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First demonstration of a lack of viral sequence evolution in a nonprogressor, defining replication-incompetent HIV-1 infection

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Abstract

It is universally acknowledged that genetic diversity is a hallmark of HIV-1 infection, and it is one of the traits that has considerably hampered the development of an effective vaccine. In a study of full-length HIV-1 genomic sequences (>9 kb), we show unique evidence for complete absence of viral evolution in an individual with truly nonprogressive infection. Gross gene defects were not detected, but the state of replication incompetence was attributed to the presence of stop codons in the structural genes *gag* p17 and p24 and in *pol RT*, which emerged as a consequence of G–A hypermutation. These inactivating mutations may have occurred early, soon after infection, during the clonal stage of primary viral replication, since these are the sole archival strains present today. This genetic homogeneity, with <1% variation between strains over an 8-year period, suggests that only limited proviral integration events occurred in this patient. Further study on the antigenic properties of this strain may assist in the development of HIV vaccines and therapeutics.

Keywords: Human Immunodeficiency virus type 1; Long-term nonprogressor; G–A hypermutation; Stop codon; Sequence evolution; Replication incompetence

Introduction

The rate of disease progression from initial infection until the development of severe immunosuppression varies greatly among infected individuals. Nonprogressors are differentiated from patients with progressive disease by maintenance of low or undetectable viral loads and stable CD4+ and CD8+ T-cell counts (Buchbinder et al., 1994; Cao et al., 1995; Sheppard et al., 1993). Although many HIV-infected individuals can survive for more than a decade, most of these long-term survivors (LTS) eventually progress to AIDS. There is a small fraction, termed "true" long-term nonprogressors (LTNP), who do not progress (constituting <0.8% of the total HIV-positive population), and represent the extreme end of the clinically asymptomatic population (Lefrere et al., 1997). These HIV-infected individuals maintain stable T cell counts, undetectable plasma RNA (<50 copies/ml) and proviral DNA (<10 copies/10^6 PBMC), and strong antiviral immune responses, in the absence of antiretroviral therapy, into their second or third decades of infection. Although many host and viral factors have been implicated in nonprogression (reviewed in Saksena et al., 2001), there is no single factor that universally determines a nonprogressive outcome.

Genetic diversity remains the hallmark of HIV disease progression. Both LTS and LTNP show similarities in terms of viral genetic diversity, immune responses, T-cell counts, and plasma viral loads during the asymptomatic stage (Cao

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et al., 1995; Pantaleo et al., 1995; Wang et al., 2000). Irrespective of the stage of disease, viral replication continues and genetic diversity increases (Shankarappa et al., 1998). Host immune selective pressure has a significant influence on viral evolution, resulting in the emergence of variants (quasispecies), particularly CTL escape mutants, which increase in dominance as disease progresses (Goulder et al., 1997b; Kelleher et al., 2001; Shankarappa et al., 1999). Rapid disease progression and late-stage disease are associated with restricted quasispecies diversity (Halapi et al., 1997; Shankarappa et al., 1998; Wang et al., 1997, 2000), and accumulation of genetic diversity indicates immunocompetence of the host (Borrow et al., 1997; Goulder et al., 1997a; Wang et al., 2000; Wolinsky et al., 1996). However, ongoing viral evolution in asymptomatic individuals eventually leads to immune escape, or increased pathogenicity, to the point that disease progression occurs (Shankarappa et al., 1999). Why this does not occur in a few rare LTNP needs to be resolved, as this may contribute to new therapeutic interventions aimed at slowing disease progression.

Truly nonprogressive HIV-infected individuals have generated enormous interest in the study of mechanisms that may confer natural control of HIV. To gain a better understanding of viral factors that may be associated with true nonprogression, we performed a detailed molecular investigation of full-length (>9000 bp) HIV-1 strains derived from a rare, truly nonprogressive individual.

Results

Clinical profile of the study subject

The clinical history of the study subject has been described in detail elsewhere (Wang et al., 2002). In brief, CD4 counts were within normal limits, while CD8 counts were elevated. Plasma viral RNA was undetectable (<50 copies) since the first available specimen, until the present (1993–2002), and proviral DNA burden was low (<10 copies/10^6 PBMC).

Viral isolation negativity

Isolation of the study subject's viral strain was attempted from each sample collected between 1998 and 2001, in 28-day co-cultures. All PBMC and CD8-depleted co-cultures were negative for virus isolation (data not shown). Increasing input cell number or length of culture did not facilitate isolation. No evidence of replicating virus was found in this individual. Viral isolation on PBMC stored between 1993 and 1998 was not attempted due to insufficient quantities of cells available.

Detection of antibodies to HIV proteins

Antibodies against all HIV proteins were detected from serum obtained between 1998 and 2001 (Fig. 1). Western blot patterns were consistently strongly reactive (4+) against all HIV-1 proteins (gp160, gp120, gp41, p55, p53, p31, p17 and p24), confirming that the study subject was infected and exposed to replicating virus.

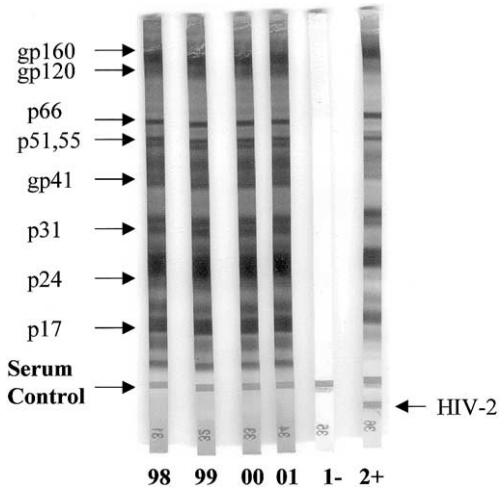


Fig. 1. Western blot reactivity of plasma collected between 1998 and 2001, compared with 1− (a HIV-negative control) and 2+ (a HIV-1-positive control). Antibody binding to all of the major HIV-1 proteins (gp160, gp120, p31, p55, p17, p24) was observed in the study subject.

PCR amplification

Attempts to amplify HIV-1 genome from plasma samples available between 1998 and 2001 failed, which was consistent with undetectable plasma viral RNA. Therefore, PBMC were used for HIV-1 gene amplification, despite the fact that the proviral DNA burden was <10 copies/10^6 PBMC. Initially, only limited gene regions (*env*, LTR, *vpr*, *nef*, and *pol*) were successfully amplified using conventional double- and triple-nested PCR, for phylogenetic analysis. Further, using primer pairs UP1A and LOW2 targeting the 5′ LTR and 3′ LTR regions, respectively, near full-length genomes were successfully amplified from PBMC samples collected between 1999 and 2001. In each case, the end 600 bp in the LTR was amplified separately to complete the full-length genome. Near full-length genomes were then used as templates for single-round amplification of individual viral genes, using gene-specific primer pairs (not shown). All attempts to amplify full-length genomes from samples collected before 1998 failed, possibly due to age or DNA degradation of these frozen specimens. High-grade genomic DNA is needed for long-range PCR. Only isolated genomic segments from *env, pol RT, vpr* and *nef* could be amplified from these older specimens, requiring double-nested PCR for *pol* and *vpr* regions, and triple-nested PCR for *env* and *nef.*

Full length HIV-1 genome analysis: gene-by-gene sequence and phylogenetic comparisons

Multiple full-length HIV-1 genomes from the study subject were sequenced to determine the level of viral evolu-

Table 1
Genetic distances (nucleotide and peptide) between different genomic segments in the full-length HIV genome from the study patient

Gene	Nucleotide sequence difference during 1999–2001	Peptide sequence difference during 1999–2001
gag	0.33%	0.4%
pol	0.25%	0.42%
*env*gp160	0.35%	0.35%
tat	0.98%	1%
rev	0.3%	0%
vif	0.18%	0
vpr	0	0
vpu	0	0
nef	0	0

tion, and whether viral gene defects could explain the lack of disease progression in this individual.

Tight sequence homology was observed in the gene segments that were studied. The maximum nucleotide variation (which includes both synonymous and nonsynonymous changes) in sequences derived from PBMC samples collected between 1999 and 2001 was 0.98%, seen in the *tat* gene (one nonsynonymous base change, which changed one of 102 amino acid residues), while there was no nucleotide variation seen in the *vpr*, *vpu*, and *nef* genes (Table 1). The maximum nonsynonymous variation seen in *gag* and *pol* was 0.41–0.42% (Table 1), yielding only <1% nonsynonymous variation detected in 3 years. Overall, <1% nucleotide variation was detected over 3 years, resulting in 0.49% non synonymous variation. Since HIV genomes have been reported to evolve *in vivo* at a rate of 1% per year and the *env* hypervariable regions at >1% (Lukashov et al., 1995), this genetic diversity over 3 years is negligible.

The HIV-1 amino acid sequences derived from the nucleotide sequences from the study subject were compared with the prototypic strain HIV-1 NL4.3. Although no major deletions were observed, a consistent feature was the presence of stop codons in Gag p17 and p24, and Pol RT (Fig. 2A and C). In addition, the start codon (ATG-methionine-M) in p17 was changed to ATA (isoleucine-I) (Fig. 2A). These *gag* gene stop codons were further verified by sequencing multiple clones from proviral genomes over time (Fig. 2B). The stop codons were a consequence of G–A hypermutation, characterized by a change of G bases to A in a particular genomic region (Fig. 2D), which usually occur in a string-like fashion. The majority of these G–A changes converted tryptophan (W) residues to stop codons in *gag*, but only one in *pol* (Figs. 2A and C).

