

Genetic Instability of Live, Attenuated Human Immunodeficiency Virus Type 1 Vaccine Strains

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Live, attenuated viruses have been the most successful vaccines in monkey models of human immunodeficiency virus type 1 (HIV-1) infection. However, there are several safety concerns about using such an anti-HIV vaccine in humans, including reversion of the vaccine strain to virulence and recombination with endogenous retroviral sequences to produce new infectious and potentially pathogenic viruses. Because testing in humans would inevitably carry a substantial risk, we set out to test the genetic stability of multiply deleted HIV constructs in perpetuated tissue culture infections. The $\Delta 3$ candidate vaccine strain of HIV-1 contains deletions in the viral long terminal repeat (LTR) promoter and the *vpr* and *nef* genes. This virus replicates with delayed kinetics, but a profound enhancement of virus replication was observed after approximately 2 months of culturing. Analysis of the revertant viral genome indicated that the three introduced deletions were maintained but a 39-nucleotide sequence was inserted in the LTR promoter region. This insert was formed by duplication of the region encoding three binding sites for the Sp1 transcription factor. The duplicated Sp1 region was demonstrated to increase the LTR promoter activity, and a concomitant increase in the virus replication rate was measured. In fact, duplication of the Sp1 sites increased the fitness of the $\Delta 3$ virus (Vpr/Nef/U3) to levels higher than that of the singly deleted Δ Vpr virus. These results indicate that deleted HIV-1 vaccine strains can evolve into fast-replicating variants by multiplication of remaining sequence motifs, and their safety is therefore not guaranteed. This insight may guide future efforts to develop more stable anti-HIV vaccines.

Relatively disappointing outcomes have been obtained thus far with a variety of anti-human immunodeficiency virus (HIV) vaccine candidates (41). However, the results of studies with live attenuated simian immunodeficiency viruses (SIVs) in monkey models are promising models for the possible use of live attenuated HIV as a protective vaccine. It has been repeatedly demonstrated that macaques or chimpanzees persistently infected with genetically attenuated, nonpathogenic isolates of SIV or HIV-1, respectively, strongly resist a subsequent challenge with pathogenic virus (2, 12, 36, 45, 47, 53, 57). In addition, there is some evidence that attenuated HIV-1 variants lacking the *nef* gene result in a benign course of infection in humans (16). These results warrant further investigation of this class of anti-HIV vaccines. However, the development of a live attenuated HIV-1 vaccine will face major safety issues, and the question has been raised of how much animal work remains to be done before human vaccine trials can proceed (7, 10, 17, 38). For instance, recent evidence suggests that SIV constructs with multiple gene deletions can be pathogenic in newborn monkeys (3, 58). Another major safety concern is the fear that the vaccine strain can evolve from an attenuated form to a more virulent, pathogenic form. The latter process of virus evolution can occur either by spontaneous mutation of one of the remaining viral functions or by recombination-mediated repair with parts of the host cell genome, e.g., endogenous retroviral sequences.

In this study, we addressed the genetic stability of multiply

deleted HIV-1 variants. To do so, we performed long-term tissue culture infections with the HIV-1 candidate vaccine strains to allow virus evolution to be studied on a laboratory timescale. Evolution of HIV-1 in a tissue culture setting has been reported repeatedly. A well-known example of tissue culture evolution occurs during culturing of primary isolates on T-cell lines, which results in the selection of “laboratory-adapted” HIV-1 variants with amino acid changes within the envelope glycoprotein that cause a shift in the host cell range and coreceptor use. Furthermore, many studies with replication-impaired HIV-1 mutants yielded revertant viruses after prolonged in vitro culturing. The analysis of revertant viruses has been used to study interactions within HIV-1 proteins, e.g., the Env glycoprotein (56) and the integrase enzyme (49). This genetic approach has been particularly useful in the dissection of complex RNA motifs, including the TAR hairpin (25, 32), the poly(A) hairpin (5, 14), and the dimerization initiation site DIS (6). Furthermore, mutations in the DIS RNA motif can be overcome by compensatory mutations within the viral Gag protein, which is most probably due to a direct interaction between the DIS RNA and the Gag protein (35). These combined results demonstrate the enormous genetic flexibility and repair capacity of the HIV-1 retroviral genome.

This genetic flexibility of HIV-1 is likely to be greater in vivo infections, where a larger number of virion particles are produced. A prominent example is the appearance of drug-resistant HIV-1 variants in patients treated with potent antiviral drugs. For resistance to drugs against the protease enzyme, there is accumulating evidence that the primary resistance mutations within the protease protein cause a partial enzyme defect, which is subsequently restored by secondary mutations in protease and/or compensatory changes within the gag-en-

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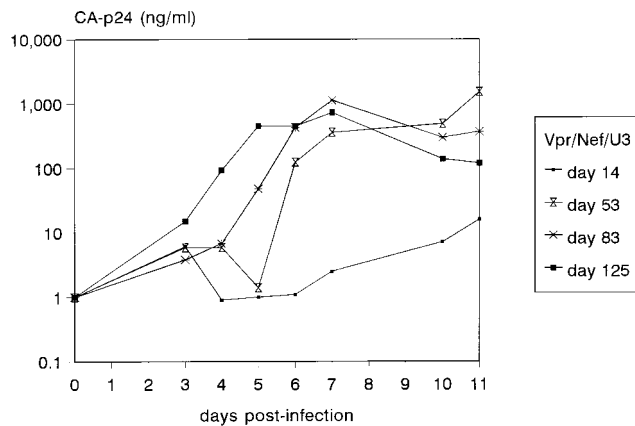


