Mutational analysis of two zinc-finger motifs in the nucleocapsid protein of simian immunodeficiency virus mac239

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To clarify the physiological function of two zinc-finger (ZF) motifs in the nucleocapsid (NC) protein of simian immunodeficiency virus (SIV), we constructed three mutant viruses with alterations in either or both motifs using a molecular clone of SIVmac (SIVmac239). An immunoblot analysis of the cell lysates transfected with DNA mutated in either the first (ZF1) or second (ZF2) motif showed that the amount of partially processed Gag products (Pr46) was greater than that produced by the wild-type (WT). The genomic RNA contents in the viral particles released from the transfected cells were measured by quantitative RT-PCR. Values for the ZF1 and ZF2 mutants and the double mutant were 26, 20 and 7 % that of the WT, respectively, indicating that the two ZF motifs of SIVmac239 NC protein function almost equivalently with respect to RNA encapsidation and processing of Gag precursors. Despite the presence of some genomic RNA in the mutant viruses, they lost all viral infectivity. To determine the reason for this, we examined (using PCR) to which step viral DNA synthesis proceeded in the mutant viruses. We did not see any block up to the step of minus-strand DNA synthesis. However, plus-strand DNA synthesis after plus-strand transfer did not occur in any of the mutant viruses. These findings indicated that the mutations in the ZF motifs of SIVmac led to a loss of infectivity due partly to impairment of DNA synthesis, in addition to inefficient encapsidation of genomic RNA.

INTRODUCTION

Retroviruses possess nucleocapsid (NC) proteins, which bind to genomic RNA in the virion cores (Meric et al., 1984; Stewart et al., 1990) and play various roles during the retrovirus life cycle. Early reports described their importance in interactions with genomic RNA such as genomic encapsidation (Aldovini & Young, 1990; Allain et al., 1994; Darlix et al., 1995; Dorfman et al., 1993; Gorelick et al., 1990, 1993; Meric & Goff, 1989), mediation of RNA dimerization (Prats et al., 1990) and annealing of the primer tRNA to its binding site on the viral RNA (Allain et al., 1994; Barat et al., 1989, 1993; Berkowitz et al., 1993; Prats et al., 1988, 1990, 1991). NC proteins of all retroviruses except spumavirus (Maurer et al., 1988) are known to possess one or two zinc finger (ZF) motifs (Cys-X2-Cys-X4-His-X4-Cys) (Berg, 1986; Covey, 1986). The function of the ZF motifs has been studied in various retroviruses. Site-directed mutagenesis of the ZF motifs in murine leukaemia virus (MuLV) (Gorelick et al., 1988; Meric & Goff, 1989), rous sarcoma virus (Meric et al., 1988) and human immunodeficiency virus type 1 (HIV-1) (Aldovini & Young, 1990; Dorfman et al., 1993; Gorelick et al., 1990) severely reduced encapsidation of genomic RNA and produced non-infectious virus particles, indicating that these motifs are involved in packaging of genomic RNA into budding-out particles at the late stage of virus replication. Moreover, recent reports have delineated the involvement of the ZF motifs in the steps of minus- and plus-strand transfer (Gorelick et al., 1996; Guo et al., 1997), formation of virions and synthesis of the end of the proviral DNA (Tanchou et al., 1998).

One approach to understanding the function of the ZF motifs of retroviruses is to analyse the ZF motifs of other retroviruses. However, the functions of the ZF motifs of other retroviruses are so diverse that it is not easy to generalize. For example, MuLV NC protein has only one motif, whereas HIV-1 NC protein has two. The two ZF motifs in HIV-1 are not functionally equivalent: the first motif (ZF1) is responsible for RNA encapsidation and viral core morphogenesis and the second (ZF2) has a role in Gag polyprotein stability (Dannull et al., 1994; Gorelick et al., 1990, 1993; Mizuno et al., 1996). Furthermore, HIV-1 and HIV-2 differ in the mechanism used for selection of genomic RNA for encapsidation (Garzino-Demo et al., 1995; Kaye & Lever, 1999), although their NC proteins have two consecutive ZF motifs and their amino acid sequences are highly homologous. Simian immunodeficiency virus (SIV) NC protein also has two ZF motifs. Although a few studies have
examined SIV NC protein (Gorelick et al., 1999; Urbanjea et al., 2000; Yovandich et al., 2001), much remains to be learned about the function of its ZF motifs, especially in comparison with those of HIV-1 and HIV-2.

