Generation of Deletion Mutants of Simian Immunodeficiency Virus Incapable of Proviral Integration

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Deletion mutants of simian immunodeficiency virus (SIVmac) which were unable to integrate into host cells were generated by removing a portion of the integrase (IN) domain of the *pol* gene. The resulting plasmid was transfected into HUT-78 and human rhabdomyosarcoma cells. In comparison with the parental plasmid DNA transfected in parallel, the deletion mutant was found to direct efficient production of virus in both cell systems. Viruses derived from wild-type and mutant proviral DNAs were also tested for their relative replicative abilities in HUT-78 and U937 cells, and the kinetics of virus production was found to vary between these two cell systems. Analysis of DNA from infected cell nuclei showed that the deletion mutant lacked the ability to integrate despite being able to produce infectious virus. Using the sensitive polymerase chain reaction technique, we have clearly demonstrated the absence of the IN domain in the deletion mutant after infection and replication in HUT-78 cells. Such mutants might form the basis for the development of an experimental live attenuated vaccine.

Various strategies to develop a vaccine for AIDS so far have either been unsuccessful (1, 8) or yielded partial protection (10). Some success has recently been obtained with an inactivated simian immunodeficiency virus (SIV) vaccine (3, 12). Although strategies based on using subunit or inactivated antigens are being thoroughly investigated, very little work is being done on a live vaccine approach because of concerns about safety. While these concerns are important, they need not halt parallel experimental exploration of the possibility of developing attenuated viral strains. Live vaccines have the potential to induce a better cell-mediated response, as the immune system is exposed to the full complement of both structural and nonstructural viral proteins.

As SIV is known to be related to human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) (2), the simian model offers a good host system with which to test candidate vaccines. Using this approach, we have made deletion mutations in the integrase portion of the *pol* gene of strain SIVmac251 with the objective of producing viruses that do not integrate into the host cell genome but have the potential to replicate from an unintegrated viral template without becoming latent, as integration provides one of the routes by which viruses can integrate and then remain silent by persisting in a latent form.

The *pol* region in the SIVmac genome is believed to code for three proteins, with a total molecular weight of 119,600 (2), which act as protease, reverse transcriptase (RT), and integrase (IN) (9). It has been shown that the reverse transcriptase is essential for the synthesis of the double-stranded DNA form and the integrase protein aids in the incorporation of this intermediate into cellular DNA (15). The protease is required for proper processing of the *gag* and *pol* polyprotein precursor into virion components during maturation (11). Since the integrase domain bestows the capacity for viral DNA to integrate into the host cell ge-

nome, this portion of the SIVmac *pol* gene was targeted for deletion. Deletion mutants of SIVmac251 lacking a large portion of the integrase domain have been introduced into three different kinds of cell systems (rhabdomyosarcoma [RD], lymphoblastoid, and monoblastoid), and we report here that all of them support the replication and production of infectious virus.

MATERIALS AND METHODS

Cells and DNA clones. HUT-78 cells (obtained from the American Type Culture Collection [ATCC], Rockville, Md.) were grown in RPMI 1640 medium with 10% fetal bovine serum, 100 U of penicillin per ml, and 100 µg of streptomycin per ml. RD cells (ATCC) were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and antibiotics, while U937 cells (provided by Eva Gonczol, Wistar Institute, Philadelphia, Pa.) were expanded in RPMI 1640 medium with 10% fetal bovine serum and antibiotics.

The SIVmac251 DNA used in this study (provided by Beatrice H. Hahn, University of Alabama, Birmingham) was cleaved as a 14-kbp insert from the vector λgtWES.λB and subcloned at the *EcoRI* site of the vector pBR322. The 14-kbp insert had cellular sequences flanking either side of the SIVmac genome. This parental plasmid clone, called pB/SIV, was subsequently used for generating deletion mutants.