FIG. 1. Improved growth kinetics of $\Delta 3$ revertant viruses. The HIV-1 samples represent the $\Delta 3$ virus after culturing on SupT1 cells for increasing periods. The perpetuated SupT1 infection was started by electroporation of 10 μ g of $\Delta 3$ construct, and at the peak of infection, virus was passaged onto fresh, uninfected SupT1 cells. Supernatant samples were taken at several days posttransfection, frozen, and subsequently used to infect SupT1 cells with the same amount of input virus (1 ng CA-p24). Cell samples were also stored for proviral DNA analysis (see Fig. 2).

coded substrate sequences (11, 59). The *in vivo* capacity for repair of small attenuating gene deletions was also demonstrated by the evolution of an SIV variant with a 12-nucleotide (nt) deletion in the *nef* gene. A sequence duplication event was observed first, and multiple mutations were subsequently selected in the "insert" to create an amino acid sequence that is virtually indistinguishable from that of the wild-type virus (55). Despite these safety concerns, it seems unlikely that HIV-1 can repair large gene deletions. Therefore, whole genes have been deleted in the current live, attenuated vaccine candidates. Nevertheless, it has been reported recently that certain HIV-1 mutants can restore their fitness by second-site changes in unrelated functions encoded by the remaining sequences in the viral genome (15). We therefore tested the genetic stability of the current generation of HIV-1 vaccine candidates in long-term tissue culture infections.

MATERIALS AND METHODS

Plasmid constructs and PCR. The HIV-1 molecular clone used in this study is the chimeric pNL4-3 plasmid (1), which contains the 5' half of proviral NY5 and the 3' half of proviral LAV sequences joined at a shared *EcoRI* site in the *vpr* gene. The different deletion mutants were described previously (21), and infectious virus was reconstructed from the 5' and 3' half genomes by *EcoRI* digestion and subsequent ligation.

Proviral DNA sequences were PCR amplified across the deletion points. To screen for the *nef*-U3 deletions, we used the primer pair NEF.SEQ and 3'TATA primer (positions 2990 to 3009 and 3756 to 3779 in plasmid p83-10 [21]). The *vif-vpr* region was analyzed by PCR with the primer pair Pol 5'FM and 6N (positions 4932 to 4964 in p83-2 and positions 213 to 237 in p83-10), and the *vpu* region was analyzed with the primer pair Tat-AUG and WS-3 (positions 88 to 107 and 801 to 820 in p83-10). The *nef*-U3 PCR fragment with an insert was introduced in a T/A cloning vector (Promega), and the sequence of multiple clones was analyzed by the *Taq* T7 Dyeprimer cycle-sequencing method (Applied Biosystems) on an Applied Biosystems 373 DNA sequencer.

Long terminal repeat (LTR)-chloramphenicol acetyltransferase (CAT) reporter constructs with either the wild-type, $\Delta 3$ mutant, or 6 \times Sp1 revertant sequences were constructed as follows. Viral DNA was PCR amplified with the upstream *XhoI*-U3 primer (CCGCTCGAGTGGGAAGGGCTAATTCAC), which places an *XhoI* restriction site (underlined) immediately upstream of the U3 region of the LTR, and the downstream U5 primer CN1. This DNA fragment was digested with *XhoI* and *HindIII* (position +77 in the R repeat region) and inserted into *XhoI*-*HindIII*-cleaved pBlue-3'LTR-CAT (33). The Tat expression vector pcDNA3-Tat and the CAT assay method were described previously (54).

The 6 \times Sp1 revertant sequence was introduced into the *nef*-U3 and $\Delta 3$ viruses as follows. First, the 6 \times Sp1 region was cloned into the 3' LTR of the *nef*-U3-

deleted 3'-half plasmid (p210-8 in reference 21) by replacement of a 216-bp 3 \times Sp1 fragment by a 255-bp 6 \times Sp1 fragment via *EcoRV* and *BspI* restriction sites in the U3-R region. Infectious virus was obtained by ligation of this 3'-half plasmid (*nef*-U3-6 \times Sp1) with a 5'-half plasmid (either the wild type or the Vpr deletion variant) as described above. Virus stocks were produced in SupT1 cells.

Cells and viruses. SupT1 cells were grown in RPMI medium containing 10% fetal calf serum and penicillin-streptomycin. Transfection of SupT1 cells by electroporation was carried out as described previously (13) with ligation mixtures that contain a total of 10 μ g of DNA. Culture supernatants containing infectious virus were harvested at the peak of infection and stored in small aliquots at -70°C . These virus stocks were carefully quantitated by CA-p24 enzyme-linked immunosorbent assay (4) and used in infections of SupT1 cells. Infections were performed in 1.5 ml of RPMI medium containing 3×10^5 cells, and virus production was monitored by measuring CA-p24 antigen production. Donor peripheral blood mononuclear cells (PBMC) were prepared, cultured, and infected with HIV-1 as previously described (4).

Long-term culturing to select for revertant viruses was initiated by massive transfection of SupT1 cells (the ligation mixture containing 5 μ g of both the 5' and 3' genome fragments was electroporated in 5×10^6 cells). In the first weeks, the cells were split when necessary. As soon as virus spread was apparent, as indicated by the presence of syncytia, the virus-containing culture supernatant was passaged onto uninfected SupT1 cells, initially with large samples of up to 1 ml and later with much smaller samples, as described previously (14).

