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Human Immunodeficiency Virus (HIV) Type 1 Proviral Hypermutation Correlates with CD4 Count in HIV-Infected Women from Kenya[▽]

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APOBEC3G is an important innate immune molecule that causes human immunodeficiency virus type 1 (HIV-1) hypermutation, which can result in detrimental viral genome mutations. The Vif protein of wild-type HIV-1 counteracts APOBEC3G activity by targeting it for degradation and inhibiting its incorporation into viral particles. Additional APOBEC cytidine deaminases have been identified, such as APOBEC3F, which has a similar mode of action but different sequence specificity. A relationship between APOBEC3F/G and HIV disease progression has been proposed. During HIV-1 sequence analysis of the *vpu*/*env* region of 240 HIV-infected subjects from Nairobi, Kenya, 13 drastically hypermutated proviral sequences were identified. Sequences derived from plasma virus, however, lacked hypermutation, as did proviral *vif*. When correlates of disease progression were examined, subjects with hypermutated provirus were found to have significantly higher CD4 counts than the other subjects. Furthermore, hypermutation as estimated by elevated adenine content positively correlated with CD4 count for all 240 study subjects. The sequence context of the observed hypermutation was statistically associated with APOBEC3F/G activity. In contrast to previous studies, this study demonstrates that higher CD4 counts correlate with increased hypermutation in the absence of obvious mutations in the APOBEC inhibiting Vif protein. This strongly suggests that host factors, such as APOBEC3F/G, are playing a protective role in these patients, modulating viral hypermutation and host disease progression. These findings support the potential of targeting APOBEC3F/G for therapeutic purposes.

APOBEC3G, originally termed CEM15, was identified as an endogenous host molecule capable of inhibiting Vif-deficient human immunodeficiency virus type 1 (HIV-1) replication in nonpermissive cells (41). It was elucidated that APOBEC3G induced HIV-1 hypermutation, causing G-to-A transitions and often the addition of stop codons (18). In the absence of the HIV-1 protein Vif, cytoplasmic host APOBEC3G is packaged into newly formed viral particles (16, 30, 31, 41). Upon infection of new cells, the HIV-1 RNA genome is reverse transcribed into DNA before proviral integration. APOBEC3G binds the single-stranded RNA genome and acts on the single-stranded DNA copy, deaminating cytosine to uracil (18, 19, 55). This results in guanine-to-adenine hypermutation in the corresponding positive DNA strand. This evidence of proviral hypermutation becomes archived in the infected cells and can be examined for evidence and elucidation of the antiviral activity, as has been demonstrated recently by Kijak et al. (23). The Vif protein of wild-type HIV-1, however, counteracts APOBEC3G activity by targeting APOBEC3G for ubiquitination and subsequent degradation and inhibits incorporation of APOBEC3G into newly formed viral particles (53). Additional APOBEC molecules have been identified, such as APOBEC3F, which although they have different sequence specificities, they act similarly and are also inhibited by Vif (2, 26, 49, 56).

The in vivo antiviral mechanism of APOBEC3F/G is not well defined. Certainly, G-to-A hypermutation would cause detrimental mutations in the viral genome, reducing or abolishing the production of viable progeny, but other antiviral APOBEC functions have also been reported. APOBEC-mediated hypermutation may trigger degradation of the nascent viral DNA by host uracil glycosylases and apurinic/apyrimidinic endonucleases, thereby inhibiting the production of provirus (51). APOBEC has also been suggested to interfere with removal of primer tRNA, as well as to inhibit DNA strand transfer and integration (27, 33). Some antiviral effects have been reported for APOBEC in the absence of hypermutation. Guo et al. reported reduced reverse transcription priming and reduced levels of viral DNA in Vif-negative virus in the absence of APOBEC3G deamination and hypermutation (15). APOBEC3G was also found to interfere with proviral integration in the absence of hypermutation (27). However, a recent publication stated that deaminase-defective APOBEC3G had antiviral activity only when it was expressed at high levels, questioning the physiological relevance of deaminase-independent activity of APOBEC3G (35).

The roles of APOBEC3F/G in HIV disease progression and pathogenesis are similarly unclear, although a relationship between the two has been suggested (1, 10, 20, 21, 29, 36).

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APOBEC3G mRNA expression level has been associated with decreased viremia and long-term nonprogression (20) and with HIV-exposed seronegative individuals (1). However, these studies have not been readily replicated (10), resulting in significant controversy over the role of APOBEC in HIV disease progression in vivo. To examine the phenomenon of APOBEC-mediated hypermutation in a clinical setting, we examined proviral DNA from 240 HIV-infected subjects from Nairobi, Kenya. Significant hypermutation of the predominate provirus was identified and found to correlate with patient CD4 count, suggesting that APOBEC-mediated hypermutation influences disease progression in a clinical setting.

MATERIALS AND METHODS

Sample selection. A total of 215 HIV-positive women enrolled in a well-described cohort of commercial sex workers with high exposure to HIV in the Pumwani area of Nairobi, Kenya, were randomly selected based on sample availability (13, 14). An additional 25 HIV-positive women from a perinatal transmission cohort of similar socioeconomic status and ethnicity but with low exposure to HIV were similarly selected (5, 12). All women are believed to be infected with HIV by heterosexual contact, and all were antiretroviral naive at the time of the study. Peripheral blood samples were obtained by venipuncture and used to isolate plasma and peripheral blood mononuclear cells (PBMCs) as previously described (24). Both the University of Manitoba and University of Nairobi ethics review panels have approved HIV-related studies involving these subjects.

DNA isolation and PCR amplification. DNA was isolated from PBMCs using the QIAamp DNA mini kit (Qiagen Inc., Mississauga, Canada). Nested PCRs were performed in order to amplify the proviral *vpu* and the first 349 nucleotides of *env*. The first reaction created an amplicon that was nearly 2 kb, using primers outer-f (5′-CAAGCAGGACATAACAAGGTAG-3′) and outer-r (5′-TGTTAT TTCTAGATCCCCTCCTG-3′) and the Expand High Fidelity PCR System (Roche Diagnostics GmbH, Mannheim, Germany) at the recommended conditions. The secondary nested reaction specifically amplified the region of interest, using primers inner-f (5′-GGCTTAGGCATCTCCTATGGCAGGAAGAAG-3′) and inner-r (5′-CGAGTGGGGTTAACTTTACACATG-3′), also with the Expand High Fidelity PCR System. Nested PCR was also used to amplify the proviral *vif* region, as described above, using outer primers 5′-AGTTATCCCA GCAGAAACAGGAC-3′ and 5′-TCGCTGTCTCCGCTTCTTCCTG-3′ and inner primers 5′-CTGCAGTTAAGGCAGCCTGTTG-3′ and 5′-CTTCAACTCC TGCCCAAGTATC-3′.