Strategy for generating an integrase domain deletion mutant. The boundary between the reverse transcriptase and integrase domains, well conserved in HIV-1, HIV-2, and SIVagm (4), is thought to be at the site of the Val-Leu and Phe-Leu codons spanning positions 4009 to 4020 (2) of SIVmac251. Restriction endonucleases *HpaI* and *BclI*, which cleave SIVmac251 DNA once, were used to delete the integrase domain (see Fig. 1A), as the DNA of pBR322 lacks restriction sites for these two endonucleases. *HpaI* cleaves at position 3888 (2), upstream of the boundary between the reverse transcriptase and integrase domains, and *BclI* cleaves at position 4622. The double digestion led to the

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formation of 17.63- and 0.73-kbp DNA fragments. After electroelution and purification of the larger DNA fragment, the recessed terminus of the *Bcl*I-cut end was filled in with all four cold triphosphates by using the Klenow (large) fragment of *Escherichia coli* DNA polymerase I. This newly created blunt end was then ligated with the blunt-end terminus of the *Hpa*I-cut site, and the DNA was used to transform *E. coli* GM2163 (*dam dcm supE*). A successful ligation resulted in the regeneration of a *Bcl*I restriction site at the site of ligation, and this was confirmed by digestion of positive clones with *Bcl*I. In addition, the resultant deletion mutant plasmid DNA, called pB/SIVΔe1, was digested with restriction endonucleases *Bgl*II (site at position 1480) and *Xba*I (with sites at positions 1399, 2694, and 4215) (2) to make sure that the sites for these two endonucleases had been lost because of the deletion of the 0.73-kbp *Hpa*I-*Bcl*I DNA fragment (data not shown). We confirmed this by agarose gel electrophoresis of the *Bgl*II and *Xba*I digests of both the pB/SIV (parental plasmid) and pB/SIVΔe1 (deletion mutant) clones. The deletion in pB/SIVΔe1 resulted in a frameshift to the -1 reading frame and a substitution of 17 amino acids followed by a translation termination codon resulting in the termination of the IN protein. After generation of the deletion mutant, we confirmed the lack of the IN domain by nucleotide sequence analysis of the entire *pol* gene of the mutant plasmid DNA (pB/SIVΔe1). The DNA stocks were also routinely checked for cross-contamination by polymerase chain reaction (PCR) analysis as described below.

Transfection and replication of cloned viruses. Five micrograms each of the parental plasmid DNA (pB/SIV) and the deletion mutant plasmid DNA (pB/SIVΔe1) were used to transfect 5×10^6 HUT-78 cells by the DEAE-dextran procedure (17), and the cells were split twice a week. The replicative potentials of the two plasmids were compared by assaying for RT activity (14) as a measure of their ability to direct efficient production of virus. Virus stocks were made by harvesting and passing cell-free supernatants through 0.45-μm-pore-size filters soon after RT activity reached the highest level. Stocks of virus were placed in liquid nitrogen.

For transfection of RD cells, the calcium phosphate coprecipitate method (5) was used. Cells (10^6) were exposed to 10 μg of the DNA precipitates for 6 h and then subjected to a 90-s glycerol shock.

To infect the cells, the equivalent of 50,000 cpm of RT activity of both the SIVmac251 and SIVΔe1 viral stocks was used for every 10^6 HUT-78 or U937 cells. Virus release was measured by detection of RT activity as described above at various intervals after infection.

