Novel, Live Attenuated Simian Immunodeficiency Virus Constructs Containing Major Deletions in Leader RNA Sequences

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Simian immunodeficiency virus (SIV) is a primate lentivirus closely related to human immunodeficiency virus type 1 (HIV-1) (14, 21, 35, 38, 41). Highly attenuated strains of SIV containing deletions in nonessential genes have been shown to elicit strong protection against pathogenic challenge in primate models (1, 7, 9, 13, 43). Although live attenuated nef-deleted viruses have protected monkeys against challenge with wild-type viruses, this type of vaccine is considered unacceptable because of reversions and safety concerns (18, 38). For example, multiply deleted SIV strains have been shown to be pathogenic in neonatal macaques (2, 3). Notably, as well, these mutants are able to replicate in permissive cell lines (15, 34). Although the relationship between viral replication capacity and pathogenesis is not always clear, high plasma viral load is strongly correlated with disease progression in the case of HIV-1 (33).

Extensive studies have shown that leader sequences within the HIV-1 genome, between the primer binding site and the major splice donor site, play a critical role in various aspects of viral replication, including packaging of viral genomic RNA, Gag protein processing, reverse transcription, and gene expression (20, 22, 24, 26, 28, 29). HIV mutants containing deletions in this region are highly attenuated in ability to grow in permissive cell lines, yet retain the ability to synthesize all viral proteins. Recent work has also revealed a similar role for the leader sequences in SIV (17).

Deletion of select areas of the leader sequences in HIV and SIV may conceivably represent a good vaccine strategy. To further study this topic, we have constructed a series of mutated SIV variants containing deletions in this region. These mutated SIV strains displayed similar patterns of impairment in both permissive cell lines and in monkey peripheral blood mononuclear cells (PBMCs), and certain of these viruses are stable in tissue culture for periods of 6 months to 1 year, with no sign that reversions or compensatory mutations have occurred. Animal trials of these novel, live attenuated viruses will be needed to delineate relationships between replication capacity and pathogenesis and to establish potential protective capacity and correlates of immunity.

MATERIALS AND METHODS

Construction of deletion mutants. The full-length infectious clone of SIV, SIVmac239/WT (17, 23), was used to construct deletion mutants. We used a PCR-based mutagenesis method to generate deletions downstream of the primer binding site; Pfu polymerase was used to increase the fidelity of the PCR. Briefly, the region between the NruI and BamHI sites in SIVmac239/WT was replaced with PCR fragments to generate mutant constructs. Figure 1 graphically illustrates the mutants generated. Primers pSD1a/pSgag1 were used for the SD1a deletion, and primers pSD1b/pSgag1 and pSD1c/pSgag1 were used for SD1b and SD1c, respectively. For construction of the SD4, SD5, and SD6 deletions, PCR fragments (pSD4/pSgag1, pSD5/pSgag1, and pSD6/pSgag1 for SD4, SD5, and SD6, respectively) were purified and were then used as mega-primers with primer pSL5. The resulting PCR fragments were then replaced to use primers between NruI and BamHI sites in SIVmac239/WT. The construction of the SD, SD1, SD2, and SD3 mutants has been described previously (17), and the sequences of novel primers used in the present work are as follows: pSD1a, 5’-GATTGGCGCCTGAAACAGGGAC/GCAGTAAGGGCGGCAGG-3’ (+301 to +321/+363 to +379); pSD1b, 5’-GATTGGCCCTGAAACAGGGAC/GCAGTAAGGGCGGCAGG-3’ (+301 to +321/+371 to +387); pSD1c, 5’-GATTGGCGCCTGAAACAGGGAC/GCAGTAAGGGCGGCAGG-3’ (+301 to +321/+380 to +396); pSD4, 5’-CTGATGTAAGGGCACTTAAGCAGCAGGCGGCGGCGG-3’ (+353 to +370/+398 to +418); pSD5, 5’-GATTGGCCCTGAAACAGGGAC/GCAGTAAGGGCGGCAGG-3’ (+301 to +321/+380 to +396); pSD4, 5’-CTGATGTAAGGGCACTTAAGCAGCAGGCGGCGGCGG-3’ (+353 to +370/+398 to +418); and pSD6,
FIG. 1. Illustration of the deletion constructs used in this study. Secondary structures of the U5-leader stem and the putative DIS stem-loop of SIVmac239 leader RNA are shown. The positions of deletion constructs are relative to the transcription initiation site and are shown next to the RNA structure. These positions are also indicated in the diagram of the secondary structure. Both the primer binding site (PBS) and the DIS palindrome sequences are highlighted.
RESULTS

