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Persistently Human Immunodeficiency Virus Type 1-infected T Cell Clone Expressing Only Doubly Spliced mRNA Exhibits Reduced Cell Surface CD4 Expression

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Several cell clones possessing the human immunodeficiency virus type 1 (HIV-1) genome, consisting of an almost full-length DNA sequence, were isolated by limiting dilution of the clonal cell line M10 derived from MT-4 that survived infection with HIV-1 *vpr* mutant (M10/*vpr*$^-$). One of the isolated clones (termed Vpr-1) expressed only doubly spliced mRNA, but not unspliced or singly spliced mRNA. Western blots of Vpr-1 revealed the presence of the *nef* translation product, although no expression of major structural genes such as *gag, pol,* and *env* was detected by indirect immunofluorescence and assay of reverse transcriptase activity. These HIV-1 phenotypes differed greatly from those of the original M10/*vpr*$^-$, most of which expressed major structural HIV-1 proteins. Despite undetectable levels of *env* expression in Vpr-1, CD4 antigens were greatly down-modulated on the surface without alteration of steady-state levels of CD4 mRNA expression, similar to M10/*vpr*$^-$. These HIV-1 phenotypes in Vpr-1 did not change after the treatment of the cells with both phorbol 12-myristate 13-acetate and phytohemagglutinin. Therefore, the abnormal HIV-1 life cycle in Vpr-1 seems to be due to some viral factor(s), as well as cellular factors. Thus, Vpr-1 could be a useful model for understanding one HIV-1 latent form.

Key words: HIV-1 — Nonproductive infection — Persistent infection — Mutation

Individuals who are infected with human immunodeficiency virus type 1 (HIV-1) remain clinically asymptomatic for an average of about 9.8 years before progression to the acquired immune deficiency syndrome (AIDS)-related complex or AIDS.[1] Recently, polymerase chain reaction (PCR) amplification of HIV-1 proviral DNA has revealed the presence of long-term silent infection with HIV-1 with no capacity for HIV-1 antigen expression or virus replication in peripheral blood mononuclear cells, in asymptomatic individuals prior to seroconversion.[2–4] Several examples of silent or nonproductive infection have been reported using cultured monocytoid and lymphoid cell lines. The monocytoid cell line U937-derived U1 cell clone expressed singly and doubly spliced mRNAs, but not unspliced mRNA.[5] On the other hand, the T cell clone ACH-2, which predominantly expressed spliced mRNAs, expressed low levels of full-length genomic RNA, produced few progeny viruses, and consecutively expressed detectable amounts of a protein reactive with an anti-HIV-1 antiserum.[6] When these cell clones were stimulated with monokines that activate T cells or with agents that activate the HIV-1 long terminal repeat (LTR), HIV-1 production was augmented. Long-term HIV-1 infection of U937 has resulted in a progressive loss of infectivity that was correlated with the accumulation of extrachromosomal forms of viral DNA.[7] In this cell system, viral latency was characterized by reduced levels of HIV-1 transcripts as a result of extensive *de novo* methylation of viral sequences present within the extrachromosomal DNA. On the other hand, mutations of both *vpr* and *vpu* genes in the HIV-1 genome resulted in the nearly complete attenuation of viral replication in primary monocytes, despite subsequent virus recovery from infected monocytes by cocultivation with uninfected peripheral blood mononuclear cells.[8] Thus, several cellular model systems for HIV-1 silent or nonproductive infection have been reported, and mechanisms involved in generating such an infection state of HIV-1 seem to differ between HIV-1 and the host cell system.

We previously reported that infection of M10 cells isolated from a human T lymphotropic virus type-I (HTLV-I)-transformed CD4-positive cell line MT-4,[9] with an HIV-1 *vif, vpr,* or *vpu* mutant, produced some survivors (termed M10/*vif*$^-$, M10/*vpr*$^-$, or M10/*vpu*$^-$, respectively) expressing HIV-1 antigens.[10] The surviving cells proliferated at a rate similar to that of uninfected M10 cells and over 90% of the cells continuously expressed HIV-1 antigens. In contrast, the HIV-1 *nef* mutant completely killed the M10 cells, as did wild-type HIV-1 under the same conditions.[10] Persistently HIV-1-infected cell clones isolated from HIV-1 antigen expression-competent M10/*vif*$^-$ and M10/*vpu*$^-$ cells were phenotypically classified into three types; a nonproducer, a low replicative

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HIV-1 producer, and a producer with a replication rate comparative to that of wild-type HIV-1.[11] In this study, we isolated several cell clones from the M10/*vpr*$^-$ cells expressing HIV-1 antigens. We found that, although the isolated clones contained an almost full-length HIV-1 DNA genome, the clones expressed only doubly spliced mRNA and no viral structural mRNAs. In addition, the HIV-1 receptor CD4 antigen level was greatly reduced on the surfaces of these clones.

