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Changes in both gp120 and gp41 Can Account for Increased Growth Potential and Expanded Host Range of Human Immunodeficiency Virus Type 1

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Virus derived from an infectious molecular clone of the ELI strain of human immunodeficiency virus type 1 (HIV-1) replicates well in peripheral blood mononuclear cells and in some CD4-positive cell lines but exhibits a delayed time course of infection in CEM and H9 cells and fails to infect SupT1 and U937 cells. If the virus that emerges from infected H9 cells is used to infect CEM and H9 cells, the time course of infection is accelerated and the virus is able to infect U937 and SupT1 cells. In this study, we used the technique of polymerase chain reaction–single-strand conformation polymorphism to localize changes in both the extracellular gp120 and the transmembrane gp41 components of the envelope gene associated with adaptation to growth in tissue culture cell lines. Specifically, mutations were identified both in a region of gp120 implicated in CD4 binding and in the amino-terminal portion of gp41 adjacent to the region involved in fusion. No changes were found in the V3 loop of gp120, a region previously shown to be involved in viral tropism. When these mutations were introduced into the original molecular clone, they conferred an enhanced replicative capacity on ELI. These results demonstrate that two additional determinants in the HIV-1 envelope protein influence viral tropism and growth in vitro. They also may have important implications for the generation of viruses with increased growth potential and expanded host range seen in the late stages of HIV disease.

Human immunodeficiency virus type 1 (HIV-1), the primary virus causing AIDS throughout the world, replicates in CD4-positive cells of both the lymphocytic and monocytic lineages. HIV isolated from different cells and tissues at various stages of disease progression from the initial seroconversion to AIDS have different properties when assayed in vitro. During the asymptomatic phase, which may last for several years, HIV isolates from peripheral blood usually replicate to low titers in peripheral blood mononuclear cells (PBMC), do not induce syncytia, and do not infect most established CD4-positive cell lines (2, 8, 13, 15, 37, 40, 41). Viruses with these properties have been termed slow/low–non-syncytium inducing (15, 40). In contrast, viruses isolated from the blood of patients with late-stage AIDS commonly replicate to higher titers in PBMC, are capable of inducing syncytia, and often are able to infect many cell lines (2, 7, 8, 13, 15, 17, 23, 40, 41). This class has been called rapid/high-syncytium inducing (15, 40). The determinants for both tropism and replicative capacity seem to reside predominantly in the envelope gene (16, 44) and not in the long terminal repeat elements (25, 35).

Although all HIV-1 isolates are able to infect primary T cells, not all are able to produce a productive infection in primary macrophages. Macrophage tropism also appears to be determined mainly by the HIV envelope (6, 29), in particular, by a region that overlaps the V3 variable region (4, 9, 26, 31, 38, 42).

Mapping of the determinants for viral growth and tropism has usually involved construction of chimeras between molecular clones that encode viruses with distinct phenotypic properties. This technique has been used successfully to delineate the *env* gene as the major host range determinant (6, 16, 29, 44). An alternative method is to use a virus derived from a single molecular clone and select for variants with enhanced replicative capacity after passage in cell lines. The determinants that account for the acquired characteristics can be mapped, and the regions that carry the mutations can be replaced in the original clone to demonstrate their function. Infection of the H9 and CEM T-cell lines with the ELI strain of HIV-1 (1, 34) results in production of high levels of virus and induction of syncytia in the cultures, but only after 20 to 30 days. This result contrasts with that obtained with other T-cell-tropic strains of HIV-1, such as LAI, in which peak reverse transcriptase (RT) activity is observed at around day 10 after infection. When the virus that emerges from infected H9 cells is used to infect H9 and CEM cells, the time of appearance of virus production is substantially accelerated and the time course of infection is close to that of LAI. In addition, the passaged virus is able to infect U937 cells (34). Therefore, ELI appears to have adapted to grow in H9 cells and the emergent virus has an expanded host range and increased replicative capacity.

