 16 adults). In contrast, although p24 antigen and antibody levels were positively and negatively related to these clinical parameters, the correlations were weaker by comparison. Like previous workers (25,26), we were able to culture cell-free virus from a significant proportion of patients who were not antigenemic (6 of 24) or who possessed detectable levels of anti-p24 antibody (11 of 21). The strong association of infectious plasma HIV-1 titers with clinical stage and the weaker association of p24 antigen and antibody with either viremia or clinical stage suggest that quantitative plasma HIV-1 cultures may provide an important marker of efficacy in future trials of antiviral therapy. In support of this, we found all 8 viremic patients to have a decrease in mean plasma virus titers from 10<sup>2.9</sup> to 10<sup>0.6</sup> following treatment with zidovudine with 5 patients clearing plasma virus altogether. Ho et al. also studied 7 patients before and after initiation of zidovudine therapy and found a mean decrease in plasma viremia titers of 1600 TCID<sub>50</sub>/ml (25). In further support of the role of zidovudine in reducing plasma viremia, the single AIDS patient in our study who was not viremic had received high-dose (1200 mg/d) zidovudine for over one year at the time of study.

In summary, the results of this study and others now provide direct virologic evidence for a model of HIV-1 natural history and pathogenesis (Figure 7). In adults, following acute HIV-1 infection by sexual or parenteral routes there is an early viremic phase (CDC stage I), followed by a prolonged period lasting years in which

viremia is generally low level or undetectable (CDC stages II and III), followed by a terminal phase of high level viremia that is associated with profound immune deficiency (CDC stage IV). Children infected in utero or perinatally differ from adults in frequently exhibiting persistent viremia and accelerated disease regardless of CD4<sup>+</sup> lymphocyte counts, duration of infection, or clinical stage, most likely as a result of a failure of the immune system to control viral replication. This model is consistent with clinical studies documenting accelerated clinical deterioration in HIV-1 infected neonates compared with adults (29), increased frequency of heterosexual viral transmission to spouses of hemophiliacs with advanced disease (30), and increased frequency of acute retroviral syndrome and progression to AIDS in recipients of HIV-1 infected blood products derived from individuals in advanced stages of illness (31). These data thus argue for the importance of the host immune system in controlling viral replication, and the need for early intervention with effective antiviral agents especially in perinatally acquired infection. They also suggest that certain individuals with high titers of cell-free virus may be at increased risk for transmission of virus, a possibility pointedly raised by the recently described case of apparent transmission of HIV-1 from a health care worker to his patient (32, 33). Finally, the demonstration of high level HIV-1 viremia in vivo, along with evidence that non-infectious HIV-1 virions exceed infectious virions by a thousandfold in tissue culture, suggest that cell free HIV-1 virions, proteins, and antigen-antibody complexes may play a direct role in the natural history and pathogenesis of HIV-1 infection as occurs in infections by equine infectious anemia virus (11).

As a second major aim of this study, we sought to better understand the

genetic and biologic complexity of HIV-1 as it exists *in vivo* as opposed to tissue culture. The premise of this work was that transmission and replication of HIV-1 (and HIV-2) in natural settings may result from complex interactions between replication competent and defective viruses and that the cell tropisms and replication patterns of these viruses *in vivo* may not be reflected fully by tissue culture. Using techniques for DNA extraction, recombinant lambda phage cloning, PCR amplification, DNA sequencing, and DNA transfection that we have already described (4), we sought to obtain full-length proviral clones from *uncultured* human brain.

Patients with AIDS dementia complex (ADC) are known to harbor considerable amounts of HIV-1 sequences within their brain, both in chromosomally integrated as well as unintegrated form (34,35). While both linear and circular viral molecules contain a complete viral genome, viral circles can be cloned in their entirety after linearization with an appropriate restriction enzyme. The abundance of circular viral DNA in the CNS of certain patients with AIDS dementia complex therefore prompted us to attempt molecular cloning of replication-competent HIV-1 genomes directly from this viral population.

Screening several brain DNAs from AIDS dementia patients, we identified one sample to be particularly suitable for lambda phage cloning. This DNA preparation was available in sufficient quantities, exhibited no signs of degradation, and contained enough HIV-1 viral sequences to be visualized by Southern blot analysis. Restriction enzyme mapping with EcoRI and SstI (alone and in combination) confirmed that EcoRI cleaved the provirus only once and indicated that viral circles comprised the majority of HIV-1 sequences within this particular sample (Figure 8). We therefore



constructed a genomic lambda phage library in  $\lambda$  gt10 using EcoRI digested brain DNA fractions 9 to 12 Kb in length (Figure 8). Screening approximately 30 genomic equivalents ( $8 \times 10^6$  lambda phage plaques), we identified ten recombinant clones which hybridized to a probe specific for HIV-1.

Detailed restriction enzyme analysis and Southern blot hybridization studies confirmed the presence of HIV-1 sequences in all ten recombinant clones and determined the length and orientation of their viral inserts (Figure 9). Nine of ten clones represented unintegrated linearized circles which were cloned in permuted form at a single EcoRI site located in the central viral region. The restriction enzyme cleavage pattern of a tenth clone (YU-6) suggested the presence of an integrated provirus (5' half) flanked by normal cellular sequences. Side-by-side comparison with a prototype HIV-1 construct known to possess a complete proviral genome (pHXB2D; 36 and 37) identified four brain clones to comprise full-length HIV-1 inserts with either one (YU-2, YU-10, YU-21) or two (YU-32) tandem copies of the viral LTR. Three clones contained internal deletions (YU-1, YU-5, YU-27), while two other clones (YU-3, YU-4) exhibited genomic rearrangements which were consistent with the presence of an extraneous LTR in reverse orientation.

Nucleotide sequence analysis was performed to further characterize all clones in regions which could not be definitively mapped by restriction enzyme analysis alone. Internal deletions of varying length were identified in three molecular clones (Figure 9 and 10). YU-5 contained a 3044bp deletion in the 3' half of its genome, which included portions of *tat* and *rev*, the entire *vif* and *env* gene, as well as the N-terminal half of *nef*. YU-27 was characterized by a less extensive deletion (549 bp)

which included the C-terminus of the *env* gene and the N-terminus of the *nef* gene. Yu-1 contained a 555 bp deletion in its *gag* gene which involved the entire p17 and a portion of the p24 protein, but did not include sequences 5' of *gag* such as the viral packaging signal. None of the deleted fragments extended to the U3 or U5 region of the viral LTR. The EcoRI cloning site was mapped within the *vpr* gene (position 5742 of the HXB2D sequence; 36).

Integration of most retroviruses involves the removal of 2 base pairs from the termini of the unintegrated linear DNA precursor, as well as a duplication of the target sequence at the insertion site (38-40). Viral circles with two LTRs generally contain those nucleotides which are lost during the integration process within their circle junction since they result from blunt-end ligation of unintegrated linear molecules. To examine whether HIV-1 proviral integration in primary tissue involved similar mechanisms, we sequenced the LTR-LTR circle junction in clone YU-32 (Figure 10). The results demonstrated the presence of four additional nucleotides between the conserved CA and TG dinucleotide that generally delineate the boundaries of integrated HIV-1 proviruses (..CA-GTAC-TG..). These data thus indicated that linear HIV-1 molecules contain two dinucleotides at each terminus which are lost before or during recombination with the host genome *in vivo*.

Nucleotide sequence analysis was also required to further characterize the nature of viral and non-viral sequences in clone YU-6. Restriction enzyme analysis indicated several enzyme site differences 5' of the YU-6 LTR which distinguished this phage clone from all other brain derived constructs. Subgenomic probes of HIV-1 failed to detect envelope sequences 5' of the LTR, whereas this same region

hybridized to nick-translated normal human PBMC DNA thus indicating the presence of human repetitive sequences (data not shown). Nucleotide sequence analysis of the LTR junction fragment of YU-6 identified the expected boundaries between an integrated HIV-1 provirus and adjoining cellular sequences (Figure 10).

Detailed restriction enzyme analysis identified two lambda phage clones (YU-3, YU-4) to exhibit extensive genomic rearrangements in the 5' halves of their genomes. Further characterization revealed that both clones contained extraneous HIV-1 LTR sequences in reverse orientation with respect to the remainder of the viral genome. To analyze more precisely the nature of these inverted LTR fragments and to elucidate the mechanisms by which they were generated, we determined their nucleotide sequences as well as the sequences of their insertion sites (Figure 11).

Sequence analysis revealed that both HIV-1 genomes contained inverted LTR sequences within their pol genes. YU-4 contained an inverted LTR which was inserted in its entirety without additional deletions or rearrangements of the target sequence except for the presence of a 5bp direct repeat (AATAC) directly adjacent to the insertion site. The boundaries of the inverted LTR itself were identical to those of an integrated HIV-1 provirus (5'-TG...AC-3'). This fact, as well as the observed duplication of sequences (AATAC) normally present only once in this region of the HIV-1 genome, indicated that the inverted LTR in YU-4 was the result of an autointegration event. Inverted LTR sequences were also identified in the pol gene of YU-3. However, this inversion was associated with deletions involving both LTR and pol sequences. The LTR itself lacked 6bp on its 3' and 3bp on its 5' end, while a major portion of the pol gene (1197bp) was also missing. No direct repeats were

identified in the vicinity of the insertion site, and there were no additional deletions or alterations within the inverted LTR. Because of the absence of the characteristic target sequence duplication, the mechanisms responsible for the genomic rearrangements in YU-3 are less clear. Interestingly, genomic inversions in both YU-3 and YU-4 involved solely LTR sequences, and did not include adjacent viral sequences as has been observed in other retroviral systems. Also, there was no evidence for consensus sequences for target site recognition in regions adjacent to both inverted LTRs.

Current data regarding the genetic heterogeneity of HIV-1 have been derived from studies of virus cultures and from direct PCR amplification of primary, uncultured tissues (4, 37, 41-44). Important differences in results from these two approaches include a lesser degree of HIV-1 genetic variation *in vitro* and a surprisingly high proportion of defective viral genomes *in vivo* (44). The availability of both recombinant lambda and PCR derived molecular clones of HIV-1 from the same brain specimen allowed us to directly compare HIV-1 variability by these two approaches. Moreover, the PCR analyses served to independently confirm the origin and authenticity of the lambda phage clones. A 510bp fragment containing the V3 loop and adjacent envelope sequences was amplified by single round PCR, subcloned into M13, and subjected to nucleotide sequence analysis. A total of 12 independent M13 clones as well as eight lambda phage clones were sequenced in the same envelope region. An alignment of their sequences is depicted in Figure 12.

Comparing a total of 20 independent envelope sequences, we identified one predominant viral genotype as well as eleven minor viral variants. Importantly, the

nucleotide sequence of the predominant genotype was identical both for lambda phage as well as PCR derived clones, thus proving unequivocally their common origin. Overall genotypic variability in 19 of 20 clones was 1.2% or less. Only one clone exhibited 8.2% nucleotide sequence differences. Lambda phage clones comprised a homogeneous group of viruses, with five clones having an identical nucleotide sequence and three others differing by only one or two point mutations (0.2% to 0.4% nucleotide sequence differences). PCR derived sequences varied more, both with respect to the number of nucleotide sequence substitutions between individual clones (0.2% to 8.2% nucleotide sequence differences) as well as with respect to the total number of distinguishable variants (8 of 12 versus 3 of 8 for lambda phage clones). In addition, only PCR derived sequences contained single base pair deletions as well as frequent G to A transitions. One PCR clone was characterized by a particularly large number of G to A changes which comprised 40 out of 42 total nucleotide substitutions.