The objective of this study was to analyse the functions of both ZF motifs of SIVmac239. We constructed three NC protein mutants of SIVmac239 with alterations in either or both of the ZF motifs in a manner similar to that used in an analysis of HIV-1 NC protein (Mizuno et al., 1996). We found that the two ZF motifs of SIVmac239 NC functioned almost equivalently in terms of RNA encapsidation, processing of Gag precursors and proviral DNA synthesis. This was in contrast to previous findings for HIV-1 NC proteins where the respective ZF motifs do not function equivalently.

**METHODS**

**Plasmid construction.** An infectious molecular clone of SIVmac239 (pMA239) (Kessler et al., 1990; Shibata et al., 1991) was used as a parent proviral DNA. The BamHI–PvuII fragment (nt 2106–2705), which includes a part of the gag open reading frame, was subcloned between the sites of BamHI and HinII in pUC119, and using this plasmid as a template, site-directed mutagenesis of the ZF motifs was performed by PCR as follows. To generate the first ZF motif mutant (designated SIVmac ZF1*), in which the first two cysteine residues in the motif were replaced by serine residues, PCR was performed using the oligonucleotide 5′-TGGGCCTGCAGATGCTCTTCTCAGATATGCCTCTTTCCAGAATTTCAGAAGATTTATAGGC-3′ (nt 2475–2535) and M13 primer M4 5′-GTITTTCAACGTCAGAC-3′. This oligonucleotide was designed to create an EcoRI site (indicated in bold) in addition to alteration of the amino acids. The underlined letters represent the changed nucleotides. The product amplified by PCR was separated by electrophoresis on an agarose gel and purified using a GENECLEAN II kit (BIO 101). The fragment was then digested with BamHI (nt 2106) and BanII (nt 2533). Another fragment obtained from the plasmid pUC119 harbouring the BamHI–PvuII fragment of SIVmac239 (nt 2106–2705) was also digested with BanII and PvuII. These two fragments, BamHI–BanII (nt 2106–2533) and BanII–PvuII (nt 2533–2705), were subcloned consecutively between the BamHI site and the HinII site of pUC119. The generated plasmid was then digested with BamHI and PvuII (nt 2698) and the obtained BamHI–PvuII fragment was reinserted into the corresponding position in a pUC119 plasmid harbouring the BamHI–Sse8387I fragment of SIVmac239 (nt 2105–3402). The generated plasmid was digested with BamHI and Sse8387I and the obtained BamHI–PvuII fragment was reinserted into the corresponding position in pMA239 to generate the full-sized genome plasmid for SIVmac ZF1*.

To generate the second ZF mutant (designated as SIVmac ZF2*), in which the first two cysteine residues in the second motif were replaced by serine residues, PCR was performed using the oligonucleotide 5′-CAGAGCCCCAAGAAGACAGATGGACATCTGGGAAMCATGAAAATCGGACC-3′ (nt 2526–2572) and M13 primer M4. This oligonucleotide was designed to create a BamHI site (indicated in bold). The underlined letters represent the changed nucleotides. The fragment generated by PCR was digested with BanII (nt 2533) and PvuII (nt 2705). Another fragment obtained from plasmid pUC119 harbouring the BamHI–PvuII fragment of SIVmac239 (nt 2106–2705) was digested with BamHI and BanII. These two fragments, BamHI–BanII (nt 2106–2533) and BanII–PvuII (nt 2533–2705), were subcloned consecutively between the BamHI site and the HinII site of pUC119. The generated plasmid was then digested with BamHI and PvuII (nt 2698) and the obtained BamHI–PvuII fragment was reinserted into the corresponding position in pMA239 in a similar manner to generate the full-sized genome plasmid for SIVmac ZF2*.

