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Vpx Is Critical for Reverse Transcription of the Human Immunodeficiency Virus Type 2 Genome in Macrophages[▽]

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The abilities of wild-type and *vpx*-defective human immunodeficiency virus type 2 (HIV-2) clones to synthesize viral DNA in human monocyte-derived macrophages (MDMs) and lymphocytic cells were comparatively and quantitatively evaluated. While the *vpx*-defective mutant directed the synthesis of viral DNA comparably to the wild-type virus and normally in lymphocytic cells, no appreciable viral DNA was detected in MDMs infected with the mutant. To substantiate this finding and to determine whether there is some specific region(s) in Vpx crucial for viral DNA synthesis in MDMs, we generated a series of site-specific point mutants of *vpx* and examined their phenotypes. The resultant five mutants, with no infectivity for MDMs, showed, without exception, the same defect as the *vpx*-defective mutant. Our results here clearly demonstrated that the entire Vpx protein is critical for reverse transcription of the HIV-2 genome in human MDMs.

Viruses of the human immunodeficiency virus type 2 (HIV-2) group carry a *vpx* gene that encodes virion-associated Vpx protein. Vpx is an accessory viral protein and is completely unnecessary and dispensable for virus replication in established cell lines and primary lymphocyte cells prepared from peripheral blood mononuclear cells (10, 21, 25). However, in human monocyte-derived macrophages (MDMs), the *vpx*-defective viruses do not grow at all (6, 20, 21, 25). Because Vpx is specifically incorporated into virions by association with Gag-p6 protein in significant quantities (1, 11, 12, 24), it has been believed that Vpx has a specific and early functional role at the Env-independent postentry replication step. In fact, there have been some articles directly addressing the early function of Vpx in the life cycle of HIV-2. Worthy of note, one report has shown that Vpx is dispensable for reverse transcription of the viral RNA genome but important for nuclear import of the viral preintegration complex in MDMs (6). But in that study (6), a unique simian immunodeficiency virus (SIV) isolated from the sooty mangabey (SIV_{SM}PBj1.9), which causes an acute fatal disease in pig-tailed monkeys (5), was used to determine the defect of *vpx* mutants in simian MDMs. Another paper has described results similar to those mentioned above, obtained for a U937 cell line growth arrested by mimosine treatment (20). Furthermore, in both studies (6, 20), the conclusions were based on the data obtained from rather qualitative PCR analysis. Therefore, quite surprisingly, virtually no studies focusing on the functional role of HIV-2 Vpx in human MDMs with clear and convincing data have been published yet. In this study, we have performed an extensive mutational functional analysis by quantitative assays of HIV-2 Vpx in human MDMs. We demonstrate here, in contrast to the previously published conclusions, that Vpx is critical for reverse transcription of the HIV-2 genome in human MDMs.

We first evaluated the extent of viral DNA synthesis by a *vpx*-defective mutant at the postentry step in human MDMs by using HSC-F cells (3, 4) as a cell control (21). The mutant used was derived from a well-characterized and widely distributed molecular clone (13, 14, 21) for easy scientific comparison. Infection of human MDMs by virus samples from 293T cells (17) transfected with proviral clones was very much inefficient and gave ambiguous data. To obtain reproducible quantitative results, we conducted the assay as follows. Virus samples (pseudotype viruses) were prepared from 293T cells cotransfected with an expression vector of the vesicular stomatitis virus G protein (pCMV-G) (23) and an *env*-defective proviral clone (pGL-Ns) (21) for the wild type (WT) or an *env*- and *vpx*-defective clone (pGL-Ns/St) (21) for the *vpx* mutant and inoculated into HSC-F and MDM cells. On day 2 postinfection, DNAs were prepared from these infected cells and subjected to real-time PCR analysis using appropriate primer pairs to detect the late reverse transcription product (U5/5′-end noncoding region) in the cytoplasm and the two-long terminal repeat (two-LTR) circle in the cell nucleus. As is clear in Fig. 1, a major replication defect in HSC-F cells of the *vpx*-defective mutant was noticed at the nuclear import process of viral DNA, in good agreement with our previous report (21). By contrast, the mutant was unable to synthesize viral DNA in MDMs, as judged by the absence of the late reverse transcription product, indicating that Vpx is crucial for reverse transcription of the viral RNA genome in a cell type-dependent manner. The same experiments were repeated, using MDMs from different individuals, with perfectly reproducible outcomes. These results prompted us to do a systemic mutational analysis of HIV-2 Vpx in MDMs to dissect its function in the virus replication cycle.