Further confirmation of a complete lack of sequence evolution is based on the highly variable envelope gene, which was devoid of any changes over time (Fig. 2E). All hypervariable domains in the *env* gene (V1–V5) showed identical peptide sequences. In addition, the C2–V5 region sequences (650 bp) between 1993 and 2001 were identical to the respective areas of the full-length sequences (Figs. 2E and 3K). The absence of evolution was further confirmed by cloning the *vpr* gene from samples obtained between 1993 and 2001, which yielded identical sequences over these 8 years (data not shown).

Tight conservation in all functional motifs in accessory (Figs. 2F–I) and regulatory (Fig. 2J and K) genes was seen in all full-length genomes, including all cysteine residues in the Vif protein, which are necessary for viral infectivity (Ma et al., 1994). Overall, there were no defects, deletions, or stop codons in these nonstructural genes, with the exception of a deletion at residue 67 in Vpu. Residue 67 in Vpu is in the critical cytoplasmic α-helical domain II (Fig. 2H). A detailed search using the HIV Molecular Database showed the variable nature of this residue between all known HIV-1 subtypes and SIVcpz, but in no sequence was deletion of this residue was seen. The biological significance of these deletions is not known. Sequence analysis of the 5′ noncoding LTR also showed complete identity of samples between 1999 and 2001, with a tight conservation of all functional motifs (Fig. 2L).

Phylogenetic analysis based on individual gene-by-gene comparisons also confirmed a lack of viral evolution (Fig. 3A–I). Analysis of partial genomic regions derived from samples obtained between 1993 and 2001 revealed that *pol*, *env*, *nef*, and *vpr* gene sequences were identical (data not shown), with strong phylogenetic clustering over time (Fig. 3J– M). This confirmed a complete lack of viral genetic diversity *in vivo* over 8 years. Of interest was the phylogenetic tree reconstructed from the *env* C2–V5 hypervariable region sequences, which showed tight clustering (Fig. 3K). Similarly, sequencing of 15 *vpr* clones from 1993 to 2001 (Fig. 3M) further confirmed a complete lack of viral quasispecies evolution consistent with the presence of a single lineage of HIV-1 *in vivo*. Phylogenetic analysis based on the full-length genomes also confirmed the authenticity of sequences originating from the study subject, evident from 100% bootstrap estimations for all three sequences between 1999 and 2001 (Fig. 3N). The tight clustering of these full-length genomes further supports an absence of evolution in this unique individual. Thus, it appears that viral replication in this individual has not occurred since at least 1993.

Discussion

Truly nonprogressing HIV-infected individuals, who constitute <0.8% of the total HIV-infected population, are a subject of intense investigation. These nonprogressors can provide valuable insight for the development of therapeutics for natural control of HIV. In this study, we have investigated the molecular structure of full-length HIV-1 genomes (>9 kb) from a rare nonprogressive individual, who, despite being infected for 14 years, satisfies all of the aforementioned criteria for a “true nonprogressor.” Based on this detailed molecular analysis, we show that genomic defects

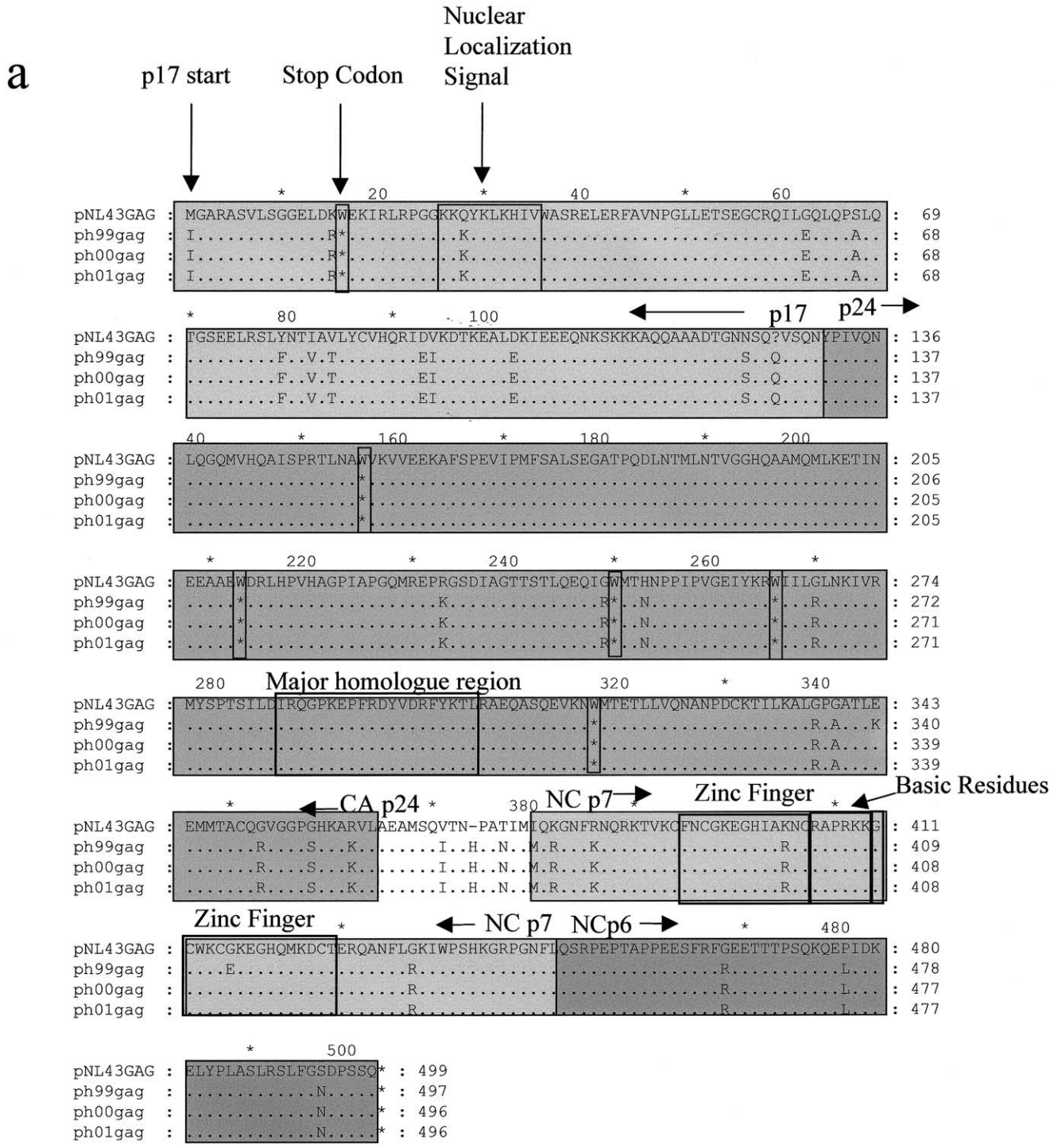


Fig. 2. Alignment of full-length gag amino acid sequences (499 residues) derived from full-length HIV-1 genomes from PBMC, by direct sequencing, showing the extent of gross defects in the *gag* gene. The tryptophan (W) codon TGG was changed throughout the *gag* p17 and p24 genomic regions to the stop codon TAG, due to high G–A hypermutation, in positions 16, 56, 213, 250, 266, and 317. The start codon ATG was absent in the p17 gene, with methionine substituted for isoleucine. Boxed areas with asterisks denote stop codons. Other boxed regions represent functional motifs/regions within Gag, as indicated. (b) Alignment of *gag* gene clones confirming the presence of stop codons in both p17 and p24 regions. (c) Alignment of full-length *pol* amino acid sequences (947 residues) derived from PBMCs, by direct sequencing. Boxed areas with asterisks denote stop codons. Other boxed regions represent functional motifs/regions within Pol, as indicated. (d) Frequency of G–A hypermutation in the *gag* gene derived from full-length HIV-1 sequences. GG-to-AG hypermutations are indicated in red, GA-to-AA in cyan, GC-to-AC. In green, and GT-to-AT in magenta. Black represents no change, while yellow represents gaps. There were 34 G-to-A hypermutations detected in *gag*. This phenomenon was identical in all three genomes from 1999, 2000, and 2001. (e) Alignment of full-length envelope amino acid sequences (849 residues). Deletions are denoted by dashes. Boxed areas represent the V1–V5 hypervariable regions, as indicated. (f–i) Alignment of amino acid sequences derived from Vif (f), Vpr (g), Vpu (h), and Nef (i), sequenced from full-length HIV-1 genomes. Highlighted areas in Vif (f) represent conserved cysteine residues. Functional domains (boxed regions) are indicated for Vpr (g); for Vpu (h), which represent the N-terminal and cytoplasmic tails; and for Nef (i). (j–l) Peptide alignment of Tat (j), Rev (k), and non-coding LTR (l) region derived from full-length HIV-1 genomes. Functional domains are highlighted.

