Characterization of Infectious Molecular Clones of Simian Immunodeficiency Virus (SIV_{mac}) and Human Immunodeficiency Virus Type 2: Persistent Infection of Rhesus Monkeys with Molecularly Cloned SIV_{mac}

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Infection of macaque monkeys with simian immunodeficiency virus (SIV) is probably the best animal model currently available for studying acquired immunodeficiency syndrome. In this report, we describe three infectious molecular clones of SIV_{mac} and one of human immunodeficiency virus type 2 (HIV-2) and their use in the study of cell and species specificity, animal infection, and the relationship of gene sequence to function. Replication of the cloned viruses in different cell lines varied dramatically. Some human $CD4^+$ cell lines (HUT 78 and MT-4) supported the replication of SIV_{mac} and HIV-2, while others (CEM and Jurkat-T) supported the replication of HIV-2 but not SIV_{mac}. Growth of cloned virus in macaque lymphocytes in vitro was predictive of macaque infection in vivo. Macaque lymphocytes supported the replication of $SIV_{mac}239$ and $SIV_{mac}251$ but not $SIV_{mac}142$ or HIV-2ROD. Using virus recovery and antibody response as criteria for infection, macaques that received cloned $SIV_{mac}251$ and $SIV_{mac}239$ became infected, while macaques receiving cloned $SIV_{mac}142$ and HIV-2ROD did not become infected. Nucleotide sequences from the envelope region of all four cloned viruses demonstrated that there is considerable flexibility in the location of the translational termination (stop) signal. These infectious molecular clones will be very useful for future studies directed at the molecular basis for persistence, pathogenicity, tropism, and cell and species specificity.

Animal models for acquired immunodeficiency syndrome (AIDS) are needed to analyze important features of pathogenesis, to study therapeutic interventions, and to examine approaches toward vaccine development. Simian immunodeficiency virus (SIV) is the closest relative to human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) identified to date (3, 10). After the initial description of SIV from rhesus macaques (5), SIV was subsequently isolated from other macaques (1, 7, 22), from mangabeys (11, 19), and from African green monkeys (8, 23). Although information on the extent of genetic variability of these viruses is minimal at present, they appear to form a quite diverse group (3, 10, 17).

SIV isolates from macaques (SIV_{mac}) have a lentivirus morphology, a $CD4^+$ cell tropism, and in vitro growth properties similar to those of HIV-1 and HIV-2 (1, 5, 7, 16) and are antigenically cross-reactive with these viruses (4, 14). Macaque monkeys inoculated with SIV_{mac} become persistently infected and develop a chronic debilitating disease remarkably similar to AIDS in humans (2, 6, 18). Clinical signs and autopsy findings in macaques that die after experimental SIV infection include the following: diarrhea and wasting; immunologic abnormalities; decreased T4-cell numbers; opportunistic infections with agents such as cytomegalovirus, adenovirus, cryptosporidia, Pneumocystis carinii, and Mycobacterium avium; and a characteristic granulomatous encephalitis very similar to that seen in humans with AIDS.

Well-characterized, infectious, molecular clones of SIV are needed not only for production of individual protein products through recombinant DNA technology but also for relating genetic sequences to in vivo functions. In this report, we describe the isolation and characterization of three such molecular clones of SIV_{mac} and one of HIV-2.

MATERIALS AND METHODS

Virus. $SIV_{mac}142$-, $SIV_{mac}251$-, and $SIV_{mac}239$-producing cell lines have been reported previously (5). HIV-2ROD-producing CEM cells (4) were provided by L. Montagnier.

Cells and cell lines. Macaque peripheral blood lymphocytes were obtained from blood samples collected in preservative-free heparin by banding over sodium diatrizoate-Ficoll (1.077 to 1.080 g/ml at 20°C; Pharmacia Fine Chemicals, Piscataway, N.J.). Peripheral blood lymphocytes were stimulated with 1 μg of phytohemagglutinin per ml for 48 h, washed free of lectin, and incubated in RPMI 1640 with 10% fetal calf serum, interleukin-2 (lectin-free T-cell growth factor; Electro-Nucleonics, Inc., Fairfield, N.J.), penicillin, and streptomycin. Continuously growing human $CD4^+$ cell lines HUT-78, CEM, Jurkat-T, and MT-4 were grown in RPMI 1640 medium with 10% fetal calf serum.

