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HIV-1 Evolves into a Nonsyncytium-Inducing Virus upon Prolonged Culture *in Vitro*

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HIV-1 LAI is a syncytium-inducing (SI) virus with a broad host cell range. We previously isolated a LAI variant that improved replication in the SupT1 T cell line due to mutations within the C1 and C4 constant regions of the Env protein. We now report that this variant exhibits a severely restricted host cell range, as replication in other T cell lines and primary cells was abolished. Several Env-mediated functions were analyzed to provide a mechanistic explanation for this selective adaptation. The change in host cell tropism was not caused by a switch to a SupT1-specific coreceptor. Biosynthesis of the variant Env glycoprotein was not improved in SupT1 cells, and in fact a small defect in intracellular Env processing was observed. SupT1 infection assays did not reveal an improved Env function either, and a dramatic loss of infectivity was measured with other cell types. The Env-mutated HIV-1 reached an approximately fivefold higher level of virus production in SupT1 cells at the peak of infection. Unlike the LAI virus, the variant did not trigger the formation of syncytia. Our combined results suggest that the HIV-1 variant allows the infected host cell to survive longer, thus producing more viral progeny. The intricate virus-cell interaction results in a balance between optimal virus replication and host cell survival, causing a cytopathic SI isolate to evolve toward a nonsyncytium-inducing (NSI) phenotype in cell culture. These findings may help explain the absence of SI variants in the initial phase of HIV-1 infection, and the results dispute the notion that HIV-1 evolution should always go from the NSI to SI phenotype.

INTRODUCTION

Primary isolates of the human immunodeficiency virus type 1 (HIV-1) consist of a complex, heterogeneous population, or so-called quasispecies (Domingo and Holland, 1997). The majority of isolates from infected asymptomatic patients replicate in macrophages, display a nonsyncytium-inducing (NSI) phenotype and generally fail to infect established $CD4^+$ T cell lines. Primary isolates that can infect and induce syncytia (SI) in the MT2 T cell line can be isolated from a significant percentage of patients that have progressed to AIDS (De Jong *et al.,* 1992; Koot *et al.,* 1992). HIV-1 uses the CD4 molecule as receptor, but coreceptors are also necessary for infection. Recent studies have indicated that the host cell tropism of HIV-1 variants reflects the ability of the viral Env protein to bind to a specific member of the chemokine receptor family. Most macrophage-tropic isolates use the CCR5 receptor, whereas the T-cell-tropic viruses employ the CXCR4 receptor (Bates, 1996; Chesebro *et al.,* 1996; Doms and Peiper, 1997). An important difference between these two systems is that HIV infection of $CD4^+$ T cells *in vitro* leads to extensive cell death, whereas HIV infection of monocytes/macrophages produces a more limited cytopathicity. The dominant killing pathway for SI viruses is syncytia-induced apoptosis, whereas NSI variants may kill host cells by an alternative mechanism (Cao *et al.,* 1996; Kolesnitchenko *et al.,* 1997). The HIV-1 cell tropism is not a fixed property and can change *in vivo* or upon passaging of the virus in cell culture. The NSI to SI shift that is observed in some patients usually coincides with a change in cell tropism and often involves amino acid substitutions in the variable V3 loop of the Env subunit gp120 (De Jong *et al.,* 1992). Interestingly, an increase in positive charge of this Env domain is usually observed that may correlate with the negative charge of CXCR4 compared with the CCR5 coreceptor (Jiang, 1997; Berkhout and Das, 1998). There are other Env determinants of cell tropism however, and it is clear that the virus-cell interaction is complex and conformational in nature (Freed and Martin, 1995) with the participation of multiple regions in the viral Env protein and the cellular CD4 and coreceptor molecules.

Passage of HIV-1 in cell lines will affect the biological properties of the virus. Cell culture will select certain phenotypes from the quasispecies population. For example, positive selection pressure will favor variants that are better able to bind to and enter the target cells, and negative pressure will eliminate variants that are excessively cytopathic. Prolonged culture will allow adaptation of the virus to the particular cell line used in that experiment. Such laboratory-adapted isolates can differ substantially from their progenitor patient viruses. The adapted viruses usually grow in an expanded repertoire of leukemic T cell lines, use the CXCR4 coreceptor, and

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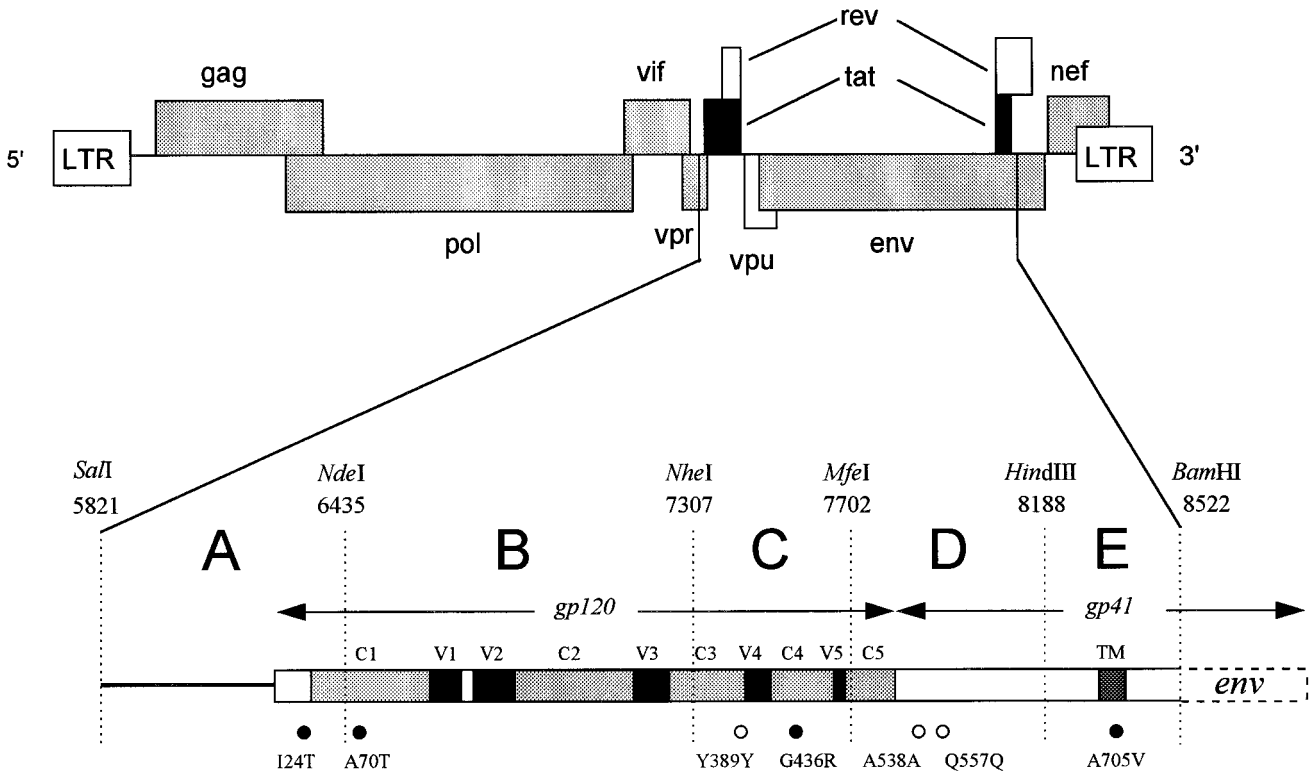

FIG. 1. Env mutations that improve replication of LAI and LAI-derived viruses on SupT1 cells. The complete *env* coding region is shown with the conserved (C) and variable (V) regions of gp120 and the transmembrane domain (TM) of gp41. Silent (open circles) and nonsilent (filled circles) mutations in the *Sal*I-*Bam*HI fragment of the mutant Env protein are indicated. The mutations were named by appending the amino acid (one-letter code) present in the LAI Env protein, the residue number, and the amino acid present in the Env revertant. The corresponding nucleotide changes are: I24T, T→C at nucleotide position 6327 (silent mutation in the *vpu* gene); A70T, G→A at 6464; Y389Y, C→T at 7423; G436R, G→A at 7562; A538A, G→A at 7870; Q557Q, G→A at 7927; A705V, C→T at 8370.

induce syncytia (Peden *et al.,* 1991; Moore and Ho, 1995; Kozak *et al.,* 1997). In general, laboratory-adapted isolates appear to be more sensitive to neutralization by a variety of reagents, including soluble CD4, anti-Env monoclonal antibodies, and sera of HIV-1-infected individuals (Daar *et al.,* 1990; Moore *et al.,* 1995; Sullivan *et al.,* 1995; Wrin *et al.,* 1995; Zhang *et al.,* 1997). Although it is evident that some questions should not be addressed with laboratory-adapted strains (e.g., vaccine tests with neutralizing antibodies), detailed analysis of such viruses may help us understand the complex interplay of the HIV-1 Env protein with multiple cell receptors in processes of virus infection and cell fusion. In this cell culture adaptation study, we present evidence that an SI virus evolves into a more fit virus with an NSI phenotype. The results suggest that HIV-1 virion production is optimized indirectly through longer survival of the cells that are infected with the adapted NSI variant.

