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Sequence Note

Evidence of Recombination between 3′ and 5′ LTRs in Macaques Inoculated with SIV DNA

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ABSTRACT

Proviral SIV DNA inoculation of macaques is an efficient method to initiate wild-type and attenuated SIV infections. However, we found that macaques inoculated with SIV DNA engineered to contain a single 105-bp deletion in the 3′ *nef*/LTR overlap region had SIV sequences subsequently isolated that had partially or fully repaired the deletion with wild-type sequence. Animals inoculated with SIV DNA containing identical deletions in both the 5′ and 3′ LTRs did not repair the deletion. Recombination events occurred early, most likely by homologous recombination with sequences from the wild-type 5′ LTR. This sequence analysis is the first demonstration of homologous recombination *in vivo* following administration of a single SIV strain.

INTRODUCTION

We have studied the initiation of wild-type and attenuated simian immunodeficiency virus (SIV) infections in macaques by administration of proviral SIV DNA.[1] Wild-type and attenuated SIV infections can be initiated utilizing as little as 15 μg of SIV DNA, providing a feasible means to manufacture and deliver live attenuated HIV vaccines should they ultimately prove sufficiently safe and effective. Humans with attenuated HIV-1 infections, including the Sydney Blood Bank Cohort, have focused attention on deletions that overlap the *nef* and 3′ long terminal repeat (LTR) genes.[2] Macaque studies have suggested some level of ongoing SIV replication is required in order to generate protective immunity.[3] In the initial considerations on the design of plasmids for the initiation of attenuated SIV infections, we hypothesized that the efficient initiation of an SIV infection might require a strong first round of viral replication from an intact 5′ LTR. We previously demonstrated a reduction in gene expression in macaques and in humans following introduction of reporter gene expression from deleted LTRs.[4] Since subsequent rounds of replication would duplicate a deleted 3′ LTR into the 5′ LTR (Fig. 2), we hypothesized that an intact 5′ LTR in the inoculated plasmid would facilitate the initial round of transcription but that subsequent rounds would contain only the attenuating deletion in both LTRs.

To assess the sequence stability of attenuated SIV plasmids inoculated into pigtailed macaques, a series of full-length SIV_{mac239} proviral plasmids were engineered. The plasmids were either wild-type sequence (pSIVmac239), contained a single in frame 105-bp deletion in the 3′ *nef*/LTR overlap region (pSIVsbbcΔ3), or contained identical deletions in the 3′ *nef*/LTR overlap region and 5′ LTR (pSIVsbbΔ3Δ5, Figs. 1 and 2).[1] The plasmids were inoculated into macaques either intramuscularly or intradermally via gene gun in studies approved by the relevant institutional animal ethics committee. To assess the stability of the introduced deletions, DNA was extracted from serial peripheral blood mononuclear cell (PBMC) samples of all SIV DNA-inoculated animals and subjected to nested PCR across the *nef*/LTR overlap region as described.[1] Representative clones were made into the pCRII-topo vector (Invitrogen, San Diego, CA) and inserts sequenced using the dideoxy method on an automated sequencer (Applied Biosystems, Foster City, CA).

All four animals inoculated with wild-type pSIVmac239 progressed to AIDS within 1 year and had SIV*nef*/LTR DNA of wild-type size amplified from PBMC by nested PCR at every time point examined following DNA inoculation.[1] Sequencing

