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Longitudinal Analysis of Human Immunodeficiency Virus Type 1 *nef*/Long Terminal Repeat Sequences in a Cohort of Long-Term Survivors Infected from a Single Source

Melissa J. Churchill,[1] David I. Rhodes,[1]† Jennifer C. Learmont,[2] John S. Sullivan,[2,3] Steven L. Wesselingh,[1,4] Ian R. C. Cooke,[1] Nicholas J. Deacon,[1]‡ and Paul R. Gorry[1,4]*

The Macfarlane Burnet Institute for Medical Research and Public Health, Melbourne, Victoria, Australia[1]; Australian Red Cross Blood Service, Sydney, New South Wales, Australia[2]; Faculty of Medicine, University of Sydney, Sydney, New South Wales, Australia[3]; and Department of Medicine, Monash University, Melbourne, Victoria, Australia[4]

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We studied the evolution of human immunodeficiency virus type 1 (HIV-1) in a cohort of long-term survivors infected with an attenuated strain of HIV-1 acquired from a single source. Although the cohort members experienced differing clinical courses, we demonstrate similar evolution of HIV-1 *nef*/long-terminal repeat (LTR) sequences, characterized by progressive sequence deletions tending toward a minimal *nef*/LTR structure that retains only sequence elements required for viral replication. The in vivo pathogenicity of attenuated HIV-1 is therefore dictated by viral and/or host factors other than those that impose a unidirectional selection pressure on the *nef*/LTR region of the HIV-1 genome.

The *nef* gene is a major determinant of virulence in primate lentiviruses. Mutations in *nef* attenuate replication capacity and pathogenicity of simian immunodeficiency viruses (SIV) (3, 7, 10, 11, 13, 19) and may promote long-term survival of human immunodeficiency virus type 1 (HIV-1) infection in humans (6, 14, 15, 18, 20).

The largest described cohort of long-term survivors is the Sydney Blood Bank Cohort (SBBC), which consists of multiple individuals who became infected with an attenuated strain of HIV-1 via contaminated blood products from a common blood donor between 1981 and 1984 (6, 16, 17). Viral attenuation has been attributed to gross deletions in the *nef*/long terminal repeat (LTR) region of the HIV-1 genome (6). Despite being infected from a single source, SBBC members comprise slow progressors (SP) and long-term nonprogressors (LTNP) (2, 4, 17). The SBBC therefore provides an unprecedented opportunity to study the pathogenesis of *nef*/LTR-deleted HIV-1 infection in a naturally occurring human setting.

Subjects. We undertook a longitudinal study of SBBC SP and LTNP. The clinical history of the study subjects from infection to 1998 has been described (17). Subjects C54, C98, C49, C64, and C135 were referred to previously as recipients 13, 7, 12, 9, and 4, respectively (17). Subjects C54 and C98 have since died from causes unrelated to HIV-1 infection. An update on the results of laboratory studies and clinical history of these two subjects and of the surviving SBBC members studied in this report is summarized in Table 1 and detailed below.

SBBC subjects with slowly progressing HIV-1 infection include the donor (D36) and transfusion recipient C98. D36 commenced highly active antiretroviral therapy (HAART) in January 1999 following HIV-associated dementia that coincided with a fall in the CD4 cell count to <200 cells/μl and the presence of high plasma and cerebrospinal fluid (CSF) HIV-1 RNA levels (4, 5). As reported previously, C98 commenced prednisone for treatment of asthma in 1995 (17). C98 was diagnosed with pulmonary amyloidosis in 1998. C98 commenced HAART in November 1999 after experiencing a steady decline in his $CD4^+$ T-cell count and a gradual increase in HIV-1 RNA from below-detectable levels to 1,500 RNA copies/ml. During 2001 his $CD4^+$ T-cell count declined and fluctuated between 213 and 484 cells/μl despite the continuance of HAART and a viral load below detectable levels. He died at the age of 64 in March 2002 of amyloidosis, which was not HIV-1 related.