A short nucleotide sequence downstream of the primer binding site plays a key role in SIV replication. We previously constructed four SIV mutants (SD, SD1, SD2, and SD3) containing large deletions within a 97-nucleotide (nt) region downstream of the primer binding site. Each of these deletions resulted in impaired viral replication in C8166 cells, and yet, in one case, compensatory mutations and restored viral replication capacity were observed over time (17). To further investigate the possibility of establishing attenuated strains that would be more permanently attenuated and to further assess which sequences were responsible for impaired virus replication, we generated six additional deletion constructs (Fig. 1). The original SD1 deletion of 22 nt at positions +322 to +344 had little long-term impact on virus replication, so we extended the deletion to lengths of 41, 49, and 58 nt, i.e., constructs SD1a (+322 to +362), SD1b (+322 to +370), and SD1c (+322 to +379), respectively (Fig. 1). RNA secondary structure analysis indicated that a nucleotide stretch downstream of the primer binding site can form a stem with an upstream sequence (i.e., the U5-leader stem) (37). A sequence homology search for a binding site for a regulatory factor revealed sequences with high homology to the SP1 binding site at positions +370 to +397 in SIV (data not shown). Accordingly, we generated the deletion mutants, termed SD4 (+371 to +397), SD5 (+380 to +397), and SD6 (+371 to +379) (Fig. 1) and studied the effect of these deletions on virus replication. The results in Fig. 2A show that both the SD1a and SD1b viruses were partially impaired, while each of the SD1c, SD4, SD5, and SD6 viruses was severely impaired in ability to replicate in C8166 cells. The SD6 construct retains the 5' portion of the exact sequence that was deleted in SD1c, but not in SD1a or SD1b. These findings suggest that the deletion of as few as 9 nt at positions +371 to +379 contributed to severely impaired virus replication. The fact that the extent of impairment of SD5 was similar to that of SD3, SD1c, and SD suggests that the sequences at positions +380 to +397 (i.e., between the U5-leader stem and the DIS stem-loop) are also important in this regard.

We have previously shown that long-term culture of cells infected by the SD2 virus resulted in reversions (17). However, maintenance of cells infected by the SD4, SD5, SD6, and SD1c viruses revealed that the SD4 and SD1c viruses appeared to be stably impaired (Fig. 2B). In contrast, the SD5 and SD6

FIG. 2. Replication capacity of mutated viruses in C8166 cells. (A) Equivalent amounts of virus from COS-7-transfected cells were used to infect C8166 cells based on levels of p27 antigen (10 ng per 10⁶ cells). Viral replication was monitored by RT assay of culture fluids. Mock transfection denotes exposure of cells to heat-inactivated wild-type (WT) virus as a negative control. (B) Replication capacity of mutated viruses during long-term tissue culture in C8166 cells.

FIG. 3. Replication capacity of mutated viruses in CEMx174 cells. (A) Equivalent amounts of virus from COS-7-transfected 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. Mock infection denotes exposure of cells to heat-inactivated wild-type (WT) virus as a negative control. (B) Replication capacity of mutated viruses during long-term tissue culture.
viruses achieved higher levels of viral replication after 9 to 10 weeks.