Sequencing and assembly. PCR amplicons were directly sequenced, using ABI Prism BigDye terminator cycle sequencing ready reaction kit v.3.1 (Applied Biosystems) and the inner PCR primers described above. The sequences were resolved on an ABI Prism 3100 genetic analyzer (Hitachi, Japan). The resulting complementary sequence electropherograms were assembled using Sequencher v.4.2 (Gene Codes Corporation, Ann Arbor, MI). Sequences were aligned using ClustalW (7).

Cloning. Samples that contained gross mutations in *vpu* and *env* were cloned into pCR4-TOPO vector (TOPO TA cloning kit for sequencing; Invitrogen Life Technologies, Carlsbad, CA). Thirteen to 20 clones, depending on cloning efficiency, were sequenced for each patient as described above using M13 forward and reverse primers. The PCR amplicon was additionally cloned for a randomly determined subset of samples, and 14 to 20 clones from each sample were sequenced and analyzed to ensure that the PCR sequence was representative of the patient proviral population.

Statistics. The statistical analyses Mann-Whitney test and test for correlation were performed using GraphPad Prism 4.

RNA isolation from plasma. Viral nucleic acids were isolated from archived heparin-plasma using NucliSens isolation reagents (bioMerieux Canada) according to the manufacturer's instructions (4). Reverse transcription-PCR (Qiagen OneStep reverse transcription-PCR kit) was performed using the specific primers outer-f and outer-r, and the viral nucleic acids were further amplified with the nested PCR strategy described above. The plasma RNA sequences were aligned with all the proviral sequences using ClustalW (7). MEGA 3 was employed for neighbor-joining phylogenetic analysis (nucleotide distance calculated by Kimura's two-parameter method) (25). MEGA 3 was employed for bootstrapping to determine whether the two independently derived sequences from the same patient significantly clustered together.

Hypermutation detection. Hypermut 2.0, available from http://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html, was employed to detect APOBEC-type hypermutation (38). Sequences generated in this study were used to generate a population-specific consensus sequence for each HIV-1 clade that was represented. The sequences were then compared on a clade-by-clade basis to the consensus sequence using Hypermut to identify G-to-A hypermutation and also to identify the nucleotide context of this hypermutation.

Determination of CD4 count. Peripheral blood T-lymphocyte subset analysis was performed as previously described (13).

Determination of plasma viral load. Viral load determinations were performed on 200 μl heparin-plasma using the NucliSens HIV-1 QT assay (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. The lower detection limit of the assay, when starting from this volume, is 125 copies/ml.

GenBank accession numbers. The genetic sequences for the *vpu*/*env* segment from the 240 proviral samples and 18 plasma RNA samples have been submitted to GenBank under accession numbers EU836326 to EU836565 and EU836566 to EU836593. Unique clones from the nonhypermutated sequences and the hypermutated sequences were submitted to GenBank under accession numbers EU875081 to EU875368. The genetic sequences for *vif* from six proviruses that were hypermutated in the *vpu*/*env* regions and eight proviruses that were not hypermutated in the *vpu*/*env* regions have been submitted to GenBank under accession numbers EU839404 to EU839417.

RESULTS

Proviral sequences contain premature stop codons due to hypermutation. HIV-1 sequence diversity was assessed by examining the predominant proviral DNA sequence in two well-characterized HIV-infected cohorts, composed of female commercial sex workers (13, 14) and women seeking antenatal care (5, 12), located in Nairobi, Kenya. A total of 240 HIV-positive women were randomly selected based on sample availability for inclusion in this study. For each participant, PBMCs were separated from whole blood and used to isolate DNA. The HIV sequence was obtained using a nested PCR approach and directly sequencing the resulting 590-nucleotide fragment of the HIV-1 proviral genome, which includes *vpu* and the first 349 nucleotides of *env*. Thirteen of the 240 sequences had significant amino acid mutations in both Vpu and Env, such as missing start codons and premature stop codons, which would presumably prevent protein translation and lead to a lethal viral phenotype (Fig. 1). Previous studies have demonstrated that hypermutation can cause premature stop codons, especially at tryptophan residues, where the transition of a G to A causes the Trp codon (UGG) to change to a stop codon (UAG, UAA, or UGA) (8, 47). To assess the possibility that the identified lethal mutations in Vpu and Env could be the result of hypermutation, the proviral patient sequences were compared to a cohort-specific consensus sequence (Fig. 1). G-to-A nucleotide transitions were noted and determined to be responsible for the observed detrimental amino acid mutations.

A subset of proviral sequences has elevated adenine proportion. As APOBEC3G and APOBEC3F are known to cause G-to-A hypermutation in HIV-1 provirus, the possibility of hypermutation due to APOBEC cytidine deamination was investigated by examining the sequences for adenine enrichment (2, 26, 49, 53, 56). The proportion of adenine nucleotides in all 240 sequences was determined and revealed a generally symmetrical bell-shaped distribution with a mean adenine proportion of 0.36 and a standard deviation of 0.018 (Fig. 2A). Fourteen sequences had values higher than 1 standard deviation above the mean. Thirteen of these were also identified as having lethal amino acid mutations in Vpu and Env. This