Nonprogressing SBBC subjects include transfusion recipients C49, C54, C64, and C135; these subjects have experienced steady $CD4^+$ T-cell counts since infection was first identified, with median values of >900, >1,000, >900, and > 500 cells/μl, respectively. HIV-1 RNA levels have remained consistently low or below detectable levels in these subjects despite being infected for up to 20 years without antiretroviral intervention. Patient C54 died aged 73 from a myocardial infarct in September 2001. C135 was not included in the present study because of the lack of consistent amplification of HIV-1 DNA.

Methods. Peripheral blood mononuclear cells (PBMC) were obtained at each of the times indicated in Table 1 according to guidelines endorsed by the Australian Red Cross Blood Service human ethics committee. The *nef*/LTR region of the HIV-1 genome was amplified from PBMC by nested PCR, as

* Corresponding author. Mailing address: Macfarlane Burnet Institute for Medical Research and Public Health, GPO Box 2284, Melbourne 3001, Victoria, Australia. Phone: 61 3 9282 2129. Fax: 61 3 9282 2100. E-mail: gorry@burnet.edu.au.

† Present address: Avexa Ltd., Richmond, Victoria, Australia.

‡ Present address: Monash University, Gippsland Campus, Churchill, Victoria, Australia.

TABLE 1. Subjects, laboratory studies, and clinical history

Subject, sex, and date of Birth[a]	Date transfused[a]	Date of blood sample[a]	Yr since infection	$CD4^+$ T-cell count (cells/μl)[b]	Viral load (RNA copies/ml)[c]	Antiretroviral drugs[d]	Clinical history[e]
D36, male, 6/4/1958	Not applicable; infected with HIV-1 via sexual transmission 12/1980 (18)	2/1996	15.2	609	1,100	ABC, AZT, NVP (1/1999–9/2004) (4, 5); ABC, NVP, 3TC (9/2004–present)	SP; diagnosed with HIVD 12/1998 (4)
		4/1996	15.3	504	7,800		
		10/1997	16.8	336	4,400		
		1/1999	18.1	210	9,900		
		9/2000	19.8	391	BD		
		4/2001	20.3	476	BD		
		2/2003	22.2	624	BD		
		2/2004	23.2	638	BD		
C49, female, 6/9/1954	6/11/1984	2/1994	9.7	1,045	BD	None	LTNP; diagnosed with age-onset diabetes in 2004, managed by diet; chronic alcoholism
		5/1994	9.8	1,458	BD		
		10/1996	12.1	1,134	BD		
		12/1997	13.5	918	BD		
		6/1999	15.0	605	BD		
		11/2001	17.4	624	BD		
		8/2002	18.2	468	BD		
		3/2004	19.7	874	BD		
C54, male, 2/17/1928	7/24/1984	7/1993	9.0	1,519	N/A	None	LTNP; IDDM; surgery for colon cancer in 1995; died 8/28/2001 from myocardial infarct; death not related to HIV-1
		6/1995	10.9	1,504	3,000		
		3/1996	11.7	1,188	1,500		
		9/1996	12.2	1,120	1,800		
		5/1997	12.8	1,286	5,500		
		8/1997	13.1	1,419	1,700		
		3/2000	15.7	840	1,600		
		5/2001	16.8	1,537	2,660		
C64, female, 3/20/1926	5/4/1983	8/1996	13.3	925	BD	None	LTNP; hypertension; hypercholesterolemia
		2/1997	13.8	851	BD		
		5/1997	14.0	1,050	BD		
		8/1997	14.3	805	BD		
		11/1997	14.5	936	BD		
		4/1999	15.9	1,026	BD		
		11/1999	16.5	1,332	BD		
		5/2000	17.0	875	BD		
C98, male, 7/11/1937	1/2/1982	7/1993	11.5	880	N/A	d4T, NVP, IND (11/1999–death)	SP; prednisone since 1995 for asthma; died 3/30/2001 from bronchial amyloidosis; death not related to HIV-1
		10/1995	13.8	576	670		
		11/1996	14.8	646	690		
		5/1997	15.3	527	760		
		2/1998	16.1	627	1,100		
		10/1998	16.8	429	1,500		
		3/2000	18.2	684	BD		
		3/2001	19.6	324	BD		