**Replication of deleted viruses in CEMx174 cells.** The B/T hybrid cell line known as CEMx174 lends itself well to the replication of SIV (39). The results of Fig. 3A show that the SD1 mutants replicated in these cells with kinetics similar to those of wild-type viruses. Although both SD1a and SD1b were partially impaired, this occurred to a lesser degree than in the C8166 cells. SD2, SD5, and SD6 displayed moderately delayed growth, but still replicated faster in CEMx174 cells than in the C8166 line. And, as with C8166 cells, the SD, SD3, SD1c, and SD4 mutants were severely impaired in ability to grow in the CEMx174 cell line.

The long-term culture of infected CEMx174 cells showed that SD4 viruses attained peak levels of RT activity at 6 weeks postinfection (Fig. 3B), while the SD, SD1c, and SD3 viruses did not show any signs of reversion after 6 months. The combined results of infections in C8166 and CEMx174 cells show that the SD2, SD5, and SD6 viruses are attenuated to an extent that is tolerated by both cell lines, while SD4 is a highly attenuated virus that can grow marginally in CEMx174 cells, but not in C8166 cells.

The potential for viral reversion over protracted periods was also investigated with CEMx174 cells. Viruses from the peaks of RT activity in the initial infection were used to infect fresh cells. The replication kinetics of these second-passage viruses are shown in Fig. 4. All of the passaged viruses still showed impaired replication kinetics compared to wild-type virus. The results of PCR and sequencing confirmed that all of these viruses retained their original deletions (data not shown).

The infectiousness of these mutated viruses was also determined by the TCID_{50} assay in CEMx174 cells and by sMAGI assay (Fig. 5). The results are consistent with those obtained by growth curve assay as described above. Therefore, the deletion of leader sequences downstream of the primer binding site adversely affected viral infectiousness.

![Fig. 4](http://jvi.asm.org/) Replication capacity of mutated virus during the second passage in CEMx174 cells. CEMx174 cells were infected with equivalent amounts of virus from the peak time of RT production after initial infection based on levels of p27 antigen (10 ng per 10^6 cells). Viral replication was monitored by RT assay of culture fluids. Mock infection denotes exposure of cells to heat-inactivated wild-type (WT) virus as a negative control.

![Fig. 5](http://jvi.asm.org/) Infectiousness of the wild type (WT) and various mutated viruses. The results shown are the averages of three independent experiments. Each of the SD, SD1c, SD3, and SD4 viruses was shown to be poorly infectious, with RT values being below the threshold sensitivity of the assay (dashed line). Mock infection represents a negative control in which cells were exposed to heat-inactivated wild-type virus. (A) TCID_{50} of the wild type and various mutated viruses were determined by infection of CEMx174 cells as described in Materials and Methods. (B) Infectivity was tested by sMAGI assays as described previously (5). Numbers of blue-stained cells were scored and plotted.
Replication of deleted viruses in macaque PBMCs. As shown in Fig. 6A, SD1 viruses (deletion of the sequence of position +322 to +344) grew in PBMCs with kinetics close to those of wild-type virus. In contrast, both SD1a and SD1b showed impairment in replication capacity, growing to only 20 to 40% of wild-type levels. The replication of the SD1c mutant was completely impaired in these studies. This again reaffirms that the 9-nt sequences at positions +371 to +379 are extremely important. This is also shown in the case of the SD6 virus, which was highly impaired in replication capacity and grew to only 5% of wild-type levels. Deletion of the sequences from +380 to +397 (SD5) only moderately impaired viral replication (i.e., 10% of the wild-type virus level), while SD2 and SD4 were even further diminished in this regard, i.e., <1% of wild-type levels. As with SD1c, the replication levels of the SD and SD3 viruses were below the limit of detection. This is in contrast to infection in C8166 cells, in which SD and SD3 produced detectable levels of viral p27 antigen (17). Therefore, these mutants were also attenuated in monkey PBMCs, in which the relative in vitro virulence of those viruses can be ranked as follows: SD, SD3, SD1c<SD4<SD2<SD6<SD5<SD1b<SD1a<SD1<wild type.