Analysis of DNA from infected cells. DNA from uninfected as well as infected HUT-78 cells was prepared as follows. The cells were spun down at 1,800 rpm for 10 min in a Sorvall RC-3B centrifuge and washed twice in ice-cold TE (10 mM Tris-Cl [pH 8.0], 1 mM EDTA). The supernatants were gently aspirated, and the cell pellets were resuspended in 0.25 ml of an ice-cold solution containing 0.15 M NaCl, 10 mM Tris-Cl (pH 8.0), 1.5 mM MgCl₂, and 0.6% Nonidet P-40. After the suspension was vortexed for 15 s, it was layered over a solution containing 25% (wt/vol) sucrose and 1% Nonidet P-40. It was then centrifuged in a Sorvall RC-3B centrifuge at 8,000 rpm for 15 min. The upper, cytoplasmic fraction was subjected to extraction with phenol-chloroform and precipitated with isopropanol. Hirt extraction (7) of the nuclear pellet was carried out, and the components were separated into Hirt supernatant and Hirt pellet fractions. The Hirt supernatant portion was mixed with the cytoplasmic

DNA and called unintegrated DNA (13), and the DNA isolated from the Hirt pellet fraction was called integrated DNA. Integrated DNA (10 μg) obtained from 10^7 infected cells was cleaved with *Bam*HI and unintegrated DNA (10 μg) obtained from a similar number of infected cells were electrophoresed in 0.8% agarose gels without any restriction endonuclease cleavage. The gel containing unintegrated DNA was run for a longer time (24 h) to resolve any difference in size between SIVmac and SIVΔe1 DNA samples. After transfer to a nylon membrane (BIOTRANS; ICN, Costa Mesa, Calif.), the digests were hybridized with a 7.3-kbp *Bam*HI-*Bam*HI SIVmac251 DNA fragment that had been labeled by nick translation. Hybridization and washing were carried out according to the manufacturer's instructions.

PCR analysis of DNA from infected cells. All of the PCR reagents and equipment were purchased from Perkin-Elmer Cetus (Norwalk, Conn.). The following primers flanking the region deleted in SIVΔe1 were used: SIV1, 5'-CATGGCAT TGACAGACTCAGGG-3' (bases 3783 to 3804 [2]); SIV2, 5'-GCCTTCTCTGTAATAGACCCG-3' (bases 4704 to 4684 [2]); and SIV5, 5'-TCTCTAGCCTCTCCGGTATCC-3' (bases 4886 to 4866 [2]). They were used in two pairwise combinations, one consisting of SIV1 and SIV2 and the other consisting of SIV1 and SIV5. Hirt supernatant DNA (unintegrated) was obtained from 10^7 SIVmac251- and 10^7 SIVΔe1-infected HUT-78 cells 30 days postinfection. Amplification was done with 1.0 μg of Hirt supernatant DNA by using 0.35 μg of each primer in a total reaction volume of 100 μl. After an initial denaturation step at 95°C for 4 min, 30 cycles of PCR were performed (2 min at 94°C for denaturation, 1 min at 55°C for annealing, and 2 min at 72°C for extension). At the end of the cycles, a 7-min soak at 72°C was carried out, and then the mixture was held at 4°C. Samples were ethanol precipitated and resuspended in 30 μl of TE. Ten microliters of each sample was electrophoresed on a 1.5% agarose gel containing ethidium bromide. The PCR products were examined under UV light. The gel was then denatured and neutralized, and the DNA was transferred to a nitrocellulose filter. Prehybridization and hybridization were done under low-stringency conditions (40% formamide, 37°C). A mixture of three ³²P-5'-end-labelled oligonucleotide probes was used for hybridization. The probes were as follows: SIV-3P, 5'-GGATGCCCTACAG AATCAGAG-3' (bases 3856 to 3876 [2]); SIV-6P, 5'-GTAG ACTACAATATGTTATGGG-3' (bases 3823 to 3845, [2]); and SIV-8, 5'-GATTGTTGAAATTGTATTTC-3' (bases 4658 to 4639, [2]). The filter was washed three times at room temperature in 1 liter of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate for 30 min and then subjected to autoradiography.

Primers SIV1 and SIV2 were capable of amplifying a 921-bp product for SIVmac251 (wild type) and a 188-bp product for SIVΔe1 (deletion mutant), whereas primers SIV1 and SIV5 were capable of amplifying a 1,103-bp PCR product for SIVmac251 and a 370-bp product for SIVΔe1. The presence of appropriate-length PCR products was confirmed by hybridization with specific probes and autoradiography.