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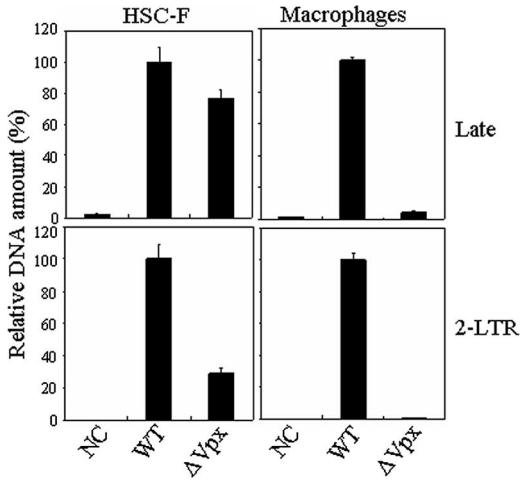

FIG. 1. Quantitative estimation of viral DNA synthesis in HSC-F and human MDM cells infected with the *vpx*-defective mutant. Cell-free virus samples (pseudotype viruses) were prepared from 293T cells cotransfected with 10 μg of pCMV-G and 10 μg of pGL-Ns (WT) or pGL-Ns/St (ΔVpx). For a negative control (NC), pGL-Ns (10 μg) and pUC19 (10 μg) were used for cotransfection. HSC-F cells (1×10^7) and confluent human MDMs, which had been prepared from peripheral blood mononuclear cells and cultured in each well of six-well tissue culture plates as previously described (7, 21) and were 95 to 97% CD68 positive and completely negative for CD3, were infected with equal amounts of these cell-free viruses (4×10^7 reverse transcriptase [RT] units [22] and 1.4 μg of Gag-p27, as determined by enzyme immunoassays of SIV Gag-p27 [Coulter, Miami, FL], for HSC-F and MDM cells, respectively) in the presence of DNase I (40 μg/ml), $MgCl_2$ (10 mM), EGTA (2 mM), and DEAE-dextran (5 μg/ml), as previously described (16). On day 2 postinfection, DNA was extracted from the infected cells and subjected to real-time PCR analysis using TaqMan probes (Applied Biosystems, Foster City, CA) to detect the late reverse transcription product (U5/5′-end noncoding region) in the cytoplasm and the two-LTR circle in the cell nucleus. The β-globin gene was amplified for normalization (19). The primers and probes used were as follows: for the late reverse transcription product (U5/5′-end noncoding region), 5′-TCCGCTTTGGGAATCCAA-3′ (forward primer), 5′-GGGCTTCTCAGTCCCTTTCAA-3′ (reverse primer), and 5′-FAM (6-carboxyfluorescein)-AAAATCCCTAGCAG GTTGGCGCCC-TAMRA (6-carboxytetramethylrhodamine)-3′ (probe); and for the two-LTR circular product (U5/U3 region), 5′-TCGCCGCCT GGTCATT-3′ (forward primer), 5′-CCCTACTGTAAAACATCCCA TCCA-3′ (reverse primer), and 5′-FAM-ACCCTGGTCTGTTAGGA CCCTTCCGC-TAMRA-3′ (probe). The reaction mixtures were heated at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

