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Nucleocapsid Protein Zinc-Finger Mutants of Simian Immunodeficiency Virus Strain Mne Produce Virions That Are Replication Defective *in Vitro* and *in Vivo*

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All retroviruses (except the spumaretroviruses) contain a nucleocapsid (NC) protein that encodes one or two copies of the Zn^{2+}-finger sequence -Cys-X_2-Cys-X_4-His-X_4-Cys-. This region has been shown to be essential for recognition and packaging of the genomic RNA during virion particle assembly. Additionally, this region has been shown to be involved in early infection events in a wide spectrum of retroviruses, including mammalian type C [e.g., murine leukemia virus (MuLV)], human immunodeficiency virus type 1 (HIV-1), *Rous sarcoma* virus, and other retroviruses. Mutations in the two Zn^{2+}-fingers of the NC protein of simian immunodeficiency virus strain Mne [SIV(Mne)] have been generated. The resulting virions contained the normal complement of processed viral proteins with densities indistinguishable from wild-type SIV(Mne). All of the mutants had electron micrograph morphologies similar to those of immature particles observed in wild-type preparations. RNA packaging was less affected by mutations in the NC protein of SIV(Mne) than has been observed for similar mutants in the MuLV and HIV-1 systems. Nevertheless, *in vitro* replication of SIV(Mne) NC mutants was impaired to levels comparable to those observed for MuLV and HIV-1 NC mutants; replication defective NC mutants are typically 10^5- to 10^6-fold less infectious than similar levels of wild-type virus. One mutant, ΔCys33–Cys36, was also found to be noninfectious *in vivo* when mutant virus was administered intravenously to a pig-tailed macaque. NC mutations can therefore be used to generate replication defective virions for candidate vaccines in the SIV macaque model for primate lentiviral diseases.

INTRODUCTION

One of the most highly conserved sequence elements of retroviruses is the Zn^{2+}-finger, which is contained in the nucleocapsid (NC) domain of the Gag precursor. It is common to all retroviruses except the spumaretroviruses (Maurer *et al.,* 1988). This element is found once or twice depending on the particular virus and consists of a 14-amino-acid sequence of invariantly spaced Cys and His residues: -Cys-X_2-Cys-X_4-His-X_4-Cys- (Berg, 1986; Covey, 1986). The ligand binding residues (Cys and His) are exquisitely sensitive to alterations. When these amino acids are mutated to nonZn^{2+} binding residues, the structure is unable to bind Zn^{2+} tightly and therefore rendered inactive (Casas-Finet *et al.,* 1997a; 1997b). Through mutagenesis and structural (DeGuzman *et al.,* 1998) studies, retroviral NC Zn^{2+}-finger structures in the Gag precursor have been implicated in genomic RNA packaging. Nonconservative mutations in the Zn^{2+}-finger motifs of avian, murine, and human retroviral NC proteins that abrogate Zn^{2+} binding results in the production of mainly RNA-deficient, noninfectious virions (Aldovini and Young, 1990; Dorfman *et al.,* 1993; Dupraz *et al.,* 1990; Gorelick *et al.,* 1988, 1990; Meric *et al.,* 1988; Sakalian *et al.,* 1994). In addition to the well established role in viral packaging, more recent studies have clearly shown that the structure of NC Zn^{2+}-fingers present in the mature NC protein function in early infection events (Gorelick *et al.,* 1996; Tanchou *et al.,* 1998).

It was of interest to compare NC mutants of simian immunodeficiency virus (SIV) to determine whether they were phenotypically similar to NC mutants in the human immunodeficiency virus type 1 (HIV-1) and Moloney murine leukemia virus (Mo-MuLV) systems. It is possible that SIV NC mutant particles could serve as effective immunogens in vaccine studies using the SIV/macaque animal model. This report describes the preparation and characterization of a number of NC protein Zn^{2+}-finger mutants that were constructed on the genetic backbone of SIV(Mne), a pathogenic strain of SIV isolated and cloned from a pig-tailed macaque (*Macaca nemestrina*) (Benveniste *et al.,* 1990, 1986; Heidecker *et al.,* 1998).

The phenotypes of SIV(Mne) NC mutants described here were similar to those previously reported for NC Zn^{2+}-finger mutants of HIV-1 (Aldovini and Young, 1990; Dorfman *et al.,* 1993; Gorelick *et al.,* 1990), with the exception of somewhat higher RNA levels in a number of

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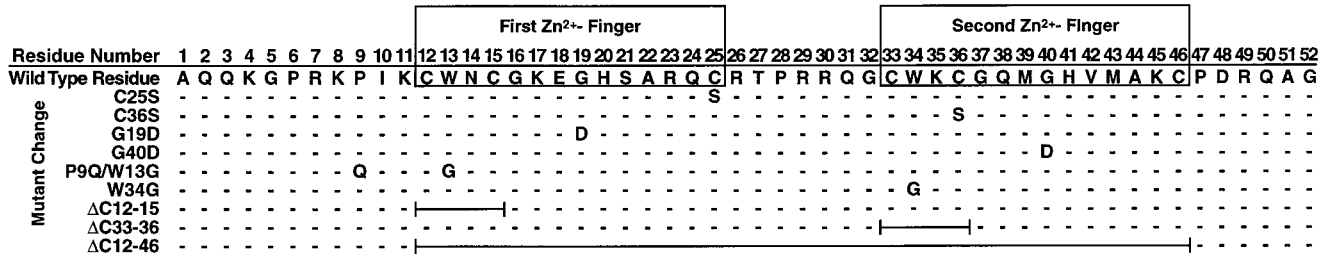

FIG. 1. Mutations introduced into the NC protein of SIV(Mne). Lines indicate amino acid residues that are the same as those in the wild-type sequence. Abbreviations are as follows: C25S, Cys25→Ser; C36S, Cys36→Ser; G19D, Gly19→Asp; G40D, Gly40→Asp; P9E/W13G, Pro9→Glu/Trp13→Gly; W34G, Trp34→Gly; ΔC12–C15, ΔCys12–Cys15 (mutation results in the deletion of amino acid residues Cys, Trp, Asn, and Cys); ΔC33–C36, ΔCys33–Cys36 (mutation deletes amino acid residues Cys, Trp, Lys, and Cys); ΔC12–C46, ΔCys12–Cys46 (the first Zn^{2+}-finger, the Arg-Thr-Pro-Arg-Arg-Glu-Gly linker, and the second Zn^{2+}-finger are deleted). Positions of the amino acids in the mature, wild-type SIV(Mne) NC protein are from Henderson *et al.* (1988) and are indicated at the top of the figure.

the SIV(Mne) mutants examined. In addition, one mutant virus (ΔCys33–Cys36) was found not to be detectably infectious after *in vivo* inoculation of a pig-tailed macaque. Proviruses with mutations in the gene coding for NC that render progeny viruses noninfectious may provide a promising novel approach for vaccine development. Particles produced from such mutant proviral clones assemble and bud from the surface of the cell in a manner similar to wild-type virus. Authentic, conformationally intact viral structures thus can be presented to the immune system in a setting devoid of the risk of spreading infection.

