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Persistence of Attenuated *rev* Genes in a Human Immunodeficiency Virus Type 1-Infected Asymptomatic Individual

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With the goal of examining the functional diversity of human immunodeficiency virus type 1 (HIV-1) *env* genes within the peripheral blood mononuclear cells of an asymptomatic individual, we substituted four complete *env* genes into the replication-competent NL4-3 provirus. Despite encoding full-length open reading frames for gp120 and gp41 and the second coding exon of *tat* and *rev*, each chimera was replication defective. Site-directed mutagenesis of codon 78 in the Rev activation domain (from a hitherto unique Ile to the subtype B consensus Leu) partially restored infectivity for two of three chimeras tested. Similarly, mutagenesis of *rev* codon 78 of NL4-3 from Leu to Ile partially attenuated this virus. Ile-78 was found in all 13 clones examined from samples taken from this asymptomatic subject 4.5 years after infection, including 9 from peripheral blood mononuclear cells and 4 from a virus isolate, as well as 4 additional clones each from peripheral blood mononuclear cells sampled 37 and 51 months later. We next examined conservation of the Rev activation domain within and among long-term survivors (LTS) and patients with AIDS, as well as T-cell-line-adapted strains of HIV-1. Putative attenuating mutations were found in a minority of sequences from all five LTS and two of four patients with AIDS. Of the 11 T-cell-line-adapted viruses examined, none had these changes. Among and within LTS, virus populations had marginally higher levels of diversity in Rev than in Env; patients with AIDS had similar levels of diversity in the two reading frames; and T-cell-line-adapted viruses had higher levels of diversity in Env. These results are consistent with the hypothesis that asymptomatic individuals harbor attenuated variants of HIV-1 which correlate with and contribute to their lack of disease progression.

Infection with human immunodeficiency virus type 1 (HIV-1) is normally characterized by progressive immune system decline, eventually leading to the development of AIDS and death. The median time from infection to overt disease is 10 years but varies from as little as 2 to 4 months to more than 13 years (55). Disease progression is reflected in a decreased $CD4^+$ cell count, and the rate of decline can be used to divide HIV-infected individuals into three general groups, (i) rapid progressors (10%), (ii) progressors (80%), and (iii) nonprogressors or long-term survivors (LTS) (10%) (55). The basis of this variability in the length of the asymptomatic period is unknown, but it has been suggested to be due to interplay between the strength of the host immune responses and the phenotypic characteristics of the initially infecting or evolving virus strain (55, 60).

While the host factors influencing the clinical course of HIV infection are as yet poorly defined, the presence of more- or less-virulent HIV strains may be of importance (55, 60), as has been demonstrated in animal model systems (13, 44). In vitro studies have demonstrated differences between HIV isolates from patients with AIDS and HIV isolates from asymptomatic infected individuals. Isolates from asymptomatic individuals tend to replicate more slowly and sometimes to lower titers and, in contrast to isolates from about 50% of patients with AIDS, have no syncytium-inducing capability when they are tested in MT-2 cells, nor do they grow in T-cell lines (4, 8, 31, 52, 59). The molecular bases of some of these phenotypic characteristics have been partially elucidated and mapped to the envelope gene. For example, certain amino acids in specific positions of the V3 loop have been shown to be important for the ability of HIV to induce syncytia in MT-2 cells and to grow in transformed T-cell lines (10, 15, 32). When viral structural genes are analyzed, the highest degree of DNA sequence diversity between viruses is found in the envelope gene. However, the envelope gene also contains the second coding exon of the regulatory genes *rev* and *tat*; it is currently unknown whether mutations in these exons have any impact on the course of HIV infection.

We undertook a study of the functional impact of sequence variation in envelope genes in vivo. Four chimeric viruses in which the gp160-encoding envelope gene from $HIV\text{-}1_{Lai}$-derived pNL4-3 was replaced by homologous segments derived from the peripheral blood mononuclear cells (PBMC) of an asymptomatic patient were generated. We report in vitro testing of these viruses and analyses of the *env* and overlapping *tat* and *rev* coding sequences. We also describe DNA sequence analysis of the second coding exon of *rev* and *tat*, obtained from

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a virus isolate from this patient, from PBMC at other time points, and from five LTS and four patients with AIDS.

MATERIALS AND METHODS

Clinical samples. PBMC were obtained in April 1989, May 1992, and July 1993 from a homosexual Caucasian male (patient MA) who had been infected with HIV-1 subtype B in late 1984 or early 1985. The clinical course of this patient and the molecular properties of his viruses have previously been described (patient 2 [27] and patient 1 [26, 33, 49]). He has remained largely asymptomatic between 1989 and 1995. His CD4 counts have ranged from 264 to 470 cells per mm^3, with most counts between 350 and 470 cells per mm^3 (mean, 414 cells per mm^3 for 14 measurements).

In 1989, PBMC samples were also obtained from HIV-infected LTS randomly chosen from the San Francisco Men's Health Study Cohort (55). The criteria for categorizing these patients as LTS were (i) verified HIV infection for more than 8 years (in 1993) and (ii) stable or increasing CD4 counts over several years. In 1992 and 1993, PBMC samples were also obtained from patients with AIDS at the Center for AIDS Research at Stanford University. In 1982, spleen samples were obtained from autopsies of American patients with AIDS, patient LA (21) and patient RF.

Construction of chimeric proviruses. Chimeric proviruses were generated between an infectious plasmid clone of HIV-1, pNL4-3 (1), and four full-length (gp160-encoding) envelope genes that had been derived from fragments of the 3′ half of the genome amplified by PCR from proviral DNA from patient MA PBMC. All patient MA envelope genes originated from the April 1989 PBMC and have been sequenced in their entirety. The amplification, cloning, and sequencing of a part of these genes (gp120) have been reported previously (33).

gp160-encoding sequences were substituted into the pNL4-3 genome at *Asp*718 restriction sites. An intermediate vector (pNLΔ5′) was generated to accept these sequences because there were additional *Asp*718 sites in pNL4-3. The 5′ half of the pNL4-3 genome was removed by *Aat*II and *Eco*RI double digest and replaced by a 469-bp *Aat*II-*Eco*RI fragment from pUC19 to generate pNLΔ5′. Next, pNLΔ5′ was digested with *Asp*718 to release the gp160 envelope fragment and religated to generate a 3′-half genome vector without an envelope gene (pNLΔ5′/Δenv).

MA clones pMA5, -7, -16, and -21 were digested with *Asp*718 to release the 2.7-kb gp160 fragment, which was purified from 1% agarose gels with Gene Clean according to the manufacturer's recommendations (Bio 101). The pNLΔ5′/Δenv vector was digested with *Asp*718, dephosphorylated with calf intestinal phosphatase, and ligated to purified 2.7-kb MA *env* fragments, generating the constructs pNLΔ5′MA5, -7, -16, and -21. Restriction enzymes *Bam*HI and *Pvu*I were used to determine the orientations of these inserts.