As shown in Fig. 2, 19 point mutations were introduced into scattered regions of WT *vpx* of an infectious HIV-2 molecular clone designated pGL-AN (13) by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). To determine the target amino acids for mutation, amino acids that are well conserved among various HIV-2 isolates were carefully selected. In addition, since the 5′ region of *vpx* encoding amino acids 1 to 58 of Vpx was overlapping with *vif*, care was taken not to change the amino acids of WT Vif. The mutants thus constructed were introduced into 293T cells, and all the mutants generated progeny virions at a normal level, with MAGI infectivity (15) comparable to that of the WT virus, as expected (data not shown). Various virus clones were then inoculated into human MDMs, and their growth properties were determined. As shown in Fig. 2, out of 19 mutants, 9 grew more poorly than the WT virus and 5 did not grow at all in MDMs. These results were confirmed in repeated experiments, using MDMs from different individuals. The mutations causing the noninfectious mutants (E15G, W24L, H39L, W49L, and Q76A) were not clustered, suggesting that there may be no specific regions or domains important for virus growth in MDMs.

There was a possibility that the damaged or noninfectious nature of the 14 mutants (Fig. 2) is due to the lack of incorporation of mutant Vpx proteins into virions. Initial attempts to detect the expression of Vpx in transfected 293T cells by Western immunoblotting were mostly unsuccessful, probably because the monoclonal and polyclonal antibodies against Vpx used for detection were insensitive. We therefore constructed a Vpx expression vector with a FLAG tag for the five noninfectious mutants and monitored the transfected 293T cells for mutant Vpx by anti-FLAG antibody. A Vpx protein level comparable to that for the WT clone was detected for each mutant (Fig. 3A). We then determined whether the mutant Vpx proteins were actually detectable in the progeny virions. We previously reported that HIV-1 virion-associated viral proteins can be examined after partial purification and concentration of the virions by ultracentrifugation (8). We applied the same method to monitor Vpx in HIV-2 virions. As controls for this experiment, we newly constructed two Gag-p6 site-specific mutants (designated p6/3AS and p6/2A) from pGL-AN, which have mutated amino acid sequences in Gag-p6 critical for the incorporation of Vpx into virions (1) (Fig. 3B). These two mutants were transfected into 293T cells, and 3 days later, virion samples for Western blot analysis were prepared as described above. As is clear in Fig. 3B, the incorporation of Vpx into virions was not detected at all for the two control mutants, as expected, indicating that the procedure used for HIV-1 can be applicable for the preparation of HIV-2 virions. Mutant virions prepared from transfected 293T cells by this method were then monitored for Vpx. As shown in Fig. 3C, the virions of the P4L (WT growth properties), P10L (intermediate growth properties [between those for WT and noninfectious viruses]), and E15G (noninfectious virus) mutants (Fig. 2) contained Vpx, like those of the WT virus. The presence of Vpx in virions of all the other mutants shown in Fig. 2 was also verified by this Western blot analysis. The percentages of specific virion incorporation of Vpx (Vpx/Gag-p27 ratio, as quantified by immunoblotting [Fig. 3C]) of the five noninfectious mutants (E15G, W24L, H39L, W49L, and Q76A) relative to the WT level were 195 ± 16, 193 ± 14, 28 ± 6, 56 ± 6, and 103 ± 19, respectively. Of note, mutant N33S, having a low level of virion-incorporated Vpx in this assay (14 ± 6), was still somewhat infectious for MDMs (Fig. 2).

We finally evaluated the abilities of the five point mutants noninfectious for MDMs (E15G, W24L, H39L, W49L, and Q76A) to synthesize viral DNA in infected human MDMs. MDMs were infected with the mutants (pseudotype viruses) as described above, and the infected cells were similarly analyzed by real-time PCR using two sets of primer pairs. As shown in Fig. 4, the reverse transcription processes at early and late phases of the five mutants were critically impaired, generating no significant quantities of viral DNA in the cell nucleus. The Q76A mutant appeared less attenuated for reverse transcrip-