Molecular cloning and restriction endonuclease mapping. Total cell DNA was prepared from SIV_{mac}-infected HUT-78 cells and HIV-2-infected CEM cells as described in Maniatis et al. (20). Isolation of the $SIV_{mac}142$ molecular clone has been described previously (3). To facilitate full-length cloning of other viral isolates, we first identified endonuclease EcoRI as a noncutter of viral sequences of $SIV_{mac}239$ and

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SIV$_{mac}$251 and BglII as a noncutter of viral sequences of HIV-2ROD. Lambda bacteriophage libraries were prepared by using sucrose density gradient size-fractionated 10- to 20-kilobase-pair DNA from EcoRI-digested total cell DNA of HUT-78-SIV$_{mac}$239 and HUT-78-SIV$_{mac}$251 and BglII-digested total cell DNA of CEM-HIV-2ROD. EcoRI-digested DNA was inserted into the EcoRI site of lambda cloning vector EMBL4, and BglII-digested DNA was inserted into the BamHI site of EMBL3. The libraries were screened by using pK2 BamA (13) as a probe, and full-length molecular clones were obtained. Physical maps of these clones were constructed by single, double, and triple digestions followed by electrophoresis and visualization of viral bands by ethidium bromide staining or Southern blot hybridization. All clones were found to contain flanking cellular sequences.

DNA transfection and growth characteristics of cloned viruses. Lambda phage DNA was transfected into cultured cells by using DEAE-dextran as described by Sompayrac and Danna (25) and Milman and Herzberg (21). Five aliquots containing a total of 3 μg of DNA were sequentially added with intermittent mixing to 1.4 ml of Dulbecco modified Eagle medium without serum containing 125 μg of DEAE-dextran per ml and 50 mM Tris (pH 7.3). Within 24 h after being split, 3 × 10^6 HUT-78 cells were washed twice with Dulbecco modified Eagle medium, suspended in the 1.4-ml DNA solution, and incubated at 37°C for 1 h. The cells were then washed with serum-free Dulbecco modified Eagle medium and serum-free RPMI 1640 medium and were finally incubated at 37°C in RPMI 1640 medium with 10% fetal calf serum. Cells were split 1:2 or 1:3 twice per week. Reverse transcriptase (RT) activity was monitored as previously described (5). Within 2 weeks after peak RT activity, cell-free virus stocks were prepared by passing the culture supernatant through a filter (pore size, 0.45 μm), and 100+ vials were stored in liquid nitrogen. A 0.5-ml portion of this stock was used to infect each of the cell lines.

Nucleotide sequencing. The nucleotide sequence of the SIV$_{mac}$142 infectious clone was described previously (3). For SIV$_{mac}$239, SIV$_{mac}$251, and HIV-2ROD, appropriate subcloned DNA fragments were derived from the infectious molecularly cloned DNAs, and the nucleotide sequences of both strands were determined by the primer-directed dideoxy-chain termination method of Sanger et al. (24). Sequenase and synthetic oligonucleotide primers were used to sequence double-stranded DNA. ^{35}S-labeled sequencing reactions were electrophoresed on a buffer gradient (0.75 to 2.5% Tris-borate buffer) 6% polyacrylamide gel with 8 M urea. Sequences were entered into an IBM-PC computer with an IBI gel reader and digitizer and were analyzed with IBI-Pustell DNA analysis software.

Experimental infection of macaques. Rhesus macaques (Macaca mulatta) negative for SIV antibody were selected from the New England Regional Primate Research Center colony. Two rhesus macaques were inoculated intravenously with 1 ml of each virus derived from transfection of cloned DNAs. Heparinized blood samples were collected from the macaques at intervals after the virus inoculation and were used for virus recovery and for monitoring antibody response.