[a] Dates shown are month/day/year. The dates refer to times when PBMC were collected for HIV-1 *nef*/LTR sequencing. The results of only those laboratory studies that correspond to these time points are shown.
[b] $CD4^+$ T-cell levels were measured by flow cytometry.
[c] Plasma HIV-1 RNA was measured using COBAS AMPLICOR HIV-1 monitor version 1.0 (Roche Molecular Diagnostic Systems, Branchburg, N.J.) prior to July 1999 and version 1.5 after July 1999. HIV-1 RNA levels of <400 copies/ml (version 1) or <50 copies/ml (version 1.5) were considered below detection. BD, below detection; N/A, not available.
[d] ABC, abacavir; AZT, zidovudine; NVP, nevirapine; 3TC, lamivudine; d4T, stavudine; IND, indinavir.
[e] HIVD, HIV-associated dementia; IDDM, insulin-dependent diabetes melitis.

described previously (4, 20). The products of six independent PCRs were pooled and cloned into pGEM-T-Easy (Promega, Madison, WI), and the nucleotide sequences of multiple independent clones were determined using a SequiTherm EXCEL II DNA sequencing kit (Epicenter Technologies, Madison, WI) and a model 4000L LI-COR DNA sequencer (LI-COR, Lincoln, NE). Nucleotide sequences were aligned and analyzed using DNAMAN software (Lynnon, Quebec, Canada).

Results. Intersubject *nef*/LTR sequences cloned from the earliest available PBMC samples were heterogenous but contained a number of common mutations (Fig. 1A): (i) deletions of various lengths in the amino terminus of the *nef* gene, (ii) at least one deletion in the *nef*/LTR overlap region, and (iii) duplication and/or rearrangement of nuclear factor-κB (NF-κB) and specificity factor 1 (Sp-1) binding sites in the LTR (Fig. 1B). The 3′-most deletion in the *nef*/LTR overlap region shared by all viruses is thought to have been present in the transmitted virus, as none of the subjects has antibodies to a peptide of the corresponding region in the Nef protein (9). None of the viruses are capable of encoding Nef, carrying either an in-phase termination codon (D36) or lacking the *nef* ATG. The duplicated or rearranged region of the LTR was

