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Genetic Characterization of Human Immunodeficiency Virus Type 1 in Elite Controllers: Lack of Gross Genetic Defects or Common Amino Acid Changes▽

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Despite reports of viral genetic defects in persons who control human immunodeficiency virus type 1 (HIV-1) in the absence of antiviral therapy, the extent to which such defects contribute to the long-term containment of viremia is not known. Most previous studies examining for such defects have involved small numbers of subjects, primarily focused on subjects expressing HLA-B57, or have examined single viral genes, and they have focused on cellular proviral DNA rather than plasma viral RNA sequences. Here, we attempted viral sequencing from 95 HIV-1 elite controllers (EC) who maintained plasma viral loads of <50 RNA copies/ml in the absence of therapy, the majority of whom did not express HLA-B57. HIV-1 gene fragments were obtained from 94% (89/95) of the EC, and plasma viral sequences were obtained from 78% (61/78), the latter indicating the presence of replicating virus in the majority of EC. Of 63 persons for whom *nef* was sequenced, only three cases of *nef* deletions were identified, and gross genetic defects were rarely observed in other HIV-1 coding genes. In a codon-by-codon comparison between EC and persons with progressive infection, correcting for HLA bias and coevolving secondary mutations, a significant difference was observed at only three codons in Gag, all three of which represented the historic population consensus amino acid at the time of infection. These results indicate that the spontaneous control of HIV replication is not attributable to shared viral genetic defects or shared viral polymorphisms.

As the human immunodeficiency virus type 1 (HIV-1) epidemic continues, it has become evident that not all infected persons exhibit a decline in $CD4^+$ T-cell numbers over time (16, 53, 55). Persons who remain free of AIDS and maintain high $CD4^+$ T-cell counts have been termed long-term nonprogressors (LTNP) or long-term survivors (LTS) (16, 53) and have been the focus of a number of studies (2, 21, 22, 32, 33, 35, 41, 45, 49, 52, 59). With the advent of viral load testing, some LTNP were observed to have detectable levels of plasma viremia, and many eventually progressed to AIDS (4, 13, 42, 56). However, a subset of HIV-1-infected persons control viremia to below the limit of detection by currently available commercial assays (<50 RNA copies/ml) without the need for medication. These individuals, referred to as HIV-1 elite controllers (EC), elite suppressors, or HIV-1 controllers, are rare and are estimated to occur at a frequency of approximately 1 in 300 infected persons (23). Some of these EC have had documented infection for more than two decades yet show no evidence of $CD4^+$ T-cell decline. Unraveling the mechanisms associated with successful HIV control in these subjects will contribute to our understanding of HIV-1 pathogenesis and may help inform vaccine strategies focused on the induction of immune responses to control disease rather than prevent the acquisition of infection (38).

Host genetics, host innate and adaptive immune responses, and viral sequence variation all have been suggested to influence the rate of disease progression in HIV-1 infection (reviewed in reference 23). Although strong virus-specific T helper cell responses are more frequent and certain HLA alleles are preferentially expressed (6, 50) in individuals who spontaneously control HIV-1 (26), studies of large numbers of EC indicate marked heterogeneity in both immunologic and host genetic factors (54). Indeed, we found that the total magnitude and breadth of HIV-specific $CD8^+$ T-cell responses were lower in EC than in persons with progressive infection (54). Recently, some studies of EC have addressed viral and host factors associated with the spontaneous control of HIV (5, 6, 9, 10), but these have been limited to small numbers of individuals and have focused primarily on persons expressing the protective HLA class I allele B57. Indeed, although the protective HLA alleles B57 and B27 are highly enriched among EC (observed in 44 and 15% of EC, respectively) (54), the presence of these protective alleles cannot explain the majority of EC cases; thus, the remainder of the cases must be

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attributable to other factors. Indeed, studies have suggested that naturally occurring human genetic polymorphisms in chemokines and their ligands (24, 25, 31, 44) and viral genetic factors attenuate infection (8, 22, 41). To date, however, there has been no large-scale evaluation of viral genetic variability in EC.

In the present study, we attempted to sequence HIV-1 from 95 EC to assess the frequency of viral genetic mutations, and we compared these results to those for 96 persons with chronic progressive HIV infection (CP). Notably, our study differs in several ways from previous reports that sought to identify viral genetic factors associated with LTNP/LTS. First, this large cohort involves subjects with undetectable plasma viremia, a substantial number of whom express no known protective HLA class I alleles, enabling us to assess viral genetics in the presence or absence of protective HLA alleles. Second, we examined all coding HIV-1 genes in these EC, rather than individual coding regions. Third, using an approach similar to that of Bailey et al. (6), we obtained plasma viral sequences that likely represent replicating virus in EC. Finally, we compared sequences between EC and CP at the single-codon level, correcting for HLA, the phylogenetic structure of the sequences, and HIV amino acid coevolution. Our results indicate that neither specific viral genetic polymorphisms nor gross viral genetic defects likely explain the ability of EC to contain HIV-1 infection.