For virus recovery, peripheral blood mononuclear cells (PBMC) were first prepared by banding over Ficoll-diatrizoate (density, 1.077 to 1.080 g/ml at 20°C). PBMC were washed with RPMI 1640, stimulated for 2 to 5 days with 1 μg of phytohemagglutinin per ml, washed free of phytohemagglutinin, and cocultivated with indicator cells (human periph-

eral blood lymphocytes growing in interleukin-2 or in HUT-78, Molt 4 clone 8, or CEM × 174 cell lines). The appearance of virus in the cell-free supernatant was monitored by measurement of RT activity (5). For determination of antibody response, pre- and postinoculation plasma samples were tested for the presence of antibody by using the enzyme-linked immunosorbent assay as previously described (7). Column-purified SIV$_{mac}$ and HIV-2 were used to coat wells of the antigen plate (6, 7).

RESULTS AND DISCUSSION

After the isolation of molecular clones containing integrated proviral DNA in lambda vectors (Fig. 1), we focused our attention on three molecular clones of SIV$_{mac}$ and one of HIV-2 that were found to be infectious. The nucleotide sequence of the infectious cloned SIV$_{mac}$142 DNA has been described previously (3). The restriction endonuclease maps of the three SIV$_{mac}$-cloned DNAs showed that they were closely related (Fig. 1). The molecular clones used previously for HIV-2 sequence analysis are not infectious (12). The clone of HIV-2ROD that we describe here was derived from the same infected cell line used by Guyader et al. (12) and is the first HIV-2 clone reported to be infectious. The restriction sites that we have mapped in the infectious HIV-2ROD clone (Fig. 1) have corresponding sites in the HIV-2ROD clones sequenced by Guyader et al. (12); however, the sequence of Guyader et al. contains one internal PstI site and two 3' PvuII sites that are absent in the infectious HIV-2ROD molecular clone. Approximately 15% of the restriction sites are conserved when SIV$_{mac}$ and HIV-2ROD are compared, which is consistent with the overall nucleotide sequence identity of 75% reported previously (3).

When the cloned DNAs were transfected into HUT-78 cells by using a DEAE-dextran procedure, the presence of virus, as detected by RT activity, could be demonstrated in the cell-free supernatant (Fig. 2A). The kinetics of virus appearance in transfected HUT-78 cells were reproducible and varied among the clones. SIV$_{mac}$142 consistently appeared first and yielded the highest RT activity, while
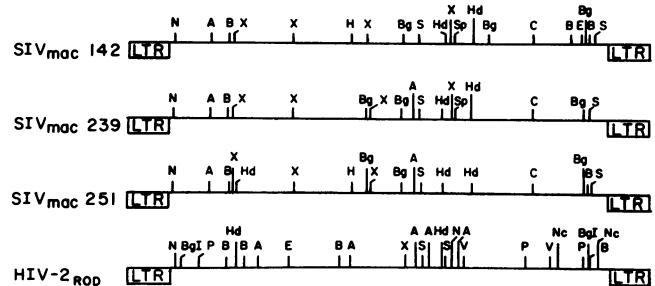

FIG. 1. Restriction endonuclease maps of infectious molecularly cloned viral DNA. All molecular clones were found to contain flanking cellular sequences outside the long terminal repeat (LTR) sequences. The locations of the long terminal repeats and the physical maps were determined by single, double, and triple endonuclease digestions followed by visualization of viral bands in agarose gels with ethidium bromide staining or Southern hybridization with ^{32}P-labeled SIV DNA fragments. A detailed map of SIV$_{mac}$142 has been published elsewhere (3). Abbreviations: A, AvaI; B, BamHI; Bgl, BglI; Bg, BglII; C, ClaI; E, EcoRI; Hd, HindIII; H, HpaI; N, NarI; Nc, NcoI; P, PstI; Sp, SphI; S, SstI; V, PvuII; and X, XbaI. Sites for NcoI, PstI, and PvuII of SIV$_{mac}$ are not shown.