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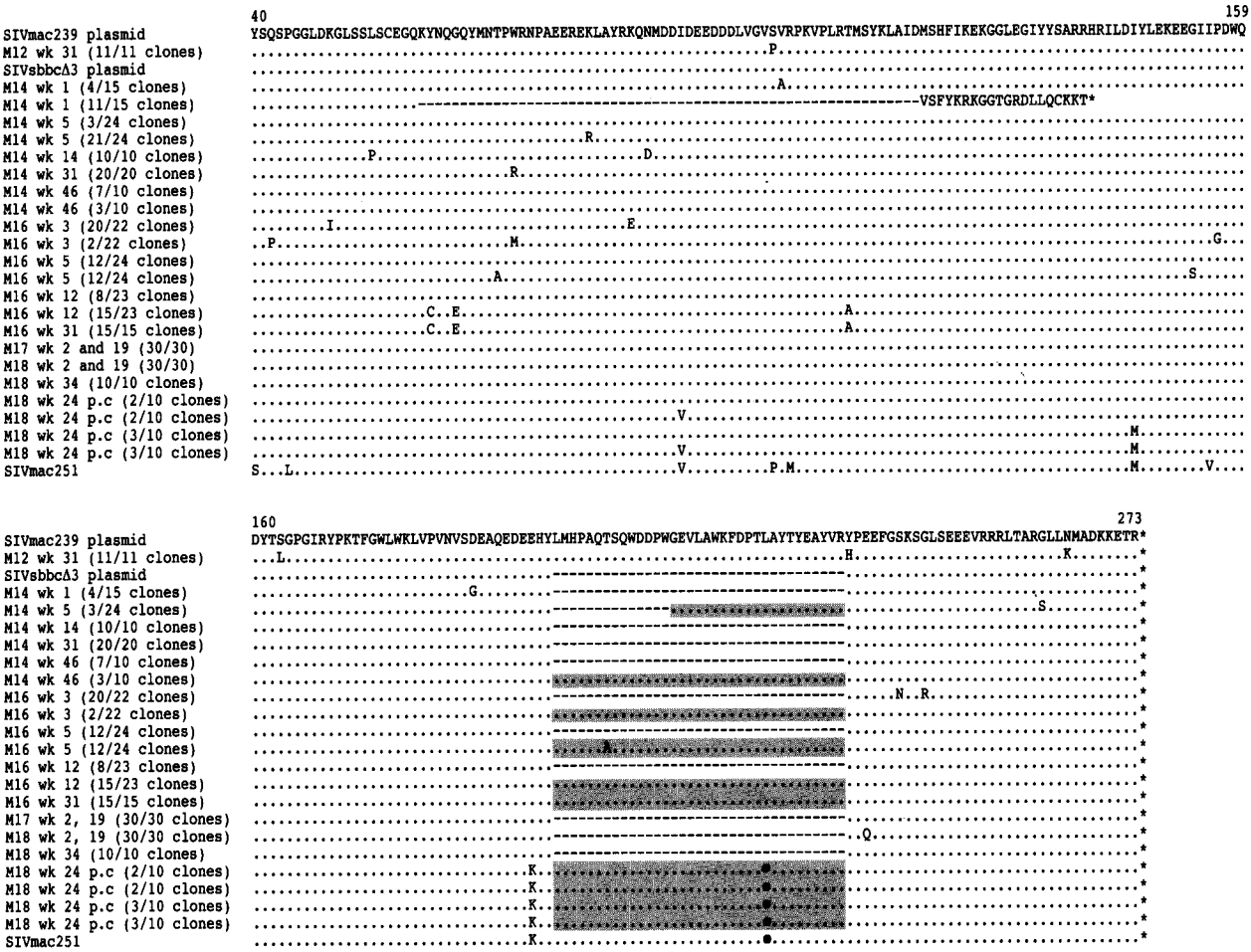

FIG. 1. Predicted amino acid sequences from the SIV *nef*/LTR region. The sequence was derived from cloned PCR products of macaque PBMC DNA following SIV DNA inoculation. The top line shows the sequence from the SIV_{mac239} plasmid as the reference, the third line the pSIVsbbcΔ3 plasmid, and the bottom line the SIV_{mac251} challenge strain. Identical sequence is annotated with a period (.), sequence deletions with a dash (–), and a stop codon with an asterisk (*). Clones were grouped according to the size of the PCR product, and the week (wk) of isolation after inoculation, or week postchallenge (wk p.c). The frequency of clones is shown in parentheses. The shaded sequence illustrates clones from animals inoculated with pSIVsbbc DNA that repaired the *nef*/LTR deletion. The SIV_{mac251} sequence at amino acid 222 within the *nef*/LTR deletion is denoted (●) to differentiate the synonymous variation between SIV_{mac239} (CTG) and SIV_{mac251} (CTA) at this position.

of the inoculated plasmid and a PBMC from one of the pSIVmac239-inoculated animals, M12 at 31 weeks postinoculation, demonstrated a wild-type sequence across the *nef*/LTR region (Fig. 1). The two animals inoculated with pSIVsbbcΔ3Δ5 containing 3′ and 5′ deletions, M17 and M18, did not progress to AIDS. PBMC from the pSIVsbbcΔ3Δ5-inoculated animals had only deleted-size *nef*/LTR amplified from PBMC by nested PCR and sequencing demonstrated the deletion was stable in these two animals (Fig. 1).