MATERIALS AND METHODS

Study subjects. Treatment-naïve chronically HIV-1-infected subjects with plasma viral loads of <50 copies RNA/ml were randomly selected from a cohort of HIV-1 EC that has been described previously (54). All studies were approved by the Institutional Review Board at Massachusetts General Hospital, and written informed consent was obtained from all participants prior to enrollment. Plasma and peripheral blood mononuclear cells (PBMC) were obtained as described previously (54) and were stored at −80°C and in liquid nitrogen, respectively.

Viral RNA isolation. Plasma (4.5 to 35.0 ml, with a mean of 19.7 ml, based on availability) from EC was centrifuged for 10 min at 1,500 rpm to remove cell debris. Virus was then concentrated by ultracentrifugation at 124,513 relative centrifugal forces for 2 h using an SW32 Ti rotor (Beckman Coulter, Fullerton, CA). Supernatant was removed, leaving 140 μl plasma, and viral RNA was extracted using the Qiagen viral RNA mini kit (Qiagen Inc., Valencia, CA). During this process, an on-column DNase treatment was performed using the Qiagen RNase-free DNase set (Qiagen Inc., Valencia, CA). Viral RNA was eluted in 80 μl of DNase- and RNase-free water and stored at −80°C.

Genomic DNA isolation. Total genomic DNA was isolated from approximately 5×10^6 previously frozen PBMC using the Qiagen DNA blood mini kit (Qiagen Inc., Valencia, CA) and was eluted in 100 to 200 μl DNase-free water. Isolated DNA was stored at −80°C.

PCR amplification. HIV-1 gene regions were amplified using nested reverse transcriptase PCR (RT-PCR) to generate either three products (*gag*, *pol*, and a 3′ genomic half) or five products (*gag*, *pol*, *vif*/*vpr*/*vpu*, *env*, and *nef*). The sequences of primers are available upon request. First-round RT-PCR was performed using the Superscript III one-step RT-PCR system with Platinum *Taq* DNA polymerase with High Fidelity (Invitrogen, Carlsbad, CA). Each 50-μl reaction mixture was composed of 10 μl of RNA, 25 μl of 2× reaction mix (buffer, deoxynucleoside triphosphate, and $MgSO_4$), 400 nM of forward and reverse outer primers, 1 μl of enzyme mix, and water. Takara EX *Taq* DNA polymerase Hot Start enzyme (Takara Bio Inc., Shiga, Japan) was used for the second-round DNA-PCR using 2 μl DNA and 600 nM of each primer according to the manufacturer's directions. To rule out the possibility that we were amplifying proviral DNA, control experiments that left out the RT step were performed using *gag* region primers, which produced the most sensitive PCR. No proviral amplification was observed in control runs. HIV-1 proviral gene sequences were amplified by nested PCR with Takara EX *Taq* DNA polymerase Hot Start enzyme and the same primer sets, except that 500 ng to 2 μg of template genomic DNA was used in each 50-μl reaction mixture.

Sequencing. PCR products were purified using the Purelink PCR purification kit (Invitrogen, Carlsbad, CA). Bidirectional cycle-sequencing reactions were performed with *Taq* DyeDeoxy terminator kits (Applied Biosystems, Foster City, CA) using 60 HIV-1-specific sequencing primers and were analyzed on an ABI 3730 PRISM automated sequencer to obtain population sequences for each product. Chromatograms were edited using Sequencher software (Gene Codes Corporation, Ann Arbor, MI). Multiple alignments were constructed using ClustalW, and HIV-1 protein sequence alignments were created using Gene Cutter (Los Alamos National Laboratories; http://www.hiv.lanl.gov). Phylogenetic trees were drawn using the maximum-likelihood method (using DNAml in PHYLIP). Proviral sequences that encoded premature stop codons as a result of apparent G-to-A hypermutations were excluded from the analysis.

Mutagenesis and replicative capacity assays. Mutant viruses were constructed by site-directed mutagenesis using a modification of the method described elsewhere (58). Briefly, mutagenesis reactions were performed on a pUC19 plasmid carrying the SacI-SbfI fragment of NL4-3 (2,353 bp containing the entire *gag* gene) using Quick Change II (Stratagene, La Jolla, CA), and mutations were confirmed by sequencing. The fragments were then ligated into full-length pNL4-3. Viruses were obtained by transfecting HEK293T cells with plasmid variants and were titrated using a CEM-derived long terminal repeat-green fluorescent protein (LTR-GFP) reporter T-cell line (GXR cells) (12). Replication capacity assays were performed in monoculture by infecting GXR cells with virus (at multiplicity of infection of 0.002), and the proportion of GFP^+ cells was measured on days 2 through 8 using a FACScalibur flow cytometer (BD Biosciences, San Jose, CA). The percentage of GFP expression was normalized to the day-2 value, and the log 10 increase (*n*-fold) was plotted for each virus. The natural log slope of each line was determined using Microsoft Excel and used to compare the replication kinetics of variant viruses. All experiments were performed in duplicate.