To obtain the 5′-half genome fragment, pNL4-3 was digested with *Aat*II and *Eco*RI and the 7-kb *Aat*II-*Eco*RI fragment was purified from 1% agarose gels by electroelution (51).

pNLΔ5′MA5, -7, -16, and -21 were digested with *Aat*II-*Eco*RI and ligated with the pNL4-3 5′-half genome fragment, and the mixture was transformed into chemically competent *Escherichia coli* HB101 cells. Full-length proviral clones were identified by restriction enzyme digestion, grown in large scale, and purified by using Qiagen-tip 500 columns (Qiagen, Inc.). Clone structure was verified by dideoxy DNA sequencing of the 3′ end of the gp41 gene and the first third of the *nef* gene. The chimeric proviral constructs were designated pNMA5, -7, -16, and -21.

Oligonucleotide-directed mutagenesis. Specific nucleotide changes were introduced into proviral constructs pNMA5, pNMA7, pNMA21, and pNL4-3 by oligonucleotide-directed mutagenesis in the second coding exon of *rev*. In the pMA clones, isoleucine 78 was changed to a leucine, and the leucine at the same position in pNL4-3 was changed to an isoleucine (see Fig. 1b). Parental plasmids were digested with *Bam*HI and *Xho*I, resulting in the generation of a 0.4-kb fragment which encompassed most of the third exon of *rev* and part of the *nef* gene and an approximately 13.5-kb fragment that contained the rest of the proviral genome and plasmid vector (pNMAΔBamHI-XhoI and pNLΔBamHI-XhoI, respectively). The 0.4-kb fragments were then cloned into the *Bam*HI-*Xho*I-digested pBluescript II KS(−) phagemid vector (Stratagene) (51). These constructs were designated pBKS5, -7, -21, and -N.

dut-ung mutagenesis was performed essentially as described by McClary et al. (40). Briefly, the pBKS5, -7, -21, and -N constructs were transformed into electrocompetent *E. coli* CJ236, and cultures were propagated. Uracil-containing single-stranded DNA packaged in phage particles was produced by superinfection of cultures with M13KO7 helper phage. A phosphorylated mutagenic oligonucleotide was annealed to purified single-stranded DNA, and a complementary strand was synthesized. The sequences of the mutagenic oligonucleotides were 5′-CAGCTACCACCGcTTGAGAGACTTACTC-3′ (Ile→Leu) and 5′-CAGCTACCACCGaTTGAGAGACTTACTC-3′ (Leu→Ile) (the lowercase letters identify introduced nucleotide changes). The resulting heteroduplexes were transformed into electrocompetent *E. coli* DH5α, and transformants were propagated and analyzed for restriction fragment length polymorphisms. Four clones from each transformation were sequenced over the site of the mutation, and the inserts of selected clones were sequenced in their entirety by dideoxy DNA sequencing. No base alterations other than the desired mutation were observed.

DNAs from mutagenized constructs were digested with *Bam*HI and *Xho*I and ligated with the respective pNMAΔBamHI-XhoI or pNLΔBamHI-XhoI vector. Ligated DNA was transformed into chemically competent *E. coli* HB101, and transformants were analyzed by using restriction enzymes to verify the plasmid structure. Selected clones were grown in larger scale, and DNA was purified and sequenced over the site of the mutation to verify that the mutagenized fragment had been inserted.

Mammalian cell culture. COS-7 cells were obtained from the American Type Culture Collection (Rockville, Md.) and maintained in Dulbecco's modified Eagle's medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM) (PSG), and 10% fetal bovine calf serum (FBS). MT-2 cells were maintained in RPMI 1640 medium containing PSG and 10% FBS. Human lymphocytes from HIV-seronegative donors were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation, resuspended at 10^6 cells per ml in RPMI 1640 medium supplemented with 20% FBS and PSG, and activated with 5 mg of phytohemagglutinin (PHA) per ml for 2 days. Nonadherent cells were harvested, resuspended at a concentration of 10^6 cells per ml, and maintained in RPMI 1640 medium supplemented as described above plus 20 U of human recombinant interleukin-2 (IL-2) (Boehringer Mannheim) per ml according to standard procedures (19). Monocyte/macrophage (M/MØ) cultures were prepared from human HIV-seronegative donor lymphocytes as described previously (61).

DNA transfection. The African green monkey kidney cell line COS-7 was chosen for DNA transfection because it does not express CD4 and therefore cannot be readily infected by HIV and because it is transfected with high efficiency. Transient DNA transfections were performed essentially as recommended for adherent cells by the manufacturer of Lipofectin (Gibco/BRL). Transfection conditions were optimized with a β-galactosidase expression plasmid (pCDM8-lac) (4a, 53), and a transfection efficiency of 50 to 60% was obtained (data not shown).

Briefly, COS-7 cells were plated on six-well tissue culture dishes and incubated until 80 to 90% confluency was achieved. Eight micrograms of proviral DNA or pUC18 DNA (negative control) was mixed with 10 μg of Lipofectin and incubated at room temperature for a minimum of 15 min. Then cells were washed three times with Dulbecco's modified Eagle's medium and incubated with 2.5 ml of serum-free medium. The DNA-Lipofectin complex was added dropwise, and cells were incubated for 13.5 h at 37°C in a humidified, 5% CO_2 incubator. Subsequently, 2.5 ml of Dulbecco's modified Eagle's medium–PSG–20% FBS was added (day 0).

MT-2 cells were transfected with Lipofectin according to the manufacturer's recommendations for nonadherent cells. Briefly, 2×10^6 MT-2 cells were transfected with 15 μg of DNA and 10 μg of Lipofectin. The serum-free incubation time was 13.5 h. Samples were collected for analysis on day 1 and every 2 days thereafter.

Virus infection studies. For peripheral blood lymphocyte (PBL) infection studies, 5×10^6 PHA-activated, IL-2-stimulated uninfected donor PBLs were added to transfected COS-7 cells on day 2. Cells were cocultured until day 4, after which PBLs were removed and cultured alone. Fresh PHA-activated, IL-2-stimulated uninfected donor PBLs were added weekly. Supernatants were collected for virus assays daily on days 1 through 4 and every 3 days thereafter until day 28 or 52.

For M/MØ infection studies, COS-7 cell supernatants from day 3 were used to infect 7-day-old M/MØs as described previously (61). Supernatants were collected every 3 days for a total of 45 days and precipitated with polyethylene glycol prior to virus assays (28).