b

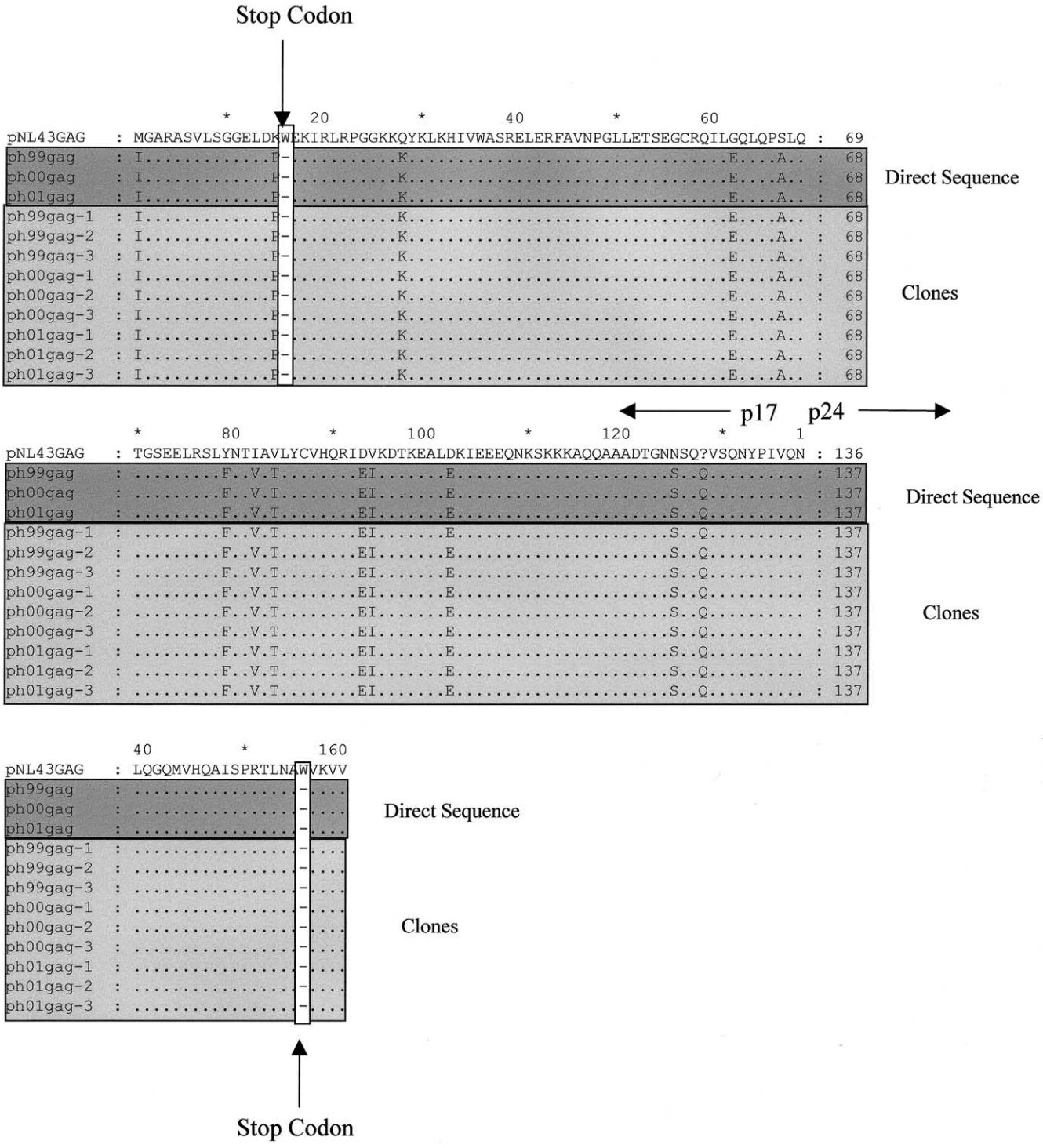
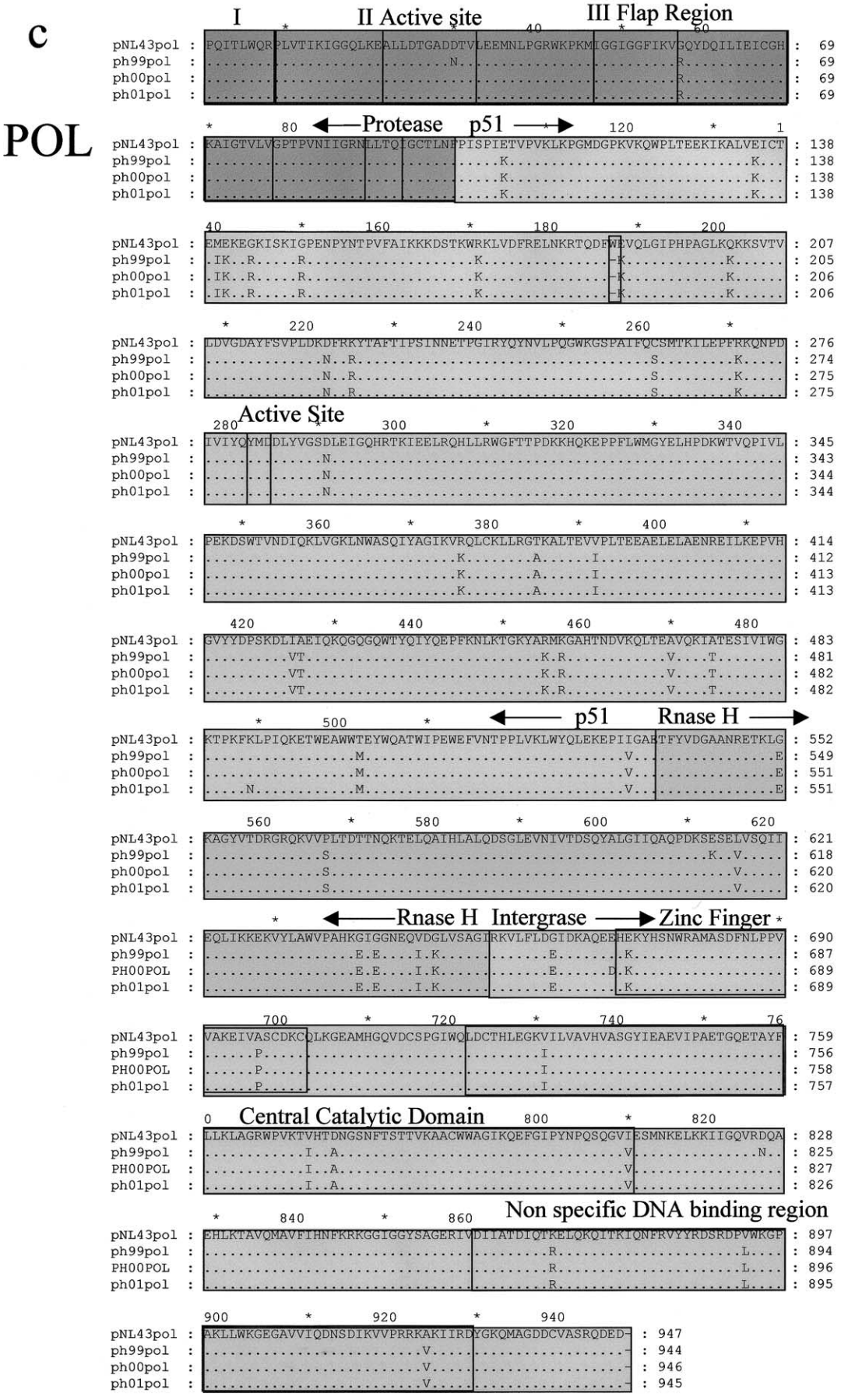


Fig. 2 (*continued*)

may have rendered the single infecting strain replication incompetent, hence inducing a state of permanent nonprogression in this unique individual.

The accumulation of genetic diversity seen in slow progressors and LTNP over time has been associated with host immune selective pressure, but this nonprogressor had no



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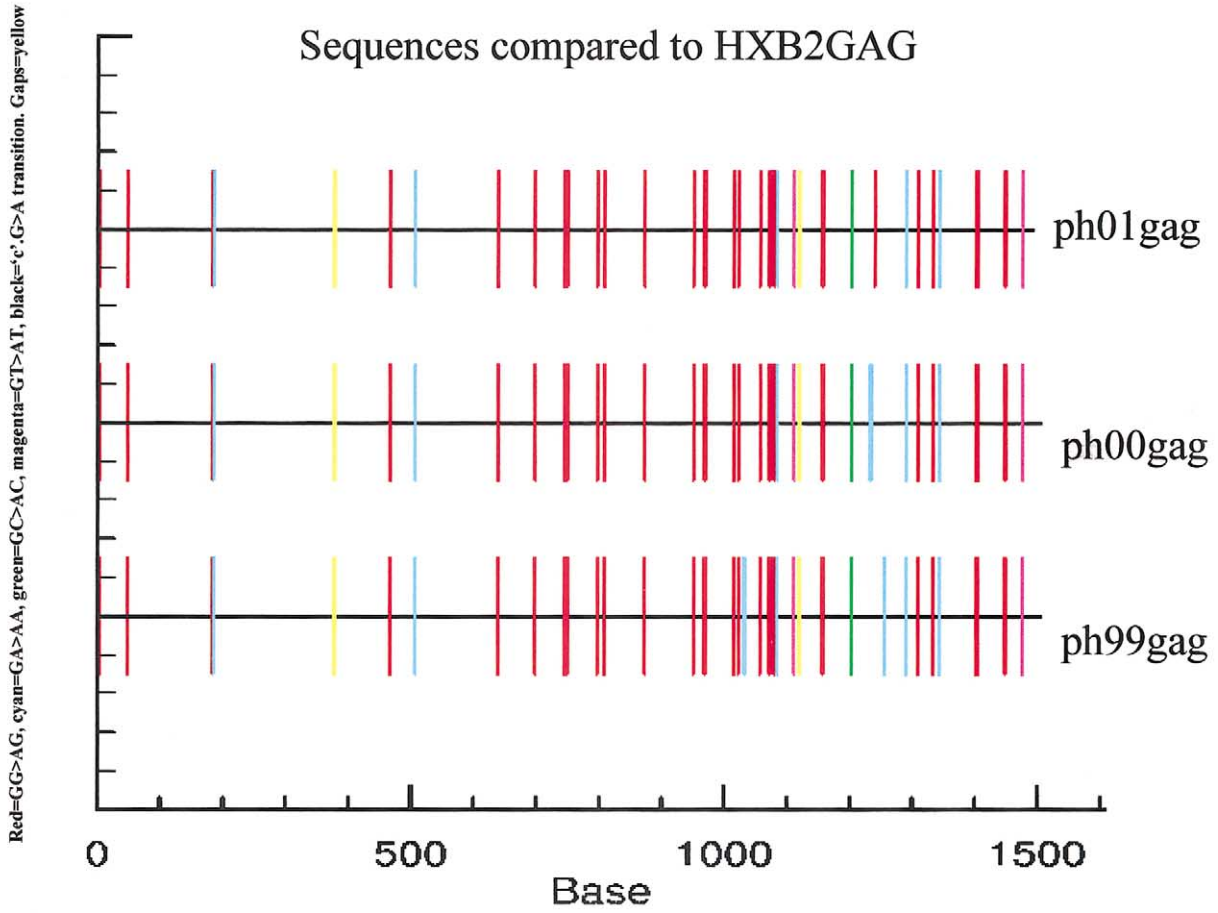
d



