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The Cytoplasmic Domain of Simian Immunodeficiency Virus Transmembrane Protein Modulates Infectivity

LISA CHAKRABARTI,[1] MICHAEL EMERMAN,[2]† PIERRE TIOLLAIS,[1] AND PIERRE SONIGO[1]*

Unite de Recombinaison et Expression Génétique (INSERM U163 and CNRS UA271)[1] and Unite d'Oncologie virale (CNRS UA1157),[2] Institut Pasteur, 25, rue du Dr Roux, 75724 Paris Cedex 15, France

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A striking characteristic of the simian immunodeficiency virus (SIV) and of the human immunodeficiency virus type 2 (HIV-2) is the presence of a nonsense mutation in the *env* gene resulting in the synthesis of a truncated transmembrane protein lacking the cytoplasmic domain. By mutagenesis of an infectious molecular clone of $SIV_{mac}142$, we investigated the function of the cytoplasmic domain and the significance of the *env* nonsense mutation. When the nonsense codon (TAG) was replaced by a glutamine codon (CAG), the virus infected HUT78 cells with markedly delayed kinetics. This negative effect was counterselected in vitro as reversion of the slow phenotype frequently occurred. The sequencing of one revertant revealed the presence of a new stop codon three nucleotides 5′ to the original mutation. Deletions or an additional nonsense mutation introduced 3′ to the original stop codon did not modify SIV infectivity. In contrast, the same deletions or nonsense mutation introduced in the clone in which the stop codon was replaced by CAG abolished infectivity. These results indicated that the envelope domain located 3′ to the stop codon is not necessary for in vitro replication. However, the presence of this domain in SIV transmembrane protein leads to a reduced infectivity. This negative effect might correspond to a function controlling the rate of spread of the virus during in vivo infection.

Simian immunodeficiency viruses (SIVs) are primate retroviruses closely related to human immunodeficiency viruses (HIVs) in their genetic structure and in their pathogenicity. SIV was first isolated from captive macaques with immunodeficiency syndrome or lymphomas (6). Subsequently, SIVs were found to infect asymptomatically a variety of Old World primate species including sooty mangabeys (11), African green monkeys (10), and mandrills (29). Therefore, SIV infection provides a valuable animal model for acquired immune deficiency syndrome, allowing the study of both susceptible and resistant monkey species.

The genomic analysis of several SIV isolates of macaque (3, 9, 15) has revealed that SIV_{mac} is much more closely related to HIV type 2 (HIV-2) than to HIV-1, with, respectively, 75% versus 40% overall nucleotidic sequence identity. The recent characterization of the *vpu* gene specific to HIV-1 (5) and of the *vpx* gene specific to HIV-2/SIV (14) further distinguishes between these virus groups.

Another remarkable difference between HIV-1 and HIV-2/SIV resides in the size of the transmembrane protein (TM) encoded by the 3′ part of the *env* gene. For the HIV-1 isolates, the apparent molecular weight of the TM is 41,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis and immunoblotting (7), which is consistent with the molecular weight calculated from sequence data. In contrast, all the SIVs isolated so far present a shorter TM with a mobility ranging from 30 to 35 kilodaltons (kDa). For HIV-2, a short form of the TM is predominant (e.g., 36 kDa for the ROD isolate) (4), but a longer form has also been observed (43 kDa for several cell clones of the slow-growing isolate ST) (17). These differences in size are likely to be due to an in-frame translation termination codon found in the SIV and HIV-2 *env* gene. Of five SIV_{mac} clones sequenced, four have a truncated *env* gene (3, 9, 15, 21). The in-frame stop codon is located precisely at the predicted splice acceptor site for the second coding exon of *tat*, or one codon 5′ to it. In one SIV clone from the African green monkey, the in-frame stop codon is found 90 nucleotides further downstream in the *env* gene (10). Two molecular clones of the HIV-2 ROD isolate differ in that one has a stop codon at the predicted splice site location while the other has no premature termination of the *env* gene (13). Thus, a truncated TM appears to be a frequent but not compulsory feature of isolates of SIV and HIV-2.

The TMs of retroviruses are membrane-spanning proteins that are organized in three functional domains. The N-terminal extracellular domain uncovalently binds to the external envelope glycoprotein (18), the major hydrophobic domain anchors the protein into the lipid bilayer (2), while the C-terminal cytoplasmic domain is of unknown function. For alphaviruses, an interaction between the cytoplasmic domain of the envelope glycoprotein and the assembled nucleocapsid has been demonstrated (30) that is possibly involved in the selective inclusion of viral proteins into the budding membrane. However, this mechanism of budding is not directly applicable to retroviruses, as a mutant of the Rous sarcoma virus that lacks the cytoplasmic domain is able to produce infectious virions (22). The lentiviruses (26), to which HIVs and SIVs belong, have a cytoplasmic domain of unusual length compared with type C and type D retroviruses (25, 27). Because SIV_{mac} and HIV-2 contain a stop codon in the *env* gene, the region corresponding to the cytoplasmic domain constitutes an independent C-terminal open reading frame (ORF), and it has been postulated that a separate function may be encoded by this part of the genome (3, 15).

We investigated the function of the SIV_{mac} envelope cytoplasmic domain by replacing the in-frame stop codon by

* Corresponding author.
† Present address: Fred Hutchinson Cancer Center, Seattle, WA 98104.

a glutamine codon to recreate a full-length TM and by introducing deletions in the *env* C-terminal region in the presence or absence of the in-frame stop codon. This study indicated that the C-terminal ORF is not necessary for in vitro replication and is unlikely to be expressed as a separate protein. However, when fused to the envelope, the cytoplasmic domain causes a reduction in the kinetics of SIV infection. This negative effect is counterselected in vitro but may be required during the latent phase of in vivo infection.

MATERIALS AND METHODS

Constructions and mutagenesis. The *Sal*I-*Aha*III insert of λSIV-1, an infectious clone of $SIV_{mac}142$ (3), was subcloned in a Bluescript vector restricted by *Sal*I and *Sma*I. The resulting plasmid, pKX10, contained a 10-kilobase provirus that lacked the first 257 nucleotides of the 5′ long terminal repeat and contained 130 nucleotides of cellular DNA after the 3′ long terminal repeat.

Mutant proviral clones derived from pKX10 are designated by pTM and the calculated length difference between their TM and that of pKX10 (e.g., pTM+146 encodes for a TM 146 amino acids [aa] longer than that of pKX10). Proviral clones mutated in the C-terminal domain in the presence of the truncating stop codon are designated by pCD followed by the length of the C-terminal domain (e.g., pCD58). Plasmids have the letter "p" before their designations (e.g., pKX10), whereas retroviruses made from those plasmids do not (e.g., KX10).

