NOTES

Role of APOBEC3G/F-Mediated Hypermutation in the Control of Human Immunodeficiency Virus Type 1 in Elite Suppressors[▽]

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While many studies show that the APOBEC3 family of cytidine deaminases can inhibit human immunodeficiency virus type 1 (HIV-1) replication, the clinical significance of this host defense mechanism is unclear. Elite suppressors are HIV-1-infected individuals who maintain viral loads below 50 copies/ml without antiretroviral therapy. To determine the role of APOBEC3G/F proteins in the control of viremia in these patients, we used a novel assay to measure the frequency of hypermutated proviral genomes. In most elite suppressors, the frequency was not significantly different than that observed in patients on highly active antiretroviral therapy. Thus, enhanced APOBEC3 activity alone cannot explain the ability of elite suppressors to control viremia.

The apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like editing complex 3, or APOBEC3, proteins are a recently discovered family of cytidine deaminases that catalyze G→A hypermutation in retroviral genomes (14–16, 22, 23, 25, 30, 32, 38, 40, 42). Although human immunodeficiency virus type 1 (HIV-1) hypermutation had been documented previously (6, 36), the discovery that APOBEC3G was responsible for rendering cells nonpermissive to *vif*-deficient viruses led to an appreciation of the role of hypermutation in innate immunity to HIV-1 (9, 14, 15, 22, 25, 30, 42).

The activity of the APOBEC3 proteins is restricted by the HIV-1 protein Vif (12, 26, 30, 31, 41), which prevents the incorporation of these deaminases into budding virions. The extent to which APOBEC3 proteins affect replication is a matter of great interest (6, 17, 19, 36). Although hypermutated viral genomes are not present in plasma, they can be detected in proviral DNA from resting CD4$^+$ T cells (20). Studies using primers based on sequences in the *pol* gene have shown that an average of 9% of integrated proviruses are hypermutated in patients on highly active antiretroviral therapy (HAART) (20).

In a subset of patients, APOBEC3 proteins may play a more significant role. Long-term nonprogressors (LTNPs) are HIV-1-infected individuals who remain asymptomatic and maintain normal CD4$^+$ counts. An inverse correlation between APBOEC3G mRNA levels and disease progression has been observed in LTNPs (18) but not in progressors (11). A finding of defective Vif in one LTNP further suggests that APOBEC3 proteins may control viral replication in vivo (13). This hypothesis is further corroborated by the finding of minimal HIV-1 evolution and characteristic APOBEC3G-induced hypermutation in one LTNP (37). In another study of three LTNPs, the frequency of defective *vif* clones amplified from PBMC was similar to that found in patients with progressive disease (33). We recently noted that proviral *gag* in elite suppressors (ESs) had substantial levels of hypermutation (2). These patients, like LTNPs, maintain normal CD4$^+$ counts but are further distinguished by undetectable viral loads (<50 copies/ml) in the absence of therapy. Recent studies have shown that some ESs are infected with replication-competent viruses, which they control through host defense mechanisms (5). Because these ESs are able to control viremia, it is important to understand the role of APOBEC3 proteins.

In this study, we examined the role of APOBEC3-induced hypermutation in ESs by directly sequencing the proviral DNA from ESs and from a control set of patients with progressive disease who had undetectable viral loads on HAART. Hypermutated sequences can be readily detected in proviral DNA from resting CD4$^+$ T cells of all infected individuals (20). This population of cells constitutes the major stable viral reservoir (7), thus providing a record of APOBEC3 activity. While it remains possible that the APOBEC3G and -F proteins are uniquely active in another compartment, this has not yet been elucidated, and the significance is unknown. Therefore, resting CD4$^+$ cells were purified from eight ESs and nine patients on HAART as previously described (1). Genomic DNA was isolated using a Puregene kit (Qiagen, Valencia, CA) (1). Limiting-dilution nested PCR (24) was then performed on the genomic DNA to amplify a region spanning the 3′ end of *env* and the 5′ start of *nef* (Fig. 1). This region was selected for its high rate of hypermutation (40, 35). Primers were designed to avoid APOBEC3G target sites. To minimize PCR error, positive reaction products were directly sequenced. Nonclonal sequences were discarded. Because amplifications were carried out at a limiting dilution, all reported sequences represent