MATERIALS AND METHODS

Cells and viruses M10,[12] a clonal cell line isolated from MT-4 cells,[9] was cultured in complete medium (RPMI-1640 medium supplemented with 10% fetal bovine serum) at 37°C in a CO_2-incubator. M10/*vpr*$^-$ cells were obtained as survivors of infection with the *vpr* mutant virus (*vpr*$^-$).[10] The *vpr*$^-$ was obtained as conditioned medium from SW480 cells transfected with pNL-Af2, constructed from the pNL432 plasmid containing full-length HIV-1 DNA[13] by inserting a frameshift mutation of 4 base pairs (bp) at the *Afl*II restriction site in the *vpr* region as described previously.[14]

Four cell clones, designated Vpr-1 to Vpr-4, were isolated from the M10/*vpr*$^-$ cells by limiting dilution in two 96-well microplates and cultured in RPMI-1640 supplemented with 20% fetal bovine serum for 2 to 3 weeks at 37°C in a CO_2-incubator.

MOLT-4/LAI cells, obtained by serial passage of the acute lymphocytic leukemia-derived cell line MOLT-4[15] after infection with the HIV-1 LAI strain,[16] were used as controls.

Assays of HIV-1 infectivity and reverse transcriptase (RT) activity HIV-1 infectivity and RT activity in the conditioned media of the isolated clones were assayed as described previously.[17] Although uninfected M10 cells harbor HTLV-I, RT activity derived from HTLV-I is distinguishable from that from HIV-1, since HIV-1 RT is Mg^{2+}-dependent, while HTLV-I RT is Mn^{2+}-dependent.[18]

Detection of HIV-1 and CD4 antigens Indirect immunofluorescence (IF) was performed as described previously[19] using an undiluted hybridoma culture fluid, producing mouse monoclonal antibodies (MAbs) to HIV-1 Gag p24 (V107) or p18 (V17).[19] Cells were fixed with cold acetone for 5 min before the reaction with antibodies. For flow cytometry, unfixed cells in the log phase of growth were reacted with 500-fold diluted serum from an HIV-1-seropositive subject (IF titer; 1:4096), anti-HIV-1 Env gp120 MAb (0.5β),[20] or anti-CD4 MAb. Two anti-CD4 MAbs were used, i.e., anti-Leu-3a (Becton Dickinson) recognizing the V1 domain[21,22] and OKT4 (Ortho-Mune) recognizing the V3 and/or V4 domain.[23]

Southern and Northern blots Southern and Northern blot analyses were carried out as described previously.[24] Total cellular DNA and RNA were extracted as described previously.[24,25] Briefly, total cellular DNAs (10 μg) or RNAs (10 μg) were separated on an agarose gel followed by blotting on a nylon membrane. A ^{32}P-labeled *Hin*dIII fragment of HIV-1 DNA containing almost the full-length HIV-1 sequence (nucleotides 531 to 9606) of pNL432 was used as the probe for both analyses. CD4 mRNA was probed with a ^{32}P-labeled CD4 *Bam*HI DNA fragment encoding the extracellular soluble region (368 amino acids) of the human CD4 gene prepared from pAcYM1, containing the human CD4 gene.[26] The β-actin cDNA probe was a ^{32}P-labeled *Hin*fI DNA fragment containing about 400 bp of the human β-actin gene (Wako Pure Chemical Industries).

PCR To examine whether the frameshift mutation at the *Afl*II restriction site within the *vpr* gene was retained in the isolated cell clones, a DNA fragment containing the *vpr* region was amplified using a primer set (nucleotides 4808 to 4829, GTACAGTGCAGGGGAAAGAATA, and 6344 to 6320, CCCCATAATAGACTGTGACCCACAA) according to the method described previously,[27] then digested with *Afl*II. Thirty PCR cycles were performed using a GeneAmp kit (Perkin Elmer Cetus Instruments). Each cycle consisted of 1 min of denaturation at 94°C, 1.5 min of annealing at 55°C, and 2.5 min of extension at 72°C. All the numbers for nucleotide sequences decribed here correspond to those of the HIV-1 DNA sequence in pNL432.[13]

To detect multiply spliced HIV-1 mRNA, RT-PCR was performed essentially according to the method of Arrigo *et al.*[28] The primer set consisted of a 5′-primer at nucleotides 689 to 710, ACGCAGGACTCGGCTTGCTGAA, located within a region between a primer binding site and a major 5′ splice junction and a 3′-primer at nucleotides 8405 to 8386, CGGGCCTGTCGGGTCCCCTC, located in the 2nd exon for *tat, rev,* and *nef* mRNAs.[29,30] Briefly, total cellular RNAs (1 μg) were reverse-transcribed by Moloney murine leukemia virus-RT (Life Technologies, Inc.) using an oligo $(dT)_{20}$ as the primer after RQ1 DNase (Promega Co.) digestion. The resulting cDNAs were used for PCR (35 cycles, each consisting of 1 min of denaturation at 94°C, 2 min of reannealing at 55°C, and 2 min of extension at 72°C). PCR products were separated by 2% agarose gel electrophoresis, blotted onto a nylon membrane, then detected by hybridization with a ^{32}P-labeled synthetic nucleotide (nucleotides 711 to 730, GCGCGCACGGCAAGAGGCGA, located in a region between the 5′-primer and the major 5′ splice junction, as shown in Fig. 5A).