Direct competition experiments with two virus variants were performed as described previously (34). We used equal amounts of the two viruses based on

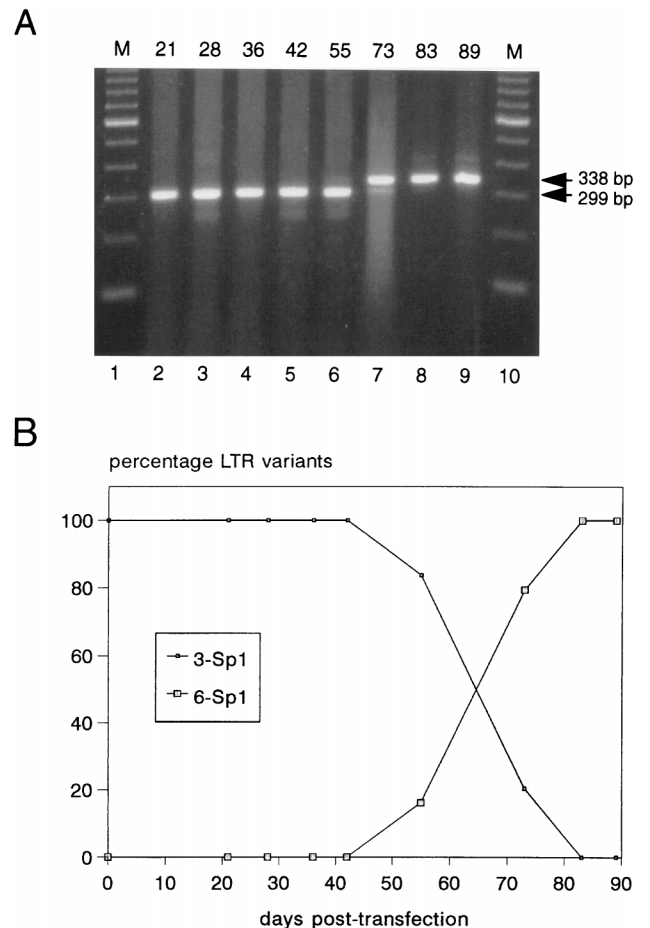


FIG. 2. The $\Delta 3$ mutant creates an LTR promoter with six Sp1 sites. (A) Cell samples taken on days 21, 28, 36, 42, 55, 73, 83, and 89 of the perpetuated SupT1 infection were used to extract cellular DNA as described previously (14), and the *nef*-LTR region of the HIV-1 genome was PCR amplified. A 299-bp fragment is produced with the $\Delta 3$ mutant template (three Sp1 sites), and a revertant fragment of 338 bp is observed (six Sp1 sites). The day of cell harvest is indicated at the top of the gel. A 100-bp DNA ladder is provided in lanes 1 and 10 (lanes labeled M). (B) Quantitation of the ethidium bromide-stained gel was performed with the Kodak digital science 1D system and used to calculate the fractions of $\Delta 3$ mutant (three Sp1 sites) and $\Delta 3$ revertant (six Sp1 sites).

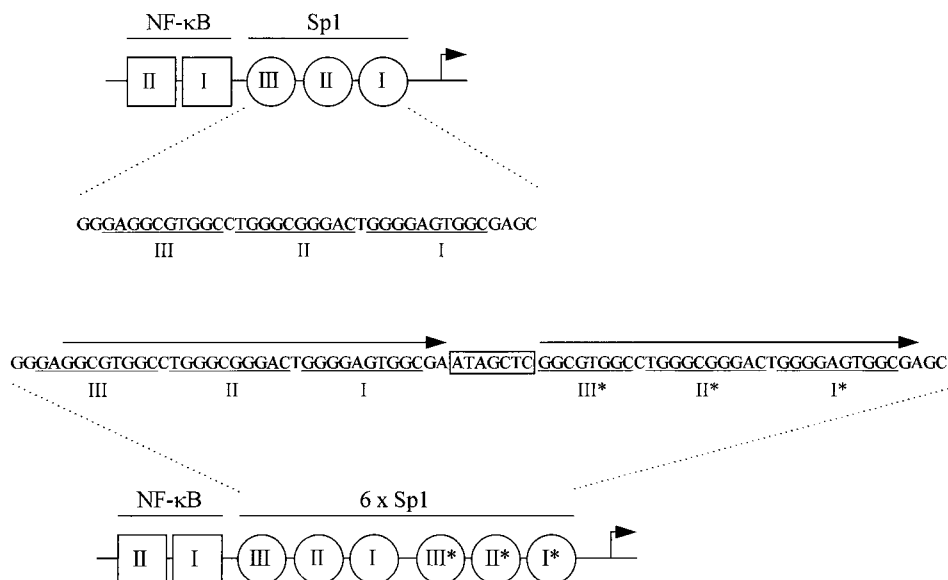


FIG. 3. Duplication of the three Sp1 sites in the LTR promoter. The wild-type LTR promoter contains two NF-κB sites (squares) at positions -105 to -96 and -91 to -82 relative to the RNA start site at $+1$ (arrow) and three Sp1 sites (circles) at positions -78 to -69 , -67 to -58 , and -56 to -47 . The $\Delta 3$ LTR carries a deletion of the upstream part of the U3 promoter region (starting at position -150). The nucleotide sequence of the three Sp1 sites is shown, with the 10-mer binding sites underlined. The lower panel shows the $\Delta 3$ revertant with the 39-nt insert. The insert consists of a 32-nt duplication (arrows) and a 7-nt sequence of unknown origin (boxed). Of the three new Sp1 motifs, the upstream site III* is a partial copy of site III, and it is therefore unknown whether site III* can bind the Sp1 factor.