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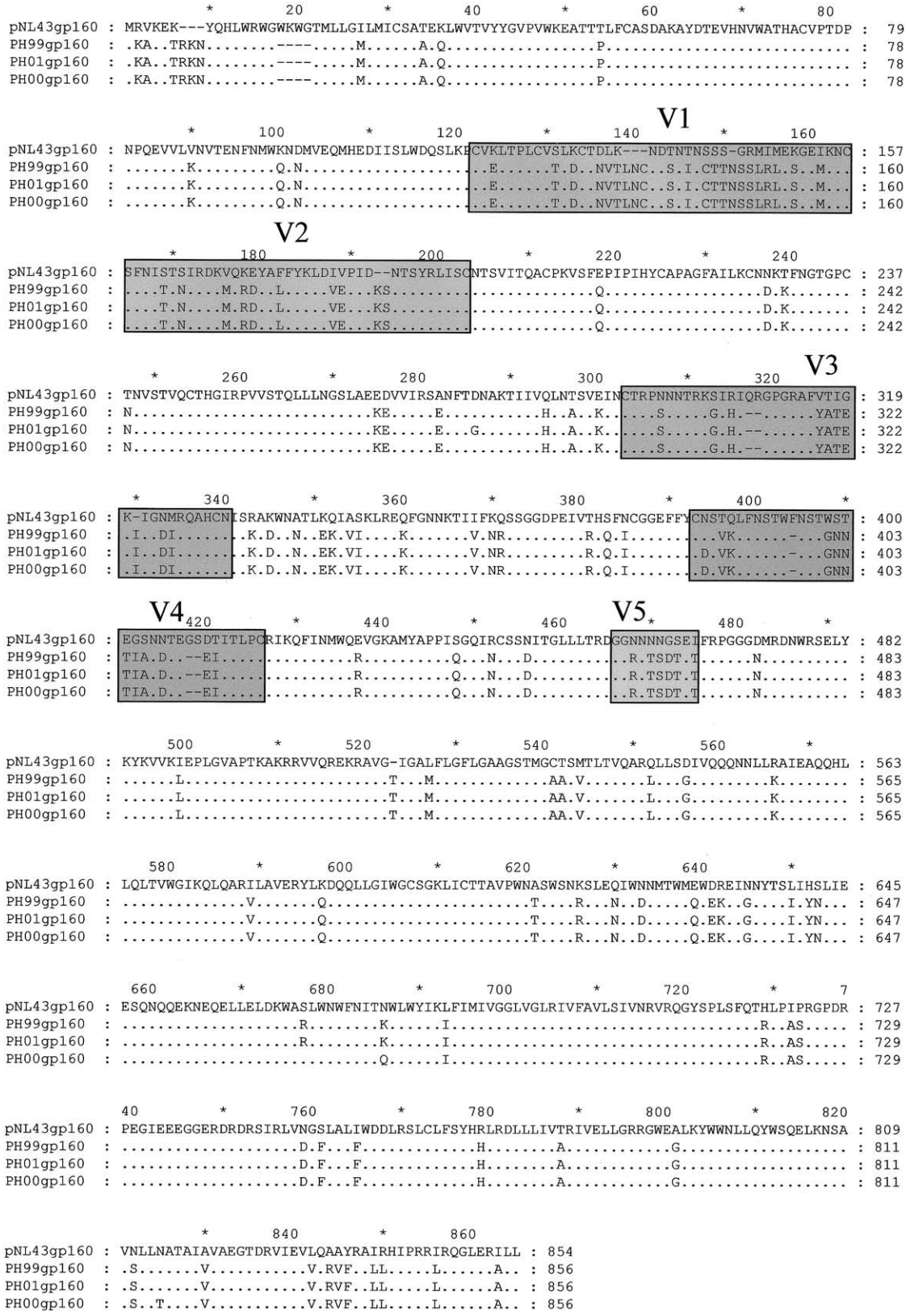
trace of viral evolution, despite potent antiviral immune responses (Wang et al., 2002). In contrast, lower variability or homogeneity in viral strains has been correlated with rapid progression to AIDS (Halapi et al., 1997; Shankarappa et al., 1998; Wang et al., 1997, 2000). Considerable genetic variation occurs in the *env* gene. Given the importance of *env* in neutralization, immune escape, viral pathogenesis, cell tropism, and viral fusion function (Fouchier et al., 1992; Hwang et al., 1991; Oka et al., 1994; Shioda et al., 1992), the marked variation seen in *env* of HIV-1 strains from individuals with different stages of HIV disease (Kuiken et al., 1992) is a biologically significant phenomenon. More importantly, this confirms that viral evolution is an integral part of HIV pathogenesis, and leads to disease progression. Therefore, the absence of genetic variability and evolution in HIV-1 strains from this immunocompetent nonprogressor is unique, and appears to be a barrier against disease progression.

From the study subject, a novel and unique nonevolving HIV-1 strain has been characterized, from uncultured PBMC, represented as an archival inactive genome. We were unable to amplify any viral sequences from plasma, consistent with his persistently undetectable plasma viremia, and the failure to isolate any virus by co-culture suggests that replication-competent genomes no longer exist in circulation. Intriguingly, this would also suggest that the mutant genomes arose very soon after infection, and have been propagated only by cell division. Alternatively, potent HIV-specific immune responses (Wang et al., 2002) may have cleared cells infected with the original infecting strain, leaving only the defective genome in resting cells, which are silent to antiviral CTL. A longitudinal comparison of various genomic segments in *pol*, *env*, *nef*, and *vpr* clearly revealed a complete lack of viral evolution, with strains retaining identical sequences over an 8-year period (1993–2001), at both the nucleotide and peptide levels. Cloning analysis further validated the absence of evolution. Further, to prove this lack of viral evolution, full-length viral genomes (>9000 bp) amplified and sequenced from uncultured PBMC obtained in 1999, 2000, and 2001 revealed identical sequences at the peptide level with maximum variation of <0.98%. This was in complete concordance

e

Env gp160



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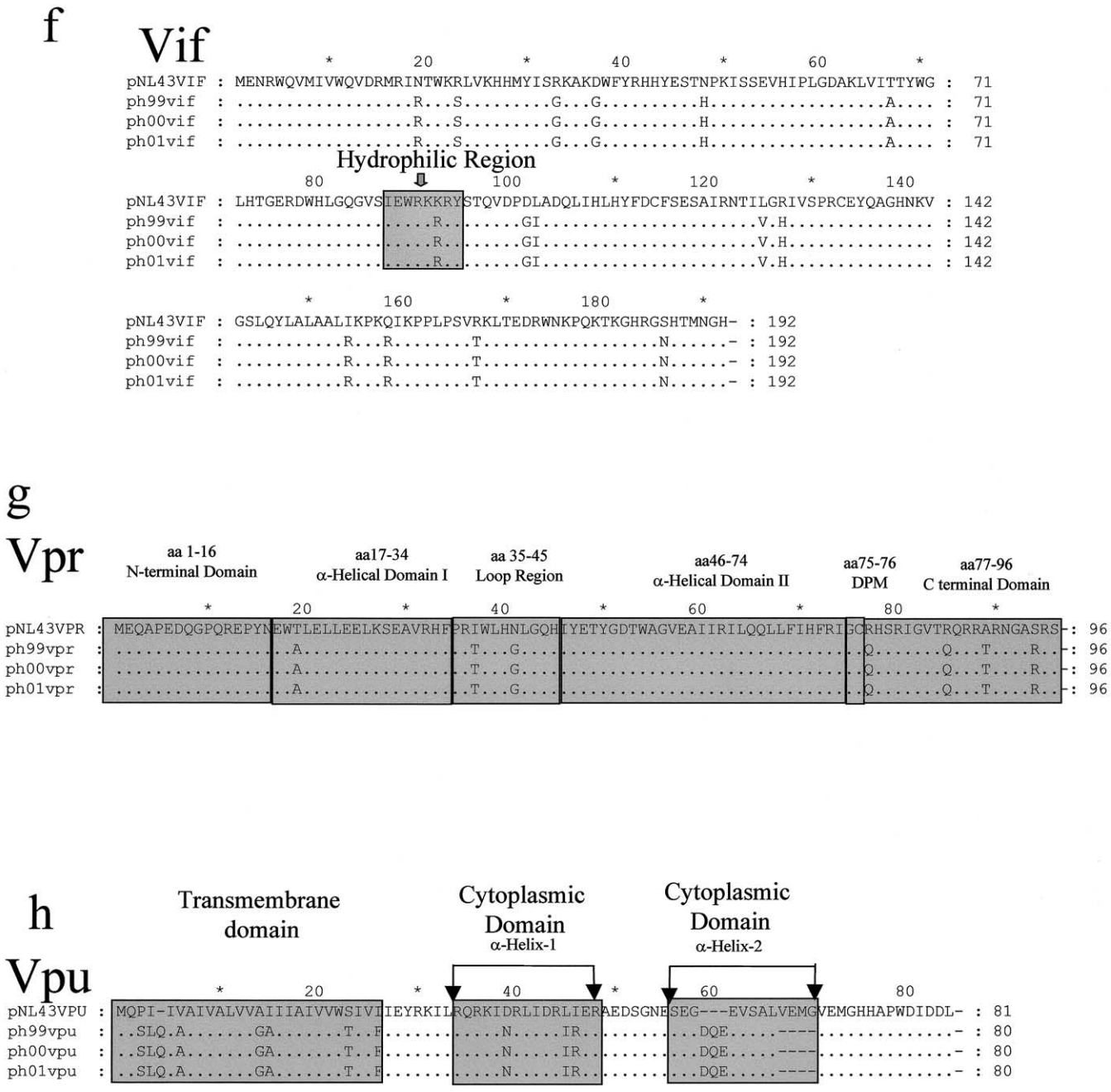