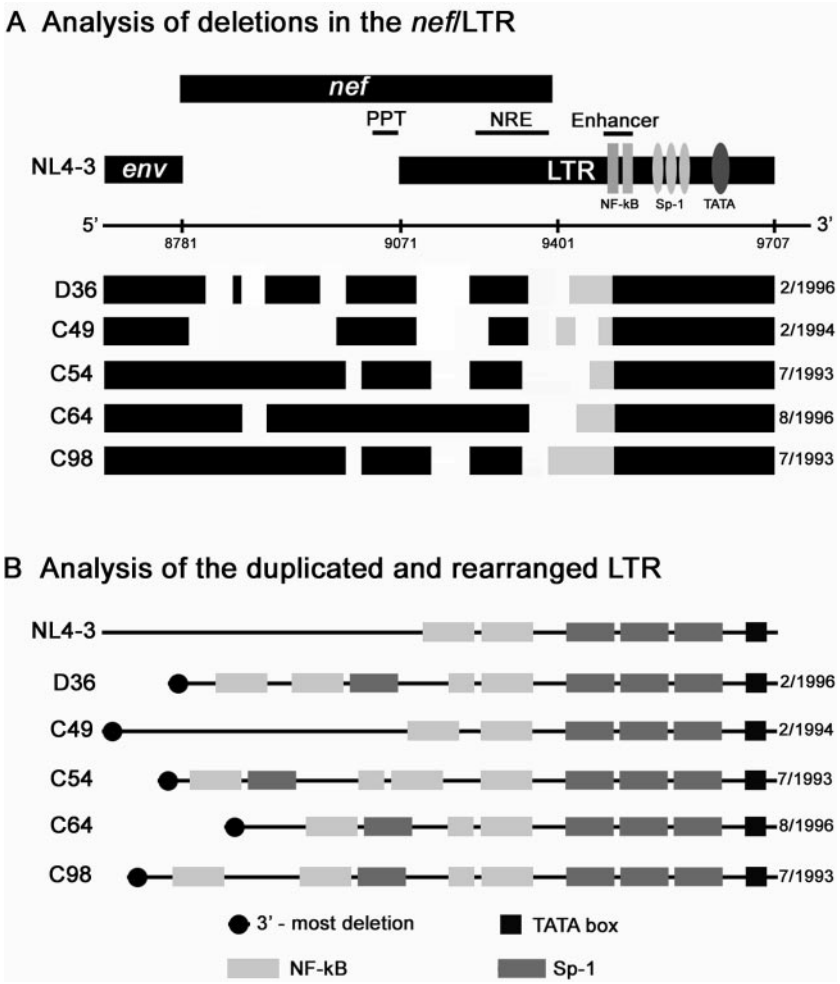

FIG. 1. Analysis of SBBC *nef*/LTR sequence. (A) Schematic representation of *nef*/LTR sequence deletions of HIV-1 cloned from the earliest available PBMC samples. The data shown represent a consensus of at least 20 independent *nef*/LTR sequences cloned from each PBMC sample. The genomic structures are compared to wild-type HIV-1 (NL4-3). Numbers refer to nucleotide positions in NL4-3. Black blocks represent intact sequence, and gaps represent deletions. Gray blocks represent the sequence area containing duplicated and rearranged NF-κB and Sp-1 binding sites in the LTR. The dates shown represent the times when PBMC were collected for analysis. (B) More-detailed analysis of the LTR, depicting transcription factor binding sites. PPT, polypurine tract; NRE, negative regulatory element.

unique to each of the cohort members and varied with respect to the number and arrangement of NF-κB and Sp-1 sites inserted (Fig. 1). Thus, despite the common origin of the viruses, the *nef*/LTR regions differed from subject to subject.

Sequential analysis of *nef*/LTR sequences spanning a 4- to 10-year period demonstrated a further loss of *nef* sequence that differed in magnitude between subjects (Fig. 2). A large deletion of 128 bp emerged in D36, effectively removing the entire *nef* gene with the exception of the region surrounding the *nef* start codon, the polypurine tract which contains terminal signals for HIV-1 integration, and a 90-bp region of the *nef*/LTR overlap region surrounding the negative regulatory element. The basal promoter and enhancer elements of the LTR were retained, but a 44-bp deletion appeared in the rearranged or duplicated NF-κB/Sp-1 motif. The pattern of *nef* sequence loss in C98 was remarkably similar to that which occurred in D36. The pattern of *nef* sequence loss in C54 was also similar but was less extensive than that observed in D36 and C98. However, the additional loss of *nef* sequence in C64 was comparatively minimal. Thus, viruses harbored by D36, C54, C98, and C64 appear to be evolving in a convergent fashion toward a highly deleted, minimal *nef*/LTR structure containing only sequence elements that are absolutely essential for HIV-1 replication. The convergent nature of the *nef*/LTR evolution is further illustrated in Fig. 3, where sequences from the earliest and most recent *nef*/LTR clones from each subject are compared. The convergent nature of the *nef*/LTR sequence changes implies the presence of strong selection pressures that maintain the ability of defective HIV-1 genomes to persist in vivo.