Western blotting Western blotting was performed as described previously.[31] Briefly, total cellular proteins

were separated by SDS-PAGE using a 10 to 15% linear gradient polyacrylamide gel according to the method of Laemmli,[32] and blotted onto a nitrocellulose membrane. The membrane was preincubated in blocking buffer (3% skimmed milk powder in phosphate-buffered saline), then incubated with a 100-fold dilution of ascites fluid containing anti-Nef mouse MAb (Du Pont). Immunoglobulins binding to the HIV-1 Nef antigen were detected using biotinylated anti-mouse IgG (H+L) horse immunoglobulin (Vector Laboratories, Inc.), the VECTASTAIN ABC kit (Vector Laboratories, Inc.), and 4-chloro-1-naphthol as a substrate. A low-molecular-weight calibration kit (Pharmacia LKB Biotechnology) was used to determine the molecular weights of proteins.

Induction of HIV-1 gene expression Vpr-1 and M10/*vpr*$^-$ cells (5×10^5 cells/ml) were cultivated in complete medium containing 50 μg/ml of phorbol 12-myristate 13-acetate (PMA) and 2 mg/ml of phytohemagglutinin (PHA) as described previously.[5] As a control, M10 cells were treated in the same manner. Aliquots of the cell suspensions were harvested after 24 h of culture. Induction of the HIV-1 genome was analyzed by Northern blotting. The RNAs on the same membrane were hybridized with a ^{32}P-labeled HIV-1 DNA or human β-actin cDNA probe as described above.

RESULTS

Isolation of cell clones with no capacity for HIV-1 production and CD4 antigen expression Cell cloning from M10/*vpr*$^-$ cells that survived extensive cytolysis after infection with the HIV-1 *vpr* mutant[10] was carried out by limiting dilution. When the cells were cultured at 10 cells per well for 2 to 3 weeks, it was found that several wells contained a single colony per well, although no colony was observed in any well in which the cells were cultured at one or two cells per well for 2 to 3 weeks. Therefore, the cells derived from a single colony per well were again subjected to the same limiting dilution procedure, and a total of four cell clones, termed Vpr-1 to Vpr-4, were finally isolated from M10/*vpr*$^-$ cells. These clones had growth rates similar to those of uninfected M10 cells

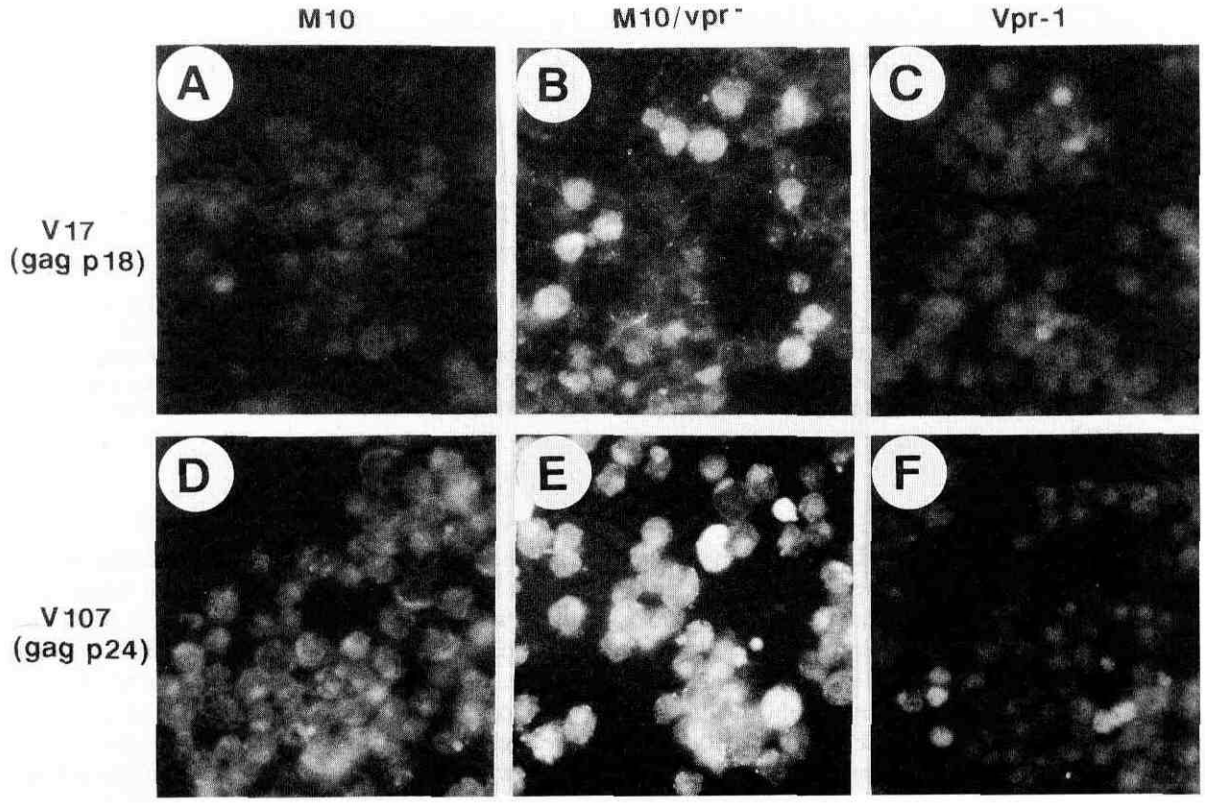

Fig. 1. HIV-1 antigen expression in the cell clone Vpr-1, determined by indirect IF. Uninfected M10 (A and D), M10/*vpr*$^-$ (B and E) and Vpr-1 (C and F) cells were fixed with cold acetone, then reacted with MAb to HIV-1 Gag p18 (V17) (A to C) or MAb to HIV-1 Gag p24 (V107) (D to F).