env

```
                1         10        20        30        40        50        60        70
consenus ATG AGA GTG ATG GGG ATA CAG AGG AAT TGT CAG CAC TTA TTG AGA TGG GGA ACT ATG ATC TTG GGG ATG ATA ATA
ML1053   ATG AGA GTG ATA AAG ATA CAG AAG AAT TGT CAA CAC TTG TTG AAA TAA AGG ACT ATG ATC TTA AAG ATG ATA ATA
ML1102   ATG AGA GTA AAG GAG ATA AAG AGG AAT TAT CAG CAC TTG TGG AGA TAA GGC ACC ATG CTC CTT AGG ATG TTG ATG
ML1230   ATA AGA GTG ATG AAG ATA CAG AAG AAT TGT CAG CAC TTA TTA AAA TGA AGA ATT ATG ATC TTG AGG ATA ATA ATA
ML1578   ATG AAA GTA AAA GAG ATA AAG AAG AAT TAT CAA CAC TTG TGG AGA TAA GGC ACC ATG CTC CTT AAG ATG TTA ATG
ML1592   ATA AAA GTA ATA AAA ACA CAA AAA AAT TAT CAA AAC TTG TAA AAA TAA GAA ACT ATA ATC TTA AAA ATA ATA ATA
ML1857   ATG AGA GTG ATG AAG ATA CAA AAG AAT TAG CAA CAA TAG TAA ATA TAA GGC ATC TTA AGC TTT TAA ATG TTA ATG
ML1942   ATA AAA GTG AGG GGG ATA CAG AGA AAT TAT CAG CAC TAG TTA ACA TAG AAA ACT ATA ATC TTA GAA ATA TTA ATC
ML1957   ATA AAA GTA ATA AAA ACA CAG AGA AAT TGT CAA CAC TTA TTA AAA TAA AGA ACA ATA ATC TTG GGT TTA ATA ATA
ML1970   ATA AGA GTG AAA AGG ATA CAG AGG AAC TCT CAA TGC TTA TTG AGA TGG AGA ACG ATA ATC TTA GGT TTG ATA ATA
ML1971   ATA AAA GTA ATA AAA ATA AAA AAA AAT TAT CAG CAT TTG TGG GGA TGG GGC ACC ATG CTC CTT AAA ATG TTA ATA
ML1975   ATG ACA GTG ATG AGG ATA CAG AGG AAT TGT CAA CTC TTG TTG AGA TGA AGA ACT ATG ATC TTA AAG ATG ATA ATA
ML2019   ATA AGA GTG AAA AAG ATA CAG AAG AAT TAT CAA CAA TAG TAG ATA TAA GGC ATC TTA GGC TTT --- --- TAG ATA
MCH4887  ATA AAA GTA AGG AGA ATA AAA AAA AAT TAT CAG CAC TTG TGA AAA TAG GGC ACC ATG CTC CTT AAA ATA TTA ATG

                1         5         10        15        20        25
consenus M R V M G I Q R N C Q H L L R W G T M I L G M I I
ML1053   M R V I K I Q K N C Q H L L K * R T M I L K M I I
ML1102   M R V K E I K R N Y Q H L W R * G T M L L R M L M
ML1230   I R V M K I Q K N C Q H L L K * R I M I L R I I I
ML1578   M K V K E I K K N Y Q H L W R * G T M L L K M L M
ML1592   I K V I K T Q K N Y Q N L * K * E T I I L K I I I
ML1857   M R V M K I Q K N * Q Q * * I * G I L S F * M L M
ML1942   I K V R G I Q R N Y Q H * L T * K T I I L E I L I
ML1957   I K V I K T Q R N C Q H L L K * R T I I L G L I I
ML1970   I R V K R I Q R N S Q C L L R W R T I I L G L I I
ML1971   I K V I K I K K N Y Q H L W G W G T M L L K M L I
ML1975   M T V M R I Q R N C Q L L L R * R T M I L K M I I
ML2019   I R V K K I Q K N Y Q Q * * I * G I L G F - - * I
MCH4887  I K V R R I K K N Y Q H L * K * G T M L L K I L M
```

FIG. 1. Sequence context of identified G-to-A hypermutation in the first 75 nucleotides of the *env* ORF from 13 dramatically mutated proviruses. The top panel displays the nucleotide sequence, while the bottom panel shows the corresponding amino acid sequence. The consensus sequence for both the DNA and protein was generated based on the 240 proviral sequences examined in this publication. The sequences are numbered from the HIV-1 *env* start. The proviral sequence was compared to the corresponding nonhypermutated plasma RNA sequence, where available, or else to a clade-specific consensus sequence. Darkened nucleotide letters in the sample sequences indicate changes from G to A in either a GG or GA context (i.e., proposed APOBEC3G or APOBEC3F hypermutation). Darkened amino acid letters in the sample sequences indicate where proposed hypermutation at the nucleotide level caused an amino acid change at the protein level.

finding provided further evidence that the lethal mutations were the result of hypermutation and merited further investigation.

Free virus RNA is not hypermutated. To determine whether hypermutation was restricted to proviral sequences or could also be observed in the RNA genomes of free virus, sequences were generated from date-matched plasma RNA for a subset of 18 subjects, including four patients with hypermutated proviral sequence. Phylogenetic analysis of the sequences determined that both samples originated from the same individual (data not shown). All the viral RNA sequences had adenine proportions similar to the overall mean observed in the proviruses and did not appear to have evidence of hypermutation (Fig. 2B). Furthermore, the plasma RNA from nonhypermutated proviral sequences had adenine proportions similar to that of the corresponding proviral DNA. However, the four sequences with hypermutated provirus sequences had significantly higher adenine proportions than the matched plasma virus sequence did ($P = 0.029$, Mann-Whitney), suggesting that the observed hypermutation was restricted to provirus only. This finding further indicates that APOBEC3-type hypermutation may be a potential explanation for these findings.

Directly sequenced PCR products show extensive hypermutation and are representative of proviral sequence. Direct sequencing of the PCR amplicon will reveal the major proviral sequence but may not be representative of intrapatient diversity. In order to examine proviral diversity within individual subjects, we sequenced 13 to 20 clones from 23 patients, including all 13 subjects with dramatically hypermutated proviral sequence and a random subset of 10 subjects with proviral sequences that were not dramatically hypermutated. The sequence data generated from the multiple clones for each subject confirmed that the detrimental mutations in Vpu and Env were not the result of sequencing or sampling errors but were in fact representative of the subjects' proviral population. For the majority of hypermutated samples, the clonal sequences showed levels of hypermutation similar to that observed in the sequence generated directly from the proviral PCR product (Fig. 3). Fifteen clonal populations showed hypermutation patterns identical to that of the direct PCR sequence for all the clones examined (including 10 out of the 13 extremely hypermutated sequences). Additionally, two clonal populations had the majority of cloned sequences (85% or greater) match the direct PCR sequence (the hypermutated sequences ML1578

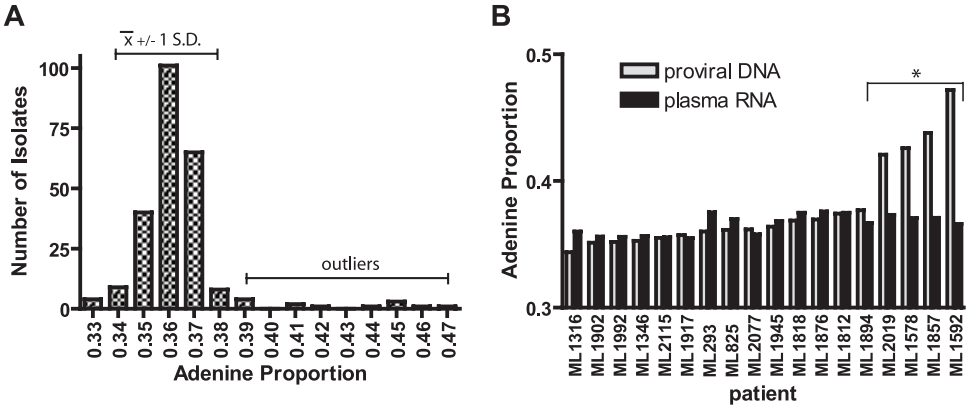


FIG. 2. Proviral adenine proportion. (A) Distribution of proviral adenine proportion in a 590-nucleotide fragment spanning *vpu* and the 5′ end of *env* for 240 HIV-1 isolates. The adenine proportion is based on proviral sequences from PBMC DNA isolated from HIV-infected women from two Kenyan cohorts. The values greater than 1 standard deviation above the mean ($\bar{x} \pm 1$ S.D.) and the outliers are indicated. (B) Adenine proportion in proviral HIV-1 sequence and plasma-derived viral RNA sequence in 18 matched samples. The same 590-nucleotide HIV-1 genomic region was examined for both proviral and viral RNA sequences. The four sequences indicated with the asterisk had hypermutated proviral *vpu*/*env* sequences.

