JOURNAL OF VIROLOGY, Feb. 1990, p. 742–747
0022-538X/90/020742-06$02.00/0

Vol. 64, No. 2

Mutational Analysis of the Human Immunodeficiency Virus Type 2 (HIV-2) Genome in Relation to HIV-1 and Simian Immunodeficiency Virus SIV_{AGM}

RIRI SHIBATA,[1] TOMOYUKI MIURA,[2] MASANORI HAYAMI,[2] KOJI OGAWA,[1] HIROYUKI SAKAI,[1] TAKAHIRO KIYOMASU,[1] AKINORI ISHIMOTO,[1] AND AKIO ADACHI[1]*

Laboratory of Gene Analysis, Department of Viral Oncology,[1] and Research Center for Immunodeficiency Virus,[2] Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606, Japan

Received 19 July 1989/Accepted 30 October 1989

We constructed an infectious molecular clone of the human immunodeficiency virus type 2 (HIV-2) and generated nine frameshift mutants corresponding to nine open reading frames identified so far. Three structural (*gag*, *pol*, *env*) and two regulative (*tat*, *rev*) gene mutants were not infectious, whereas *vif*, *vpx*, *vpr*, and *nef* genes were dispensable for infectivity. All of the mutants except *env* and *rev* were cytopathic in $CD4^+$ human leukemia cells. In transfection assays, the expression of HIV-2 long terminal repeat was activated by infectious clones of HIV-1, HIV-2, and simian immunodeficiency virus from African green monkey but not by the *tat* mutants. However, an HIV-2 *tat* mutant could produce small amounts of virus proteins and particles in contrast to a *rev* mutant, which directed no detectable synthesis of virus proteins and virions.

Human immunodeficiency virus type 2 (HIV-2) shares many of the same biological properties with the well-studied HIV-1. Both are associated with acquired immunodeficiency syndrome (AIDS) in humans, are cytopathic in tissue culture, and have a tropism for $CD4^+$ subset of T cells, macrophages, and other cells (6, 23). Concordantly, their genome organization is quite similar and unique among retroviruses (15, 18, 21, 22, 30, 34). The common feature of HIV genomes is the abundance of open reading frames (ORFs) not found in other retroviruses. In addition to three structural genes (*gag*, *pol*, *env*), there are several extra ORFs. ORFs designated as *vif*, *vpr*, *tat*, *rev*, and *nef* are common to HIV-1 and HIV-2. Functional analyses of the HIV-1 genome have revealed the functions of these ORFs (3–5, 8, 9, 17, 19, 24–26, 28, 29). In spite of the structural similarity of the genome, the nucleotide sequences and predicted amino acid sequences of these two viruses differ significantly, and furthermore, there is an unique ORF in each virus (*vpu*, unique to HIV-1 [27, 30], and *vpx*, unique to HIV-2 [14, 15]). Characterization of the HIV-2 genome by recombinant DNA and molecular biological techniques to understand precisely the relationship between the two viruses has not yet been reported, with the exception of the *vpx* gene (14, 33).

To gain an insight into the biology and natural history of primate lentiviruses, we recently determined the complete nucleotide sequence of a simian immunodeficiency virus from a naturally infected African green monkey (SIV_{AGM}) (11) and of HIV-2 from an AIDS patient in Ghana (A. Hasegawa, H. Tsujimoto, N. Maki, K. Ishikawa, T. Miura, M. Fukasawa, K. Miki, and M. Hayami, AIDS Res. Hum. Retroviruses, in press). We further characterized the SIV_{AGM} genome by genetical methods and have shown that the essential or dispensable genes for infectivity are the same as those for HIV-1 (23a). For the purpose of comparative virology on the basis of gene functions, we have constructed nine mutants corresponding to all of the identified ORFs from an infectious DNA clone of HIV-2 and characterized the mutants biologically and biochemically.