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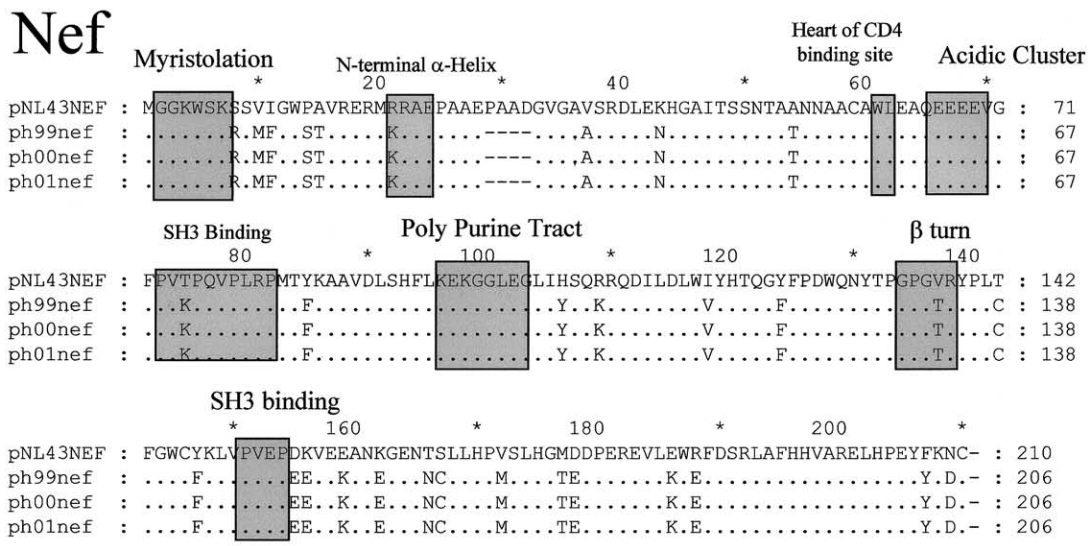
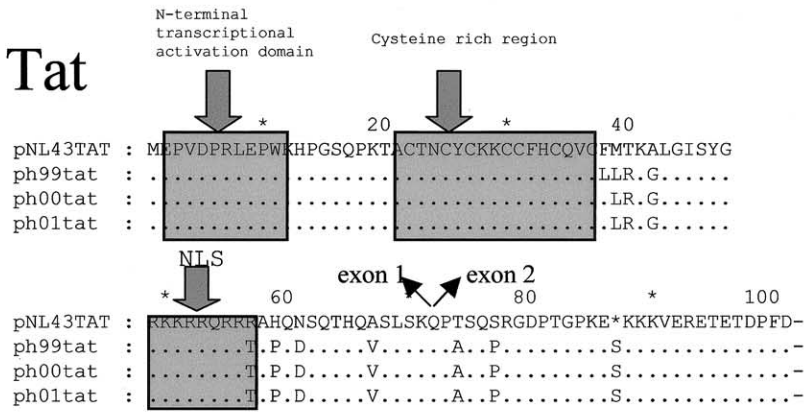
with sequences we obtained from small genomic segments throughout the whole 8-year period. This also suggests an early eradication of all infectious genomes, probably at the initial stages of infection, during the clonal expansion stage of viral replication, after antigenic exposure was sufficient to induce the development of a full Western blot pattern. In addition, these data provide evidence for limited integration events of proviral DNA in this individual.

The lack of sequence evolution demonstrated in the study subject has not been described before. Conventionally, the HIV-1 genome undergoes mutation rates of 1% or more per year (Lukashov et al., 1995). Thus, genomic variability of <1% over 8 years based on the entire genome (especially in the *env* hypervariable regions), and partial gene segments between 1993 and 2001, is a unique biological and molecular phenomenon. In addition, we could not rule out errors in Taq polymerase being responsible for some of these apparent sequence changes. The underlying reasons for this lack of variability may be a consequence of defects, primarily stop codons that we observed in p17 and p24 regions of *gag*, and the RT region of *pol*, which may have eliminated replicative potential of this strain. These stop codons were created via systematic elimination of tryptophan residues (W) by a process of "G-to-A hypermu-

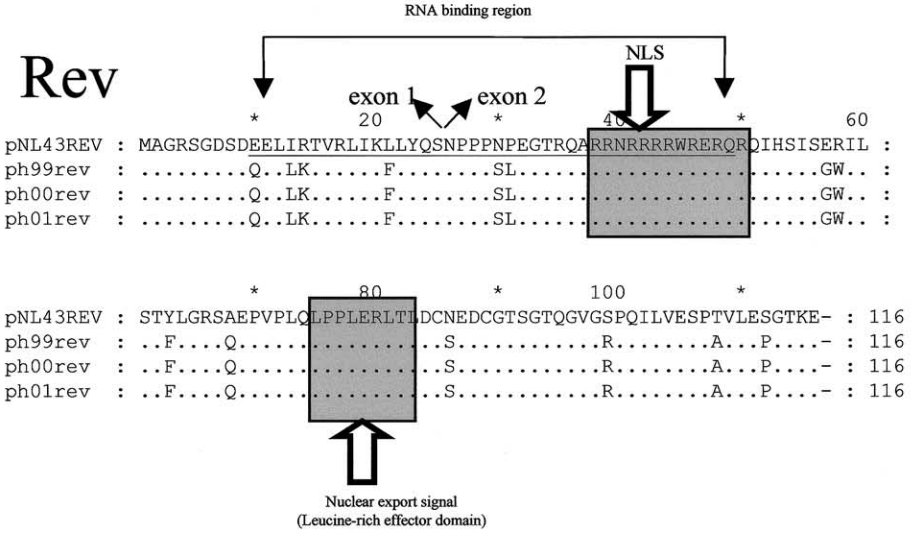
i



j



k



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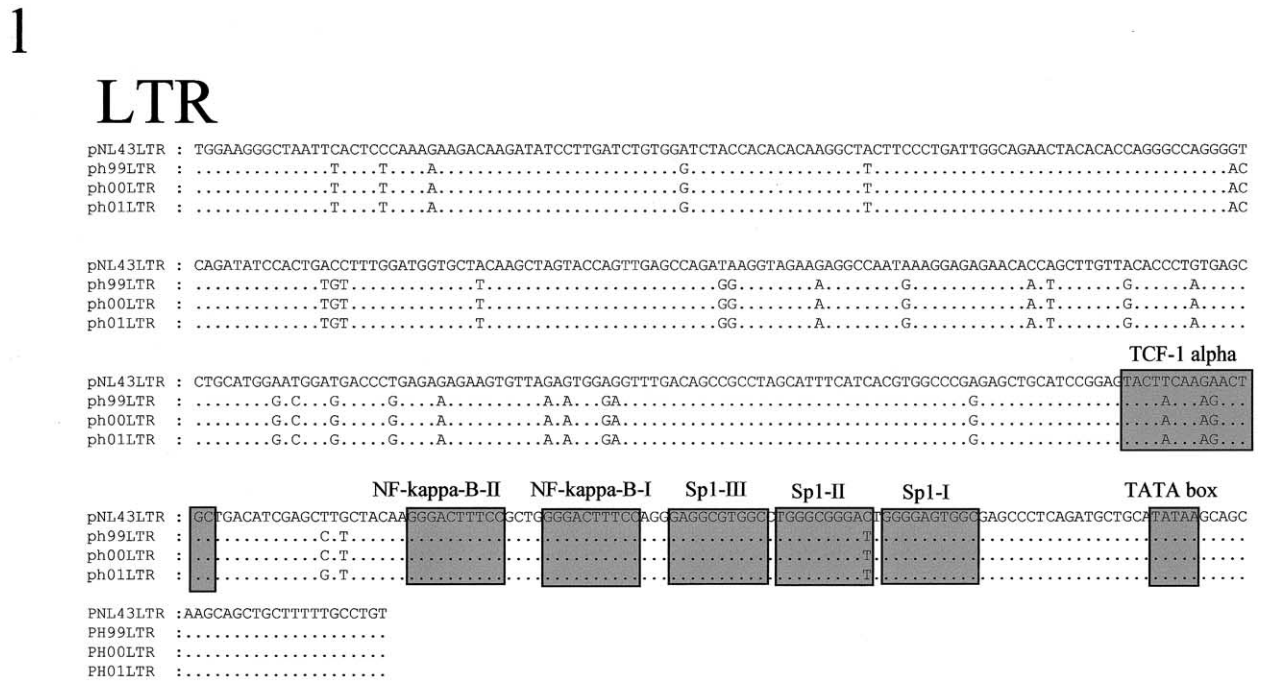