RESULTS

The SupT1-adapted HIV-1 LAI variant

By long-term cultivation of a replication-impaired HIV-1 mutant with a translation defect (mLAI) on SupT1 cells, we previously obtained a variant Env protein that improved viral replication in these cells (Das *et al.,* 1998). This variant Env protein contains four amino acid changes (indicated as closed circles in Fig. 1) and improved replication of the wild-type LAI virus (Fig. 2, top left, compare LAI and LAI^{env}, see also Das *et al.,* 1998). Although the gain of replication capacity is relatively small, we observed the same pattern in multiple independent transfections. The increase in replication capacity is more obvious when the variant Env gene is present in the poorly replicating mLAI mutant (Fig. 2, top left, compare mLAI and mLAI^{env}). The SupT1-adapted Env fragment was split into five subfragments (Fig. 1, fragments A–E) that were introduced individually into the mLAI genome. The replication of this set of viruses was tested in SupT1 cells (Fig. 2, top right). Fragment C with the G436R mutation improved replication profoundly. The B fragment with the A70T mutation also improved virus replication but to a lesser extent. The other fragments did not improve viral replication. In summary, these results indicate that two Env amino acid changes in the C1 and C4 domains are responsible for the increased replication of HIV-1 in SupT1 cells.

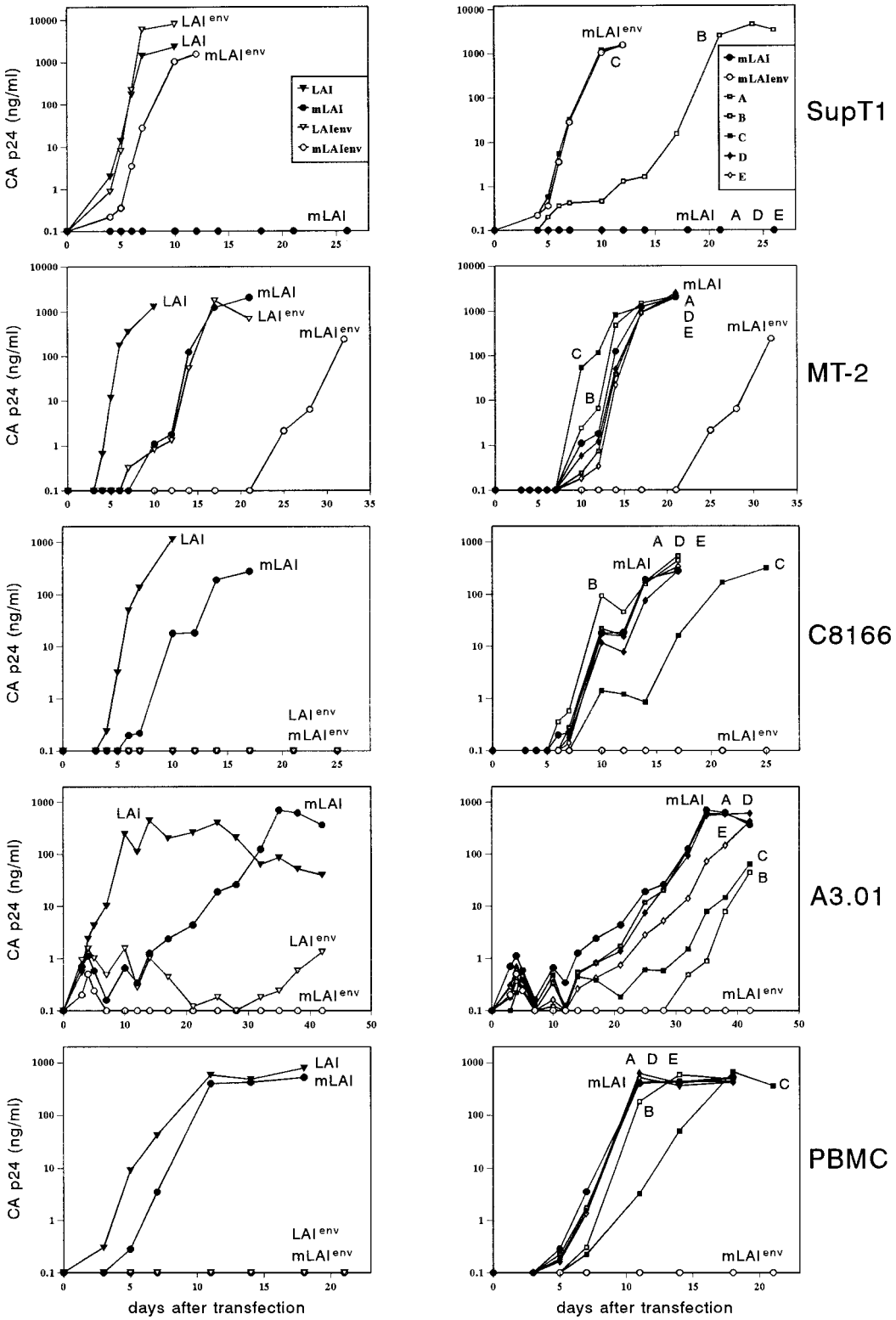

FIG. 2. Env mutations that stimulate replication in SupT1 cells reduce replication in other cell types. Proviral clones with a wild-type *env* gene (LAI and mLAI) or the mutant *env* gene (LAI^{env} and mLAI^{env}) were transfected into different cell types (left). The revertant env fragment was split into five subfragments (Fig.1, fragments A–E) that were used to replace the corresponding sequences in the mLAI mutant. Replication of these clones (A–E) was assayed by transfection of different cell types (right). Note that the top right panel has been published as part of the preceding study in which the variant Env function was selected (Das *et al.,* 1998). We included this graph for comparison. DNA was transfected into the T-cell lines SupT1, MT-2, C8166 (all 1 μg) and A3.01 (2.5 μg), and primary PBMC (5 μg). CA-p24 levels were measured in the culture supernatant at several days posttransfection.

TABLE 1

Replication of Wild-Type and Env-Mutated HIV-1 in Different Cells

Virus cell	SupT1	MT-2	C8166	A3.01	PBMC
LAI	++++	++++	++++	++++	++++
LAI^{env}	++++	++	−	+	−
mLAI	−	++	++	++	++
mLAI^{env}	+++	+	−	−	−
mLAI^{envA}	−	++	++	++	++
mLAI^{envB}	+	++/+++	+++	+	+
mLAI^{envC}	+++	+++	+	+	+
mLAI^{envD}	−	++	++	++	++
mLAI^{envE}	−	++	++	+/++	++

Adaptation of HIV-1 Env to SupT1 cells leads to reduced replication in other cell types

We assayed the effect of the modified Env protein on viral replication in other T-cell lines (MT-2, C8166, A3.01) and peripheral blood mononuclear cells (PBMC). Replication of the LAI and mLAI viruses with the wild-type and variant Env fragment was compared (Fig. 2, left). The mLAI mutant demonstrated a partial replication defect in all these cells. Surprisingly, the new Env fragment further reduced the replication capacity of this virus. Moreover, this Env segment also strongly interfered with replication of the LAI virus. This inhibitory effect of the modified Env protein in these cells contrasts with the stimulatory effect observed in SupT1 cells. The cell-type-dependent effects we observed for the complete Env fragment are summarized in Table 1.

We also tested the A–E subfragments of the Env protein for their effect on replication of the mLAI virus in different cell types (Fig. 2, right). The mutant subfragment C, which was responsible for the major improvement of replication in SupT1 cells, also stimulated replication in MT-2 cells but reduced replication in C8166, A3.01, and PBMC. The mutant subfragment B, which demonstrated a minor stimulatory effect in SupT1 cells, stimulated replication in MT-2 and C8166 but reduced replication in A3.01 and PBMC. The other subfragments (A, D, and E) demonstrated no effect on HIV-1 replication in all cell types, with one exception. Subfragment E moderately inhibited replication in A3.01 cells. These rather complicated phenotypes of the Env subdomains are summarized in Table 1. Despite the stimulatory effect of the B and C subfragments in some cell types, the combined *env* mutations reduced replication in all non-SupT1 cells. Thus these modified Env residues represent adaptations of HIV-1 for optimal replication in SupT1 cells, and the combined mutations severely reduce the capacity to replicate in other T-cell lines and primary T cells. Interestingly, whereas only one mutation seems largely responsible for improved replication in SupT1 cells (subfragment C), several mutations seem required for a complete loss of infectivity on the other cell lines. Although speculative, these differences may be relevant for discerning the mechanism(s) behind each phenotype.