MATERIALS AND METHODS

Cell culture and DNA transfection. $CD4^+$ human leukemia cell lines, A3.01 (10), Molt-4-clone 8 (M4-8) (16), Molt3, and TALL-1, were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. Molt3 and TALL-1 cells were obtained from the Japanese Cancer Research Resources Bank. A human colon carcinoma cell line, SW480 (ATCC CCL228), was maintained in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum. For transfection, uncleaved plasmid DNA was introduced into SW480 cells by the calcium coprecipitation method (13, 31).

Infection. The infectivity of progeny virions produced in transfected SW480 cells was assayed in $CD4^+$ leukemia cells. Culture supernatants were filtered (pore size, 0.2 μm), and appropriate volumes were added to 10^6 cells as previously described (10). When necessary, transfected cells were cocultivated with $CD4^+$ leukemia cells to determine infectivity of progeny virions as previously described (1).

RT assays. Virion-associated reverse transcriptase (RT) activity was measured as described previously (32).

CAT assays. Chloramphenicol acetyltransferase (CAT) assays have been previously described (12). Equivalent amounts of cell lysates from transfected SW480 cells were used for determination of CAT activity.

Dot-blot and Northern (RNA) blotting analyses. Total cellular RNA was prepared from transfected SW480 cells by the guanidinium isothiocyanate-cesium chloride method, electrophoresed (20 μg of RNA) through 1% agarose gel containing 2.2 M formaldehyde, and analyzed by Northern blot hybridization as previously described (20). For dot-blot analysis, 3 μg of RNA was used. A cutout DNA fragment (about 1 kilobase), which encompassed the complete 3′ long terminal repeat (LTR) with a 175-base-pair viral flanking sequence, was labeled with ^{32}P in a multiprime DNA labeling system (Amersham, Buckinghamshire, England) and used as a probe.

* Corresponding author.

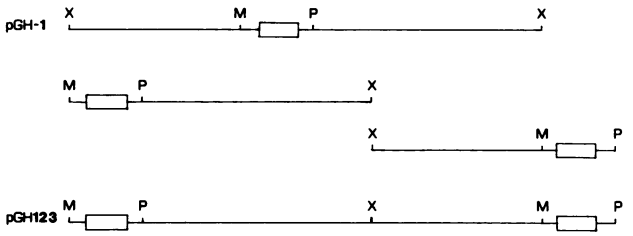

FIG. 1. Construction of a full-length molecular clone of HIV-2 with proviral structure. Two fragments were cut out from the unintegrated form of virus DNA cloned in a plasmid (pGH-1) and rejoined into pGH-123. Abbreviations: M, *Mlu*I; P, *Pma*CI; X, *Xba*I.

Western blotting. Lysates of transfected SW480 cells were prepared as previously described (32), and proteins were resolved on 12% sodium dodecyl sulfate-polyacrylamide gels and then electrophoretically transferred to nitrocellulose membranes. The membranes were incubated overnight at room temperature with sera from individuals infected with HIV-2 and then were incubated with ^{125}I-protein A for 3 h, washed, and visualized by autoradiography (32).

DNA constructs. The full-length molecular clone of HIV-2, designated pGH-1, is a subclone of λ GH-1, which was derived from extrachromosomal closed-circular virus DNA of HIV-2 from an AIDS patient in Ghana (A. Hasegawa et al., in press). To facilitate transfection analysis, pGH-1 was reconstituted as outlined in the legend to Fig. 1 to generate pGH-123. All of the mutants were constructed from pGH-123 by recombinant DNA techniques (see Fig. 3). Frameshift mutations were introduced into the sites (see Fig. 3) by cleaving plasmid DNAs with the restriction enzymes, blunt ended by T4 DNA polymerase, and resealed by T4 DNA ligase. When necessary, the appropriate DNA fragment was first subcloned into pUC19, the mutation was introduced, and the mutated DNA was put back into pGH-123. Thus, pGH-Hi, pGH-Bg, pGH-Xb, pGH-St, pGH-Ec, pGH-Av, and pGH-MI all have a 4-base-pair insertion, whereas both pGH-Ns and pGH-Ps contain a 4-base-pair deletion. CAT constructs under the control of virus LTR were made by placing LTR fragments in front of the CAT gene of pUC19 that contains the *Hin*dIII-*Bam*HI fragment of pSV$_2$CAT (12). Each construct contains complete LTR (from viral or cellular flanking sequence, less than 200 base pairs, to the *Nar*I site located in the primer binding region) derived from infectious clones pNL432 (1), pSA212 (23a), and pGH-123 (this report). Expression of pH1-CAT, pH2-CAT, and pSA-CAT is directed by the LTR of HIV-1, HIV-2, and SIV$_{AGM}$, respectively.