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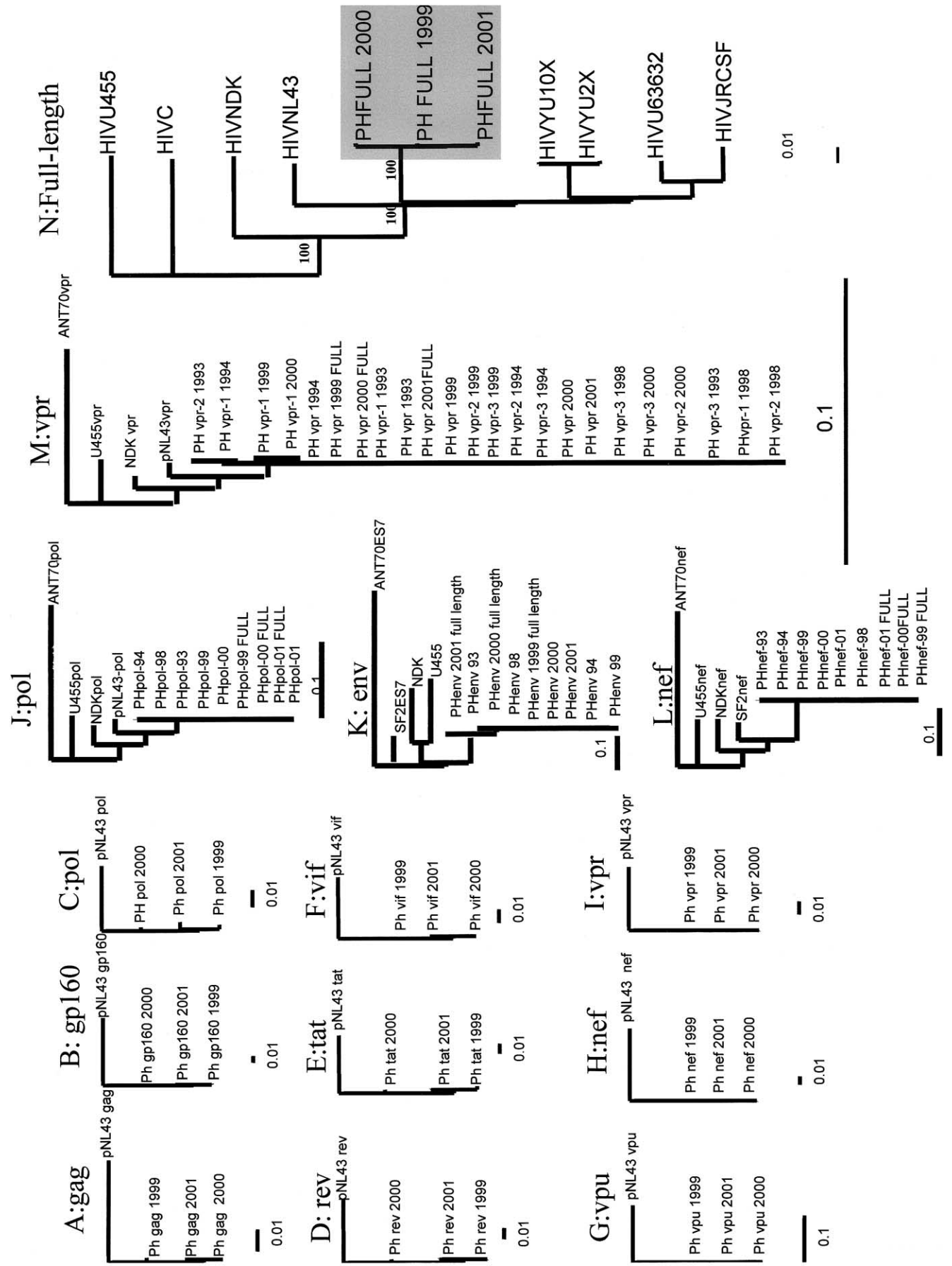
tation" (Pathak and Temin, 1990a,b) in which G-to-A transitions far exceed all other mutations in viral sequences (Borman et al., 1995). G-to-A hypermutation was recently described to be existing in 38% of asymptomatic individuals and 50% of seroconverters (Janini et al., 2001). However, the functional relevance between G-to-A hypermutation and the clinical stage of disease remains poorly understood. Further, it is unclear whether hypermutation is influenced by viral escape or if it is a function of the cell population harboring the virus. The loss of coding potential of these hypermutated HIV sequences strongly suggests that they are incapable of generating progeny virions and cannot contribute to the HIV gene pool (Janini et al., 2001). In the case of the study subject, the extent of G-to-A hypermutation was significantly higher in *gag* and *pol* genes, in which we observed stop codons. PBMC-derived sequences may display a higher incidence of G–A hypermutation (Janini et al., 2001). However, our recent longitudinal analysis of full-length HIV-1 genomes from an American cohort of 20 slow progressors and nonprogressors (Wang et al., 2000, and unpublished data) did not show a predominance of G–A hypermutation in the nonprogressors, thereby suggesting a minimal role of this phenomenon in defining the rate of disease progression. Whether G–A hypermutation is a transitory phenomenon or is permanent remains unclear. It was suggested that G–A hypermutation detected after culture may be a transitory phenomenon, and can be induced by mitogens (Janini et al., 2001). Thus, based on the uniform presence of a strain bearing G–A hypermutation in the study subject, we hypothesize that G–A hypermutation may play a significant role in rendering HIV genomes defective, and may be important in containing HIV disease progression in some cases.

The persistence of defects in the genome throughout the study period suggests that no new integrants were generated during this period. Together, the absence of plasma viremia and viral evolution and the presence of strong anti-HIV immune responses (Wang et al., 2002) may have contributed to truly nonprogressive HIV disease in this individual. Based on broad potent immune responses against multiple Gag antigens, cells expressing intact antigens may have been eradicated long ago, leaving only cells containing defective HIV genomes, being replicated only by normal cell mitotic processes, not by viral replication.

Based on the prevalence of stop codons in HIV-1 genomes amplified from the study subject over 8 years, it is unlikely that the synthesis of normal p17, p24, and Pol RT proteins is occurring *in vivo.* The p17 matrix protein is critical in the transport of the pre-integration complex to the host cell nucleus (Bukrinsky et al., 1992), the p24 core protein for viral maturation, budding, and viral infectivity, and RT for integration of the provirus into the genome. Therefore, stop codons in these genes would interfere with expression of functional proteins, which would render the virus both infection and replication incompetent. Of particular interest is the recognition of antibodies to p17 and p24 by Western blot. Sustained antibody responses to an apparently defective HIV-1 protein have also been observed in studies of a New York patient (Binley et al., 1998). Similarly, the detection of antibody responses to both p17 and p24 proteins by Western blot from samples taken from the study subject between 1998 and 2001 does not imply that


A:gag
pNL43 gag
Ph gag 1999
Ph gag 2001
Ph gag 2000
0.01
B: gp160
pNL43 gp160
Ph gp160 2000
Ph gp160 2001
Ph gp160 1999
0.01
C:pol
pNL43 pol
PH pol 2000
Ph pol 2001
Ph pol 1999
0.01
D: rev
pNL43 rev
Ph rev 2000
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Ph rev 1999
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E:tat
pNL43 tat
Ph tat 2000
Ph tat 2001
Ph tat 1999
0.01
F:vif
pNL43 vif
Ph vif 1999
Ph vif 2001
Ph vif 2000
0.01
G:vpu
pNL43 vpu
Ph vpu 1999
Ph vpu 2001
Ph vpu 2000
0.1
H:nef
pNL43 nef
Ph nef 1999
Ph nef 2001
Ph nef 2000
0.01
I:vpr
pNL43 vpr
Ph vpr 1999
Ph vpr 2001
Ph vpr 2000
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J:pol
ANT70pol
U455pol
NDKpol
pNL43-pol
PHpol-94
PHpol-98
PHpol-93
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PHpol-00
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PHpol-00 FULL
PHpol-01 FULL
PHpol-01
0.1
K: env
ANT70ES7
SF2ES7
NDK
U455
PHenv 2001 full length
PHenv 93
PHenv 2000 full length
PHenv 98
PHenv 1999 full length
PHenv 2000
PHenv 2001
PHenv 94
PHenv 99
0.1
L:nef
ANT70nef
U455nef
NDKnef
SF2nef
PHnef-93
PHnef-94
PHnef-99
PHnef-00
PHnef-01
PHnef-98
PHnef-01 FULL
PHnef-00FULL
PHnef-99 FULL
0.1
M:vpr
ANT70vpr
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PH vpr-2 1993
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PH vpr 2001FULL
PH vpr 1999
PH vpr-2 1999
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PH vpr-2 1994
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PH vpr 2000
PH vpr 2001
PH vpr-3 1998
PH vpr-3 2000
PH vpr-2 2000
PH vpr-3 1993
PHvpr-1 1998
PH vpr-2 1998
0.1
N:Full-length
HIVU455
HIVC
HIVNDK
HIVNL43
PHFULL 2000
PH FULL 1999
PHFULL 2001
HIVYU10X
HIVYU2X
HIVU63632
HIVJRCSF
100
100
100
0.01

these proteins are currently being produced. Binley et al. proposed a number of hypotheses to explain how antibody responses could persist in the absence of apparent viral replication, including presentation of remnant virus on follicular dendritic cells; long-lived memory and persistence of antibody-secreting B cells; and presence of intact virions replicating within some privileged site that is not in rapid equilibrium with blood. While we have not been able to obtain lymph node or bone marrow biopsies from the study subject primarily due to the patient's personal decision, our comprehensive study of the immunobiological mechanisms operating in the study subject (Wang et al., 2002) proposed different reasons for the persistence of a broader T- and B-cell antiviral memory. One speculative possibility is that the defective genomes are harbored by a population of activated T cells, perhaps HIV-specific CD4+ T cells, that may be producing defective viral antigen. This also provides a mechanism for the clonal nature of this strain's DNA replication, without retroviral reinfection and mutation. This scenario is the subject of ongoing studies.