the CA-p24 measurements. The compositions of the $3\times\text{Sp1}$ and $6\times\text{Sp1}$ virus mixtures were analyzed by PCR amplification of the proviral genomes to monitor the LTR length polymorphism. These data were used to calculate the relative fitness as described previously (26). In brief, the relative fitness of the $6\times\text{Sp1}$ virus was approximated from the equation $p/q = [p(0)/q(0)] \times (\text{fitness})^T$, where p is the proportion of $6\times\text{Sp1}$ virus, q is the proportion of $3\times\text{Sp1}$ virus, 0 indicates time zero, and T is the time in viral generations (2 days per generation).

RESULTS

We analyzed the in vitro replication capacity of a set of 20 HIV-1 variants with single or multiple deletions of accessory genes (*vif*, *vpr*, *vpu*, and *nef*) and of the upstream part of the U3 promoter region (21). Most variants with multiple deletions were severely replication impaired in primary human lymphocytes, e.g., *vpr-nef-U3* ($\Delta 3$), *vpr-vpu-nef*, and all viruses with the *vif* deletion (data not shown; see also reference 21). The evolutionary capacity of such crippled viruses will be severely limited because the generation of new variants depends on random mutations introduced during viral replication by the error-prone reverse transcriptase. Therefore, we tested the deleted HIV-1 strains in a human T-cell line in which the defects caused by inactivation of some of the HIV-1 accessory genes are less pronounced (51). For instance, the $\Delta 3$ vaccine strain replicated with delayed kinetics in the SupT1 T-cell line but was eventually able to produce massively infected cultures as measured by the CA-p24 levels in the culture supernatant and the appearance of syncytia (data not shown). This optimized culture system was used for the long-term evolution studies of several HIV-1 constructs with multiple deletions. Virus was passaged onto uninfected cells for 4 months to select for faster-replicating revertants, and increased virus replication was noticed in several cultures. To accurately compare the replication kinetics of viruses sampled over time, we used identical amounts of these virus samples to infect SupT1 cells. The results obtained with the samples of the $\Delta 3$ virus are shown in Fig. 1 and indicate that replication improved dramatically after approximately 2 months of culturing. A similar gain of repli-

cation capacity was observed with other HIV-1 deletion constructs (e.g., *vif-vpu* and *vif-nef-U3*) but not with all variants.

One possibility for repair of deleted gene functions is the insertion of a cellular gene with a similar function through recombination. To check whether the deletions in the HIV-1 genome were maintained, we performed PCR analyses across the deletion sites for all viruses that were cultured for 4 months. No insertions were observed for any of the virus samples, except for the $\Delta 3$ virus, in which a large insert appeared over time in the *nef*-LTR region. To analyze this process in more detail, SupT1 cell samples taken at different times were used to extract genomic DNA and to amplify the *nef*-LTR region of integrated proviruses (Fig. 2A). The 299-bp fragment predicted for the $\Delta 3$ virus was observed in the first 2 months of culturing, but a larger fragment appeared around day 55. This new fragment became the most prominent band at day 73 and completely replaced the original fragment at later times. Quantitation of the data indicated that the size variant was able to efficiently outgrow the $\Delta 3$ virus, with an increase in relative concentration from 16 to 80% in only 18 days (Fig. 2B).

The complete *Nef*-LTR region of the $\Delta 3$ revertant was sequenced (Fig. 3). Interestingly, both deletions in the *Nef* and U3 region were still present, but a 39-nt fragment was inserted in the promoter region containing the Sp1 sites that bind the constitutively expressed Sp1 transcription factor (31). The insert consists of a 32-nt duplication and a 7-nt sequence of unknown identity. It seems likely that the Sp1 region was duplicated during reverse transcription of the viral genome. Tandem repeat sequences such as the Sp1 sites are known to be subject to deletion or duplication by a slippage-realignment mechanism (42). Inspection of the nucleotide sequence of the insert clearly indicates that the whole Sp1 region was copied in a single step. The alternative, i.e., multiple rounds of duplication of a single Sp1 site, can be excluded also because no PCR products of intermediate length were observed in the evolution experiment (Fig. 2A). Thus, a novel LTR promoter configura-