In this report, we describe how mutations can be readily mapped in the genome of HIV-1_{ELI} that has acquired different growth properties following passage on a T-cell line. The method uses the polymerase chain reaction (PCR)–single-strand conformational polymorphism (SSCP) method of Orita et al. (33) on DNA extracted from HIV-infected cells. Changes were found to reside in two important regions of the envelope protein—a region of gp120 shown to be involved with CD4 binding and the region adjacent to the fusogenic peptide of gp41. The association of these two regions with tropism and replicative capacity suggests that the mechanism for the enhanced replicative capacity and extended host range found with adaptation of HIV-1_{ELI} to growth in vitro involves virus uptake. Although these results were obtained by using cell lines, it is possible that similar

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adaptation occurs in vivo to contribute to the generation of the highly virulent variants observed in AIDS.

MATERIALS AND METHODS

Nomenclature. Plasmid molecular clones of HIV are indicated by the letter p preceding the strain designation, e.g., pELI and pLAI. For the viruses derived from molecular clones, the p is omitted, e.g., ELI and LAI. Molecular clones of the same strain with different genotypes are designated pELI1, pELI2, etc. The pELI plasmid described by Peden et al. (34) is now called pELI1.

Bacterial strain and plasmids. Throughout this work, *Escherichia coli* DH5 was used. It was purchased as competent cells from Bethesda Research Laboratories (Gaithersburg, Md.) and transformed as described by Hanahan (24). Infectious molecular clones of HIV-1_{LAI} (pLAI) and HIV-1_{ELI} (pELI1) have been described previously (34). The nucleotide numbers of HIV start at the first nucleotide of the 5′ long terminal repeat of the proviral genome. Plasmid p347 consists of the *Bam*HI-to-*Xho*I fragment (nucleotides 5832 to 8890) of pELI1 cloned in pKS (Stratagene, La Jolla, Calif.).

Preparation of plasmid DNAs. Plasmid DNAs from both small-scale (1.5-ml) and large-scale (300-ml) cultures were prepared by using an alkaline lysis procedure (27), followed by banding twice in CsCl-ethidium bromide gradients, proteinase K digestion, phenol extractions, and alcohol precipitations.

Cells and media. HeLa cells were obtained from the laboratory of Barrie Carter, National Institutes of Health, Bethesda, Md., and grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The sources of the CD4-positive lymphocyte cell lines (CEM clone 12D7, HUT78 clone H9, SupT1, and Jurkat clone E6-1), PBMC, and promonocyte line U937 have already been described (34). The cells were grown in RPMI 1640 with 10% fetal bovine serum and 2 μg of Polybrene per ml (RPMI-10/2).

Assays for biological activity. The methods used to assay biological activity have been described previously (34). Briefly, HeLa cells (ca. 5×10^5) in T25 flasks were transfected with plasmid DNA (15 to 20 μg) by using calcium phosphate coprecipitation methods (19, 22, 43). RT activity was measured 24, 48, and 72 h after transfection. Supernatants generally contained maximum activity between 24 and 48 h, and then the activity decreased. For infection of cell lines, amounts of virus corresponding to 5×10^5 cpm of RT activity were added to 2×10^6 cells in 1 ml of RPMI-10/2 (approximate multiplicity of infection, 0.01). Virus adsorption was for 2 h at 37°C, after which time the cells were diluted in RPMI-10/2. At 2-day intervals, the cells were fed with RPMI-10/2 and samples were taken for RT activity determination. Cultures were monitored daily for the presence of syncytia.

DNA sequence analysis. DNA sequencing was done by the chain termination method (36) adapted for use with plasmid DNA (5) and with [α-^{35}S]dATP as the labelled nucleotide (3). Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) was used as the DNA polymerase.

Isolation of cellular DNA and SSCP analysis. DNA was isolated from infected cells as follows. Cells were pelleted by centrifugation at 1,500 × *g* for 10 min at room temperature, washed in phosphate-buffered saline, and suspended in 0.2 M NaCl–10 mM Tris-HCl (pH 7.2)–1 mM EDTA. Sodium dodecyl sulfate was added to 0.5%, proteinase K was added to 100 μg/ml, and the sample was incubated overnight at 37°C. The DNA was extracted once with phenol, once with phenol-$CHCl_3$-isoamyl alcohol (50:48:2), and finally with $CHCl_3$-isoamyl alcohol (24:1). It was precipitated once with isopropanol and once with ethanol before being dissolved in 10 mM Tris-HCl (pH 7.4)–1 mM EDTA (TE).