This is the first demonstration of a complete absence of HIV-1 evolution and replication in a human, along with evidence of gene defects that may have induced replication incompetence of the study subject's viral strain. The persistence of gene defects over 8 years demonstrates that genetic repair of these regions does not occur. This is starkly different from the PBMC-derived HIV-1 strains from the Sydney Blood Bank Cohort members, in which the virus underwent slow but continual change, leading to resumption of pathogenic potential and eventually to disease progression in two individuals (Deacon et al., 1995; Learmont et al., 1999). The strain described in this study, which may be able to induce a protective immune response due to some antigen production, while the ability to replicate and evolve is ablated by the gross gene defects, could prove to be an ideal candidate for a DNA-based HIV vaccine. Further biological studies of cloned versions of this defective provirus are needed to confirm protein expression *in vitro.*

Materials and methods

Study subject

A 53-year-old man, presumed infected in December 1988, from a partner who died of AIDS in 1992, was diagnosed HIV-positive in 1992. He has remained asymptomatic after up to 14 years of infection. While he has not received combination antiretroviral therapy, he has participated in numerous therapeutic interventions. These and other clinical details have been reported (Wang et al., 2002).

Viral load measurements

HIV-1 plasma RNA load was determined using the Amplicor HIV-1 Monitor "ultrasensitive" Version 1.5 (Roche Diagnostics Systems, Branchburg, NJ, USA), according to the manufacturer's protocol.

PBMC isolation and genomic DNA extraction

Fresh EDTA blood was centrifuged at 1800 rpm for 10 min to separate plasma, and PBMC were fractionated from the remaining blood cells by Ficoll–Hypaque gradient centrifugation. Genomic DNA was extracted from 5×10^6 PBMC using the QIAmp Blood kit (Qiagen, GmBh, Germany) according to the manufacturer's protocol.

Virus isolation

PBMC 1×10^7 were co-cultured in RPMI 1640 + 10% FCS and 10% IL-2 (IL-2 medium) with an equal number of phytohemagglutinin (PHA)-stimulated uninfected donor PBMC. Viral isolation in the study subject was also attempted using 5×10^7 CD8-depleted PBMC (using CD8 Dynal beads, according to the manufacturer's protocol). Cultures were maintained twice weekly by addition of fresh PHA-stimulated HIV-negative donor PBMC and IL-2 medium, for a period of 4 weeks. Viral replication was measured by assaying p24 antigen in culture supernatants according to the manufacturer's protocol (Beckman Coulter, USA).

Polymerase chain reaction

Amplification of proviral DNA was conducted using an external and internal PCR. For external reaction, 5 μl of 10× buffer, 5 μl of $MgCl_2$ (25 mM), 0.8 μl (100 mM) of dNTP, and 1 μl (20 pmol) of oligonucleotide primers (external) 1 and 2 were mixed together with 0.5 μl (2.5 U) of *Taq* DNA polymerase and 10 μl (equivalent to 1 μg) DNA template. The PCR was run for 35 cycles (94°C for 30 s, 55°C for 1 min, and 72°C for 2 min), after an initial 94°C for 5 min before the first cycle. The internal reaction conditions were identical to those of the first PCR round, but

Fig. 3. Neighbor joining phylogenetic reconstructions based on complete nucleotide sequences for *gag* (A), *env*gp160 (B), *pol* (C), *rev* (D), *tat* (E), *vif* (F), *vpu* (G), *nef* (H), and *vpr* (I), amplified from the full-length genomes, from the PBMCs, obtained between 1999 and 2001. Figures J, K, L, and M show the comparison of small genomic segments amplified from proviral DNA from 1993-2001 against the segments amplified from the full-length HIV-1 genomes in the *pol* (J), *env* (K), *nef* (L), and *vpr* (M) regions over time. Multiple clones of *vpr* (M) were created to assess diversity *in vivo*. Phylogenetic tree (N) based on full-length nucleotide sequences (>9000 bp). Other non-B subtypes (retrieved from Los Alamos Database, Kuiken et al., 2001) used for comparison clustered away from the study subject's viral strain.

only 25 cycles. PCR products were subsequently run on a 1.5% agarose gel.

RNA extraction from plasma and reverse transcription PCR

Viral RNA from plasma was extracted using Tri-Reagent (Sigma, St. Louis, MO, USA) and cDNA synthesis was conducted using the Reverse Transcriptase System (Promega, Madison, WI, USA) with a total of 2 μg RNA in a final volume of 20 μl. The reaction mixture contained 10× reaction buffer, 2 μl dNTP mix (10 mM), 40 units (U) of RNasein ribonuclease inhibitor, 15 U AMV reverse transcriptase (AMV-RT, a high-fidelity enzyme), and 0.5 μg oligo(dT); the remaining volume was made up to 20 μl using nuclease-free water. The reaction mixture was then incubated at 42°C for 30 min and heated at 99°C for a further 5 min. Conditions for the amplification of cDNA were identical to those used for regular PCR as mentioned in the previous section.

Amplification of full-length HIV-1 genomes

The GeneAmp XL PCR kit (Perkin-Elmer, Emeryville, CA, USA) was used to amplify near full-length HIV genomes involving a two-layered PCR. Two primers were used for the amplification of full-length genomes from low copy numbers: UP1A (5′-AGTGGCGCCCGAACAGG-3′), directed at the 5′ LTR, and LOW2 (5′-TGAGGCTTAAGCAGTGGGTTTC-3′), directed near the end of *nef* to capture the 3′ LTR. To minimize PCR mispriming and primer oligomerization in the internal reaction, a hot start technique was applied using the lower reagent. After allowing the wax to harden into a solid separating layer, the upper layer comprising 18 μl of 3.3× XL buffer, mixed with 2 μl of rTh DNA polymerase (XL), was added. One microgram of DNA template, in addition to PCR water to make a final volume of 60 μl, was added. Following addition of the upper layer to the sample tube of wax the mixture was heated at 94°C for 1 min followed by 94°C for 15 s, 68°C for 10 min, running for 25 cycles. An extra 12 cycles were added using 94°C for 15 s, 68°C for 10 min, and an increment of 15 s Ten to fifteen microliters per cycle. of amplified product was used for gel electrophoresis. Near full-length genomes were then used as a template for the amplification of individual genes. Before sequencing, all PCR products were purified using the Millipore multiscreen manifold system according to the manufacturer's protocol. Sequencing reactions were carried out using dye terminators (Version 3 Big Dye Kit, Perkin Elmer), and were purified on Sephadex 50 using the Millipore multiscreen manifold system.

Ligation of PCR products and transformation and screening of positive clones

Purified PCR fragments were ligated with the PGEM-T vector system (Promega), and ligated products were used in transforming competent JM109 *Escherichia coli* cells, provided with the kit, according to the manufacturer's protocol. One hundred microliters of the resuspended cells in SOC medium were gently spread on X-Gal (40 μl of 40 mg/ml) IPTG (20 μl of 200mg/ml) and ampicillin (100 μl of 100 mg/ml) plates. Plates were incubated at 37°C overnight. White clones were handpicked, and resuspended in 30 μl sterile PCR water. Sample tubes were heated at 93–95°C for 10 min to lyse the cells, followed by centrifugation at 14,000 for 5 min. Three microliters of supernatant was used to amplify the desired viral genomic region(s) to verify positive clones, which were then cultured in 2 ml Luria broth at 37°C overnight. Plasmid DNA was extracted as previously described (Wang et al., 1996) and purified plasmid preparations (as described above) were used for sequencing.

Sequence and phylogenetic analysis

Sequence obtained from the study subjects were first BLAST searched and also compared against all lab-derived HIV-1 sequences to rule out any possibility of contamination. Multiple nucleotide and peptide sequence alignments were generated using CLUSTAL W v1.7 (Thompson et al., 1994), from the GCG package, with default settings, and improved manually. The pairwise nucleotide distance was calculated using the Kimura two-parameter model in PHYLIP. Phylogenetic reconstructions were performed with the neighbor-joining method, also using the Kimura two-parameter distance matrix, with a transition to transversion ratio of 2. Analysis was performed with DNADIST and NEIGHBOR, part of the PHYLIP program. One hundred bootstrap replications were applied to generate phylogenetically significant temporal relationships, using Seqboot. G–A hypermutation was anlyzed using Hypermut, accessed through the Los Alamos HIV database web site (www.hiv.lani.gov/HYPERMUT/hypermut.html) (Rose and Korber, 2000).

Western blot analysis

Serum or plasma samples were heat inactivated at 56°C for 30 min. Reactivity to HIV-1 Env, Gag, and Pol proteins was confirmed using the Genelabs Diagnostic HIV Blot 2.2 Kit (Genelabs Diagnostic, Singapore) as per the manufacturer's instructions. Appropriate controls (internal sample control IgG to minimize false negatives and HIV-1-positive control) provided with the kit were run. The Western blot was considered positive for antibody to HIV if bands corresponding to gp160, 120, or 41 were present in combination with p66, p51, p31, and p24, or p55 and p17.

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References

Binley, J.M., Jin, X., Huang, Y., Zhang, L., Cao, Y., Ho, D.D., Moore, J.P., 1998. Persistent antibody responses but declining cytotoxic T-lymphocyte responses to multiple human immunodeficiency virus type 1 antigens in a long-term nonprogressing individual with a defective p17 proviral sequence and no detectable viral RNA expression. J. Virol. 72, 3472–3474.

Borman, A.M., Quillent, C., Charneau, P., Kean, K.M., Clavel, F., 1995. A highly defective HIV-1 group O provirus: evidence for the role of local sequence determinants in G → A hypermutation during negative-strand viral DNA synthesis. Virology 208, 601–609.