Env mutations do not change the coreceptor usage

The T-cell tropic LAI virus uses the CXCR4 protein as coreceptor for infection. Because the Env mutations may have resulted in the adaptation to another coreceptor that is specific for the SupT1 cell line, we tested the coreceptor usage of LAI^{env}. We first assayed the ability of LAI^{env} to replicate in a set of CD4-positive human osteosarcoma (HOS) cells expressing different chemokine receptors (CCR2b, CCR3, CCR4, CCR5, and CXCR4) (Deng *et al.,* 1996). However, this approach failed because LAI^{env} did not replicate in any of these cells, including the HOS-CXCR4 cell line. Apparently, the Env mutations interfere with virus replication in HOS cells, similar to the effects seen in PBMC and all T cell lines other than SupT1 (Fig. 2). We therefore used an alternative approach to test whether a coreceptor switch had taken place in LAI^{env}. The chemokine SDF-1, the natural ligand of the CXCR4 receptor, can inhibit replication of CXCR4-dependent HIV-1 isolates (Bleul *et al.,* 1996; Oberlin *et al.,* 1996). We examined the effect of SDF-1 on the replication of LAI and LAI^{env} virus. SupT1 cells were infected with two different amounts of these viruses in the presence or absence of 100 nM SDF-1, and virus production was measured after 6 days. In the absence of SDF-1, increased virus production was observed for LAI^{env} compared with LAI (compare Figs. 3A and 3B for the 5 ng infection and Figs. 3C and 3D for the 0.5 ng infection; note the differences in CA-p24 values plotted on the *y* axes). This observation is consistent with the previous results in SupT1 cells (Fig. 2). As expected, infection and replication of the wild-type LAI virus was strongly inhibited by SDF-1 (Figs. 3A and 3C), but a very similar pattern was observed for LAI^{env} (Figs. 3B and 3D). In a control experiment, CCR5-using primary isolates were not found to be sensitive to this CXCR4-specific chemokine (results not shown). This result strongly suggests that both LAI and LAI^{env} viruses use the CXCR4 coreceptor for infection.

Env mutations affect Env biosynthesis

The efficiency of Env protein synthesis may differ significantly among different cell types (Koken and Berkhout, 1994; Shahabuddin *et al.,* 1996), and we previously reported that intracellular trafficking of the wild-type LAI Env protein is inefficient in SupT1 cells (Koken and Berkhout, 1994). This raised the possibility that the observed Env adaptation may improve this process, which should result in enhanced Env production. We therefore examined the effect of the mutations on Env protein synthesis. First, Env protein was expressed upon transient transfection of the CD4$^-$ C33A cervix carcinoma cell line and

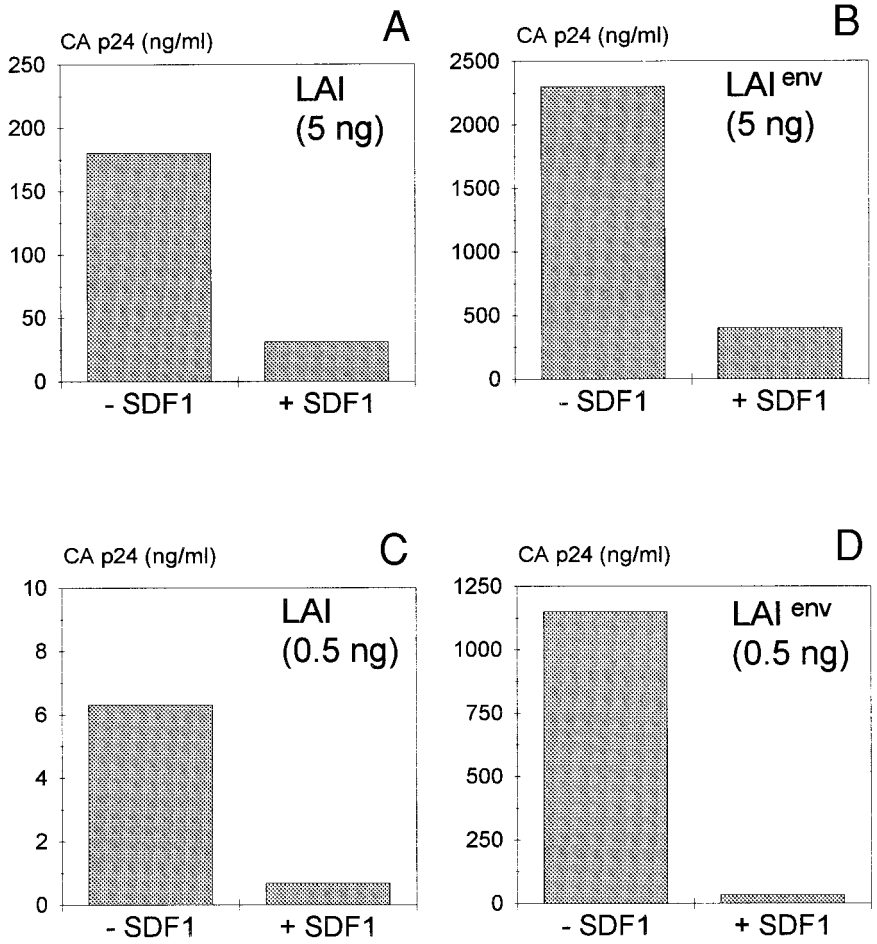

FIG. 3. LAI and LAI^{env} are similarly inhibited by the chemokine SDF-1. SupT1 cells were infected with equal amounts of LAI or LAI^{env} virus (5 or 0.5 ng CA-p24) in the presence or absence of 100 nM SDF-1 1–67 (Bleul *et al.,* 1996; Oberlin *et al.,* 1996). CA-p24 production was measured in the culture supernatant at 6 days after infection.

analyzed by Western blotting with a monoclonal antibody that detects both gp160 precursor and processed gp120 Env proteins (Fig. 4). The wild-type LAI and translation-impaired mLAI constructs, both expressing the wild-type Env protein, demonstrated a gp120 signal that is more intense than the gp160 precursor band. In contrast, the ratio of the two Env forms was reversed for the LAI^{env} and mLAI^{env} constructs. These results suggest that processing of the gp160 precursor in these non-T cells was affected by the Env mutations. Despite this difference in gp160 processing, Western-blot analysis of virion particles showed a similar gp120 content of wild-type and mutant virions (not shown).

To follow the fate of the wild-type and variant Env molecules in SupT1 cells, we performed pulse–chase labeling experiments. SupT1 cells were transfected with pLAI or pLAI^{env} and cultured for 2 days. The cells were pulse-labeled with [^{35}S]methionine/cysteine for 30 min and subsequently chased by addition of an excess of unlabeled amino acids. At various times (0–16 h), cell and culture medium samples were taken. The viral proteins in both fractions were immunoprecipitated with polyclonal antibodies present in the serum of an HIV-1-infected individual and analyzed by reducing denaturing

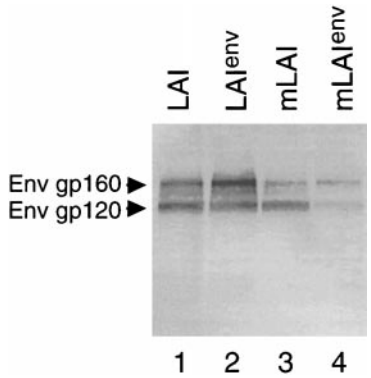

FIG. 4. Western blot analysis of Env proteins produced in C33A cells. C33A cells were transfected with wild-type (LAI) and mutant proviral constructs (lane 1, LAI; lane 2, LAI^{env}; lane 3, mLAI; lane 4, mLAI^{env}). At 3 days posttransfection, total cellular extracts were prepared and analyzed. Env gp160 and gp120 proteins were identified with an anti-Env MAb.