The 1.8-kilobase *Hin*cII fragment (8028 to 9860) of pKX10 was subcloned into an M13mp18 vector for oligonucleotide-directed mutagenesis (35). In mutant pTM+146, the stop codon TAG at position 8298 was replaced by CAG by using oligonucleotide 5′-TCCAGCAGACCCATACCC-3′ (the mutagenized nucleotide is underlined). In mutant pTM−1, a stop was reintroduced one codon upstream at position 8295 by mutating pTM+146 with oligonucleotide 5′-CCTCTTAT TTCTAGCAGACC-3′. In mutant pCD58, a stop codon was introduced at position 8473 with oligonucleotide 5′-CTTGCT ATAGAGAGCATACC-3′. In the pF− mutant, the octamer *Xho*I linker 5′-CCTCGAGG-3′ was inserted in *nef* at the *Pvu*II site at 8791.

Deletions in the C-terminal domain were made by digesting the *Eco*RI (8530)-*Xba*I (9925) subclone of pKX10 with two restriction enzymes and by reparing the ends with the Klenow fragment of DNA polymerase I prior to ligation. Mutant pCD110 has a *Bgl*II (8618)-*Sac*I (8725) deletion and mutant pCD335 has a *Sac*I (8722)-*Nco*I (8776) deletion. The mutated fragments were sequenced by the dideoxynucleotide-chain termination method of Sanger et al. (24) and reintroduced into pKX10.

Mutant pTM-122 was derived from an *env* subclone that had a spontaneous GG deletion at positions 7924 and 7925. The *Cla*I (7567)-*Eco*RI (8530) fragment of this subclone was sequenced and then exchanged with that of pKX10 to give pTM-122.

In double mutants pTM+110, pTM+335, and pTM+F− the *Eco*RI (8530)-*Eag*I (in Bluescript polylinker) 1.4-kilobase fragment of pTM+146 was replaced with that of mutants pCD110, pCD335, and pF−, respectively. In double mutant pTM+58, the *Bam*HI (8315)-*Eco*RI (8530) fragment of pTM+146 was replaced with that of mutant pCD58.

Cell culture and DNA transfection. SW480, a colon carcinoma cell line obtained from the American Type Culture Collection, Rockville, Md. (ATCC CCL228), was maintained in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum. At 14 to 16 h before transfection, cells were trypsinized and plated at 10^6 cells per 25-cm^2 flask. The cultures were transfected with 15 μg of plasmid DNA by the calcium phosphate precipitation method (32). Supernatants were harvested 24 and 48 h after transfection.

HUT78, a human CD4+ lymphocytic cell line (12), was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Cells were transfected with 3 μg of supercoiled plasmid by the DEAE-dextran method as described by Peterlin et al. (23).

Infections. The infectivity of progeny virions generated from SW480 cell transfections was assayed by infection of the HUT78 cell line. SW480 culture supernatants were diluted to normalize the amount of reverse transcriptase (RT) activity used for each infection. A total of 4×10^6 cells were suspended in 1 ml of supernatant supplemented with 10 μg of Polybrene and incubated for 1 h at 37°C. Cells were pelleted and suspended in 6 ml of RPMI medium containing 10% fetal calf serum and 2 μl of Polybrene per ml. The infected cells were maintained in the same medium at a density of 0.5×10^6 to 1×10^6 cells per ml for at least 3 weeks. RT activity was assayed as described by Willey et al. (33). Briefly, 10 μl of culture supernatant was mixed with 50 μl of an RT reaction mixture containing poly(A) as the template and [^{32}P]dTTP as the substrate. After a 40-min incubation at 37°C, 10 μl of the reaction mixture was spotted onto DE81 paper and washed six times for 5 min each time in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) before drying. The spots were visualized by autoradiography or counted in a beta scintillation counter.

Immunostaining. Samples of infected HUT78 cells were taken every 2 or 3 days to observe the percentage of cells expressing viral antigens. For each time point, 2×10^5 cells were washed in phosphate-buffered saline, spotted on a slide, dried for at least 2 h, and fixed for 15 min in acetone. The cells were labeled by a two-stage immunoperoxidase technique. The slides were first incubated in a 1/1,200 dilution of a human anti-HIV-2 serum and then with a 1/100 dilution of rabbit anti-human antibodies conjugated with peroxidase (Diagnostic Pasteur). Incubations were for 30 min at room temperature, followed by extensive washing in phosphate-buffered saline. Cells were then stained with diaminobenzidine (0.6 mg/ml) and hydrogen peroxide (0.01%) and rinsed in distilled water.

Antipeptide sera. The peptide KYGRWILAIPRRIRQG LELTLL (Neosystem Laboratories) was coupled to keyhole limpet hemocyanin and used as an immunogen in rabbits. Two rabbits were subjected to four injections with the equivalent of 200 μg of peptide each time. Sera were obtained 30 days after the last injection.

Radioimmunoprecipitation of viral proteins. Infected HUT78 cells were preincubated for 1 h in serum-free Eagle medium without cysteine and methionine. The cells were then labeled for 14 to 16 h in the same medium supplemented with 100 μCi each of [^{35}S]cysteine and [^{35}S]methionine per ml. After labeling, cells were pelleted and suspended at a concentration of 5×10^6 cells per 50 μl of NTE buffer (20 mM Tris hydrochloride [pH 7.6], 150 mM NaCl, 1 mM EDTA, 0.05% aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, 7 mM 2-mercaptoethanol). After 5 min on ice, 50 μl of lysis buffer (2% Triton X-100 in NTE) was added and the tube was left on ice for 5 min before centrifugation at 12,000 rpm for 5 min in an Eppendorf centrifuge. The soluble fraction was stored at −70°C and used for immunoprecipi-