A. HUT-78 TRANSFECTION
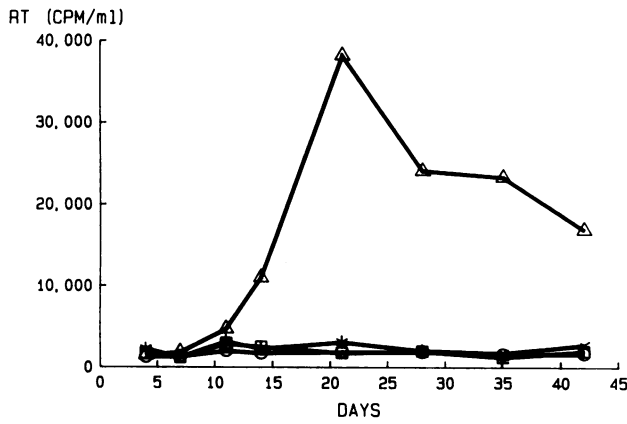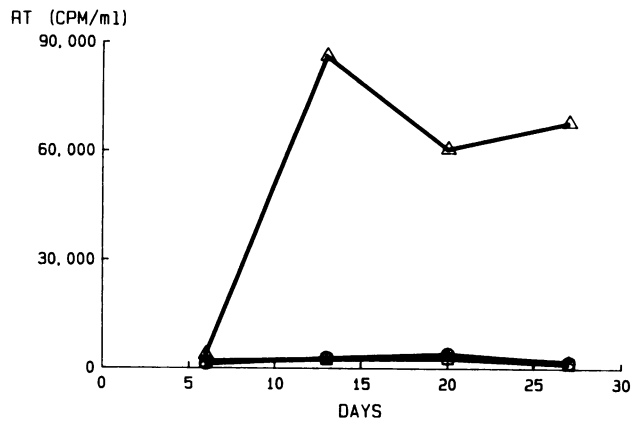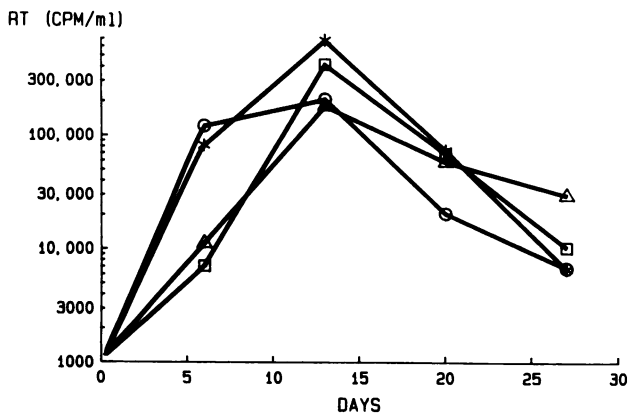
RT (CPM/ml)

B. HUT-78

RT (CPM/ml)

C. CEM

RT (CPM/ml)

D. JURKAT-T

RT (CPM/ml)