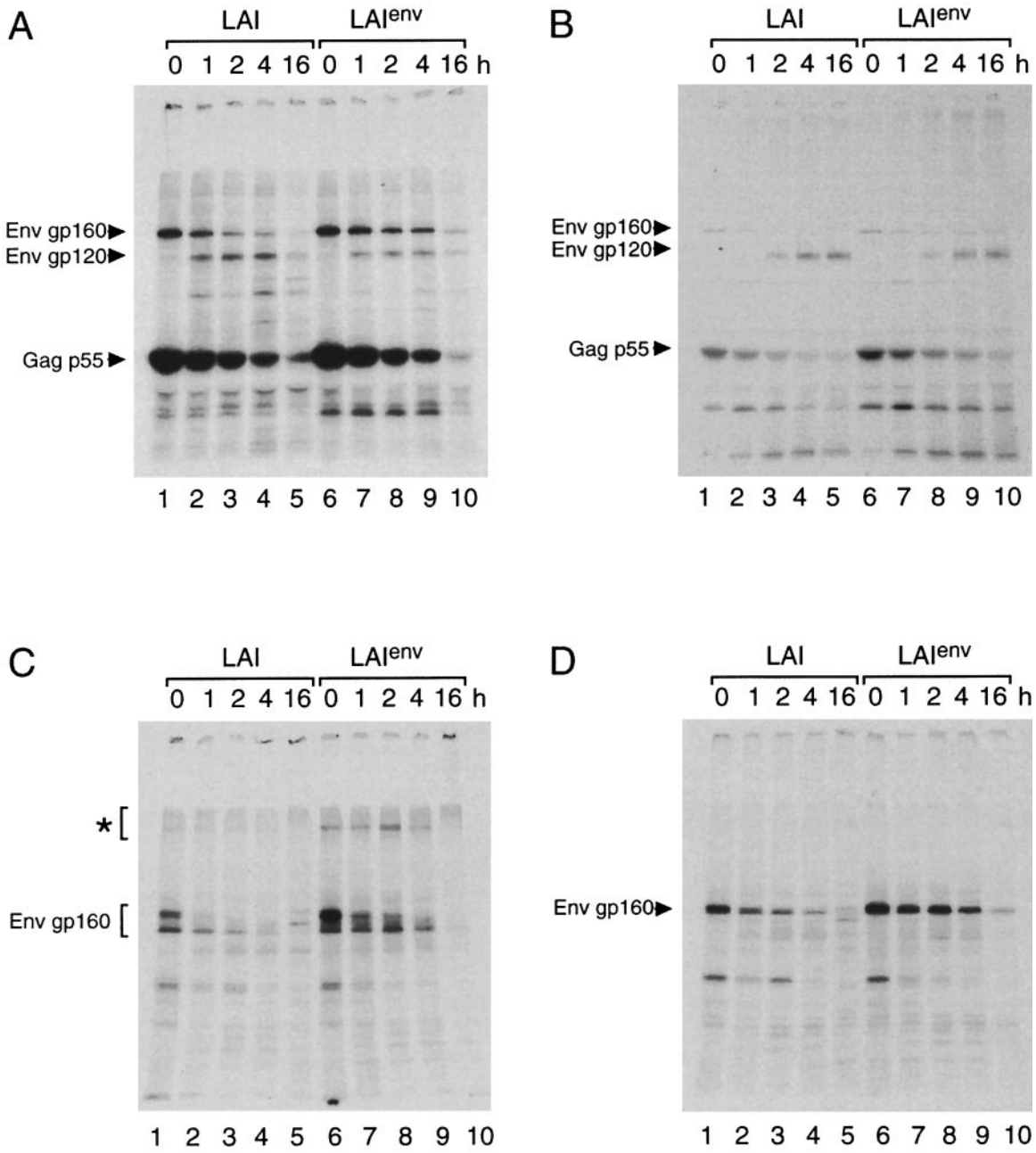

FIG. 5. Metabolic labeling of viral proteins. Two days after transfection of SupT1 cells with LAI or LAI^{env} proviral constructs, cells were pulse-labeled with [^{35}S]methionine/cysteine for 30 min, and labeled protein was chased by adding an excess of unlabeled amino acids. At various times (0–16 h), cell and culture medium samples were taken. (A and B) Viral proteins in both fractions were immunoprecipitated with polyclonal antibodies present in the serum of an HIV-1-infected individual and analyzed by reducing SDS–PAGE [(A) cell samples; (B) culture medium samples]. The position of the gp160 precursor and the processed gp120 Env molecules, and the Gag p55 protein are indicated. (C and D) Cellular Env gp160 protein was immunoprecipitated with a gp160-specific rabbit antiserum, deglycosylated by treatment with endoglycosidase F as described underMaterials and Methods, and analyzed via nonreducing (C) or reducing SDS–PAGE (D). In (C), Env gp160 aggregates are indicated with an asterisk (*).

polyacrylamide gel electrophoresis (Figs. 5A and 5B for the cell and supernatant samples, respectively). Directly after pulse-labeling, a similar Env gp160 level was observed in the cells transfected with the wild-type LAI and the mutant LAI^{env} construct (Fig. 5A, lanes 1 and 6). Due to processing of this gp160 precursor into gp120 and gp41, Env gp120 molecules were detectable at later time points. The majority of LAI gp160 was cleaved into gp120 already after a chase period of 2 h, but uncleaved LAI^{env} gp160 remained the most prominent form for ≥4 h. Apparently, processing of the wild-type precursor was more efficient than processing of the mutant protein. Processing of the Gag p55 protein proceeded at a similar rate for both viruses, indicating that processing of the viral pro-

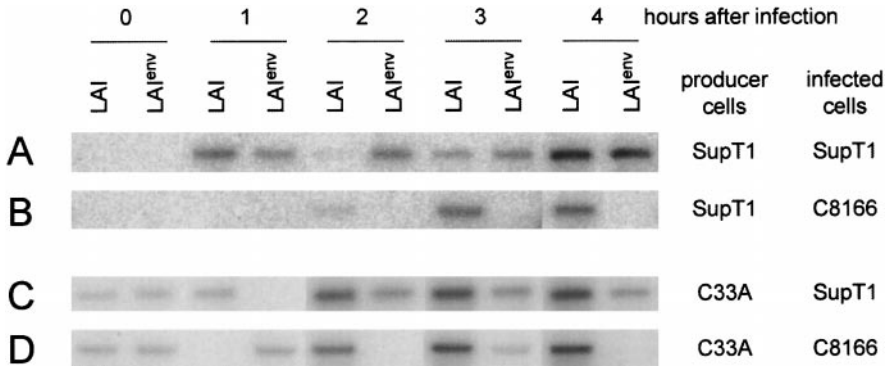

FIG. 6. Env mutations do not stimulate infection of SupT1 cells and abolish infection of C8166 cells. Equal amounts of LAI and LAI^{env} virions produced in SupT1 (A and B) or C33A cells (C and D) were used to infect SupT1 (A and C) or C8166 cells (B and D). At different times (0–4 h) postinfection, reverse transcription products were detected by PCR and visualized on a Southern blot probed with a ^{32}P-labeled HIV-1 probe. Hybridization signals were quantitated with a PhosporImager and are presented in Fig. 7.

teins was not affected generally for the Env-mutated construct. Although the rate of processing of Env protein was slower in LAI^{env}, a similar amount of Env gp120 ended up in the culture medium for the wild-type and mutant constructs (Fig. 5B). This result indicates that assembly of Env-containing virions was not significantly affected by the mutations in LAI^{env}. We also quantitated the Gag p55 and Env gp120 signals to determine the ratio of the two viral proteins in virions (not shown). We measured a similar ratio for the wild-type and mutant virus samples, indicating that these virion particles have the same composition.

To investigate in more detail the biosynthetic defect of the mutant Env protein, we analyzed the formation of disulfide bridges in the gp160 protein. This assay is a measure for the folding kinetics of the Env protein. To follow disulfide bond formation in newly synthesized Env protein, the immunoprecipitates first were treated with endoglycosidase F to increase the electrophoretic mobility of the protein and then analyzed under nonreducing conditions (Braakman *et al.,* manuscript in preparation). Newly synthesized Env protein has fewer disulfide bonds than the mature, folded Env protein, and this more reduced conformation has a lower electrophoretic mobility because it is less compact. Cell lysates were immunoprecipitated with an Env-specific rabbit antiserum that recognizes early forms of gp160 particularly well. The samples were analyzed with SDS–PAGE under nonreducing and reducing conditions (Figs. 5C and 5D, respectively). The nonreducing gel indicates that two Env forms are predominantly present in the cell lysate of LAI immediately after the pulse (Fig. 5C, lane 1). These forms represent a relatively reduced, unfolded Env protein and a relatively mature Env form that contains disulfide bonds. The more reduced Env form was more prominent in the LAI^{env} sample immediately after the pulse (Fig. 5C, lane 6). Within a 1-h chase, the more reduced form of gp160 (LAI, lane 2) had disappeared almost completely and only the oxidized form of gp160 was visible. In contrast, for LAI^{env} the reduced form of gp160 was still detectable after ≥2 h of chase (lane 8), indicating that disulfide bond formation was slower in LAI^{env} gp160 than in LAI gp160. Under reducing conditions, the two Env forms comigrated as reduced gp160 (Fig. 5D). Again, reduced processing of the LAI^{env} gp160 is apparent from the decrease of the gp160 signal. Note that gp120 is not recognized by the antibody used in this experiment. In summary, formation of disulfide bonds, and therefore folding of gp160, in addition to processing into gp120 and gp41 was less efficient for the mutant Env than for the wild-type protein. This phenomenon was observed both in SupT1 and C33A cells.