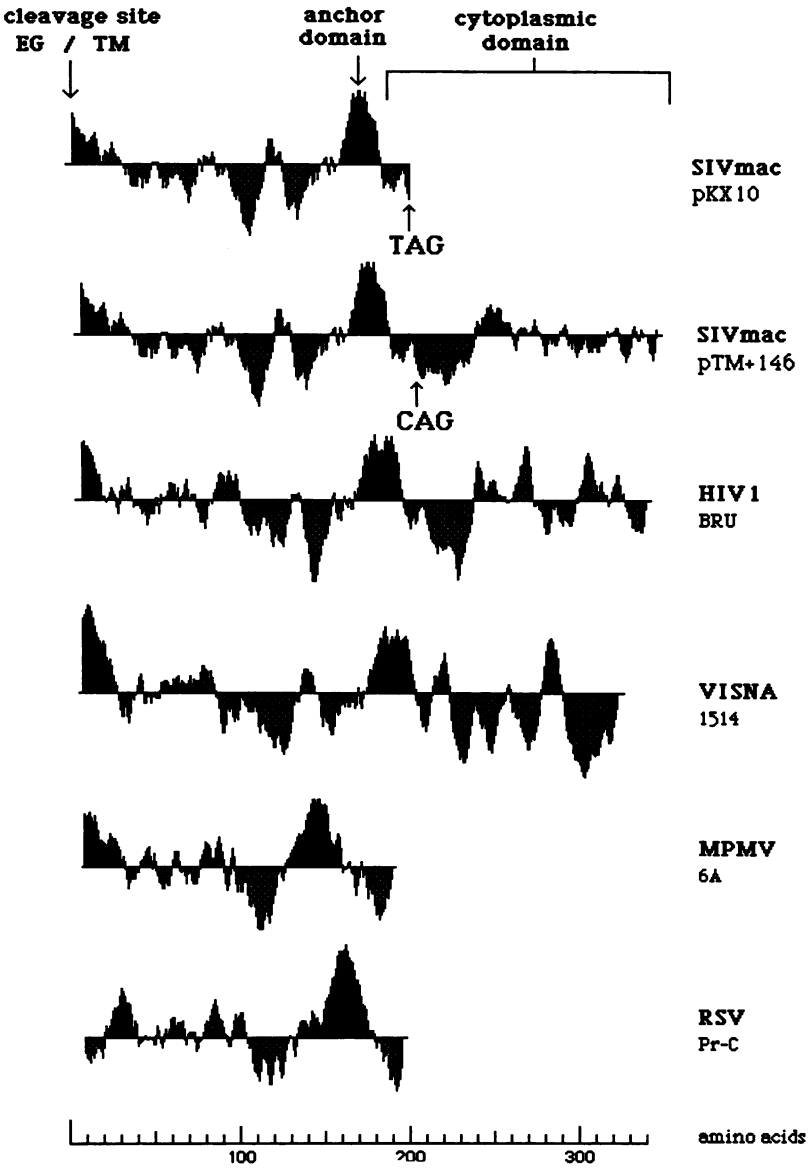

FIG. 1. Hydrophobicity profile of the TM for different retroviruses: SIV_{mac} (3), HIV-1 (31), the ungulate lentivirus visna (26), and the Mason-Pfizer monkey virus (MPMV) (27) representative of type D retroviruses and Rous sarcoma virus (RSV) (25) representative of type C retroviruses. For SIV_{mac}, the *env* in-frame stop codon TAG of clone pKX10 and the corresponding mutation CAG of pTM+146 are indicated. The profile was obtained by the method of Kyte and Doolittle (19) with a step size of 1 and an averaging window of 10. Hydrophobic regions appear above the line, and hydrophilic regions are below. The two major hydrophobic domains are represented in black.

tation. For each reaction, 25 μl of cell extract was preincubated for 2 h at 4°C with a human control serum, cleared with 25 μl of protein A-Sepharose suspension, and then incubated overnight at 4°C with 3 μl of the indicated serum (a human anti-HIV-2 serum or a rabbit antipeptide serum). A 50-μl portion of protein A-Sepharose suspension was added for 2 h, pelleted, and washed four times in buffer (1% Nonidet P-40, 0.2% sodium deoxycholate, 120 mM NaCl, 20 mM Tris hydrochloride, pH 8.0). The precipitated proteins were solubilized by boiling in 50 μl of loading buffer (20% glycerol, 10% 2-mercaptoethanol, 4.6% sodium dodecyl sulfate, 125 mM Tris hydrochloride, pH 6.8) and resolved on a sodium dodecyl sulfate-7.5% polyacrylamide gel.

Molecular cloning of one revertant virus. Unintegrated DNA (16) was obtained from one of the TM+146-infected cultures which had phenotypically reverted. The SIV_{mac} envelope fragment from positions 8235 to 8739 was amplified by the polymerase chain reaction technique. The coding strand primer 5′-ATGCTAGCTAGGTTAAGACAGGGG-3′ contained an *Nhe*I site and the reverse strand primer 5′-CTCACAAGAGAGTGAGCTCAAGCC-3′ contained a *Sac*I site. The polymerase chain reaction was performed with Taq DNA polymerase (Cetus) according to the recommendations of the manufacturer. The samples were subjected to 40 cycles of amplification with an annealing temperature of 50°C. Uninfected HUT78 DNA and water were tested in every experiment as negative controls. The amplification product was restricted by *Nhe*I and *Sac*I prior to ligation in an M13 vector (M13mp18 or M13mp19) restricted by *Xba*I and *Sac*I. Five M13 recombinant clones issued from two separate amplification reactions were sequenced to characterize the reversion.

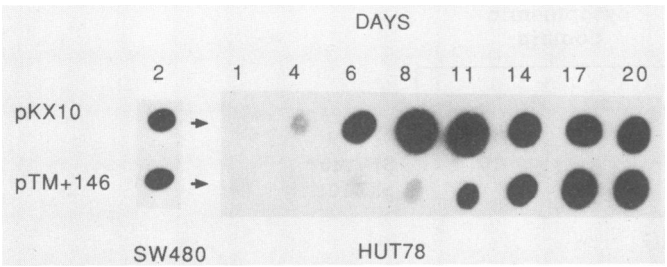

FIG. 2. Comparison of the infectivity of virions recovered after transfection of SIV proviral clones with (pKX10) and without (pTM+146) the in-frame stop codon in the envelope. RT activity was detected in 10-μl supernatant samples after calcium phosphate-mediated transfection of SW480 cells. Day 2 supernatants containing the same amount of RT activity were used to infect HUT78 cells, which are susceptible to SIV infection.

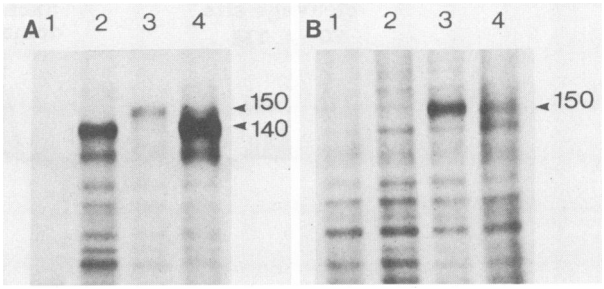

FIG. 3. Immunoprecipitation analysis of the envelope precursor of SIV clone KX10 and of mutant TM+146. ^{35}S-labeled protein extracts from infected HUT78 cells were immunoprecipitated and resolved on a 7.5% acrylamide gel. (A) Immunoprecipitation with a human anti-HIV-2 serum. (B) Immunoprecipitation with an antipeptide antibody raised against the last 20 aa of the C-terminal domain of SIV envelope. Lanes: 1, mock-infected cells; 2, SIV KX10; 3, mutant TM+146; 4, revertant of TM+146. The positions of the 140-kDa form and of the mutant 150-kDa form of the envelope precursor are indicated by arrowheads.