RESULTS

Viral protein analyses of mutant and wild-type SIV(Mne) viruses

The mutations introduced into the full-length proviral clone of SIV(Mne) clone 8 (Benveniste *et al.,* 1990; Heidecker *et al.,* 1998) in the gene coding for the NC protein are summarized in Fig. 1. Mutant and wild-type proviruses were introduced into the vector, pSVori/*neo*. When transfected into 293T or 293 cells (adenovirus transformed human fetal kidney cells with or without expression of the large T antigen, respectively), the clones transiently expressed virus particles. Mutant and wild-type viruses from cells transfected with the proviral plasmids listed in Table 1 were used in this study.

Table 1 also lists reverse transcriptase (RT) activities and $p28^{CA}$ levels of mutant and wild-type viruses obtained after a typical transfection. Samples were adjusted to equal levels of RT activity and fractionated by SDS–PAGE. Immunoblot analysis revealed the presence of similar levels of processed $p28^{CA}$ protein (Fig. 2A). Envelope proteins were also observed at comparable levels in mutant and wild-type preparations (Fig. 2B). Taken together, the immunoblot, $p28^{CA}$ ELISA, and RT analyses roughly show that *gag*-to-*pol* as well as *env*-to-*pol* gene product ratios were comparable between the mutant and wild-type viruses. These data also indicate that the *pol* gene products RT and protease (PR) are functional in the mutant viruses. Some uncleaved precursor species that react with the $p28^{CA}$ monoclonal antibody were observed in several of the mutants (Fig. 2A), suggesting incomplete processing of the Gag precursor by the viral PR in most of the mutants but not the wild-type or Gly40→Asp mutant viruses.

RNA blot analysis of transiently expressed mutant and wild-type virus particles from 293T cells

Mutant and wild-type SIV particles were prepared and analyzed for full-length SIV genomic RNA using a random primed ^{32}P-labeled 8.2-kbp *Dra*III–*Bgl*I SIV(Mne) probe (Fig. 3). Samples were adjusted for equal RT levels, fractionated by formaldehyde–agarose gel electrophoresis, and analyzed as de-

TABLE 1

Properties of Mutant and Wild-Type SIV(Mne) Particles

Mutation	Plasmid designation	RT activity[a] (cpm/ml) × 10^6	$p28^{CA}$ (μg/mL)[b]
(−) Control[c]		0[d]	0
Wild type	pRB86	6.55	1.50
Cys25 → Ser	pRB119	4.61	2.55
Cys36 → Ser	pRB121	3.96	1.70
Gly19 → Asp	pRB123	4.28	1.19
Gly40 → Asp	pRB124	6.74	3.03
Pro9 → Glu/Trp13 → Gly	pRB127	2.83	1.38
Trp34 → Gly	pRB128	3.44	0.95
ΔCys12-Cys15	pRB129	1.85	0.62
ΔCys33-Cys36	pRB130	1.48	0.76
ΔCys12-Cys46	pRB131	0.44	0.25

[a] RT activity is expressed as cpm of [^{3}H]TMP incorporated per milliliter of culture fluid.

[b] Determined using the Retro-Tek SIV-1 Antigen ELISA from Cellular Products, Inc. (Buffalo, NY).

[c] Samples from 293T cells transfected with sheared salmon sperm DNA.

[d] A background of 1623 cpm/ml has been subtracted from the RT values shown.

scribed previously (Gorelick *et al.,* 1996). The Cys25→Ser, ΔCys33–Cys 36, and ΔCys12–Cys46 mutants packaged less than wild-type levels of full-length viral RNA. For comparison, HIV-1 NC mutants in which ligand binding residues were replaced with Ser had greatly reduced levels of full-length viral RNA (Aldovini and Young, 1990; Dorfman *et al.,* 1993; Gorelick *et al.,* 1990); they packaged <20% wild-type levels of their genomes. All other mutants in this study reproducibly packaged levels of full-length genomic RNA that were comparable to or greater than that for the wild-type virus. Note that it has been shown previously that the majority of the smeared material that hybridizes with the ^{32}P-radiolabeled probe is degraded DNA from the original transfection. This degraded DNA copurifies with the particles and results in the contamination of the RNA sample (Gorelick *et al.* 1996).

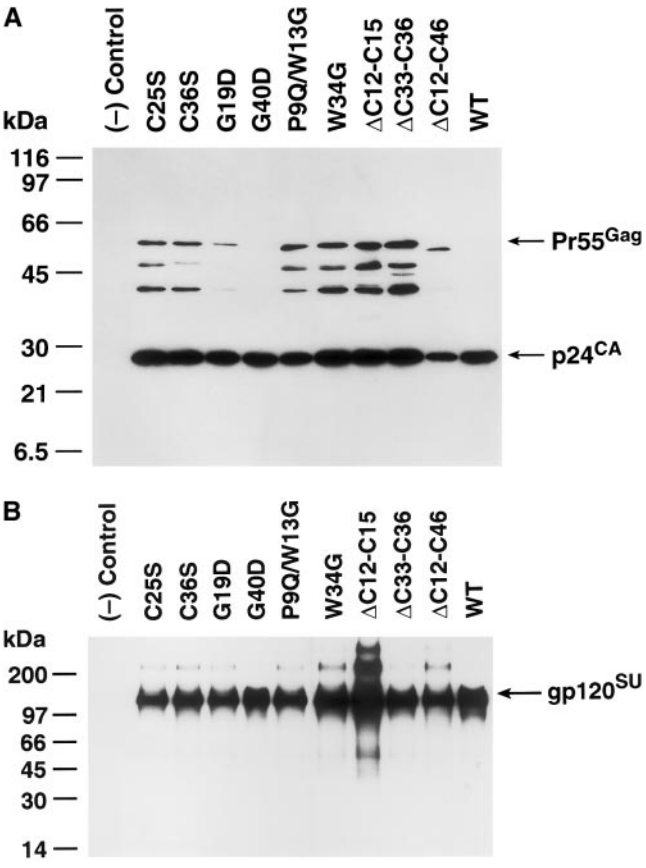

FIG. 2. Protein immunoblot analysis of wild-type and mutant SIV(Mne) virus particles transiently expressed in 293T cells. Clarified supernatants were centrifuged at 120,000*g* for 1 h at 4°C. Samples were adjusted for equal RT levels, fractionated by SDS–PAGE, and treated as described under Materials and Methods. (A) Immunoblot using 4.6×10^6 cpm RT activity of mutant and wild-type virus, visualized using mouse monoclonal antibody against $p28^{CA}$ [AIDS Vaccine Program, Biological Products Laboratory (AVP-BPL) inventory no. 96–0020 89A P9B5 H8B1]. (B) Immunoblot using 4.0×10^6 cpm of RT activity of mutant and wild-type virus, detected with polyclonal rabbit antiserum against $gp120^{SU}$ (AVP-BPL inventory no. DJ-36603 OD3545). Marker proteins are indicated on the left, and positions of (A) $p28^{CA}$, $Pr55^{Gag}$, and (B) $gp120^{SU}$ are indicated on the right of each panel. (−) Control and WT indicate pellets from centrifuged supernatants from 293T cells transfected with sheared salmon sperm and wild-type SIV(Mne) DNA, respectively.