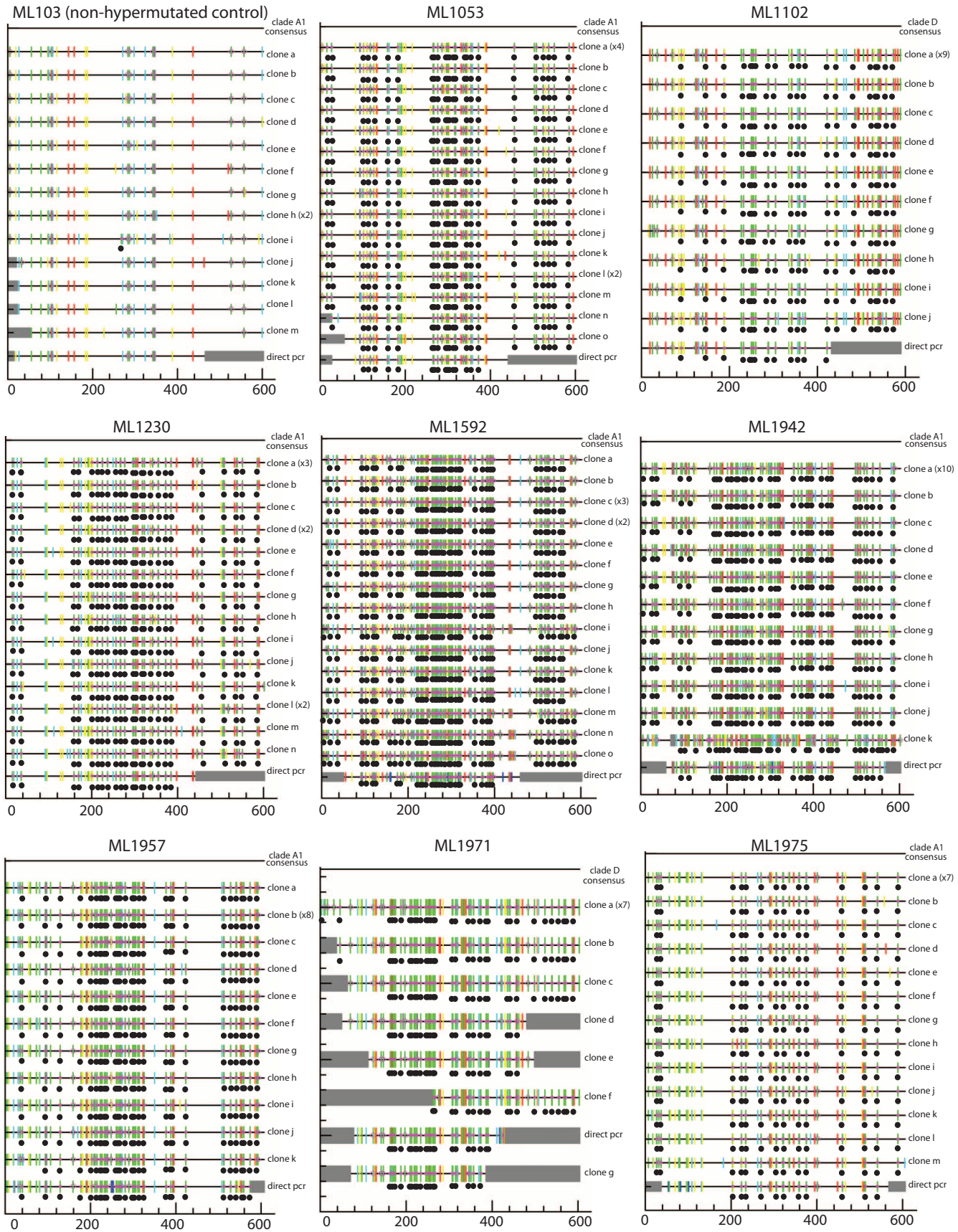
and ML1970). In three clonal populations, half of the clones matched the direct PCR sequence, including the hypermutated sequence ML1857. In three clonal populations, the hypermutation patterns in the clones were generally similar, though not identical, to that observed in the direct PCR sequence. Overall, the vast majority of clonal and proviral sequences from the samples display almost identical patterns of hypermutation; for example, ML103 shows no hypermutation in the direct PCR sequence and only one site of hypermutation in 1 of the 14 clones examined (Fig. 3). Similarly, ML1053 shows many hypermutation sites in the direct PCR sequence, and these are shared with and between all 19 clones examined. However, the levels of hypermutation of 3 out of 16 clonal sequences from ML1578 and 9 out of 17 clonal sequences from ML1857 were lower than the level of hypermutation of the proviral sequence. Conversely, 2 out of 19 clonal sequences from ML1970 had higher levels of hypermutation than did the proviral sequence. These data suggest that the direct PCR sequence is generally representative of the predominant proviral sequence; however, as expected, subtle diversity can be observed in some patients.

Observed hypermutation characteristic of APOBEC3G/3F. All 240 proviral sequences were examined for hypermutation characteristic of APOBEC3F/G deamination activity using Hypermut 2.0 (http://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html), which examines the sequence connotation of nucleotide changes (38). Using the conservative default settings to examine APOBEC-type G-to-A hypermutation, we identified 17 sequences out of our 240 that had significant APOBEC-type hypermutation ($P < 0.05$). Thirteen of the 17 sequences were previously identified as both having an adenine proportion higher than 1 standard deviation above the mean and containing lethal mutations in Vpu and Env. Thus, these 13 represent the patients with the most hypermutated dominant proviral sequences in this population (Table 1).