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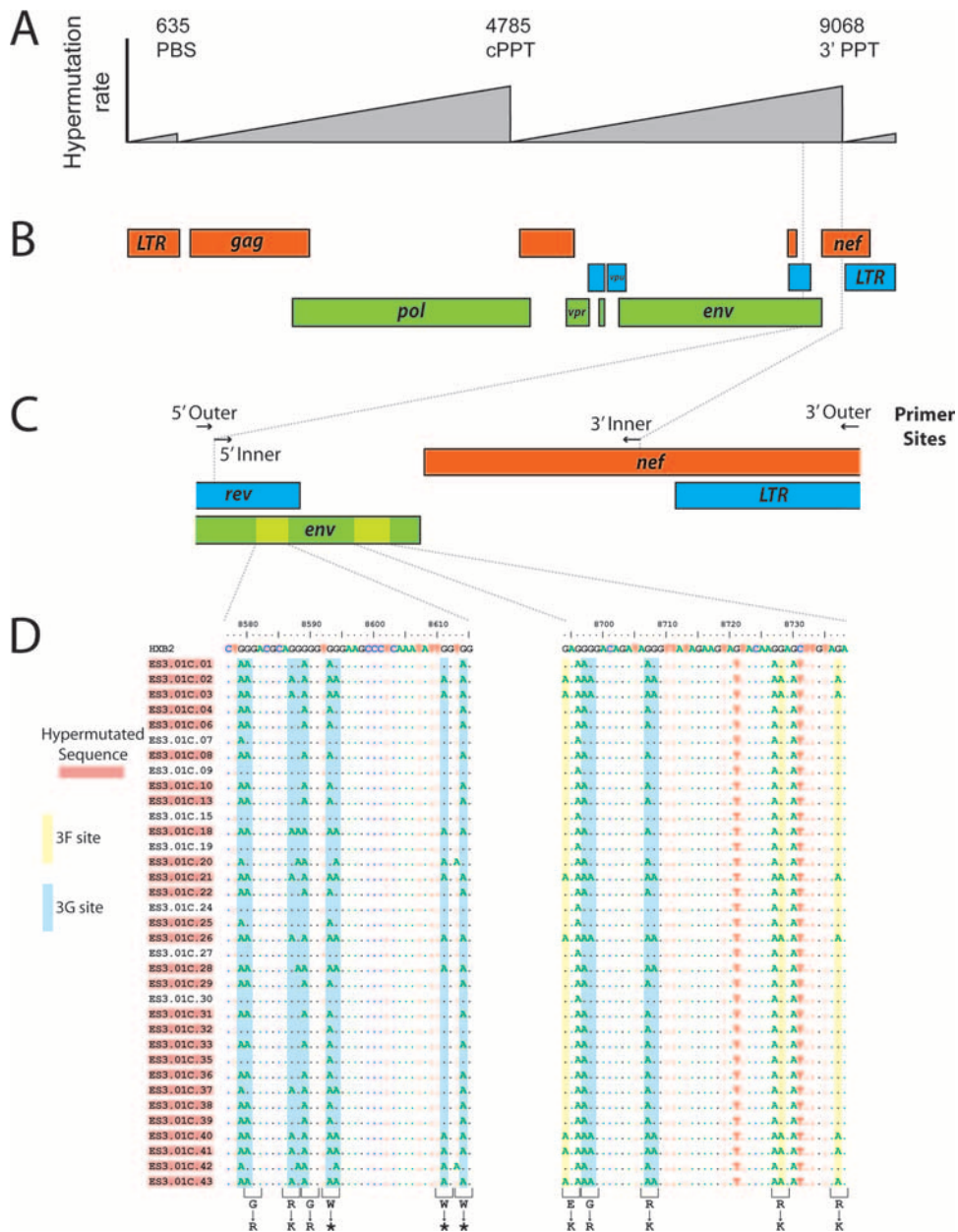
FIG. 1. Detection of hypermutated proviral sequences in ESs. (A) Gradients of APOBEC3-mediated hypermutation as defined by Yu et al. (40) and Suspene et al. (35). The gradients peak just 5′ of the primer binding site (PBS), the central polypurine tract (cPPT), and the 3′ polypurine tract (3′ PPT). (B) Map of the HIV-1 genome indicating the region selected for analysis. This region is at the peak of the a gradient of hypermutation frequency. (C) Enlargement of the region amplified showing outer and inner PCR primers. Primers were chosen at sites lacking APOBEC3G consensus hypermutation sites. Sequences from two regions in the *env* gene are shown in detail in panel D. Plus-strand sequences for the indicated regions of *env* from 35 independent proviral sequences from ES3 are shown. APOBEC3F and APOBEC3G hypermutation sites are in yellow and blue boxes, respectively. Amino acid changes resulting from hypermutation are indicated at the bottom of the figure. *, stop codon.