RESULTS AND DISCUSSION

The strategy that was devised and used for generating an integrase-negative SIVmac251 clone is outlined in Fig. 1A. After confirmation that we had an authentic deletion mutant by restriction enzyme mapping, PCR, and sequence analysis

FIG. 1. Generation of an integrase domain deletion mutant of SIV_{mac251} and transfection of HUT-78 cells with parental plasmid pB/SIV and mutant plasmid pB/SIVΔe1. (A) Schematic representation of the construction of the integrase-negative deletion mutant. After transformation of competent *E. coli* GM2163 cells, positive deletion clones with a regenerated *Bcl*I restriction site (circled) were obtained and named pB/SIVΔe1. dNTPs, deoxynucleoside triphosphates. (B) Kinetics of virus production by the parental and deletion mutant plasmids in HUT-78 cells. At specific intervals after transfection, cell-free supernatants were obtained by centrifugation and assayed for reverse transcriptase activity (14) with poly(A) · poly(dT)₁₀ (Boehringer Mannheim) as the primer and [α -³²P]TTP (Amersham).

(see Materials and Methods), the ability of the mutant plasmid (pB/SIVΔe1) to produce virus was compared with that of the parental plasmid (pB/SIV). When these two plasmid DNAs were used to transfect HUT-78 cells, the appearance of viral particles in the cell-free supernatants was assessed at 5-day intervals by performing the RT assay (Fig. 1B). As shown in Fig. 1B, peak RT activity was displayed by pB/SIV and pB/SIVΔe1 25 days after transfection. The integrase-negative deletion mutant was capable of proper replication, although the level of RT activity detected was lower than the level of RT activity expressed by the parental plasmid. To show that the deletion did not affect

FIG. 2. Production of virus in RD cells transfected with the parental plasmid and the deletion mutant. RD cells were transfected with 10 μ g of DNA of pB/SIV or pB/SIVΔe1, and cell-free supernatants from each were assayed for RT activity.

specific RT activity, we made dilutions (1:2, 1:10, and 1:20) of the cell-free supernatants from pB/SIV- and pB/SIVΔe1-transfected cells and found that RT activity varied linearly with dilution for both plasmid clones. Using the p27 core antigen assay (Coulter Immunology, Hialeah, Fla.) specific for SIV, we found that cell-free supernatants of pB/SIV- and pB/SIVΔe1-transfected cultures contained 1,090 and 360 pg of p27 antigen per ml, respectively, 25 days posttransfection. These results were reproducible and thus established that the integrase-negative deletion mutant could replicate in a CD4⁺ host cell system.

Similar results were obtained when RD cells were transfected with the two plasmid clones, pB/SIV and pB/SIVΔe1. Maximal virus production for the undeleted parental plasmid was reached by 3 days posttransfection (Fig. 2), as observed for HIV in earlier studies (18), whereas the deletion mutant displayed peak RT activity by 6 days after transfection. Since this pattern was reproducible and equal amounts of wild-type and deletion mutant DNAs were transfected by an identical procedure, it can be concluded that the kinetics of appearance of virus in RD cells is more rapid for SIV with an intact integrase domain than for SIV with an integrase domain with deletions or alterations.

To examine whether the viruses released from cells transfected with the two infectious molecular clones (pB/SIV and pB/SIVΔe1) could cause an infection and produce virus again, the respective virus stocks were used to infect HUT-78 cells (50,000 cpm of RT activity per 10⁶ cells) and monitored over a period of 6 weeks. Virus production was monitored by assaying for RT activity, and peak RT activity was reached by 5 weeks postinfection for both SIV_{mac251} and SIVΔe1 (Fig. 3A). Though the SIVΔe1 clone showed a similar kinetic profile, the level of RT activity detected was lower. When U937 (monoblastoid) cells were infected with the two viral stocks, essentially similar results were obtained (Fig. 3B). The deletion mutant (SIVΔe1) replicated quite well, and maximum virus production was observed by 15 days after infection. These results once again demonstrated that the integrase-negative mutant could replicate in yet another cell system, U937 cells.