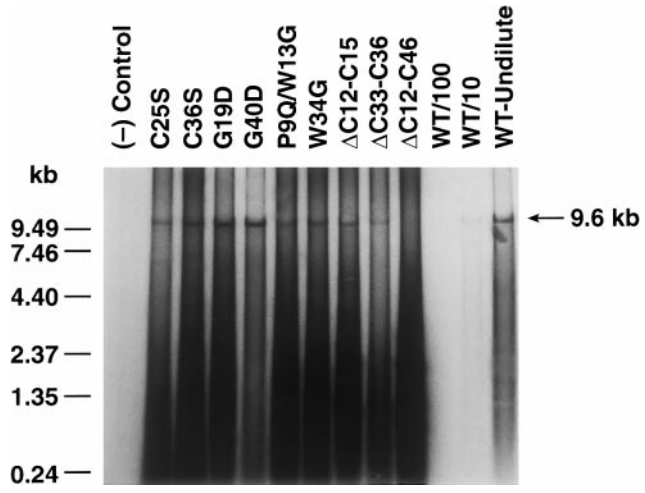

FIG. 3. RNA blot analysis of wild-type and mutant SIV(Mne) virus particles transiently expressed in 293T cells. Particles were analyzed for full-length SIV(Mne) genomic RNA using a ^{32}P-labeled 8.2-kbp *Dra*III–*Bgl*I SIV(Mne) probe. All samples were adjusted for equal RT levels (7.9×10^5 cpm), fractionated, and treated as described under Materials and Methods. Dilutions (10- and 100-fold) of the wild-type sample were also tested. (−) Control indicates samples isolated and prepared from centrifuged supernatants from 293T cells transfected with sheared salmon sperm DNA. The RNA markers are indicated on the left, and the size of full-length SIV(Mne) genomic RNA is noted on the right.

Real-time RT-PCR determination of RNA content of mutant and wild-type viruses

Viral RNA levels were also determined by real time RT-PCR as described previously (Suryanarayana *et al.,* 1998). RNA samples were prepared from mutant and wild-type viral pellets as described under Materials and Methods. DNase treatment of all samples was required before RT-PCR to remove contaminating DNA. The majority of the DNA observed in the samples probably originated from the proviral plasmid clones used in the transfections to obtain the viral particles. Table 2 summarizes the levels of viral RNA contained in the mutants. The Cys36→Ser, ΔCys33–Cys36, and ΔCys12–Cys46 mutants were found to contain less than wild-type levels of genomic RNA. All other mutants packaged viral RNA at levels comparable to or greater than wild-type levels.

Electron micrograph analysis

Transfected 293T cells produced abundant levels of mutant and wild-type SIV(Mne) particles. Thin sections of cells transfected with proviral clones were examined at a magnification of 90,000×. Electron micrographs from wild-type SIV(Mne), as well as the Cys36→Ser and ΔCys12–Cys46 mutants, are given (see Fig. 5). Wild-type virus preparations contain uni-

TABLE 2

Relative RNA Content of Viruses Derived from Transfections with Wild-Type or NC Mutant SIV(Mne) Proviral Clones

Mutation	RT adjusted			$p28^{CA}$ adjusted		
	RNA content[a]	n[b]	Standard error[c]	RNA content[a]	n[b]	Standard error[c]
(−) Control[d]	0			0		
Wild type	100			100		
Cys25 → Ser	96	3	52	82	2	27
Cys36 → Ser	73	3	45	42	2	26
Gly19 → Asp	189	3	31	251	2	111
Gly40 → Asp	62	2	41	65	2	3
Pro9 → Glu/Trp13 → Gly	109	2	22	122	1	
Trp34 → Gly	77	3	19	87	2	17
ΔCys12-Cys15	96	2	5	106	1	
ΔCys33-Cys36	39	2	4	54	1	
ΔCys12-Cys46	8	3	4	14	2	5

[a] Nominal RNA copies/ml of culture supernatant were determined by real-time RT-PCR (Suryanarayana *et al.,* 1998). The RNA copy number per ml of culture fluid was then divided by either the RT activity or $p28^{CA}$ content per ml of culture fluid for the same sample for each of the mutants. The percent wild-type level of RNA in the mutants was then calculated by dividing this parameter for the mutants by the same parameter calculated for the wild-type virus and then multiplying by 100.

[b] *n* = number of samples examined.

[c] Standard error = standard of the mean.

[d] (−) Control = samples isolated, prepared and analyzed from 293T cells transfected with sheared salmon sperm DNA.

formly sized particles of ~100 nm in diameter. In addition, both mature and immature particles are evident (see Fig. 5A) with ~30% of the particles being of a mature morphology. The Gly40→Asp mutant preparations also contained mature particles because it was replication competent (see below); ~20% of the Gly40→Asp particles were of a mature morphology. All of the mutant clones (except Gly40→Asp and ΔCys12–Cys46) produced particles that had predominantly the immature morphology. An example of the mutant particles is shown (see Fig. 5B). These particles are derived from 293T cells transfected with the Cys36→Ser proviral clone. All mutant particles (except the ΔCys12–Cys46 particles) are uniform in diameter (~100 nm) and had morphologies identical to those of Cys36→Ser particles. Well defined spikes are easily visualized on the surface of all of the mutant and wild-type particles, identical to those seen (see Figs. 5A and 5B). Presumably, these are the SIV envelope glycoproteins $gp120^{SU}$. These structures were not as well defined in transmission electron micrographs of HIV-1 NC mutant or wild-type particles (Gorelick *et al.,* 1993). The ΔCys12–Cys46 mutant particles are shown (see Fig. 5C). These particles are highly variable in both diameter and shape, with a majority of the particles being larger than normal. In addition, the outer ring of electron dense material appears to be less diffuse in the ΔCys12–Cys46 particle compared with the outer ring seen in the immature particles from the other mutant and wild-type particles. It should be noted that the densities of all viruses examined in this work were 1.16 ± 0.02 g/ml as determined by isopycnic sucrose density gradient centrifugation (data not shown), performed as described previously (Gorelick *et al.,* 1988).

Infectivity analyses of mutant and wild-type virus

Supernatants containing mutant or wild-type virus from 293T transfections were clarified and used to infect AA2-clone 5 cells. Supernatants were harvested weekly, and RT activity was measured. Figure 4A shows the results of an 8-week cultivation of mutant and wild-type SIV. All of the mutants were replication defective except for Gly40→Asp. The titers of the wild-type and the replication competent mutant viruses were determined by 10-fold limiting serial dilutions (Figs. 4B and 4C, respectively). The Gly40→Asp mutant was infectious at a dilution of $1{:}10^4$ but not at a dilution of $1{:}10^5$. Wild-type virus obtained by transfection could infect the AA2-clone 5 culture at a dilution of $1{:}10^5$ but not at a dilution of $1{:}10^6$.