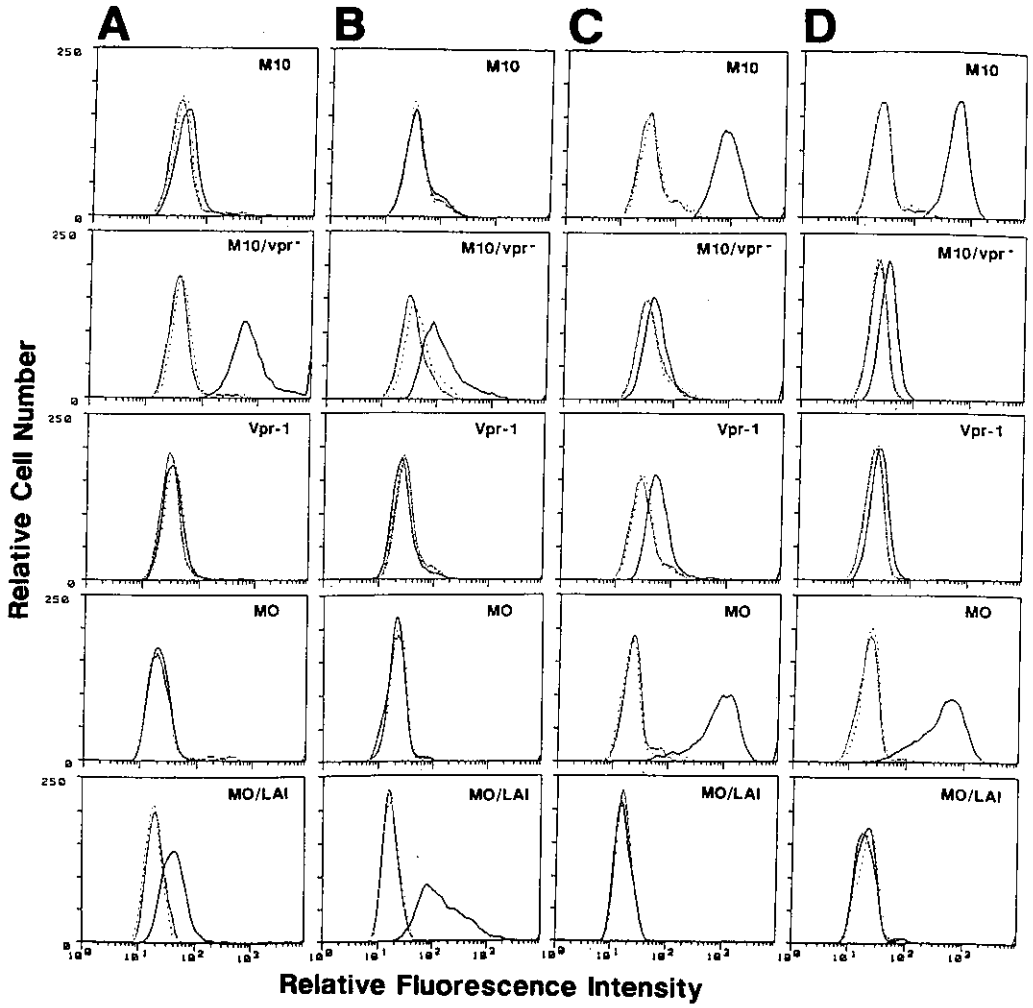

Fig. 2. Cell surface expression of HIV-1 and CD4 antigens on cell clone Vpr-1 determined by flow cytometry. Uninfected M10, M10/*vpr*⁻, Vpr-1, uninfected MOLT-4 (MO) and MOLT-4/LAI (MO/LAI) cells were reacted with serum from an HIV-1-seropositive subject (A), MAb to HIV-1 Env gp120 (0.5β) (B), or MAb to CD4 (C, anti-Leu-3a; and D, OKT4). After formalin fixation, 5×10^3 cells were analyzed using a Becton Dickinson FACScan system. The data are presented as histogram profiles comparing relative fluorescence with total cell numbers (—). Normal human or mouse serum (···) and PBS (-·-) were used as negative controls.