DNAs extracted from infected cells were amplified with the pairs of primers indicated in Fig. 1. PCRs were carried out in a total volume of 10 μl containing 50 ng of extracted DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, each deoxynucleoside triphosphate at 0.2 mM, 0.5 μCi of [^{32}P]dCTP (3,000 mCi/mmol), 5 pmol of each primer, and 0.5 U of *Taq* DNA polymerase (Perkin Elmer Cetus). Conditions were 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. In some cases, the [^{32}P]dCTP was omitted from the PCR and the nonradioactive PCR product was used for primer extension so that only one strand of the amplified DNA was visualized. The amplified fragments were mixed with an equal volume of 95% formamide–20 mM EDTA (pH 8.0)–0.05% xylene cyanol–0.05% bromophenol blue, denatured at 85°C for 5 min, and analyzed by electrophoresis on a 5% nondenaturing polyacrylamide gel as previously described (33). Electrophoresis was carried out at 4°C with a constant voltage of 400 V for 12 to 15 h or 1,100 V for 3 to 5 h.

Reconstruction of mutant clones. PCR-amplified fragments (nucleotides 6945 to 8499) were digested with *Eco*RI (position 7358) and *Bgl*II (position 8066), gel purified, and ligated into p347 digested with the same enzymes. The sequence of the region from the *Eco*RI site to the *Bgl*II site was determined, the resulting plasmids carrying the mutations were digested with *Dra*III and *Xho*I, and the fragment was inserted into pELI to produce the mutant proviral clones.

RESULTS

To determine whether the adaptation of HIV-1_{ELI} to growth in certain cell lines was a consequence of genetic changes, DNAs were isolated from cultures of H9 and U937 cells that had been infected with this adapted ELI virus. For analysis of mutations, the technique of Orita et al. (33), termed PCR-SSCP, was applied. In this method, the region of the genome of interest is amplified by using the PCR in the presence of [α-^{32}P]dCTP and the products are heat denatured and subjected to nondenaturing polyacrylamide gel electrophoresis. Under these conditions, single nucleotide substitutions can frequently lead to altered mobility of the single-strand fragments. Therefore, this method was chosen for rapid analysis of large regions of the HIV genome for sequence variations. Clearly, mutant fragments can be detected only if they are present in significant proportions and only those mutations that cause a difference in the migration of the fragments can be identified.

We focused on the 3′ half of the genome, because previous work had identified the major determinants of tropism to be in the *env* gene (4, 6, 16, 29, 44). Multiple overlapping regions of approximately 300 bp spanning the 3′ half of the ELI genome from the *vif* gene to the 3′ long terminal repeat were examined (indicated by the bars in Fig. 1A). Most of the fragments amplified from infected H9 and U937 cells migrated indistinguishably from the corresponding fragments amplified from parental clone pELI1. One region of the genome consistently and unequivocally showed heterogeneity by PCR-SSCP. This was from nucleotides 7687 to 7930 and encompasses the amino terminus of gp41 (indicated by the thicker bars in Fig. 1A).

PCR-SSCP analysis of parental pELI1 plasmid DNA (considered the wild-type [WT] pattern) contains two SSCP