E. MT-4

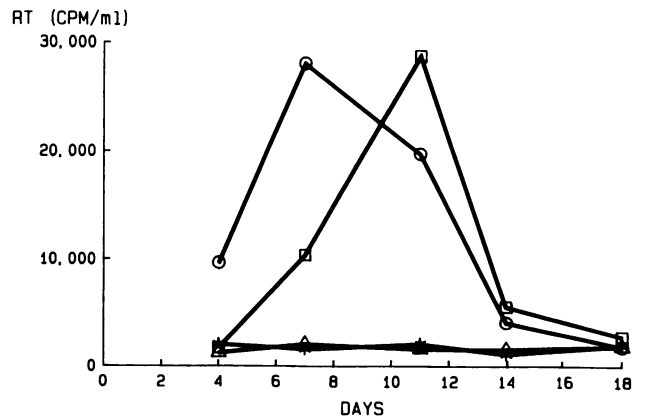RT (CPM/ml)
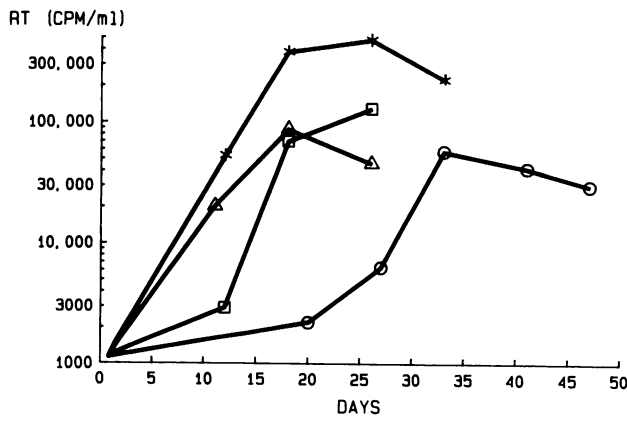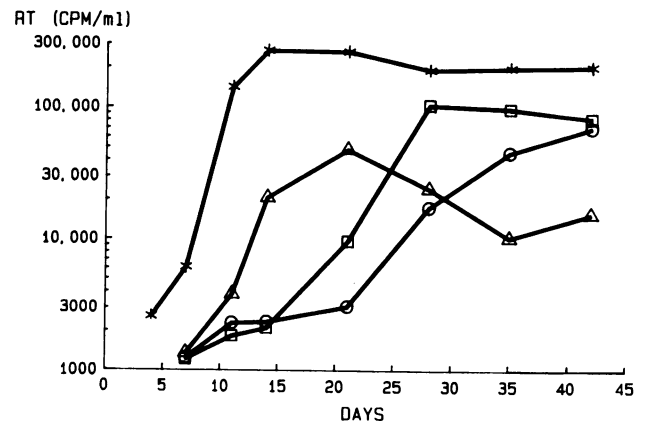
F. MACAQUE LYMPHOCYTES

RT (CPM/ml)

FIG. 2. Replication of cloned viruses in different cell lines. After transfection of cloned DNA into HUT-78 cells, RT activity in the cell-free supernatant was monitored (A). Virus derived from these molecular clones was used to infect different cell lines (B through F). Symbols: O, SIV$_{mac}$239; □, SIV$_{mac}$251; △, HIV-2ROD; *, SIV$_{mac}$142.

	8270	8280	8290	8300	8310	8320	8330	8340	8350	8360	8370	8380

SIV$_{mac}$ 142 CCA GTG TTC TCT TCC CCA CCC TCT TAT TTC CAG▨▨ACC CAT ACC CAA CAG GAT CCG GCT CTG CCA ACC AAA GAA GGC AAA AAA GGA GAC GGT GGA GGC AGC GGT GGC AAC AGC TCC TGG
Pro Val Phe Ser Ser Pro Pro Ser Tyr Phe Gln▨▨Thr His Thr Gln Gln Asp Pro Ala Leu Pro Thr Lys Glu Gly Lys Lys Gly Asp Gly Gly Gly Ser Gly Gly Asn Ser Ser Trp

SIV$_{mac}$ 251 CCA GTG TTC TCT TCC CCA CCC TCT TAT TTC▨▨CAG ACT CAT ACC CAA CAG GAC CCG GCA CTG CCA ACC AGA GAA GGC AAA GAA GGA GAC GGT GGA GAA GGC GGT GGC AAC AGC TCC TGG
Pro Val Phe Ser Ser Pro Pro Ser Tyr Phe▨▨Gln Thr His Thr Gln Gln Asp Pro Ala Leu Pro Thr Arg Glu Gly Lys Glu Gly Asp Gly Gly Glu Gly Gly Gly Asn Ser Ser Trp