Virus assays with RT and p24 antigen enzyme-linked immunosorbent assays (ELISA). Virion-associated reverse transcriptase (RT) activity was essentially measured as previously described (28). Briefly, 15 μl of viral supernatant was mixed with 50 μl of an RT reaction mixture which contained a poly(A)·poly$(dT)_{10}$ template primer (0.3 U/μl) (Sigma Chemical Co.) in 50 mM Tris-HCl (pH 7.8)–75 mM KCl–2 mM dithiothreitol–5 mM $MgCl_2$–0.05% Nonidet P-40–10 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′,*N*′,*N*′-tetraacetic acid]–20 mCi of [^{32}P]TTP (3,000 Ci/mmol) per ml. Following a 2-h incubation at 37°C, 15 μl of the reaction mixture was spotted onto DEAE ion-exchange paper (Whatman) and washed to remove unincorporated nucleotides with 1× SSC (0.15 M NaCl, 0.015 M sodium citrate) five times for 5 min each, with two subsequent 3-min washes in 95% ethanol. Filters were dried and counted in a scintillation counter. All RT reactions were performed in triplicate or quadruplicate. RT values that exceeded the background by more than 3 times were considered positive.

ELISA for the detection of p24 antigen were performed by using a HIVAG-1 ELISA kit according to the manufacturer's recommendations (Abbott Laboratories). All ELISA values reported are the means of duplicate dilutions of each sample.

PCR amplification of the third exon region of *rev*. Amplimer sequences for nested PCR were chosen from conserved areas immediately flanking the second coding exon of *rev*. The first-round amplimers were 5′-GCTGTACTTTCTATAGTGAATAGAGTTAG-3′ (positions 8312 to 8339 in pNL4-3) (Alrev1) and 5′-TAAGATGGGTGGCAAGTGGTCACGCGGCCGCATGCTGAC-3′ (positions 8783 to 8804) (Alrev2). The second-round amplimers were 5′-GTGAATAGAGTTAGGCAGGG-3′ (Alrev3) (positions 8327 to 8346) and 5′-TCTGTCCCCTCAGCTACTG-3′ (positions 8676 to 8694) (Alrev4). The HIV provirus

copy number in patient samples was established through dilution series by PCR. First- and second-round PCR mixtures of 100 μl contained patient sample DNA (round 1) or 2.5 μl of round 1 product (round 2), 10 (round 1) or 50 pmol (round 2) of each primer, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 (round 1) or 1.8 mM (round 2) $MgCl_2$, 200 mmol of all four deoxynucleoside triphosphates, 0.01% Triton X-100, and 2.5 U of Amplitaq polymerase (Perkin-Elmer Cetus). Thirty-five and 30 cycles of amplification were performed in rounds 1 and 2, respectively, in a Perkin-Elmer Gene Amp PCR System 9600. Each cycle consisted of 1 min at 94°C, 30 s at 57°C, and 1 min (round 1) or 45 s (round 2) at 72°C. The last cycle was followed by a 5-min incubation at 72°C. Each PCR mixture contained patient DNA equivalent to 4 to 5 HIV copies, and five separate PCRs were performed for each sample.

Cloning of PCR amplicons. PCR amplicons were cloned directly from pooled PCRs from each patient by using a TA cloning kit according to the manufacturer's recommendations (Invitrogen Corp.). Transformants were propagated and analyzed by restriction site analysis. Five to eight clones from each patient sample were grown in larger scale and processed for sequencing.

DNA sequencing. Dideoxy DNA sequencing was performed by using a Sequenase-2 kit (U.S. Biochemical Corp.) and ^{35}S-dATP according to instructions provided by the manufacturer. Automated sequencing was performed with Sp6 and T7 sequencing primers and dye-labeled terminators on a model 370A DNA Sequencer (Applied Biosystems, Inc.) according to the manufacturer's recommendations (DyeDeoxy terminators [Applied Biosystems, Inc.]).

Sequence analyses. Basic sequence manipulations were performed on a SUN 4/260 workstation by using the IG suite programs (Intelligenetics). Sequences were aligned by using GENALIGN and improved by eye with MASE (14), and divergence analysis was performed with DOTS (55a).

All 74 *rev* gene sequences found in the HIV-1 database (42) were used in the sequence analysis described in Results. Twenty-nine originated in North America (MN, ALA1, BRVA, SC, SF2, NY5NEW, JFL, ADA, WMJ12, SF162, JRCSF, P28TZ, HXB2CG, REHTLV3, 1SG3X, BAL2A, SEGP, JH32, ENVBA1A, CDC42, ENV, SF33, SF2B13, PROV, YU2X, YU10X, SFAAA, RF, and WMj22). Thirty-three *rev* sequences were from Europe (WMJ22, ENVVG, ENVVH, ENVVE, ENVVF, ENVVD, ENVVC, ENVVB, ENVVA, ENV3L, ENV3B, ENV4B, ENV4L, ENV20, ENV22, ENV28, ENV27B, ENV27L, HIVHN53, HAN, IO1038_REV, H3BH8, SIMI84, 5393ENV, IHB101, ENVBD, ENVBB, ENVBF, ENVBC, ENVBE, ENVBA, CAM1, and BRU), three were from Japan (GUNAB, GUNAA, and ETR), and 12 were from Africa (ELI, OYI, Z2, NDK, JY1, MAL, Z321, SF1702, SF1703, U455, ANT70, and MVP5180). All of the American, European, and Japanese *rev* sequences originated from envelope subtype B HIV. The African *rev* sequences were derived either from subtype A (U455, Z321, SF1702, and SF1703), B (OYI), or D (ELI, Z2, NDK, JY1, and MAL) or from group O (ANT70 and MVP5180).

Nucleotide sequence accession numbers. DNA sequences have been submitted to GenBank under accession numbers U30730 through U30786.

RESULTS

Four complete HIV-1 envelope genes from the PBMC of an asymptomatic man at one time point (33) were inserted into the infectious provirus NL4-3 (1). They were chosen since it had previously been shown that the gp120-coding portion of each contained no known inactivating mutations and that these four corresponded to the breadth of *env* gene diversity found among the 13 clones examined (33). Furthermore, after the completion of sequence analysis, no out-of-frame deletions, insertions, or in-frame stop codons were found in any of the chosen full-length *env* or overlapping *rev* and *tat* gene sequences (data not shown).

Infectivity and tropism of chimeric (pNMA) viruses. Each chimeric provirus (pNMA5, -7, -16, and -21) was found to produce viral p24 Gag antigen upon transfection into COS-7 cells (data not shown). Only pNMA5 and -21 consistently produced amounts of p24 equivalent to that produced by the positive control, pNL4-3. Two days following transfection, PBLs were added to COS-7 cells, and virus was propagated in COS-PBL coculture for 2 days. Subsequently, PBLs were removed and cultured alone with weekly supplementation of donor lymphocytes. Culture supernatants were analyzed every third day over either 30 days for the presence of p24 antigen or 52 days for the presence of RT activity.