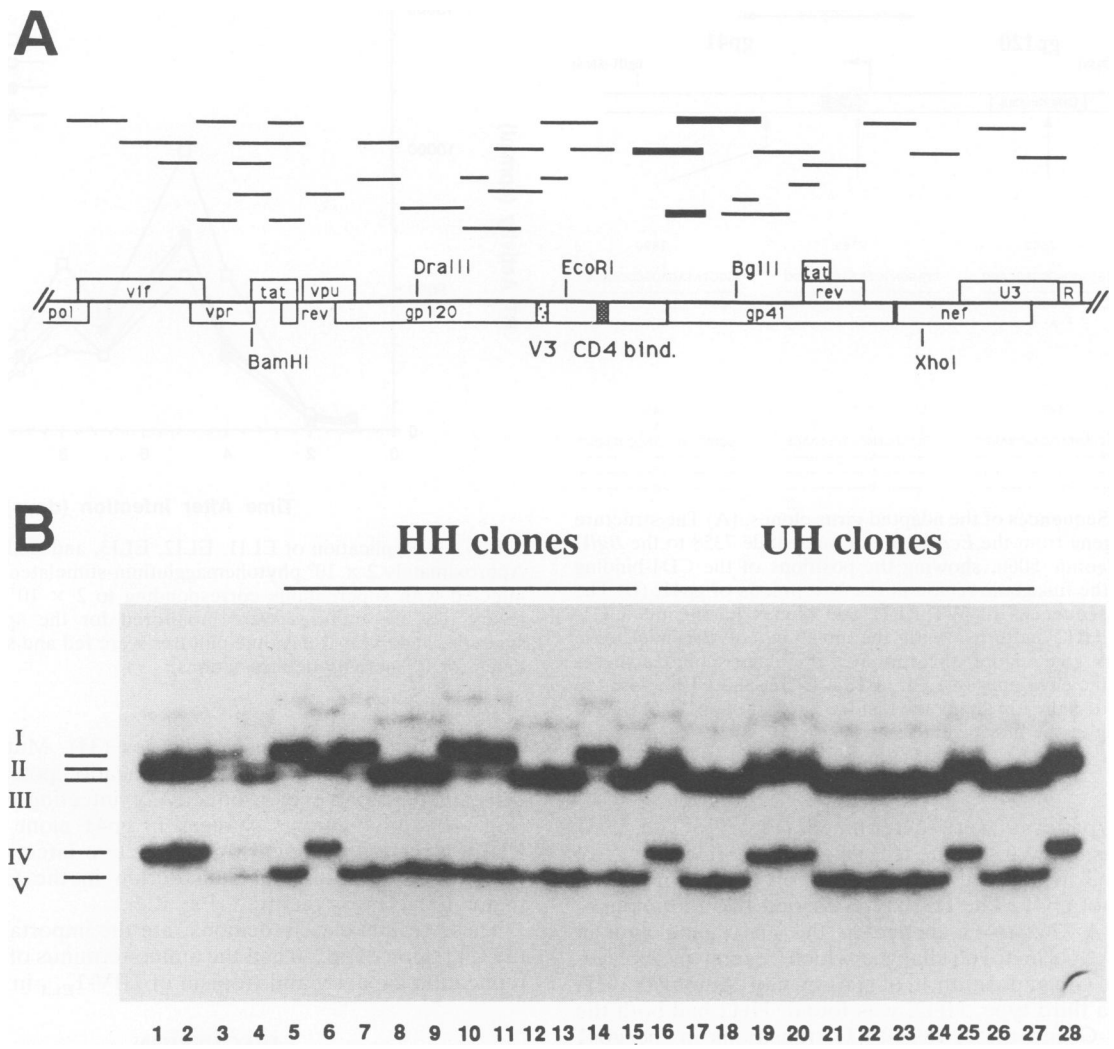

FIG. 1. PCR-SSCP analysis of infected cell DNA and plasmid clones. (A) The HIV-1 genome from the *vif* gene to the 3′ long terminal repeat is shown, with the known open reading frames and some relevant restriction enzyme sites. The positions of the immunodominant V3 epitope and the CD4-binding region are indicated in gp120. Across the top are positions of the regions amplified by PCR for SSCP analysis, with the thicker bars indicating the fragments that were polymorphic. (B) Nondenaturing 5% acrylamide gel analysis of the PCR products. Lanes: 1, 2, and 28, pELI1 plasmid DNA; 3, cell DNA extracted from ELI-infected H9 cells; 4, cell DNA extracted from ELI-infected U937 cells; 5 to 15, individual plasmid clones prepared from infected H9 cell DNA; 16 to 27, individual plasmid clones prepared from infected U937 cell DNA. Since labelled dCTP was incorporated during the PCR reaction, both strands of the denatured DNA are visible in the SSCP analysis.

bands, labelled II and IV in Fig. 1B, lanes 1, 2, and 28. With the DNA of ELI-infected H9 cells (HH DNA), five bands were found, the most intense of which were I and V. Of the remaining three bands, two (bands II and IV) corresponded to those of the WT. PCR-SSCP analysis of the DNA from infected U937 cells (UH DNA) produced four bands, of which two (II and IV) corresponded to the WT and the other two (bands III and V) corresponded to the bands in the HH DNA. Thus, three distinct SSCP patterns were detected: the WT pattern (bands II and IV), the pattern from HH DNA (bands I, II, III, IV, and V), and the pattern from UH DNA (bands II, III, and IV).