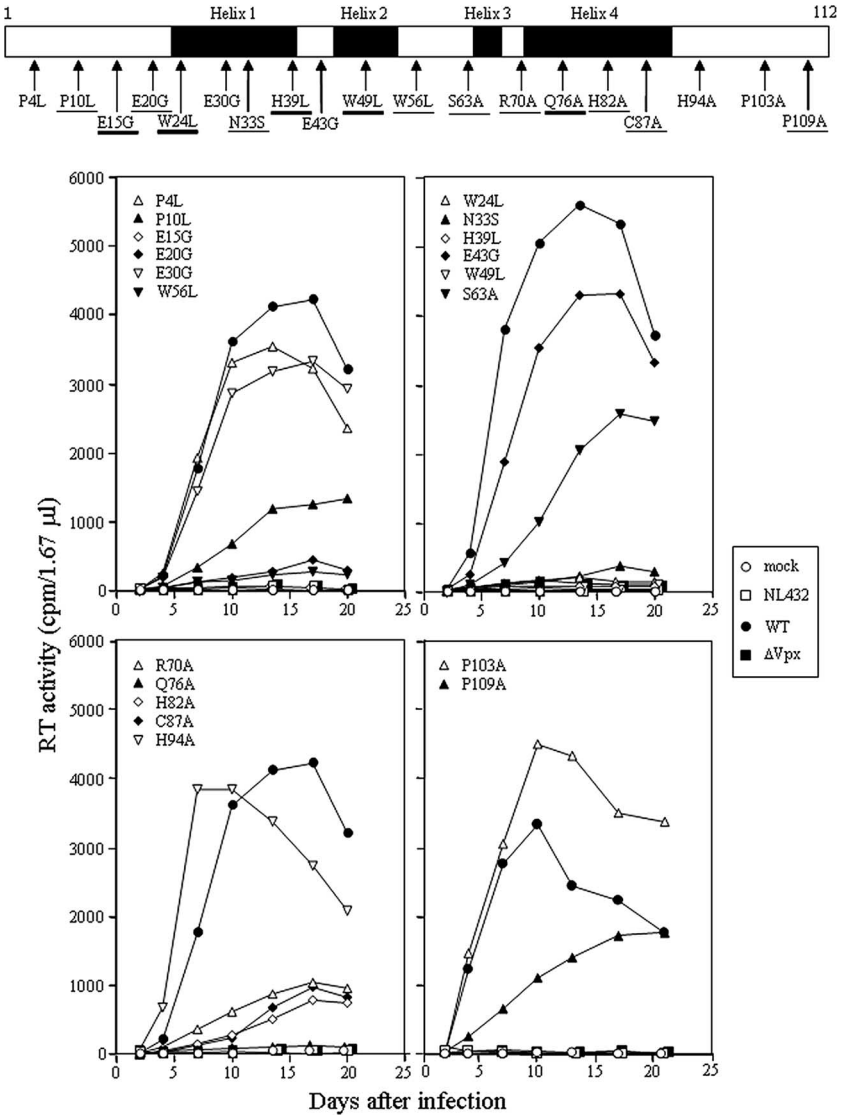

FIG. 2. Growth kinetics in human MDMs of various *vpx* point mutants. Confluent MDMs in each well of 24-well tissue culture plates prepared as described in the legend to Fig. 1 were infected with equivalent numbers of RT units of cell-free viruses (6×10^5) in the presence of DEAE-dextran (5 μg/ml), and viral replication was monitored at intervals by determining RT production in the culture supernatants (22). Input viruses were prepared from 293T cells transfected with 20 μg of pGL-AN, its *vpx* mutants, or the HIV-1 infectious clone pNL432 (2) as a negative control. At the top, the locations of the point mutations in pGL-AN Vpx, consisting of 112 amino acids (GenBank accession no. M30895) with four predicted helices (14), and the standard designations of the *vpx* mutants are indicated. The noninfectious and growth-defective mutants are indicated by bold and thin underlines, respectively. Mock, pUC19; WT, pGL-AN (13); ΔVpx, pGL-St (13).

tion than the other four mutants. The experiment for Fig. 4 was repeated extensively, with reproducible results.