As expected, NL4-3 virus produced in COS-7 cells was able to grow in COS-PBL coculture (Fig. 1a [open circles]). Depending on the source of donor lymphocytes, two patterns of infection were observed with this virus. Either a peak level of p24 (or RT [data not shown]) was observed on day 3 or 4 with a transient decrease and subsequent increase in p24 or RT, or a gradual increase in the level of p24 or RT took place throughout the initial week of infection. The pattern was consistent within duplicate experiments with the same donor lymphocytes but varied between experiments with different donor lymphocytes. A peak in p24 or RT production was observed following weekly lymphocyte supplementation of all cultures during the first 3 weeks. The p24 or RT levels peaked between days 20 and 25 and then decreased despite additional supplementation with donor lymphocytes. Coincident with the decline, gross cytopathological changes (syncytia and cell fragmentation) were observed.

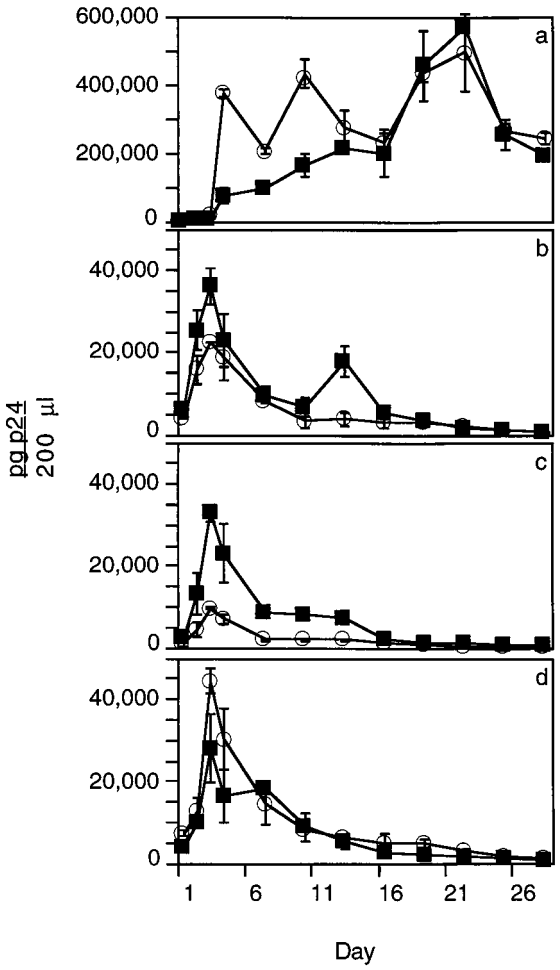


FIG. 1. Growth in PBMC of recombinant clone-derived HIV-1 strains NL4-3 and NL (Leu-78→Ile) (a), NMA5 and NMA5 (Ile-78→Leu) (b), NMA7 and NMA7 (Ile-78→Leu) (c), and NMA21 and NMA21 (Ile-78→Leu) (d). The growth curves of wild-type virus (open circles) and mutated virus (filled squares) are shown. Experimental points were determined in duplicate in each experiment, and error bars refer to the standard deviations of the means of two independent experiments. Proviral constructs were transfected into COS-7 cells, PBLs were added on day 2, and cells were cocultured for 2 days. Then PBLs were removed from the COS-7 cells and cultured alone for 28 days. Cultures were supplemented with fresh PHA-activated, IL-2-stimulated HIV-negative donor PBLs weekly and assayed for HIV-1 p24 antigen as described in Materials and Methods.

In contrast, the chimeric viruses NMA5, -7, -16, and -21 did not spread in COS-PBL or PBL culture. After an initial p24 antigen (or RT [data not shown]) peak following COS-7 cell transfection, the levels decreased continuously (Fig. 1b

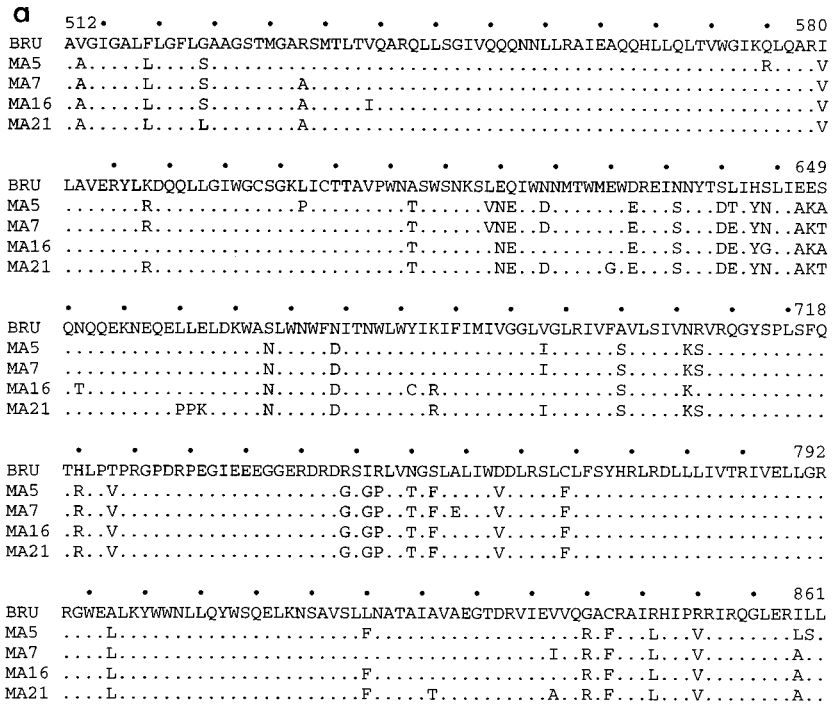


FIG. 2. Amino acid sequence alignments of full-length gp41 sequences from patient MA (a) and of Rev (b) and the corresponding gp41 region (c) derived from patient MA, LTS (patients 1 [50824], 2 [50306], 3 [50333], 4 [50365], and 5 [50823]), patients with AIDS (patients 6 [923064], 7 [931395], 8 [RF], and 9 [LA]), and laboratory-adapted viruses, including the prototype HIVBRU sequence used as a reference. Only amino acids that differ from the BRU sequence are shown. Identical amino acids (dots) and gaps introduced to maintain alignment (dashes) are indicated. X, not determined. A number to the right of a sequence signifies the total number of identical sequences obtained from that patient. The amino acid residues involved in the Rev activation domain are indicated above the alignment in panel b. Amino acids 75 to 83 denote the activation domain as defined by Malim et al. (38), and amino acids 78 to 93 denote the activation domain as defined by Weichselbraun et al. (63). The conserved leucine residues within this domain are underlined.

through d [open circles]) (data for NMA16 not shown). Similarly, when these COS-7 cell supernatants or NL4-3 supernatants were used to infect M/MØs, a continuous decrease in RT activity, consistent with dilution of RT due to the maintenance of cultures and degradation of protein over time, was observed. No positive RT values were observed during the 52-day culture period with M/MØs (data not shown).