In addition to examining the 240 sequences for general APOBEC activity with Hypermut 2.0, we also looked for APOBEC3F and APOBEC3G activity individually, as these two enzymes have distinct sequence specificities; APOBEC3G causes GG-to-AG mutations, while APOBEC3F causes GA-to-AA mutations (26, 52). This approach has not been validated by Hypermut, so although suggestive, the results are likely less informative than those generated by examining the sequences for general APOBEC3F/G activity. Twenty-four sequences were identified as having significant hypermutation characteristic of APOBEC3F and/or APOBEC3G activity. All 17 sequences previously identified as containing general APOBEC-type hypermutation by Hypermut 2.0 also had significant specific hypermutation for APOBEC3F and/or APOBEC3G, suggesting that the observed hypermutation was due to one or both of these enzymes (Table 1).

To further examine the roles of these enzymes in the 13 proviral sequences with the most pronounced hypermutation, we examined the dinucleotide sequence connotation of the G-to-A hypermutation. Figure 4 shows that most of the G-to-A nucleotide changes occurred where the original sequence was a GA or GG, suggestive of APOBEC3F or APOBEC3G involvement, respectively. Comparatively fewer changes occurred at GC or GT dinucleotides. It is interesting to note that patients with a higher proportion of G-to-A nucleotide changes occurring at a GG dinucleotide context have a comparatively lower proportion of changes occurring at a GA dinucleotide context. The reverse is also true. In patient ML1970, however, the proportions of G-to-A nucleotide changes occurring at GG and GA dinucleotides are similar.

Proviral hypermutation associated with increased CD4 counts. To ascertain whether the observed hypermutation has any clinical significance, the subjects' CD4 counts, measured at the time the blood sample was collected, were examined as a surrogate marker for disease progression. CD4 counts from the 17 patients with significant hypermutation, as identified by Hypermut 2.0, were significantly higher than those for the other subjects ($P = 0.009$) (Fig. 5A). Thirteen patients met all of the following criteria for hypermutation: general APOBEC3F/G-type hypermutation as determined by Hypermut 2.0 analysis, adenine proportion higher than 1 standard deviation above the mean for


ML103 (non-hypermutated control)
clade A1 consensus
clone a
clone b
clone c
clone d
clone e
clone f
clone g
clone h (x2)
clone i
clone j
clone k
clone l
clone m
direct pcr
0
200
400
600
ML1053
clade A1 consensus
clone a (x4)
clone b
clone c
clone d
clone e
clone f
clone g
clone h
clone i
clone j
clone k
clone l (x2)
clone m
clone n
clone o
direct pcr
0
200
400
600
ML1102
clade D consensus
clone a (x9)
clone b
clone c
clone d
clone e
clone f
clone g
clone h
clone i
clone j
direct pcr
0
200
400
600
ML1230
clade A1 consensus
clone a (x3)
clone b
clone c
clone d (x2)
clone e
clone f
clone g
clone h
clone i
clone j
clone k
clone l (x2)
clone m
clone n
direct pcr
0
200
400
600
ML1592
clade A1 consensus
clone a
clone b
clone c (x3)
clone d (x2)
clone e
clone f
clone g
clone h
clone i
clone j
clone k
clone l
clone m
clone n
clone o
direct pcr
0
200
400
600
ML1942
clade A1 consensus
clone a (x10)
clone b
clone c
clone d
clone e
clone f
clone g
clone h
clone i
clone j
clone k
direct pcr
0
200
400
600
ML1957
clade A1 consensus
clone a
clone b (x8)
clone c
clone d
clone e
clone f
clone g
clone h
clone i
clone j
clone k
direct pcr
0
200
400
600
ML1971
clade D consensus
clone a (x7)
clone b
clone c
clone d
clone e
clone f
direct pcr
clone g
0
200
400
600
ML1975
clade A1 consensus
clone a (x7)
clone b
clone c
clone d
clone e
clone f
clone g
clone h
clone i
clone j
clone k
clone l
clone m
direct pcr
0
200
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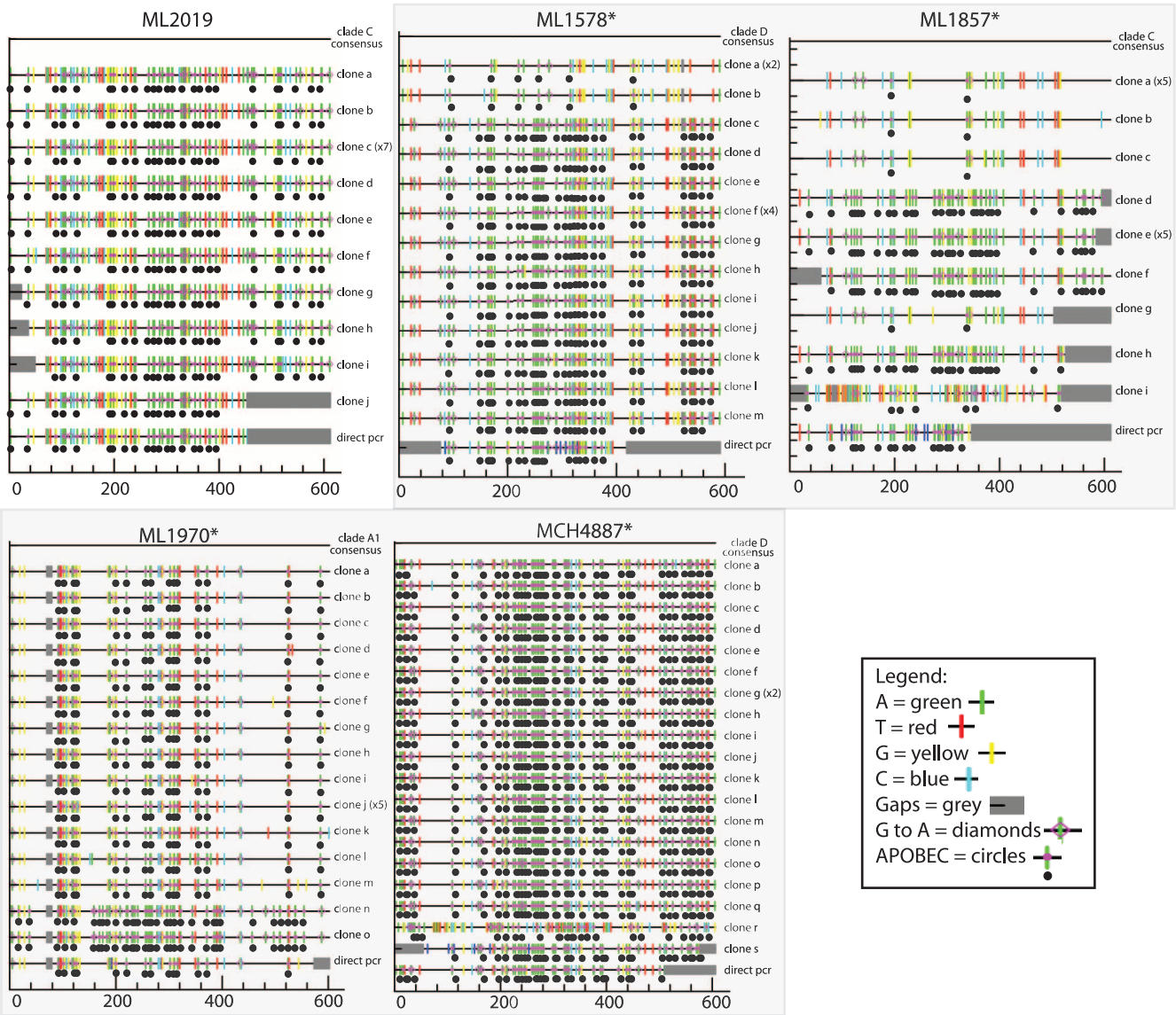