We further examined the relative replication capacity of these mutants in monkey PBMCs by using blood samples from the same animal harvested 6 months apart as well as blood from another monkey. The results in Fig. 6B and C show that our mutant viruses were similarly impaired in replication capacity, as were the viruses studied in Fig. 6A, except that the SD2 virus displayed marginally greater replication in the PBMCs of monkey 2 than in those of monkey 1.

To investigate the potential for phenotypic reversion of these mutants after replication in monkey PBMCs, several cell-free viruses harvested at 7 days after infection of PBMCs (Fig. 6A) were used to infect new PBMCs from the same animal as well as CEMx174 cells. As shown in Fig. 7A and B, these passaged viruses still showed impaired replication kinetics similar to those seen during the initial infection of the PBMCs (Fig. 6A) and CEMx174 cells (Fig. 3A).

Deletions of sequences at positions +322 to +418 affect both packaging of viral genomic RNA and processing of Gag precursor protein. We previously showed that a large deletion in each of the SD, SD2, and SD3 constructs could adversely impact packaging of viral genomic RNA (17). To study this subject mechanistically in these and our more stably attenuated viruses, we next analyzed the efficiency of viral RNA packaging in the mutated viruses containing small deletions in the leader region. RT-PCR was employed to amplify a region of the gag gene as previously described (17). The results in Fig. 8 show that both the SD1a and SD1b mutants packaged viral RNA with efficiency similar to that of wild-type virus, while the deletions in the SD1c, SD4, SD5, and SD6 constructs reduced packaging to 19, 21, 35, and 33% of wild-type levels, respectively. Thus, the sequences at both positions +371 to +379 and +380 to +397 apparently play a key role in viral RNA packaging, while those at positions +322 to +370 do not.

To shed further light on these deficits, we also examined the processing of Gag precursor proteins through short-term radiolabeling and immunoprecipitation experiments, since previous work with HIV-1 had shown that leader sequences are important for protein processing (28, 29). Immunoprecipita-
tion of viral proteins in cell lysates was achieved through use of MAbs against SIV p27 (CA); this permitted identification of the Gag precursor Pr55, the intermediate proteins p40 and p28, and the mature p27 product. The amount of each protein was quantified by densitometry, and for each virus, the percentage of each band relative to total protein was plotted (Fig. 9). We found that the SD1 mutant possessed proportions of these four proteins similar to those of wild-type virus, while all of the other mutants displayed an accumulation of each of the Pr55, p40, and p28 proteins and diminished levels of p27. Both the SD1a and SD1b mutants appeared to suffer only minor impairment in the processing of Gag precursor proteins, while SD1c and SD6 were moderately affected in this regard. In contrast, the deletions in SD2, SD3, SD4, SD5, and SD resulted in severely impaired processing of Gag precursor proteins.

To further study this topic, 35S-labeled viral progeny that were released during 1 h from 293T cells were purified at 24 h after transfection; their protein band patterns are shown in Fig. 10. Seven of the protein bands were quantified by densitometry, and for each virus, the percentage of each band versus all seven proteins was plotted (Fig. 10). The seven bands include the Gag precursor Pr55 (band 1), the Gag intermediate proteins p40 (band 3) and p28 (band 6), and the mature p27 product (CA, band 7). The other three bands, with molecular masses of 43 (band 2), 36 (band 4), and 34 (band 5) kDa, cannot be easily identified, but most likely represent proteins that are found to a limited extent in virions (e.g., the integrase, the transmembrane envelope protein, or the Gag intermediate proteins present in immature virions). We found that the SD1 virus displayed a protein pattern similar to that of wild-type virus, while all of the other mutated viruses appeared to be modified, particularly with regard to bands 2 and 4, which were represented only to a limited extent.