To generate the mutant of both ZF motifs (designated as SIVmac ZF1*2*), the BamHI–BanII (nt 2106–2533) and BanII–PvuII (nt 2533–2705) fragments possessing the respective mutations were subcloned consecutively between the BamHI site and the HinII site of pUC119. The generated plasmid was then digested with BamHI and PvuII and the obtained BamHI–PvuII fragment (nt 2106–2698) was reinserted into the corresponding position in pMA239 to generate the full-sized plasmid for SIVmac ZF1*2*.

The sequences of the three generated full-sized genome plasmids were confirmed by sequence analysis in the mutated regions from the BamHI site to the PvuII site (nt 2106–2705) and by digestion with EcoRI and BamHI, which can differentiate the respective genomic constructs. The EcoRI and BamHI sites were purposely incorporated without any change of amino acid sequence.

**Cell culture and transfections.** COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% foetal bovine serum (FBS). The cells were transfected by the DEAE-dextran method (Naïda et al., 1988) with 5 μg of wild-type (WT) or mutant viral plasmid DNAs for a reverse transcriptase (RT) assay to analyse the amount of p27CA using ELISA and for immunoblot analysis of transfected cells. For immunoblot analysis of virus particles and Northern blot analysis of viral RNA, the cells were transfected with a FuGENE 6 Transfection Reagent kit (Roche Diagnostics) with 20 μg of the WT or mutant viral plasmid DNAs following the manufacturer’s recommendations.

**Reverse transcriptase assay and ELISA.** The RT assay was performed as described previously (Willey et al., 1988). The amount of p27CA in the culture supernatant was measured with an ELISA kit (Coulter SIV core antigen assay; Coulter).

**Immunoblot analysis.** The immunoblot analysis was performed as described previously (Mizuno et al., 1996). For immunoblot analysis of transfected cells, the cells were washed three times 3 days after transfection, suspended in lysis buffer and boiled for 3 min. The samples were fractionated by 5–20% gradient SDS-PAGE (Atto) and transferred to an Immobilon polyvinylidene difluoride membrane (Millipore). Plasma from a SHIV-NM-3rN-infected monkey (Kuwata et al., 1995) was used to provide the first antibody, since high titres of antibodies against SIVmac 239 Gag proteins have been raised in this monkey. For visualization, an ECL system (Amersham) was used with goat anti-human immunoglobulins linked to horseradish peroxidase. To analyse the processing of virus particle-incorporated proteins, the transfected culture supernatant was filtered through a 0.45 μm pore size filter. Each sample was adjusted to contain an equal amount of RT activity in the supernatant. The released viral particles were pelleted by centrifugation at 14,000 r.p.m. for 2 h at 4 °C. The pelleted virions were lysed in lysis buffer and subjected to immunoblot analysis.

**Quantitative RT-PCR.** For measurement of the genomic RNA in the viral particles and in the cell, we performed quantitative RT-PCR. The transfected culture supernatant was filtered through a 0.45 μm pore size filter and viral RNA was extracted using the QIAamp viral RNA Mini Kit (Qiagen). For the measurement of viral RNA in the cell, the genomic RNA was extracted with Trizol (Gibco BRL). To remove contaminating plasmid DNA, the precipitated RNA was digested with DNase I (Gibco BRL) for 15 min at 37 °C, followed by heat treatment (70 °C, 15 min) to inactivate DNase I. DMEM containing 10 μg pMA239 was also digested with DNase I as a negative control to ensure complete removal of contaminating plasmid DNA.
plasmid DNA. RT reactions and subsequent PCR conditions were performed with a Taqman RT-PCR kit (Perkin Elmer) according to the manufacturer’s recommendations. For gag region measurement, SIVII-696F 5'-GGAAAATCCCCAGTACCAAAATA-GC-3' and SIVII-784R 5'-TCTATCATTTTAACAGGCTTTA-3' were used as primers and the labelling probe SIVII-731 5'-FAM-CCGTCTGGAGATCTGCGACAGAGAC-TAMRA-3'. Each sample was adjusted to contain an equal amount of RT activity in the supernatant before RT-PCR measurement.