Analysis of MA gp41 sequences. To understand the lack of infectivity of NMA proviruses, we studied their nucleotide and deduced amino acid sequences in detail. As mentioned above, each clone had previously been shown to encode an open gp120-coding sequence (33). The same was true for the sequences of the gp41-coding region (Fig. 2a) and the overlapping Rev (Fig. 2b) and Tat (data not shown). A series of mutations that were consistent among MA gp41 sequences but distinct from the NL4-3 (BRU) sequence were found in the fusion peptide, leucine zipper domain, and transmembrane domain, but many were common to other infectious virus sequences (Fig. 2a and c). The average diversity between pNMA gp41 nucleic acid sequences was 2.4%, and the average diversity was between 7 and 10.5% when these sequences were compared with other envelope sequence subtype B viruses.

Analysis of the MA RRE sequence and the second coding exon of *rev* and *tat*. The Rev-responsive element (RRE) begins approximately 12 nucleotides downstream from the cleavage site of gp120 and gp41 and spans 234 bases. This region has the potential of forming four stem-loops and one branched stem-loop (34, 36). The MA RRE sequences were very similar to those observed for other HIV-1 sequences, with only one pair of changes unique to the MA clones. Nucleotides 26 and 27 were changed from the conserved GG sequence to either TC (pNMA5, -7, and -16) or TT (pNMA21). This change affects stem-loop I and a potential Rev binding site (30). However, it has been demonstrated that stem-loop I is not essential for RRE function, even though it does affect the level of biological activity of the RRE (9, 39).

The second coding exon of *tat* in MA sequences is invariant and identical to that of HIVNY5NEW except for position 5, at which the MA sequences encode a serine instead of a proline (data not shown). However, both amino acids are commonly found at this position in other strains (42).

Within Rev, three functional domains are recognized. An arginine-rich motif (amino acids 35 to 50 [Fig. 2b]) appears to be necessary for Rev nuclear or nucleolar localization and specific binding to the RRE (36). Sequences flanking the arginine-rich motif (approximately amino acids 18 to 56) are essential for multimerization of Rev on the RRE target sequence (18, 37, 64). The arginine-rich motifs in the MA sequences were identical to those of HIVBRU, but four consistent changes were observed in the flanking region, Asn-30→Ser, Gln-51→Arg, His-53→Arg, and Ser-54→Thr. All but the His-53→Arg mutation have been found in other strains. A highly conserved, leucine-rich activation domain is positioned in the C-terminal part of Rev and has been mapped to amino acids 75 to 83 (37, 38) and 78 to 93 (63), thus including either four (Leu-75, Leu-78, Leu-81, and Leu-83) or three (Leu-78, Leu-81, and Leu-83) leucines (Fig. 2b). In all of the PBMC Rev sequences determined from this time point ($n = 9$), Leu-78 was replaced by an isoleucine. The causative nucleotide substitution (C-8525→A) does not change an amino acid in the envelope reading frame and is situated outside the Tat coding region.