In contrast, only the WT SSCP pattern was found in the DNA from ELI1-infected PBMC, and no SSCP pattern differences were observed between the DNAs isolated from LAI-infected H9 and CEM cells and the original plasmid pLAI (data not shown). These results demonstrate that sequence variants are not detectable when adaptation is not evident.

The results from the analysis of total DNA were corroborated by analyzing the SSCP patterns of individual clones. A region of the HIV genome including the PCR-SSCP heterogeneity (nucleotides 6945 to 8499) was PCR amplified from HH DNA and UH DNA, digested with *Eco*RI and *Bgl*II, and cloned into p347. Plasmids were screened by PCR-SSCP for the presence of mutations (Fig. 1B). Eleven HH clones yielded three different SSCP patterns corresponding to the bands found in HH DNA: WT (II and IV; lane 6), HH1 (I and V; lanes 5, 7, 10, 11, and 14), and UH1 (III and V; lanes 8, 9, 12, 13, and 15). Among the 12 clones derived from UH DNA, only two SSCP profiles were found: WT (lanes 16, 19, 20, and 25) and UH1 (lanes 17, 18, 21, 22, 23, 24, 26, and 27).

The DNA sequences of several plasmids with the UH1

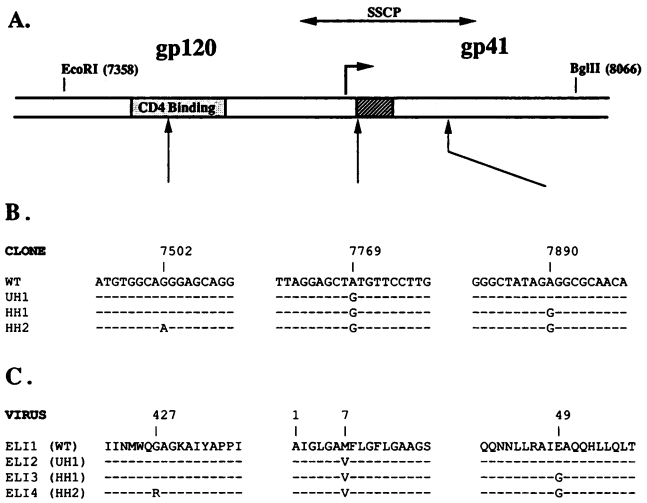

FIG. 2. Sequences of the adapted virus clones. (A) The structure of the *env* gene from the *Eco*RI site at nucleotide 7358 to the *Bgl*II site at nucleotide 8066, showing the positions of the CD4-binding region and the fusogenic region at the N terminus of gp41. (B) The nucleotide sequences of WT ELI1 and clones having the UH1, HH1, and HH2 patterns, with the positions of the nucleotide substitutions given along the top. (C) Predicted amino acid sequences of the envelopes of ELI1, ELI2, ELI3, and ELI4, with the numbers across the top giving the residue number of gp120 (427) and gp41 starting at the first residue.

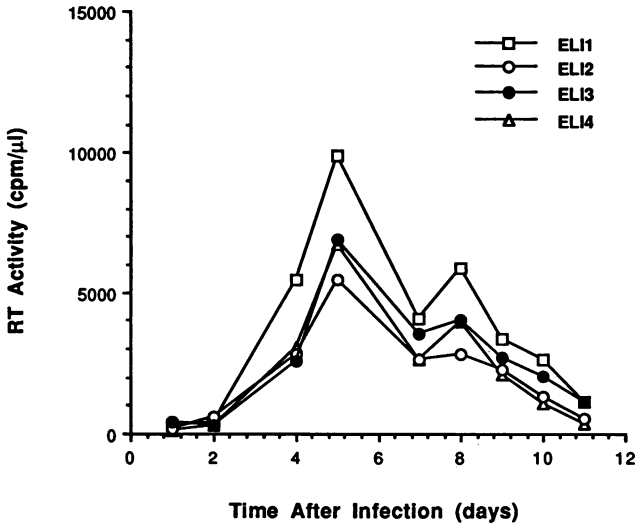

FIG. 3. Replication of ELI1, ELI2, ELI3, and ELI4 on PBMC. Approximately 2×10^6 phytohemagglutinin-stimulated PBMC were infected with supernatants corresponding to 2×10^5 cpm of RT. Every day the cultures were monitored for the appearance of syncytia, and every 2 days the cultures were fed and samples were taken for RT activity determinations.