**RNA blot analysis.** Virus particles were pelleted by ultracentrifugation using an SW41 rotor (Beckman Fullerton) at 27000 r.p.m. for 2 h at 4 °C. Each sample was adjusted to contain an equal amount of RT activity in the supernatant before centrifugation. The viral RNA was extracted with Trizol (Gibco BRL). To remove contaminating plasmid DNA, the precipitated RNA was digested with DNase I (Gibco BRL) and incubated for 15 min at 37 °C and then 1 µl 25 mM EDTA was added to inactivate the DNase I. Each sample was denatured, electrophoresed and transferred to a nitrocellulose filter. The KpnI–KpnI fragment of pMA239 (nt 1198–4714) was 32P-labelled by the random probing method using a commercial labelling kit (Ambion) and used as a hybridization probe.

**Viral DNA detection.** M8166 cells (5 x 10⁵ cells), a human CD4-positive lymphoid cell line, were infected with the virus stock for 4, 8 and 48 h at 37 °C in a six-well plate. Each virus stock was collected from the supernatant of the Cos-1 cells transfected with either the WT or the mutant full-sized genomic plasmid and each stock was filtered through a 0.45 µm pore size filter and adjusted to contain an equal amount of viral RNA in the supernatant. For adjustment of the amount of viral RNA in the each stock, quantitative RT-PCR in the gag region was performed before infection. After 4, 8 and 48 h infection, M8166 cells were washed three times with PBS, mixed with 2 U DNase I (Gibco BRL) and incubated for 15 min at 37 °C to remove contaminating plasmid DNA. DNA was then extracted using the QIAamp DNA Mini kit (Qiagen). PCR was carried out using extracted DNA obtained from 5 x 10⁴ cells using the following five primer pairs: (i) R/U5 PCR of viral DNA [synthesis of minus strong-stop DNA (−ssDNA)] by LTR RT 5'-TCTTCTTCCAGC ACTAGCGGTAGCCGGGTCTTGCTTCGTCG-3' (nt 816–855) and LTR U5r 5'-CAGGGCGCAATCTGCTAGGGATTTTCTGCTTC-3' (nt 1054–1086) primers; (ii) U3/U5 PCR of viral DNA (elongation of minus-strand DNA after the minus-strand transfer) by LTR 2F 5'-GGAGCGGGTCGAGGATTTTCTGCTTGATGTAT-3' (nt 712–746) and LTR U5r primers; (iii) CA/MA minus-strand DNA by HP1 5'-TGAAGCAGCACTCCGGCCGAAGA-3' (nt 1364–1387) and MG1655R 5'-TGCATAGGCCGTGGATGTCGCCACAC-3' (nt 1883–1910) primers (end of the minus-strand DNA synthesis); (iv) U3/CA of viral DNA by LTR 2F and MG1655R primers (elongation of plus-strand DNA after plus-strand transfer); and (v) 2-LTR PCR of viral DNA by LTR 2F and MLTR 5'-CCCCGTCGAG TGGTATGATGCCTTCTCCTTCTTCTAAGTA-3' (nt 304–343) primers. The primer pairs in (ii), (iv) and (v) have been used previously (Xiao et al., 2000). After denaturation of the template DNA at 96 °C for 5 min, the DNA was amplified for 40 cycles under the following conditions: denaturation at 96 °C for 60 s, annealing at 60 °C for 60 s and extension at 72 °C for 60 s. For the primer pairs in (iv), extension at 72 °C was prolonged for 120 s. A final extension at 72 °C for 5 min was added to the last cycle.