RESULTS

SIV without stop codon has delayed kinetics of infection. In a first series of experiments, we compared the infectivity of SIV_{mac} with a truncated form and SIV_{mac} with a full-length form of TM. The original clone pKX10 encodes for a truncated TM of 208 aa, which is comparable in size to the TM of type D and type C retroviruses (Fig. 1). By oligonucleotide-directed mutagenesis, the *env* in-frame stop codon TAG of pKX10 was replaced by a glutamine codon CAG at position 8473 (as numbered in the SIV_{mac}142 sequence [3]). CAG was chosen as a replacement codon in pTM+146 because the same glutamine codon is found at the equivalent position in an HIV-2 clone (13). The resulting mutant clone pTM+146 could encode for a TM 146 aa longer, equivalent in size to that of HIV-1 and of ungulate lentiviruses (Fig. 1).

The infectivity of the SIV with or without the stop codon was assayed in two steps. First, the colon carcinoma cell line SW480 was transfected with the proviral DNA clones. This cell line allows a transient viral expression at a high level but is not efficiently infected by the progeny virions. In a second step, the supernatant of the SW480 transfected cell culture was used to infect the T-cell line HUT78, which is susceptible to SIV infection. In this way, we assayed first viral gene expression and then the cell-free infectivity of the viruses.

In four separate experiments, an equivalent amount of RT activity was recovered at day 2 in the supernatant of SW480 cells transfected with pKX10 and with pTM+146 (Fig. 2). This indicates that, after transient expression, the number of released viral particles is equivalent for the original clone and for the mutant. SW480 cell supernatants containing an equal amount of RT activity were used to infect HUT78 cells. In each of four separate infections, we found that the kinetics of infection was significantly delayed for the mutant TM+146 compared with the wild-type KX10. The mean delay was 5 days for the appearance of syncytia in the culture and 16 days for the peak of RT activity. Figure 2 shows the RT activity obtained for a representative experiment. The RT assay was quantified by determining the amount of radioactivity in each spot in a scintillation counter (see Fig. 5A). For this experiment, the peak of RT activity is at day 8 for KX10 and at day 23 for TM+146. When the initial inoculum was lowered by one-half, the difference in the kinetics of infection was increased: the RT activity peaked at day 10 for KX10 and at day 33 for TM+146 (data not shown).

Infectivity was also assayed by direct transfection of HUT78 cells. This method allows a massive infection of the culture and initial cell-to-cell propagation of the virus. In the transfected HUT78 cultures, the RT activity peaked at day 7 for KX10 and at day 12 for TM+146 (data not shown). Thus, when the initial number of infected cells was high, the difference in the kinetics of infection by the wild-type SIV clone and by mutant TM+146 decreased.

The rate of infection was observed by immunostaining cells for SIV-encoded antigens. For each time point, we counted the percentage of cells reactive with a human HIV-2 antiserum that cross-reacts with SIV_{mac} antigens. We found that the number of cells expressing viral antigens increased much more slowly for mutant TM+146 than for the wild-type virus. As shown in Fig. 5B, 50% of the cells were labeled at day 6 after infection by KX10, while it took until day 17 to reach the same number of infected cells after infection by TM+146. Taken together, these results show that the SIV mutant without the stop is less infectious than the wild-type clone.

To ensure that no spontaneous mutation occurred during plasmid construction and was responsible for the phenotype of mutant TM+146, we replaced the *Cla*I (7567)-*Eco*RI (8530) fragment of pTM+146 with that of pKX10. The resulting clone, pKX22, gave the same kinetics of infection as pKX10 (data not shown). The 1-kilobase *Cla*I-*Eco*RI fragment of pTM+146 was also cloned and resequenced. No mutation was found other than that introduced at the stop codon location.

SIV viruses with a truncated form of TM are selected for during tissue culture. Supernatants of a HUT78 cell culture infected with mutant TM+146 were obtained at days 15, 21, and 27 and measured for RT activity. The same amount of counts per minute for each supernatant was used to reinfect HUT78 cells. The infection with the day 15 supernatant had the slow kinetics characteristic of TM+146 (the RT activity peaked at day 20), while the infections with day 21 and 27 supernatants had the same kinetics as obtained with the original virus KX10 (the RT activity peaked at day 7). Thus, virus taken at late times after infection had reverted to a rapid phenotype.

The revertant viruses could be characterized by radioimmunoprecipitation with an HIV-2 antiserum (Fig. 3A). As the TM of the SIVs is weakly radiolabeled with [^{35}S]cysteine and [^{35}S]methionine, we compared the size of the envelope precursor which includes both the extracellular glycoprotein

and the TM. Two forms of the envelope precursor could be separated on a 7.5% acrylamide gel. In cells infected with SIV KX10, the envelope precursor was found to have an apparent molecular weight of 140,000. In cells infected with mutant TM+146, the precursor had an apparent molecular weight of 150,000, consistent with the C-terminal domain being fused to the rest of the envelope. In another batch of cells infected with TM+146 (Fig. 3A, lane 4), the two forms of precursor coexisted, with a predominance of the 140-kDa protein. This mixed population indicated the occurrence of a revertant with a truncated TM. To confirm these results, we immunoprecipitated the same infected cell extracts with a rabbit antiserum raised to the last 20 aa of the C-terminal domain. This serum (Fig. 3B) was specific only for the large form of the envelope precursor found for mutant TM+146.

In repeated experiments, we found that reversion of the slow phenotype frequently occurred after 3 weeks of infection. Of seven TM+146-infected cultures analyzed between days 21 and 30 of infection, three had only the 150-kDa envelope precursor and four had recovered a 140-kDa form of envelope precursor or a mixture of the two forms.

To characterize the reversion at the genotype level, we extracted the unintegrated viral DNA from one of the TM+146-infected cultures which exhibited a rapid phenotype and a truncated envelope. We used the polymerase chain reaction to amplify a 500-base-pair fragment between the *Nhe*I site (8237) and the *Sac*I site (8725) of SIV_{mac} envelope. This fragment was cloned in an M13mp18 vector and sequenced. We found that the original stop-to-glutamine mutation at 8298 was conserved. However, a C-to-T transition at 8295 created a new stop codon immediately 5′ to the glutamine codon. These results showed that in-frame stop codons in the SIV envelope are selected for during in vitro infection.

C-terminal ORF not necessary for in vitro infectivity. Although the full-length TM is not needed in culture, it remained possible that sequences 3′ to the stop codon contained an exon of an independent gene needed for viral growth. Thus, we constructed three mutants of SIV pKX10 to inactivate possible products of the C-terminal ORF (Fig. 4). For pCD58, a second termination codon was introduced at 8473, truncating the C-terminal ORF after 57 aa. The mutation was chosen to keep the amino acid sequence of the overlapping *rev* reading frame unmodified. Mutant pCD110 has a 36-aa deletion before the last 5 aa of the C-terminal ORF, which also deletes 36 aa of the overlapping *nef* gene. In mutant pCD335, the deletion leads to a fusion of the first 140 aa of the C-terminal ORF with the last 193 aa of *nef* (an extra alanine codon being created at the junction).