All 12 distinguishable HIV-1 genotypes were also unique in their deduced amino acid sequence, indicating that the majority of nucleotide changes were non-silent in nature. Three of eight lambda phage clones differed from the predominant genotype by only one or two amino acid sequence changes, none of which interrupted the envelope open reading frame. Three of eight PCR derived clones varied from the predominant strain by a similar number of substitutions, while five other PCR clones contained frame shift mutations and/or in frame stop codons. This indicated that at least 42% of the PCR derived clones would correspond to defective viral genomes.

To investigate whether any of the four full-length HIV-1 genomes were replication competent, we isolated viral inserts from YU-2, YU-10, YU-21 and YU-32, self-ligated these inserts to generate viral genomes in nonpermuted orientation, and transfected them into Cos-1 cells. Primary transfectants were subsequently co-cultivated with PHA-stimulated normal donor PBMCs or immortalized T-cell lines in an attempt to transmit replication competent virus. Cultures were inspected daily for the appearance of virally-induced syncytia and monitored for retroviral activity by p24 antigen capture and reverse transcriptase assays. Virus positive culture supernatants were filtered and transmitted to additional target cells to determine cell free infectivity and host cell range.

The results of these studies (Figure 13) demonstrated that only one of the four self-ligated inserts (YU-2) reproducibly yielded a productive viral infection in primary lymphocytes, monocytes and Molt4(clone8) cells (4, 45). YU-2 derived virus was also cell-free transmissible to these cell types, but failed to replicate in SupT1 and CEMx174 cells. Cos-1 cells transfected with self-ligated YU-10 insert produced p24 transiently and formed syncytia after cocultivation. However, repeated attempts to establish a productive infection in primary PBMC or immortalized T-cell lines failed, suggesting a defect in one of the major viral proteins of YU-10. Self-ligated inserts of YU-21 and YU-32 failed to yield virus in all cell types tested including primary transfected Cos-1 cells.

To improve the transfection efficiency, we reconstructed two of the four full-length lambda phage clones (YU-2 and YU-10) to generate viral genomes in nonpermuted orientation. This was achieved by subcloning a SalI/EcoRI fragment

(containing the 5' half of the virus) and a EcoRI/SphI fragment (containing the 3' half of the virus) into the same plasmid vector (Figure 13). Subsequent transfection experiments confirmed the results utilizing self-ligated lambda phage inserts. pYU-2 derived virus replicated well in primary lymphocytes and monocytes and induced syncytia when cocultivated with Molt4(clone8) cells (Figure 13). Electron microscopy demonstrated normal particle morphology, and Western blot analysis confirmed the presence and appropriate size of all virus specific proteins. Importantly, pYU-2 derived virus infected primary macrophages and replicated to high titers, exhibiting growth characteristics similar to those previously identified for macrophage-tropic HIV-1 strains (4, 45, 46). Transfection of pYU-10 failed to result in a productive viral infection even in the nonpermuted plasmid construct. This strongly suggested that YU-10 was replication defective, although certain viral genes, including the gag and the env gene, appeared to be functional since transient p24 production and syncytium formation was observed. Thus, the results of this analysis revealed for the first time that replication competent HIV-1 genomes, complex mixtures of defective viral forms, and chromosomally integrated provirus persist *in vivo*. In addition, the brain-derived viral clones were expected to prove valuable for future studies of macrophage and neurotropism, as well as for the analysis of other viral properties which are subject to *in vitro* selection pressures.

The final aim addressed during the first half of the contract period was to use PCR amplification and "universal" (consensus) oligonucleotide primer sequences as an approach to identify, clone, and characterize divergent or novel human retrovirus in high risk populations. Current understanding of the biology and origins of human

immunodeficiency virus type 2 (HIV-2) has derived from studies of cultured isolates from urban populations experiencing epidemic infection and disease (46-53). To test the hypothesis that such isolates might represent only a subset of a larger, genetically more diverse group of viruses, we used nested polymerase chain reaction (PCR) to characterize HIV-2 sequences in uncultured blood mononuclear cells of two healthy Liberian agricultural workers (F0784; 2238) from whom virus isolation was repeatedly unsuccessful and from a culture-positive symptomatic urban dweller (7312A). Previously, we had conducted a seroepidemiological survey in Liberia, West Africa to estimate the prevalence of HIV-1 and HIV-2. Serum samples were collected from 372 healthy adults living in remote villages of northern Liberia, 944 rubber plantation workers attending outpatient clinics in rural areas of central Liberia, and 366 adults from the capital city of Monrovia and surrounding urban areas. Three individuals were seropositive for HIV-1. Five other individuals, all healthy inhabitants of rural villages, were seropositive for HIV-2. Two of these individuals (F0784 and 2238) could be relocated for additional serologic testing, virus culture, and PCR analysis. Both were male rubber plantation workers, ages 46 and 47, with normal physical examinations, no history of sexually transmitted disease, chronic illness, blood transfusion, or homosexuality. For comparison, a third HIV-2 seropositive but symptomatic man from Abidjan, Côte d'Ivoire was also studied. He was 32 years old, had lymphadenopathy, cutaneous anergy and recurrent skin abscesses, and admitted to frequent sexual encounters with urban prostitutes. Blood specimens from all three subjects were obtained on two separate occasions and processed for virus isolation and PCR analysis (54,55). Mononuclear cells (PBMCs) were cultured alone,



in combination with normal donor lymphocytes and macrophages, and with immortalized T-cell lines (Molt4 clone8, CEMx174, H9, SupT1). HIV-2 was successfully isolated from subject 7312A on each of two occasions but not from subjects F0784 and 2238, despite excellent cell growth and viability.

To determine the genetic identity of viral sequences in subjects F0784, 2238, and 7312A, we employed a highly-sensitive nested PCR technique to amplify viral sequences directly from uncultured PBMC DNA (54,55). Using primer pairs designed according to HIV-2/SIV<sub>MAC</sub>/SIV<sub>SM</sub> consensus sequences (56), 708bp *pol* and 453bp *env* fragments were amplified (Figure 14) and sequences corresponding to a total 34,770 nucleotides determined. This analysis revealed viral mixtures, or quasispecies, of varying complexity for each subject (Figure 15). F0784 harbored the largest number of variants, most of which represented defective (prematurely terminated) viral genomes which resulted from G to A hypermutation. Such G to A changes, which were found in two different genes (*pol* and *env*) and in blood samples obtained four months apart, accounted for 66% to 87% of all nucleotide substitutions in F0784 and resulted in sequence differences among individual clones as high as 11.5%. *Env* and *pol* regions from 2238 and 7312A exhibited much less intrastrain variability (< 0.3%) and no G to A hypermutation, which together with our previous studies of SIV<sub>AGM</sub> (54), indicated that *Taq* polymerase or sequencing errors did not contribute significantly to the G to A changes observed in F0784.

To determine the phylogenetic relationships of HIV-2<sub>F0784</sub>, HIV-2<sub>2238</sub>, HIV-2<sub>7312A</sub>, and other HIV-2/SIV<sub>SM</sub>/SIV<sub>MAC</sub> strains, we constructed evolutionary trees for their *pol* and *env* sequences using both the neighbor-joining approach (58,59) (a reliable method

which allows for unequal rates of evolution) and the maximum parsimony algorithm of PAUP (60). In both *pol* (Figure 16A) and *env* (Figure 16B) regions, seven previously reported prototype isolates of HIV-2 clustered in a closely related group. In contrast, the three strains reported here branched quite differently. HIV-2<sub>2238</sub> was most closely related to a single divergent HIV-2 isolate, HIV-2<sub>D205</sub> (52), although these two viruses differed from each other to a greater extent than was typical for prototypic HIV-2 strains. HIV-2<sub>7312A</sub> appeared to have a mosaic genome: its *pol* sequence was most closely related to HIV-2<sub>D205</sub> (Figure 16A), yet its *env* sequence clustered with the prototypic HIV-2 isolates (Figure 16B). These discordant relationships for HIV-2<sub>7312A</sub> *pol* and *env* regions were strongly supported by bootstrap analyses (61) and most likely reflect recombination between phylogenetically divergent strains.

The most striking finding from the phylogenetic analysis was that HIV-2<sub>F0784</sub> *pol* and *env* sequences clustered with the simian viruses, SIV<sub>SM</sub> and SIV<sub>MAC</sub>, rather than with other HIV-2 strains. This relationship was found in the majority, though less than 95%, of bootstrap resamples, and so to substantiate the phylogenetic position of HIV-2<sub>F0784</sub> we amplified and analyzed two additional regions of its genome: a much larger 1972bp *pol* fragment encoding reverse transcriptase (RT) and a 717bp *nef*/LTR fragment (Figure 14). Phylogenetic analysis of the RT fragment (Figure 16C) demonstrated clustering of HIV-2<sub>F0784</sub> with the SIV lineage in more than 99.9% of bootstrap resamples. The *nef*/LTR region of HIV-2<sub>F0784</sub> chosen for analysis because it encompasses a 40-44bp "signature" sequence that is present in all published clones of HIV-2 (including HIV-2<sub>D205</sub>) but absent from all SIV<sub>SM</sub>/SIV<sub>MAC</sub> genomes (56), lacked

this sequence (Figure 17) which therefore no longer distinguishes between viruses that infect man and monkeys. Overall, our phylogenetic analyses thus indicated that HIV-2 in man and SIV in mangabeys and captive macaques represent genetically-diverse members of a *single* group of viruses.

For several reasons, we consider it most likely that the natural reservoir for the entire group of HIV-2-related viruses is the sooty mangabey and that HIV-2 infection of man represents a zoonosis (62). First, SIV<sub>SM</sub> is apathogenic in sooty mangabeys (63,64) but is pathogenic in man and macaques (65, 67), as is the case for many zoonotic infections which cause less severe or even no disease in their natural hosts (62). Second, the natural habitat of the sooty mangabey generally coincides with the geographic pattern of HIV-2 endemicity in West Africa (64). Third, close contact between mangabeys and man and between mangabeys and captive macaques is well-documented (64,67,68). These observations, together with phylogenetic data showing a close overall genetic relationship between HIV-2 and SIV<sub>SM</sub> (68) yet deep and roughly equidistantly branching lineages for the different subgroups (Figure 16), argue for multiple introductions of genetically-diverse SIV<sub>SM</sub> viruses into different human and simian populations.

It is notable that most previously studied isolates of HIV-2 were obtained by virus culture from individuals who were generally symptomatic and resided in areas where HIV-2 was spreading epidemically (46-53). In contrast, HIV-2<sub>F0784</sub> and HIV-2<sub>2238</sub> could not be cultured (despite several attempts) from healthy inhabitants of rural areas where HIV seroprevalence was low and AIDS had not been recognized clinically. This raises the possibility that certain naturally-occurring strains of HIV-

2 may replicate less efficiently and cause less virulent infection. In this regard, it is of interest that the majority of HIV-2 sequences from subject FO784 contained multiply-defective genomes and an inordinate number of G to A substitutions. Biased G to A hypermutation has previously been described for HIV-1 (69), but not to the extent found in HIV-2<sub>FO784</sub>. Studies are underway to determine the molecular basis for G to A hypermutation and whether, in the extreme case, it could result in attenuated or even abortive viral infection.

Finally, a recent suggestion (70) that SIV<sub>SM</sub> may have been accidentally transmitted to man by inoculation with infected monkey blood probably cannot explain the diversity now recognized for HIV-2. Results of our studies suggest that viruses from feral monkey populations and humans living in remote areas of Africa should be targeted in a search for the origins of simian and human immunodeficiency viruses and the events leading to their recent epidemic spread.