RESULTS

Construction and characterization of an infectious clone. pGH-1 plasmid DNA was reconstituted for transfection experiments (Fig. 1). A 6.7-kilobase *Mlu*I-*Xba*I fragment and a 5.0-kilobase *Xba*I-*Pma*CI fragment were cut out from pGH-1 and cloned into pUC19 cleaved with *Nde*I-*Hin*dIII (*Mlu*I, *Pma*CI, *Nde*I, and *Hin*dIII sites were blunt ended). The resultant pGH-123 was introduced into SW480 cells that are known to be very sensitive to transfection analysis (1). pGH-123 could produce progeny virions, as determined by RT assay, comparable with those of pNL432 (1), the infectious clone of HIV-1 (not shown). The infectivity of pGH-123 was tested in several human leukemia cell lines with

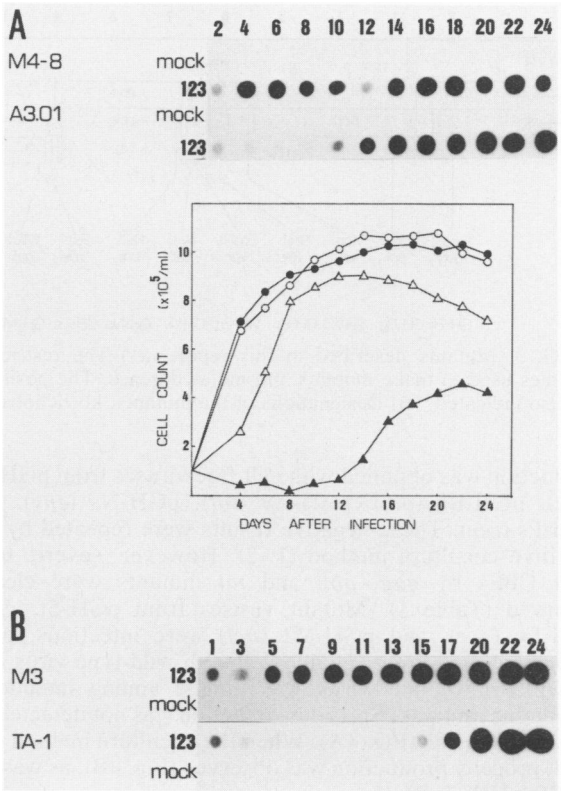

FIG. 2. Infection kinetics in human CD4$^+$ cells inoculated with virus particles derived from pGH-123. (A) RT production in M4-8 and A3.01 cells (top). Viable cell counts as determined by trypan blue staining are also shown (bottom). Closed symbols indicate the number of viable cells infected with virus from pGH-123, and open symbols show that of mock-infected cells. Circles and triangles represent A3.01 cells and M4-8 cells, respectively. (B) RT production in Molt3 cells (M3) and TALL-1 cells (TA-1). The values above the autoradiograms indicate the day after infection.

virus particles from transfected SW480 cells. Figure 2 shows the kinetics of infection by virus from pGH-123. RT production was observed in all four cell lines tested with different time courses. Clear cytopathic effects (CPEs), including fusion, ballooning, and cell death, were also noted, except in A3.01 cells. Viable cell counts confirmed this observation (Fig. 2A). Many of the M4-8 cells were killed shortly after being infected, in contrast to A3.01 cells. However, M4-8 cells began to grow 12 days after being infected and continued to produce progeny viruses. The results shown in Fig. 2 clearly indicate that pGH-123 is a biologically active molecular clone.