In vivo infectivity analysis of SIV(Mne)-ΔCys33–Cys36

The mutant ΔCys33–Cys36 virus was injected intravenously into a juvenile pig-tailed macaque (Washington Regional Primate Research Center, Seattle, WA; animal identification number M91182) to determine whether an SIV(Mne) NC mutant was infectious in an animal from which the wild-type virus was originally obtained. The stock of the noninfectious SIV(Mne) ΔCys33–Cys36 mutant injected into a macaque contained 28 μg of $p28^{CA}$, the equivalent of an infectious stock with a titer of $\sim 1.4 \times 10^7$ AID, had this virus actually been infectious. An AID was determined previously by titering SIV(Mne) viral

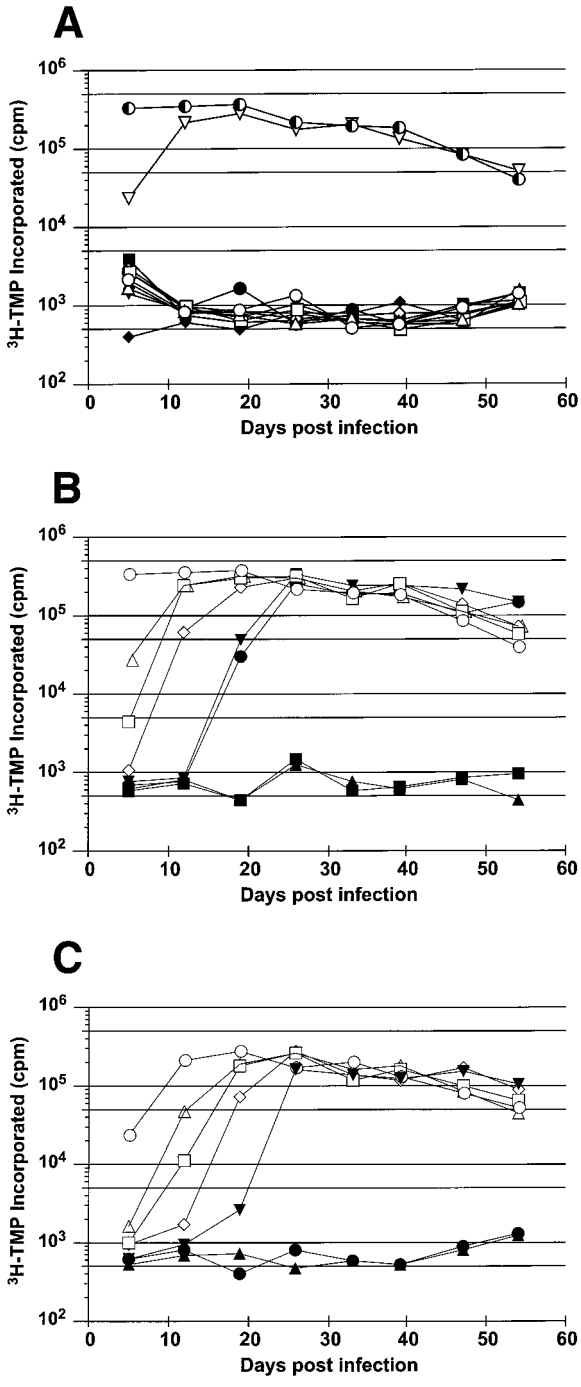

FIG. 4. Infectivity analysis of mutant and wild-type SIV(Mne) viruses in AA2-clone 5 cells. Mutant and wild-type viruses were used to infect AA2-clone 5 cells. RT activities, corrected for background (in cpm [^{3}H]TMP incorporated/ml) of starting inoculum, are as follows: negative control (AA2-clone 5 cells infected with clarified supernatants from 293T cells transfected with sheared salmon sperm DNA), 0 cpm; Cys25→Ser, 9.6×10^5 cpm; Cys36→Ser, 5.3×10^5 cpm; Gly19→Asp, 9.2×10^5 cpm; Gly40→Asp, 2.1×10^6 cpm; Pro9→Glu/Trp13→Gly, 6.9×10^5 cpm; Trp34→Gly, 1.3×10^6 cpm; ΔCys12–Cys15, 6.7×10^5 cpm; ΔCys33–Cys36, 9.2×10^5 cpm; ΔCys12–Cys46, 6.3×10^5 cpm; and wild-type SIV(Mne), 1.7×10^6 cpm. (A) Undilute SIV(Mne) mutant and wild-type viruses. Symbols are as follows: negative control (◆), Cys25→Ser (■), Cys36→Ser (▲), Gly19→Asp (●), Gly40→Asp (▽), Pro9→Glu/Trp 13→Gly (▼), Trp34→Gly (◇), ΔCys12–Cys15 (□), ΔCys33–Cys36 (△), ΔCys12–Cys46 (○), and wild-type SIV(Mne) (circle with left half filled). (B) Wild-type SIV(Mne) end point dilution analysis. Dilutions are $1{:}10^7$ (■), $1{:}10^6$ (▲), $1{:}10^5$ (●), $1{:}10^4$ (▼), $1{:}10^3$ (◇), $1{:}10^2$ (□), $1{:}10^1$ (△), and undilute (○). (C) Infectious titer of Gly40→Asp mutant. Virus was diluted as follows: $1{:}10^6$ (▲), $1{:}10^5$ (●), $1{:}10^4$ (▼), $1{:}10^3$ (◇), $1{:}10^2$ (□), $1{:}10^1$ (△), and undilute (○).

stocks in three species of macaques (Benveniste *et al.,* 1994; Kuller *et al.,* 1994; Tsai *et al.,* 1993). The infectious titer of these stocks *in vivo* range from 10^4 to 10^5 AID/ml. Because these stocks typically contain 20–200 ng/ml $p28^{CA}$, an animal infectious dose corresponds to ~2 pg of $p28^{CA}$.

Longitudinal samples were analyzed over a period of 118 weeks postinoculation, after which the animal was euthanized. At no time could virus be isolated from 8×10^6 PBMC that were analyzed at each sampling, nor could SIV proviral sequences be detected by the sensitive nested PCR method (Benveniste *et al.,* 1988; 1994). By nested PCR, proviruses could not be detected in inguinal, axillary, or mesenteric lymph nodes or in spleen tissue obtained at necropsy, nor could proviruses be detected by nested PCR in PBMC obtained at various times after inoculation. The animal did not develop any measurable antibodies to SIV viral proteins and was clinically normal when euthanized.

DISCUSSION

At this laboratory, we are currently using the SIV/macaque model to examine candidate vaccines against immunodeficiency viruses and are exploring a novel vaccine approach. It was therefore of interest to characterize the behavior of SIV NC mutants, particularly in relation to previously described HIV-1 and MuLV NC mutants. The phenotypes of the SIV(Mne) NC mutants described in this study are in fact reminiscent of those for similar Mo-MuLV and HIV-1 mutants. Transfected mutant clones transiently produced virions that were replication defective (except for the Gly40→Asp mutant) and, in a number of cases, viral RNA deficient. Comparable exogenous template RT activities were observed in mutant and wild-type virions (Table 1). Protein expression and processing in the mutants were also similar to those of wild-type virus (Fig. 2). The presence of uncleaved precursors in the mutant virus preparations is probably due to decreased processing efficiency related to changes in the conformation of the Gag precursor rather than to a decrease in the activity of the viral PR protein. The ΔCys12–Cys46 mutant has less of a Gag precursor processing defect than the other mutants. This phenotype resembles that observed for a similar mutant, the RSV Myr1.ΔNC mutant reported by Krishna et al. (1998). Additionally, the Gly40→Asp mutant does not show processing defects; this mutant is replication competent. In contrast to similar HIV-1 and Mo-MuLV mutants that produce RNA-deficient virions, a number of the SIV(Mne) NC mutants package greater quantities of viral RNA.