For the mutations of pCD110 and pCD335, we needed to distinguish between the effects on the C-terminal and on the *nef* ORF. Therefore, we made a frameshift mutant with *nef* inactivated but without changes in the C-terminal ORF. pF− was derived from pKX10, with an octamer linker inserted after the first 73 aa of *nef* so that the protein would stop after three supplementary linker-encoded amino acids. We also constructed the double mutant pTM+F−, which has both the *env* in-frame stop codon removed and the linker inserted in *nef*.

We found that the F− mutant had the same kinetics of infection as the wild-type KX10. The double mutant TM+F− had a slow kinetics of infection similar to that of TM+146, as measured by RT activity or by immunostaining (Fig. 5A and B). Thus, in contrast to what has been reported for *nef* mutants of HIV-1 (20), *nef* inactivation in this SIV_{mac} clone did not lead to a higher infectivity.

Since the mutations in *nef* did not modify the kinetics of infection, we could evaluate separately the effect of the C-terminal ORF mutations. Each of the mutants CD58, CD110, and CD335 was found to be infectious by DEAE-dextran transfection of HUT78 cells. When biological activity was assayed by SW480 transfection and then infection of HUT78 with the supernatants, the three mutants displayed the same kinetics of infection as the wild-type SIV KX10 (Fig. 5C and D). In addition, we could not detect a viral protein specific to the C-terminal ORF by radioimmunoprecipitation with the antipeptide serum (data not shown). These results indicated that the C-terminal ORF is not necessary to SIV infectivity in vitro.

Length of SIV TM is critical for infectivity. To further assess the extent to which the structure of the TM modulates SIV infectivity, we constructed mutants with a TM of intermediate length. As a negative control, we made a mutation that severely deleted the TM. pTM−122 had a frameshift mutation at 7924 that caused the TM to stop after 86 aa, preventing the anchor domain from being translated. As expected, mutant TM-122 was noninfectious (Fig. 5A and B), indicating that the TM is needed for SIV infectivity.

To construct a mutant with a TM shorter by 1 aa than that of SIV KX10, we made a second point mutation in the sequence of pTM+146 which restored an *env* in-frame stop codon one codon 5′ to the original one. Mutant TM-1 was found to have the same kinetics of infection as the wild type (Fig. 5A and B), which showed that some flexibility is allowed in the stop codon location for full infectivity. Furthermore, *env* in-frame stop codons located as in mutant TM-1 can be selected for during in vitro culture, as the revertant of TM+146 we characterized (see above) had the same sequence as mutant TM−1.

To consider the effects of a partially truncated cytoplasmic domain, we constructed double mutants with both the *env* in-frame stop codon replaced by CAG and the C-terminal region mutated. The fragments mutated in pCD58, pCD110, and pCD335 were used to replace the equivalent unmutated fragments in pTM+146 to give pTM+58, pTM+110, and pTM+335, respectively. These mutant clones can encode for a TM of increasing length (Fig. 4).

None of the mutants TM+58, TM+110, and TM+335 was found to be infectious by direct transfection of HUT78 cells (data are shown). When transfected in SW480 cells, they gave the same amount of RT activity in the supernatant as the wild-type, but these supernatants were not infectious for HUT78 cells (data are shown only for TM+58; Fig. 5A and B). Since the immunostaining technique allows one to distinguish one stained cell from 10,000 cells spotted on each slide, the fact that no infected cell could be detected by this method indicates that infectivity is reduced by at least 10^4 for mutants TM+58, TM+110, and TM+335. The unexpected finding was that TM+58 and TM+110 were noninfectious, whereas TM+146 was still infectious. Therefore, the fusion of a truncated cytoplasmic domain to SIV TM seems more detrimental to infectivity than the fusion of an entire cytoplasmic domain.

DISCUSSION

A distinctive feature of the sequence of several infectious clones of SIV is the presence of a stop codon in the ORF encoding the envelope gene. The stop codon separates the cytoplasmic domain of the TM from the rest of the envelope. We investigated the significance of this naturally occurring stop codon in the SIV envelope gene and the role of the