## Conclusions

In the first half of the contract period of project DAMD17-90-C-0064, we made substantial progress toward achieving its goals. We showed for the first time, the dynamic changes in viral replication that occur during the natural course of HIV-1 infection from acute (CDC stage I) through chronic (CDC stages II-IV) disease; characterized the remarkable changes in genotypic complexity that occur during sexual transmission of HIV-1 and the subsequent establishment of viral persistence; obtained the first full-length replication competent HIV-1 proviral clones from *uncultured* human tissue and used these to determine the molecular characteristics of HIV-1 *in vivo*,

including viral determinants of cell tropism; developed a novel PCR approach for amplifying and cloning divergent HIV strains from uncultured human tissues; and discovered a strain HIV-2 in West African people that is genetically and phylogenetically indistinguishable from SIV<sub>SM</sub> from sooty mangabeys thereby providing direct evidence for HIV-2 as a zoonotic infection of man. Each of these accomplishments represent specific aims of the current contract. In the second half of the contract period, we will pursue these studies to their completion. In addition to these studies, we also proposed to develop quantitative assays of HIV-1 RNA and DNA in order to characterize viral replication and load as determinants of pathogenesis. We will continue to pursue this aim with high priority and with an overall long-range goal of defining the molecular basis of HIV-1 and HIV-2 pathogenesis.

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## Appendix



**Table 1.** Human immunodeficiency virus (HIV) type 1 plasma titers and clinical characteristics in adults.

| Virus titer,<br>patient | Date of<br>evaluation<br>(1989) | CDC<br>stage | CD4<br>cells/mm <sup>3</sup> | HIV p24           |                    | Therapy |
|-------------------------|---------------------------------|--------------|------------------------------|-------------------|--------------------|---------|
|                         |                                 |              |                              | Antibody<br>titer | Antigen<br>(pg/ml) |         |
| No growth (negative)    |                                 |              |                              |                   |                    |         |
| EDRI-0025               | 01/19                           | II           | 1904                         | 88                | 26                 | D       |
| RIPH-0179               | 08/24                           | II           | 1080                         | 0                 | 0                  | N       |
| MDJO-0252               | 07/20                           | II           | 996                          | QNS               | 0                  | N       |
| GICL-0061               | 05/10                           | IVC2         | 798                          | 0                 | 37                 | N       |
| ATKA-0381               | 12/28                           | II           | 760                          | 414               | 0                  | N       |
| HOJU-0143               | 06/28                           | II           | 720                          | 14,763            | 0                  | N       |
| WHMA-0128               | 09/14                           | II           | 713                          | QNS               | 0                  | Z       |
| VAST-0181               | 07/20                           | IVC2         | 710                          | 1                 | 0                  | Z       |
| MAMA-0341               | 10/25                           | II           | 705                          | 64                | 0                  | N       |
| ADFR-0194               | 05/10                           | II           | 703                          | 387               | 0                  | N       |
| BECH-0171               | 03/09                           | II           | 678                          | 596               | 0                  | N       |
| MALE-0264               | 07/26                           | III          | 644                          | 3138              | 0                  | N       |
| ROJO-0331               | 11/29                           | II           | 640                          | 574               | 0                  | N       |
| BAMA-0037               | 03/22                           | II           | 624                          | 0                 | 0                  | N       |
| ADDI-0101               | 03/15                           | II           | 616                          | 1709              | 0                  | N       |
| ALFR-0229               | 08/23                           | IVC2         | 615                          | 390,625           | 0                  | N       |
| CADA-0309               | 11/08                           | IVC2         | 595                          | 872               | 0                  | N       |
| KEBO-0042               | 06/21                           | II           | 594                          | 20                | 0                  | N       |
| ERBR-0339               | 10/25                           | II           | 589                          | 6                 | 0                  | N       |
| GIRO-0137               | 10/11                           | II           | 585                          | 290               | 0                  | N       |
| WIMI-0366               | 10/31                           | II           | 572                          | 9092              | 0                  | N       |
| CADA-0024               | 05/17                           | IVC2         | 529                          | 1                 | 0                  | N       |
| SIWE-0201               | 05/03                           | II           | 528                          | 22                | 0                  | N       |
| BRST-0214               | 05/17                           | III          | 522                          | 98                | 0                  | N       |
| DALE-0069               | 12/21                           | III          | 520                          | 1                 | 72                 | N       |
| COBI-0232               | 05/31                           | III          | 504                          | 8243              | 0                  | N       |
| PORA-0355               | 11/01                           | III          | 483                          | 0                 | 32                 | N       |
| FOMA-0117               | 03/08                           | IVC2         | 476                          | 0                 | 10                 | N       |
| HUAL-0211               | 05/03                           | III          | 468                          | 758               | 0                  | N       |
| SCRI-0023               | 03/01                           | II           | 462                          | 259               | 0                  | N       |
| AXBO-0085               | 02/16                           | IVC2         | 441                          | 0                 | 0                  | N       |
| SMMA-0222               | 05/17                           | IVC2         | 440                          | 900               | 0                  | N       |
| LIWE-0187               | 09/27                           | IVC2         | 435                          | 17                | 0                  | N       |
| JAJO-0083               | 05/24                           | II           | 432                          | 41,311            | 0                  | N       |
| RYJE-0273               | 08/23                           | III          | 420                          | 0                 | 36                 | N       |
| KOFL-0311               | 11/15                           | III          | 396                          | 359               | 0                  | N       |
| CASH-0292               | 10/04                           | IVC2         | 378                          | 1                 | 6                  | N       |
| JUJA-0156               | 05/18                           | III          | 360                          | 2889              | 0                  | H       |
| HATI-0385               | 12/28                           | IVC2         | 341                          | 6402              | 0                  | N       |
| WAFR-0077               | 10/04                           | IVC2         | 312                          | 65                | 0                  | N       |
| NADA-0234               | 10/03                           | III          | 311                          | 46                | 0                  | N       |
| PAAL-0266               | 09/06                           | IVC2         | 273                          | 2506              | 0                  | N       |
| Hoke-336                | 11/29                           | IVC2         | 240                          | 0                 | 62                 | N       |
| MMRA-0007               | 01/26                           | IVC1         | 239                          | 51                | 51                 | Z       |
| SATI-0088               | 04/04                           | IVC2         | 234                          | 15,135            | 0                  | N       |
| STDA-0183               | 07/27                           | IVC2         | 234                          | 390,347           | 0                  | Z       |
| PLJO-0293               | 10/18                           | IVC2         | 228                          | 270               | 0                  | N       |
| REJA-0225               | 07/06                           | IVC2         | 220                          | 358               | 0                  | N       |
| CHPA-0351               | 11/21                           | IVC2         | 220                          | 1                 | 0                  | N       |
| DAAD-0212               | 09/06                           | IVC2         | 78                           | 1                 | 9                  | N       |
| 10 <sup>1</sup> TCID/ml |                                 |              |                              |                   |                    |         |
| COWI-0019               | 03/29                           | IVC1         | 5                            | 16                | 0                  | N       |

## HIV Viremia in Adults and Children

Table 1. Continued.

| Virus titer,<br>patient | Date of<br>evaluation<br>(1989) | CDC<br>stage | CD4<br>cells/mm <sup>3</sup> | HIV p24           |                    | Therapy |
|-------------------------|---------------------------------|--------------|------------------------------|-------------------|--------------------|---------|
|                         |                                 |              |                              | Antibody<br>titer | Antigen<br>(pg/ml) |         |
| 10 <sup>2</sup> TCID/ml |                                 |              |                              |                   |                    |         |
| ISBU-0089               | 03/23                           | IVAB         | 3                            | 0                 | 31                 | N       |
| GIRO-0005               | 02/02                           | IVC1         | 9                            | 0                 | 14                 | Z       |
| REMI-0022               | 03/15                           | IVD          | 11                           | 29                | 0                  | N       |
| TIMI-0018               | 03/02                           | IVC1         | 14                           | 0                 | 29                 | N       |
| HADO-0110               | 05/11                           | IVC2         | 20                           | 5                 | 21                 | N       |
| GOJU-0183               | 03/29                           | IVC1         | 23                           | 500               | 0                  | N       |
| LUDA-0153               | 03/22                           | IVC1         | 71                           | 0                 | 74                 | N       |
| WERI-0233               | 06/28                           | IVC2         | 286                          | 0                 | 30                 | N       |
| MCSE-0176               | 05/24                           | IVC2         | 360                          | 1                 | 960                | N       |
| 10 <sup>3</sup> TCID/ml |                                 |              |                              |                   |                    |         |
| NAPH-0073               | 03/16                           | IVC1         | 14                           | 0                 | 70                 | N       |
| LAJO-0060               | 04/04                           | IVC1         | 19                           | 108               | 3                  | Z       |
| WIAL-0014               | 01/26                           | IVC1A        | 20                           | 0                 | 40                 | N       |
| BIJA-0205               | 05/11                           | IVC1         | 32                           | 7                 | 770                | N       |
| 10 <sup>4</sup> TCID/ml |                                 |              |                              |                   |                    |         |
| DEDA-0006               | 03/15                           | IVC1         | 57                           | 1                 | 0                  | N       |
| SMDO-0157               | 03/02                           | IVC2         | 282                          | 1                 | 250                | N       |
| 10 <sup>5</sup> TCID/ml |                                 |              |                              |                   |                    |         |
| DODO-0116               | 01/19                           | IVC1         | 50                           | 0                 | 98                 | N       |
| 10 <sup>8</sup> TCID/ml |                                 |              |                              |                   |                    |         |
| JOJI-0070*              | 02/10                           | IVC1         | 4                            | 0                 | 250                | N       |

NOTE. Virus titer = quantitative plasma virus titer log base 10 (TCID/ml of plasma); CDC = Centers for Disease Control (stage II, asymptomatic; III and IVC2, AIDS-related complex; IVAB, IVC1, and IVD = AIDS); therapy = antiviral therapy at time of evaluation (N = none, Z = zidovudine, H = hypericin, D = ditiocarb). QNS = quantity not sufficient for analysis.

**Table 2.** Human immunodeficiency virus (HIV) type 1 plasma titers and clinical characteristics in children.

| Virus titer,<br>patient | Date of<br>evaluation<br>(1989) | Age at<br>evaluation | Risk | CDC<br>stage | CD4<br>cell/mm <sup>3*</sup> | HIV p24           |                    |
|-------------------------|---------------------------------|----------------------|------|--------------|------------------------------|-------------------|--------------------|
|                         |                                 |                      |      |              |                              | Antibody<br>titer | Antigen<br>(pg/ml) |
| No growth (negative)    |                                 |                      |      |              |                              |                   |                    |
| WIBB-C013               | 10/31                           | 1 day                | P    | P0           | 1025                         | 0                 | 0                  |
| CADE-C009               | 09/20                           | 8 months             | P    | P0           | 536                          | 0                 | 0                  |
| JERO-C012               | 10/18                           | 18 months            | P    | P0           | 2848                         | 0                 | 0                  |
| MIDA-C014               | 12/06                           | 18 months            | P    | P0           | 904                          | 0                 | 0                  |
| BRRO-C011               | 10/11                           | 2 years              | P    | P0           | 1355                         | 0                 | 0                  |
| CACH-C016               | 12/20                           | 9 years              | H    | P2A          | 359                          | 8096              | 0                  |
| LACL-C008               | 10/18                           | 11 years             | H    | P1B          | 407                          | 3970              | 1.0                |
| WOME-C242               | 06/28                           | 14 years             | H    | IVC2         | 228                          | 1                 | 28.6               |
| STDA-C007               | 07/13                           | 5.5 years            | T    | P2F          | 180                          | 0                 | 71.2               |
| 10 <sup>1</sup> TCID/ml |                                 |                      |      |              |                              |                   |                    |
| GRAL-C015               | 12/13                           | 3 years              | P    | P2D2F        | 1628                         | 9                 | 0                  |
| 10 <sup>3</sup> TCID/ml |                                 |                      |      |              |                              |                   |                    |
| HUJO-C005†              | 08/02                           | 6 weeks              | P    | P1A          | 2227                         | 822               | 0                  |
| MUWI-C003               | 05/25                           | 6 months             | P    | P2D1         | 150                          | QNS               | 345.0              |
| MOBG-C004               | 06/21                           | 10 months            | P    | P2F          | 572                          | QNS               | 6.4                |
| WHRO-C002               | 06/21                           | 14 months            | P    | P2ABD3       | 42                           | 0                 | 54.0               |

NOTE. \*Virus titer = quantitative plasma virus titer log base 10 (TCID<sub>50</sub>/ml of plasma); risk = mode of infection or exposure (P = perinatal, T = transfusion recipient, H = hemophiliac); CDC = Centers for Disease control classification of HIV-1 infection in children <13 years [22]; therapy = antiviral therapy at time of evaluation; QNS = quantity not sufficient for analysis.