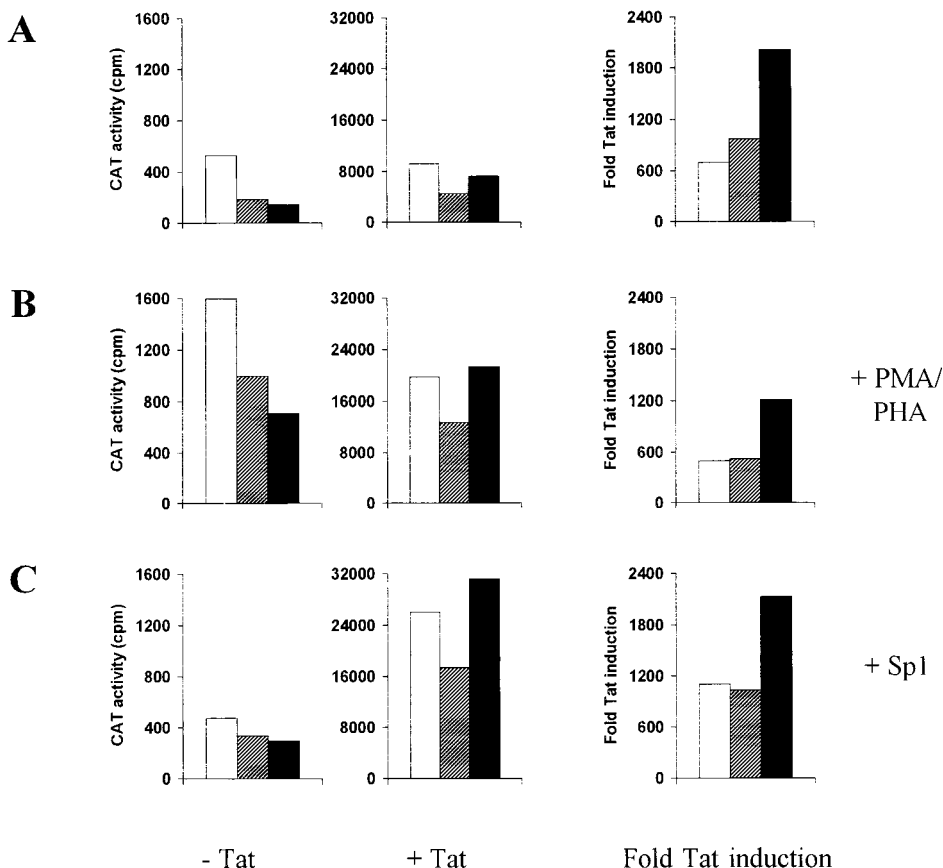


FIG. 4. The Δ3 LTR promoter gains activity by duplication of the Sp1 sites. (A) SupT1 T cells (5×10^6) were electroporated with 40 μg of LTR-CAT reporter construct (wild type [open bars], Δ3 mutant [hatched bars], and Δ3 revertant [solid bars]) in the absence of Tat (left) or with 1 μg of LTR-CAT plasmid in combination with 2.5 μg of pDNA3-Tat (middle). The cultures were harvested on day 3 for CAT assays. The fold Tat-mediated activation of LTR-transcription was calculated and is plotted (right). (B and C) Parallel transfections were performed on cells that were treated with PMA-PHA (final concentrations 25 ng/ml and 1 μg/ml, respectively) on day 1 posttransfection (B), and transfections were repeated in the presence of Sp1 expression plasmid pSVSp-1 (44a) (C). A representative experiment is shown, and similar results were obtained in four independent transfections. Furthermore, similar results were obtained in transfections with other cell types, including non-T cells. The basal and Tat-induced promoter activities cannot be compared directly because different amounts of LTR-CAT plasmid were used. When the results were corrected for this difference, an approximately 200-fold induction of LTR activity was measured.

tion consisting of two NF-κB and six Sp1 binding sites was created during replication of the Δ3 virus.

To test whether duplication of the three Sp1 motifs improved the transcriptional activity of the Δ3 promoter, we constructed a set of LTR-CAT reporter plasmids, including the wild-type LTR promoter, the Δ3 mutant lacking the upstream part of the U3 region, and the Δ3 revertant with six instead of three Sp1 sites. These plasmids were transfected into SupT1 cells in the presence or absence of a second plasmid encoding the viral Tat *trans*-activator protein. The results of a representative experiment are shown in Fig. 4A (left and middle), and the fold transcriptional activation was calculated (right). To boost the low level of basal LTR transcription, similar transfections were performed in cell cultures that were activated with phorbol myristate acetate and phytohemagglutinin (PMA-PHA) on day 1 posttransfection (Fig. 4B) and in the presence of additional Sp1 encoded by an expression plasmid (Fig. 4C). Comparison of the wild-type and Δ3 promoters indicated an approximately twofold reduction of transcriptional activity upon deletion of the upstream U3 sequences, in both the absence and presence of Tat. A further reduction of the LTR activity in the absence of Tat was measured for the Δ3 revertant with six Sp1 sites. However, Tat-activated transcription of the Δ3 revertant was improved relative to that of the Δ3 mu-

tant, and an expression level comparable to that of the wild-type LTR was reached. Similar results were obtained in cells activated with PMA-PHA and upon overexpression of Sp1. Our finding that the six Sp1 sites are beneficial only in the presence of Tat is consistent with the proposed functional interaction between the Tat and Sp1 proteins during LTR-mediated transcription (30, 39, 48).

Enhanced LTR promoter activity of the 6×Sp1 variant is consistent with the improved replication of the Δ3 revertant virus, but it cannot be excluded that other genomic changes contribute to the reversion phenotype. To unequivocally prove that the duplication of the Sp1 region is responsible for the observed fitness gain, we introduced the 6×Sp1 sites in the 3' LTR region of two molecular clones, the *nef*-U3 plasmid and the *vpr-nef*-U3 (Δ3) variant. Virus stocks were produced and used to infect SupT1 cells. We used equal amounts (based on CA-p24) of the 6×Sp1 viruses and the appropriate control viruses (Fig. 5). The contribution of the six Sp1 sites in the *nef*-U3 background is shown in Fig. 5A for three infections with a variable amount of input virus (top, 0.2 ng; middle, 1.0 ng; bottom, 5.0 ng). Introduction of the six Sp1 sites improved the replication of the *nef*-U3 virus to a level very similar to that of the wild-type control. The increase in virus replication capacity is even more prominent in the Δ3 background (Fig. 5B).