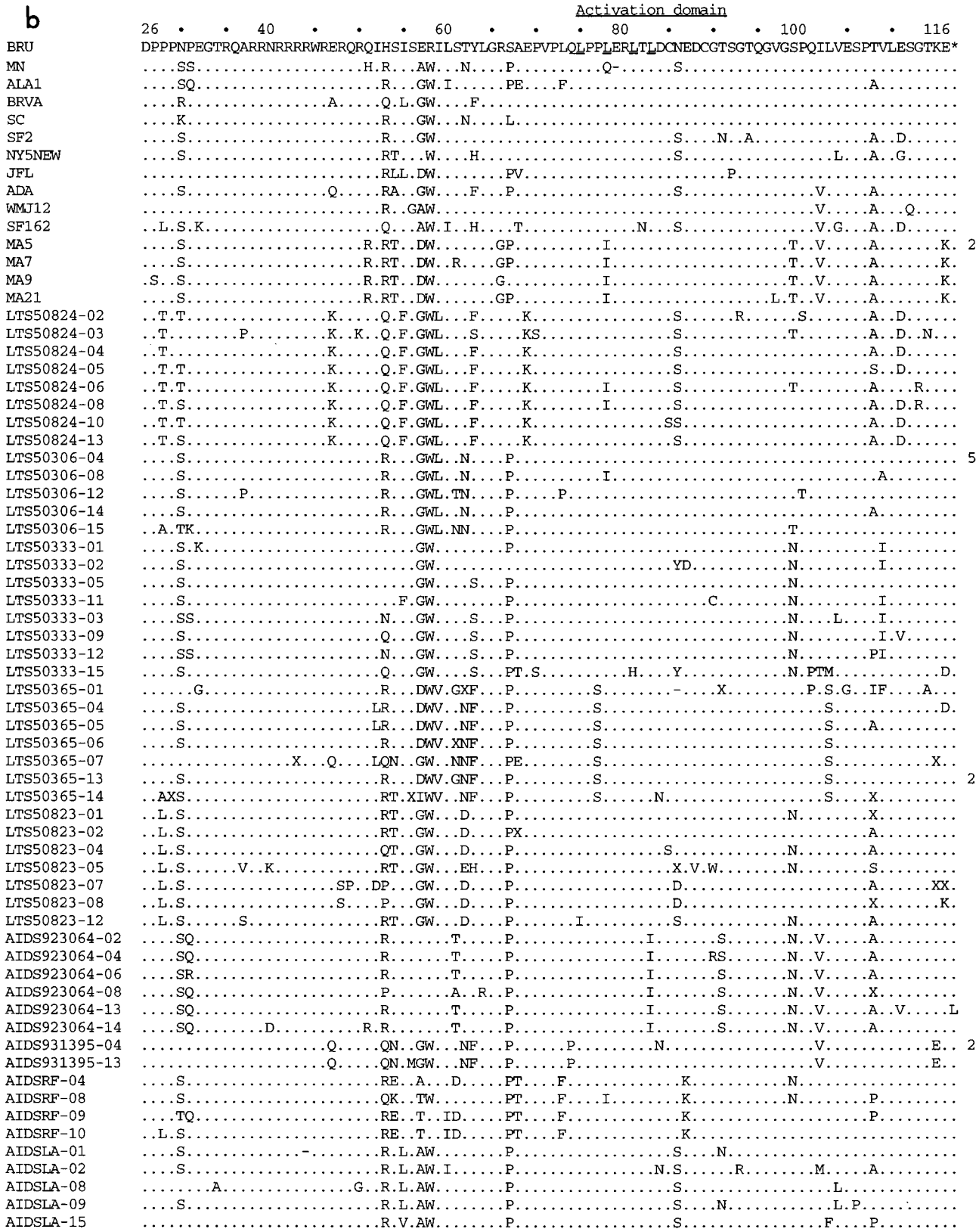


FIG. 2—*Continued.*

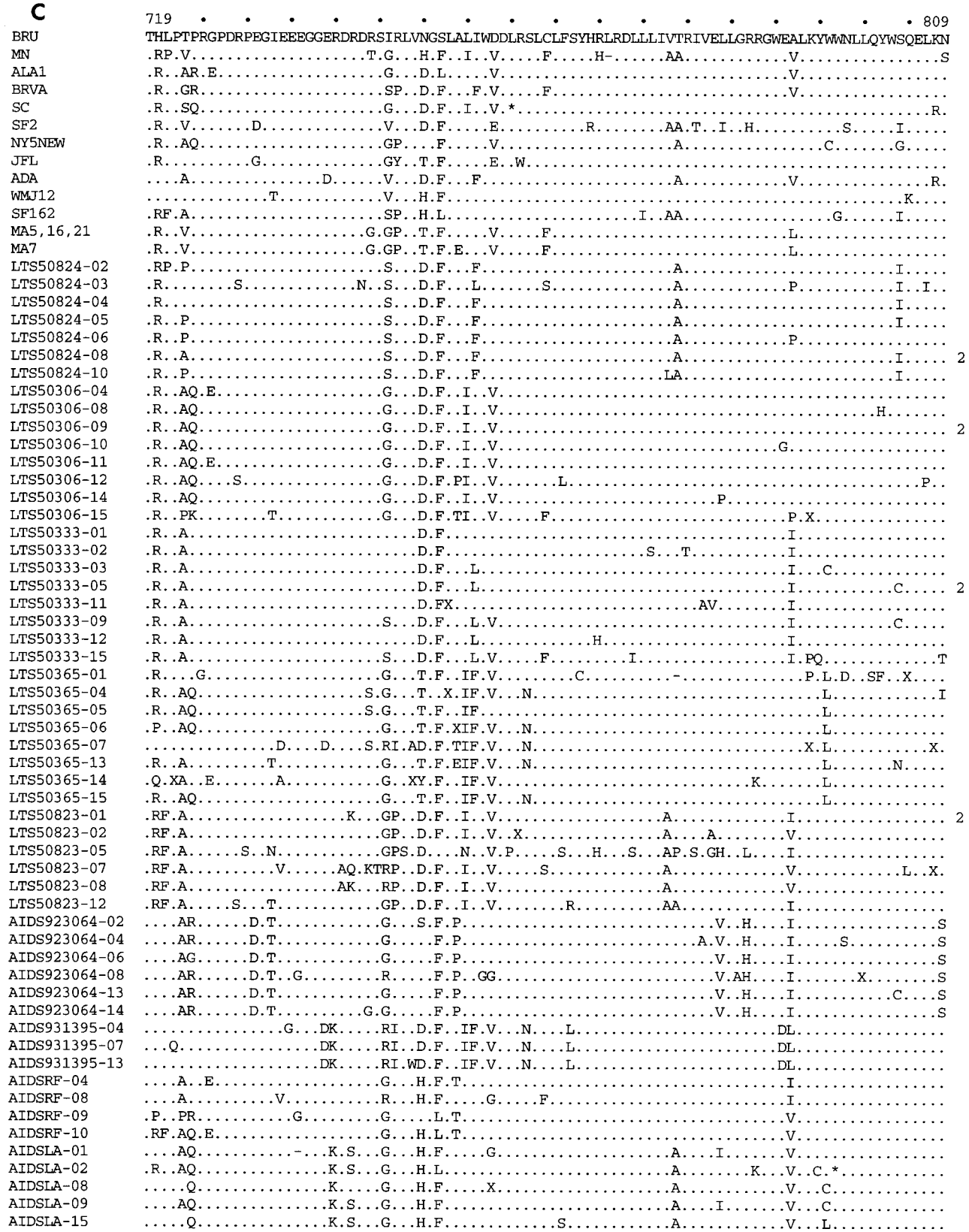


FIG. 2—*Continued.*

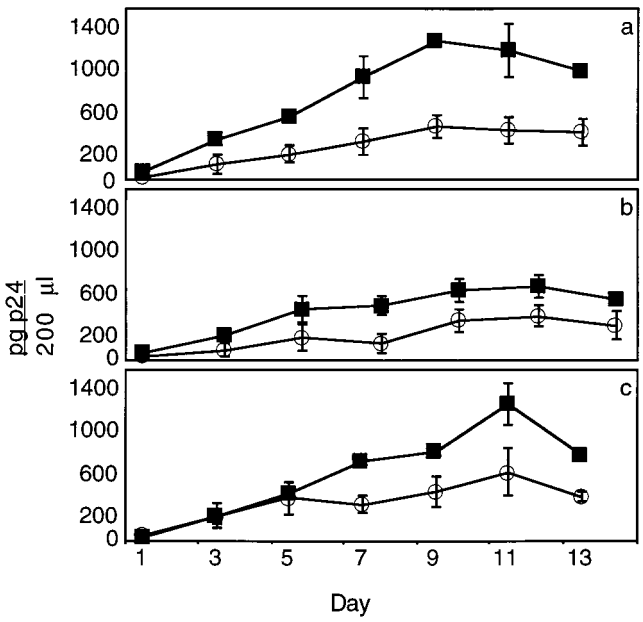


FIG. 3. Comparison of the growth of NMA5 and NMA5 (Ile-78→Leu) (a), NMA7 and NMA7 (Ile-78→Leu) (b), and NMA21 and NMA21 (Ile-78→Leu) (c) in MT-2 cells. The growth curves of wild-type virus (open circles) and mutated virus (filled squares) are shown. Experimental points were determined in duplicate in each experiment, and error bars refer to the standard deviations of the means of two independent experiments.

Functional consequences of Rev changes. Because DNA sequence analysis demonstrated that the MA Rev activation domains carried a unique Leu-78→Ile substitution, the possibility that a Rev malfunction was responsible for the lack of NMA infectivity was addressed. Chimeric proviruses MA5, -7, -16, and -21 and NL4-3 were transfected into MT-2 cells. These cells are productively infected with human T-cell leukemia virus type 1 (HTLV-1) and contain the HTLV-1 regulatory proteins Rex and Tax that can substitute for HIV-1 Rev and Tat (6, 48, 56, 65). In contrast to PBL cultures, NMA viruses did replicate in MT-2 cells, as shown by continuous increases in p24 antigen levels in all cultures during the first 2 weeks (Fig. 3). Syncytia were observed in NL4-3 cultures from day 3 and increased in size and number during the culture period. Small syncytia consisting of three to seven cells were observed in all NMA virus cultures from day 5 and increased slightly in number over time. No syncytia were observed in the plasmid-transfected negative control culture. The virus growth curve and absolute amount of p24 produced in NMA virus cultures varied but were comparable to those obtained during coculture of infected PBMC and MT-2 cells (45). In conjunction with analyses of *tat*, *rev*, and *env* genes, these studies suggested that the lack of growth of NMA viruses in PBLs was due to a deficient Rev.