DISCUSSION

The RNA genomes of both HIV-1 and SIV contain a long 5’ untranslated leader sequence that is crucial for viral replication. Although the 5’ untranslated leader sequence of SIV has little sequence homology with that of HIV-1, similar secondary structures have been predicted for both (37). Studies with HIV-1 have shown that the leader sequences between the primer binding site and the major splice donor site are important for viral replication and are involved in packaging of viral RNA, gene expression, the processing of viral core protein, and reverse transcription (20, 22, 24–29). In the case of SIV,

<table>
<thead>
<tr>
<th>Template</th>
<th>WT</th>
<th>SD5</th>
<th>SD4</th>
<th>SD1c</th>
<th>SD1a</th>
<th>SD1b</th>
<th>SD6</th>
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<td>150</td>
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<td>80</td>
<td>20</td>
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<td>-</td>
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<tr>
<td>Relative digestion</td>
<td>1.0</td>
<td>0.35±0.08</td>
<td>0.21±0.04</td>
<td>0.19±0.06</td>
<td>1.01±0.04</td>
<td>0.98±0.04</td>
<td>0.33±0.02</td>
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FIG. 8. Viral RNA packaging in the wild type (WT) and mutated viruses. Equivalent amounts of virus derived from transfected COS-7 cells, based on levels of p27 antigen, were used to prepare viral RNA, which was then used as a template for quantitative RT-PCR to detect the full-length viral RNA genome in an 18-cycle PCR. Relative amounts of a 114-bp DNA product were quantified by molecular imaging, with wild-type values arbitrarily set at 1.0. Reactions run with RNA template, digested by DNase-free RNase, served as a negative control for each sample to exclude any potential DNA contamination. Relative amounts of viral RNA that were packaged were determined on the basis of four different experiments.
this region is also involved in the packaging of viral RNA (17). The present work extends these observations by showing that deletion of the upper part of the U5-leader stem did not impair viral RNA packaging (SD1a, SD1b), but did affect both the patterns of viral proteins and Gag precursor processing.

The lower part of the stem-loop is even more important, since all deletions in this region resulted in severely impaired replication capacity, as well as decreased packaging of viral RNA, delayed processing of Gag precursor proteins, and changed patterns of viral proteins. It may be that a stable U5-leader stem is essential for virus replication and that this stem is destroyed by deletions in its lower part, but not upper part. Our results also show that the sequence between the U5-leader stem-loop and the putative DIS stem-loop is important. Deletion of this region adversely affected packaging of viral RNA, processing of Gag proteins, and patterns of viral proteins. From a mechanistic standpoint, it is known that viral RNA packaging is a process involving specific recognition between viral proteins and viral RNA elements.

Studies with HIV-1 have shown that the viral nucleocapsid (NC) protein, which is required for packaging of viral RNA, can bind to viral leader RNA sequences with high affinity in cell-free assays (8). An encapsidation signal, located at the 5’ end of the viral genome, consists of four RNA stem-loop structures, termed SL1 to SL4, and of these, SL1 and SL3 are the major elements that bind NC proteins (6, 19, 32). Deletions of SL1 in HIV-1 were shown to impair viral replication, as well as cause delayed processing of Gag proteins and decreased levels of viral RNA packaging (26, 28). Compensatory point mutations in four distant Gag proteins, i.e., NC, MA, CA, and p2, were able to restore these deficits (27, 29). Similar studies with SIV have also shown that deletions of leader sequences that affect viral RNA packaging can be rescued by compensatory point mutations in Gag proteins (17). These observations and the present work suggest the likelihood of important functional interactions between Gag proteins and leader sequences in both HIV-1 and SIV. Both the processing of Gag proteins and encapsidation of viral RNA may require that leader RNA sequences exist within constraints of proper tertiary structure, which are highly conserved in both HIV-1 and SIV (37). The changes reported here with regard to patterns of viral proteins may be the consequence of impaired processing of Gag proteins. Such delayed processing, caused by our deletions, may result in abnormal incorporation of proteins into virions, although other mechanisms to explain the attenuation effects seen here are also possible and are under investigation.