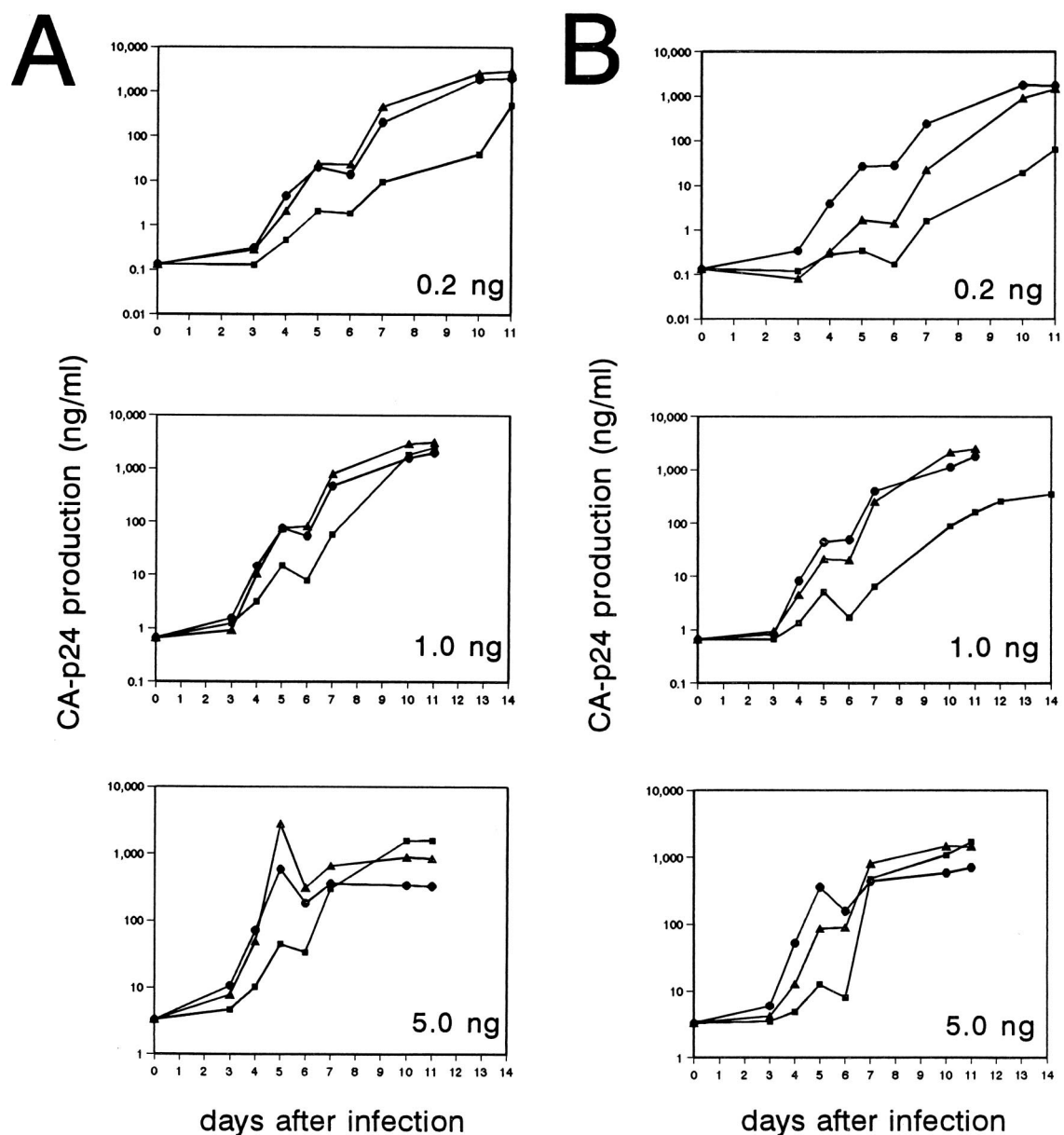


FIG. 5. The reconstituted $6\times Sp1$ virus replicates with wild-type kinetics. Virus production in SupT1 cultures after infection with wild-type virus (▲) and the *nef-U3* (■) and *nef-U3-6×Sp1* (●) variants (A) and the *vpr* single-deletion mutant (▲), the *vpr-nef-U3 Δ3* mutant (■), and the *vpr-nef-U3-6×Sp1* revertant (●) (B). The infections were performed in triplicate with different amounts of input virus: 0.2 ng of CA-p24 (top), 1.0 ng of CA-p24 (middle), and 5.0 ng of CA-p24 (bottom). Virus replication was monitored by measuring CA-p24 production in the culture supernatant. The cultures were split 1:5 at several times postinfection to sustain cell growth and virus replication; this resulted in small decreases in CA-p24 values.