Construction and characterization of mutant clones. On the basis of the nucleotide sequence of HIV-2 (GH-1 strain) (A. Hasegawa et al., in press), frameshift mutations were introduced into the restriction sites indicated in Fig. 3. The resultant mutants were evaluated for transient expression of RT in SW480 cells and for infectivity in CD4$^+$ cells. RT production was observed in the SW480 cells transfected with *vif*, *vpx*, *vpr*, *tat*, *env*, or *nef* mutants (Table 1). RT production by a *tat* mutant was significantly low and correlated well with the reduced synthesis of RNA and protein (see below). Growth abilities of progenies produced in transfected cells were tested in M4-8 cells with equivalent amounts (RT units for RT producers and all of the culture fluids for nonproducers) of virus. No evidence of infectivity as judged by RT

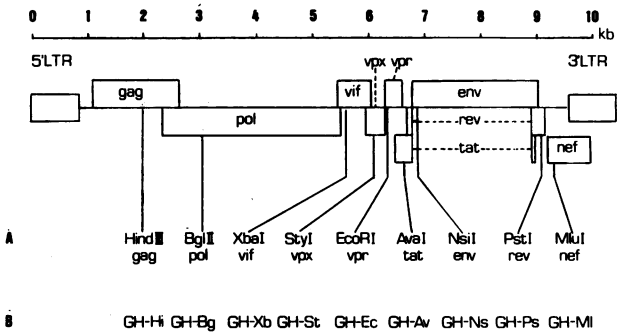

FIG. 3. Mutants described in this report. (A) The restriction enzymes used to make mutants and mutated genes. The positions are also indicated. (B) Designations of the mutants. kb, Kilobases.

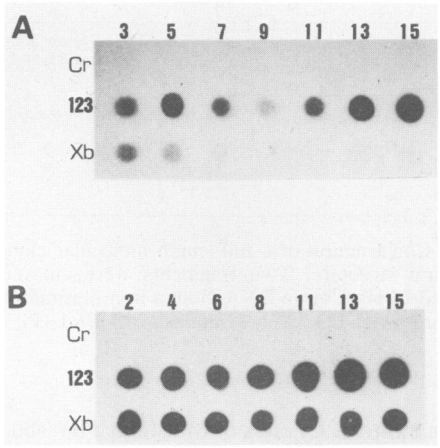

FIG. 4. Kinetics of RT production by a *vif* mutant. (A) M4-8 cells were infected with equal amounts of cell-free virus from wild-type DNA (123) or a *vif* mutant (Xb), and RT production was monitored at intervals with a negative control (mock infected; Cr). (B) Transfected SW480 cells were cocultured with Molt3 cells, and RT activity in the culture fluids was determined. Values above the autoradiograms indicate the day after infection (A) or the day after coculture was initiated (B).

production was obtained with cell-free viruses from pGH-Hi (*gag*), pGH-Bg (*pol*), pGH-Av (*tat*), pGH-Ns (*env*), and pGH-Ps (*rev*). These negative results were repeated by the sensitive coculture method (1, 2). However, severe, transient CPEs by *gag*, *pol*, and *tat* mutants were clearly observed (Table 1). Mutant viruses from pGH-St (*vpx*), pGH-Ec (*vpr*), and pGH-M1 (*nef*) were infectious, were cytopathic, and grew equally well with wild-type virus (not shown). *vif* mutant virus was unique among infectious, cytopathic mutants. Spreading infection was not detected by cell-free *vif* virus (Fig. 4A). When the coculture method was used, progeny production was observed (Fig. 4B), as was the case for HIV-1 (26).