Analysis of sequences. EC viral sequences were compared to viral sequences obtained from 96 CP from the greater Boston area (87 to 96 CP [complete fragments were obtained from 70 to 87 CP], depending on HIV genes) (28 and unpublished data). The majority of CP were untreated. The median viral load was 48,950 RNA copies/ml (interquartile range, 9,266 to 181,250 RNA copies/ml), and the median duration of infection was 7.7 years (IQR, 1.5 to 9.8 years). The comparison of length variation between EC and CP was limited to areas of known-length polymorphisms, including the C terminus of p17 (amino acids [aa] 109 to 133 in HXB2); the V1 (aa 131 to 149), V2 (aa 158 to 197), V3 (aa 296 to 331), V4 (aa 385 to 418), and V5 (aa 460 to 471) loops in gp120; the N terminus of gp120 (aa 1 to 35), Vpr (aa 79 to 91), and Vpu (aa 55 to 83); and the N terminus of Nef (aa 20 to 32). Statistical analysis was performed by the Mann-Whitney U test. Comparisons of viral sequences from EC and CP at each individual codon were performed, excluding any insertions that had been compared to HXB2, and analyzed initially using a likelihood ratio test. Likelihoods were adjusted to account for HLA class I-associated mutations, phylogenetic relationships among the sequences, and viral sequence covariations (7, 14, 17, 18). In order to construct phylogenetic trees, corresponding gene sequences were used for *gag*, *gp120*, *gp41*, and *nef*. For protease, RT, and integrase, the shorter *pol* sequences (excluding the p6 sequence) were used. For *vif*, *vpr*, *vpu*, *tat1*, and *rev1*, sequences extending from the initiation codon of *vif* to the stop codon of *vpu* were used. Finally, for *tat2* and *rev2*, the corresponding *gp41* sequences were used. A q-value approach was employed (61) to correct for multiple comparisons. Associations with $q < 0.2$ (indicating a 20% false discovery rate) were considered significant.

Nucleotide sequence accession numbers. Sequences determined in this work have been submitted to GenBank under accession numbers EU517721 to 518128.

RESULTS

Amplification and sequencing of plasma and proviral HIV-1 from EC. To address the possibility that viral genetic defects contribute to the ability of some persons to control HIV-1 to undetectable levels (<50 RNA copies/ml plasma), we attempted to amplify and sequence plasma viral RNA and/or proviral DNA from a total of 95 individuals randomly selected from an established cohort of HIV-1 EC (54), and we obtained plasma RNA sequences from 78 EC and proviral

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TABLE 1. Sequences obtained from HIV-1 EC[a]

HIV-1 gene	No. of sequences[b] from:		Total no. of subjects[c]
	Plasma	PBMC	
gag	60 (55)	37 (33)	78 (71)
pol			
Protease	46 (46)	18 (18)	59 (59)
RT	46 (46)	18 (18)	59 (59)
Integrase	45 (43)	18 (17)	58 (56)
vif	45 (45)	22 (22)	58 (58)
vpr	45 (45)	22 (22)	58 (58)
vpu	45 (44)	21 (17)	58 (54)
rev			
Exon 1	45 (45)	24 (24)	61 (61)
Exon 2	34 (33)	13 (13)	43 (42)
tat			
Exon 1	45 (45)	23 (23)	59 (59)
Exon 2	33 (31)	12 (12)	41 (39)
env			
GP120	40 (19)	13 (11)	49 (27)
GP41	34 (29)	13 (11)	42 (33)
nef	47 (44)	31 (28)	64 (60)

[a] Sequences that span more than half of the corresponding gene were counted.
[b] The numbers in parentheses are the numbers of complete fragments.
[c] The number of EC from whom at least one of the HIV-1 gene fragments was amplified.

DNA sequences from 71 EC. Of the 95 subjects studied, at least one gene fragment was obtained from 89 subjects (94%), including 78% (61/78) from plasma and 76% (54/71) from PBMC (Table 1). Full HIV genome sequences without any breakpoints were obtained from 17 EC. Sequences were obtained from individuals carrying known protective HLA class I alleles (i.e., HLA-B27, HLA-B51, or HLA-B57) and from persons lacking these alleles at similar frequencies. Potential contamination among the samples was ruled out by phylogenetic analysis, and when both plasma and proviral sequences were available from the same subjects, these were confirmed to cluster together in the tree (data not shown). The V3 loop sequences of gp120 from the 41 subjects from whom Env sequences were obtained indicated that all were infected with R5 viruses (http://indra.mullins.microbiol.washington.edu/pssm/) (37). A summary of the obtained sequences for each coding gene is shown in Table 1.

We further analyzed these sequences using the entire *gag* sequence (71 EC and 96 CP) to construct a phylogenetic tree, including HIV-1 group M ancestral, HIV-1 subtype B ancestral, and other HIV-1 subtype reference sequences from the LANL database, and rooted it to a group M ancestral sequence (Fig. 1). Plasma viral sequences were used in those cases in which plasma and proviral sequences were available from the same subject. In all but two cases, *gag* sequences indicated that these EC recruited in the United States were infected with clade B virus strains. Two of the non-clade B cases were infected with CRF_02 or clade F virus, which were confirmed by using the REGA HIV subtyping tool, version 2.0 (http://www.bioafrica.net/subtypetool/html/). In one additional subject for whom the *gag* sequence was not available, a unique recombinant form, which could not be categorized to any subtypes, was indicated by the same Web-based tool using the *vif*, *vpr*, and *vpu* gene sequence (data not shown). We analyzed phylogeny in the context of HLA alleles (Fig. 1) and likewise found no evidence of a common ancestor among EC that did or did not express a protective HLA allele.