(data not shown). The assays of HIV-1 infectivity and RT activity in the conditioned media of these clones revealed no HIV-1 infectivity (less than $10^{0.5}$ $TCID_{50}$/ml) and no RT activity (similar level to the conditioned medium of uninfected M10 cells) (data not shown), although both activities in the conditioned medium of M10/*vpr*⁻ cells were positive, that is, infectivity with $10^{5.5}$ $TCID_{50}$/ml. The RT activity was almost comparable with that of M10 cells infected with wild-type HIV-1.[10]

HIV-1 antigen expression in the isolated clones was examined by indirect IF (Fig. 1) using MAbs to HIV-1 Gag p24 (V107) and p18 (V17). However, apparent HIV-1 antigen expression was not detected in any of the clones (Fig. 1C and 1F), since the IF intensity in all of them was similar to that in uninfected M10 cells (Fig. 1A and 1D). Representative results using Vpr-1 are shown in Fig. 1. On the contrary, high levels of HIV-1 antigen expression were detected in over 90% of uncloned M10/*vpr*⁻ cells (Fig. 1B and 1E). Similar results were obtained by flow cytometry. No significant reaction was identified in Vpr-1 cells using serum from an HIV-1-seropositive subject (Fig. 2A) or an MAb against HIV-1 Env gp120 (0.5β) (Fig. 2B). In contrast, the level of HIV-1 antigen expression on the surface of uncloned M10/*vpr*⁻ cells

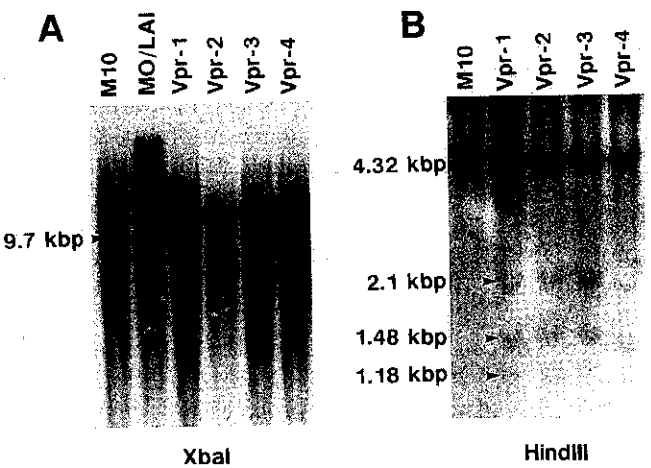

Fig. 3. Southern blots of proviral DNAs in cell clones isolated from M10/*vpr*⁻ cells. Total cellular DNAs (10 μg) extracted from uninfected M10, Vpr-1, Vpr-2, Vpr-3, and Vpr-4 cells were digested with *Xba*I (A) or *Hin*dIII (B), then separated on a 0.45% or 0.6% agarose gel, respectively. The membranes blotted with these DNAs were hybridized with a ^{32}P-labeled *Hin*dIII DNA fragment containing a nearly full-length HIV-1 DNA. The total cellular DNA from MOLT-4/LAI (MO/LAI) cells was used as uncloned controls.

was somewhat higher than that in control cells, MOLT-4/LAI (Fig. 2A and 2B). Thus, there was no apparent Env expression on the surface of Vpr-1. However, CD4 expression was greatly reduced on the cell surface (Fig. 2C and 2D). Flow cytometry using two anti-CD4 MAbs, anti-Leu-3a and OKT4, revealed high expression on uninfected M10 and MOLT-4 cells, but greatly reduced expression on MOLT-4/LAI cells. In Vpr-1, the amount of the CD4 antigen reactive with both anti-Leu-3a and OKT4 MAbs was reduced to less than 10%, compared with that of uninfected M10 cells. The results from Vpr-2, Vpr-3 and Vpr-4 were similar to those of Vpr-1 (data not shown). The expression level of CD4 on M10/*vpr*⁻ cells was similar to that of Vpr-1.

DNA analysis of the isolated clones The presence of HIV-1 DNA in each clone was examined by Southern blotting with a ^{32}P-labeled *Hin*dIII DNA fragment of HIV-1 DNA encompassing almost the entire HIV-1 genome (nucleotides 531 to 9606) as a probe. The total cellular DNAs from uninfected M10 cells and uncloned MOLT-4/LAI cells were used as controls. When total cellular DNAs were digested with the restriction endonuclease *Xba*I, clones Vpr-1 to Vpr-4 gave single sharp bands of identical length which were apparently larger