In fact, the $\Delta 3$ virus with six Sp1 sites replicated faster than did the singly deleted *vpr* virus, which was included as a control. We also performed mixed-infection experiments with pairs of viruses to demonstrate the gain of fitness by duplication of the Sp1 region. Infections were initiated with equal amounts of the $6\times Sp1$ virus and the $3\times Sp1$ control, proviral samples were analyzed over time by LTR PCR amplification, and the composition of the viral mixture was determined by size separation of the LTR fragments on a gel as in Fig. 2 (data not shown). Rapid outgrowth of the $6\times Sp1$ variant was observed in both the *nef-U3* and *vpr-nef-U3 Δ3* contexts. Based on the results of this internally controlled competition experiment, we calculated a relative gain of virus fitness of 30 and 60%, respectively.

There is abundant evidence that certain LTR promoter-enhancer motifs can affect virus replication in a cell-type-specific manner (8, 9, 29, 40, 43). Although we showed a gain of fitness of the $6\times Sp1$ variant virus in the SupT1 cell line that was used for the evolution experiment, we also wanted to know whether this promoter adaptation is beneficial in primary cells. PBMC were infected with equal amounts (10 ng of CA-p24) of the $6\times Sp1$ viruses (in both the *nef-U3* and $\Delta 3$ backgrounds) and the appropriate control viruses (Fig. 6). Interestingly, the $6\times Sp1$ sites did not significantly improve replication in the *nef-U3* background (Fig. 6, left), and a small negative effect was measured in the $\Delta 3$ context. This effect was verified in more sensitive competition experiments (data not shown).

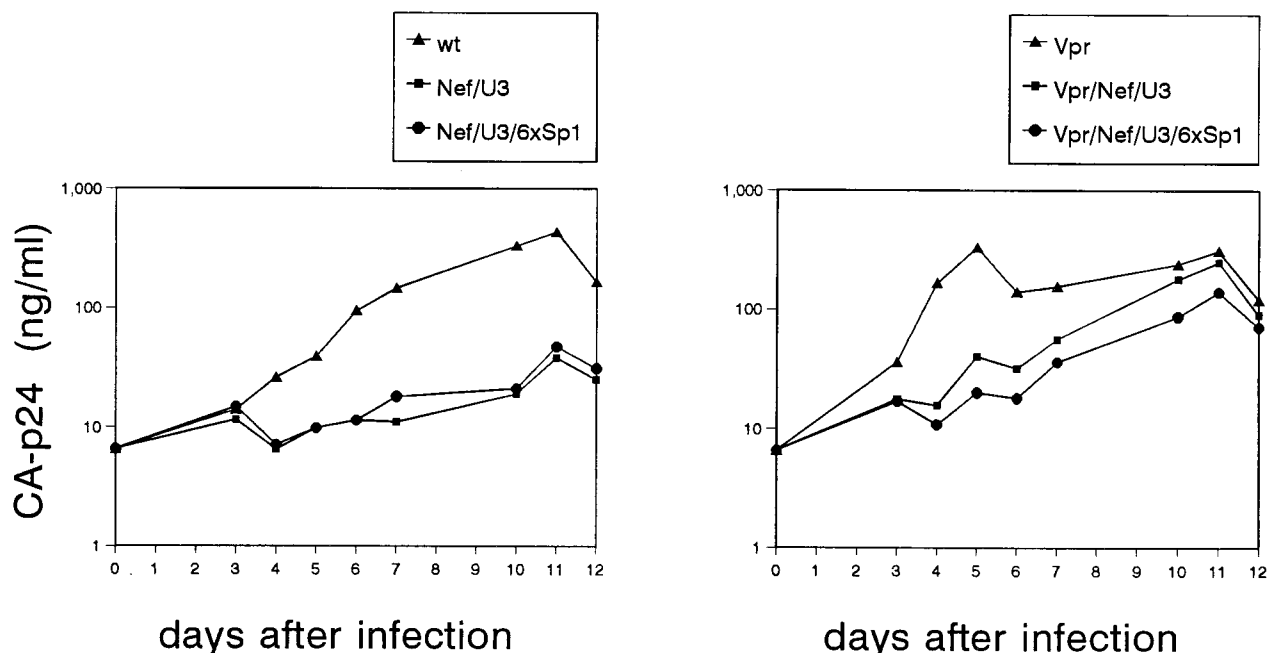


FIG. 6. The 6 \times Sp1 variant does not improve replication in primary cells. PBMC cultures were infected with the wild-type virus and the *nef*-U3 and *nef*-U3-6 \times Sp1 variants (left) and the *vpr* single deletion mutant, the *vpr*-*nef*-U3 (Δ 3) mutant, and the *vpr*-*nef*-U3-6 \times Sp1 revertant (right). Equal amounts of input virus was used (10 ng of CA-p24). Virus replication was monitored by measuring CA-p24 production in the culture supernatant.