The development of a safe, effective vaccine to protect against infection by HIV-1 must be regarded as a top priority.

FIG. 9. Processing of SIV Gag precursor proteins. 293T cells transfected with wild-type (WT) or mutated SIV constructs were radiolabeled and viral proteins in the cell lysates were then immunoprecipitated with MAbs against SIV p27 as described in Materials and Methods. The positions of viral Gag proteins are shown on the right side of the gel (A). Mo, mock transfection control. The percentage of each viral protein relative to all viral proteins detected was calculated with the NIH Image program. The results are illustrated as well by a bar graph (B) showing the different percentages of each band associated with each of the constructs studied, as well as by a line chart (C), showing a steady change in band representation from wild-type virus to the mutated constructs.
in public health. In monkeys, live attenuated SIVs have been shown to induce a protective immune response against pathogenic strains (1, 7, 9, 13, 43). Most of this work has involved deletions of accessory genes, such as nef, vpr, or vpx. However, these mutated viruses have retained replication capacity similar to that of wild-type virus in permissive cell lines (15, 34). In addition, multiply deleted SIV variants have been shown to be pathogenic in neonatal macaques (2, 3).

In contrast, our group has studied viruses containing deletions in the noncoding leader regions of both HIV-1 and SIV (17, 25–29). Unlike the SIV strains that have been mutated in accessory genes, our deleted viruses are significantly impaired in replication capacity in cell lines, yet they retain all viral genes, including accessory genes. This may be important, since the Nef protein and other viral nonstructural proteins are important targets of antiviral immune responses (42, 45). In the present work, we have constructed a series of SIV mutants that are attenuated to different extents in replication capacity in both human T-cell lines and macaque PBMCs. Next, we wish to evaluate the safety and protective capacity of these constructs in a macaque monkey model.

The attenuation strategies employed in our work versus deletions in accessory genes also have different mechanistic consequences. Deletion of accessory genes can compromise the ability of the virus to replicate under certain circumstances (10), but such attenuation is relatively inefficient, since the vi-
ruses retain replication capacity in cell lines and because multiply deleted SIV mutants are still pathogenic (2, 3, 37). Of viruses retain replication capacity in cell lines and because multiple replication steps may impair multiple steps in the viral life cycle, since the deleted sequences are contained in all viral RNA species (both full-length viral RNA and spliced viral RNA) and are involved in multiple functions (17, 20, 22, 24–29).

Studies of SIV infection of rhesus macaques have indicated that the intrinsic susceptibility of monkey PBMCs to infection with SIV in vitro was predictive of relative viremia after SIV challenge (16, 30, 40). However, significant animal-to-animal variation exists, and it is difficult to identify viruses that fit the “window” between levels of replication that elicit a protective immune response and those that result in disease (38). Our panel of novel mutants displayed a wide range of replication levels, i.e., from minor to severe impairment, in both T-cell lines and monkey PBMCs. This constitutes a major advantage of our novel, live attenuated viruses compared with multigene-deleted SIVs that retain high levels of replication competence in T-cell lines and often in PBMCs as well (15, 34). In vivo analysis of our panel of mutants may identify viruses that do fit the “window” required for identification of the requisite level of viral replication in outbred hosts.

A major safety concern with regard to live attenuated viruses as vaccine candidates is the problem of phenotypic reversion. Our experiments with PBMCs from two different monkeys as well as separate time points showed that our mutants were stably attenuated in these cells. In contrast, some of our constructs did show a potential for reversion in a T-cell line; however, such reversion appeared only gradually and involved several additional mutations (17). Replication studies with PBMCs showed no reversion of viruses that had been passaged in either PBMCs or CEMx174 cells. Hence, we hope that the potential for reversion in vivo, under the pressure of an immune response, would be minimal and that it might not result in disease.

Of all the attenuated constructs that we have developed, we believe that SD4 holds the most potential for work in animals. Further studies will hopefully be directed toward assessing its stability and ability to induce immune responsiveness in both newborn and adult rhesus macaques.

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