SIV$_{mac}$ 239 CCA GTG TTC TCT TCC CCA CCC TCT TAT TTC CAG CAG ACC CAT ATC CAA CAG GAC CCG GCA CTG CCA ACC AGA GAA GGC AAA GAA AGA GAC GGT GGA GAA GGC GGT GGC AAC AGC TCC TGG
Pro Val Phe Ser Ser Pro Pro Ser Tyr Phe Gln Gln Thr His Ile Gln Gln Asp Pro Ala Leu Pro Thr Arg Glu Gly Lys Glu Arg Asp Gly Gly Glu Gly Gly Gly Asn Ser Ser Trp

HIV-2 ROD CCT GTT TTC TCT TCC CCC CCC GGT TAT ATC CAA CAG ATC CAT ATC CAC AAG GAC CGG GGA CAG CCA GCC AAC GAA GAA ACA GAA GAA GAC GGT GGA AGC AAC GGT GGA GAC AGA TAC TGG
Pro Val Phe Ser Ser Pro Pro Gly Tyr Ile Gln Gln Ile His Ile His Lys Asp Arg Gly Gln Pro Ala Asn Glu Glu Thr Glu Glu Asp Gly Gly Ser Asn Gly Gly Asp Arg Tyr Trp

FIG. 3. Nucleotide and predicted amino acid sequences from a region of the envelope transmembrane protein. Nucleotide numbers correspond to those of Chakrabarti et al. (3). Translational termination signals are shaded. Additionally, no in-frame stop signals were found within 319 nucleotides upstream or 351 nucleotides downstream of the sequences shown for SIV$_{mac}$239 or 160 nucleotides upstream of the sequences shown for HIV-2ROD. SIV$_{mac}$142, SIV$_{mac}$251, and SIV$_{mac}$239 are three infectious molecular clones of different SIV isolates; HIV-2ROD is the infectious molecular clone of HIV-2.

SIV$_{mac}$239 appeared last after transfection. Vials (100+) of each cloned virus were stored in liquid nitrogen within 2 weeks after reaching the peak RT activity shown in Fig. 2A. This stored virus was competent for infection (see below) and was used for all subsequent studies.

The kinetics of virus production were essentially reproduced when frozen virus stocks derived from the infectious molecular clones were used to infect HUT-78 cells (Fig. 2B). The kinetics of virus production, however, varied dramatically with the cell line used. For example, the cloned SIV$_{mac}$239 virus replicated most rapidly of the four in MT4 cells (Fig. 2E). CEM and Jurkat-T cells supported the replication of the cloned HIV-2 virus, but they did not significantly support replication of the cloned SIV$_{mac}$ viruses (Fig. 2C and D). Thus, while some human cell lines (HUT-78 and MT4) supported the replication of cloned SIV$_{mac}$ and HIV-2 viruses, others (CEM and Jurkat-T) supported the replication of HIV-2 but not of SIV$_{mac}$. The most dramatic cell killing was observed with MT4 cells. By 14 days post-infection, most of the MT4 cells were killed by all four cloned viruses without evidence of syncytium formation; RT activity declined dramatically after that time (Fig. 2E). Some syncytium formation was observed in HUT-78 cells. The ability of HUT-78 and MT4 cells to support replication of all four cloned viruses, the ability of CEM and Jurkat-T to support replication of cloned HIV-2ROD but not of SIV$_{mac}$, and the killing effects of all four cloned viruses with MT4 cells essentially mimic the properties of the uncloned virus stocks from which the clones were derived.

When normal rhesus macaque lymphocytes growing in the presence of interleukin-2 were tested for their ability to support the replication of cloned viruses, significant production was detected only with SIV$_{mac}$239 and SIV$_{mac}$251 (Fig. 2F). Since SIV$_{mac}$142 was originally obtained from an infected rhesus macaque (5, 7), it was surprising that we were not able to detect replication of the cloned SIV$_{mac}$142 virus in rhesus macaque lymphocytes. However, all of these viruses were originally isolated and grown in human cells. It is conceivable that the SIV$_{mac}$142 clonal derivative adapted to growth in human cells in such a way that it no longer had the ability to grow in macaque lymphocytes.