To evaluate the relationship between the suspected Rev malfunction and the consistent Leu-78→Ile-78 substitution in the MA *rev* activation domain, four mutant *rev* viruses were made by site-directed mutagenesis. The Ile-78 in constructs pNMA5, -7, and -21 was changed to a leucine, and the Leu-78 in pNL4-3 was changed to an isoleucine. When pNMA5 (Ile-78→Leu) was transfected into COS-7 cells and cocultured with PBLs, peaks of p24 antigen (Fig. 1b [filled squares]) and RT activity (data not shown) were observed on day 13. Transfection with pNMA7 (Ile-78→Leu) resulted in steady levels of p24 and RT during days 4 through 11 (Fig. 1c), whereas transfection with pNMA21 did not result in increases in p24 or RT (Fig. 1d). Higher levels of p24 and RT were also generated when NMA Rev mutant viruses were grown in MT-2 cells (Fig. 3). In contrast, the pNL4-3 (Leu-78→Ile) virus did not grow as well as wild-type pNL4-3 in the PBMC assay during the first 10 to 18 days (Fig. 1a); thereafter, equivalent amounts of p24 and RT were produced for 6 days, coincident with cytopathological changes in both cultures. As previously observed, the levels of p24 and RT decreased after day 24, despite supplementation of cultures with fresh donor lymphocytes.

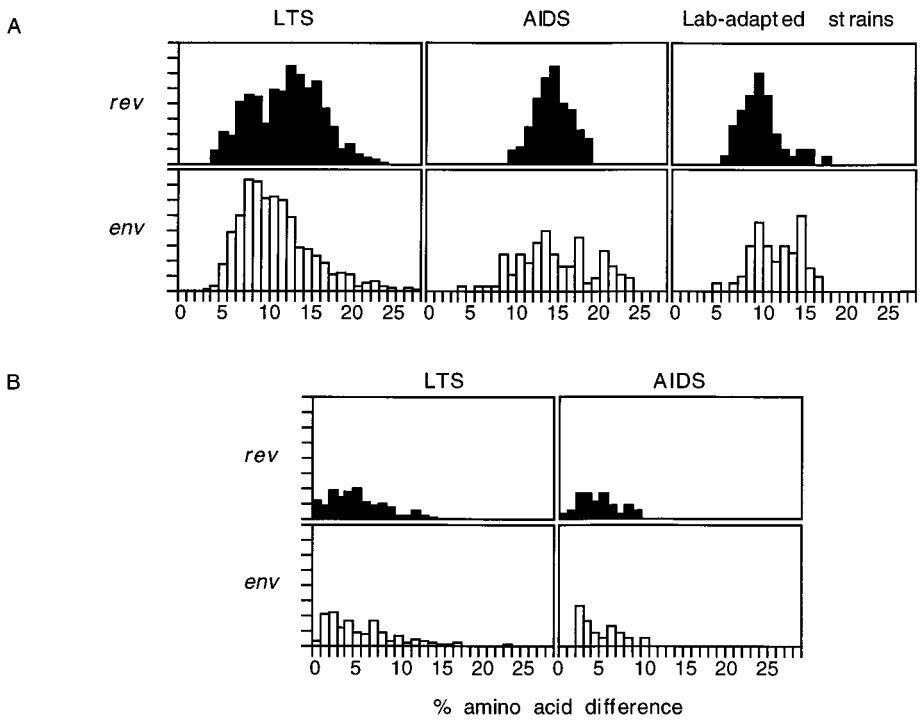
Prevalence of Rev-deficient HIV-1 genomes from subject MA in culture and PBMC over time. Since our data strongly suggested that NMA viruses were defective for growth in PBMC coculture at least in part because of a deficient Rev and since patient MA has (at this writing) remained asymptomatic for 10 years, we hypothesized that the prolonged asymptomatic stage is due in part to the persistent replication of attenuated HIV strains within him. Recent evidence suggests that the turnover of proviral sequences in PBMC lags behind that found in cultured isolates (12). We therefore examined the Rev activation domain coding sequences of four clones from a cultured virus isolate derived from the initial time point studied, as well as two PBMC samples taken 37 and 51 months later. Fragments (367 bp) encompassing the second coding exon of *rev* were amplified by PCR, cloned, and sequenced. All 12 sequences retained the Leu-78→Ile codon in Rev and were otherwise identical to the original sequences from this patient for the Rev activation domain (data not shown).

Rev gene variation within distinct clinical groups of HIV-infected individuals. As a further examination of the hypothesis that a prolonged asymptomatic state in HIV-1-infected individuals is due to virus growth attenuation resulting from mutations in the Rev activation domain, we evaluated *rev* gene variation within HIV-infected individuals belonging to distinct clinical groups. PBMC samples were obtained from five HIV-infected LTS (Fig. 2c) and two patients with AIDS, while spleen samples were obtained at the autopsies of two other patients with AIDS (patients LA and RF).

Fragments (367 bp) covering the Rev activation domain were again determined (four to eight clones per individual). Comparisons of the nucleic acid sequences demonstrated that none of the clones were identical (data not shown). The translated Rev activation domains and corresponding gp41-encoding sequences are presented in Fig. 2b and c. Included in the alignments for comparison are 11 Rev sequences from independent, laboratory-adapted subtype B virus isolates.

No known inactivating mutations were observed in any sequence obtained from patients 1 through 8; however, in a sequence from patient 9, a deletion of Arg-47 in the Rev reading frame (clone 901) and an in-frame stop codon at codon 290 in the gp41 reading frame (clone 902) were observed. Selection for maintenance of continuous Rev and Env reading frames was suggested by the low ratios of nonsynonymous to synonymous mutations, 1.7 in the Rev reading frame and 1.8 over this region of the Env reading frame, compared with 2.5 over full-length MA gp41 genes and 1.5 in gp41 genes from tissue culture-adapted viruses.

Within the individual groups of LTS, patients with AIDS, and laboratory-adapted HIV-1 strains, all pairwise comparisons of sequences were made in terms of the Rev and Env reading frames (Fig. 4). A trend of slightly higher divergence in Rev than in Env was noted for the LTS group, equivalence was noted for the group of patients with AIDS, and higher divergence in Env was noted for the laboratory strain group, although none of these differences were statistically significant at the 95% level.



C

Group	Rev	Env
LTS (n=10)	13.7	12.4
AIDS (n=6)	17.2	17.5
Lab. strains (n=6)	11.4	12.9
Total (n=22)	14.1	13.9

FIG. 4. Distribution comparison of the percentages of amino acid differences between pairs of Rev and gp41 sequences for sets of sequences obtained from five LTS, four patients with AIDS, and 11 independent laboratory-adapted HIV strains (HIVBRU, HIVMN, HIVALA1, HIVBRVA, HIVSC, HIVSF2, HIVNY5NEW, HIVADA, HIVJ12, HIVSF162, and HIVJRCSF). (A) Interpatient HIV strain differences; (B) intrapatient differences. The *y* axis is constant within a group but different between groups. (C) The average pairwise divergence was calculated by using independent pairs of sequences in a two-factor analysis of variance.