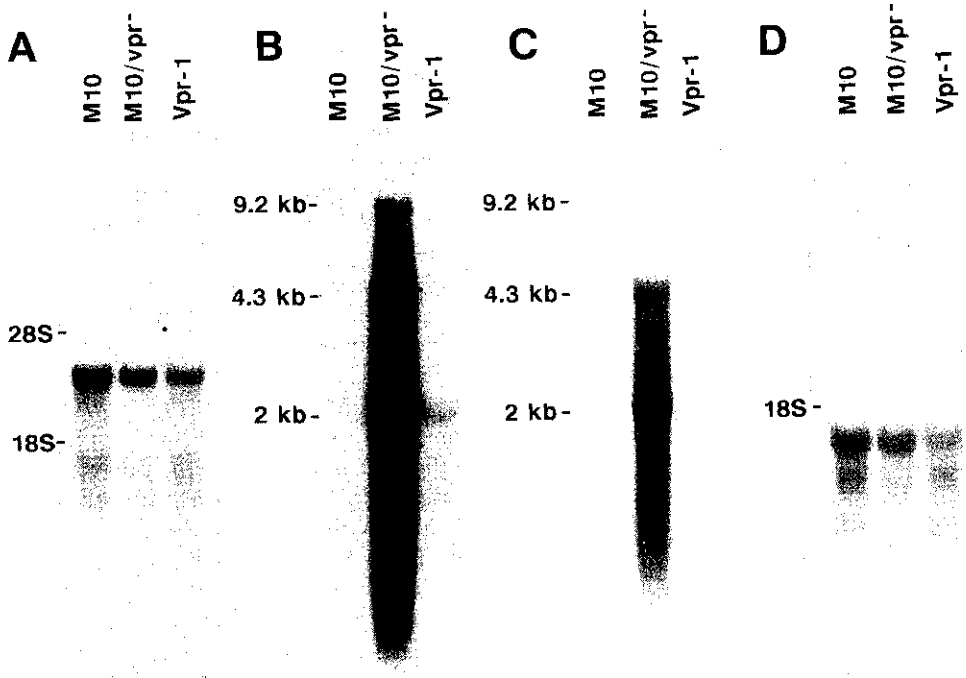

Fig. 4. Northern blot analyses of HIV-1 and CD4 mRNAs in cell clone Vpr-1. Probes: CD4 DNA (A), HIV-1 DNA (B and C), and human β-actin cDNA (D). The same membrane was used for all hybridizations successively, after removal of each probe. Exposure times for autoradiography were 20 and 4 h for B and C, respectively.

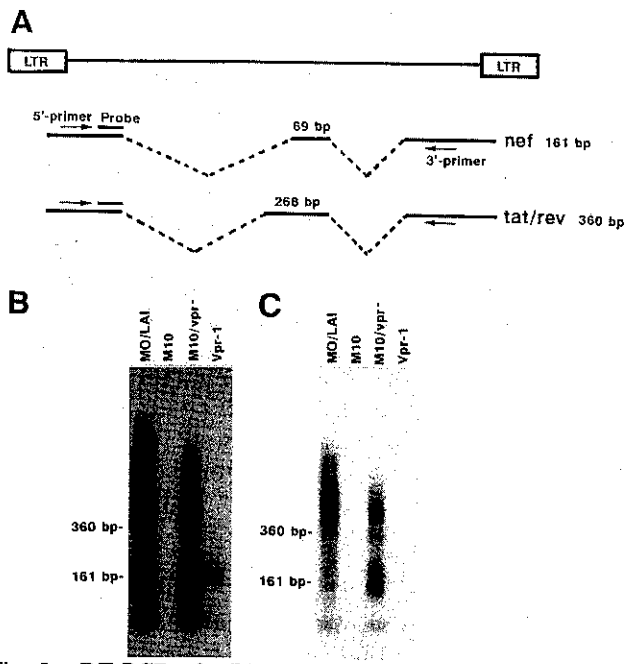

Fig. 5. RT-PCR of mRNA extracted from cell clone Vpr-1. The PCR strategy is illustrated in A. An autoradiogram of the Southern blot hybridization using a 1/10 aliquot of PCR products is shown in B and C. Exposure times for autoradiography were 3 and 1 h for B and C, respectively.

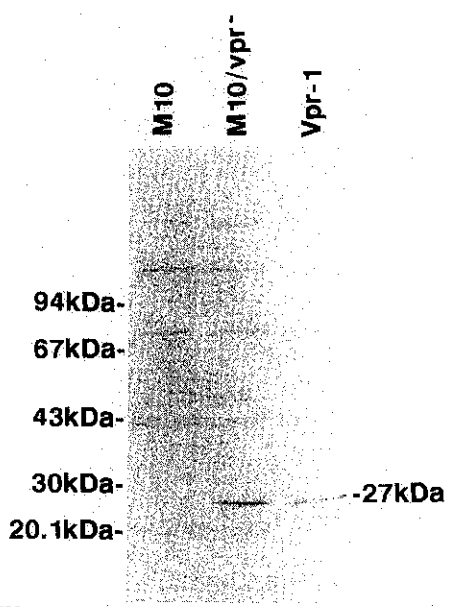

Fig. 6. Western blot analysis of HIV-1 antigen in cell clone Vpr-1. Uninfected M10, M10/*vpr*$^-$, and Vpr-1 cells were solubilized with 0.5% Nonidet P-40 then separated by SDS-PAGE. After proteins were blotted onto a nitrocellulose membrane, Nef antigen that reacted with anti-Nef mouse MAb was detected using a biotin-avidin system.

than the 9.7 kbp band of the HIV-1 proviral genome, being about 11 kbp (Fig. 3A). This restriction profile of single 11 kbp fragments observed in Vpr-1 to Vpr-4 was reproducible even after serial passage for more than one year. On the other hand, uncloned MOLT-4/LAI gave heterogeneous bands longer than 9.7 kbp (Fig. 3A). Next, Southern blot hybridization using the same HIV-1 DNA probe as for Fig. 3A was performed after digestion of the DNAs with *Hin*dIII, which has restriction sites at five locations, two being at nucleotides 531 and 9606 in the 5′- and 3′-LTRs, respectively, in the HIV-1 DNA sequence of pNL432 (Fig. 3B).[13] Therefore, four (4.32, 2.1, 1.48, and 1.18 kbp) among six *Hin*dIII fragments should be detectable with the probe used here. All clones gave four *Hin*dIII DNA fragments of the same size, indicating the presence of an almost full-length HIV-1 proviral genome within nucleotides 531 to 9606 without any detectable deletion.