FIG. 3. Clones and directly sequenced PCR product are compared to a population-specific, clade-specific consensus sequence. The nucleotides are numbered across the bottom of each panel, indicating position within the 590-nucleotide region. ML103 was selected as a representative nonhypermutated sequence. Changes in the patient sequence compared to the consensus sequence are indicated by colored bars, as indicated in the legend. The representations were generated with the Highlighter tool available from http://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/highlighter.html. Black circles below hypermutation sites were added manually to ease visualization. The sequences are ordered from top to bottom from the most similar to the consensus sequence to the least similar. Identical sequences are shown once, with the number of times the sequence was retrieved listed beside the clone name. Patients with an asterisk showed intraclonal diversity.

the 240 proviral sequences examined, and lethal mutations in the two open reading frames (ORFs) examined. These 13 patients also had significantly higher CD4 counts than the other participants did ($P = 0.005$) (Fig. 5B).

After determining that there was a relationship between hypermutation and CD4 count for the subset of patients with evidence of dramatic proviral hypermutation, the entire data set was examined to see whether the relationship persisted. CD4 count and proviral adenine proportion were correlated in an unbiased, casewise comparison for all samples where CD4 counts were available ($n = 208$) and were found to be significantly correlated with adenine proportion ($P = 0.042$) with a Spearman r value of 0.1411 (Fig. 6).

Viral load was measured from archived samples where date-matched plasma samples were available ($n = 138$). Although the 13 patients infected with hypermutated provirus had lower average viral loads than the other patients ($\bar{x}$ of 4.07 $\log_{10}$ copies/ml versus 4.78 $\log_{10}$ copies/ml, respectively), the distribution was not statistically significant ($P = 0.82$). However, viral load determination was conducted on archived samples, not freshly isolated plasma. Sample storage is known to affect viral load measurement, whereas the CD4 counts were mea-

TABLE 1. Hypermutation criteria for proviral HIV-1 sequences that were significant in at least one category[a]

Patient identifier	Fatal mutations in Vpu and Env	Adenine proportion	General Hypermut 2.0 significance	Specific Hypermut 2.0 significance		All categories
				A3G	A3F	
MCH4887	Yes[b]	0.4560[c]	1.012E−13[d]	0.00560[e]	2.709E−14[e]	Yes
ML1592	Yes	0.4688[c]	2.660E−10[d]	0.0175[e]	9.277E−11[e]	Yes
ML1942	Yes	0.4510[c]	6.420E−10[d]	0.0042[e]	5.693E−10[e]	Yes
ML1957	Yes	0.4353[c]	5.911E−9[d]	0.0251[e]	6.44E−10[e]	Yes
ML1971	Yes	0.4533[c]	3.085E−8[d]	0.4574	2.039E−10[e]	Yes
ML1230	Yes	0.3937[c]	2.772E−7[d]	1.734E−9[e]	0.0013[e]	Yes
ML1578	Yes	0.4212[c]	1.708E−5[d]	1.214E−5[e]	0.0014[e]	Yes
ML1975	Yes	0.3865[c]	8.894E−5[d]	5.915E−5[e]	0.0038[e]	Yes
ML1857	Yes	0.4520[c]	1.425E−4[d]	1.456E−4[e]	0.0038[e]	Yes
ML2019	Yes	0.4148[c]	6.901E−4[d]	1.361E−7[e]	0.1554	Yes
ML1053	Yes	0.3932[c]	0.0012[d]	6.704E−5[e]	0.0644	Yes
ML1102	Yes	0.3828[c]	0.0029[d]	1.466E−4[e]	0.1321	Yes
ML1970	Yes	0.3861[c]	0.0058[d]	0.00350[e]	0.0633	Yes
ML2209	No	0.3635	0.0030[d]	0.0910	0.0017[e]	No
ML1649	No	0.3605	0.0113[d]	0.0494[e]	0.0228[e]	No
ML1903	Only Env	0.3784	0.0153[d]	0.0021[e]	0.2276	No
ML1419	No	0.3643	0.0287[d]	0.2019	0.0243[e]	No
ML1992	No	0.3519	0.0508	1	0.0149[e]	No
ML602	No	0.3596	0.0619	0.0397[e]	0.2397	No
ML1155	No	0.3727	0.0764	0.5218	0.0356[e]	No
ML1894	Only Vpu	0.3728	0.0982	0.0277[e]	0.4758	No
ML2115	No	0.3551	0.1039	1	0.0268[e]	No
ML790	No	0.3513	0.1076	0.0229[e]	0.5607	No
ML49	No	0.3539	0.1163	1	0.0453[e]	No
MCH5736	Only Vpu	0.4061[c]	0.3199	0.1815	0.5891	No

[a] The total numbers of patients meeting the criteria were as follows: 13 patients had fatal mutations in Vpu and Env, 14 patients had adenine proportion higher than 1 standard deviation above the mean, 17 patients had general Hypermut 2.0 significance, 24 patients had specific Hypermut 2.0 significance, and 13 patients met all the criteria.

[b] These samples had mutations, such as missing start codons and premature stop codons, in the proviral ORFs examined.

[c] Adenine proportion higher than 1 standard deviation above the mean (i.e., >0.382751).

[d] Hypermut 2.0 analysis for general APOBEC-type hypermutations (default settings of G-to-A subsitutions with a downstream context of RD, where R is either A or G and D is A, T, or G) that gave a significant P value ($P < 0.05$).

[e] Hypermut 2.0 analysis for specific APOBEC3G (settings of G-to-A substitutions with a downstream context of GD) or APOBEC3F (settings of G-to-A substitutions with a downstream context of AD) hypermutations that gave a significant P value ($P < 0.05$).

sured on site from fresh blood and are likely more accurate (6).