Variability in the four leucine positions (Leu-75, Leu-78, Leu-81, and Leu-83) in the Rev activation domain was found in a minor subset of clones from four of five LTS and two of four patients with AIDS (Fig. 2b). The change found in the clones from LTS patients 1 (two of eight clones) and 2 (one of nine clones) and patient 8 with AIDS (one of four clones) was a Leu-78→Ile substitution identical to the change observed in MA sequences. For LTS patient 5, a Leu-75→Ile substitution was observed in one of seven clones, and for LTS patient 3, a Leu-81→His substitution was found in one of eight clones. Finally, all six clones from patient 6 with AIDS carried a Leu-83→Ile substitution. Clones from two patients with AIDS and LTS patient 4 displayed no variability in the four leucine positions, but all eight clones from LTS patient 4 had a Pro-77→Ser substitution.

DISCUSSION

We have demonstrated that an observed leucine-to-isoleucine substitution in the activation domain of the HIV-1 Rev protein in an asymptomatic subject contributes to virus attenuation in vitro. Combined with the data of Malim et al. (38), the data suggest that this position (amino acid 78) is a determinant of the stability and/or activity of the protein. However, this substitution alone does not fully account for the lack of growth of NMA viruses in PBMC and M/MØ, because the replication of NL4-3 carrying the same mutation is only partially inhibited and growth of the mutated NMA viruses is still relatively attenuated. No individual changes or groups of changes distinguishing the MA sequences from those of previously studied strains or suggesting that they account for this attenuation were found in the V1 (58), V2 (2, 20, 58), or V3 (10, 15, 41) of gp120 or the fusion peptide (16), leucine zipper (11, 17), or transmembrane regions (5, 25) of gp41.

This Rev activation domain mutation persisted in cultured virus and PBMC for at least another 4.3 years. It is interesting that this particular culture was only poorly infected with a highly clonal virus population, as indicated by a near homogeneous gp120-coding sequence population (33) and infection in only approximately 1 per 31,000 cells (data not shown). These results suggest that the viruses under study are not defective but highly attenuated for growth in vitro, yet they persist in vivo. Therefore, a larger spectrum of replication-competent viruses may exist in vivo than is normally identified by isolation in vitro.

We decided to address the speculative but potentially significant hypothesis that the Rev-attenuating mutations commonplace in the virus from patient MA represent a mechanism

by which some LTS achieve a prolonged asymptomatic state. Analyses of Rev activation domain sequences in the HIV database, with regard to the leucines at positions 75, 78, 81, and 83, demonstrated only one divergent sequence (HIVMN) within the subtype B viruses ($n = 65$) as well as four divergent sequences (HIVU455 [A], HIVZ321 [A], HIVANT70 [O], and HIVMVP5180 [O]) within non-B viruses ($n = 13$). The two group O sequences, whose replicative capacities are unknown, carried a Leu-83→Ile substitution similar to that found in replication-competent HIV2ROD (24, 62). HIVMN and HIVU455 are replication defective (23, 42, 43), while a chimeric HIVHXB2-HIVZ321 virus replicated more slowly than did HXB2 (57), compatible with our pNL (Leu-78→Ile) results. The virtual homogeneity of subtype B Rev sequences in the database was intriguing. Most were obtained from virus isolates from patients with AIDS. Such patients generally have high viral loads (3, 22, 35, 50), possibly as a result of the outgrowth of fast-replicating viruses carrying well-functioning Rev molecules, and cultured viruses are selected for in culture partially on the basis of replicative capacity.

To obtain a more representative sampling of *rev* variation and address the hypothesis that Rev attenuation is more common in asymptomatic individuals, sequences were obtained by direct PCR amplification of HIV in PBMC DNAs from individuals at different stages of disease. However, variability within the Rev activation domain was found only in a minor proportion of sequences from all five LTS and two of four patients with AIDS. The majority of amino acid changes were found at the four leucine positions, at which substitutions have been shown to give rise to Rev molecules with either attenuated (position 75) or *trans*-dominant, negative phenotypes (positions 78, 81, and 83) (36, 38, 47). However, the HIV-2 Rev activation domain consensus sequence has an isoleucine at position 83 and is interchangeable with HIV-1 (18). The proline-to-serine change at position 77 might also result in attenuated Rev molecules, as a proline-to-aspartic acid substitution at this position has been demonstrated to result in a 40% decrease in Rev activity (38).

Pairwise divergence analyses were also performed with the 93 amino acid sequences encompassing the second translated exon of *rev* in both the Rev and Env reading frames from LTS, patients with AIDS, and laboratory-adapted strains. Only a weak trend toward higher diversity in *rev* than in *env* was noted for the LTS group. This and the slightly higher frequency of Rev-attenuating mutations in the activation domain are consistent with, but do not provide compelling evidence in support of, the hypothesis that Rev attenuation is commonly associated with virus attenuation or a prolonged asymptomatic state.

Studies of the RNA splicing pattern in HIV-infected individuals have revealed a picture of clinical HIV-1 infection characterized by two patterns of infection that can be explained by differences in Rev function (54). One pattern is prevalent in patients with AIDS and is indicative of Rev activity; it is characterized by productively infected cells that harbor a high ratio of unspliced viral RNA to multiple spliced viral RNA. The other pattern is characterized by a low unspliced-to-multiple spliced viral RNA ratio and suggests less Rev activity. The latter pattern is common in asymptomatic HIV-1-infected individuals and has been called blocked early-stage latency. A more recent study failed to confirm these patterns but did show that abundant HIV mRNA expression in PBMC preceded disease progression within 2 years, whereas mRNA levels were undetectable in PBMC of long-term asymptomatic infected individuals (50). Similarly, enhanced expression of HIV *gag* mRNA was demonstrated to correlate with a decline in $CD4^+$ cells and the development of AIDS (22). It was suggested that the differences that arise between the patterns found in LTS and patients with AIDS are due to either a particularly effective anti-HIV immune response or a lower replicative potential for the virus.

Thus, one hypothesized difference between LTS and patients with AIDS is that LTS harbor a higher proportion of viruses with defective and/or attenuated *rev* genes, resulting in undetectable or lower levels of HIV mRNA production in a large percentage of infected cells. Rather than latency, we call this "attenuated productive viral infection." This attenuation may be due to changes in the second coding exon of *rev*, possibly complemented, as indicated in our study of the MA gp160 genes, or substituted for by additional attenuating changes elsewhere in the viral genome. During this study, we determined the nucleotide sequence of the first third of the *nef* gene in four MA clones and found one stop codon (in clone MA16). Therefore, *nef* gene inactivation may also be implicated in a persistent asymptomatic state, as suggested also by the consistent finding of a defective *nef* in one of five LTS examined by Kirchhoff et al. (29). Our hypothesis is further supported by recent studies detailing the low viral load and poor replicative potential of virus in LTS (7, 46). Thus, the level of viral replication within an infected individual would result not only from a balance between the immune system and the HIV population but also from a balance within the HIV population between viruses with different growth potentials that carry regulatory genes with various degrees of transactivating or *trans*-dominant inhibitory ability.

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