The highly evolved *nef*/LTR sequences harbored by D36, C54, and C98 are strikingly similar to those that have remained dominant in C49 for at least 10 years (Fig. 2). The presence of Nef antibodies directed against peptides spanning the entire Nef protein (with the exception of the common, 3′-most deletion in the *nef*/LTR overlap region) in C49 (9) suggests that prior to 1994 a near-complete *nef* sequence existed and that the bulk of *nef*/LTR evolution occurred during the first 10

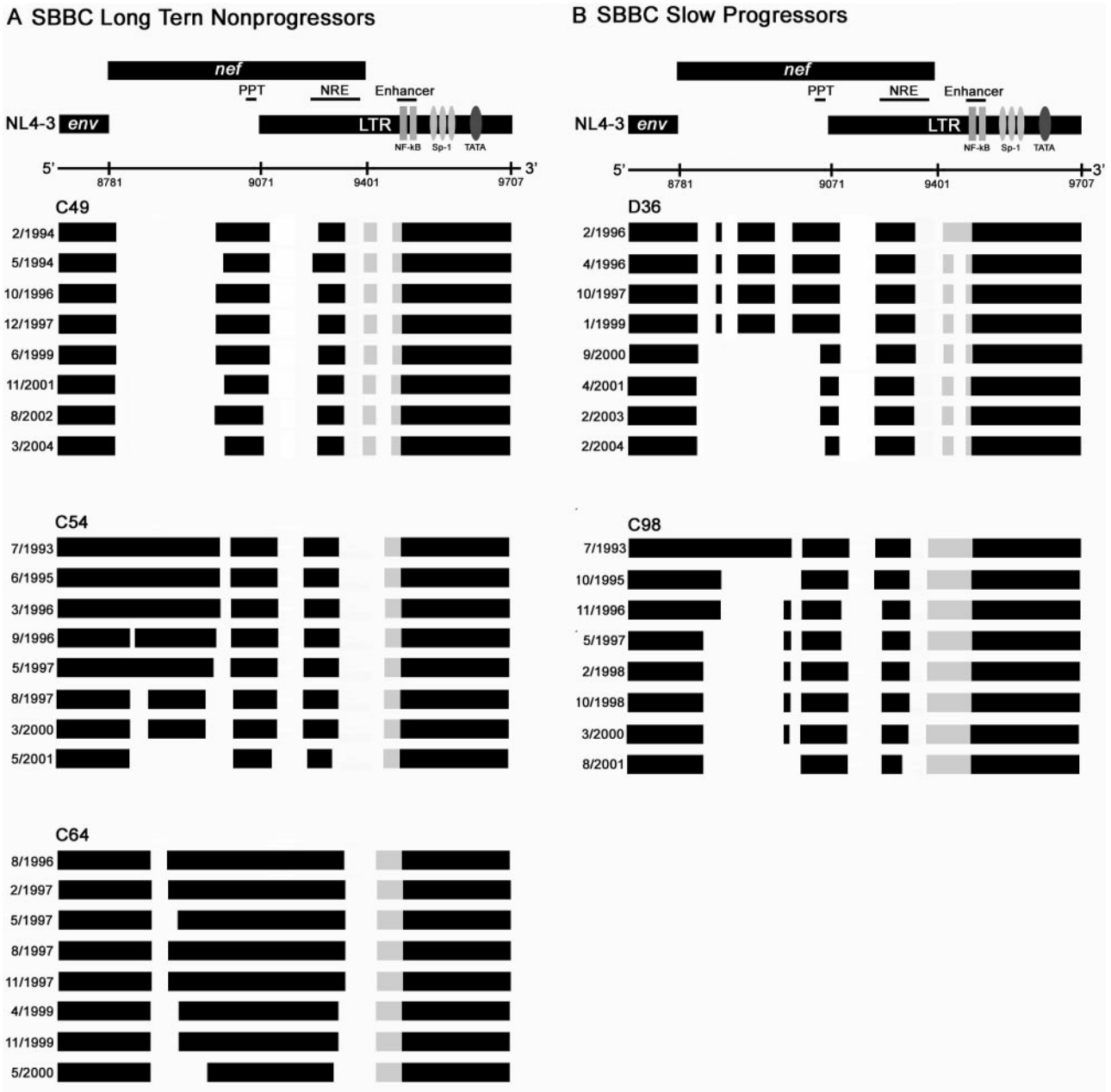

FIG. 2. Evolution of SBBC *nef*/LTR sequence deletions. Schematic representation of *nef*/LTR sequence deletions of HIV-1 cloned from longitudinally collected PBMC samples from SBBC long-term nonprogressors (A) and slow progressors (B). The data shown represent a consensus of at least 20 independent *nef*/LTR sequences cloned from each PBMC sample. The genomic structures are compared to wild-type HIV-1 (NL4-3). Numbers refer to nucleotide positions in NL4-3. Black blocks represent intact sequence, and gaps represent deletions. Gray blocks represent the sequence area containing duplicated and rearranged NF-κB and Sp-1 binding sites in the LTR. The dates shown represent the times when PBMC were collected for analysis. PPT, polypurine tract; NRE, negative regulatory element.