and HH1 patterns were determined (Fig. 2). The UH1 pattern was found to be due to a single nucleotide change of A-7769 to G, which causes a substitution of Val for Met at position 7 of gp41. The HH1 type carried two mutations—the same A-7769–to–G change in the gp41 gene and an additional A-7896–to–G change, which results in replacement of the Glu at position 49 of gp41 by Gly. Among the HH plasmids, a third type, HH2, was found. HH2 had both the A-7769–to–G and the A-7896–to–G transitions in the gp41 gene and a third mutation of G-7502 to A, resulting in a substitution of Arg for Gly at position 427 in the C4 region of gp120, a region implicated in the binding of Env to the virus receptor, CD4 (12, 28, 32).

To determine whether these mutations conferred an advantage in the tropism of ELI, they were introduced separately into WT pELI1 to produce pELI2, pELI3, and pELI4 (Fig. 2). Stocks of ELI1, ELI2, ELI3, and ELI4 were used to infect both PBMC and CD4-positive cell lines. In most of the experiments, LAI was included for comparison. The Env mutations did not affect the ability of ELI to replicate in PBMC, since there was little difference in the time course of infection or in the amount of virus produced (Fig. 3).

CEM and H9 cells were infected with the four ELI viruses to examine whether the Env changes allowed virus replication in these cells without the usual 20- to 30-day delay. Changes in gp41, either the single Met-7–to–Val change (ELI2) or both Met-7 to Val and Glu-49 to Gly (ELI3), were able to overcome the initial growth delay in both CEM and H9 cells (Fig. 4A and B, respectively). Substitution of Gly-427 with Arg in the CD4-binding region in the background of the two gp41 mutations (ELI4) further increased the replication capacity.

The original virus, ELI1, was unable to replicate in the SupT1 T-cell line and the promonocytoid U937 cell line. After passage through H9 cells, ELI acquired the replication capacity for both of these cell lines (34). Mutants ELI2, ELI3, and ELI4, with enhanced replication potential for H9 cells, were capable of productively infecting SupT1 cells (Fig. 4C). In contrast, changes in gp41 alone (ELI2 and ELI3) were not sufficient for productive infection of U937 cells, and the additional substitution in the CD4-binding region (ELI4) was required (Fig. 4D).

These results clearly demonstrate the importance of both the C4 region of gp120 and the amino terminus of gp41 to the replication capacity and tropism of HIV-1_{ELI} in vitro.

DISCUSSION

Passage in tissue culture of ELI1, the virus derived from a molecular clone of HIV-1_{ELI} leads to production of variants that have improved replication capacity and extended host range (34). These results had demonstrated that selection can occur even in the absence of the immune system. Since the viruses that emerged originally came from a molecular clone, we reasoned that the variants would differ from the parental virus at only a few positions, and therefore the regions of the HIV genome accounting for the altered phenotype could be determined. Furthermore, it was hoped that the positions of the mutations would be informative as to the mechanism of the improved growth potential.

By using the PCR-SSCP method of Orita et al. (33) with the DNA of infected cells, mutations were detected in a fragment spanning the amino terminus of gp41. These mutations were introduced into pELI1 by transferring a fragment whose sequence differed only at the nucleotides indicated in Fig. 2 to produce the following viruses: ELI2 (Met-7 to Val in gp41), ELI3 (Met-7 to Val and Glu-49 to Gly in gp41), and ELI4 (Met-7 to Val and Glu-49 to Gly in gp41 and Gly-427 to Arg in gp120). Viruses derived from the resulting clones were found to have altered phenotypes compared with parental virus ELI1. Although they replicated similarly to ELI1 on PBMC, demonstrating that neither the gp41 nor the gp120 substitutions had affected the capacity to replicate in