In contrast, of the two animals inoculated with pSIVsbbcΔ3, one animal, M16, progressed to AIDS after 60 weeks following inoculation.[1] PBMC DNA from animal M16 amplified wild-type size SIV*nef*/LTR by nested PCR from week 3 following SIV inoculation and thereafter. Sequencing of the *nef*/LTR PCR product from serial M16 PBMC samples showed that rare clones with an exact rebuilding of the entire 105-bp *nef*/LTR deletion to wild-type SIV_{mac239} sequence appeared by week 3, both at the nucleotide level (not shown) and predicted amino acid sequence (Fig. 1). The wild-type clones gradually became more predominant at weeks 5 and 12 following inoculation and, effectively competing out the attenuated sequence, became the sole isolates by week 31 following inoculation, coincident with a decline in $CD4^+$ T cells.

Additionally, animal M14, also inoculated with pSIVsbbcΔ3, had rare (3 of 24) clones isolated 5 weeks following inoculation in which the 105-bp *nef*/LTR deletion had been partially rebuilt with a 63-bp insertion exactly homologous with the wild-type sequence (Fig. 1). At 46 weeks after DNA inoculation, 3 of 10 clones showed that the original 105-bp deletion had been completely and precisely rebuilt with the wild-type sequence, although these species never became the predominant species and the animal did not experience a decline in $CD4^+$ T cells. This animal demonstrated a high level of T cell immunity to SIV proteins by interferon (IFN)-γ ELISPOT[1] and thus could presumably protect it-