Hypermutation is not associated with Vif mutations. In order to determine the role Vif sequence polymorphisms may be playing in the observed APOBEC3F/3G-like cytosine deamination hypermutation, a subset of Vif sequences was examined from both highly hypermutated and nonhypermutated proviruses. Intact starting methionine residues, no stop substitutions, and a low or undetectable level of hypermutation was found in the *vif* genes of all samples examined. Amino acid polymorphisms in the Vif sequences were identified, but none in regions that had been previously identified as critical for Vif interaction with APOBEC (28, 32, 34, 39, 46, 50, 54). Additionally, only conservative substitutions were identified at single amino acid residue locations that had been suggested to have an impact on APOBEC interaction (43). Overall, Vif from highly hypermutated provirus was markedly similar to Vif from the other samples in this study and to published consensus sequences, suggesting that Vif polymorphisms are not responsible for the increased APOBEC3-type hypermutation observed within this population.

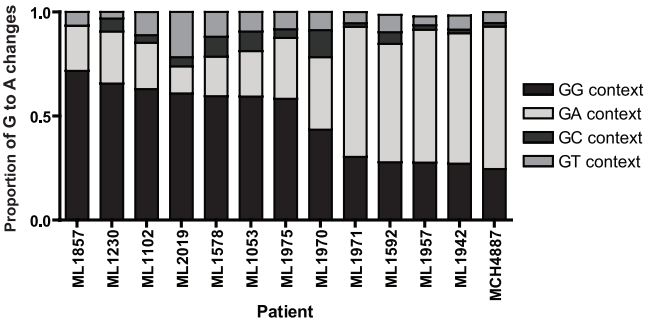

FIG. 4. Dinucleotide context of G-to-A hypermutation in 13 patients. The proportion of G-to-A hypermutation in each proviral sequence occurring at the dinucleotides GG, GA, GC, and GT is indicated. Hypermutation context was determined through comparison to a clade-specific consensus sequence, using Hypermut 2.0.

DISCUSSION

This study of viral diversity in 240 HIV-1 proviral sequences identified premature stop codons in the examined ORFs of a number of isolates. Further examination revealed that the detrimental amino acid changes were in fact the result of G-to-A hypermutation in the DNA coding sequence. Examination of the corresponding viral RNA, however, showed a lack of hypermutation. This suggested that the mechanism of hypermutation was not global, such as an imbalance in nucleotide pools, but rather a targeted phenomenon, such as APOBEC-medi-

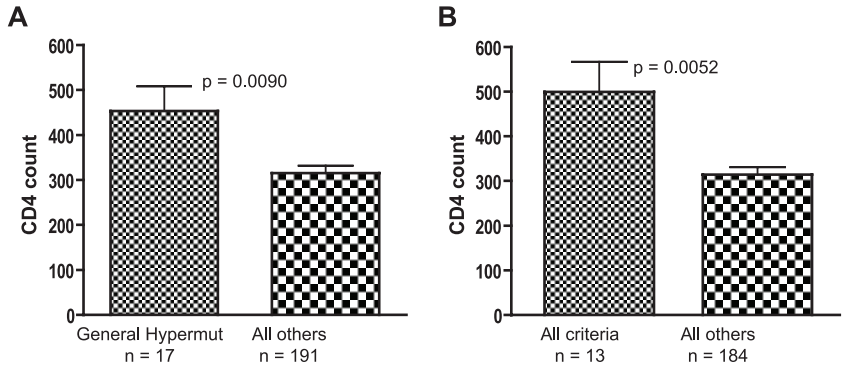

FIG. 5. Association of higher CD4 count with hypermutated sequences. CD4 counts are compared in patients with hypermutated provirus and patients without hypermutated provirus. (A) Hypermutation was defined as significant general APOBEC cytidine deaminase activity, as determined by Hypermut 2.0. (B) Hypermutation was defined as sequences with mutations in Vpu and Env, proviral adenine proportion greater than 1 standard deviation above the mean, and significant Hypermut 2.0 hypermutation. Groups are compared by a Mann-Whitney test. The height of the bar indicates the mean, and the error bar represents the standard error of the mean.

ated hypermutation. This finding agrees with a survey of more than 2,000 plasma virus sequences from nine HIV-positive patients that found no evidence of hypermutation in RNA sequences, although over 6% of the proviral DNA sequences from the same patients were hypermutated (22). We agree with the authors' hypothesis that the hypermutated proviruses were unable to produce virus particles, due to the fatal mutations caused by the hypermutation, explaining the absence of hypermutated plasma RNA sequences. These nonhypermutated RNA sequences that were isolated from subjects infected with hypermutated provirus may have originated in an unsampled tissue compartment or reservoir where hypermutation has not occurred.

The sequence context of the observed hypermutation further suggested that the host antiviral APOBEC3F and APOBEC3G proteins were involved. The Hypermut 2.0 tool identified significant general APOBEC3-type hypermutation in these sequences, as well as specific APOBEC3F and APOBEC3G hypermutation; APOBEC3F causes GA-to-AA nucleotide changes, while APOBEC3G causes GG-to-AG transitions (26, 52). It is interesting to note that 10 samples showed significant Hypermut 2.0 hypermutation characteristic of both APOBEC3G and APOBEC3F, suggesting that these proteins may be working in concert in these patients. Examination of the dinucleotide context for the G-to-A hypermutation in 13 patients with dramatic hypermutation supported these observations. An elevated GG context of the hypermutation would be expected to be due to APOBEC3G involvement, while an elevated GA context would be caused by APOBEC3F involvement. In contrast, the GC and GT context should be representative of the background mutation rate. In most of the examined sequences, both the GG and GA context were elevated compared to GC and GT, again suggesting coinvolvement of APOBEC3F and APOBEC3G. Indeed, it has been shown that these proteins can form heteromultimers (17, 48, 49).

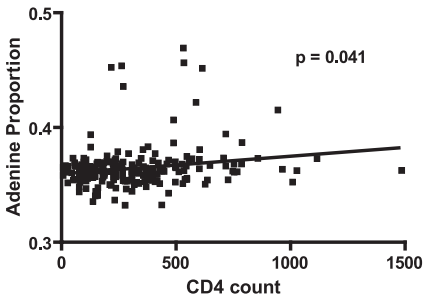

FIG. 6. Correlation of adenine proportion with CD4 count. CD4 counts were available for 208 subjects. Adenine proportion was measured from the 590-nucleotide *vpu*/*env* HIV-1 proviral region. These two measures were significantly positively correlated ($P = 0.041$).