A DNA fragment containing the *vpr* regions amplified from the total cellular DNAs of four clones by PCR as described in "Materials and Methods" was not digested with *Afl*II, indicating that all clones retained the frameshift mutation at the *Afl*II restriction site in the *vpr* region (data not shown).

Thus, it is suggested that the 11 kbp *Xba*I fragment in all clones contains flanking cellular sequences in addition to the nearly full-length HIV-1 DNA with frameshift mutation in the *vpr* gene, since *Xba*I does not have a restriction site within the HIV-1 DNA sequence.[13] In addition, it is also suggested that all these clones might be derived from a single M10/*vpr*$^-$ cell. Therefore, Vpr-1 was used as a representative line for further characterizations.

Nef expression in Vpr-1 The mRNA expression of CD4 and HIV-1 in Vpr-1 as a representative clone was characterized by Northern blotting (Fig. 4), using β-actin mRNA expression as an internal control (Fig. 4D). The same membrane blotted with total cellular RNAs was repeatedly used after removal of each probe. The level of CD4 mRNA in Vpr-1 was similar to those in uninfected M10 and M10/*vpr*$^-$ cells (Fig. 4A), although expression of cell surface CD4 was greatly down-modulated (Fig. 2C and 2D). On the other hand, there was only a trace of mRNA(s) of about 2 kb in Vpr-1 after hybridization with the HIV-1 DNA probe (Fig. 4B), although no mRNA could be detected in an autoradiogram with a shorter exposure time (Fig. 4C). No other mRNAs, such as 9.2 and 4.3 kb, were detected in this cell clone (Fig. 4B). In contrast, 9.2, 4.3, and 2 kb mRNAs were found in uncloned M10/*vpr*$^-$ cells, but not in uninfected M10 cells (Fig. 4B and 4C).

RT-PCR was performed to determine whether the 2 kb mRNA detected in Vpr-1 encodes for *tat*, *rev*, or *nef* (Fig. 5). According to Arrigo *et al.*,[28] 360 bp cDNA

fragments derived from *tat* and *rev* mRNAs or a 161 bp cDNA fragment derived from *nef* mRNA should be amplified in the RT-PCR as illustrated in Fig. 5A. Alternatively, *rev* mRNA might be amplified as a cDNA fragment of 183 bp.[29,30] Hybridization of a membrane blotted with PCR-amplified cDNAs using a ^{32}P-labeled synthetic nucleotide as a probe revealed predominant hybridization with a 161 bp band in Vpr-1 (Fig. 5B), while no hybridizing band with the same probe was detected at a shorter exposure time (Fig. 5C). In contrast, several hybridizing bands, including 360 and 161 bp DNA fragments, were observed in M10/*vpr*$^-$ and MOLT-4/LAI cells as controls, but not in uninfected M10 cells (Fig. 5B and 5C). Thus, it was indicated that the mRNA of about 2 kb detected in Vpr-1 (Fig. 4B) is predominantly a *nef* transcript.

Western blotting confirmed that the *nef* mRNA detected in Vpr-1 (Fig. 5B) was translated into Nef protein (Fig. 6). A 27 kDa protein reactive with anti-Nef MAb was identified in both Vpr-1 and M10/*vpr*$^-$ cells.

No induction of HIV-1 gene expression in Vpr-1 HIV-1 gene expression in Vpr-1 was examined after incubation with both PMA and PHA as described in "Materials and Methods." The cells were harvested every 4 days after the treatment. However, IF of harvested cells with the serum from an HIV-1-seropositive individual showed no induction of HIV-1 antigen expression (data not shown). Expression levels of HIV-1 (Fig. 7A) and β-actin (Fig. 7B) mRNAs were next examined by Northern blotting. However, the level of HIV-1 mRNA expression in Vpr-1 after incubation with both PMA and PHA for 24 h was almost the same as that in untreated Vpr-1, although the level of HIV-1 mRNA expression in M10/*vpr*$^-$ was increased 2- to 3-fold by the same treatment.