Based on the results described above, we concluded that Vpx is crucially required for reverse transcription of the HIV-2 RNA genome in human MDMs. We also claim here, by our mutational analysis, that a specific region or domain(s) in Vpx may not be responsible for the Vpx activity shown in this report; rather, the entire structure of Vpx is important. Of the five mutations that completely abrogate viral infectivity in MDMs, H39L and W49L might affect the stability of mutant proteins and give the phenotype shown in Fig. 2 and 4. However, this was quite unlikely, because the mutant proteins were stably expressed in cells by a FLAG tag expression vector (Fig. 3). Furthermore, the N33S mutant, which contains a smaller amount of Vpx in virions than the H39L and W49L mutants, still retained viral infectivity. In any case, our main conclusion, that the overall structure of Vpx is crucial for reverse transcription of the HIV-2 genome in human MDMs, is unchanged. Whether inactive or defective mutants other than the five noninfectious mutants shown in Fig. 2 display the defect in nuclear import of viral DNA is another intriguing question to address, and this needs to be determined. Determination of the subcellular localizations of these mutant Vpx proteins could explain their biological differences, if there are any.

Our results described in this report are quite distinct from those previously published (6, 20) but not inconsistent. Clearly,

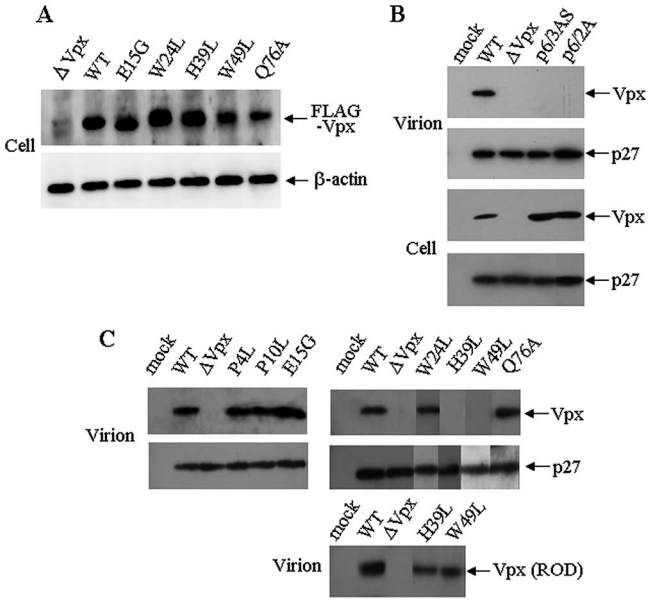

FIG. 3. Immunoblot analysis of mutant Vpx proteins in cells and virions. 293T cells were transfected with 10 μg of an expression vector of WT Vpx designated pME18Neo-Fvpx (14) and its mutants or 20 μg of WT proviral clone pGL-AN (13, 21) and its mutants, as previously described (2), and on day 2 or 3 posttransfection, cell or virion lysates were prepared as previously described (8, 22). The lysates normalized by β-actin or RT activity were then analyzed by Western immunoblotting (7, 8, 14) with the HIV-2 Vpx monoclonal antibody 6D2.6 (Vpx) (NIH AIDS Research and References Reagent Program, catalog no. 2710), an HIV-2 ROD Vpx polyclonal antibody (ROD) (catalog no. 2609), and an antiserum to SIV-p27 (p27) (NIBSC Centralised Facility for AIDS Reagent, repository reference no. ARP414). Commercially available monoclonal antibodies were used for detection of FLAG-Vpx (ANTI-FLAG M2; Sigma-Aldrich, St. Louis, MO) and β-actin (anti-β-actin clone AC-15; Sigma-Aldrich). The results obtained for the mutant Vpx proteins in cells, for the Vpx-defective-virion mutants as predicted by their Gag-p6 amino acid sequences (p6/3AS and p6/2A), and for the mutant Vpx proteins in virions are shown in panels A, B, and C, respectively. Gag-p6 mutants designated p6/3AS and p6/2A have A^{17}, S^{19}, A^{22}, and A^{23} (instead of D^{17}, A^{19}, L^{22}, and L^{23}, respectively) and A^{22} and A^{23} (instead of L^{22} and L^{23}, respectively) in the Gag-p6 amino acid sequence. Because the monoclonal antibody 6D2.6 did not react with the H39L and W49L mutant proteins, the polyclonal antibody was used to detect them as shown in panel C. Mock, pUC19.