Gross genetic defects. We next examined HIV-1 coding genes for evidence of gross genetic defects, excluding the highly variable regions (see Materials and Methods). Insertions/deletions (In/dels) were observed in a minority of persons and mostly were limited to 1 or 2 aa. We found a comparable frequency of such short In/dels in the sequences from EC and CP (Table 2). Although gross defects in the *nef* gene have been described for LTNP/LTS (22, 41), only 3 of 63 (4.8%) EC for whom the *nef* gene was sequenced showed deletions of 9 aa or greater within codons 33 to 85, where such defects previously had been reported (Table 2). One of the three showed a 21-aa deletion (codons 54 to 74 in HXB2), which corresponds to a known functional motif involved in CD4 and major histocompatibility complex class I down-regulation, as well as cellular activation by Nef (30). We found a 16-aa deletion (codons 34

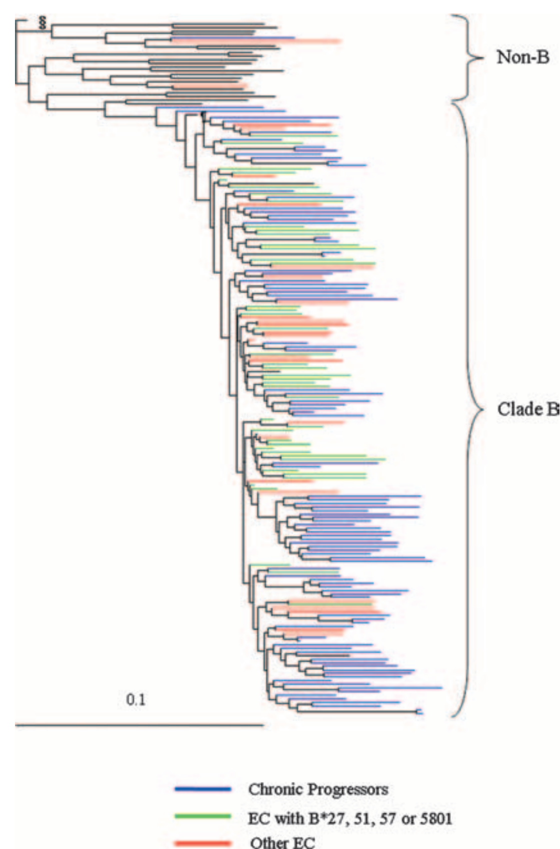

FIG. 1. Maximum-likelihood tree of HIV-1 from EC and CP. Trees were computed with DNAml (in PHYLIP) using 1,500 bases of *gag* nucleotide sequences and were rooted to M ancestral sequences. Two of each subtype reference sequence in LANL were included. Gap was proportionally distributed. Blue lines indicate chronic progressors. Green and red lines indicate EC with protective major histocompatibility complex class I alleles (B*27, B*51, B*57, or B*5801) and the other EC, respectively. Only complete fragments were used for this analysis. § indicates the M ancestral sequence; ¶ indicates the B ancestral sequence.

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TABLE 2. In/dels observed in conserved regions of the HIV-1 genome in EC and CP

Protein	HXB2 codon position	Consensus B sequence	In/del	No. and length of In/dels[a]				P value[b]
				EC		CP		
				No. observed/total no. of subjects	aa length (no. of subjects)	No. observed/ total no. of subjects	aa length (no. of subjects)	
Gag	340	A	Deletion	1/77		0/96		0.445
Gag	427–428		Insertion	1/77 (S)		1/96 (N)		1.000
Gag	443–444		Insertion	1/77 (G)		1/96 (G)		1.000
Vif	60–61		Insertion	1/58 (D)		2/96 (D or EA)		1.000
Vpu	7–8		Insertion	1/58 (I)		0/96		0.377
GP41	4–5		Insertion	8/36	1 (8)	10/94 (A [3], T [6], V [1])		0.097
GP41	103–110	SWSNKSLDE	Insertions	2/42	2 (1), 1 (1)	4/96	2 (2), 1 (2)	1.000
GP41	268–271	TRIV	4-aa deletion	1/42	4 (1)	0/96		
						2/96	4 (1), 6 with stop[c] (1)	0.304
GP41	328–333	AILHIPR	7-aa deletion	1/42	7 (1)			1.000
Nef	33–85	Not shown	Deletions	10/63	21 (1),16 (1), 4 and 5 (1), 2 (7)	6/96	2 (6)	0.061, 0.249[d]
Nef	152–161	Not shown	Deletions	6/64	3 (2), 4 (2), 5 (1), 1 (1)	5/96	1 (2), 2 (2), 6 (1)	0.349
Rev (exon 2)	86–89	NEDC	Deletions	1/43		0/96		0.309
Tat (exon 2)	87	S	Stop	1/41		0/96		0.299

[a] The letters in the parentheses indicate the observed amino acids, and the numbers in parentheses indicate the number of subjects.
[b] The frequency of In/dels was examined by Fisher's exact probability test regardless of specific amino acid change or the length of In/dels.
[c] Stop, premature stop codon.
[d] The frequency of 2-aa deletions.