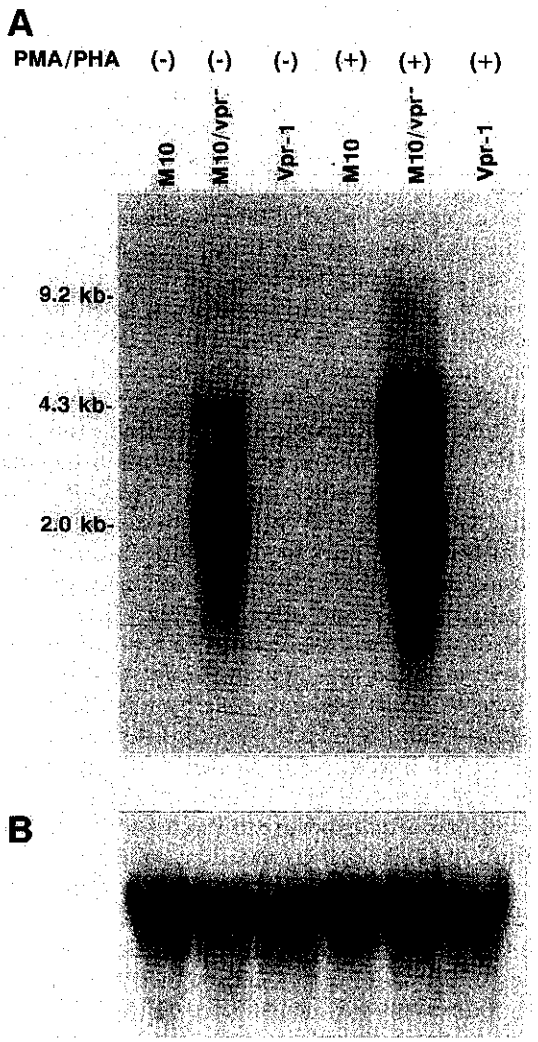

Fig. 7. Northern blot analysis of mRNA after treatment of Vpr-1 cells with PMA and PHA. Total cellular RNAs (about 10 μg) were extracted from uninfected M10, M10/*vpr*$^-$, and Vpr-1 cells incubated for 24 h in complete medium in the presence (+) or absence (−) of PMA and PHA. RNAs were separated on a 0.6% agarose gel and blotted onto a nylon membrane, which was hybridized with ^{32}P-labeled HIV-1 DNA (A) or a human β-actin cDNA (B) probe.

DISCUSSION

This report describes the isolation of cell clones Vpr-1 to Vpr-4 possessing an almost full-length HIV-1 DNA genome (Fig. 3) and expressing only doubly spliced mRNA (Fig. 4), unlike UI cells, which express small amounts of singly and doubly spliced HIV-1 mRNA.[5] Therefore, no expression of major structural proteins such as Gag, Pol, and Env was observed (Figs. 1 and 2). Thus, the HIV-1 phenotypes in the clones greatly differed from those of the original M10/*vpr*$^-$ cells, most of which expressed major structural HIV-1 antigens.[10] However, each clone, which was isolated from a single colony grown in microplates in which M10/*vpr*$^-$ cells were cultured at 10 cells per well, similarly gave a single sharp band of about 11 kbp by Southern blot hybridization after *Xba*I-digestion (Fig. 3A). The frameshift mutation at the *Afl*II restriction site seemed to be conserved in their HIV-1 genomes (data not shown). These results suggest that the isolated clones are derived from a single M10/*vpr*$^-$ cell. On the other hand, in our previous report, cell clones were easily isolated from M10/*vif*$^-$ and M10/*vpu*$^-$ cells by limiting dilution in microplates containing a single cell per well and the HIV-1 phenotypes of most of the isolated clones were essentially the same as those of the original M10/*vif*$^-$ and M10/*vpu*$^-$ cells, respectively.[11] In addition, survivors could be produced by culturing M10 cells at a higher cell density after

infection with *vpr*$^-$, compared with *vif*$^-$ or *vpu*$^-$.[10] Thus, the clones isolated here seem to be derived from a minor population of M10/*vpr*$^-$, which does not express major structural HIV-1 antigens and has higher colony-forming efficiency, but not from a predominant population of M10/*vpr*$^-$, which expresses major structural HIV-1 antigens and has lower colony-forming efficiency.

Vpr-1 expressed only the doubly spliced 2 kb mRNA containing at least the *nef* transcript (Figs. 4 and 5), which could be translated into Nef (Fig. 6). There are two possible explanations for this restricted expression of the HIV-1 antigen in Vpr-1. One is the induction of minor mutation(s), such as a point mutation, somewhere other than in the *vpr* gene. Support for this idea also comes from a recent finding that infection of monocytes with HIV-1 containing a genetic mutation in both *vpr* and *vpu*, but not in either *vpr* or *vpu*, leads to silent infection.[8] Otherwise, HIV-1 DNA modification such as methylation might be an important mechanism for regulating HIV-1 replication in Vpr-1 as described by Singh and Pauza.[7] The other possibility is the involvement of host cell factors. Zack *et al.*[33] and Stevenson *et al.*[34] have reported that low levels of HIV-1 replication in quiescent T cells was inducible by mitogenic or antigenic stimulation. Similarly, silently infected cell clones U1 and ACH-2 were inducible with PMA and PHA.[5] Unlike these cells, the HIV-1 gene expression was not induced in Vpr-1 after exposure to PMA and PHA (Fig. 7). Therefore, it is likely that persistent infection of HIV-1 with restricted expression in the human lymphoid cell line M10 involves the former mechanism.

The CD4 antigen level was greatly reduced on the cell surfaces of Vpr-1 (Fig. 2), although the level of CD4 mRNA expression was not affected (Fig. 4). Generally, CD4 is down-modulated in cells acutely infected or persistently infected with HIV-1.[35–39] It is believed that down-modulation of CD4 is induced as a consequence of inefficient transport of CD4 to the cell surface, since newly synthesized CD4 forms a complex with precursor Env gp160 prior to transport from the endoplasmic reticulum and is retained within the endoplasmic reticulum.[40] However, CD4 down-modulation in Vpr-1 could be due to another mechanism(s), since *env* expression in Vpr-1 was undetectable (Figs. 2 and 4). Recently, it has been reported that the reduction in the cell surface CD4 level is inversely related to *nef* gene expression, although the *nef* expression did not alter the steady-state levels of CD4 mRNA or CD4 protein.[41] Thus, our results on CD4 down-modulation from the cell surface and expression of the *nef* gene in Vpr-1 may support this notion.

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