independent templates present in vivo (24). The extent of hypermutation was determined using the Hypermut 2.0 tool (www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html), which can distinguish plus-strand APOBEC3G/F hypermutation from background levels of mutation. Previous studies have established a dinucleotide sequence specificity of 5′-GG-3′ and 5′-GA-3′ for APOBEC3G and APOBEC3F, respectively, where the underlined plus-strand dG is complementary to the targeted minus-strand dC. APOBEC3G-mediated hypermutation does not occur when dC is in a downstream +2 position on the plus strand (3, 20, 21, 40). Thus, target sites were defined as either 5′-GG-3′ or 5′-GA-3′, depending on the enzyme being studied, and the control sequences were defined as 5′-GYN-3′ or 5′-GRC-3′. A sequence was considered hypermutated if it registered a *P* value of <0.05 on the Fisher exact test that compared the number of G→A changes in APOBEC3 versus control contexts.

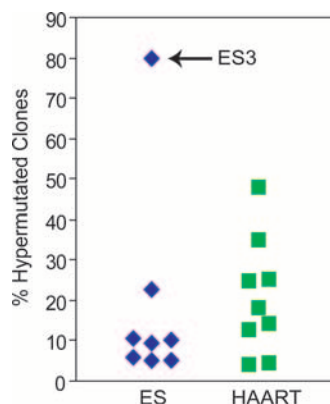As shown in Fig. 1D, hypermutated proviral sequences are

FIG. 2. Rates of hypermutation in ESs versus patients on HAART. The percentage of hypermutated genomes amplified from resting CD4$^+$ T cells from ESs and patients on HAART is shown. Patient ES3 had an unusually high frequency of hypermutated proviral sequences.

readily detected by this method. A total of 978 independent proviral sequences were obtained by limiting dilution PCR, for an average of 57.5 per patient (range 35 to 99). The frequency of hypermutation is indicated in Fig. 2. Except for one outlier (ES3), ESs had levels of hypermutated proviral sequences that were comparable to or slightly lower than those of patients on HAART. As shown in Table 1, the average fraction of hypermutated clones for all ESs was $18.6\% \pm 25.5\%$, compared to $20.9\% \pm 14.6\%$ for control patients. Sequences obtained from three ESs and four patients on HAART at a second time point

confirmed that the rate of hypermutation is invariant with time, demonstrating the stability of the reservoir (average of 30.6 clones analyzed per patient; rate of hypermutation was not significantly different [data not shown]).

The observed level of hypermutation is higher than previous estimates (20) because of the use of unbiased primers targeting the most heavily hypermutated region of the genome. It is also important to note that the levels of hypermutation observed in sequencing of proviruses may represent an underestimate of the actual fraction of viral cDNAs that are inactivated by APOBEC3 proteins because of both nonmutagenic viral control by APOBEC3 (4, 8, 32) and the fact that hypermutated genomes destroyed through UNG-dependent mechanisms (29) are not detected.

Within both populations of patients, however, there was significant patient-to-patient variation in the levels of hypermutation (Fig. 2; Table 1). In patients on HAART, the percentage of hypermutated proviruses ranged from 4% to 48%. In ESs, the range was broader due to a single outlier (ES3, 80%). Moreover, the patients also varied in the type of hypermutation they exhibited (Table 1; Fig. 1D). Overall, APOBEC3G patterns of hypermutation dominated, being present in 97% of pooled hypermutated sequences from all ESs and 94% in patients on HAART. APOBEC3F patterns, on the other hand, were present in 56% of pooled hypermutated sequences from ESs and 66% in patients on HAART. Interestingly, 95% of all clones with APOBEC3F patterns of hypermutation also had evidence of APOBEC3G hypermuta-