Borrow, P., Lewicki, H., Wei, X., Horwitz, M.S., Peffer, N., Meyers, H., Nelson, J.A., Gairin, J.E., Hahn, B.H., Oldstone, M.B.A., Shaw, G.M., 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. Nat. Med. 3, 205–211.

Buchbinder, S.P., Katz, M.H., Hessol, N.A., O'Malley, P.M., Holmberg, S.D., 1994. Long-term HIV-1 infection without immunologic progression. AIDS 8, 1123–1128.

Bukrinsky, M.I., Sharova, N., Dempsey, M.P., Stanwick, T.L., Bukrinskaya, A.G., Haggerty, S., Stevenson, M., 1992. Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. Proc. Natl. Acad. Sci. USA 89, 6580–6584.

Cao, Y., Qin, L., Zhang, L., Safrit, J., Ho, D.D., 1995. Virologic and immunologic characterization of long term survivors of human immunodeficiency virus type 1 infection. N. Engl. J. Med. 332, 201–208.

Deacon, N.J., Tsykin, A., Solomon, A., Smith, K., Ludford-Menting, M., Hooker, D.J., McPhee, D.A., Greenway, A.L., Ellet, A., Chatfield, C., Lawson, V.A., Crowe, S., Maerz, A., Sonza, S., Learmont, J., Sullivan, J.S., Cunningham, A., Dwyer, D., Dowton, D., Mills, J., 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science 270, 988–991.

Fouchier, R.A., Groenink, M., Kootstra, N.A., Tersmette, M., Huisman, H.G., Miedema, F., Schuitemaker, H., 1992. Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. J. Virol. 66, 3183–3187.

Goulder, P., Price, D., Nowak, M., Rowland-Jones, S., Phillips, R., McMichael, A., 1997a. Co-evolution of human immunodeficiency virus and cytotoxic T-lymphocyte responses. Immunol. Rev. 159, 17–29.

Goulder, P.J.R., Phillips, R.E., Colbert, R.A., McAdam, S., Ogg, G., Nowak, M.A., Giangrande, P., Luzzi, G., Morgan, B., Edwards, A., McMichael, A.J., Rowland-Jones, S., 1997b. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat. Med. 3, 212–217.

Halapi, E., Leitner, T., Jansson, M., Scarlatti, G., Orlandi, P., Plebani, A., Romiti, L., Albert, J., Wigzell, H., Rossi, P., 1997. Correlation between HIV sequence evolution, specific immune response and clinical outcome in vertically infected infants. AIDS 11, 1709–1717.

Hwang, S.S., Boyle, T.J., Lyerly, H.K., Cullen, B.R., 1991. Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. Science 253, 71–74.

Janini, M., Rogers, M., Birx, D.R., McCutchan, F.E., 2001. Human immunodeficiency virus type 1 DNA sequences genetically damaged by hypermutation are often abundant in patient peripheral blood mononuclear cells and may be generated during near-simultaneous infection and activation of CD4(+) T cells. J. Virol. 75, 7973–7986.

Kelleher, A.D., Long, C., Holmes, E.C., Allen, R.L., Wilson, J., Conion, C., Workman, C., Ahaunak, S., Wulfestieg, K., Ogg, G., Sullivan, J.S., Dyer, W., Jones, I., McMichael, A.J., Rowland-Jones, S.L., Phillips, R.E., 2001. Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27 restricted CTL responses. J. Exp. Med. 193(3), 375–386.

Kuiken, C., Foley, B., Hahn, B., McCutchan, F., Mellors, J., Mullins, J., Sodroski, J., Wolinsky, S., Korber, B., 2001. HIV sequence compendium. HIV Database, Los Alamos, NM.

Kuiken, C.L., de Jong, J.J., Baan, E., Keulen, W., Tersmette, M., Goudsmit, J., 1992. Evolution of the V3 envelope domain in proviral sequences and isolates of human immunodeficiency virus type 1 during transition of the viral biological phenotype. J. Virol. 66, 5704.

Learmont, J.C., Geczy, A.F., Mills, J., Ashton, L.J., Raynes-Greenow, C.H., Garsia, R.J., Dyer, W.B., McIntyre, L., Oelrichs, R.B., Rhodes, D.I., Deacon, N.J., Sullivan, J.S., 1999. Immunologic and virologic status after 14 to 18 years of infection with an attenuated strain of HIV-1: a report from the Sydney Blood Bank Cohort. N. Engl. J. Med. 340, 1715–1722.

Lefrere, J.J., Morand-Joubert, L., Mariotti, M., Bludau, H., Burghoffer, B., Petit, J.C., Roudot-Thoraval, F., 1997. Even individuals considered as long-term nonprogressors show biological signs of progression after 10 years of human immunodeficiency virus infection. Blood 90, 1133–1140.

Lukashov, V.V., Kuiken, C.L., Goudsmit, J., 1995. Intrahost human immunodeficiency virus type 1 evolution is related to length of the immunocompetent period. J. Virol. 69, 6911–6916.

Ma, X.Y., Sova, P., Chao, W., Volsky, D.J., 1994. Cysteine residues in the Vif protein of human immunodeficiency virus type 1 are essential for viral infectivity. J. Virol. 68, 1714–1720.

Oka, S., Ida, S., Shioda, T., Takebe, Y., Kobayashi, N., Shibuya, Y., Ohyama, K., Momota, K., Kimura, S., Shimada, K., 1994. Genetic analysis of HIV-1 during rapid progression to AIDS in an apparently healthy man. AIDS Res. Hum. Retroviruses 10, 271–277.

Pantaleo, G., Menzo, S., Vaccarezza, M., Graziosi, C., Cohen, O.J., Demarest, J.F., Montefiori, D., Orenstein, J.M., Fox, C., Schrager, L.K., Margolick, J.B., Buchbinder, S., Giorgi, J.V., Fauci, A.S., 1995. Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. N. Engl. J. Med. 332, 209–216.

Pathak, V.K., Temin, H.M., 1990a. Broad spectrum of in vivo forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: substitutions, frameshifts, and hypermutations. Proc. Natl. Acad. Sci. USA 87, 6019–6023.

Pathak, V.K., Temin, H.M., 1990b. Broad spectrum of in vivo forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: deletions and deletions with insertions. Proc. Natl. Acad. Sci. USA 87, 6024–6028.

Rose, P.P., Korber, B.T., 2000. Detecting hypermutations in viral sequences with an emphasis on G → A hypermutation. Bioinformatics 16, 400–401.

Saksena, N.K., Wang, B., Dyer, W.B., 2001. Biological and molecular mechanisms in progression and non-progression of HIV disease. AIDS Rev. 3, 3–10.

Shankarappa, R., Gupta, P., Learn, G.H., Rodrigo, A.G., Rinaldo, C.R., Gorry, M.C., Mullins, J.I., Nara, P.L., Ehrlich, G.D., 1998. Evolution of human immunodeficiency virus type 1 envelope sequences in infected individuals with differing disease progression profiles. Virology 241, 251–259.

Shankarappa, R., Margolick, J.B., Gange, S.J., Rodrigo, A.G., Upchurch, D., Farzadegan, H., Gupta, P., Rinaldo, C.R., Learn, G.H., He, X., Huang, X.L., Mullins, J.I., 1999. Consistent viral evolutionary changes

associated with the progression of human immunodeficiency virus type 1 infection. J. Virol. 73, 10489–10502.

Sheppard, H.W., Lang, W., Ascher, M.S., Vittinghoff, E., Winkelstein, W., 1993. The characterization of non-progressors: long-term HIV-1 infection with stable CD4+ T cell levels. AIDS 7, 1159–1166.

Shioda, T., Levy, J.A., Cheng-Mayer, C., 1992. Small amino acid changes in the V3 hypervariable region of gp120 can affect the T-cell-line and macrophage tropism of human immunodeficiency virus type 1. Proc. Nati. Acad. Sci. USA 89, 9434–9438.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence allgnment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Wang, B., Ge, Y.C., Palasanthiran, P., Xiang, S.H., Ziegler, J., Dwyer, D.E., Randle, C., Dowton, D., Cunningham, A., Saksena, N.K., 1996. Gene defects clustered at the C-terminus of the vpr gene of HIV-1 in long-term nonprogressing mother and child pair: in vivo evolution of vpr quasispecies in blood and plasma. Virology 223, 224–232.

Wang, B., Ge, Y.C., Jozwiak, R., Bolton, W., Palasanthiran, P., Ziegler, J., Chang, J., Xiang, S.H., Cunningham, A.L., Saksena, N.K., 1997. Molecular analyses of human immunodeficiency virus type 1 V3 region quasispecies derived from plasma and peripheral blood mononuclear cells of the first long-term-nonprogressing mother and child pair. J. Infect. Dis. 175, 1510–1515.

Wang, B., Spira, T.J., Owen, S., Lal, R.B., Saksena, N.K., 2000. HIV-1 strains from a cohort of American subjects reveal the presence of a V2 region extension unique to slow progressors and non-progressors. AIDS 14, 213–223.

Wang, B., Dyer, W.B., Zaunders, J.J., Mikhail, M., Sullivan, J.S., Williams, L., Haddad, D.N., Harris, G., Holt, J.A.G., Cooper, D.A., Miranda-Saksena, M., Boadle, R., Kelleher, A.D., Saksena, N.K., 2002. Comprehensive analyses of a unique HIV-1-infected nonprogressor reveal a complex association of immunobiological mechanisms in context of replication-incompetent infection. Virology 304, 246–264.

Wolinsky, S.M., Korber, B.T., Neumann, A.U., Daniels, M., Kunstman, K.J., Whetsell, A.J., Furtado, M.R., Cao, Y., Ho, D.D., Safrit, J.T., 1996. Adaptive evolution of human immunodeficiency virus-type 1 during the natural course of infection. Science 272, 537–542.