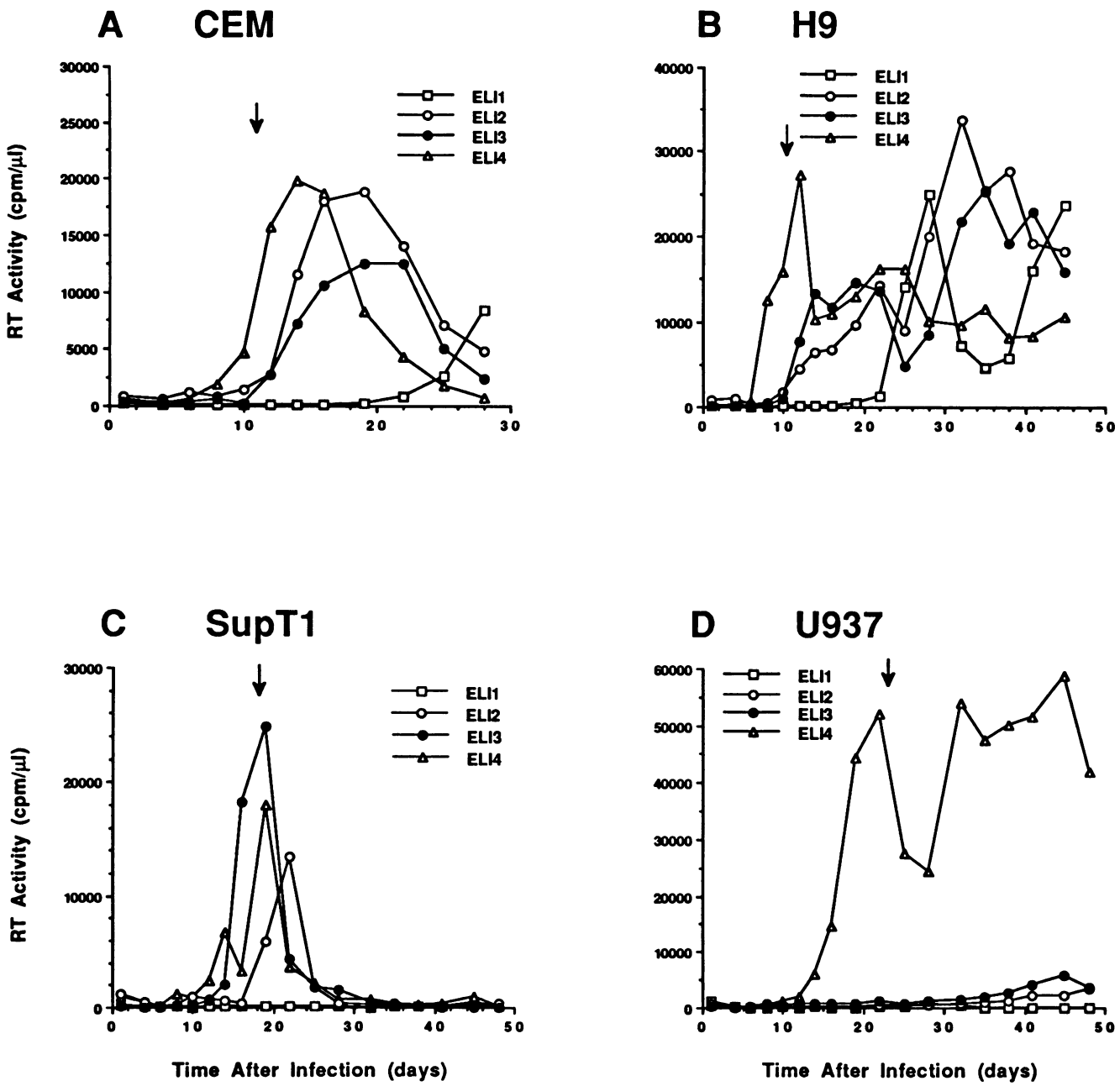

FIG. 4. Replication of ELI1, ELI2, ELI3, and ELI4 in cell lines. Approximately 2×10^6 MT-4, Jurkat, CEM, H9, SupT1, and U937 cells were infected with supernatants corresponding to 5×10^5 cpm of RT. Every day the cultures were monitored for the appearance of syncytia, and every 2 days the cultures were fed and samples were taken for RT activity determinations. Panels: A, infection of CEM cells; B, infection of H9 cells; C, infection of SupT1 cells; D, infection of U937 cells. The arrows indicate positions of peak RT activity for LAI virus infection.

primary T cells, in their activities on T-cell lines and promonocytic cell line U937 they differed from ELI1 and from each other. For example, ELI2 and ELI3 were able to induce a productive infection in H9 and CEM cells without the delay seen with ELI1, whereas an additional substitution in the C4 region (ELI4) was necessary to establish a productive infection in U937 cells. Therefore, these mutations were sufficient to convert the original virus, ELI1, into viruses with properties similar to those of the adapted viruses present after passage in H9 cells.