to 49) in a second EC and a 9-aa deletion (codons 36 to 40 and 48 to 51) in a third EC. Neither of these deletions occurred in known functionally important regions. One of 42 EC encoded a plasma viral sequence with 4-aa (codons 268 to 271) and 7-aa (codons 328 to 334) deletions in gp41, but 4- to 6-aa deletions at position 328 to 334 were seen in 2 of the 96 CP samples. Although the 4-aa deletion (codons 268 to 271) was not found in CP, it did not fall within any known functional motif in gp41. This 4-aa position also corresponds to codons 86 to 89 of Rev (in exon 2), which is located inside the Rev activation domain, though whether they are essential codons for Rev function is not known (46, 66). One EC plasma viral sequence had a premature stop at codon 87 of Tat (in exon 2). However, the *tat* exon 2 of HIV-1 is not required for the *trans*-activation activity of Tat protein (65). Taking these results together, we conclude that there were no common gross viral genetic defects in *nef* or other HIV-1 coding genes among EC.

Length polymorphisms. In addition to analyzing the conserved regions of the genome for genetic defects, we also examined the more variable regions of the genome, in this instance the regions of known length polymorphism in the HIV-1 coding sequence (see Materials and Methods). Variation in Gag p6 has been associated with changes in viral replication capacity (48). Other studies have shown an association between long-term nonprogression and the extension of the V2 loop sequence in gp120 (60, 64). Examining the length of variable regions (see Materials and Methods) in sequences between EC and CP, we observed statistically significant differences at four sites, which were located in the C terminus of p17 (codons 109 to 133 in HXB2), the V1 (codons 131 to 149) and V2 (codons 158 to 197) loops in gp120, and the N terminus of Nef (codons 20 to 32) (P values of <0.0001, 0.0004, 0.0114, and <0.0001, respectively). The majority of EC maintained gene lengths comparable to that of the consensus B sequence, whereas samples from CP displayed extensions in these four regions. In addition, CP tended to have longer Gag p6 sequences, though this was not statistically significant (P = 0.0568) (Fig. 2). There were no statistically significant differences in the lengths of other variable regions, including Vpr (79 to 91), Vpu (55 to 83), the N terminus of gp120 (1 to 35), V3 (296 to 331), V4 (385 to 418), and V5 (460 to 471) (Fig. 2 and data not shown). Since length polymorphisms in the C terminus of p17 may be associated with protease inhibitor resistance mutations (3), we examined the resistance mutation profile of the protease gene in CP since some had a history of antiretroviral treatment. We found that 10 of 96 harbored major resistance mutations, while 26 had minor resistance mutations (data not shown) (http://hivdb.stanford.edu/pages/algs/HIVdb.html). However, there was no difference in the length of the C termini of p17 between protease inhibitor-sensitive and -resistant progressors (data not shown). Therefore, neither extensions nor deletions in these highly variable regions of HIV genes were likely associated with elite control.

Codon-by-codon comparison between EC and CP. To assess the possibility that shared amino acid polymorphisms explain the ability of some individuals to control HIV-1 infection, we examined whether any single amino acid usage was statistically associated with the EC phenotype or the CP phenotype by using a likelihood ratio test, correcting for known HLA-associated mutations, phylogenetic relationships among the sequences, and coevolving secondary mutations (see Materials and Methods). Since particular HLA alleles, like B57, B27, and

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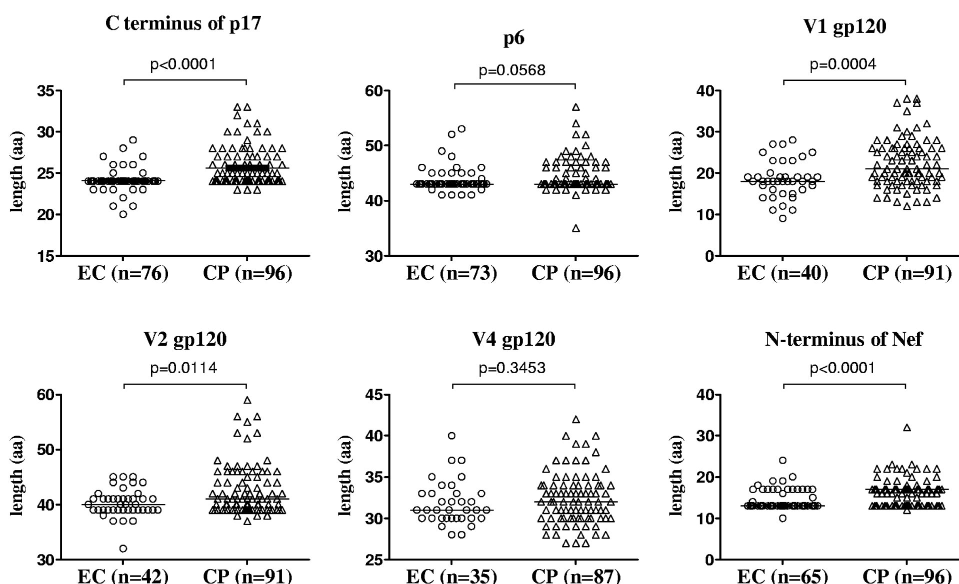

FIG. 2. Comparison of length polymorphisms between EC and CP. The lengths of the C terminus of p17 (codons 109 to 133 in HXB2; 24 aa), p6 (codons 1 to 43; 43 aa), V1 (codons 131 to 149; 19 aa), V2 (codons 158 to 197; 39 aa), and V4 (codons 385 to 418; 26 aa) of gp120 and the N terminus of Nef (codons 20 to 32; 13 aa) were compared between EC and CP. Statistical analysis was performed by a Mann-Whitney U test. Black lines indicate median values.