\* Normal ranges (mean  $\pm$  2 SD) for CD4-lymphocyte numbers in children 0-6 months, 3166  $\pm$  2100/mm<sup>3</sup> (n = 106); 6-12 months, 2828  $\pm$  2300 (n = 28); 12-24 months, 2306  $\pm$  1890 (n = 46); 24-74 months, 1688  $\pm$  1190 (n = 29); adults, 1027  $\pm$  770 (n = 327).

<sup>†</sup> Treated with zidovudine. All other children were untreated.

**Table 3.** Sequential and replicated plasma human immunodeficiency virus (HIV) titer determinations.

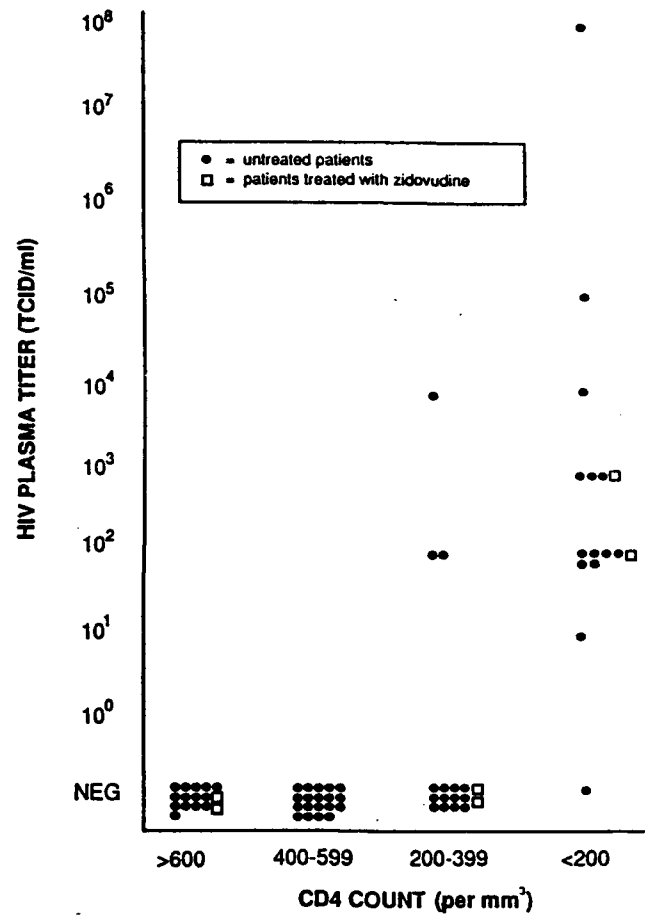
| Patient   | Date of evaluation (1989) | Normal PBL donor | HIV p24 antigen level (pg/ml) | Plasma virus titer |
|-----------|---------------------------|------------------|-------------------------------|--------------------|
| BIJA 0205 | 05/11                     | D1               | 770                           | 3                  |
|           | 05/23                     | D2               | 75                            | 2                  |
|           | 05/23                     | D3               |                               | 3                  |
|           | 05/23                     | D4a              |                               | 3                  |
|           | 05/23                     | D4b              |                               | 3                  |
|           | 05/23                     | D4c              |                               | 3                  |
|           | 12/14                     | D5               | 293                           | 4                  |
|           | 02/01                     | D6               | 49                            | 5                  |
| DODO 0116 | 02/08                     | D7               | 98                            | 3                  |
|           | 03/02                     | D8               | 250                           | 4                  |
| SMDO 0157 | 08/15                     | D9               | 998                           | 4                  |
|           | 03/15                     | D10              | 0                             | 4                  |
| DEDA 0006 | 05/31                     | D11              | 0                             | 3                  |
|           | 06/21                     | D12              | 54                            | 3                  |
| WHRO C002 | 11/15                     | D13              | 165                           | 2                  |
|           | 03/16                     | D14              | 70                            | 3                  |
| NAPH 0073 | 07/11                     | D15              | 27                            | 2                  |
|           | 07/11                     | D16              |                               | 1                  |
|           | 07/11                     | D17a             |                               | 2                  |
|           | 07/11                     | D17b             |                               | 2                  |
|           | 07/11                     | D17c             |                               | 2                  |

NOTE. Normal peripheral blood lymphocytes (PBL) D1-D17 are from 17 different normal donors; D4a, D4b, D4c and D17a, D17b, and D17c are PBL from individual donors that were cultured in triplicate sets of wells. Plasma virus titer = titer of HIV type 1 cultured from plasma (log base 10; TCID<sub>50</sub>/ml of plasma). No subject except NAPH received antiviral therapy.

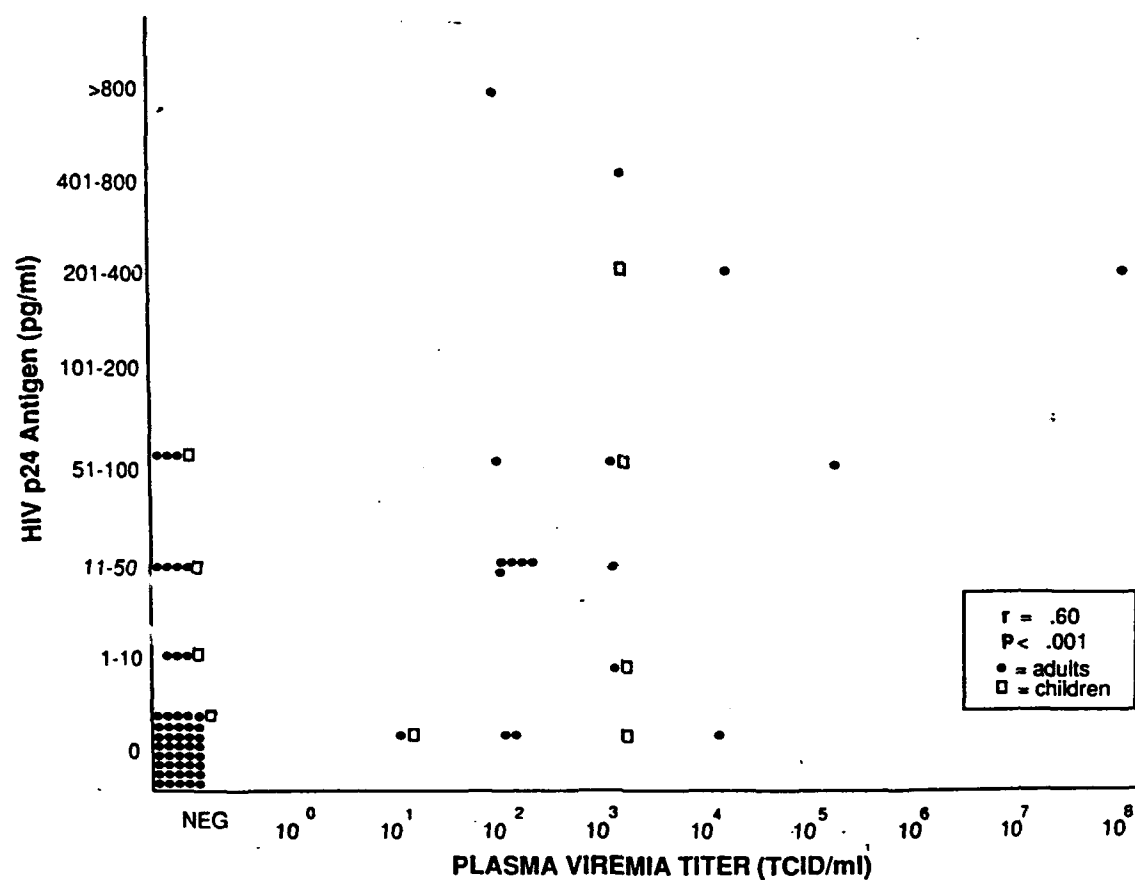
**TABLE 4: Signs and symptoms of acute HIV-1 infection.**

| <b>SIGN OR SYMPTOM</b>                 | <b>FREQUENCY (%)</b> |
|----------------------------------------|----------------------|
| Fever                                  | 97                   |
| Adenopathy                             | 77                   |
| Pharyngitis                            | 73                   |
| Rash                                   | 70                   |
| Myalgia or arthralgia                  | 58                   |
| Thrombocytopenia                       | 51                   |
| Leukopenia                             | 38                   |
| Diarrhea                               | 33                   |
| Headache                               | 30                   |
| Elevated serum aminotransferase levels | 23                   |
| Nausea or vomiting                     | 20                   |
| Hepatosplenomegaly                     | 17                   |
| Oral thrush                            | 10                   |
| Encephalopathy                         | 8                    |
| Neuropathy                             | 8                    |

\* As identified in a review of 139 reported cases (ref 2). Also reported, though less frequently, were esophageal ulceration, esophageal candidiasis, vasculitis, nephritis, rhabdomyolysis with acute renal failure, hypoxemia, and fatal aplastic anemia.