The places where the mutations were found—one of the regions in gp120 implicated in binding to CD4 and the amino-terminal region of gp41—immediately suggest possible mechanisms for the acquired increased replication capacity of these viruses. Two lines of evidence implicate the amino terminus of gp41 in virus-cell fusion. (i) There is a region in HIV-1, HIV-2, and simian immunodeficiency virus that is similar to the region of the hemagglutinin of the paramyxoviruses known to be involved in fusion of these viruses with cells (20, 21). (ii) Substitution of hydrophilic

amino acids for hydrophobic residues in this region of gp41 severely impaired the syncytium-inducing capacity of these envelopes (14, 18). In the variants that arose on passage in tissue culture, the Met at position 7 of gp41 has been changed to Val, a more hydrophobic amino acid, and perhaps, therefore, the fusion reaction of ELI2, ELI3, and ELI4 has been enhanced. Interestingly, this position in other HIV-1 isolates has been found to vary. Five amino acids have been found at this position: Met, Val, Leu, Phe, and Ile, with Met being the most common (30). While it is not possible to correlate growth potential with the amino acid at this position, some of the viruses with a Met at position 7 of gp41 do not have as wide a host range or as high a replication capacity in cell lines (e.g., MAL and ELI1) as those with a hydrophobic residue at this position (e.g., the LAI group) (34).

Substitution of Arg for Gly at position 427 of gp120 in concert with the two mutations in gp41 produces a virus, ELI4, with the highest replication capacity and broadest host range. Among the isolates sequenced, Gly (ELI1) is infrequently found, and the charged Glu, Lys, and Arg (ELI4) residues are more common (30). Since this residue is in a part of the extracellular Env implicated in CD4 binding (12, 28, 32), altered affinity of HIV-1 to its receptor may cause the phenotype. However, Cordonnier et al. (11) noted that while substitution of Thr for Ile at position 427 in the gp120 of HIV-1_{LAI} (equivalent to position 419 of ELI) did not alter the binding of gp120 to CD4, it did alter the tropism of the virus for cell lines. The mutant virus was no longer able to infect U937 cells, whereas its replicative capacity on SupT1 cells was unaffected. Therefore, mutations in a CD4-binding region of gp120 can influence the host range of HIV-1 without necessarily affecting the affinity of gp120 for its receptor.

That mutations were identified in two regions of Env does not exclude the possibility that additional mutations were also present in the *env* gene or elsewhere but were not detected by the PCR-SSCP method used here. In fact, failure to detect some mutations may explain the fact that, while plasmids with the UH1 sequence were obtained from infected U937 cells, a virus with this change alone (ELI2) did not grow well in this cell line. Nevertheless, the mutations identified do confer many of the characteristics of the virus stock that emerged from H9 cells. While the relevance of these changes to pathogenesis requires further study, these results have shown that the amino-terminal part of gp41 and a region of gp120 involved with CD4 binding are two additional determinants for virus growth and cell tropism.

Significantly, no changes were found in the immunodominant V3 loop region of gp120, either by SSCP analysis or by sequencing of several PCR clones. This region has been implicated in the tropism and growth potential of HIV-1 (4, 9, 10, 26, 38, 39, 42), but changes in the V3 loop were not required for phenotypic changes in this system. One possible reason why changes in this immunodominant region of Env were not seen is that such changes are driven by a combination of both immune selection and replication selection. The absence of the immune system in the in vitro system allows for selection solely for those mutants that are able to replicate better. It is possible that nonimmune selection for viruses with increased replicative capacity also occurs in vivo, especially in the late stages of AIDS, when the immune system is compromised. PCR-SSCP analysis of clinical specimens taken at various stages of disease will help to address this issue.

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