B51, were enriched in EC, it was necessary to exclude HLA-associated mutations from this analysis. Also, in order to detect primary mutations that are associated with EC, secondary or compensatory mutations that tracked with primary mutations were taken into consideration (17). We limited our analysis to viral sequences with known HLA class I types both for EC and CP and to plasma viral sequences for EC, since there was a report that cytotoxic T-lymphocyte (CTL) escape mutations are seen in plasma viral sequences but are seen less frequently in proviral sequences in EC (6). Indeed, we observed a similar finding in our EC data set (data not shown). CP sequences used in this analysis were mostly from proviruses, which tend to be highly concordant with plasma virus sequences in viremic subjects (data not shown). In/dels that differed from the HXB2 reference sequence were removed for this analysis. Table 3 shows candidate mutations for which the q value was <0.2. After correction for multiple comparisons, only three mutations in Gag (67A, 102E, and 389I) and one in gp41 (125S) remained statistically significant. In particular, S67A and D102E showed a strong association with the EC phenotype. We next constructed S67A and D102E Gag mutant NL4-3 viruses and compared their viral replication capacities to that of wild-type NL4-3 (Fig. 3). However, no measurable defects in viral replication capacity were observed. We next questioned whether these amino acid differences simply reflect the infection of EC with older strains of HIV-1, since most of these individuals had been infected for more than a decade. To examine this, we stratified EC for whom years-after-diagnosis data were available, and we compared viral sequences between the EC diagnosed within the past 10 years (EC $<$ 10) and the EC diagnosed more than 10 years ago (EC $>$ 10). Both S67A and D102E were observed in 6 of 15 (40.0%) EC $<$ 10. However, 18 of 25 (72.0%) EC $>$ 10 had S67A, and 21 of 25 (84.0%) EC $>$ 10 had D102E (P = 0.094 and P = 0.0063, respectively; Fisher's exact test). Therefore, 67A and 102E mutations were much more common in EC infected for more than a decade. However, there was no difference in the frequency of the 389I mutation of Gag between the groups (10/15 and 13/25; P = 1.0). To further address the issue of HIV-1 population sequence evolution, we examined U.S. clade B se-

TABLE 3. Polymorphisms associated with EC[a,b]

Protein	Codon	No. of EC with polymorphism	No. of EC without polymorphism	No. of CP with polymorphism	No. of CP without polymorphism	*N*	*P* value	q value
Gag	67A	29	21	8	78	136	4.64E–08	0
Gag	67S	21	29	77	10	137	1.54E–07	0
Gag	102E	39	14	23	60	136	9.37E–05	0.1
Gag	102D	14	39	60	23	136	9.37E–05	0.1
Gag	389I	30	21	21	64	136	0.000398	0.181818
GP41	125S	5	19	2	69	95	0.00018	0.076923

[a] Comparisons were performed using only complete clade B fragments.
[b] Mixtures were excluded from the analysis, which led to variation in the total numbers across tests. For example, when both N and T were present at a position, the observation was excluded when the amino acid N or T was tested. In contrast, the observation was included (as a negative) when an amino acid other than N or T was tested.

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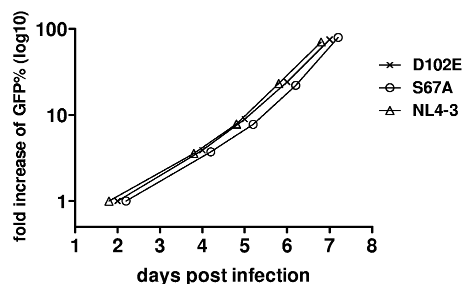

FIG. 3. Viral replication capacity of S67A and D102E Gag mutant NL4-3. The percentage of GFP expression was normalized to day-2 values and are shown on a log 10 scale. In order to avoid overlay, they were nudged a little bit in the direction of the *x* axis. The experiment was duplicated, and the representative data are shown.

quences deposited in the LANL database since 1982. During the period between 1982 and 1995, 11 of 32 sequences had 67A (34.3%), 17 of 32 had 102E (53.1%), and 13 of 32 (40.6%) had 389I. In contrast, after 1995, 5 of 22 (22.7%) had 67A, 5 of 22 (22.7%) had 102E, and 7 of 22 (31.8%) had 389I. Therefore, each of the three Gag mutations appeared to be more common in circulating HIV-1 isolates before 1996 in the United States. From the data on replication capacity and sequence frequency, we conclude that the common Gag mutations identified in EC merely reflect the relative proportion of these amino acids in circulating strains at the time of infection, rather than revealing a specific biological difference between viruses in EC and CP.