This report, to our knowledge, presents the first char-

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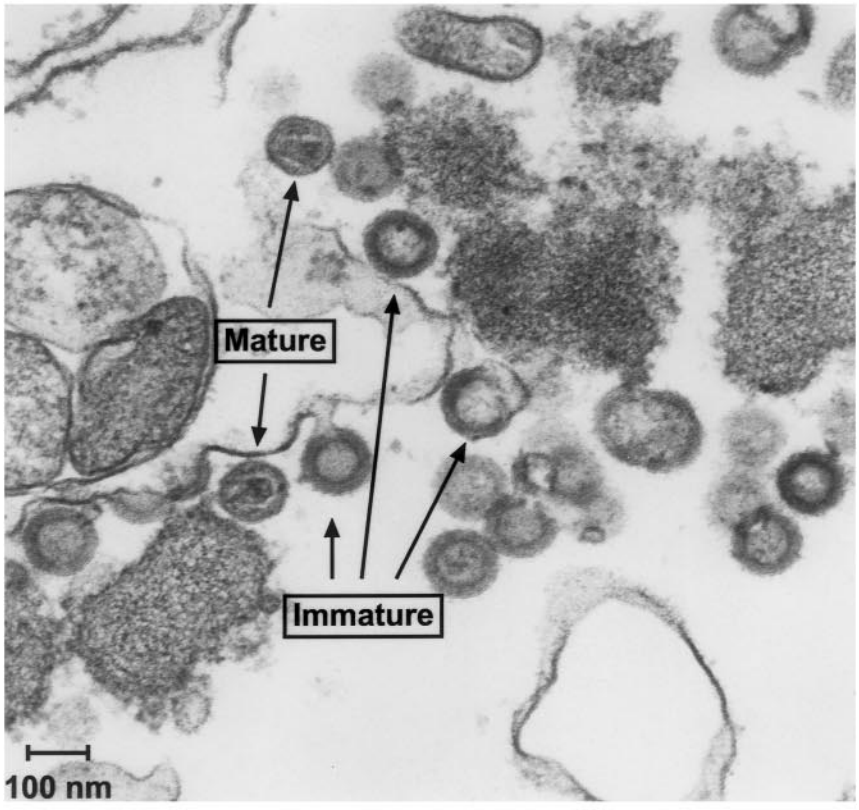

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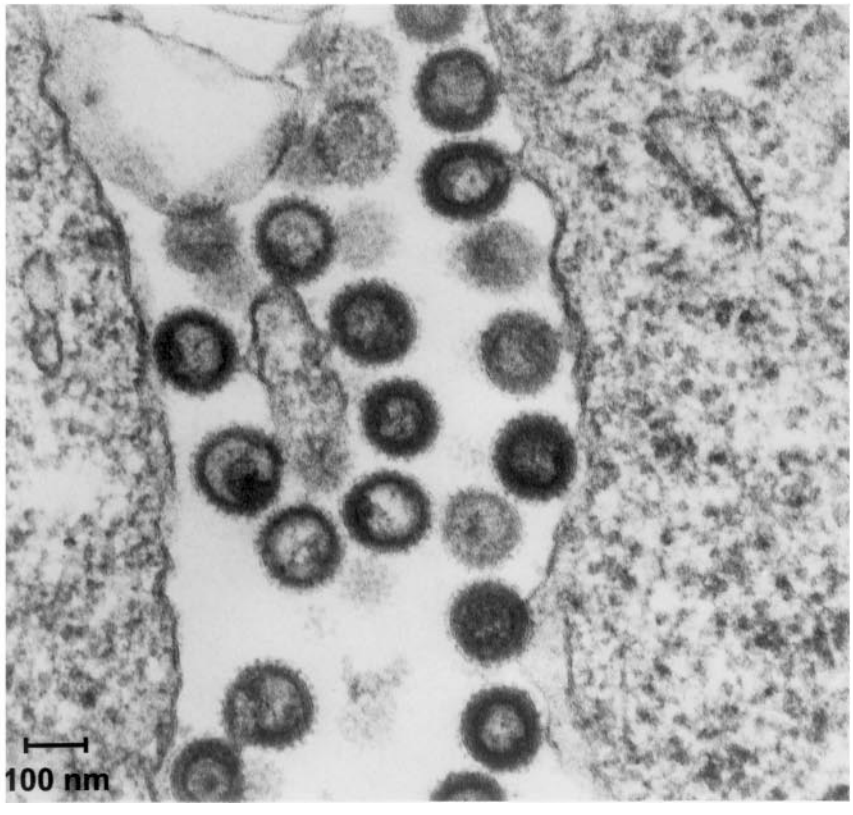

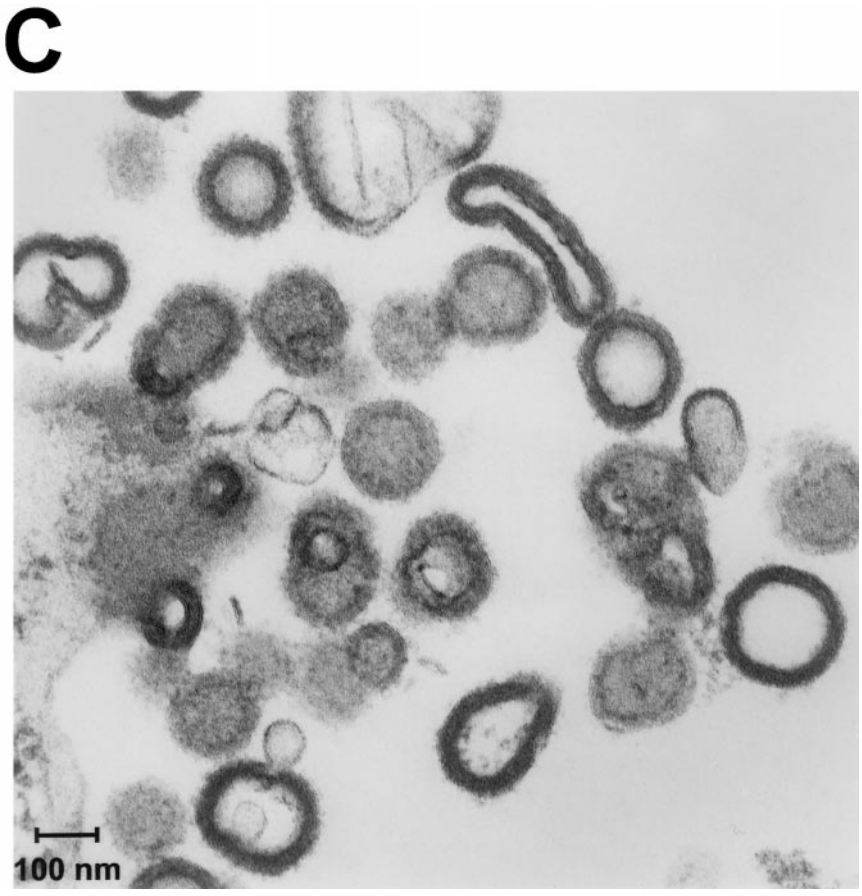

FIG. 5. Electron micrographic analysis of mutant and wild-type SIV(Mne) particles. The 293T cells were transfected with mutant and wild-type proviral clones. At 48 h after transfecting, cells were fixed in glutaraldehyde and prepared for thin section transmission electron microscopy. Particles were visualized at 90,000×, and the distance corresponding to 100 nm is indicated in the lower left corner of each panel. (A) Wild-type SIV(Mne) particles from transfected 293T cells. Mature and immature particles are denoted in the figure. (B) Particles from 293T cells transfected with the Cys36→Ser proviral DNA clone. (C) 293T cells expressing ΔCys12–Cys46 mutant particles.

acterization of the full-length genomic RNA content of an SIV preparation. The Cys36→Ser, ΔCys33–Cys36, and ΔCys12–Cys46 mutants of SIV(Mne) packaged reduced levels of full-length genomes as assessed by Northern blot (Fig. 3), and this reduction was also observed when viral RNA content was measured by real-time RT-PCR (Suryanarayana *et al.,* 1998). For these three mutants, this result was similar to results obtained with other retroviral NC mutants that alter ligand-binding residues to non-Zn^{2+}-coordinating residues (e.g., changes in Zn^{2+}-binding residues to those other than Cys or His or that delete part or all of the Zn^{2+}-finger; Aldovini and Young, 1990; Dorfman *et al.,* 1993; Dupraz *et al.,* 1990; Gorelick *et al.,* 1988, 1990; Meric *et al.,* 1988).