The mutant Env protein abolishes infection of non-SupT1 cells but does not improve SupT1 infection

Because the Env protein mediates the binding of the virion to the host-cell receptors, we next examined the effect of the Env mutations on virus infectivity. Virions produced in SupT1 cells were harvested and quantitated by CA-p24 ELISA. Equal amounts of LAI and LAI^{env} virions were used to infect SupT1 and C8166 cells. Infection of these cells was monitored by detection of reverse transcription products at different times (0–4 h) postinfection. With SupT1 cells, cDNA products first were detected 1 h after infection by the wild-type virus and increased levels were observed over time (Fig. 6A, quantitated signals are shown in Fig. 7A). Although considerable variation was observed for the different time samples, we measured no significant difference for the two HIV-1 variants. In contrast, infection of the C8166 cells yielded only cDNA products for the wild-type LAI virus (Figs. 6B and 7B). Even at 4 h p.i., no cDNA products could be detected for the mutant LAI^{env}. Apparently, the mutant virions cannot infect C8166 cells, which explains the observed replication defect of the LAI^{env} and mLAI^{env} viruses on these cells (Fig. 2). Thus whereas the Env

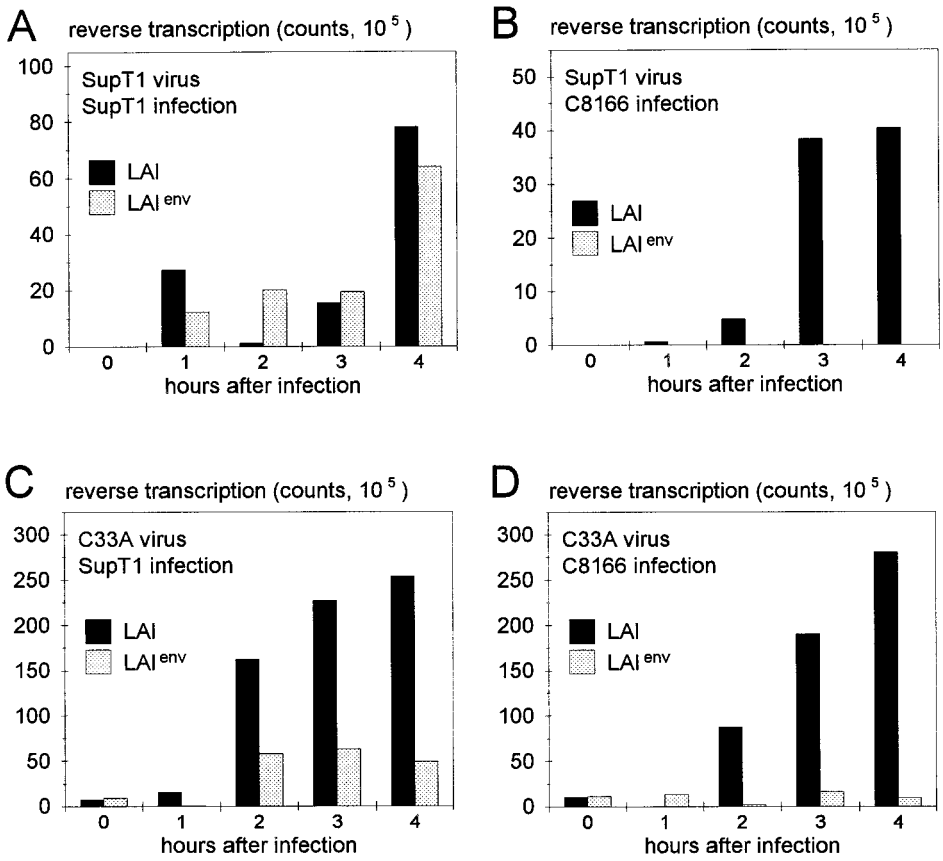

FIG. 7. The effect of the Env mutations on infection. See legend to Fig. 6 for details.

mutations do not affect infection of SupT1 cells, they abolish infection of C8166 cells.

We also assayed the infectivity of virions produced by C33A non-T cells. Upon infection of C8166 cells with the C33A-produced virions, strongly reduced cDNA production was measured for LAI^{env} mutant (Figs. 6D and 7D). Thus the Env mutations significantly reduced infection of C8166 cells, both for virions produced in T cells (SupT1) and non-T cells (C33A). Upon infection of SupT1 cells, reduced cDNA production was observed for the LAI^{env} mutant compared with the LAI wild-type virus (Figs. 6C and 7C). Thus SupT1 infectivity of the C33A-produced virions is reduced by the Env mutations. Because such an effect was not observed with SupT1-produced virions (Figs. 6A and 7A), this inhibitory effect is apparently producer-cell specific.

Reduced syncytium-inducing capacity of the mutant Env protein

So far, we investigated several Env-mediated steps of the viral replication cycle and found that the Env mutations reduced folding and processing of gp160 in both SupT1 and C33A cells. Furthermore the Env mutations abolished infection of C8166 cells and reduced infection of SupT1 cells when virions were produced in C33A cells. These defects can explain the reduced replication of Env-mutated virus on cells other than SupT1, but there is no apparent explanation for the improved replication on SupT1 cells. Both the assembly of SupT1-produced virions and the infection of SupT1 cells by these virions were not affected by the Env mutations.

The HIV-1 LAI virus is able to induce syncytia, and infected SupT1 cells readily fuse to form multinucleated cells. Thus the virus can spread in the culture not only by the infection route but also by this cell-fusion route (Sato *et al.,* 1992). When culturing LAI and LAI^{env} viruses in SupT1 cells, we consistently observed that LAI^{env} produced approximately fivefold more progeny than LAI at the peak of infection (Fig. 2, and results not shown). In fact, examination of these cultures revealed the presence of large syncytia in the LAI cultures and the absence of such large multinucleated cells in LAI^{env}-infected cultures. We therefore examined the effect of the Env mutations on cell fusion in single-cycle assays.

SupT1 cells that express wild-type or mutant Env protein and all other HIV-1 proteins (including Tat) were produced by transfection with pLAI or pLAI^{env}, respectively. Two days after transfection, cells and supernatant were separated, and each was mixed with SupT1 cells containing a Tat-responsive LTR–CAT reporter gene con-

struct. Upon fusion of Tat-expressing cells with these reporter cells, the LTR promoter will be activated and CAT enzyme will be produced. Formation of syncytia was analyzed by light microscopy after 24 h and quantitated by measurement of the CAT activity in cell extracts (Fig. 8A). The cells expressing the wild-type Env protein fused efficiently with the reporter cells, resulting in high CAT levels. In contrast, for the cells expressing mutant Env, no syncytia were observed and a low CAT level was measured. Mixing the reporter cells with the virus-containing supernatant did not result in CAT production, indicating that transmission of the Tat protein indeed was mediated by cell fusion and not by virus infection.

In an alternative assay, SupT1 cells that express the HIV-1 Tat protein and either the wild-type or mutant Env protein were produced by transfection with $pLAI_{\Delta gagpol}$ or $pLAI^{env}_{\Delta gagpol}$. These proviral constructs lack the gag/pol genes and do not produce virions. These cells were mixed with LTR–CAT-containing reporter cells. Syncytium formation was assayed after 24 and 48 h by light microscopy and by measuring the induction of CAT activity (Figs. 8B and 8C). Syncytium formation was not detected with the Env-mutated construct, whereas cells expressing wild-type Env produced large syncytia. Accordingly, high CAT activity levels were observed for the wild-type Env constructs, whereas at least a 10-fold drop in activity was measured for the mutant Env constructs. These combined results indicate that the Env mutations abolish cell-fusion, which explains the absence of syncytia in LAI^{env}-infected cultures.