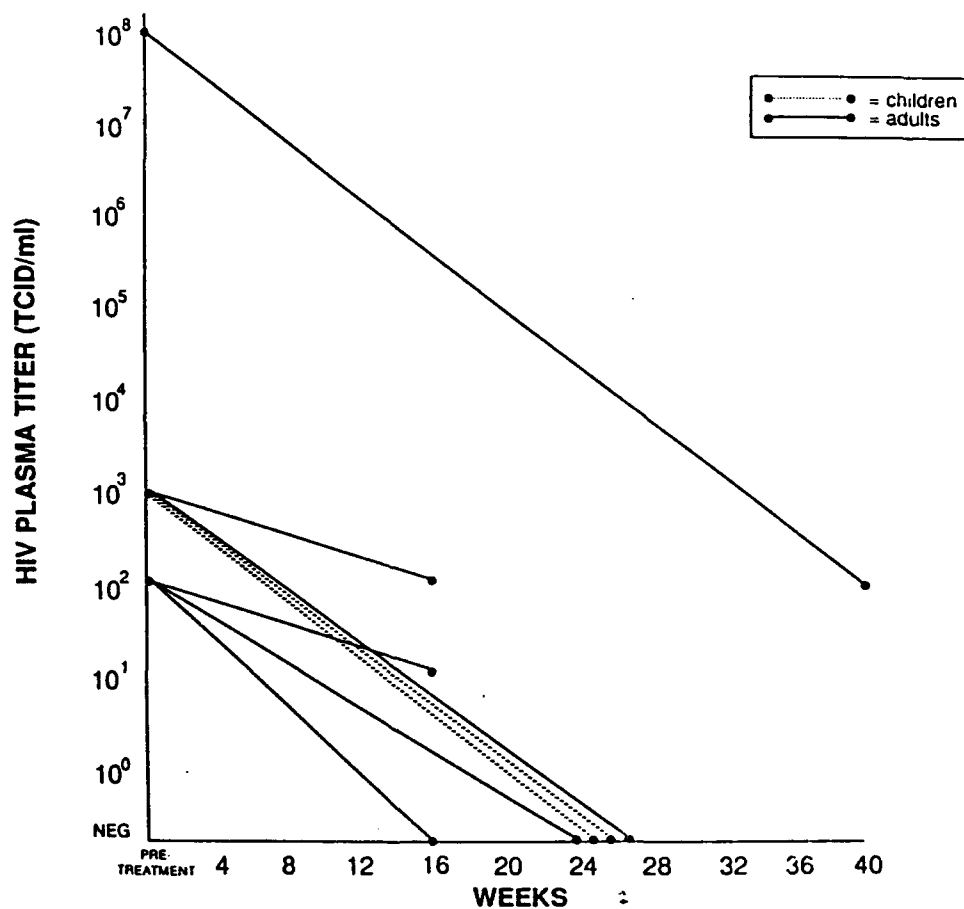


**Figure 1.** Plasma titers of human immunodeficiency virus (HIV) type 1 in adults. Magnitude of plasma viremia by CD4 counts (cells/mm<sup>3</sup>).



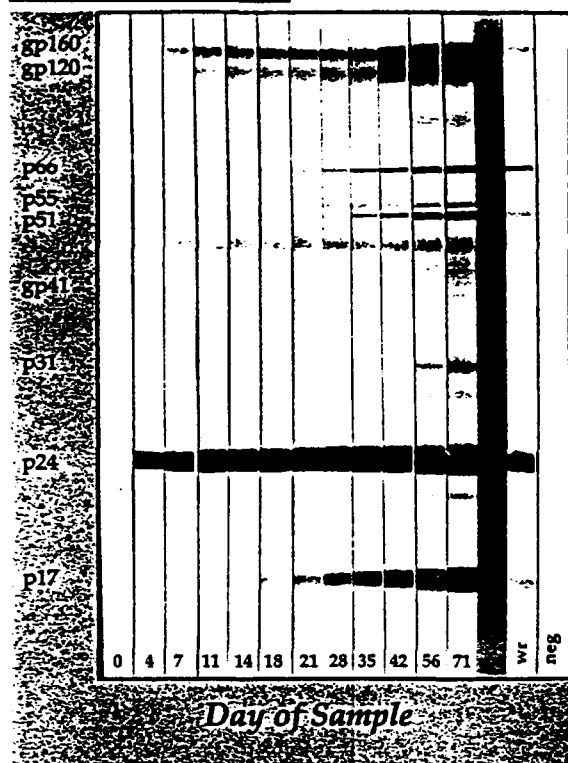
**Figure 2.** Comparative analyses of human immunodeficiency virus (HIV) type 1 plasma viremia titers and p24 antigen levels in adults and children. Neg. no growth.

**Figure 3.** Changes in human immunodeficiency virus (HIV) type 1 plasma viremia titers in adults and children before and after treatment with zidovudine. Neg. no growth.

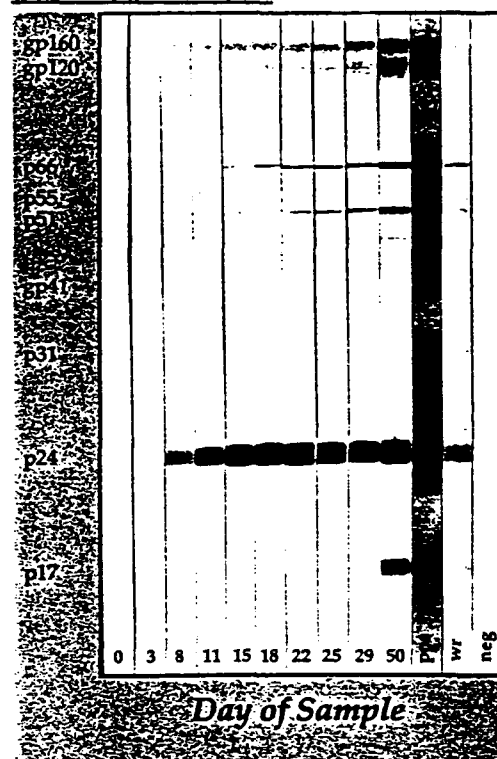




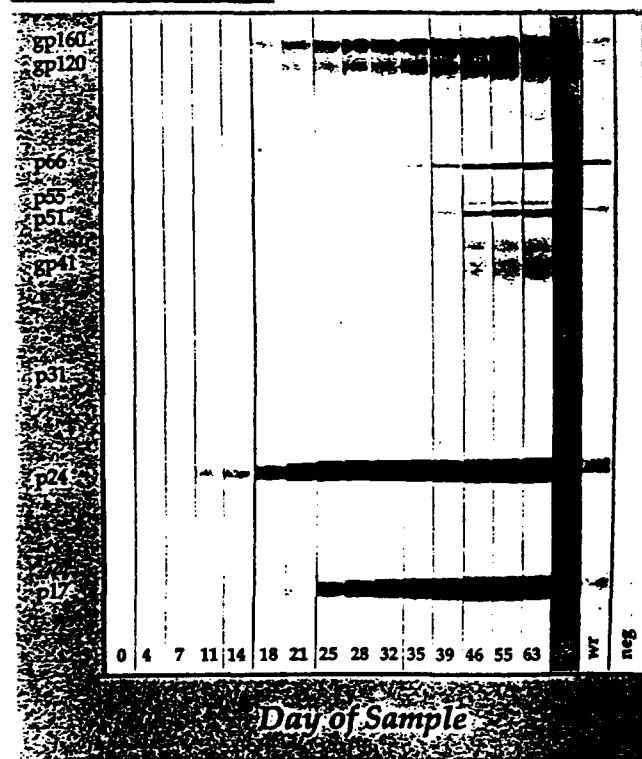
**Patient #1: WEAU**



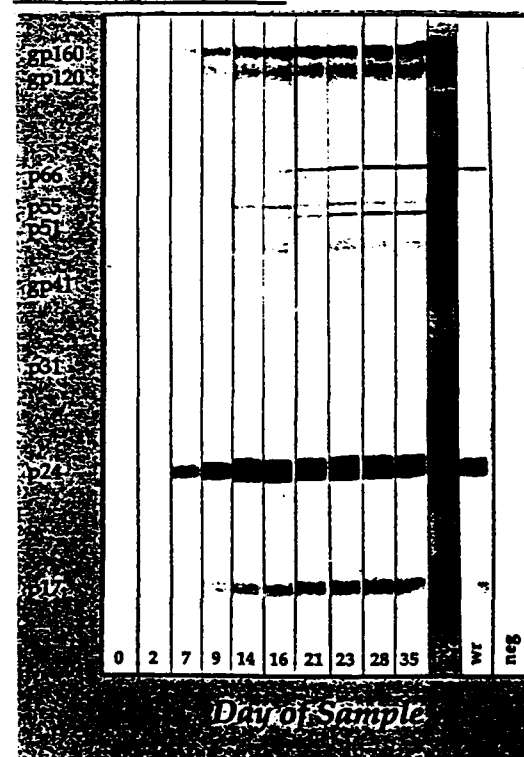
**Patient #3: BORI**



**Patient #2: INME**



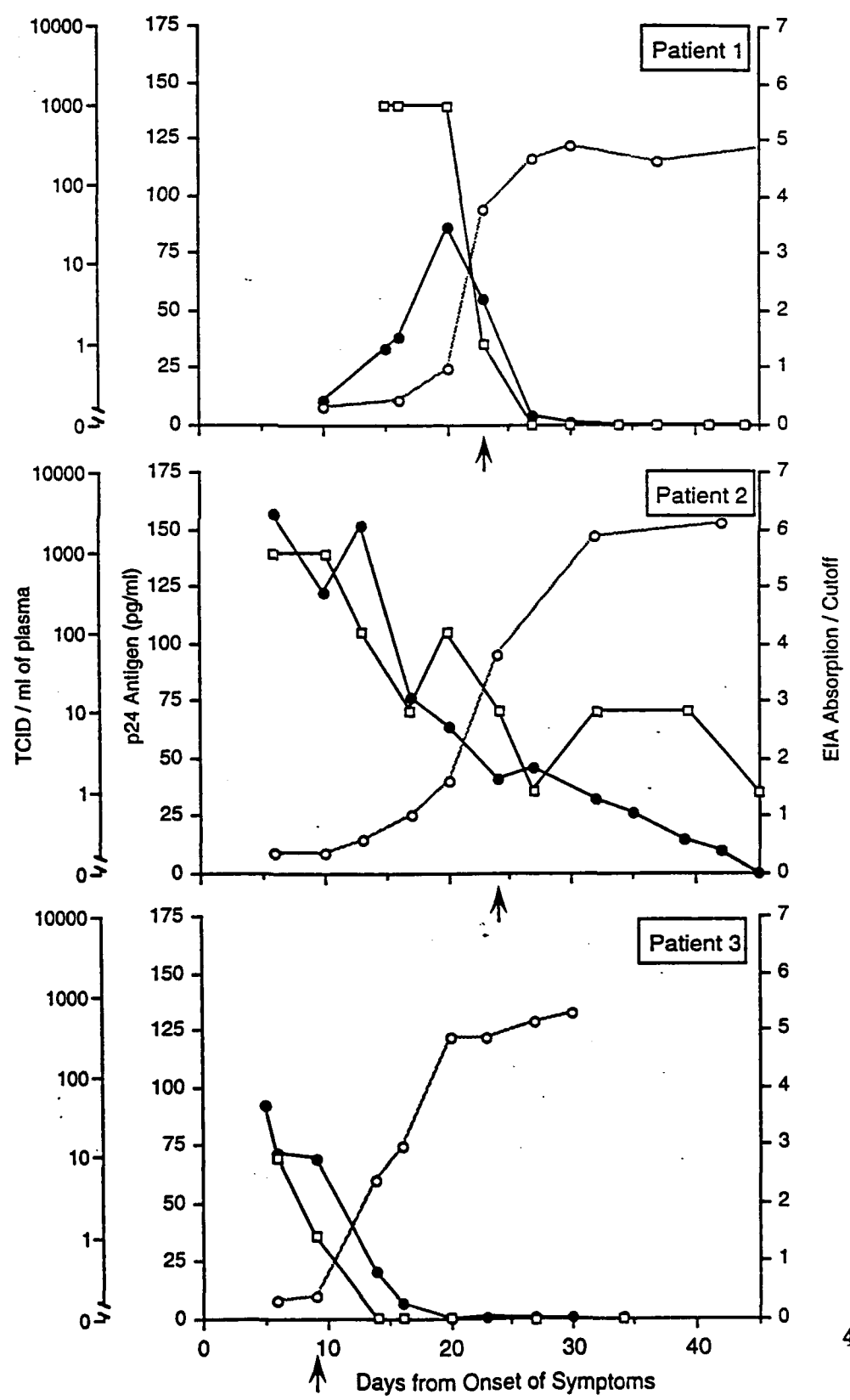
**Patient #4: SUMA**



**FIGURE 4:** Seroconversion profiles for four subjects with acute HIV-1 infection resulting from sexual transmission.

FIGURE 5:

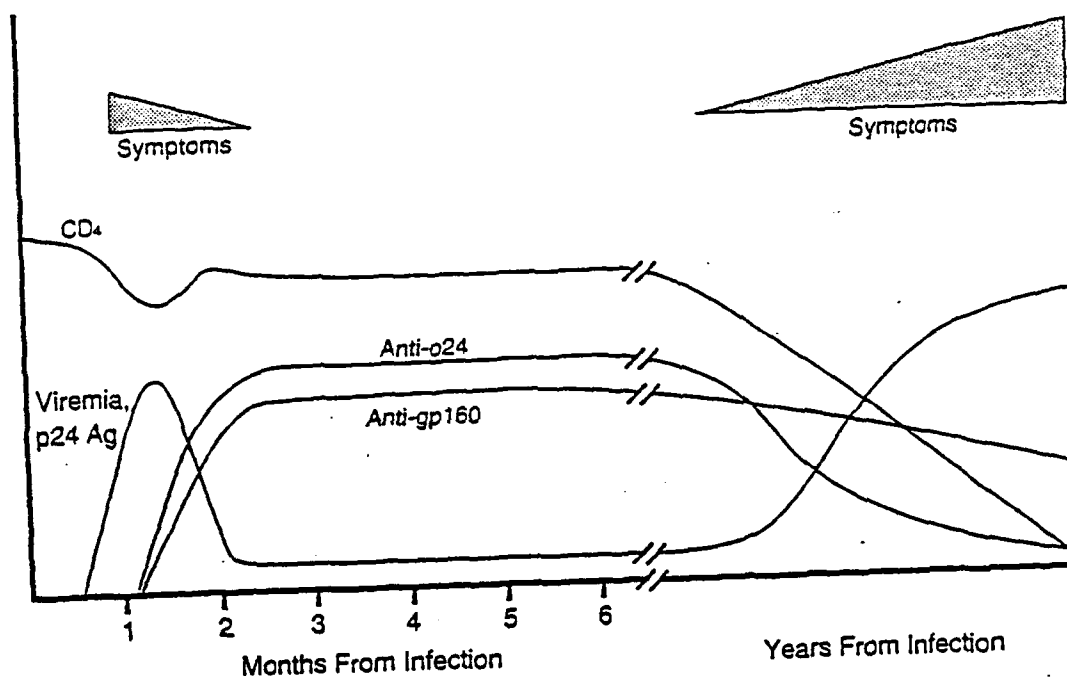
Plasma virus titers (squares) are expressed as tissue-culture-infective doses (TCID) per milliliter, p24 antigen titers (solid circles) as picograms per milliliter, and antibody titers (open circles) as ratios of ELISA absorbance to cutoff. Arrows indicate the time of first detection of both HIV-1 p24 and gp160 antibody by Western blot analysis.



| <u>Patient</u>      | <u>Date</u> | <u># of clones</u> |                                     |
|---------------------|-------------|--------------------|-------------------------------------|
| RIER<br>(Donor)     | 3/18/90     | 9                  | CTRPNNNTRKKITLGPGRVLYTTGEIIGDIRRAHC |
|                     |             | 7                  | -----RS--I-----AF-R--Q-----         |
|                     |             | 1                  | -----H-----RS--I-----AF-R--Q-----R- |
|                     |             | 1                  | -----I-----I-----VS-----R-          |
|                     |             | 1                  | -----VS-----VS-----                 |
|                     |             | 1                  | -----G-----                         |
|                     |             | 1                  | -----                               |
| WEAU<br>(Recipient) | 5/30/90     | 24                 | CTRPNNNTRKKITLGPGRVLYTTGEIIGDIRRAHC |
|                     | 6/21/90     | 17                 | -----                               |
|                     |             | 1                  | -----M-----                         |
|                     |             | 1                  | -----R-----                         |
|                     |             | 1                  | -----V-----                         |
|                     | 1/18/91     | 10                 | -----                               |
|                     |             | 11                 | -----I-----                         |
|                     |             | 1                  | -----I-----I-----                   |
|                     |             |                    | -----                               |
|                     |             |                    | -----                               |

FIGURE 6: Evolution of V3 envelope loop sequences associated with HIV-1 transmission and acute infection.

FIGURE 7: Natural history model of HIV-1 infection.



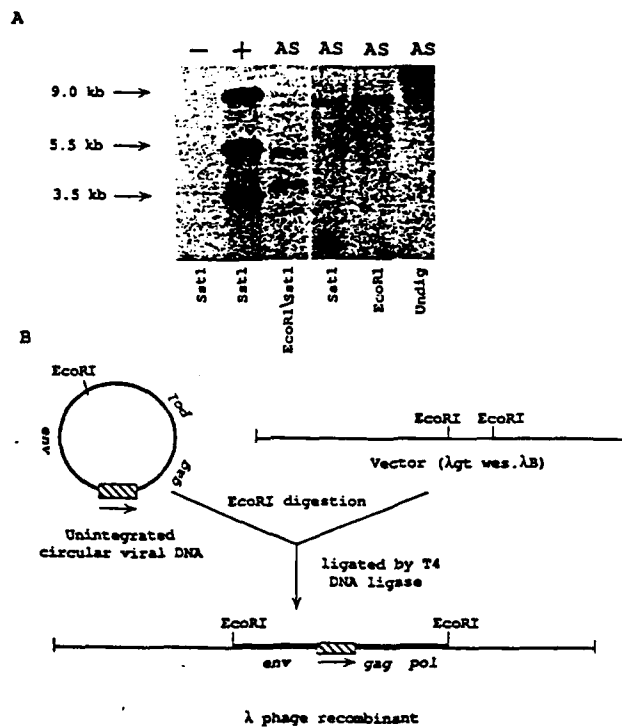


FIG. 8 Molecular cloning of full-length HIV-1 genomes from uncultured human brain. (A) Southern blot analysis of high-molecular-weight brain DNA derived from a patient with ADC. The restriction enzyme cleavage patterns and relative intensities of HIV-1 viral sequences in uncultured brain DNA (AS) are compared with those of positive (+; H9/HTLV-IIIb) and negative (-; uninfected PBMCs) control DNAs. Undig, undigested. (B) Schematic representation of the cloning strategy. Unintegrated circular viral DNA was linearized with *Eco*RI and subsequently cloned in permuted form into *Eco*RI-cleaved phage arms of  $\lambda$ gtWES- $\lambda$ B.

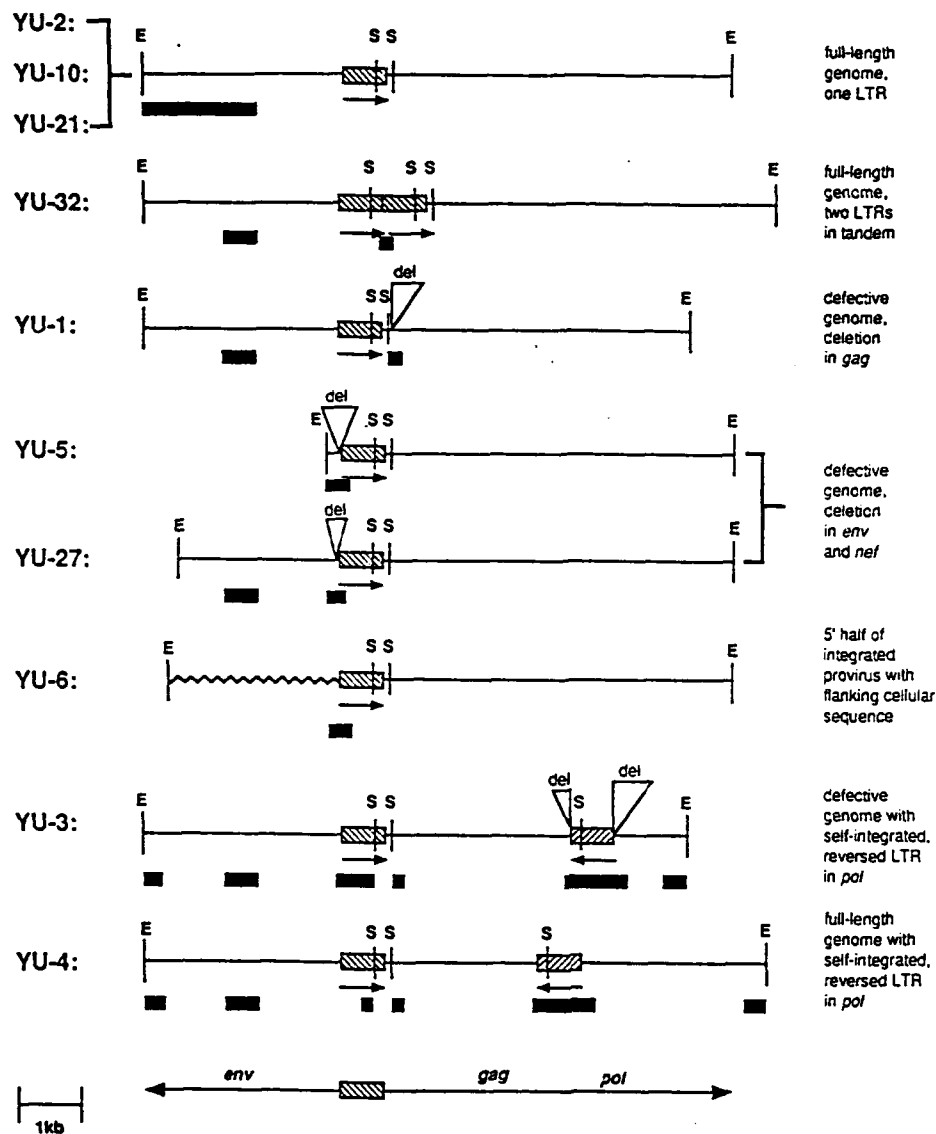


FIG. 9 Recombinant lambda phage clones obtained from a genomic library of uncultured brain DNA. Restriction enzyme analysis identified nine clones as containing unintegrated HIV-1 circles in permuted orientation and one clone as representing an integrated proviral half with flanking cellular sequences (wavy lines). Hatched boxes represent LTR sequences; arrows indicate the transcriptional direction. Triangles depict the positions of internal deletions. Locations of restriction enzymes sites (E, *EcoRI*; S, *SstI*) and relative positions of major HIV-1 open reading frames are shown. Genomic regions confirmed by nucleotide sequence analysis are shaded.

