The M184V Mutation in Reverse Transcriptase Can Delay Reversion of Attenuated Variants of Simian Immunodeficiency Virus

James B. Whitney,1,2 Maureen Oliveira,1 Mervi Detorio,1 Yongjun Guan,1† and Mark A. Wainberg1,2*

McGill University AIDS Centre, Lady Davis Institute-Jewish General Hospital, Montreal, Quebec, Canada H3T 1E2,1 and Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada H3A 2B42

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We previously constructed a series of simian immunodeficiency virus (SIV) mutants containing deletions within a 97-nucleotide region of the SIVmac239 untranslated region or leader sequence. However, as is common with live attenuated viruses, several of the mutants exhibited a moderate propensity for reversion. Since the M184V mutation in human immunodeficiency virus type 1 reverse transcriptase is associated with diminished fitness as well as lamivudine resistance, we introduced this substitution into several of our deletion mutants to determine its effects on viral replication and compensatory reversion. Our results indicate that M184V impaired viral fitness in pair-wise comparisons of mutants that contained or lacked this substitution. We also observed that M184V significantly impaired the potential for both compensatory mutagenesis and reversion in these mutants both in cell lines and in peripheral blood mononuclear cells.

Genetic variation in human immunodeficiency virus type 1 (HIV-1) and other retroviral lineages has been associated with multiple factors, which include viral recombination, reverse transcriptase (RT) infidelity, and both viral and cellular factors that can influence mutation rates (10, 15, 21, 25, 33). The generation of viral quasispecies as a result of RT infidelity is largely due to the lack within viral RT of a 3′-to-5′ proofreading ability, combined with high rates of viral replication (8, 31).

The catalytic domains of the RTs of HIV-1 and its simian counterpart, simian immunodeficiency virus (SIV), both include a highly conserved YMDD motif. This domain is common throughout the polymerase family of enzymes (26), and mutations within this region are commonly lethal. However, viruses resistant to antiretroviral nucleoside 2′,3′-dideoxy-3′-thiacytidine (3TC) harbor a single M184V substitution within the aforementioned motif (32). This same substitution in RT is associated with resistance to 3TC in the case of SIV (7).

In HIV-1, it is well documented that the M184V mutation also confers a deficit in fitness that is most apparent in primary cell lines (2). The reasons for this are multifaceted and include the fact that RT enzymes containing M184V are associated with diminished processivity (2, 3, 23, 28), diminished nucleotide primer unblocking (11), and diminished ability to initiate reverse transcription (M. Gotte, X. Wei, K. Diallo, B. Marchand, A. Schaffer, and M. A. Wainberg, 5th Int. Workshop HIV Drug Resist. Treat. Strateg., abstr. 46, 2001). These events are also modulated by intracellular deoxynucleoside triphosphate substrate availability (3).

In the aftermath of deletion mutagenesis, leading to attenuation of replication, genetic variation requires passage through the constraints of an artificially produced bottleneck. Under these conditions, the spectrum of compensatory mutations is likely to be restricted (29, 30). In this study, we demonstrate that the M184V substitution can impair the viral capacity for reversion in the context of specific deletions within the 5′ leader regions of a series of attenuated SIVmac239 constructs. As well, the presence of the M184V substitution may affect the process of compensatory mutagenesis in regard to codon change.

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Several of our viral deletion mutants were previously shown to display moderate reversion kinetics over serial passage (12, 13), i.e., constructs SD2, SD5, and SD6. The M184V mutation was introduced into the RTs of these constructs by site-directed mutagenesis of the pCRII vector containing 1.7 kb of the SIV RT coding region as described previously (7). The coding sequence for the recovered M184V-containing RT fragment was then inserted between the NarI and BamHI sites in the full-length wild-type (WT) and mutant SIV proviral clones. All recombinant viruses were confirmed by sequencing.

After transfection of COS-7 cells with appropriate plasmid DNA using lipofectamine (GIBCO, Burlington, Ontario, Canada), viral supernatants were recovered and the concentration of p27 antigen in these stocks was quantified with a Coulter SIV core antigen assay kit (ImmuneTech Inc., Westbrook, Maine) as described previously (12).

Viral replication assays. Viral inocula for each construct, equivalent to 10 ng of p27 CA antigen, were treated with DNase I and used to infect CEMx174 cells (12). RT assays were used as a surrogate for viral replication and revealed that the presence of M184V together with the various deletions in the 5′ leader resulted in an additional impairment in viral replication compared to when M184V was not present. These results were observed consistently in replicate experiments,
regardless of which leader mutant was studied. The impairment for each mutant virus containing M184V was further amplified by the presence of 8 μM 3TC, which further constrained viral replication by an additional 2 to 4 days (results not shown). This may have been due to additional selective pressure by 3TC to maintain the M184V mutation and prevent the outgrowth of revertant viruses.

To establish the potential for viral reversion over protracted periods, we performed serial passage or "forced evolution" of our mutant constructs using the CEMx174 cell line. Typically, aliquots of viral supernatants were taken at the observed peak of infection, and these samples were then used to infect fresh CEMx174 cells at doses equivalent to 10 ng per 10^6 cells. Infected cells were grown over protracted periods, and culture fluids were monitored by RT assay. Mock infection denotes exposure of cells to heat-inactivated WT virus as a negative control. A representative example of the SD2-M184V and SD6-M184V variants at the fourth passage is shown (the experiment was performed three times with similar results).

To determine the propensity for phenotypic reversion in PBMCs, additional passages were performed with supernatants taken at the peak levels of viral p27 antigen production, at 21 days after infection. These were used to infect fresh stimulated PBMCs from the same animal, i.e., donor B. This second passage of mutant virus harboring the M184V substitution exhibited levels of antigen production nearly identical to that seen during the first infection (not shown), suggesting that no increase in replication capacity occurred in this case.