DISCUSSION

In this study, we analyzed in detail a variant of the HIV-1 LAI virus that was obtained upon prolonged culture of a poorly replicating LAI mutant in the SupT1 T cell line (Das *et al.,* 1998). Mutation of two highly conserved amino acids within the C1 and C4 domain of the Env protein was found to be responsible for the improved replication in SupT1 cells. Interestingly, this adaptation to the SupT1 T cell line coincided with a loss of infectivity in other T cell lines and primary cells. The restriction of the host range was not caused by a switch in coreceptor usage, as both the LAI virus and the LAI^{env} variant were sensitive to inhibition by the CXCR4-specific chemokine SDF-1. To identify which step of the viral replication cycle was improved by the modified Env molecule, we analyzed Env protein biosynthesis and Env-mediated virus infection. Surprisingly neither Env function was found to be improved in SupT1 cells, and even minor defects were apparent for the mutant Env protein. Such defects can explain the inability of this virus to replicate on other cell types but do not explain the improved replication of the LAI^{env} virus on SupT1 cells. The mutant Env protein was found to exhibit a gross defect in mediating cell fusion and the induction of large multinucleated syncytia.

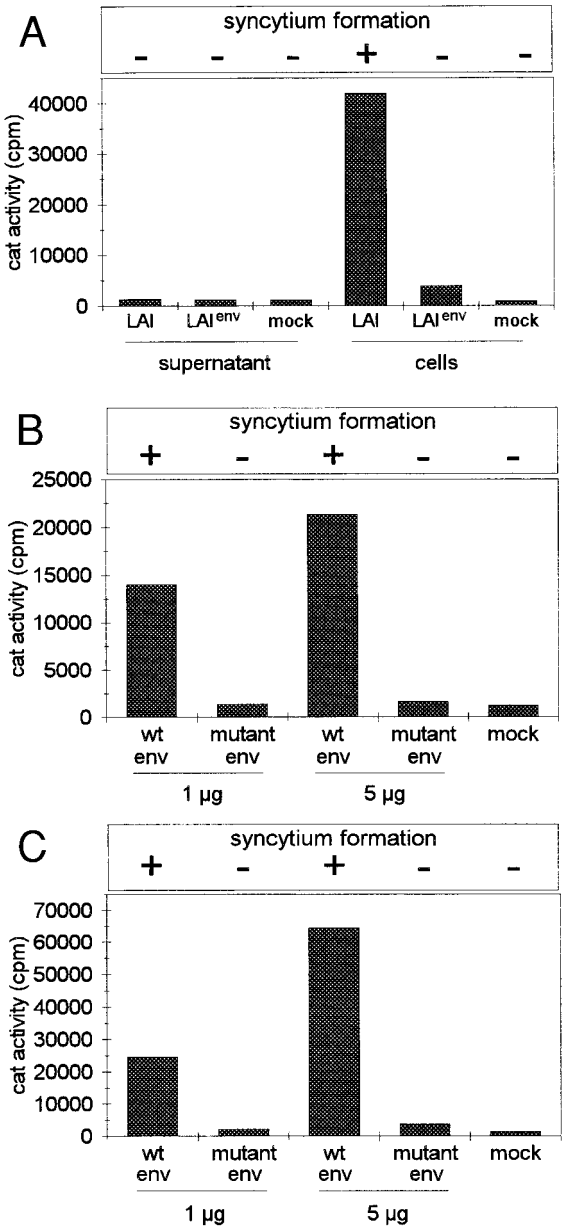

FIG. 8. Env mutations inhibit syncytia formation. (A) SupT1 cells were transfected with LAI, LAI^{env}, or pBluescript (mock). Two days after transfection, cells and supernatant were separated, and each was mixed with SupT1 cells containing a Tat-responsive LTR–CAT reporter gene construct. After 24 h, formation of syncytia was analyzed by light microscopy (+, syncytia; −, no syncytia), and quantitated by measurement of the CAT activity in cell extracts. (B and C) SupT1 cells were transfected with a Tat-expression vector and $pLAI_{\Delta gagpol}$ (1 or 5 μg), $pLAI^{env}_{\Delta gagpol}$ (1 or 5 μg), or no additional plasmid (mock). Two days after transfection, these cells were mixed with LTR–CAT-ransfected SupT1 cells. Syncytium formation was assayed after 24 (B) and 48 h (C).

Combining these results, we propose that the mutant virus postpones the death of the infected host cell because syncytia are not formed, thereby increasing the production of progeny. Consistent with this idea, no syncytia were observed in SupT1 cultures infected with the LAI^{env} virus, and the final virus level in these cultures was

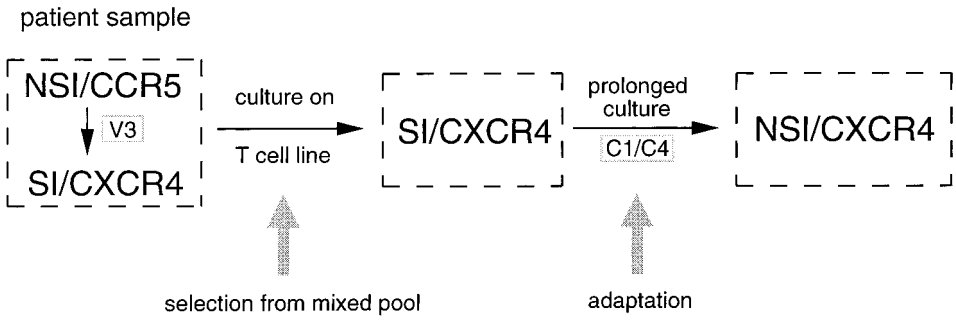

FIG. 9. HIV-1 evolution *in vivo* and *in vitro*.

approximately fivefold higher than in cultures infected with the wild-type LAI virus. Apparently, lengthening the period of virus production before the infected cell dies can improve viral replication so efficiently that it even rescues replication of otherwise defective viruses (e.g., mLAI). This evolutionary pathway demonstrates the delicate balance between viral replication rate and the deleterious effect of the virus on the host cell. Moreover, our *in vitro* evolution studies reveal that HIV-1 can evolve toward a virus that is less cytopathic, in this case by switching from the SI to the NSI phenotype. Please note that the SI-NSI nomenclature was based on infection experiments in SupT1 cells, whereas this phenotype is defined based on infection of MT2 cells.

A general viral strategy is to protect the host cell from being killed too early, and the timing of cell death can be an important determinant of viral replication. For instance, adenoviruses increase their "burst size" by delaying death of the infected cell (Kirn *et al.,* 1998). Because cellular defense mechanisms are designed to remove infected cells, e.g., by means of programmed cell death, some viruses have evolved ways to actively counteract these measures. Such viruses encode specific proteins to suppress cell suicide, which would normally curtail the infection (McFadden, 1998; Shisler *et al.,* 1998). Furthermore virulent viruses evolve to become relatively harmless to their host, as was described for the highly virulent myxomavirus that was introduced in Australia to control rabbit populations (Fenner and Ratcliffe, 1965). Similarly, simian immunodeficiency viruses (SIV), which have been in equilibrium with their simian hosts for a much longer time than HIV with its human host, have evolved to a nonpathogenic state. The SI-inducing HIV-1 variants also may be suppressed *in vivo* because of their cytopathicity. Indeed, SI variants are underrepresented or not detectable in most HIV-infected individuals, in particular in the asymptomatic phase of the infection (Fauci and Desrosiers, 1997). The preferential occurrence of NSI variants in transmission and in the early course of HIV-infection has been suggested to reflect the availability of $CCR5^+$ cells at the site of transmission, as there is some evidence that the initial target cell population during transmission consists of $CCR5^+/CXCR4^-$ Langerhans cells (Zaitseva and *et al.,* 1997). The observation that HIV-1 does not evolve toward the SI phenotype in some patients, and only partially and in a delayed manner in other patients, may be related to the increased cytopathicity of such variants.

The SI to NSI switch presented in this study seems to dispute the general notion that primary HIV-1 isolates usually change from the NSI to the SI phenotype both *in vivo* and in cell culture. Several reports suggest that laboratory adaptation involves an increased ability of the virus Env molecule to interact with the CD4 receptor, and this change usually coincides with an increase in syncytium-inducing capacity (Fujita *et al.,* 1992; Kozak *et al.,* 1997; Moore and Ho, 1995). However, most of these studies do not test for virus adaptation, but rather represent selection schemes that favor outgrowth of the CXCR4/SI variants from the mixed quasispecies of a primary isolate (Fig. 9). Real adaptation studies are rare, but two previous studies did present a virus evolution pathway that is comparable with the one described in this study. Prolonged passage of primary isolates in the H9 T cell line rendered the viruses sensitive to neutralization by human sera, which coincided with reduced cytopathicity (Wrin *et al.,* 1995). A more detailed study demonstrated adaptation of the ELI strain for growth on T cell lines, which resulted in a virus with a broadened host range and a reduced capacity for syncytium formation (Peden *et al.,* 1991). Several amino acid substitutions were present in the selected Env protein, and the loss of the SI phenotype was demonstrated to be mediated by an E49G substitution in the fusion domain of gp41 (Kozak *et al.,* 1997). In general, the evolutionary route chosen by a virus will critically depend on the virus–host combination used. For instance, the selection of less virulent HIV-1 variants as described in this study will be seen only with virus–cell combinations that exhibit gross cytopathic effects. Alternative routes toward a less cytopathic variant are also possible, as exemplified by the NDK isolate that evolved a CD4-independent phenotype upon prolonged culturing *in vitro* (Dumonceaux *et al.,* 1998).