Biochemical characterization of mutants. Synthesis of virus RNA and protein in transfected SW480 cells was examined in order to learn more about the nature of mutations. Figure 5A shows the amount of virus RNA synthesized in the cells as monitored by dot-blot hybridization. Compared with wild-type virus (pGH-123) and a *rev* mutant (pGH-Ps), approximately 10% of RNA was synthesized in the cells transfected with a *tat* mutant (pGH-Av). When the sizes of virus RNAs were analyzed by Northern blotting (Fig. 5B), three major species of RNAs as previously described with HIV-1 (7) could easily be seen in SW480 cells producing wild-type virus and, to a much lesser extent, a *tat* mutant. Major species of RNAs synthesized by a *rev* mutant were around 2 kilobases in size, and relative intensities of the bands were very different from those observed with wild-type virus and a *tat* mutant. Next, virus-specific protein synthesis was analyzed by Western blotting (Fig. 6). Serum samples from four individuals infected with HIV-2 were checked for reactivity by our system, and all of them recognized mainly *gag*-related proteins of HIV-2, as determined by the size and cross-reactivity with HIV-1 (our unpublished observations). Three *gag* proteins could be seen in the lysates from SW480 cells transfected with wild-type DNA (Fig. 6, lane b), whereas reduced amounts of the proteins were seen in lysates of cells transfected with a *tat*

TABLE 1. Characterization of HIV-2 mutants

Mutant	Mutated gene	RT production[a]	CPEs[b]	Infectivity[c]
pGH-123	None	++	+	+
pGH-Hi	*gag*	−	+	−
pGH-Bg	*pol*	−	+	−
pGH-Xb	*vif*	++	+	+
pGH-St	*vpx*	++	+	+
pGH-Ec	*vpr*	++	+	+
pGH-Av	*tat*	+	+	−
pGH-Ns	*env*	++	−	−
pGH-Ps	*rev*	−	−	−
pGH-Ml	*nef*	++	+	+

[a] Virus production was assayed by the presence of particle-associated RT activity in the culture supernatants of transfected SW480 cells. Symbols: ++, similar level (50 to 120%) of RT production by wild-type DNA; +, 10 to 20% of wild type; −, no detectable RT.

[b] CPEs (M4-8 cells) caused by virus were monitored daily by microscopic observation and trypan blue staining. Symbols: +, evidence of CPEs; −, no evidence of CPEs.

[c] M4-8 cells were infected with cell-free virus stock, and RT production was assayed at intervals for 21 days. In the cases of pGH-Hi, pGH-Bg, pGH-Av, pGH-Ns, and pGH-Ps, infectivity was also determined by RT production in the coculture of transfected SW480 cells and Molt3 cells. As for the infectious mutants, genome structures of progeny viruses produced in the infected cells were confirmed by hybridization mappings. Symbols: +, evidence of infectivity; −, no evidence of infectivity.

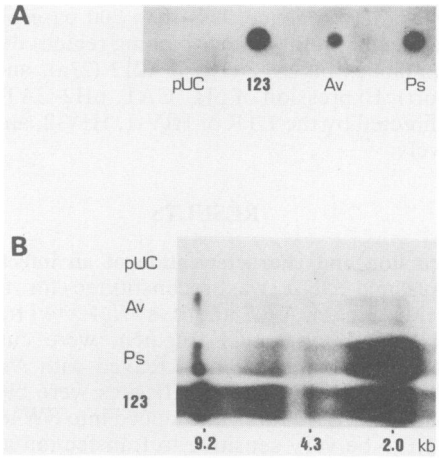

FIG. 5. Examination of virus RNA synthesis in SW480 cells transfected with various DNAs by dot-blot (A) and Northern (B) blot hybridization. Abbreviations: pUC, pUC19; 123, wild type; Av, *tat* mutant; Ps, *rev* mutant.