We also used viral supernatants collected at the peak of the first passage in monkey PBMCs, again from donor B, to infect fresh CEMx174 cells. The results show that increased viral replication occurred in variants that lacked the M184V mutation (Fig. 2C). In contrast, replication in M184V-harboring fresh CEMx174 cells showed undetectable levels of viral replication during 2 months of passage as assessed by RT assay.

RNA dimerization. We also assessed the ability of the SD2 mutant virus to properly incorporate a mature RNA dimer. Nondenaturing Northern analysis of purified RNA preparations had indicated that deletion of the sequence between nucleotides +398 and +418 in SD2 completely eliminated viral RNA dimerization. The additional presence of the M184V mutation together with the leader mutation did not appear to outwardly affect RNA dimerization (unpublished data).

Those viruses that were continually passaged in the CEMx174 cell line were sequenced by PCR amplification of proviral DNA recovered from cells isolated at the peak of the fourth round of infection. The sequencing of the complete SD2-M184V untranslated region (UTR) and gag regions showed numerous point mutations in all clones. Despite this variability, one point mutation that corresponded to a G-to-A transition in MA, encoding a change from a threonine to isoleucine at residue 70 (T70I), was found in all six sequenced clones.

To assess the relevance of this mutation, site-directed mutagenesis was performed with the SD2-M184V clone to produce the variant termed SD2-MA-M184V. Infectious inocula were produced in COS-7 cells and were then used to infect CEMx174 cells in parallel with controls, which included two
previously described SD2 reversion mutants (12, 14). The compensatory mutations that restored the SD2 virus to replication competence had been shown to be members of two distinct sets of mutations within the putative dimerization initiation site (DIS) loop (A423G) and within several different Gag proteins, i.e., NC (E18G and G31K) or CA (K197R) and p6 (E49K). These amino acid changes are responsible for restoration of viral RNA packaging and viral fitness (14). A similar situation has been observed for deletions within the SL1 region of HIV-1 (20).

Figure 3A also shows that the T70I mutation in MA was sufficient to confer a replicative advantage in the context of SD2-M184V. This codon change had no observable effect on a WT virus (not shown). MA is known to be involved in the targeting of both Gag and genomic RNA to the cell membrane and in the formation and stabilization of genomic RNA dimers (6, 9). The potential role of the T70I MA mutation in the rescue of viral replication is also suggested by recent studies on a role for upstream leader sequences and the MA coding sequence in formation of an extended RNA pseudoknot structure. Extended interactions involving a region of MA and the pol open reading frame have also been reported (24, 27). Additional in vitro evidence also supports a role for higher-order structures in the regulation of viral replication (16).

To further explore the notion that reversion of SD2-M184V virus was impaired specifically as a result of M184V, we inserted this substitution into the two aforementioned SD2 reversion mutants, termed SD2-DIS-NC1+2-M184V and SD2-DIS-CA-P6-M184V. Both these variants were impaired in replication ability in CEMx174 cells compared with equivalent constructs that lacked M184V (Fig. 3B).

In summary, the M184V mutation in RT adversely impacts the replicative fitness of a number of SIV constructs. Furthermore, SIVs containing both the M184V and DIS mutations are less able to effect repair through compensatory mutagenesis than are viruses containing a WT RT. Our results also show that viral species that harbored both the M184V mutation and deletions in the region of the DIS displayed reduced replication capacity over multiple passages. Similar results have been reported with HIV-1 viruses containing the M184V mutation in RT (17, 19). In nonhuman primate studies, M184V-containing SIV failed to revert to WT and may have been initially impaired in its ability to multiply to high titer. However, this replication deficit may have been corrected over time, as a consequence of a distinct compensatory mutation within RT (22).

We should point out that attenuation of the SD2-M184V variant may be partly attributable to synergy between the 5′ UTR and the Gag-Pol region in regard to both structure and function. HIV-1 RTs that harbor M184V suffer from diminished ability to initiate reverse transcription and to participate in the elongation phase of minus-strand DNA synthesis (5, 18).
In addition, the UTRs of both HIV and SIV play key roles in RNA dimerization and strand transfer (1, 4, 18).

Finally, we have shown that both RT and UTR sequences are necessary for restoration of viral replication, and our experiments suggest that viral recombination is involved in the process of compensatory mutagenesis. Mutants that are presumed to lack this function in the process of reversion might be relegated to fixing advantageous mutations in an iterative fashion, likely imparting delays to restoration of a WT replication phenotype.

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FIG. 3. (A) Reversion of the SD2-M184V variant after replication in CEMx174 cells. Growth curves of reverted viruses in CEMx174 cells. Equivalent amounts of virus from transfected COS-7 cells were used to infect CEMx174 cells based on levels of p27 antigen (10 ng per 10⁶ cells). Viral replication was monitored by RT assay of culture fluids. Shown is a representative growth curve of experiments conducted in triplicate. Mock infection denotes exposure of cells to heat-inactivated WT virus as a negative control. (B) The M184V mutation restricts compensatory mutagenesis in the case of the SD2 variant. Viruses derived from COS-7 cells were standardized on the basis of p27 CA antigen and used to infect 10⁶ CEMx174 cells. RT activity of culture fluids was used to monitor replication. Shown is a representative growth curve of experiments performed in triplicate. Mock infection denotes exposure of cells to heat-inactivated WT virus as a negative control. Note that the scales of the ordinates are logarithmic in both panels.
human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. J. Virol. 69:5087–5094.