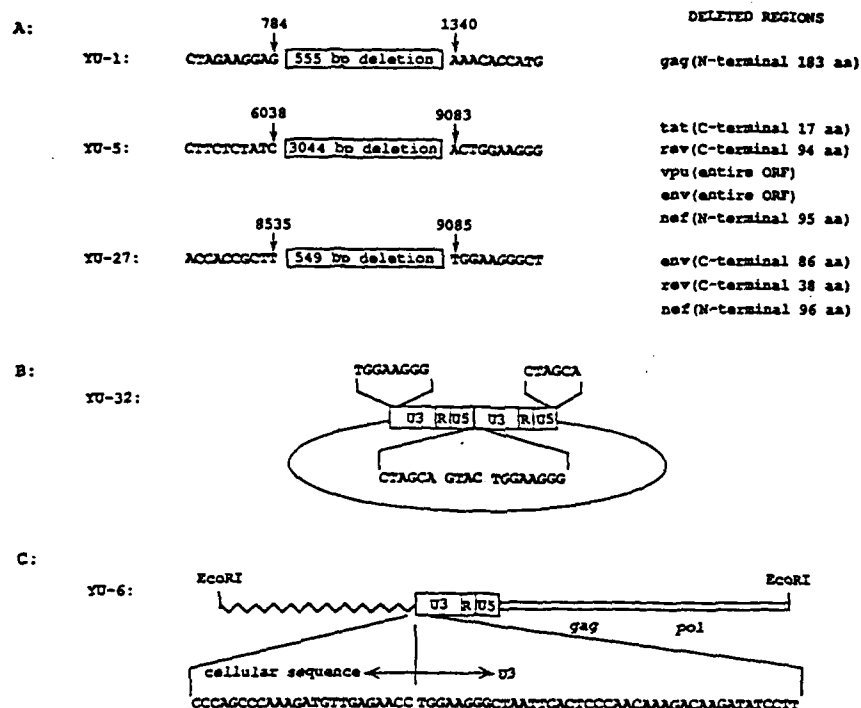


FIG. 10 Nucleotide sequence analysis of selected genomic regions in brain-derived HIV-1 genomes. (A) Molecular characterization of internal deletions. The extents and locations of internal deletions in clones YU-1, YU-5, and YU-27 are depicted. Sequences are numbered according to the HXB2 reference clone (36). (B) Schematic representation of the circle junction in clone YU-32. The orientation of both LTRs was confirmed by sequence analysis. An additional 4 bp located between the 3' terminus of the 5' LTR and the 5' terminus of the 3' LTR are shaded. (C) Sequence analysis of the junction between HIV-1 proviral DNA and flanking cellular sequences in clone YU-6. The boundaries of the integration site are depicted. aa, amino acids.

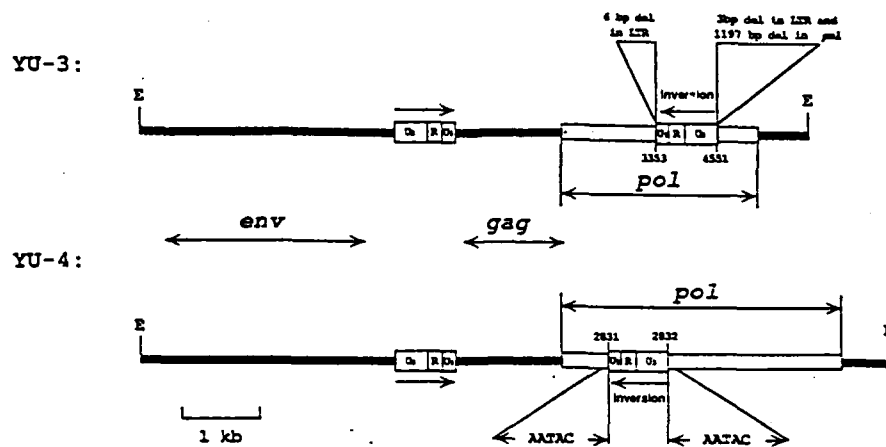


FIG. 11 Identification of inverted LTR sequences in the *pol* gene of two unintegrated HIV-1 circles. Nucleotide sequence analysis confirmed the boundaries of the insertion sites. YU-3 contains deletions in both inverted LTR and adjacent *pol* sequences. YU-4 is characterized by an intact inverted LTR flanked by a 5-bp direct repeat (AATAC) immediately adjacent to the insertion site. Sequences are numbered according to the HXB2 reference clone (36).



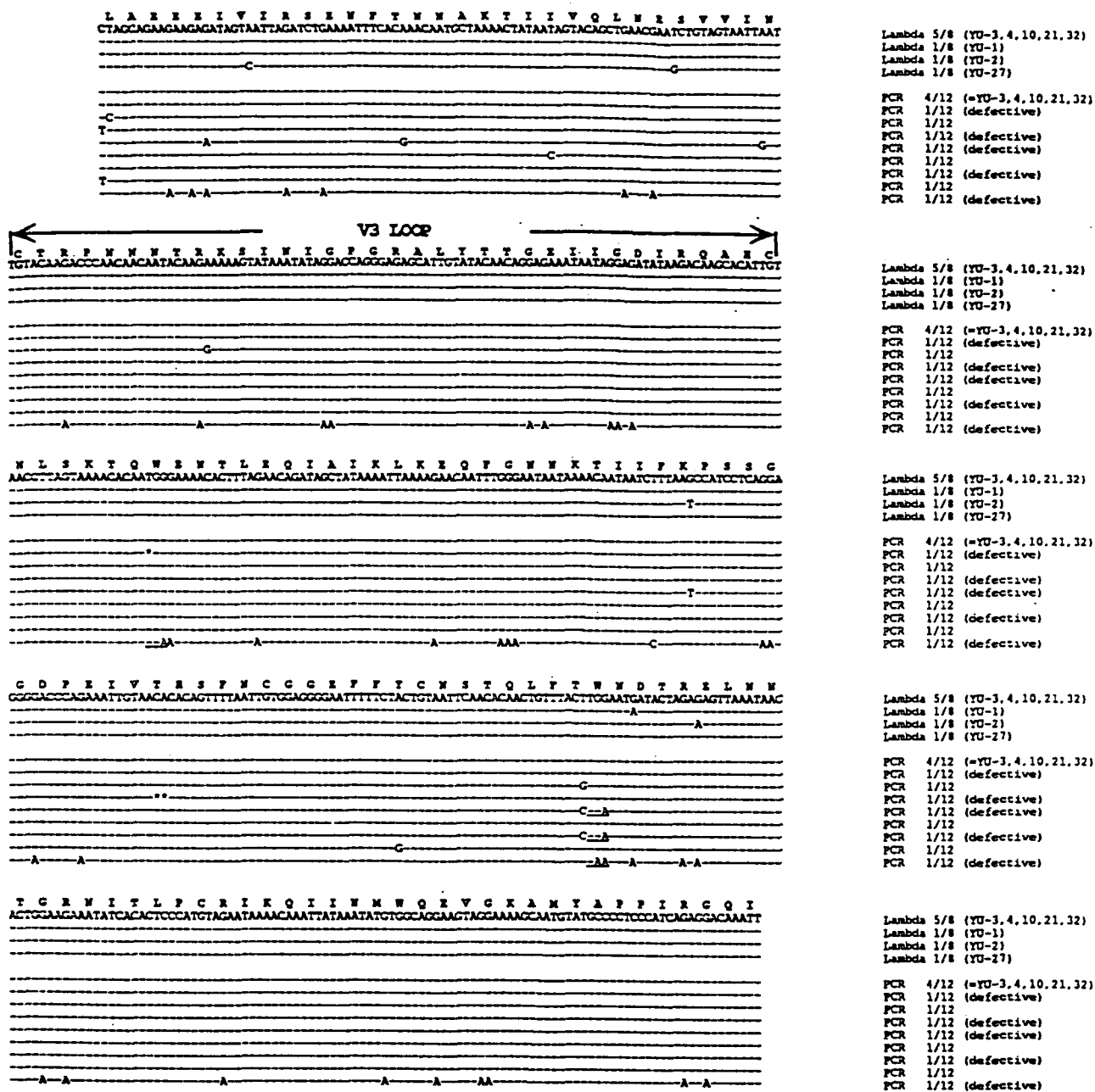


FIG. 12 Genetic variation of HIV-1 in primary human brain. A 510-bp stretch of envelope sequence is aligned between 8 lambda phage-derived and 12 PCR-derived clones. The deduced amino acid sequence, including the position of the major V3 neutralizing epitope, is shown above the nucleotide sequence. Sequence changes are depicted in reference to the predominant viral form, which represents five lambda phage-derived and four PCR-derived clones. Dashes indicate nucleotide sequence identity; asterisks represent single-base-pair deletions. Underlined nucleotides indicate the positions of in-frame translational stop codons. The frequencies of lambda phage- and PCR-derived clones with identical sequences along with the total number of clones analyzed are shown.

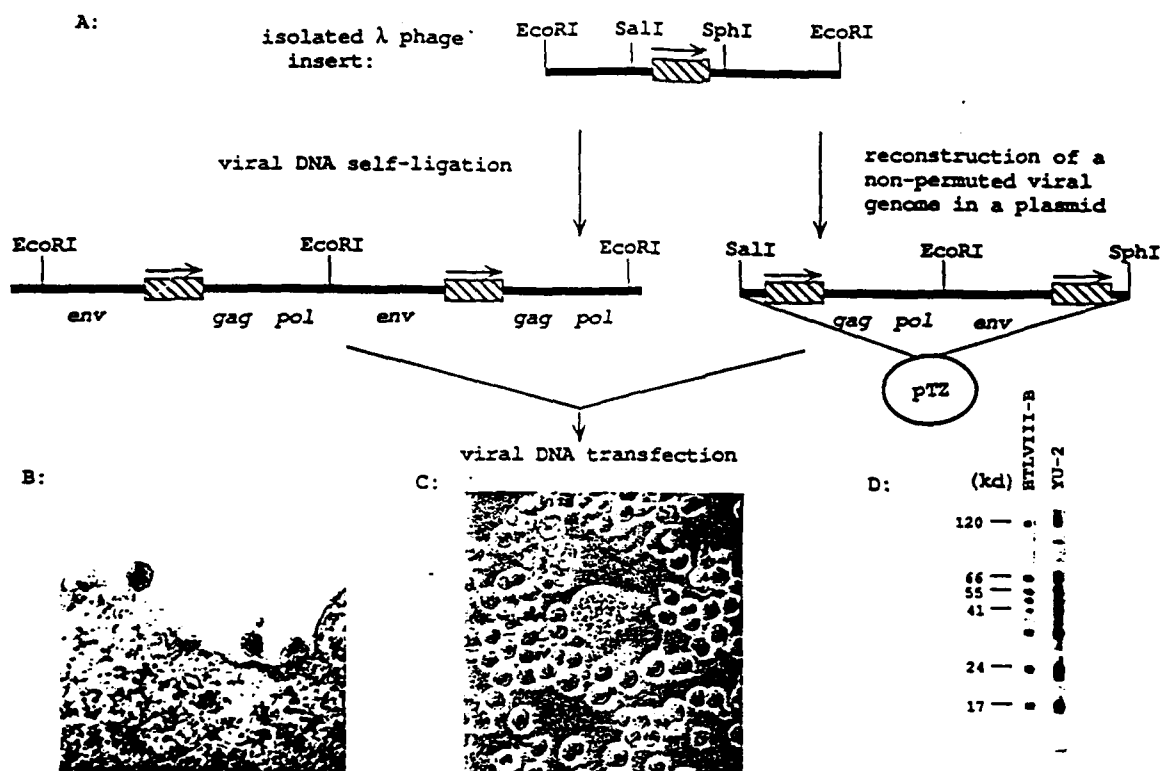
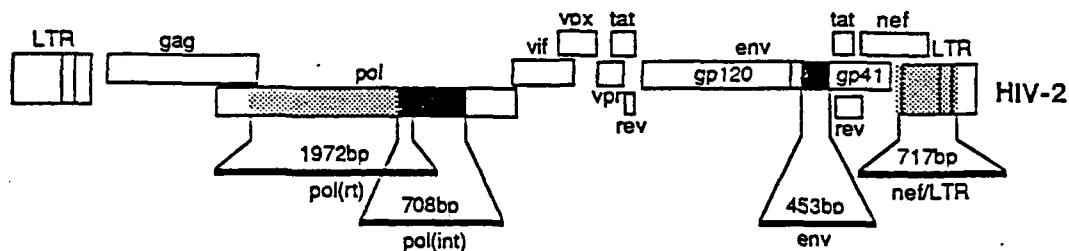
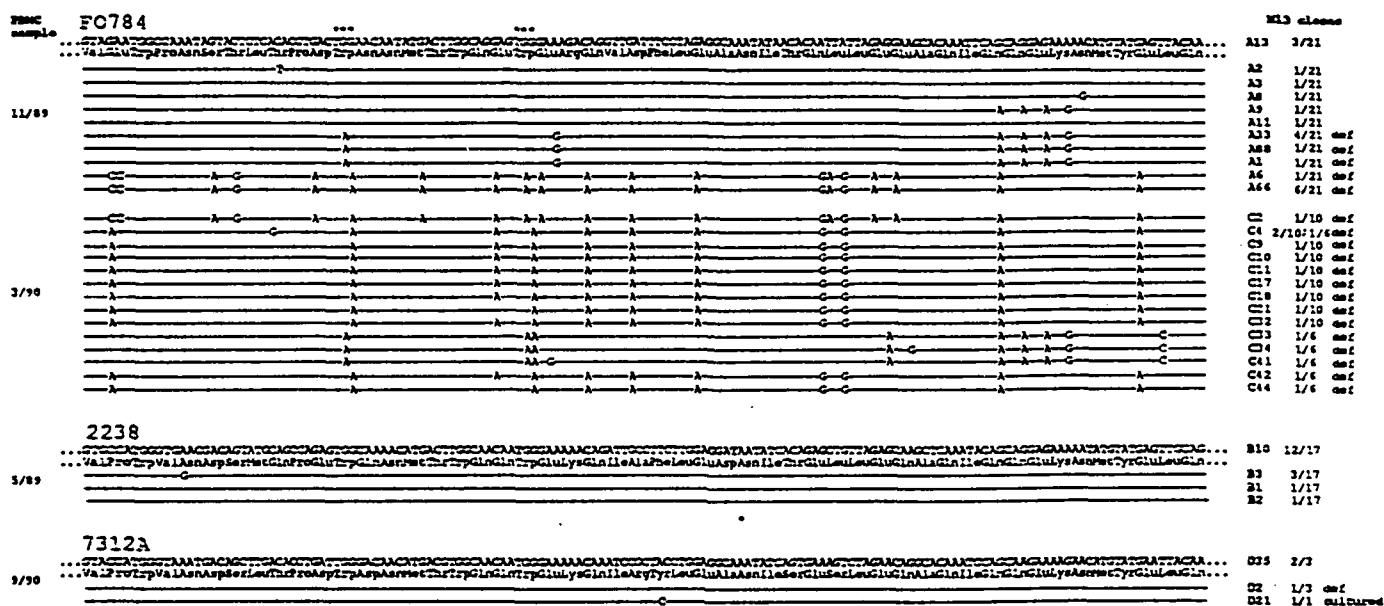