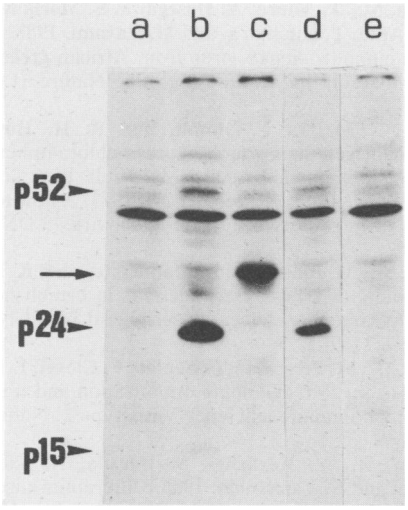

FIG. 6. Detection of *gag* proteins produced in transfected SW480 cells by Western blotting with sera from HIV-2-infected patients. Cell lysates were prepared from the cells transfected with pUC19 (lane a), pGH-123 (wild type) (lane b), pGH-Hi (*gag*) (lane c), pGH-Av (*tat*) (lane d), and pGH-Ps (*rev*) (lane e).

mutant (Fig. 6, see p52 and p24, lane d) and no specific reactivity was seen in lysates of cells transfected with a *rev* mutant (Fig. 6, lane e). A *gag* mutant produced abnormal protein p33 (Fig. 6, lane c, arrow). This truncated, uncleaved form of *gag* product was predictable from the location of mutation (Fig. 3).

Transactivation of HIV-1, HIV-2, and SIV_{AGM} LTRs. Our characterization of mutants derived from infectious clones of HIV-1 (A. Adachi, K. Ogawa, H. Sakai, R. Shibata, T. Kiyomasu, and A. Ishimoto, manuscript in preparation), HIV-2 (this report), and SIV_{AGM} (23a) has revealed that these three primate lentiviruses are functionally very related to each other. To see if *tat* gene products can be exchangeable, transactivation potential was evaluated by cotransfection of LTR-CAT constructs and wild-type or mutated DNAs into SW480 cells, followed by monitoring of CAT activity in the cell lysates. All of the wild-type DNAs activated every LTR compared with corresponding *tat* mutant DNAs (Fig. 7). However, the extent of activation varied among each pair of LTR and *tat* product-producing DNA. pNL432 (HIV-1) was the best transactivator of HIV LTRs, and the highest augmentation of the expression of SIV_{AGM} LTR was obtained with pSA212 (SIV_{AGM}). In general, pNL432 highly activated LTR expression while pGH-123 (HIV-2) did relatively poorly.