Previous sequencing of SIV has revealed the presence of a premature translation termination signal which results in a truncated form of the env transmembrane protein (3, 9, 13). The premature stop codon in the SIV$_{mac}$142 infectious clone was described previously in a report of its entire nucleotide sequence (3). A similar stop codon was found in a noninfectious integrated provirus of HIV-2ROD (12). We thus examined the nucleotide sequences in the corresponding regions of the SIV$_{mac}$251, SIV$_{mac}$239, and HIV-2ROD infectious clones to determine whether the presence of this stop codon was a universal feature of replication-competent SIV$_{mac}$ and HIV-2 viruses.

Appropriate subclones of SIV$_{mac}$239, SIV$_{mac}$251, and HIV-2ROD were derived, and nucleotide sequences were determined by the primer-directed dideoxy-chain termination method (Fig. 3). The SIV$_{mac}$251 infectious clone has two nucleotide differences in the region corresponding to the premature stop codon in SIV$_{mac}$142: a T-to-C change results in glutamine rather than stop; however, a C-to-T change results in stop rather than glutamine in the codon immediately preceding it (Fig. 3). Thus, the SIV$_{mac}$142 and SIV$_{mac}$251 infectious clones have premature stop codons in env that differ in location by one codon. The corresponding regions in SIV$_{mac}$239 (CAG CAG) and HIV-2ROD (CAA CAG) have no such stop codon. In fact, 280 nucleotides of flanking sequences in HIV-2ROD and 790 nucleotides of flanking sequences in SIV$_{mac}$239 that were analyzed had no stop codons in this reading frame (Fig. 3). The only stop codon in the envelope reading frame of SIV$_{mac}$239 was found in a position corresponding to its position in HIV-1, which corresponds to nucleotides 8736 to 8738 in the SIV$_{mac}$142 sequence (3; data not shown). These results suggest that replication-competent SIV$_{mac}$ and HIV-2 viruses have considerable flexibility in the location of their env stop codon.

Two rhesus macaques (M. mulatta) were inoculated intravenously with 1 ml of each cloned virus. Blood samples were taken at intervals after virus inoculation, and infection was monitored by the ability to recover virus from PBMC and by antibody response. PBMC were prepared from blood samples by banding over Ficoll-diatrizoate and were cocultivated with human peripheral blood lymphocytes or HUT-78, Molt 4 clone 8, or CEM × 174 cells for virus recovery as described in Materials and Methods. Antibody response was measured by whole-virus enzyme-linked immunosorbent assay (Fig. 4). No antibody response was detected in macaques inoculated with SIV$_{mac}$142 and HIV-2ROD (Fig. 4B and D). An anti-SIV antibody response did clearly develop, however, in macaques that were inoculated with SIV$_{mac}$251 and SIV$_{mac}$239 (Fig. 4A and C). The results shown in Fig. 4 are from plasma obtained 18 weeks postinoculation; similar results were also obtained with plasma taken at 13 weeks postinoculation. Virus was not recovered on all seven attempts from PBMC from each of the macaques that received SIV$_{mac}$142 and HIV-2ROD. Virus was recovered, however, from each of the SIV$_{mac}$239-inoculated macaques on six
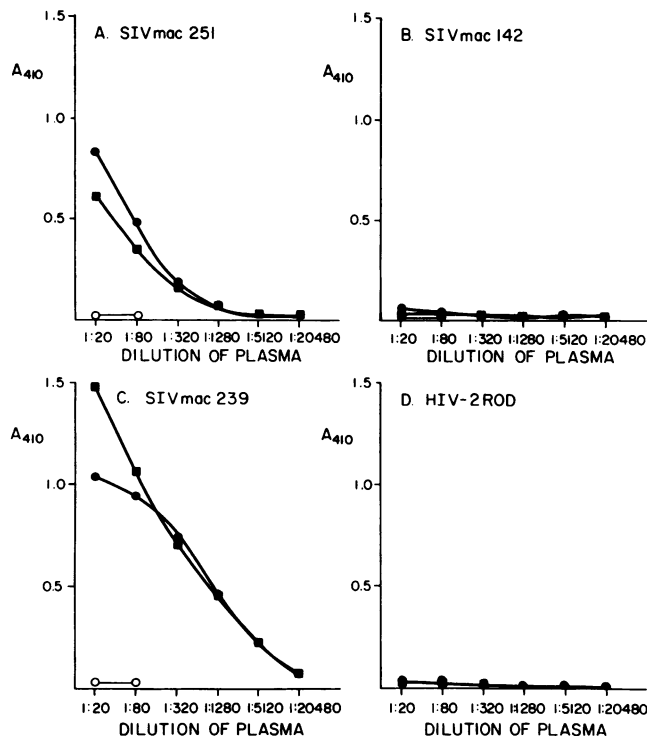
FIG. 4. Antibody responses in rhesus macaques inoculated with cloned virus. Plasma was taken from each macaque on the day of virus inoculation (O) and 18 weeks later (● and ■). Plates coated with SIV$_{mac}$ and HIV-2ROD were prepared for enzyme-linked immunosorbent assay, and the indicated dilutions of plasma were tested for antibody reactivity to SIV$_{mac}$ (A through C) and HIV-2ROD (D) antigen as described previously (7). A_{410} was recorded with a Dynatech enzyme-linked immunosorbent assay reader.