**RESULTS**

**Plasmid construction**

To investigate the individual roles of the two ZF motifs (Cys-X2-Cys-X4-His-X4-Cys) in virus replication, we constructed a series of mutant proviral DNAs with alterations to amino acid residues in the respective motifs. To minimize the effects that these changes had on the conformation of the NC protein, we replaced the first two cysteine residues in the respective fingers with serine residues because of the similarity between the two side-chains. The mutants for ZF1 and ZF2 and the double ZF mutant were named SIVmac ZF1*, SIVmac ZF2* and SIVmac ZF1*2*, respectively.

**Viral protein production**

To examine viral protein production by the mutant proviral DNAs, Cos-1 cells were transfected with each plasmid, and the RT activity and amount of p27CA in the supernatants were measured. Table 1 summarizes the RT activity and amounts of p27CA 3 days after transfection. In comparison with the WT, we observed approximately the same or slightly lower levels of RT activity and amounts of p27CA in SIVmac ZF1* and SIVmac ZF2*. However, the level of RT activity and amount of p27CA were extremely low in SIVmac ZF1*2* (approximately 20% that of the WT).

**Viral protein processing in transfected cells and in particles**

Processing of the viral proteins by the mutant proviral DNAs in Cos-1 cells was examined by Western immunoblot

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**Table 1. Production of SIVmac239 viral proteins in supernatants of transfected cells**

<table>
<thead>
<tr>
<th>Days after transfection</th>
<th>10⁻² × RT activity (c.p.m. ml⁻¹)†</th>
<th>p27 (ng ml⁻¹)‡</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>ZF1*</td>
</tr>
<tr>
<td>1</td>
<td>80</td>
<td>62</td>
</tr>
<tr>
<td>2</td>
<td>214</td>
<td>161</td>
</tr>
<tr>
<td>3</td>
<td>283</td>
<td>183</td>
</tr>
</tbody>
</table>

†The values given are the mean of three experiments.
‡The amount of p27CA was measured using an SIV p27CA antigen capture assay kit. The values given are the mean of two experiments.
analysis. Cos-1 cells were transfected with equal amounts of the WT and mutant DNAs. Three days after transfection, the cells were lysed. As a source of first antibody, we used plasma from an SHIV-NM-3rN-infected monkey. Antibodies in this plasma can detect the SIVmac239 Gag precursor protein, Pr56, and a partially processed precursor, Pr46 (p17MA and p27CA), as well as p27CA, since SHIV-NM-3rN is a chimeric virus and its gag region is derived from SIVmac239. As shown in Fig. 1, the amounts of Pr46 in the cell lysates transfected with both SIVmac ZF1* and SIVmac ZF2* were greater than that for the WT. The cell lysates transfected with SIVmac ZF1*2* contained greatly reduced levels of Pr46 and p27CA.

To investigate the effects of the mutations on particle maturation after the budding step, virus particles produced by the mutant DNAs were pelleted and subjected to immunoblot analysis. The samples were adjusted so that they had equal amounts of RT activity prior to the immunoblot analysis. Each of the mutant particles was found to contain amounts of p27CA, p17MA and unprocessed Gag precursor proteins approximately equal to the WT (Fig. 2).

Taken together, these results indicate that the viruses were influenced by the ZF mutations shown by the delayed processing of Gag precursors in the transfected cells before budding out. However, viral particles in all the mutants appeared to incorporate viral proteins that were processed normally during the budding step.

**Analysis of genomic RNA**

Based on an analysis of the gag region of genomic RNA by quantitative RT-PCR, the RNA contents in the viral particles produced by the SIVmac ZF1*, ZF2* and ZF1*2* DNAs were 26, 20 and 7 % that of the WT, respectively (Fig. 3). A similar analysis based on the nef region of genomic RNA by quantitative RT-PCR gave similar results (data not shown). We also measured the genomic RNA level in the cells 3 days post-transfection and found no significant difference in the RNA levels among the three mutants and the WT (data not shown). A Northern blot analysis was attempted to detect unspliced viral RNA. Unspliced genomic RNA was seen for the WT. However, unspliced genomic RNA was not clearly shown.