years after infection. C49 has had below-detectable HIV-1 RNA levels since February 1994 (Table 1) and persistent HIV-1 cytotoxic T-cell responses since monitoring of the SBBC began in 1992 (2, 8, 17). Thus, our findings suggest that the highly evolved *nef*/LTR structure is stable and, in the case of C49, does not increase pathogenicity.

Discussion. In this study, we demonstrated a progressive loss of *nef*/LTR sequence in an epidemiologically-linked cohort of long-term survivors who have been infected with attenuated HIV-1 for up to 25 years. The intersubject evolution of *nef*/LTR sequence loss appears convergent, tending toward retention of only those sequence elements that are absolutely required for HIV-1 replication. The highly evolved, minimal *nef*/LTR structure appears stable, being present as the dominant strain in C49 for at least 10 years. Its persistence in this subject, who has consistently had a below-detectable viral load in the absence of HAART (Table 1), initially suggested that evolution to the minimal *nef*/LTR sequence may result in a

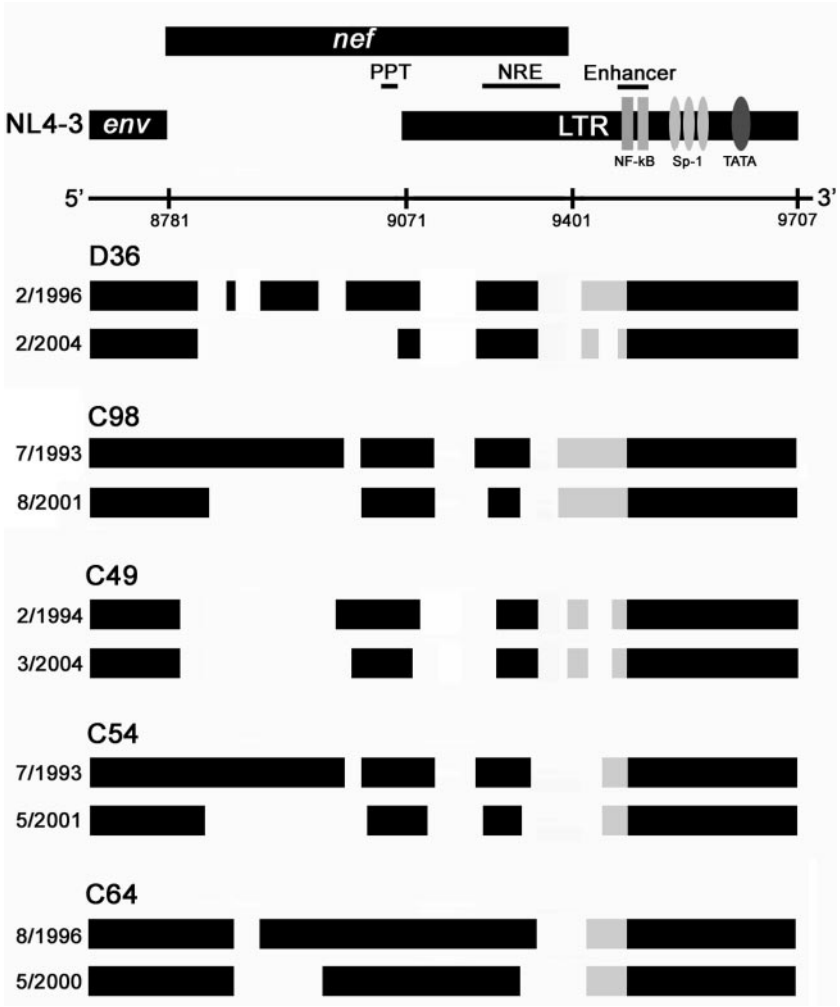

FIG. 3. Convergent evolution of SBBC *nef*/LTR sequences to a minimal *nef*/LTR structure. Comparisons of the genomic structures of *nef*/LTR sequences cloned from the earliest available and most recently obtained PBMC samples are shown. The genomic structures are compared to those of wild-type HIV-1 (NL4-3). Numbers refer to nucleotide positions in NL4-3. Black blocks represent intact sequence, and gaps represent deletions. Gray blocks represent the sequence area containing duplicated and rearranged NF-κB and Sp-1 binding sites in the LTR. The dates shown represent the times when PBMC were collected for analysis. PPT, polypurine tract; NRE, negative regulatory element.

further-attenuated, even less-pathogenic virus. However, we found no association between evolution toward the minimal *nef*/LTR sequence and the clinical status of the subject. For example, both progressing (D36, C98) and nonprogressing (C54, C49) subjects harbored highly evolved forms of *nef*/LTR, and a nonprogressing subject (C64) harbored a significantly less evolved *nef*/LTR. Thus, other viral and/or host factors are likely to contribute to modulating the in vivo pathogenicity of HIV-1 strains with *nef*/LTR deleted.