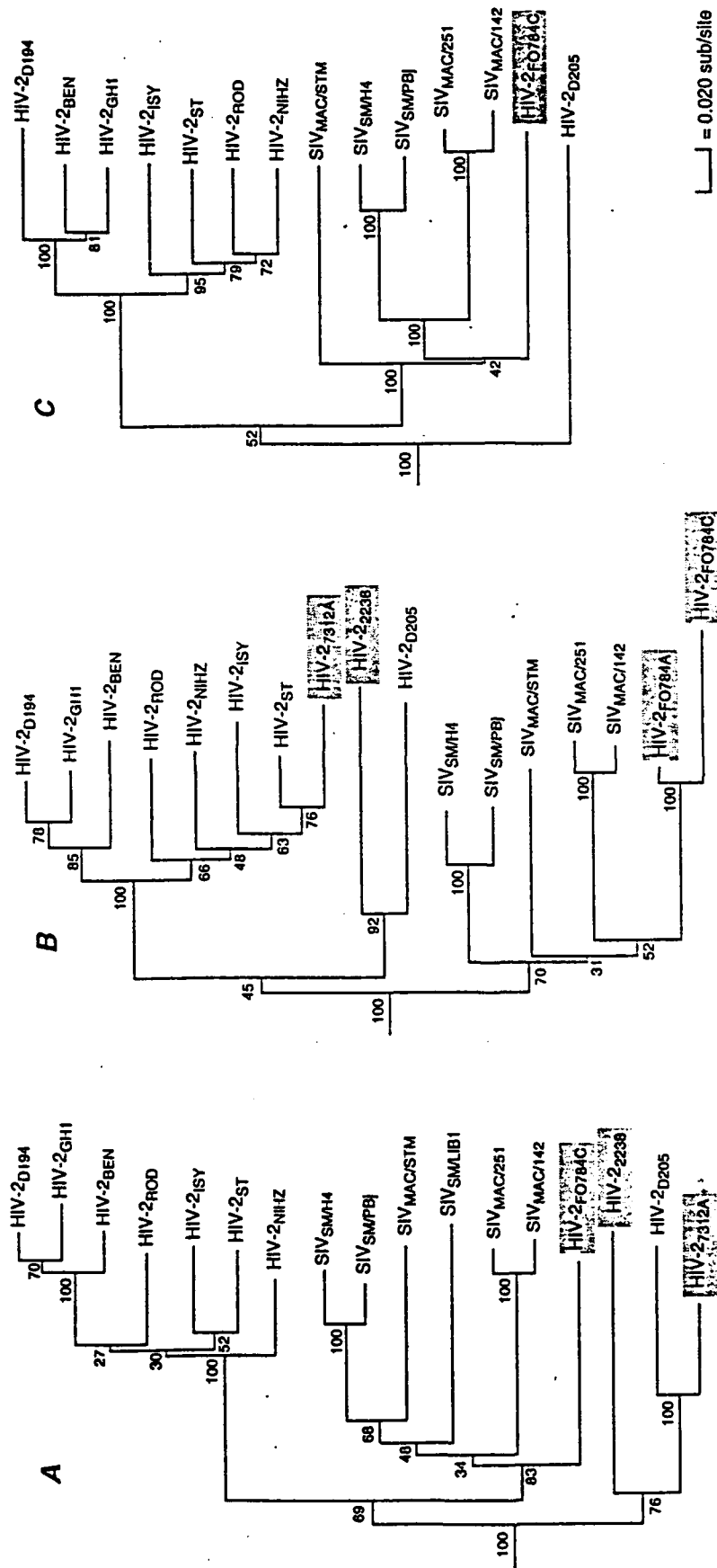
FIG. 13 Biological analysis of a replication-competent HIV-1 genome cloned directly from uncultured human brain. (A) Reconstruction of viral genomes in nonpermuted orientation. Lambda phage inserts were isolated following digestion with *EcoRI* and subsequently self-ligated. Correctly oriented viral inserts were also obtained by ligating a *SalI-EcoRI* fragment and an *EcoRI-SphI* fragment into the plasmid vector pTZ19R. (B) Electron micrograph of transfection derived YU-2 virus in normal donor PBMCs. (C) Virus-induced syncytium formation after cocultivation of YU2-infected PBMCs with Molt-4 (clone 8) cells. (D) Western blot analysis of cell-free YU-2 virus. All major viral gene products are compared with respect to presence and size with HIV-1 from H9/HTLV-IIIb-infected cells.



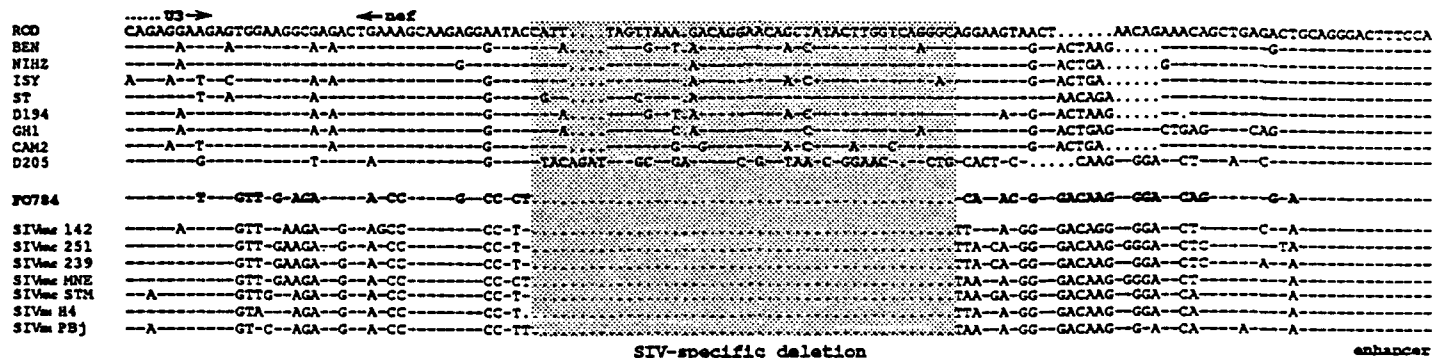
**FIGURE 14.** Location of HIV-2 sequences amplified from uncultured PBMC DNA. Dark shaded areas highlight *pol* (integrase; 708 bp) and *env* (453 bp) fragments amplified from all three study subjects (FO784, 2238, 7312A). Lighter shaded areas indicate *pol* (reverse transcriptase; 1972 bp) and *nef*/LTR (717 bp) fragments amplified only from subject FO784.



**FIGURE 15.** Intrastrain variability and G to A hypermutation in HIV-2. An alignment of 150 bp *env* sequences is shown. Nucleotide substitutions in this segment are representative of the extended *env* and *pol* sequences. Individual clones are compared to a predominant, non-truncated clone with dashes indicating sequence identity. The total number of recombinant M13 clones analyzed, the number of distinguishable clones per amplification reaction, and the number of defective clones (def) with premature truncations are indicated. Asterisks indicate premature stop codons resulting from G to A hypermutation.



**FIGURE 16.** Phylogenetic relationships among (A) *pol*-integrase sequences, (B) *env* sequences, and (C) *pol*-reverse transcriptase sequences from HIV-2<sub>F0784C</sub>, HIV-2<sub>2238</sub>, and HIV-2<sub>7312A</sub> (highlighted) and other HIV-2, SIV<sub>SM</sub>, and SIV<sub>MAC</sub> isolates (56). Horizontal branch lengths are to scale; vertical separation is for clarity only. Numbers at each node indicate the percentage of bootstrap resamples (out of 5000) in which the cluster to the right is supported. The trees are rooted using HIV-1, SIV<sub>AGM</sub>, and SIV<sub>MND</sub> sequences (not shown); the branch lengths from the nearest node to the roots of the trees shown are 0.08, 0.11, and 0.11 substitutions per site for the *pol*-integrase, *env*, and *pol*-RT trees, respectively.



**FIGURE 17.** Identification of an SIV<sub>SM</sub>/SIV<sub>MAC</sub> "signature" sequence in HIV-2<sub>F0784</sub>. HIV-2<sub>F0784</sub> LTR sequences are aligned with prototype HIV-2 and SIV sequences in a region of the LTR encompassing a specific 40-44 bp insertion that is present in all previously reported HIV-2 viruses but absent in all SIV<sub>SM</sub>/SIV<sub>MAC</sub> viruses (56). Sequences are compared to HIV-2<sub>ROD</sub>(46) as a reference sequence with dashes (-) indicating sequence identity and periods (.) indicating gaps introduced for optimal alignment. Enhancer sequences and the *nef* termination codon are indicated. HIV-2<sub>F0784</sub> (boldfaced), like viruses of monkey derivation, lacked the 40-44bp insertion.