![Fig. 1. Immunoblot analysis of processing of Gag proteins by WT and mutant DNAs in transfected Cos-1 cells. At 72 h post-transfection, Cos-1 cells were suspended in lysis buffer. The samples were fractionated by 5–20% gradient SDS-PAGE. Lane 1, WT; lane 2, SIVmac ZF1*; lane 3, SIVmac ZF2*; lane 4, SIVmac ZF1*2*; lane 5, mock-infected cells. Molecular mass markers (Da) are shown on the left.](image1)

![Fig. 2. Immunoblot analysis of virus particles produced by WT and mutant DNAs. Culture supernatants were collected 24–72 h post-transfection, filtered and adjusted to contain equal amounts of RT activity. The released viral particles were pelleted by centrifugation. The pelleted virions were lysed and fractionated by 5–20% gradient SDS-PAGE. Lane 1, WT; lane 2, SIVmac ZF1*; lane 3, SIVmac ZF2*; lane 4, SIVmac ZF1*2*; lane 5, mock-infected cells. Molecular mass markers (Da) are shown on the left.](image2)

![Fig. 3. Viral RNA content in the WT and mutant viral particles measured by quantitative RT-PCR. The average amount of viral RNA in the WT was arbitrarily set at 100%. Each average value and standard deviation was determined from four independent experiments.](image3)
detected in any of the three mutants due to the low sensitivity of this technique (data not shown).

**Infectivity of ZF mutant viruses and proviral DNA synthesis**

We next determined whether or not the mutant DNA plasmids produced infectious virus. CD4-positive M8166 cells were infected with the supernatants of transfected Cos-1 cells containing equal amounts of viral RNAs, as measured by quantitative RT-PCR. Conspicuous syncytium formation was observed in the M8166 cells infected with the supernatant from cells transfected with WT DNA. The culture supernatant of these cells accumulated RT activity. In contrast, there was no indication of infectivity in the cells infected with the supernatants from the three mutant DNAs during the observation period of more than 1 month. Thus, alteration of the ZF motifs resulted in total loss of viral infectivity.

According to a recent report (Guo et al., 1997), alterations in ZF motifs appear to affect not only the packaging ability of genomic RNA but also the ability to synthesize DNA at the step of strand transfer. To analyze DNA synthesis in SIVmac, we determined the levels of the different forms of DNA (Fig. 4A) by PCR. The genomic DNA was extracted from M8166 cells 4, 8 and 48 h after infection with the WT and mutant viruses containing the same amount of viral RNA, and five different regions were amplified to monitor to which step viral DNA synthesis proceeded: (i) R/U5 LTR (synthesis of −ssDNA); (ii) U3/U5 (synthesis of minus-strand DNA after minus-strand transfer); (iii) MA/CA (synthesis of the end of minus-strand); (iv) U3/CA (synthesis of the plus-strand DNA after plus-strand transfer); or (v) 2-LTR form. As shown in Fig. 4(B), −ssDNA was synthesized in the WT and all mutant viruses. After the step of minus-strand transfer, synthesis of the minus-strand DNA was completed in all the viruses. Interestingly, after the plus-strand transfer, synthesis of the plus-strand DNA failed in all the mutants. The 2-LTR form was not detected in any of the mutants, while it was detected in the WT at 48 h post-infection (data not shown). The detection limit of the PCRs (i)–(v) was found to be 100 copies of the target molecules in a reaction mixture by measuring serially diluted standard solutions of the parental plasmid (a representative result is given in Fig. 4C). The results indicated that the ZF motifs contribute to the efficient synthesis of viral DNA at the step of plus-strand transfer and that the roles of ZF1 and ZF2 were almost the same. Thus, all the SIVmac ZF mutants appeared to be non-infectious due to inefficient or incomplete synthesis of viral DNA.