We next sought to determine whether integration into cellular DNA had occurred by analyzing proviral DNA from infected HUT-78 cells. DNA was extracted from nuclear and cytoplasmic fractions (see Materials and Methods). The detection of integrated viral DNA (from the Hirt pellet

FIG. 3. Time course of RT activity in cell-free supernatants of HUT-78 cells (A) and U937 cells (B) infected with SIVmac251 (SIV) or SIVΔe1 derived from infectious molecular clones.

fraction) was carried out by digestion with the restriction endonuclease *Bam*HI. *Bam*HI cleaves SIVmac251 DNA (Fig. 1A) at two internal sites (positions 1346 and 8682 [2]), resulting in a unique 7.3-kbp fragment and two flanking DNA fragments of variable sizes based on the sites for *Bam*HI in the flanking host cell DNA. Thus, integrated DNA would be detected as a 7.3-kbp fragment in SIVmac251-infected cells, while a 6.6-kbp fragment would be observed in SIVΔe1-infected cells because of the 0.73-kbp deletion in the integrase domain. After the *Bam*HI digests of integrated DNA were electrophoresed and transferred to a nylon membrane, they were hybridized to a ³²P-labelled SIVmac251 7.3-kbp *Bam*HI-*Bam*HI probe. A band of about 7.3 kbp was observed in the DNA isolated from the nuclei of HUT-78 cells infected with SIVmac251 (Fig. 4A, lane 1) but not observed in the DNA derived from the nuclei of SIVΔe1-infected cells (Fig. 4A, lane 2). To show that digestion, labelling, and transfer had been carried out properly, we hybridized the same blot with a *v-jun* probe and found a similar 5- to 6-kbp band in all three lanes at about the same intensity (bottom of Fig. 4A). When identical amounts (10 μg) of unintegrated viral DNA (from Hirt supernatant fractions) were analyzed without any restriction endonuclease digestion, we observed a 10-kbp band in the DNA from SIVmac251-infected cells, a slightly smaller DNA fragment (~9.3 kb) in DNA from SIVΔe1-infected cells, and no hybridization with DNA from uninfected cells (Fig. 4B). The sensitivity of the hybridization procedure used was more than adequate to detect a single copy of integrated SIV DNA per cell, and this conclusion was reached by carrying out reconstruction experiments, as described elsewhere (6), in which we mixed a

FIG. 4. (A) Detection of integrated viral DNA in HUT-78 cells. Ten micrograms of DNA from the nuclei of 10⁷ SIVmac251-infected cells (lane 1), 10⁷ SIVΔe1 deletion mutant-infected cells (lane 2), or 10⁷ uninfected cells (lane 3) was digested with *Bam*HI, electrophoresed in a 0.8% agarose gel, and subjected to Southern blot analysis. Hybridization was carried out with a ³²P-labelled 7.3-kbp *Bam*HI-*Bam*HI SIVmac251 DNA fragment as the probe. After the blot was stripped, it was hybridized to a *v-jun* probe to show proper digestion, transfer, and hybridization (bottom). (B) Unintegrated viral DNA in HUT-78 cells. Ten micrograms of DNA isolated from Hirt supernatants of 10⁷ SIVmac251-infected cells (lane 1), SIVΔe1-infected cells (lane 2), and uninfected cells (lane 3) was electrophoresed for 24 h in a 0.8% agarose gel without restriction endonuclease digestion, transferred to a nylon membrane, and hybridized to the same probe used for panel A. λ DNA (*Hind*III-cut) markers were run in parallel for size estimation.

constant amount of uninfected cell DNA with the equivalent of 0.5 copy of viral DNA per cell and 0.25 copy of viral DNA per cell separately, digested them with *Bam*HI, and electrophoresed them in a gel with *Bam*HI-digested integrated viral DNA from the nuclei of infected cells (positive control). We found that we could detect as little as 0.25 copy of viral DNA per cell (data not shown).