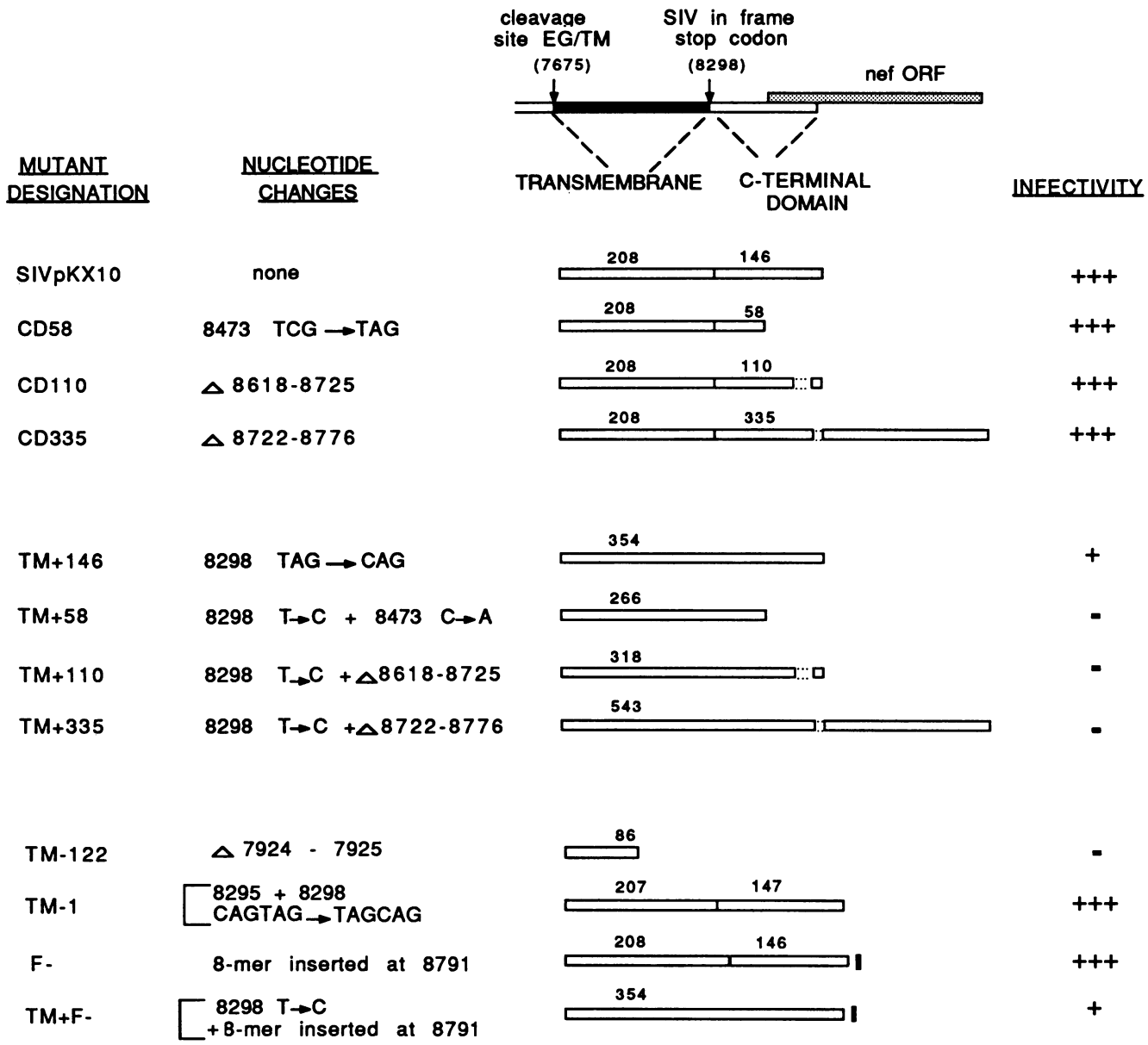

FIG. 4. Nucleotide changes and schematic representation of the SIV mutants derived from clone pKX10. The position of each mutation is numbered according to the $SIV_{mac}142$ nucleotidic sequence (3). The regions of *env* coding for the TM and for the C-terminal domain are represented by open boxes, and their calculated length in amino acids is reported above each box. The level of infection of HUT78 cells by the SIV mutants is summarized on the right side of the figure. Δ, Deletion.

cytoplasmic domain by comparing viruses derived from mutated infectious clones. We showed that while the C-terminal ORF (defined as those sequences 3′ to the stop codon in the same phase as the envelope gene) is not essential for growth of the virus in a human T-cell line, when these sequences are fused to the rest of the envelope gene by removing the stop codon, they can inhibit virus growth.

The C-terminal ORF delineated by the *env* in-frame stop codon contains no methionine that could initiate a protein. Nonetheless, because the ORF is intact after the stop codon, it was suggested that an independent product is encoded by this region. However, we found that mutants with premature termination (CD58) or internal deletions (CD110, CD335) are fully infectious in vitro (Fig. 5C and D). Furthermore, an antipeptide serum raised against the predicted C-terminal amino acids of the ORF reacted with the envelope precursor produced by the virus in which the stop codon was removed but failed to detect a specific product of the virus with the stop codon (Fig. 3). Thus, it is unlikely that the C-terminal ORF is expressed as a separate protein that would result from unusual splicing or from a suppression event at the stop codon location. While our results do not rule out that a minor product of the C-terminal ORF is produced that we do not detect, they do show that this region is not necessary in tissue culture.

We found that the cytoplasmic domain has a inhibitory effect on infectivity since virus in which the stop codon was replaced such that the entire TM would be produced (mutant TM+146) grew slowly in culture (Fig. 2). Revertant viruses that had mutated to recover a stop codon frequently over-