**DISCUSSION**

In our previous analysis of HIV-1 NC (Mizuno et al., 1996), the first two cysteine residues in both ZF1 and ZF2 mutants (HIV-1 ZF1* and HIV-1 ZF2*) were replaced by serine residues. In this study, we modified the ZF motifs of SIVmac in the same way to compare the functions of the two motifs in HIV-1 and SIVmac. The genomic RNA content of HIV-1 ZF1* was about 10% that of the WT, whereas the genomic RNA content of HIV-1 ZF2* was almost as much as that of the WT. An immunoblot analysis of the virus particles showed that HIV-1 ZF2* particles contained some proteins with smaller molecular sizes than expected. These were thought to be degradation products of p27CA. HIV-1 ZF1* particles did not appear to have such degradation products. These results indicated that the ZF1 of HIV-1 is primarily responsible for RNA encapsidation while ZF2 is required for stabilization of virus particles.

The present results contrast with these previous results obtained for HIV-1. SIVmac ZF1* and ZF2* had approximately the same reduction in genomic RNA content compared with the WT. The genomic RNA content of SIVmac ZF1* was about 26% and SIVmac ZF2* was about 20% that of the WT. These results indicate that the two ZF motifs of SIVmac239 NC protein function almost equivalently with respect to RNA encapsidation. It also means that ZF2 of SIVmac might bind the genomic RNA slightly more strongly than ZF1. Urbaneja et al. (2000) reported that, with respect to the isolated SIVmne ZF1 and ZF2 peptides, their intensity of binding to nucleic acids was close to half that seen for SIVmne NCp8 and that the SIVmne ZF2 peptide bound the nucleic acids slightly more strongly than the SIVmne ZF1 peptide. The finding of this report is thus similar to our result. Yovandich et al. (2001) reported that the SIVmne ZF2 mutant resulted in an approximately 30% reduction in the level of genomic RNA, while the SIVmne ZF1 mutant had a genomic RNA similar to that of the WT virus. They suggested that SIVmne ZF2 appears to function more significantly in genomic RNA encapsidation than SIVmne ZF1. With respect to the encapsidation of genomic RNA in HIV-2, both ZF1 and ZF2 motifs interact specifically with viral RNA (Komatsu et al., 1996), which is in marked contrast to HIV-1, where the ZF2 motif does not interact specifically with viral RNA (De Rocquigny et al., 1992; Mizuno et al., 1996). The functions of the two ZFs of HIV-2 are thus similar to those of SIVmac. This similarity is not surprising since SIVs are genetically more closely related to HIV-2 than to HIV-1. An immunoblot analysis of the transfected cells showed that SIVmac ZF1* and ZF2* expressed the Gag precursor protein, Pr46, at approximately the same level, indicating that the ZF motifs of SIVmac239 NC protein function almost equivalently with respect to the processing of Gag precursors, as well as RNA encapsidation. These results also suggest that the functions of the two ZFs in SIVmac are slightly different from those in HIV-1. Interestingly, it has been reported that HIV-1 and HIV-2 differ in the process of genomic RNA packaging (Kaye & Lever, 1999), with genomic RNA being captured in cis in HIV-1, whereas unspliced RNA is selected in trans by the Gag protein in HIV-2. We cannot tell whether that is true or not from our results, but we consider that the difference in selection of genomic RNA might be related to the difference in the ZF functions between HIV-1 and HIV-2/SIV.
In order to distinguish between a drop in packaging efficiency and a decrease in the amount of RNA available for encapsidation as a result of the mutations in the ZF motifs, we measured the genomic RNA levels in the cell lysates. We found no significant differences in the RNA levels of the three mutants and the WT, indicating that the introduction of mutations in the ZF motifs did not cause a drop in the levels of genome RNA available for encapsidation.