separate occasions, the most recent being 49 weeks postinoculation. Virus was recovered from one of the SIV$_{mac}$251-inoculated macaques at 6 weeks postinoculation and from both of the SIV$_{mac}$251-inoculated macaques at 55 weeks postinoculation; however, the intervening 11 attempts were unsuccessful. These results indicate that macaques inoculated with SIV$_{mac}$239 and SIV$_{mac}$251 became infected, while macaques inoculated with SIV$_{mac}$142 and HIV-2ROD were not infected. Thus, growth of cloned virus in macaque lymphocytes in vitro (Fig. 2F) was predictive of macaque infection in vivo. These results also provide further evidence that the SIV$_{mac}$142 clonal derivative has lost its ability to replicate in macaque cells. The infection with SIV$_{mac}$251 differed somewhat from that of SIV$_{mac}$239 and differed also from what we have seen previously with uncloned SIV$_{mac}$ stocks (6), in that we were not able to reproducibly recover virus over time from the macaques inoculated with cloned SIV$_{mac}$251. Walker et al. (26) and Kannagi et al. (15) have described the use of CD8$^+$ cell depletion to more readily recover virus from CD4$^+$-enriched cell populations in individuals from whom virus recovery was difficult by standard direct cocultivation procedures. At week 32 postinoculation, virus recovery from all eight macaques was attempted by using this technique; virus was recovered from both macaques that received SIV$_{mac}$239 but not from the other six macaques. None of the inoculated macaques that received cloned virus have developed signs of AIDS as yet; the elapsed time (56 weeks) is similar to the median time of death

(>50 weeks) in similar studies with uncloned HUT-78-grown SIV$_{mac}$ (6).

The infectious molecular clones described in this report are likely to prove useful in future studies directed at the molecular basis for pathogenicity, persistence, tropism, and cell and species specificity of the primate lentiviruses. Construction of recombinants between clones with different biological properties will enable us to delineate the molecular basis for differences in these biological properties. The differences in location of the premature stop codon in *env* indicate that there is a certain amount of flexibility in its positioning; how much flexibility and any possible function for the potential coding sequences downstream of the stop codon in the same reading frame must be subject to further investigation. The SIV$_{mac}$239 and SIV$_{mac}$251 infectious molecular clones will be particularly useful for future studies because they induce long-term persistent infection in macaques, as we have seen previously with uncloned SIV$_{mac}$ stocks and as is observed in humans infected with HIV-1. The possible emergence of genetic and antigenic variants with the time of in vivo infection and the effects of mutations on in vivo infection can now be analyzed with this system.

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