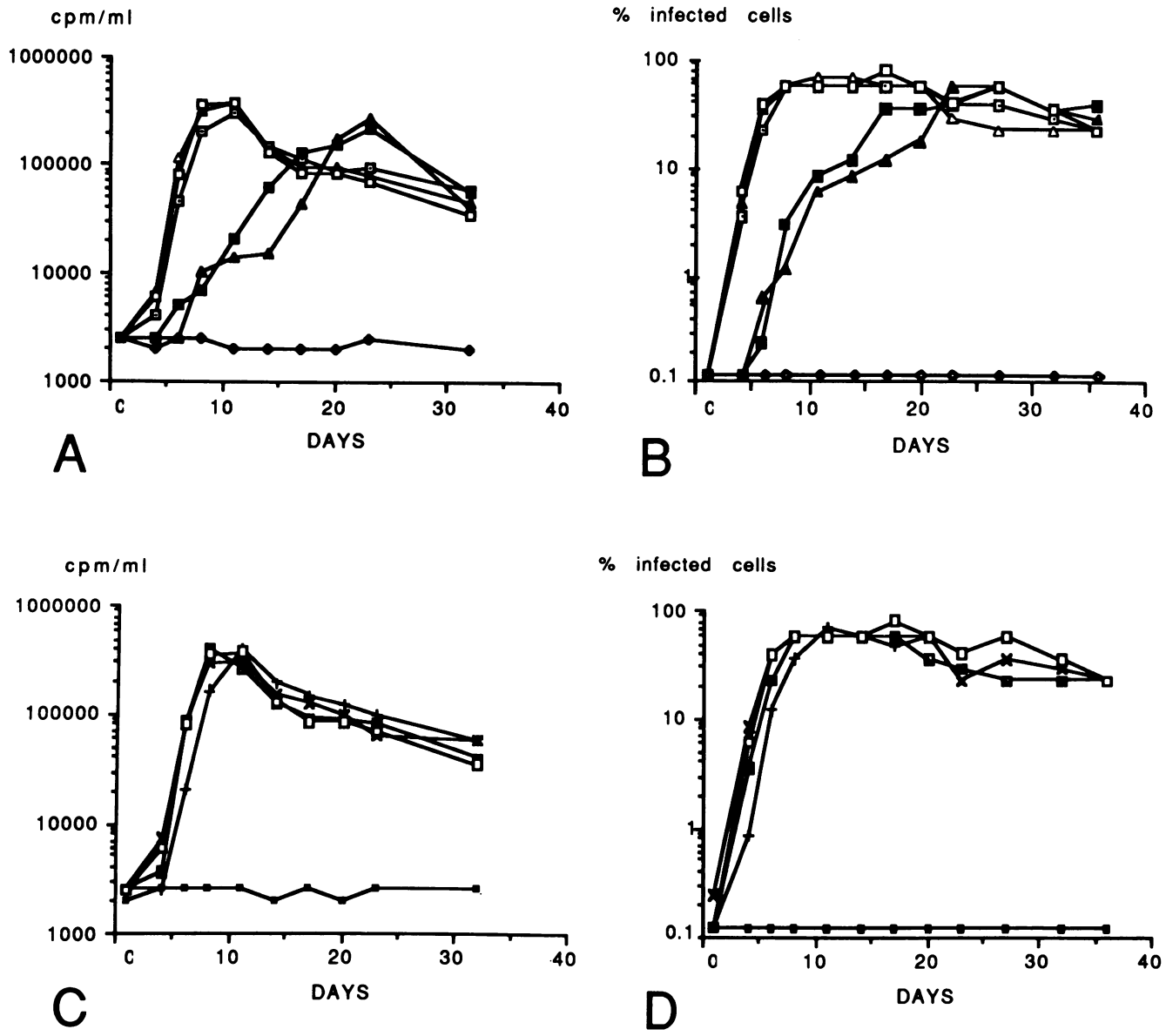

FIG. 5. Kinetics of infection of HUT78 cells by the SIV mutants. Supernatants recovered from transfected SW480 cells were used to infect the T-cell line HUT78. For this representative experiment, infectivity was assayed for the wild-type SIV clone pKX10 and for nine of the mutants. Results are reported twice for pKX10 to allow comparisons between the top and bottom part of the figure. The infection was monitored by assaying the RT activity in the culture supernatants (A and C) and by determining the number of cells expressing viral antigens by immunostaining with an anti-HIV-2 serum (B and D). Symbols: □, pKX10; △, pF−; ⊡, pTM−1; ■, pTM+146; ▲, pTM+F−; ◇, pTM−122; +, pCD58; ×, pCD110; □, pCD335; ▪, pTM+58.

grew the original population (Fig. 3). These results suggest that the stop codon truncating the SIV *env* gene arises by in vitro selection for fast-growing viruses in the T-cell line HUT78. A comparable phenomenon was found for the *nef* gene of HIV-1, a negative regulator (1, 20) which is also mutated in most of the molecular clones obtained after in vitro culture. Thus, selection for growth in vitro may significantly alter the population of rescued virus from an infected host.

Although not necessary in a human cell line, it is quite possible that the cytoplasmic domain of the envelope protein has an essential role in vivo. The fact that a long cytoplasmic domain distinguishes the TM of lentiviruses from other retroviruses (Fig. 1) suggests that this region carries a function which is important in lentiviral infection. The facts that at least one molecular clone of SIV does not contain the stop codon (21) and that the cytoplasmic domain of SIV envelope is highly conserved at its most 3′ end with both HIV-1 and HIV-2 further support this hypothesis. It may be significant that while virus produced from the SIV-142 molecular clone (which contains the stop codon) grows to high titers in tissue culture, it fails to infect macaque monkeys (21). The down-modulatory effects of the cytoplasmic domain that we observed in a T-cell line in vitro may correspond to a similar effect in the infected host and may serve as a mechanism controlling the rate of spread of the

virus. That is, in vivo, there could be a selection against virus which grew too quickly, perhaps because this virus fails to establish a latent phase and is eliminated by the host immune response.

The mechanism by which the cytoplasmic domain of the TM has a inhibitory effect on virus growth is unknown at present. It does not act at the level of general viral protein expression, since clones with or without the truncating stop codon produced the same amount of RT activity after transient expression in SW480 cells (Fig. 2). However, the presence of the cytoplasmic domain may delay the processes leading to the production of infectious viral particles because of impaired intracellular transport of the TM or impaired conformation of an important functional domain (for example, the one that mediates cell fusion at the N-terminal extremity of the TM). Alternatively, the cytoplasmic domain itself may have a down-regulatory function (for example, because of its location, in the transduction of signals through the membranes of infected cells).

We observed that SIV mutants containing a partially truncated cytoplasmic domain have completely lost infectivity (Fig. 5C and D). This seems paradoxical compared with the observation that SIV deprived of the entire cytoplasmic domain is fully infectious and SIV with a full-length cytoplasmic domain retains a reduced infectivity. However, similar results have been described for mutants of the Rous sarcoma virus: a punctual mutation introducing a stop codon just before the cytoplasmic domain did not modify viral infectivity (22), whereas progressive deletions in the cytoplasmic domain correlated with a reduction in the rate of intracellular transport of the truncated protein (34). These results suggest that the cytoplasmic domain is composed of several complementary subdomains which must be either simultaneously present or simultaneously absent.

The stop codon of the original clone pKX10 is located next to the predicted splice acceptor site used for the *tat* and *rev* and mRNAs. However, it is unlikely that the replacement of the stop codon TAG by a glutamine codon CAG modified the balance of splicing for these regulatory genes because mutant TM-1, which has also the T-to-C transition in the splice consensus sequence, retained full infectivity (Fig. 5A and B). The stop codon would not be expected to be found further upstream, as this would truncate the hydrophobic anchor domain of the TM, nor further downstream, as this mutation would not be viable (Fig. 4).

A regulatory role for the cytoplasmic domain of the envelope of HIV-1 has also been suggested (8, 28). Genetic studies showed that a mutant deleted of the last 14 aa of the cytoplasmic domain was impaired for T-cell killing (8), while a mutant deleting the last 43 aa of envelope had a markedly reduced infectivity (28). However, the absence of envelope truncation in any sequenced HIV-1 infectious clone suggests that the function of the TM or, more likely, the structure-function relationships of the different envelope domains are different in HIV-1 than in SIV/HIV-2. The elucidation of the functions of the different domains of the envelope gene in vivo will be important to understanding the pathogenesis of the immunodeficiency viruses.