It can thus be concluded that mutagenesis in the ZF motifs of SIVmac NC protein resulted in loss of viral infectivity, and that this loss was partly attributable to a reduction in the packaging efficiency of genomic RNA. However, this does not explain fully the results, since the ZF mutant viruses lost all viral infectivity, even though they retained some genomic RNA (20% or higher). In this context it is noteworthy that SIVmne ZF mutants with no viral infectivity produced immature or morphologically aberrant particles (Gorelick et al., 1999; Yovandich et al., 2001) which retained genomic RNA to some extent (some contained almost WT levels of genomic RNA). To elucidate the reason for this, we focused on the roles of the ZF motifs of SIVmac NC in viral DNA synthesis. In fact, the NCs of MuLV and HIV-1 are reported to increase the efficiency of synthesis of DNA products (Allain et al., 1994; Berthoux et al., 1997; Gonsky et al., 2001; Gorelick et al., 1996; Wu et al., 1999; You & McHenry, 1994; Yu & Darlix, 1996). Guo et al. (1997) reported that alterations in ZF motifs of HIV-1 NC affected the ability to

![Fig. 4. PCR analysis of the four steps of viral DNA synthesis.](image)

(A) Schematic drawings of the steps: (i) ssDNA synthesis, which is detected by the LTR Rf/LTR U5r primer pair; (ii) minus-strand DNA synthesis after the minus-strand transfer, which is detected by the LTR 2F/LTR U6r primer pair; (iii) the end of the minus-strand DNA synthesis, which is detected by the HP1/MG1655R primer pair; and (iv) plus-strand DNA synthesis after the plus-strand transfer, which is detected by the LTR 2F/MG1655R primer pair. Primer pairs are shown in italics and their complementary sequences are shown by horizontal arrows. (B) PCR results obtained for each of the four steps using M8166 cells 4 h (left panels) and 8 h (right panels) after being infected with the WT and mutant viruses. The amount of each type of virus used to infect the cells was the same. Lane 1, WT; lane 2, SIVmac ZF1*; lane 3, SIVmac ZF2*; lane 4, SIVmac ZF1*2*; lane 5, mock-infected cells. (C) Sensitivity of the PCR used in the analysis of DNA synthesis. Quantified copy numbers of SIVmac plasmid (pMA239) were amplified with U3/CA viral DNA detection primers LTR 2F and MG1655R, which detect elongation of plus-strand DNA after the plus-strand transfer. Lanes 1–8 correspond to copy numbers of $10^8$, $10^6$, $10^4$, $10^3$, $10^2$, 10, 1 and 0, respectively.
synthesize DNA at the step of strand transfer. Recently Guo et al. (2000), using an in vitro model system, reported that the ZF motifs of HIV-1 NC were required for efficient minus- and plus-strand transfer and that these motifs were involved in the reaction by which NC completely removes the tRNA primer during plus-strand transfer. In this study, it was clarified that the minus-strand transfer was completed, but the plus-strand transfer seemed to have failed in all the ZF mutants of SIVmac. This finding suggests that the mutations in ZF motifs of SIVmac led to a loss of infectivity partly because of impairment of DNA synthesis. The removal of tRNA primer during the plus-strand transfer might be a critical step in the replication of all the SIV ZF mutants, since the presence of the tRNA primer attached to the template RNA genome obviously blocks a smooth move to the next step of elongation of plus-strand DNA synthesis.

In conclusion, the present study has revealed that the ZF motifs of SIVmac NC protein are required in the final step of DNA synthesis, suggesting an important role for the NC protein in viral DNA synthesis, in addition to its importance in genome packaging, which is perhaps not surprising considering that it is the protein that interacts most closely with genomic RNA. Further studies of how NC proteins of HIV and SIV perform these multiple tasks, especially at the molecular level, are necessary to understand fully the mechanism of replication of the primate lentiviruses.

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