TABLE 1. Interpatient variability in extent of hypermutation

Clinical status and patient ID	No. of clones		Fraction (%) of clones			Fraction (%) of sites with G→A	
	Sequenced	Hyper mutated	Hypermutated	With 3G pattern[a]	With 3F pattern[b]	3G	3F
HAART[c]							
99	57	8	14	100	25	44	27
108	50	9	18	100	67	64	30
134	55	7	13	100	57	54	27
136	57	21	37	90	81	70	34
139	70	3	4	100	33	33	30
140	46	22	48	95	55	64	35
148	48	2	4	100	100	70	48
154	56	14	25	86	93	72	34
147	61	15	25	93	67	61	28
Mean ± SD	55.6 ± 7.3	11.2 ± 7.2	20.9 ± 14.6	96.0 ± 5.3	64.2 ± 25.1	59.1 ± 13.0	32.7 ± 6.5
ES[d]							
ES8	62	14	23	100	43	47	24
ES1	76	7	9	100	57	45	45
ES3	35	28	80	100	39	54	28
ES5	80	4	5	100	100	73	37
ES11	47	5	11	100	100	16	65
ES6	99	6	6	100	100	65	27
ES7	40	4	10	50	75	62	40
ES4	39	2	5	100	50	49	64
Mean ± SD	59.8 ± 23.3	8.8 ± 8.6	18.6 ± 25.5	93.8 ± 17.7	70.5 ± 26.7	51.3 ± 17.3	41.2 ± 15.9

[a] An APOBEC3G pattern was defined as a G→A mutation in a 5′-GG-3′ dinucleotide context.
[b] An APOBEC3F pattern was defined as a G→A mutation in a 5′-GA-3′ dinucleotide context.
[c] Patient characteristics were described previously (27).
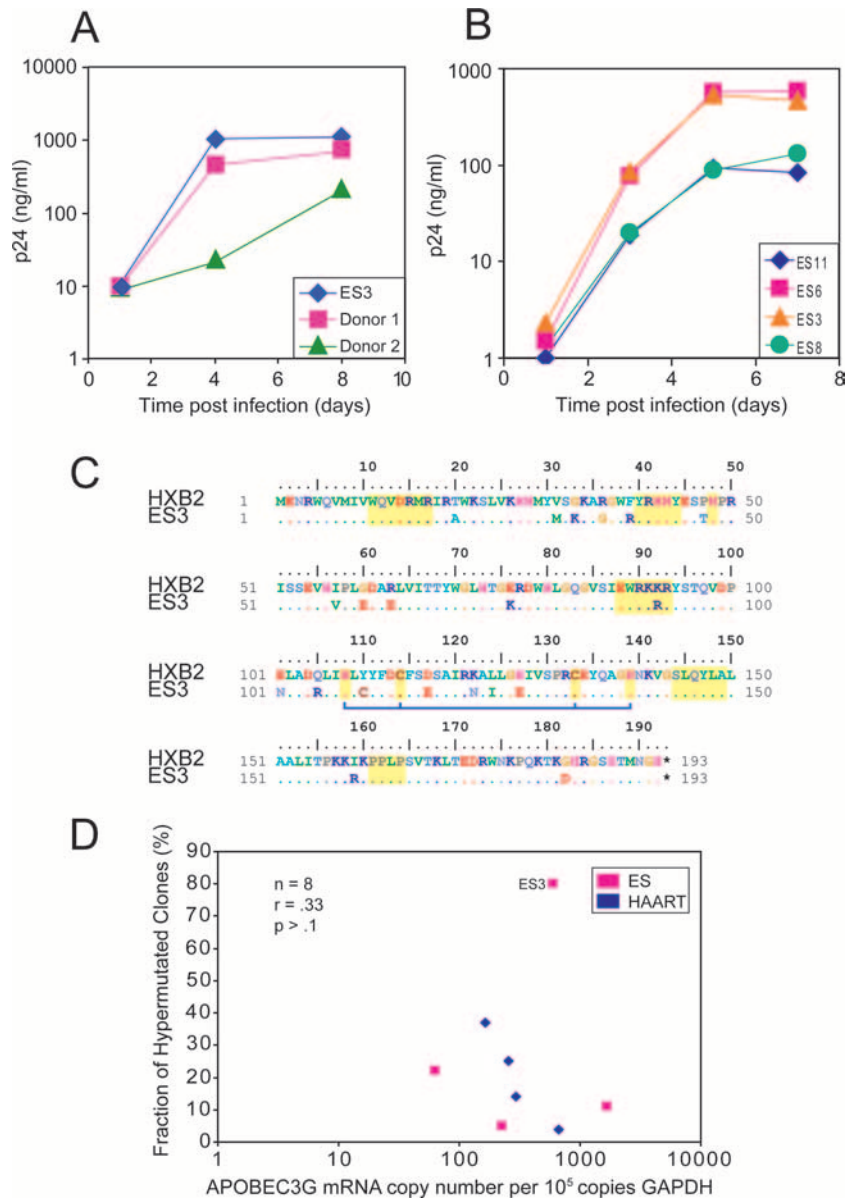[d] Patient characteristics were described previously (1, 2).