It is surprising that a number of mutants that were replication defective were found to contain viral RNA levels comparable to or greater than levels found in the wild-type virus. This suggests that the NC domain of SIV(Mne) is not as sensitive to alteration with respect to genomic RNA packaging as are HIV-1 or MuLV. In addition, there is agreement between the Northern blot analysis that examines full-length viral RNA genomes and the real-time RT-PCR method that samples total viral RNA (full-length as well as degraded RNA) containing the target *gag* sequence (Suryanarayana *et al.,* 1998) in the virions. This reinforces the conclusion that mutations in the NC domain of the Gag precursor actually affect genome packaging (Aldovini and Young, 1990; Dorfman *et al.,* 1993; Dupraz *et al.,* 1990; Gorelick *et al.,* 1988, 1990; Meric *et al.,* 1988; Rein *et al.,* 1994; Sakalian *et al.,* 1994) as opposed to altering protection of the viral RNA by the NC protein (Aronoff *et al.,* 1993).

An interesting observation was made regarding the affinity of SIV(Mne) core proteins for the viral RNA genome. Although viral RNA can be isolated from Mo-MuLV or HIV-1 by treatment of virus with 1% SDS and subsequent phenol extraction (Gorelick *et al.,* 1993, 1988, 1990), it cannot be isolated from SIV(Mne) mutant or wild-type viruses by this method as was previously observed (Benveniste *et al.,* 1986). A Proteinase K digestion step (Fu *et al.,* 1994; Fu and Rein, 1993; Stewart *et al.,* 1990) is required with SIV(Mne), after which viral RNA can readily be isolated from particles after phenol–chloroform extraction. This indicates that there is a very tight association of SIV viral core proteins with the RNA genome that is not disrupted by the addition of SDS alone. When SIV

preparations were treated only with SDS and then extracted with phenol–chloroform, the RNA partitioned in the organic layer with tightly bound viral proteins.

Due to the tight interaction between the viral core proteins and the viral RNA, additional properties of the mutant and wild-type SIV particles were examined. However, there were no surprising results when the densities of these particles were determined by equilibrium density gradient centrifugation on 10–50% isopycnic sucrose gradients (data not shown). Additionally, electron micrographs of mutant and wild-type particles (Figs. 5A and 5B) were not out of the ordinary, except for the ΔCys12–Cys46 mutant. The mutant and wild-type particles were uniform in diameter with replication competent viruses [Gly40→Asp and wild-type SIV(Mne)], having mature and immature morphologies, and the mutant viruses showed mainly immature morphologies.

The SIV(Mne) ΔCys12–Cys46 particles were interesting because they had a normal density but were larger in size and had irregular shapes, as seen in Fig. 5C. This phenotype is similar to the M1.HNCΔ mutant reported by Garnier *et al.* (1998). The M1.HNCΔ clone produced particles of normal density but were larger in size as determined by velocity gradient centrifugation. It appears that the mutation introduced into the SIV(Mne) ΔCys12–Cys46 mutant altered a particle size determinant, as had the mutation introduced into the M1.HNCΔ particles that removed the carboxyl-terminal half of the NC sequence (including the second Zn^{2+}-finger) and the first four residues of the spacer peptide SP2 (Garnier *et al.,* 1998).

The infectious titers of the SIV(Mne) mutants are reminiscent of similar MuLV and HIV-1 NC mutants (Fig. 4A). All mutations appear to render SIV(Mne) particles replication defective with one exception. The Gly40→Asp mutation has a titer of 10^4 tissue culture infectious doses (TCID)/ml (Fig. 4C). This mutant was constructed and examined because several SIV proviral clones have an Asp residue replacing the conserved Gly residue adjacent to the conserved His in the second Zn^{2+}-finger of SIV NC (e.g., SIV strains Mm251, Mm32H, Mm1A11, Mm239, and Mm142; Korber *et al.,* 1997). We wished to see if there was any detriment to replication if the Gly adjacent to the conserved His in either the first or second position finger (amino acid residue 19 and 40, respectively; Fig. 1) was replaced with Asp. Like the SIV strains with the Gly-to-Asp change, the distal finger of SIV(Mne) can accommodate such a change, whereas the finger in the first position cannot. This suggests that for SIV(Mne), alterations in the finger in the first position are less tolerant of alteration than the second position finger with respect to preservation of viral replication, as was observed with the HIV-1 NC Zn^{2+}-finger mutants reported by Gorelick *et al.* (1990). In the pNC1/1, pNC2/1, and pNC2/2 mutants of HIV-1, only those that contained the first position NC Zn^{2+}-finger amino acid sequence in the first NC Zn^{2+}-finger position were replication competent to varying degrees [e.g., pNL4–3 (wild-type HIV-1) and pNC1/1 (Gorelick *et al.* 1993)]. Figure 4B shows that wild-type SIV(Mne) had a titer of 10^5 TCID/ml. Even though the mutant particles were able to package significant levels of full-length genomic RNA in some cases, a significant defect in replication was nonetheless evident because they were $\geq 10^5$-fold less infectious than wild-type virus. A similar phenotype was observed in the His23→Cys and Cys34→His mutants of MuLV. Both mutants packaged wild-type levels of full-length genomic RNA but were nonetheless replication defective (Gorelick *et al.,* 1996). These results parallel previous observations that additional functions are performed by the retroviral NC protein, beyond its well established role in genomic RNA recognition and packaging (Gorelick *et al.,* 1988, 1990, 1996; Poon *et al.,* 1996, 1998; Tanchou *et al.,* 1998). It should also be noted that there is a strong correlation between mutants that are replication defective and those that show incomplete processing of the Gag precursor (Fig. 2A). This suggests that the replication defect may be attributable in part to changes in the conformation of the mature viral core.

SIV(Mne) ΔCys33–Cys36 mutant virus particles were also inoculated into a pig-tailed macaque to examine *in vivo* infectivity. The animal did not become infected as determined by virus isolation (Benveniste *et al.,* 1988), nested PCR procedures (Benveniste *et al.,* 1994), or the development of antibodies to SIV proteins, despite intravenous inoculation with the equivalent of 1.4×10^7 AID. A dose of 1.4×10^7 AID of wild-type SIV(Mne) virus would certainly result in the productive infection of a macaque. The infectivity results in this study parallel results from similar mutants in HIV-1 (Aldovini and Young, 1990; Dorfman *et al.,* 1993; Gorelick *et al.,* 1990). Alterations to the NC Zn^{2+}-fingers of these viruses, especially the ligand-binding residues, result in virions that are replication defective and do not revert over extended culture periods or, as shown in this work, do not revert in an animal. In addition, the ΔCys33–Cys36 NC mutant of SIV(Mne), which is noninfectious *in vitro* and *in vivo* but produces wild-type levels of viral proteins, could be a potentially valuable immunogen for vaccine studies.