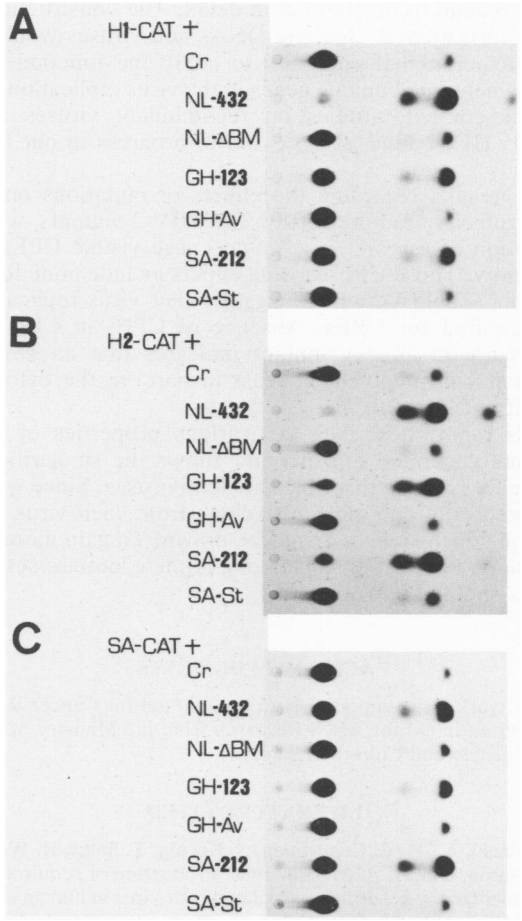

FIG. 7. Determination of transactivation of HIV-1 (A), HIV-2 (B), and SIV_{AGM} (C) by CAT assays. Abbreviations: NL-432, HIV-1 wild type; NL-ΔBM, HIV-1 *tat* mutant; GH-123, HIV-2 wild type; GH-Av, HIV-2 *tat* mutant; SA-212, SIV_{AGM} wild type; SA-St, SIV_{AGM} *tat* mutant; Cr, control DNAs, for which the same LTR constructs without CAT gene were used.

DISCUSSION

Recombinant DNA techniques have been used with the HIV-2 genome to analyze the effects of mutations in a specified region on various activities of the virus. This is the first report on systematic, genetic analyses of the second AIDS virus, HIV-2.

The phenotypical characteristics of HIV-2 mutants have been compared with those of HIV-1 (A. Adachi et al., in preparation) and SIV_{AGM} (23a) mutants in the same experimental system we are using. Mutations affecting *gag*, *pol*, *env*, *tat*, and *rev* did abolish virus infectivity as expected (Table 1). Results obtained from biochemical characterization of HIV-2 *rev* and *tat* mutants (Fig. 5, 6, and 7) are consistent with previously published data on HIV-1 (4, 5, 7, 9, 24, 28) and with our data on SIV_{AGM} (23a). *rev* gene mutation resulted in the accumulation of small mRNAs and no particle production. Our CAT analysis showed that gene expression directed by HIV-1 LTR was more dependent on *tat* products than were the other two LTRs (see the result of homologous pair in Fig. 7). Nonetheless, CAT expression driven by HIV-1 LTR could be augmented by HIV-2 and SIV_{AGM} *tat* products, which suggests no essential difference of mechanism of LTR activation.

Among the genes dispensable for virus infectivity, *vif* and *nef* are commonly present in the genomes of HIV-1, HIV-2, and SIV_{AGM}. Mutations in the *nef* genes did not affect much the replication rates of the viruses. In contrast, *vif* mutants of HIV-1 and HIV-2 (Fig. 4) showed very retarded growth when cell-free virus stocks were used as inocula. No effects were observed with SIV_{AGM} mutants. Other genes not essentially required for infectivity include *vpx* (unique to HIV-2 and SIV_{AGM}), *vpr* (unique to HIV-1 and HIV-2), and *vpu* (unique to HIV-1). Growth properties of *vpx* and *vpr* mutants of HIV-2 were not distinct from its wild type like a *vpx* mutant of SIV_{AGM} in established cell lines. However, *vpr* mutant viruses of HIV-1 grow significantly more slowly than do wild-type viruses (19). The functions of these three

genes remain to be clarified in detail. The construction and characterization of chimeric DNAs and viruses would be a good experimental approach to study the function of the above-mentioned unique genes in the virus replication cycle. In this context, studies on recombinant viruses among HIV-1, HIV-2, and SIV_{AGM} are in progress in our laboratory.

The results regarding the effects of mutations on cytopathogenicity are noteworthy. Our HIV-2 mutants, with the exception of *rev* and *env*, could cause visible CPEs quite efficiently. Those CPE-causing effects include noninfectious mutants (Table 1), which suggests that virus replication is not required for CPEs. Absence of CPEs in $CD4^+$ cells infected with an *env* mutant indicates that an *env* gene product(s) is involved, at least in part, in the cytopathogenicity of HIV-2.

This report describes the various properties of HIV-2 mutants generated in vitro and shows the similarities and differences among three primate lentiviruses. Since we have analyzed only one molecular clone from each virus group, general conclusions may not be drawn. To gain more information on the relationship among primate lentiviruses, more clones should be investigated.

ACKNOWLEDGMENTS

This work was supported by a grant-in-aid for Cancer Research and a grant-in-aid for AIDS Research from the Ministry of Education, Science and Culture of Japan.

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