The LAI adaptation described in this study was caused by two mutations in conserved Env domains:

G436R in the C4 domain and, although to a lesser extent, A70T in the C1 domain. Each individual mutation was able to improve LAI replication on SupT1 cells. The same two Env mutations negatively affected replication on most other cell types (Table 1). Our combined results suggest that alteration of one Env-mediated mechanism is responsible for both improved replication in SupT1 cells and loss of function in other cells. The ELI adaptation experiment mentioned above (Peden *et al.,* 1991; Kozak *et al.,* 1997) yielded an Env protein variant with broadened host range and increased affinity for CD4. This phenotype was determined by acquisition of a G427R mutation, and this C4 amino acid corresponds to LAI position E434, which is in close proximity to the G436 position that was found to be altered in this study. Because the C4 region has been implicated as a contact site for CD4 (Lasky *et al.,* 1987; Cordonnier *et al.,* 1989; Olshevsky *et al.,* 1990; Robey *et al.,* 1996), these changes may directly affect the ability of Env to interact with CD4 and thereby influence the cell–cell and virus–cell interactions. The X-ray structure of the HIV-1 Env protein (Kwong *et al.,* 1998; Rizzuto *et al.,* 1998) is consists with this possibility because amino acid 436 is located within the CD4 binding domain. We measured a dramatic loss of cell-fusion capacity of the Env variant in SupT1 cells, and the infectivity of these virions was severely affected in all other T cell lines and primary cells. Alternatively, the C4 and C1 mutations may affect the Env-CD4 interaction in a more indirect manner through an effect on the folding of this complex glycoprotein. For instance, there is ample evidence for complex intramolecular interactions within the Env protein, and the C4 domain has been reported to interact with the V1/V2 and V3 domains (Willey *et al.,* 1989; Freed and Martin, 1995; Wang *et al.,* 1996). The observation of a partial folding/processing defect of the mutant Env protein (Figs. 4 and 5) may be indicative of such an effect.

The results presented in this study underscore the notion that HIV-1 Env-mediated phenotypic changes can vary significantly among different host cell types. The LAI^{env} variation influenced virus production in SupT1 cultures in a positive manner, whereas strong negative effects were scored in all other cell types. The deleterious effects seen in most cells are most likely due to the compromised infection function of the Env protein. We think that this same Env molecule can be beneficial in SupT1 cells for two reasons. First, the loss of infection function is not apparent in SupT1 cells, although the infection efficiency was not improved either. Second, virus production is significantly increased due to reduced cell death. We do not currently understand what is special about the SupT1 T cell line such that it is less sensitive to the reduced infection capacity of the modified Env protein. It could be high surface expression of the CD4 receptor on SupT1 cells somehow can bypass the requirement for an optimally active Env protein for virus infection. Indeed, FACS analysis indicated the highest CD4 staining of SupT1 cells among the panel of T cell lines used in this study: SupT1 (mean intensity 29.3) > MT2 (21.3) > A3.01 (15.4) > C8166 (12.4) (see also Koken and Berkhout, 1994). We cannot exclude either that the CD4 or CXCR4 receptor of SupT1 cells contains a unique mutation that triggered adaptation of the viral Env protein. Also an as yet unidentified accessory component of the cell membrane may interact with Env and thereby influence virus infection in a cell-type-specific manner.

The Env variant efficiently infected SupT1 cells but demonstrated a complete loss of cell-fusion capacity. Apparently, we could separate the role of Env in virus infection (virus–cell fusion) from its role in syncytium formation (cell–cell fusion), indicating that the two mechanisms are dissimilar. This is supported by a microscopic study that followed the redistribution of fluorescent dyes, which demonstrated that the process of Env-induced cell–cell fusion occurs much more slowly than virus–cell fusion (Dimitrov and Blumenthal, 1994).

In summary, it turns out that we have selected for a virus that acquired increased fitness on SupT1 cells by means of a loss of Env function. This expertise may help us design new evolutionary strategies that will allow the selection of altered Env molecules that exhibit a gain of function. They may be used to target new cell types through binding of Env to novel coreceptors or through virus entry in a CD4-independent manner. The use of an HIV-1 Env protein with a modified host cell range can add certain safety features to live attenuated HIV-1 vaccines. Modified Env reagents also may be useful in gene therapy protocols to target-specific host cells with HIV-based vectors (Naldini *et al.,* 1996). It is clear that optimized virus–host systems may provide efficient means to produce high amounts of virus. As an example, we routinely reach amounts of virus of ≤10.000 ng/ml CA-p24 in SupT1 cultures infected with the LAI^{env} variant, which to our knowledge is significantly higher than any previously reported level of virus production.

MATERIALS AND METHODS

DNA constructs

The full-length molecular clone pLAI (Peden *et al.,* 1991) of the HIV-1 isolate LAI (Wain-Hobson *et al.,* 1985, 1991) was used to produce wild-type and mutant viruses. Nucleotide numbers refer to the LAI proviral DNA, with position 1 being the first nucleotide of the 5′ LTR. One sequence difference was found between the pLAI molecular clone that we used and the published LAI sequence (GenBank Accession No. K02013) at position 7868–7869 (GC instead of the reported sequence CG), changing Env amino acid 538 from Arginine (R) to Alanine (A) (Das *et al.,* 1998). The sequence of pLAI at this position now conforms to the consensus sequence for HIV-1 type B (Myers *et al.,* 1995). Construction of the

mLAI mutant, in which a short open reading frame had been introduced in the leader region of the HIV-1 transcript, was described previously as the uAUG mutant (Das *et al.,* 1998).

Selection of the variant Env protein by culturing of the mLAI virus on SupT1 cells for 110 days and introduction of the mutant *env* gene in pLAI were described previously (Das *et al.,* 1998). In the pLAI^{env} and mLAI^{env} plasmids, nearly the complete *env* gene of the adapted HIV-1 variant had been inserted as a *Sal*I-*Bam*HI fragment (position 5821–8522). In addition, we constructed a set of five subclones with small portions of the *env* gene (Das *et al.,* 1998). Fragment A (*Sal*I-*Nde*I, position 5821–6435) contains the Env I24T substitution; fragment B (*Nde*I-*Nhe*I, position 6435–7307) encodes the A70T change in the C1 domain, fragment C (*Nhe*I-*Mfe*I, position 7307–7702) encodes the C4 mutation G436R and a silent codon change, fragment D (*Mfe*I-*Hin*dIII, position 7702–8188) contains two silent Env codon changes, and fragment E (*Hin*dIII-*Bam*HI, position 8188–8522) has a A705V substitution in the transmembrane domain.

The Env-expression vector pLAI$_{\Delta gagpol}$ was derived from pLAI by deletion of the *gag-pol* domain by *Cla*I-*Nco*I digestion (position 829–5710), filling-in of the recessive ends with Klenow DNA polymerase in the presence of dNTPs, and religation of the vector. The same strategy was used to convert pLAI^{env} into pLAI$^{env}_{\Delta gagpol}$. The LTR–CAT reporter plasmid and pcDNA3-Tat expression vector used in the cell-fusion assays were described previously (Verhoef *et al.,* 1997).

Cells, DNA transfection, and virus infection

T cell lines (SupT1, MT-2, C8166, A3.01) were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS) at 37°C and 5% CO_2. Donor PBMC were prepared and cultured as previously described (Back *et al.,* 1996). Cells were transfected with HIV-1 molecular clones by means of electroporation. Briefly, 5×10^6 cells were washed in RPMI 1640 with 20% FCS, mixed with 1–5 μg DNA in 0.4 cm cuvettes and electroporated at 250 V and 960 μF, followed by resuspension of the cells in RPMI 1640 with 10% FCS. T cell lines and PBMC were split twice a week (1–10 and 1–2, respectively). To monitor virus production, the CA-p24 level in the culture supernatant was measured by ELISA (Abbott) as described previously (Back *et al.,* 1996).