MATERIALS AND METHODS

Cell lines

The 293 cells (adenovirus-transformed human embryonic kidney cells) were obtained from American Type Culture Collection (Rockville, MD). These cells were cultured in minimal essential medium with Earle's salts (E-MEM), containing 10% heat-inactivated fetal bovine serum. The 293 cells, which express the large T antigen (293T), were obtained and cultured as described previously (Gorelick *et al.,* 1996). This line was maintained in Dulbecco's modified Eagle's medium (E-MEM) containing 2 mM l-glutamine and 10% heat-inactivated fetal

bovine serum. AA2-clone 5 cells were cultured in RPMI 1640 medium containing 2 mM l-glutamine, 10% heat-inactivated bovine calf serum, and 2 μg/ml hexadimethrine bromide (Polybrene; Sigma Chemical Co., St. Louis, MO). The AA2-clone 5 line is very sensitive to HIV-1 and SIV infection. AA2-clone 5 is a single cell clone developed in the laboratory of R. Benveniste that is derived from the AA2 cell line (American Type Culture Collection) that is an EBV^+ B lymphoblastoid cell line containing high levels of CD4 (Chaffee *et al.,* 1988). All cell lines were maintained in an atmosphere of 7% CO_2 at 37°C.

Plasmids and mutagenesis

SIV(Mne), originally isolated from a pig-tailed macaque with lymphoma, was cultured on HuT 78 cells (Benveniste *et al.,* 1986). A single cell clone of infected HuT 78 cells, E11S, was used as the source of DNA to obtain the full-length proviral clone, SIV(Mne) clone 8 (Benveniste *et al.,* 1990; Heidecker *et al.,* 1998). Clone 8 is flanked on both the 3′ and 5′ ends by ~1 kbp of uncharacterized cellular DNA from HuT 78 cells. Each flanking region contains a *Sal*I restriction endonuclease site. SIV(Mne) clone 8 was used for constructing the mutant and wild-type full-length proviral plasmids described below.

Site-directed mutagenesis was performed on a subclone (designated pPP1) containing the SIV(Mne) 3998-bp *Kpn*I fragment [nucleotide (nt) positions 418–4416] cloned into the pBluescript $KS(^-)$ [pBSKS(−)] plasmid (Stratagene, La Jolla, CA). Nucleotide positions refer to the position in the SIV(Mne) provirus (Benveniste *et al.,* 1990; Heidecker *et al.,* 1998; GenBank Accession No. M32741). The following mutagenic oligonucleotides were obtained from Marilyn Powers of the DNA Support Laboratory to ABL-Basic Research Program [SAIC-Frederick, National Cancer Institute–Frederick Cancer Research and Development Center (NCI–FCRDC)] and were used to mutate the gene for the SIV(Mne) NC protein: OSL2512, 5′-CAC TCT GCA AGG CAA TCC AGA-3′ (Cys25→Ser, 5′ end corresponds to nt 1733); OSL2514, 5′-GGC TGC TGG AAA AGT GGA CAA-3′ (Cys36→Ser, 5′ end corresponds to nt 1769); OSL2695, 5′-AAT TGT GGG AAA GAA GAC CAC-3′ (Gly19→Asp, 5′ end corresponds to nt 1715); OSL2516, 5′-AAA TGT GGA CAA ATG GAC CAT-3′ (Gly40→Asp, 5′ end corresponds to nt 1778); OSL2735, 5′-AAG CAA ATT AAG TGT GGG AAT-3′ (Pro9→Glu/Trp13→Gly, 5′ end corresponds to nt 1697); OSL2736, 5′-AGA AGA CAG GGC TGC GGG AAA-3′ (Trp34→Gly, 5′ end corresponds to nt 1760); OSL3124, 5′-CCA AGA AAG CCA ATT AAG GGG AAA GAG GGA CAC TCT-3′ (ΔCys12–Cys15, 5′ end corresponds to nt 1691, nt 1709–1720 deleted); OSL3123, 5′-ACC CCA AGA AGA CAG GGC GGA CAA ATG GGC CAT GTT-3′ (ΔCys33–Cys36, 5′ end corresponds to nt 1754, nt 1772–1783 deleted); and OSL3125, 5′-CCA AGA AAG CCA ATT AAG CCA GAC AGA CAG GCA GGT-3′ (ΔCys12–Cys46, 5′ end corresponds to nt 1691, nt 1709–1813 deleted). The amino acid residue numbers refer to positions in the mature SIV(Mne) NC protein (Henderson *et al.,* 1988), and the underlined nucleotides indicate the changes in the oligonucleotide sequences used to introduce point mutations in the NC gene.

The oligonucleotides were used in conjunction with the Amersham Oligonucleotide-Directed In Vitro Mutagenesis System, Version 2 (Arlington Heights, IL). Once mutations were verified by sequencing of the pPP1 plasmids, *Bam*HI–*Nsi*I fragments (wild-type fragment is 561 bp and corresponds to nt position 1329–1886) were cloned into the corresponding sites of a subclone designated pPP4. The plasmid pPP4 with the fragment from the wild-type provirus contains a 3898-bp *Bam*HI–*Sac*I fragment (nt position 1329–5223) cloned into pBKS(−). The resulting *Bam*HI–*Sac*I fragments containing the various mutations were then ligated into homologous sites of the original λ-phage EMBL3 clone containing the SIV(Mne) clone 8 provirus.

For ease in the production of proviral DNA, the *Sal*I fragments containing mutant and wild-type full-length SIV(Mne) proviruses were transferred from the λ-phage EMBL3 clones into a pBR322-based vector designated pSV*ori/neo*. This vector was constructed as follows. To obtain the SV40 origin of replication, a 2.0-kbp *Pvu*II fragment from SV40 viral DNA was ligated using *Eco*RI linkers into the *Eco*RI site of pBR322. pSV*ori* was constructed by ligating the resulting 342-bp *Eco*RI–*Hin*dIII fragment, containing the SV40 origin of replication from this last construct, into the homologous sites of pBR322. A 2.2-kbp *Hin*dIII–*Bam*HI fragment containing the *neo* gene from pSV2*neo* (Southern and Berg, 1982) was cloned into the *Hin*dIII and *Bam*HI sites of pSV*ori*, next to the SV40 origin of replication, to create the vector pSV*ori/neo*. The mutant and wild-type clones used for transfections were constructed by ligating the *Sal*I fragment of the full-length SIV(Mne) clone 8 proviruses from the λ-phage EMBL3 vector into the single *Sal*I site of pSV*ori/neo*. The resulting plasmid designations are listed in Table 1.