FIG. 3. Characterization of ES3. Susceptibility of CD4$^+$ T cells from ES3 to spreading HIV-1 infection. CD4$^+$ T lymphoblasts from ES3 and from two normal controls (donors 1 and 2) (A) and three ESs (ES5, ES8, and ES11) (B) were isolated and infected with Ba-L. Virus growth was measured by quantitating HIV-1 p24 antigen in the culture supernatant. (C) Sequence of the *vif* gene from ES3. No novel substitutions, insertions, or deletions were found. There were no significant changes at sites that are known to be important in Vif functionality (regions indicated in yellow). The blue bar denotes amino acids that constitute a zinc binding motif important for APOBEC3G degradation (28, 33, 39). (D) Expression of APOBEC3G mRNA was measured using a real-time PCR assay in four ESs (ES3, ES5, ES8, and ES11) and four HAART-treated patients (patients 99, 136, 147, and 148). mRNA levels were calculated as copies of APOBEC3G per 100,000 copies of GAPDH. No correlation between the fraction of hypermutated clones and expression of APOBEC3G was seen.

tion, possibly indicating some degree of cooperation between the two enzymes.

ES3 exhibited a statistically significant elevated level of hypermutated sequences (80%, $P < 0.01$) compared to other ESs (Z test, $z = 2.38$) and thus warranted further investigation (Table 1; Fig. 3). To test whether this patient's CD4$^+$ cells were permissive for viral replication, CD4$^+$ T cells from ES3 were activated with phytohemagglutinin and infected with the Ba-L isolate of HIV-1 as previously described (5). At various times after infection, p24 levels in the supernatant were mea-

sured. As shown in Fig. 3A and B, there was no difference in the kinetics of Ba-L replication between CD4$^+$ T cells from ES3 and those from three other ESs who had lower levels of proviral hypermutation. In addition, the virus replicated as well in cells from ES3 as it did in cells from two seronegative donors. To determine whether *vif* was defective in this patient, we amplified this gene from the low level of free virus in the patient's plasma (viral load, <50 copies/ml). Figure 3C shows that were no stop codons, insertions, deletions, or novel mutations in this gene. In addition, there were no substitutions at

amino acid positions known to be important for Vif function (13, 28, 33), although we were unable to definitively rule this out with a phenotypic analysis of the protein. Furthermore, no polymorphisms were identified in the APBOEC3G gene amplified from mRNA from this patient (data not shown).

To investigate the levels of APOBEC3G mRNA expression in the rate of hypermutation, we performed real-time PCR analysis on four ESs and four patients on HAART from our study. We found no correlation between APOBEC3G expression and the rate of hypermutation (Fig. 3D). Because APOBEC3G and -F are coordinately regulated (11), no association would be expected for APOBEC3F either. Additionally, no relationship was seen between disease status and APOBEC3G mRNA levels, contrary to previous findings (18).

While these findings suggest that innate host mechanisms involving APOBEC3 proteins are not solely responsible for the control of viremia in ES3, further investigation is warranted. Interestingly, Greene and colleagues have demonstrated that in activated T cells, APOBEC3G is in high-molecular-mass complexes with limited enzymatic activity (10, 34). We have shown that ES3, like other ESs, has a low level of free virus in the plasma that can be detected with extremely sensitive RT-PCR techniques. Nonhypermutated *gag* (2), *pol* (2), *env* (1), and *nef* sequences have been amplified from the patient's plasma. Furthermore, the plasma *gag* clones show evidence of viral evolution in response to selective pressure from CD8^{+} T cells (2). These findings strongly suggest that APOBEC3-mediated hypermutation does not completely inhibit viral replication in this ES. This patient also is positive for the HLA-B*57 allele (2), which is overrepresented in ESs, and thus, it is likely that multiple factors contribute to the control of viral replication.

In summary, we found that hypermutation occurs in ~20% of proviral sequences with significant patient-to-patient variability. As previously noted, APOBEC3G patterns of mutation dominate, being present in 95% of hypermutated sequences. Our sequencing data indicate that, despite previous reports of elevated levels of APOBEC3G in LTNP, hypermutation cannot explain the control of viremia in the majority of ESs. Suppression in these patients is probably maintained via a combination of other viral and host factors, including CTL responses. While APOBEC3 proteins do not appear to be solely responsible for control of viral replication in ESs, our study does not rule out a contribution of these cytidine deaminases in ES status.

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in GenBank under accession numbers EU383053 to EU384245.

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