In terms of the *env*-associated polymorphisms detected by this codon-by-codon approach, insufficient sequences were available with reported dates of isolation to allow for a meaningful comparison. The amino acid 125S in gp41 was seen in only 7 of 163 (4.3%) U.S. clade B sequences with a known sampling year, of which four were sampled after 1995. Therefore, we were unable to examine this substitution in circulating virus by this approach. It will be necessary to expand the number of EC gp41 sequences to evaluate this association further, but the data thus far indicate that specific genetic polymorphisms, as assessed by codon-by-codon analysis, do not account for EC.

DISCUSSION

The generation and analysis of viral sequences derived from HIV-1 EC represents a substantial challenge, since PCR amplification from such small quantities of starting material is problematic. Despite these difficulties, we were able to obtain viral genetic information from >90% of EC subjects analyzed, including >75% of EC plasma samples. These results confirm that the vast majority of EC harbor ongoing low levels of HIV-1 replication. In this large cohort, however, we observed that many of the previously reported genetic defects identified in small-scale studies were not present at significant levels, including a lack of gross genetic defects in Nef or other genes, with no single-amino-acid changes that were predictive of elite control.

Our study attempted to address several limitations that have hampered previous studies on HIV-1 viral genetics in LTNP/LTS. First, most studies analyzed only a small number of individuals (often fewer than 10), and analyses to date have focused primarily on EC who express HLA-B57. Here, we were able to obtain sequences from 89 of 95 EC tested, the majority of whom did not express HLA-B57. As reported by Bailey et al. (6), we could obtain plasma viral sequences from most EC by concentrating virus from a large volume of plasma (up to 35 ml). Second, previous studies typically focused on individual genes, making it difficult to assess the relative importance of viral sequence alterations in the context of other genes. Here, we were able to examine sequence variation in all HIV-1 proteins, comparing between 27 and 71 independent sequences from each coding gene from EC. Finally, the majority of studies have used proviral DNA extracted from PBMC. Amplified proviral genes may not represent actively replicating viruses in these individuals and may, rather, reflect only latent or defective viruses that were archived in PBMC, such as hypermutated sequences (6).

Taking these concerns into account, we analyzed our EC samples for evidence of viral genetic defects. In the phylogenetic studies of our samples, we did not observe the clustering of EC viruses, suggesting that there was no common genetic ancestor to account for control by these individuals. Nef deletions, which had been linked to nonprogressive infection in a cluster of common-source infections in Australia (22) and have been noted in other studies (11, 36, 40, 41, 47, 62), were likewise infrequent. In the cohort, only 3 of 63 EC sequences examined encoded moderate-length deletions in the conserved region of the *nef* gene, and two were observed in plasma sequences. Deletions in other genes likewise were uncommon. One EC plasma virus had a previously undescribed 4-aa deletion at codons 86 to 89 in Rev, which corresponded to codons 268 to 271 in gp41. Although it was inside of the activation domain of Rev, it was unknown whether this deletion affected Rev function or not. Previously, unique 1- to 2-aa deletions located in the C terminus of p17 and p6 in Gag were reported to be seen in LTNP/LTS sequences (2). However, both of these regions were seen to have highly variable lengths. Therefore, they are unlikely to explain successful spontaneous viremia control. Similarly, a deletion in the C terminus of Vpr reported for LTNP/LTS (63) also appears to fall within a region of length polymorphism. Finally, a case report identified a 2-aa insertion between codons 60 and 61 in Vif, which was seen in a mother-child pair displaying long-term nonprogression (1). In our data, we also identified an EC with a single-amino-acid insertion at this position; however, two progressors had either a 1- or 2-aa insertion at this site, suggesting that insertions at this position represent naturally occurring HIV polymorphisms unrelated to viremia control. We have not assessed viral LTR sequences in these subjects, so it remains possible that LTR function is altered in EC. It will be necessary to investigate this issue in future studies. Based on our comprehensive analysis of viral sequences in EC subjects, we conclude that gross viral genetic defects in coding sequences contribute little to the development of the EC phenotype.

These studies also allowed us to examine regions with reported length variability, and we found that the majority of EC maintained normal gene lengths. However, EC expressing HLA-B27, HLA-B51, HLA-B57, or HLA-B5801 differed from other EC in the length of the C terminus of p17. On the contrary, compared to that of EC, CP encoded extensions of the C terminus of p17, the V1 and V2 regions of gp120, and the N terminus of Nef, and there was a similar trend in the p6