Transfections

The 293 cell line (without the large T-antigen) was transfected with the pRB130 clone (ΔCys33–Cys36 mutant) using Lipofectin reagent (Life Technologies, Gaithersburg, MD). To obtain a large quantity of the mutant virus, 50% confluent monolayers in 150-cm^2 flasks were rinsed twice with 10 ml of Hanks' balanced salt solution. Then, 20 μg of DNA diluted to 8.0 ml with OptiMEM (Life Technologies) was added to each monolayer and incubated at room temperature for 30 min. Next, 75 μl of Lipofectin reagent (Life Technologies) diluted to 2.0 ml with OptiMEM was added to the monolayer (without removal of the DNA–OptiMEM solution), and the mixture was incubated overnight at 37°C in a 7% CO_2 atmo-

sphere. The next day, culture fluids were changed to 30 ml of E-MEM containing 10% fetal bovine serum and incubated at 37°C. At 48 h later, the cell culture fluid was removed, and virus was harvested. Next, 30 ml of medium was added to the cell monolayers, and they were incubated for an additional 48 h at 37°C. Culture fluids were again harvested, and virus was isolated. The transiently expressed virus from each harvest was clarified by centrifugation and purified as described previously (Bess *et al.,* 1992). The ΔCys33–Cys36 mutant virus preparation was used for intravenous injection of a macaque as described below.

For viral particle analysis, mutant and wild-type proviral clones were transfected using the Calcium Phosphate Mammalian Cell Transfection Kit from 5 Prime-3 Prime (Boulder, CO). Log phase 293T cells, grown in 150-cm^2 flasks, were transfected, and virus was harvested as described previously (Gorelick *et al.,* 1996). Briefly, culture supernatants were clarified by low-speed centrifugation. The clarified supernatants were centrifuged at 120,000*g* for 1 h at 4°C in a Beckman SW 28.1 rotor.

Exogenous template RT assays

For virus characterization, RT assays were performed on clarified supernatants as described previously (Gorelick *et al.,* 1990). Exogenous template RT assays used in virus isolation experiments were performed as described previously (Benveniste *et al.,* 1986).

Viral protein analysis

Virus was harvested as described previously (Gorelick *et al.,* 1988). Immunoblot analysis was performed as described (Gorelick *et al.,* 1993) using monoclonal antibody to SIV $p28^{CA}$ and rabbit polyclonal antibody to $gp120^{SU}$. $p28^{CA}$ levels were determined on clarified viral supernatants using the reagents and protocols from the Retro-Tek SIV-1 Antigen ELISA Kit (Cellular Products, Buffalo, NY).

Viral RNA analysis

RNA was isolated as described previously (Fu *et al.,* 1994). Briefly, virus pelleted from 30 ml of culture fluid was disrupted in 100 μl of sterile buffer containing 50 mM Tris buffer, pH 7.4, 100 mM NaCl, 10 mM EDTA, 1.0% (w/v) SDS, 50 μg of yeast tRNA/ml (Life Technologies), and 100 μg of Proteinase K/ml (Life Technologies). Samples were incubated at 37°C for 2–4 h. For RNA blot analysis, samples were extracted twice with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1, v/v) and then ethanol precipitated. Samples were adjusted for equal levels of particles based on RT activity before fractionation by denaturing agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized as described previously (Gorelick *et al.,* 1990). The hybridization was performed using an 8.2-kbp *Dra*III–*Bgl*I SIV(Mne) ^{32}P-labeled probe that was prepared using the DECAprime II DNA Labeling Kit (Ambion, Austin, TX).

For viral particle RNA genome estimation, the Proteinase K-treated samples were ethanol precipitated and dried. RNA pellets were dissolved in 0.1 ml of buffer containing 40 mM Tris–HCl, pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$, and 10 U of RQ1 RNase-free DNase (Promega, Madison, Wi) to remove contaminating DNA. Samples were incubated at 37°C for 1 h, and reactions were stopped with the addition of 4.0 M guanidine thiocyanate, 0.3 M sodium acetate, pH 6.0, and 0.12 M β-mercaptoethanol. Samples were ethanol precipitated, rinsed with 70% ethanol, dried, and resuspended in 50–100 μl of sterile RNase-free water containing 400 U of RNASEOUT RNase inhibitor/ml (Life Technologies). Real-time RT-PCR for determining viral RNA levels were performed essentially as described by Suryanarayana *et al.* (1998). Briefly, in a single reaction, viral RNA is first reverse transcribed, and then the cDNA is amplified by PCR using the following oligonucleotide primers and probe. The forward primer (S-GAG03) has the sequence 5′-CAG GGA AII AAG CAG ATG AAT TAG-3′, and the reverse primer (S-GAG04) has the sequence 5′-GTT TCA CTT TCT CTT CTG CGT G-3′. The 5′ ends of these oligonucleotides correspond to nucleotide positions 558 bp and 818 bp, respectively, in the SIV(Mne) nucleotide sequence (GenBank accession no. M32741). The probe, which was labeled with 6-carboxyl-fluorescein (FAM) and 6-carboxyl-tetramethyl-rhodamine (TAMRA) on the 5′ and 3′ ends, respectively, has the sequence 5′-ATT TGG ATT AGC AGA AAG CCT GTT GGA G-3′ (5′ end corresponds to nt position 661 of the SIV(Mne) nucleotide sequence). The Applied Biosystems (Foster City, CA) Prism 7700 thermocycler was used for real-time quantification of cDNA as described previously (Gibson *et al.,* 1996; Heid *et al.,* 1991; Suryanarayana *et al.,* 1998).

Electron microscopy

Electron micrographs were performed by K. Nagashima (Laboratory of Cell and Molecular Structure, SAIC-Frederick, NCI–FCRDC). The 293T cells transfected with mutant and wild-type proviral clones were fixed 48 h post-transfection with 1.25% (v/v) glutaraldehyde in PBS. Cell pellets were embedded and processed as described previously (Gonda *et al.,* 1976). Embedded cell pellets were sectioned and examined in a Hitachi H-7000 electron microscope operated at 75 kV.

In vitro infectivity analysis

Infectivity analysis of transiently expressed virus was performed as described previously (Gorelick *et al.,* 1993) except that the $CD4^+$ cell line AA2-clone 5 was used instead of H9 cells. Briefly, 1 ml of fresh mutant or

wild-type viral supernatant from each of the 293T transfections was clarified of cells and added to 2×10^6 AA2-clone 5 cells in the presence of 2 μg/ml hexadimethrine bromide. Samples were incubated overnight, diluted to 10 ml with medium, and cultured over an 8-week period. Samples were harvested weekly, and RT levels were determined and used as an indication of virus replication.

In vivo infectivity analysis

The SIV(Mne)–ΔCys33–Cys36 mutant virus, produced from transfected 293 cells, was concentrated 10-fold by centrifugation of virus-containing supernatants in a Beckman Ty50Ti rotor at 35,000 rpm for 100 min, underlaid with a pad of 20% v/v glycerol, 50 mM Tris–HCl, and 100 mM KCl, pH 7.8. Viral pellets were resuspended in PBS. The mutant particle preparation had an estimated 28 μg of $p28^{CA}$ based on a calibrated immunoblot using dilutions of purified $p28^{CA}$ (data not shown). Mutant virus in PBS was injected intravenously into a juvenile pig-tailed macaque, and the animal was monitored for the presence of virus and viral DNA sequences as described previously (Benveniste *et al.,* 1988, 1994).

PCR analysis of pig-tailed macaque PBMC

PBMC were isolated from heparinized whole blood as described previously (Benveniste *et al.,* 1993), and DNA was extracted according to standard procedures. DNA (350 ng) was used as template for a two-step amplification by PCR. A nested set of oligonucleotides specific for the long terminal repeat regions of SIV(Mne) were used as primers as described previously (Benveniste *et al.,* 1994).

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