DISCUSSION

We described a dramatic gain of fitness by the Δ 3 candidate vaccine strain (*vpr*-*nef*-U3) in prolonged tissue culture infections. In particular, this Δ 3 virus restored LTR-mediated transcription and virus replication by multiplication of the Sp1 binding sites in the core promoter. Similar replication gains were observed for other multiply deleted HIV-1 variants (e.g., *vif*-*vpu* and *vif*-*nef*-U3 [data not shown]). Although we did not analyze the latter revertant viruses in detail, they do not have the characteristic duplication of Sp1 sites that we observed for the Δ 3 revertant. Thus, HIV-1 exhibits an enormous evolutionary potential to restore replication. This may not come as a surprise, because there is ample evidence that HIV-1, which replicates as a quasispecies, is capable of overcoming a variety of selective pressures that are intended to limit its replication, including potent antiviral drugs (18). We demonstrate that replication-impaired HIV-1 variants with multiple gene deletions can improve their fitness within a relatively short culture period in an optimized in vitro system. We are obviously unable to directly translate the evolutionary potential of the Δ 3 virus as measured in tissue culture to HIV-1 infections in humans. In fact, we found that this particular LTR modification does not improve virus replication in primary cells, suggesting that we may have selected for a SupT1-specific promoter change. Other LTR promoter motifs have also been demonstrated to function in a cell-type-specific manner (9, 40, 43). Nevertheless, because many more viruses are usually replicating in the in vivo situation, it seems unavoidable that other escape routes will be found in vivo.

Although the precise correlation between replication and pathogenicity is unknown, it is likely that a virus revertant with improved fitness will also regain pathogenic potential. For instance, the *vpr*-*nef*-U3-6 \times Sp1 revertant replicates more efficiently than does the singly deleted *vpr* virus in SupT1 cells, and an SIV variant with a single Vpr gene deletion induces AIDS in rhesus monkeys (20, 28). These combined results cast

serious doubts on the safety of the current generation of multiply deleted HIV-1 vaccine strains. Most importantly, our results indicate that virus strains with multiple gene deletions can apparently restore their replication capacity without repairing the deleted gene functions. Can we mechanistically explain the reversion of a virus with deletions of the *vpr*-*nef*-U3 functions by acquisition of additional Sp1 sites in the core LTR promoter? First, the LTR-CAT transcription assays (Fig. 4) indicate that the twofold inhibition of LTR activity caused by the deletion of the upstream U3 region is compensated for by the six Sp1 sites. Multiplication of the Sp1 motifs may be particularly beneficial in SupT1 cells because these cells contain extremely low levels of the NF- κ B transcription factor (9). Second, because one role of *vpr* is to maintain the host cell in a stage of the cell cycle where viral gene expression is optimal (22), changes in the LTR motifs may also indirectly compensate for a *vpr* defect. Consistent with this idea, expansion of the Sp1 region caused a more dramatic gain of fitness in the *vpr*-*nef*-U3 mutant than in the *nef*-U3 mutant in SupT1 cells (Fig. 5). Thus, part of the reversion is likely to occur at the level of viral gene expression. The Δ 3 revertant with six Sp1 sites does not fully regain the wild-type fitness, which may be due in part to the inability to rescue the deleted Nef function.

There is some precedent for variation in the number of Sp1 binding sites in the LTR promoter of HIV-1. Several HIV-infected individuals were found to contain isolates with four Sp1 sites (34), and one natural isolate with five Sp1 sites was recently identified (44). It was shown that introduction of a fourth Sp1 site has a small but significant effect on LTR transcription and virus replication in SupT1 cells (34). Multiple Sp1 binding sites are found in various viral and cellular promoters, but the number of motifs varies widely, with up to eight sites in the cardiac α -actin gene (24). There is ample evidence for changes in host cell tropism or modulation of the viral oncogenic or pathogenic properties by variation in LTR promoter motifs in animal retroviruses (reviewed in reference 52).

For instance, a point mutation in the Moloney murine leukemia virus LTR was shown to increase transcription and enable replication in embryonal cells because of the generation of an Sp1 binding site (23). There are also numerous examples of more blatant LTR rearrangements, including deletion and duplication of motifs in different clades of HIV-1 (37). Finally, we should also mention the reversion analysis performed with enhancer mutants of the DNA virus simian virus 40. Similar to our results, duplication of existing elements was the predominant mechanism for regaining promoter function (27). This apparent evolutionary flexibility of eukaryotic promoters is largely due to the modular architecture of these elements (19).

These *in vitro* studies demonstrate that multiply deleted HIV-1 strains are genetically unstable and therefore potentially unsafe. At the same time, these *in vitro* observations may also guide us toward the construction of improved HIV-1 vaccine candidates. Removal of all accessory genes from HIV-1 creates a replication-incompetent virus that will be useless as a vaccine. However, the $\Delta 3$ revertant virus described in this study may allow the removal of two additional genes (*vpu* and *vif*) without leading to complete loss of replication capacity. Subsequently, another round of *in vitro* evolution can be used to optimize the replication capacity of this $\Delta 5$ virus. Thus, repeated cycles of gene deletion and optimization of replication by means of forced evolution in tissue culture are proposed to generate a replication-competent version of HIV-1 that encodes only the basic set of retroviral proteins (Gag, Pol, Env, and perhaps Tat and Rev). This strategy may allow one to convert the complex HIV-1 genome into the form of a simple retrovirus, a vaccine approach that was originally proposed by Temin (50). This study indicates that such an evolutionary strategy should ideally be performed with primary cells, otherwise the selected variants may have an unpredictable *in vivo* replication phenotype. Other safety features can be added to such a mini-HIV backbone. For instance, insertion of the herpes simplex virus thymidine kinase gene will allow the elimination of cells carrying proviruses by treatment with ganciclovir (46). It remains to be tested whether such HIV-1 variants have lost their pathogenicity and whether humans can mount a response that protects against wild-type HIV-1 infection. Besides its use as a live, attenuated virus vaccine, the mini-HIV construct could be used as inactivated virus vaccine.

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