In order to confirm that SIVΔe1 was IN domain deficient, PCR analysis was performed with Hirt supernatant (unintegrated) DNA prepared from 10⁷ SIVmac251- and 10⁷ SIVΔe1-infected HUT-78 cells (30 days postinfection). The results of the hybridization of the Southern blot of the PCR products are shown in Fig. 5. As described in Materials and Methods, the expected-length PCR products of 1,103 and 370 bp were observed for DNA from SIVmac251- and SIVΔe1-infected cells, respectively, when the primer pair consisting of SIV1 and SIV5 was used. Similarly, appropriate-length products of 921 and 188 bp were observed for DNA from SIVmac251- and SIVΔe1-infected cells, respectively, when the primer pair consisting of SIV1 and SIV2 was used (data not shown). A known SIVmac251 DNA (positive control) and a water control amplified with the primer pair SIV1 and SIV2 were included in the PCR analysis (Fig. 5), and the positive control showed the appropriate-size band of 921 bp. These findings clearly showed that the SIVΔe1 mutant was IN deficient. Taken together, all these data established (i) that the deletion in the mutant (SIVΔe1) was retained after infection and replication and (ii) that only SIVmac251 with an intact integrase domain had the potential to integrate. It is pertinent to mention here that the intensity of the PCR-amplified band observed for SIVΔe1 was not much lower than that of SIVmac251. This correlates with the observation of a two- to threefold difference in virus production between SIVmac251- and SIVΔe1-infected cells,

FIG. 5. Blot analysis of PCR products generated from HUT-78 cells. PCR using appropriate primers was carried out according to the kit manufacturer's instructions (Perkin-Elmer Cetus), and the products were run on a 1.5% agarose gel, transferred to nitrocellulose, and hybridized to a mixture of three oligonucleotide probes (SIV-3P, SIV-6P, and SIV-8) ³²P-labelled at the 5' end. UN, uninfected-cell DNA; WT, wild type (SIVmac251); Δe1, SIVΔe1 deletion mutant; H₂O, negative water control; and Control, a known SIVmac251-positive control. The primers used for each PCR are given at the top, while the bars on the right indicate the positions of φX174 DNA (*Hae*III-cut) markers in base pairs.

as shown by both RT and SIVmac-specific p27 antigen assays. It is possible that the unintegrated viral DNA found in SIVΔe1-infected cells is not able to set up a productive infection (like SIVmac251) because of the lack of integration into the host cell genome.

In conclusion, these data suggest that the capacity to integrate has been lost by the deletion mutant virus (SIVΔe1) without significantly affecting its ability to replicate and produce infectious virus. We speculate that SIV has the potential to express unintegrated viral DNA in infected cells. Similar suggestions have been made for HIV-1 (16, 19), visna virus (a lentivirus) (6), and spleen necrosis virus (13). In contrast to a recent report (19) that integrase-negative mutants of HIV-1 could not generate infectious virus, our results show that an integrase-negative SIV mutant can, upon infection of appropriate cells, produce infectious virus. Though these results differ from those of a study carried out with Moloney murine leukemia virus (15), in which an integrase-defective mutant was shown to be about 300 times less efficient than the wild type at establishing a productive infection, studies of spleen necrosis virus (13) have shown that integration of this avian retrovirus was not required for virus replication, though there was a reduction in virus production. An SIV deletion mutant of the type generated in this study could be readily tested for virulence in animals such as rhesus monkeys. Mutants that are unable to produce persistent infection may be candidate live virus vaccines.

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