Reversion to pathogenicity by SIV with *nef* deleted has been associated with restoration of a truncated Nef protein (21), acquisition of further deletions in the *nef*/LTR overlap region (1), and/or duplications of NF-κB binding sites in the LTR (1). In contrast to the SIV studies, the in vivo evolution of HIV-1 with *nef*/LTR was unidirectional toward a smaller *nef*/LTR sequence and the majority of the additional sequence loss was within the *nef*-alone region. Furthermore, none of the clones were capable of encoding Nef. In addition, the presence of duplicated NF-κB binding sites in the LTR was not associated with the clinical status of the SBBC subjects. Therefore, it is likely that any viral factors that modulate the in vivo pathogenicity of HIV-1 with *nef*/LTR deleted will be distinct from those in SIV with *nef* deleted. Interestingly, the unidirectional evolution toward the minimal *nef*/LTR sequence observed in SBBC members is strikingly similar to the pattern of evolution in a slow progressor infected with a *nef*/LTR deletion variant of HIV-1 circulating recombinant form 01_AE (15). The convergent pattern of *nef*/LTR evolution among viruses harbored by SBBC members is therefore unlikely to be due to a unique property of the infecting strain but rather likely reflects an intrinsic instability of HIV-1 with *nef*/LTR defects that is common across clades.

The molecular mechanisms underlying the increased or acquired pathogenicity of *nef*/LTR-deleted HIV-1 harbored by slow progressors D36 and C98 remain to be determined, but possibilities include changes in LTR or Env function that may contribute to increasing replicative capacity or cytopathicity, respectively. In support of this hypothesis, enhanced transcrip-

tional activity of LTR clones isolated from CSF of D36 was shown to contribute to high CSF HIV-1 RNA levels and the development of HIV-associated dementia (4). Moreover, enhanced coreceptor usage by HIV-1 isolated from D36 when $CD4^+$ cell counts fell below 200/μl contributed to enhanced cell killing in ex vivo human tissue cultures (12). Further studies on the function of sequential LTR and Env clones from SBBC subjects are in progress to elucidate their role in the pathogenesis of *nef*/LTR-deleted HIV-1 infection.

In conclusion, while our studies affirm that *nef* is important for HIV-1 pathogenesis, convergent evolution of HIV-1 in vivo toward a minimal and apparently stable *nef*/LTR structure via extensive loss of *nef* sequence was not associated with HIV-1 progression. Factors other than *nef* sequence therefore contribute significantly to HIV-1 progression in the SBBC of long-term survivors.

Nucleotide sequence accession numbers. The *nef*/LTR nucleotide sequences reported here have been assigned GenBank accession numbers DQ287272 to DQ287311.

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