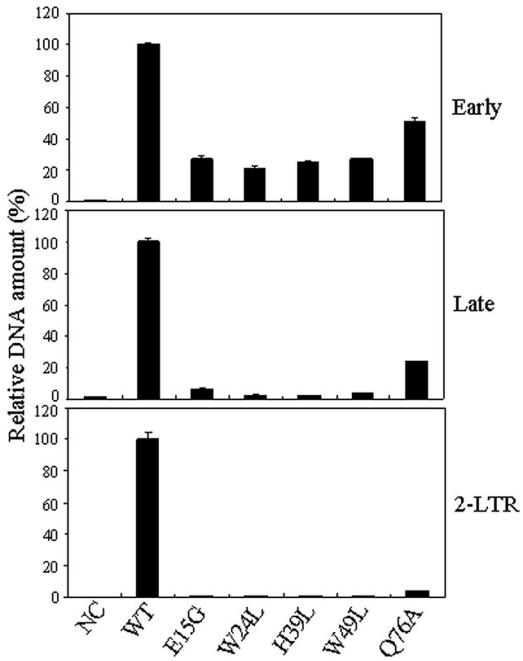

FIG. 4. Quantitative estimation of viral DNA synthesis in human MDMs infected with *vpx* point mutants. Pseudotype viruses were prepared by transfection and inoculated into human MDMs as described in the legend to Fig. 1. The procedures for real-time PCR analysis for Fig. 1 were also used, but the early reverse transcription product (R/U5 region) was additionally monitored here. The primers and probe for the early reverse transcription product (R/U5 region) were as follows: 5′-CAAGT TAAGTGTGTGTTCCCATCTCT-3′ (forward primer), 5′-CCAGGGT CTTGTTATTCAGATGAA-3′ (reverse primer), and 5′-FAM-CTAGT CGCCGCCTGGTCATTCGG-TAMRA-3′ (probe).

we and they have used different experimental systems, including different methods for infection, virus clones, cell types, and methods for analysis of viral DNAs. Therefore, the data obtained could be different. Interestingly, one report has described reproducible reductions in the abundances of reverse transcription products in MDMs infected with *vpx*-defective mutants (6). In agreement with this and our results here, it has recently been demonstrated that Vpx of SIV_{MAC} of the HIV-2 lineage plays an essential role for the reverse transcription process in human dendritic cells (9).

Determination of the molecular basis underlying the macrophage-specific requirement of Vpx for reverse transcription of the viral genome is virologically very important. In this regard, two recently published articles are quite provocative. Goujon et al. reported that Vpx may counteract a restriction factor present in human dendritic cells to escape the proteasome-mediated degradation pathway (9). Le Rouzic et al. showed that Vpx binds to DCAF1/VprBP, an adaptor molecule of the ubiquitin ligase complex (18). These findings have raised the possibility that there is a proteasome-dependent factor(s) in a certain cell type that suppresses reverse transcription. It is not unreasonable to assume that HIV-2 Vpx antagonizes such a factor, thus efficiently promoting viral replication. In addition, it has been well established that innate antiretroviral factors, such as TRIM5α and APOBEC3G/F, target the step of viral DNA synthesis. The association of HIV-2 Vpx with the reverse transcription process of the viral RNA genome needs to be biochemically proved to clarify the early events of HIV-2 replication precisely.

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