A subset of subjects that were determined to have dramatic levels of hypermutation (as defined by the presence of premature stop codons, elevated adenine proportion, and a significant APOBEC3F/G sequence connotation to the hypermutation) had significantly higher CD4 cell counts than the other subjects did. These subjects represent an extreme, where severely hypermutated provirus appears to be the predominant proviral species. A previous study of nine patients with long-term viral suppression due to highly active antiretroviral therapy demonstrated that hypermutation could be identified, albeit at a minority, in the proviral sequences from all the subjects (22). Entertaining the likelihood, therefore, that all HIV-positive patients have a variable amount of proviral hypermutation, adenine proportion in the dominant proviral sequence was used to estimate the level of hypermutation in all subjects and was indeed found to be positively correlated with CD4 cell count in all 240 subjects. This indicates that as the dominant proviral HIV-1 sequence is increasingly hypermutated, the subjects' CD4 counts similarly increase, both for patients with dramatic hypermutation and patients with subtle hypermutation. Although correlation does not necessarily indicate causation, this supports the hypothesis that with increasing hypermutation, fewer viable viral progeny are produced, protecting infected cells from direct viral cytopathic effects (22). This work thus also supports the antiviral role of APOBEC-mediated hypermutation, which has been recently debated (15, 27, 33, 35, 51).

An alternative explanation of the association of hypermutation with CD4 count is that a strong host cytotoxic T lympho-

cyte (CTL) response targets cells that are infected with replication-competent virus for destruction. As a result, cells infected with non-replication-competent HIV-1, such as virus affected by the observed hypermutation, would be comparatively enriched in patients with strong, protective immune responses and therefore higher CD4 cell levels. However, if this were true, a general enrichment of mutation would be expected, due to the error-prone nature of HIV-1 reverse transcriptase, not just G-to-A hypermutation. Yet, all the proviruses that had detrimental mutations in Vpu and Env were hypermutated, and all the mutations were due to nucleotide G-to-A hypermutation. Also, the G-to-A changes in the dinucleotide context of GG and GA, hallmarks of APOBEC3G and APOBEC3F activity, respectively, were elevated above the dinucleotide context of GC and GT, which would represent non-APOBEC-mediated mutations (26, 52). In the absence of APOBEC-specific hypermutation, the levels would presumably be similar. Furthermore, Vif, which is located at a local minimum of APOBEC hypermutation, was not significantly mutated (44). This also would not be expected if another mutating factor had a significant effect on these viruses. Nevertheless, APOBEC may work in cooperation with strong CTL responses to control HIV-1 replication, highlighting the need for continued research about the cooperation between host innate, intrinsic, and adaptive immunity.

The HIV-1 protein Vif is known to counteract APOBEC3F and APOBEC3G, but examination of Vif sequences from subjects did not reveal detrimental mutations that would explain the seemingly increased APOBEC3 hypermutation activity. Furthermore, hypermutation was not observed in the proviral *vif* sequences, although sequences were obtained from subjects that had significant proviral hypermutation in the *vpu*/*env* portion of the HIV-1 genome. This lack of Vif mutation is in contrast to a study by Pace et al. of 127 clade B HIV-1-infected patients from an Australian cohort, which found a correlation between hypermutated proviral sequences and viral load and attributed the relationship to stop mutations due to hypermutation in the infecting viral Vif that disabled this viral defense against cellular APOBEC (36). Priming for DNA synthesis occurs at fixed locations in the HIV-1 genome, and therefore, not all sites are single stranded for the same period of time, suggesting that as APOBEC3G acts only on single-stranded DNA, hypermutation will not occur uniformly across the genome (19, 44). In fact, Vif is located at a local minimum of predicted mutation, suggesting a mechanism that explains our findings (44).

To explain our observations, we hypothesize that subjects infected with more dramatically hypermutated HIV-1 provirus have elevated APOBEC3F/G activity. Potential causes for this elevated activity include upregulation of APOBEC3, such as by gamma interferon, which has been recently shown to be a potent inducer, allowing APOBEC to overcome Vif inhibition (3, 37, 40, 45), or the enzyme may exist in a more enzymatically active form (for example, a higher proportion of APOBEC3G may be present in its low-molecular-mass, active form) (9). Alternatively, the infecting viruses may encode a Vif protein that is inefficient or unable to target APOBEC3F and APOBEC3G for degradation or prevent their packaging into nascent viral particles, although our data do not support this hypothesis (11, 28, 32, 34, 42, 46, 50, 53, 54) In either scenario, we theorize that as a result of increased APOBEC3 or decreased Vif level/activity, the newly budded viral particles contain higher levels of APOBEC3F and APOBEC3G, ultimately resulting in hypermutation during infection of new cells. It seems likely that the resulting provirus is sufficiently hypermutated that replication-competent, hypermutated progeny cannot be generated, as illustrated by the lack of hypermutated RNA sequences. Importantly, the cells infected with hypermutated provirus would not be subjected to HIV's direct cytopathic effects, nor would they be a target of CTLs, likely explaining the observed higher CD4 counts and presumable decrease in disease progression in patients with increased proviral hypermutation (22).

Two conflicting papers highlight that the role of APOBEC3 in HIV disease progression has not yet been resolved. In their study using stimulated PBMCs, Jin et al. (20) found that APOBEC3G mRNA levels were positively correlated with patient CD4 count and inversely correlated with viral load, while Cho et al. (10), using unstimulated PBMCs, found that neither APOBEC3G nor APOBEC3F mRNA correlated with CD4 count and viral load. The hypermutation observed in the present study is likely a direct effect of APOBEC3F/G cytidine deamination, whereas mRNA expression levels, which were examined in these previous studies, may not directly correlate with enzymatic activity due to translational and posttranslational regulation. This direct assessment of sequence hypermutation, likely the result of APOBEC3 cytidine deaminase activity, found that as the level of hypermutation in the subjects' predominant proviral sequence increased, so did the subjects' CD4 counts, suggesting an in vivo role for APOBEC in disease progression.

APOBEC3G/3F proviral hypermutation likely exists in a spectrum. The 13 subjects with dramatically hypermutated provirus had detectable viral loads, intact viral RNA sequence, and significantly depressed CD4 counts due to HIV infection compared to patients not infected with HIV, suggesting that there may be a tissue or cellular compartment where APOBEC3 is not packaged into viral progeny, leading to the production of nonhypermutated virus, which in turn leads to nonhypermutated infectious viral progeny, sustaining the infection. On the other end of the spectrum, patients without hypermutation in the dominant provirus may have minority sequences that are hypermutated (22). Indeed, the examination of clonal sequences indicated some degree of variability within patients. The data presented illustrate a correlative relationship between this spectrum of hypermutation, likely the direct result of APOBEC3F/3G activity and CD4 cell count in vivo in the absence of obvious Vif polymorphism. These findings highlight the potential for enhancing host APOBEC3F and APOBEC3G for therapeutic purposes and suggest that even small increases in APOBEC activity may attenuate HIV-1 replication and disease progression.

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