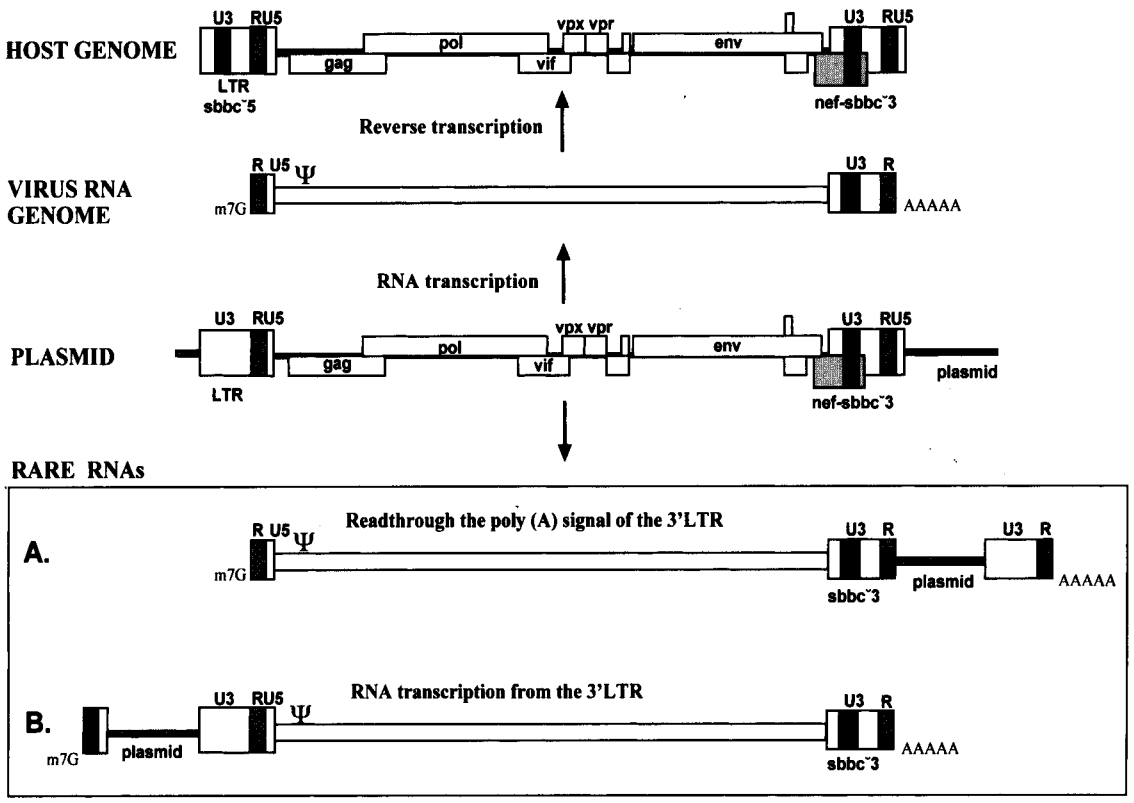

FIG. 2. Possible outcomes of inoculation with SIV DNA plasmid encoding a deletion only in the U3 of the 3′ LTR. The upward arrows show the common processing events leading to the duplication of the 3′ LTR deletion (in black) into the 5′ LTR following the first round of transcription. Rare events that could lead to generation of wild-type SIV may occur as depicted in **A** by readthrough of the poly(A) signal of the 3′ LTR or **B** by RNA transcription starting from the 3′ LTR.

self from this "autologous" challenge with the recombinant wild-type SIV_{mac239} at this later time following inoculation.

To assess the potential for attenuated SIV infections to protect from a virulent challenge, the pSIVsbbc-inoculated animals were challenged intrarectally with SIV_{mac251}. Animals M14 and M17 were protected from challenge but M18 was not protected and experienced a decline in CD4 T cells.[1] Sequencing of PCR clones from M18 PBMC postchallenge demonstrated an SIV_{mac251} sequence within the *nef*/LTR deletion was present following challenge in all 10 clones examined (Fig. 1). Sequences of PCR clones from M18 in regions flanking the *nef*/LTR deletion showed sequences of both SIV_{mac251} and SIV_{mac239}, suggesting evidence of multiple recombination events between SIV_{mac239} and SIV_{mac251} (Fig. 1).

The rapid and accurate repair of the 3′ *nef*/LTR deletions in pSIVsbbcΔ3-inoculated macaques M14 and M16 is consistent with homologous recombination with wild-type sequences derived from the 5′ LTR (which had not been mutated in an attempt to efficiently initiate transcription). This supposition is supported by the fact that infection of macaques with proviral DNA in which the *nef*/LTR deletions had been engineered into both 3′ and 5′ LTR regions did not generate any wild-type virus sequence. *In vitro* transfection studies with deleted HIV-1 DNA plasmids have demonstrated that homologous recombinations frequently occur, presumably by copackaging of heterologous RNA.[5] Homologous recombination events between attenuated vaccine strains and challenge strains of SIV have occurred when two SIV strains have been inoculated concurrently, or between attenuated vaccine strains and challenge strains of SIV, as was observed in animal M18 in this study (Fig. 1).[6,7] However, this is the first demonstration of homologous recombination *in vivo* following administration of a single SIV strain. The mechanism driving these recombination events is unclear but possibilities include (1) recombination with a copackaged rare RNA transcript that reads through the deleted 3′ LTR and terminates at the poly(A) signal site in the R of the wild-type 5′ LTR in the plasmid, thereby including a wild-type U3 (Fig. 2A), (2) recombination with copackaged transcripts initiating from the 3′ LTR and reading through the 5′ U3 to the poly(A) signal sequence in the 3′ LTR (Fig. 2B), or (3) recombination between the wild-type 5′ LTR of plasmid DNA and viral DNA or RNA forms.[8]

The principal implication of this finding is that if the desired attenuation of these lentiviruses depends wholly or in part from mutations within the LTR (either of the LTR region itself or of the *nef* gene overlapping the LTR), such mutations will have to be engineered into both proviral LTRs to prevent rapid recombination to wild-type virus during *in vivo* replication. Since wild-type 5′ LTR promoter activity is not required for efficient initiation of infection *in vivo*, and it poses a major safety hazard, it argues that any mutations in the LTR region used in live attenuated vaccines should be made in both proviral LTRs.

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