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protein as well. Interestingly, two studies described an association between V2 elongation and slow disease progression (60, 64); however, both studies compared LTNP/LTS to rapid progressors. Recently, Sagar et al. demonstrated that V2 loop extension occurs progressively during the course of infection (57). Taken together, the current data suggest that the V2 loop extends over the course of HIV infection as a result of immune pressure exerted by neutralization antibodies (NAb). Since rapid progressors likely generate weak antibody responses (19), viruses are not forced to evade NAb pressure and may, therefore, maintain shorter V2 loops. The observation that EC viruses maintain shorter V2 loops than CP suggests that they are subjected to less NAb pressure, which is consistent with recent reports of low-magnitude NAb responses in EC (54). This feature seems to distinguish EC viruses from previous descriptions of LTNP/LTS subjects, who typically display higher plasma viremia. Although we examined heterologous viral neutralization by plasma from EC in a previous study (54), we are currently planning a study for autologous envelope neutralization using envelope PCR products obtained for the present study, which hopefully will add further insight about the role of NAb in EC, particularly in those who do not express protective HLA alleles. Mechanisms to explain genetic extensions at the other three sites are unknown. Polymorphism in the V1 loop may function similarly to the V2 extensions described above. Very recently, Cao et al. reported the ability of HIV-1 to escape CTL pressure through an insertion in p6 Gag that disrupted an epitope in the Gag-Pol frameshift region (15). Gag and Nef are highly targeted by CTL during HIV-1 infection (29, 39, 43). It is possible that viral escape from immune selection pressure in the C terminus of p17 and the N terminus of Nef also explain the differences we observed in these regions, but this will require more study.

We did not observe gross genetic differences between EC and CP; however, it is possible that even single-amino-acid changes can result in defective virus. To assess this, we compared viral sequences between EC and CP at the single-codon level. Kirchhoff et al. analyzed Nef codon sequences between LTNP/LTS and progressors (40), but we know of no similar studies on other HIV-1 genes or EC using this approach. Moreover, we controlled for HLA-mediated selection, phylogenetic relationships among the sequences, and HIV amino acid covariation to reduce potential confounding by these factors (61). After correction for multiple comparisons, we observed significant differences at only three codons in Gag and one codon in gp41. However, all three Gag substitutions represented a historical consensus residue, reflecting an earlier date of infection in EC, rather than true association with elite control. Codon 125S in gp41 also was associated with elite control, but the paucity of data regarding the timing of infection precluded an appropriate analysis. Larger studies therefore are warranted to elucidate whether this substitution is truly related to elite control.

We also investigated the occurrence of mutations previously associated with LTNP/LTS in our data set. Lum et al. reported that Vpr R77Q was more frequently observed in LTNP/LTS than in progressors and demonstrated that the 77Q mutant resulted in a reduction in the apoptosis of HIV-infected target cells (45). However, this result remains controversial (20, 27, 51). Our data indicated that 27 of 59 (45.8%) EC encoded Q at codon 77, but this mutation actually was seen more frequently in CP (56/97, 57.7%), and the difference was not statistically significant ($P = 0.86$; Fisher's exact probability test). Of note, the 77Q residue is present in the current consensus B sequence in the LANL database. We conclude that 77Q is not associated with long-term nonprogression or elite viremia control. Similarly, Hassaine et al. reported that an R132S mutation in Vif diminished viral replication capacity and was more frequently seen in subjects with lower plasma viral loads (34). However, we failed to observe a difference between EC and CP at this residue, with 132S present in 22 of 54 EC (40.7%) and 33 of 93 CP (35.5%) ($P = 0.602$). Kirchhoff et al. compared Nef protein sequences between LTNP/LTS and progressors and observed five amino acid changes (15T, 51N, 102H, 170L, and 182E) associated with LTNP/LTS (40). Following correction for multiple comparisons, none of these changes was statistically significant in our analysis ($q > 0.2$). However, the uncorrected P value of 15T was significant; therefore, we compared the frequency of 15T in U.S. clade B sequences deposited in the LANL database prior to 1990 with sequences from 1990 onward. 15T was more frequently observed in the period before 1990 (30 of 56; 53.6%) than for later samples (11 of 35; 31.4%) ($P = 0.05$), suggesting that 15T is a sampling artifact and is unlikely to be associated with LTNP/LTS or elite control. We conclude that these previously reported amino acid changes are unlikely to explain elite control and, rather, are due to differences in transmitted strains. Since LTNP/LTS/EC who have been living with HIV-1 for decades are infected with old strains, one must be careful to interpret past and future reports addressing this issue with this bias in mind. Even with the large numbers evaluated here, however, larger datasets may be required to detect statistically significant differences following multiple comparisons. Therefore, further work with even larger numbers of viral sequences is necessary.

Although we observed neither common gross genetic defects nor common amino acid changes among EC, it is possible that some particular combination of variables contributes to strict viremia control. However, in order to enable such an analysis, significantly higher numbers of sequences, particularly full-genome viral sequences, would be necessary. We are currently planning to obtain full-length viral genome sequences from over 500 EC, which hopefully will resolve this issue and add more insight regarding viral genetic factors in elite control.

In conclusion, we observed little evidence that shared viral genetic defects are associated with the EC phenotype. We rarely observed gross genetic defects in plasma or PBMC viral sequences of EC, and a codon-by-codon analysis corrected for multiple comparisons and taking into account biases resulting from HLA expression and amino acid covariation failed to identify signature amino acid changes associated with elite control. EC viruses did not cluster phylogenetically and, therefore, did not appear to share a common ancestor. The conserved length of variable genomic regions in EC, such as the C terminus of p17, the V1 and V2 loops in gp120, and the N terminus of Nef, suggests that these viruses have been under limited immune selection pressure. Further experiments will be necessary to characterize the evolution of plasma viruses in

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EC and to examine the impact of these sequence changes on viral replication capacity and pathogenesis.

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