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LITERATURE CITED

1. **Ahmad, N., and S. Venkatesan.** 1988. Nef protein of HIV-1 is a transcriptional repressor of HIV-1 LTR. Science **241**:1481–1485.
2. **Berman, P. W., W. M. Nunes, and O. K. Haffar.** 1988. Expression of membrane-associated and secreted variants of human immunodeficiency virus type 1 in vitro and in continuous cell lines. J. Virol. **62**:3135–3142.
3. **Chakrabarti, L., M. Guyader, M. Alizon, M. D. Daniel, R. C. Desrosiers, P. Tiollais, and P. Sonigo.** 1987. Sequence from simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. Nature (London) **328**:543–547.
4. **Clavel, F., D. Guétard, F. Brun-Vésinet, S. Chamaret, M.-A. Rey, M. O. Santos-Ferreira, A. Laurent, C. Dauguet, C. Katlama, C. Rouzioux, D. Klatzmann, J. L. Champalimaud, and L. Montagnier.** 1986. Isolation of a new retrovirus from West African patients with AIDS. Science **233**:343–346.
5. **Cohen, E. A., E. F. Terwilliger, J. G. Sodroski, and W. A. Haseltine.** 1988. Identification of a protein encoded by the *vpu* gene of HIV1. Nature (London) **334**:532–534.
6. **Daniel, M. D., N. L. Letvin, N. W. King, M. Kannagi, P. K. Sehgal, R. D. Hunt, P. J. Kanki, M. Essex, and R. C. Desrosiers.** 1985. Isolation of a T-cell tropic HTLVIII-like retrovirus from macaques. Science **228**:1201–1204.
7. **DiMarzoveronese, F., A. L. De Vico, T. D. Copeland, S. Oroszlan, R. C. Gallo, and M. G. Sarngadharan.** 1985. Characterization of gp41 as the transmembrane protein coded by the HTLV-III/LAV envelope gene. Science **229**:1403–1405.
8. **Fisher, A. G., L. Ratner, H. Mitsuya, L. M. Marselle, M. E. Harper, S. Broder, R. C. Gallo, and F. Wong-Staal.** 1986. Infectious mutants of HTLV-III with changes in the 3′ region and markedly reduced cytopathic effects. Science **223**:655–659.
9. **Francini, G., C. Gurgo, H.-G. Guo, R. C. Gallo, E. Collati, K. A. Fargnoli, L. F. Hall, F. Wong-Staal, and M. S. Reitz, Jr.** 1987. Sequence of simian immunodeficiency virus and its relationship to human immunodeficiency viruses. Nature (London) **328**: 539–543.
10. **Fukasawa, M., T. Miura, A. Hasegawa, S. Morikawa, H. Tsujimoto, K. Miki, T. Kitamura, and M. Hayami.** 1988. Sequence of simian immunodeficiency virus from African green monkey, a new member of the HIV/SIV group. Nature (London) **333**: 457–461.
11. **Fultz, P. N., H. M. McLure, D. C. Anderson, R. B. Swenson, R. Anand, and A. Srinivasan.** 1986. Isolation of a T-lymphotropic retrovirus from naturally infected sooty mangabey monkeys (*Cercocebus atys*). Proc. Natl. Acad. Sci. USA **83**:5286–5290.
12. **Gazdar, A. F., D. N. Carney, P. A. Bunn, E. K. Russell, E. S. Jaffe, G. P. Schechter, and J. G. Guccion.** 1980. Mitogen requirements for the *in vitro* propagation of cutaneous T-cell lymphomas. Blood **55**:409–417.
13. **Guyader, M., M. Emerman, P. Sonigo, F. Clavel, L. Montagnier, and M. Alizon.** 1987. Genome organization and transactivation of the human immunodeficiency virus type 2. Nature (London) **326**:662–669.
14. **Henderson, L. E., R. C. Sowder, T. D. Copeland, R. E. Benveniste, and S. Oroszlan.** 1988. Isolation and characterization of a novel protein (X-ORF product) from SIV and HIV2. Science **241**:199–201.
15. **Hirsch, V., N. Riedel, and J. I. Mullins.** 1987. Genome organization of STLV-3 is similar to that of the AIDS virus except for a truncated transmembrane protein. Cell **49**:307–319.
16. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:364–369.
17. **Kong, L. I., S.-W. Lee, J. C. Kappes, J. S. Parkin, D. Decker, J. A. Hoxie, B. H. Hahn, and G. M. Shaw.** 1988. West African HIV2-related human retrovirus with attenuated cytopathogenicity. Science **240**:1525–1529.
18. **Kowalski, M., J. Potz, L. Basiripour, T. Dorfman, W. C. Goh, E. Terwilliger, A. Dayton, C. Rosen, W. Haseltine, and J. Sodroski.** 1987. Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. Science **237**:1351–1355.
19. **Kyte, J., and R. Doolittle.** 1982. A simple method for displaying

the hydrophobic character of a protein. J. Mol. Biol. **157:** 105–132.

20. **Luciw, P. A., C. Cheng-Mayer, and J. A. Levy.** 1987. Mutational analysis of the human immunodeficiency virus: the *orf-B* region down-regulates virus replication. Proc. Natl. Acad. Sci. USA **84:**1434–1438.
21. **Naidu, Y. M., H. W. Kestler III, Y. Li, C. V. Butler, D. P. Silva, D. K. Schmidt, C. D. Troup, P. K. Sehgal, P. Sonigo, M. D. Daniel, and R. C. Desrosiers.** 1988. 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}. J. Virol. **62:**4691–4696.
22. **Perez, L. G., G. L. Davis, and E. Hunter.** 1987. Mutants of the Rous sarcoma virus envelope glycoprotein that lack the transmembrane anchor and cytoplasmic domains: analysis of intracellular transport and assembly into virions. J. Virol. **61:**2981–2988.
23. **Peterlin, B. M., P. A. Luciw, P. J. Barr, and M. D. Walker.** 1986. Elevated levels of mRNA can account for the transactivation of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **83:**9734–9738.
24. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
25. **Schwarz, D. E., R. Tizard, and W. Gilbert.** 1983. Nucleotide sequence of the Rous sarcoma virus. Cell **32:**853–869.
26. **Sonigo, P., M. Alizon, K. Staskus, D. Klatzmann, S. Cole, O. Danos, E. Retzel, P. Tiollais, A. Haase, and S. Wain-Hobson.** 1985. Nucleotide sequence of the visna lentivirus: relationship to the AIDS virus. Cell **42:**369–382.
27. **Sonigo, P., C. Barker, E. Hunter, and S. Wain-Hobson.** 1986. Nucleotide sequence of Mason-Pfizer monkey virus: an immunosupressive D-type retrovirus. Cell **45:**375–385.
28. **Terwilliger, E., J. G. Sodroski, C. A. Rosen, and W. A. Haseltine.** 1986. Effects of mutations within the 3′ *orf* open reading frame region of human T-cell lymphotropic virus type III (HTLV-III/LAV) on replication and cytopathogenicity. J. Virol. **60:**754–760.
29. **Tsujimoto, H., R. W. Cooper, T. Kodama, M. Fukasawa, T. Miura, Y. Ohta, K.-I. Ishikawa, M. Nakai, E. Frost, G. Roelants, J. Roffi, and M. Hayami.** 1988. Isolation and characterization of simian immunodeficiency virus from mandrills in Africa and its relationship to other human and simian immunodeficiency viruses. J. Virol. **62:**4044–4050.
30. **Vaux, J. T., A. Helenius, and I. Mellman.** 1988. Spike-nucleocapsid interaction in Semliki Forest virus reconstructed using network antibodies. Nature (London) **336:**36–42.
31. **Wain-Hobson, S., P. Sonigo, O. Danos, S. Cole, and M. Alizon.** 1985. Nucleotide sequence of the AIDS virus, LAV. Cell **40:**9–17.
32. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlsaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenine phosphoribosyl transferase locus into mammalian cells. Proc. Natl. Acad. Sci. USA **76:**1373–1376.
33. **Willey, R. L., D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin.** 1988. In vitro mutagenesis identifies a region within the envelope gene of human immunodeficiency virus that is critical for infectivity. J. Virol. **62:**139–147.
34. **Wills, J. W., R. V. Srinivas, and E. Hunter.** 1984. Mutations of Rous sarcoma virus *env* gene that affect the transport and subcellular location of the glycoprotein products. J. Cell Biol. **99:**2011–2023.
35. **Zoller, M. J., and M. Smith.** 1984. Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single stranded DNA template. DNA **3:**479–488.