C33A cervix carcinoma cells (ATCC HTB31) (Auersperg, 1964) were grown as a monolayer in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% FCS and MEM nonessential amino acids at 37°C and 5% CO_2. C33A cells were transfected by the calcium phosphate method. Cells were grown to 60% confluency in 24-well multidish plates or 75-cm^2 cell-culture flasks. For transfection of the 24-well plates, 1 μg DNA in 22 μl water was mixed with 25 μl 2[mult] HBS (50 mM HEPES pH 7.1, 250 mM NaCl, 1.5 mM Na_2HPO_4) and 3 μl 2 M $CaCl_2$. For transfection of 75-cm^2 flasks, 40 μg DNA in 880 μl water was mixed with 1 ml 2× HBS and 120 μl 2 M $CaCl_2$. The mixtures were incubated at room temperature for 20 min and subsequently added to the culture medium. The culture medium was changed after 16 h.

Chemokine inhibition assays

To measure the effect of the chemokine SDF-1 on the replication of LAI and LAI^{env} virus, the corresponding molecular clones were transfected into SupT1 cells. Viruses produced at 3 days posttransfection were quantitated by CA-p24 ELISA, and equal amounts of virus (0.5 or 5 ng CA-p24) were added to 1×10^6 SupT1 cells in the presence or absence of 100 nM SDF-1 1–67 (Bleul *et al.,* 1996; Oberlin *et al.,* 1996). This synthetic peptide was kindly provided by Dr. Ian Clark-Lewis, University of British Columbia, Vancouver. Cells were incubated at 37°C and 5% CO_2 for 2 h, washed twice with 8 ml phosphate-buffered saline (PBS; 10 mM Na-phosphate pH 7.4, 150 mM NaCl) and once with 2 ml complete medium with or without SDF-1, and then cultured for 6 days in 1.5 ml of the same medium.

Reverse transcription analysis upon infection of T cells

Virus stocks were prepared by transfection of C33A or by infection of SupT1 cells. Three days posttransfection of C33A cells and 4 days p.i. of SupT1 cells, the culture medium was centrifuged at 4000 rpm for 30 min to remove cells. The virus-containing supernatant was subsequently filtered through a 0.45-μm-pore-size filter (Schleicher and Schuell) and stored at −70°C. Contaminating DNA present in this supernatant was digested by incubation with 50 U DNaseI (RNase-free, Boehringer Mannheim) per ml and 10 mM $MgCl_2$ at 37°C for 1 h. SupT1 or C8166 cells (8×10^6 cells in 5 ml medium) were incubated with equal amounts of wild-type and mutant viruses (normalized by CA-p24 levels: 250 ng for C33A-produced virus, 4 ng for SupT1-produced virus) for 1 h at 37°C. A control sample was incubated at 0°C to prevent reverse transcription. Viruses were removed by extensive washing in medium. The cells that had been incubated at 37°C were either harvested directly (1 h sample) or incubated for an additional 1–3 h (2-, 3-, and 4-h samples). The control cells that had been incubated at 0°C were harvested directly (0-h sample). Infected cells were made into pellets by centrifugation at 4000 rpm for 4 min and washed with PBS buffer. DNA was solubilized by resuspending the cells in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.5% Tween 20, followed by incubation with 200 μg Proteinase K per ml at 56°C for 30 min and at 95°C for 10 min. Reverse transcription products were amplified by PCR with a 5′ primer identical to *tat* sequences (KV1, position 5821/5839) and a 3′ primer com-

plementary to *env* sequences (WS3, position 6579/6598). PCR products were analyzed by agarose gel electrophoresis and Southern-blotted onto nylon membrane (Zeta probe, Bio-Rad). To quantitate the PCR products, the filters were hybridized with a ^{32}P-labeled HIV-1 probe (LAI positions 5821/6379) in 0.5 M Na-phosphate (pH 7.2), 7% SDS, 1 mM EDTA and 50 μg salmon testis DNA per ml at 65°C for 16 h. Membranes were washed in 40 mM Na-phosphate (pH 7.2), 1% SDS at 65°C, three times for 5 min and once for 15 min and for 5 min in the same buffer at room temperature. Hybridization signal were quantitated with a PhosphorImager (Molecular Dynamics).

Cell-fusion assays

In the assay shown in Fig 6A, SupT1 cells (5×10^6) were transfected with 5 μg pLAI, pLAI^{env}, or the control plasmid Bluescript. The cells were cultured for 2 days, and cells and supernatant were separated by centrifugation at 1200 rpm for 10 min. Both cell and supernatant samples were mixed with a SupT1 culture that was transfected the previous day with 5 μg LTR–CAT reporter. Saquinavir (1 μM) was added after 1 h to inhibit subsequent rounds of viral replication. Cells were cultured for 24 h, and intracellular CAT activity was measured as described (Verhoef *et al.,* 1997).

In the assay shown in Figs. 6B and 6C, 5×10^6 SupT1 cells were transfected with 5 μg pcDNA3Tat and 0, 1, or 5 μg pLAI$_{\Delta gagpol}$ or pLAI$^{env}_{\Delta gagpol}$. The cells were cultured for 2 days, washed, and mixed with LTR–CAT-transfected SupT1 cells (see above). Cells were cultured and intracellular CAT activity was measured after 24 and 48 h.

Western blot analysis

C33A cells were transfected with the proviral clones. At 3 days posttransfection, cells were trypsinized and collected by low-speed centrifugation (1500 rpm, 10 min). Pelleted cells were washed with PBS buffer and resuspended in reducing SDS sample buffer (50 mM Tris–HCl pH 7.0, 2% SDS, 10% β-mercaptoethanol, 5% glycerol). Proteins were resolved in a 10% SDS–polyacrylamide gel and transferred to an Immobilon-P membrane (16 h, 60 V). The membrane was blocked with PBS containing 5% nonfat dry milk, 3% BSA, and 0.05% Tween 20 and subsequently incubated with the monoclonal anti-Env antibody ADP332 (Thiriart *et al.,* 1989) (dilution 1 in 100) for 1 h at room temperature, washed, incubated with rabbit anti-mouse IgG-alkaline phosphatase conjugate (Bio-Rad), and developed with the 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium protocol (Sigma).

Metabolic labeling and immunoprecipitations

SupT1 cells (15×10^6) were transfected with 15 μg pLAI or pLAI^{env} and cultured in 15 ml medium for 2 days. Cells were centrifuged at 1200 rpm for 10 min and washed with 30 ml PBS buffer. Cells were starved for 15 min at 37°C in 10 ml methionine/cysteine-free RPMI 1640 medium with 10 mM HEPES pH 7.4. The cells were centrifuged and resuspended in 150 μl of the same, prewarmed medium. Pulse-labeling was started by addition of 250 μCi [^{35}S]methionine/cysteine (1000 Ci/mmol, Amersham), and cells were incubated for 30 min at 37°C. Radiolabeled proteins were chased by addition of 1500 μl prewarmed RPMI 1640 medium, supplemented with 5 mM methionine, 5 mM cysteine, 20 mM HEPES pH 7.4, and 0.5 mM cycloheximide. Samples (300 μl) were taken at various times and placed on ice. Cells were collected by centrifugation (6 s in Eppendorf centrifuge at maximum speed), lysed in 600 μl of 1% Triton X-100 in MNT (20 mM MES, 100 mM NaCl, and 30 mM Tris–HCl pH 7.4), containing protease inhibitors (chymostatin, leupeptin, antipain, pepstatin, PMSF, and EDTA) and 20 mM iodoacetamide to block free sulfhydryl groups (Braakman *et al.,* 1991). Virus-containing supernatant samples were lysed by addition of an equal volume of 2[mult] concentrated lysis buffer.

The Env protein was immunoprecipitated at 4°C from cell lysates (75–150 μl per sample) and medium lysates (400 μl per sample) as described (Braakman *et al.,* 1991), using either an antiserum from an HIV-1-infected individual or a rabbit antiserum raised against HIV-1 LAI Env. Immunoprecipitates were washed twice at room temperature, once with 150 mM NaCl and 1 mM EDTA in 10 mM Tris–HCl pH 8.0, once with 0.1% SDS, 0.05% Triton X-100, and 300 mM NaCl in 10 mM Tris–HCl pH 8.6. One portion of the immunoprecipitates was treated with endoglycosidase F. All samples were analyzed using nonreducing and reducing SDS–PAGE (7.5%) followed by fluorography as described before (Braakman *et al.,* 1991).

Endoglycosidase F treatment

The pellet from the washed immunoprecipitates was resuspended in 15 μl of 0.2% SDS in 100 mM sodium acetate pH 5.5 and heated to 95°C for 5 min. After cooling, an equal volume of 100 mM sodium acetate pH 5.5 was added and the sample was incubated for 90 min at 37°C with 0.025 U of endoglycosidase F (Boehringer Mannheim). Laemmli sample buffer was added to a final concentration of 1.5% SDS, 10% glycerol, 0.004% bromphenol blue in 200 mM Tris–HCl pH 6.8.

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