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Clonal Expansion of Human T-Cell Leukemia Virus Type I-Infected Cells in Asymptomatic and Symptomatic Carriers without Malignancy

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Human T-cell leukemia virus type I (HTLV-I) is associated with adult T-cell leukemia/lymphoma and tropical spastic paraparesis/HTLV-associated myelopathy. Both diseases are usually preceded by a long clinically asymptomatic period. PCR amplification of the HTLV-I proviral integration sites shows that clonal expansion of HTLV-I-bearing T cells, rather than being an occasional phenomenon in nonmalignant disease, is the norm for both symptomatic and asymptomatic carriers. Sequencing of 100 molecular clones derived by PCR amplification of part of the envelope gene from two asymptomatic carriers revealed almost no genetic variation. Viral amplification via clonal expansion, rather than by reverse transcription, would explain this remarkable genetic stability.

Adult T-cell leukemia/lymphoma is usually characterized by a monoclonal expansion of the malignant cell with respect to the integrated human T-cell leukemia virus type I (HTLV-I) provirus (31). Among the virological hallmarks of tropical spastic paraparesis/HTLV-associated myelopathy (TSP/HAM) are elevated proviral copy numbers. Recent work suggests that during the clinically asymptomatic period preceding disease, the proviral load may be considerable, with approximately one copy per 25 to 1,000 peripheral blood mononuclear cells (PBMC) (8, 9, 20, 27). However, the proviral load in patients with TSP/HAM may be as high as one cell per 10 to 100 PBMC (16, 20, 27). The finding of an elevated proviral load in the absence of malignancy contrasts with the remarkable genetic stability of HTLV-I and related viruses, HTLV-II and bovine leukemia virus (BLV). It has been estimated that the rate of fixation of base substitutions for HTLV-I is on the order of 1% per 1,000 years (3, 19).

By contrast, late-stage human immunodeficiency virus (HIV) infection is accompanied by heavy proviral loads (<5% of PBMC [5]) and phenomenal intrapatient (≤15% nucleic acid divergence [13, 15]) and interpatient (≤30% [13, 15]) variation. In fact, the genetic variation within a single HIV-infected patient is greater than the variation among the entire collection of HTLV-I sequences identified to date (13, 15). There is ample evidence that RNA virus replication and genetic diversity go hand in hand (6). Retroviruses are no exception, with the double-stranded provirus resulting from reverse transcription of the single-stranded virion RNA, a process which occurs in the absence of proofreading, as the viral reverse transcriptase is devoid of 3′ exonucleolytic activity.

Therefore, in terms of genetic variability, it appears that HTLV-I is an exception. Either the mutation rate per cycle of the HTLV-I reverse transcriptase must be particularly high or the virus replicates in concert with the cell, i.e., via mitosis. As RNA viral variation and genetic variation are synonymous, the latter hypothesis seems to pertain. Indeed, this conclusion is supported by the recent determination of the overall mutation rate per cycle for BLV and HIV type 1 (HIV-1) (10, 11). The mutation rate for BLV was 4.8×10^{-6} per base per cycle and that for HIV-1 was 3.5×10^{-5}, while the mutation rate per cycle for spleen necrosis virus, an avian retrovirus distinct from BLV and HIV-1, was 1.2×10^{-5}. Such small differences are insufficient to explain the extraordinary genetic stability of HTLV and BLV.

TSP/HAM was originally shown to be accompanied by polyclonal integration of the HTLV provirus in PBMC (4), although more recently it has been shown to be accompanied by occasional oligoclonal integration of the HTLV provirus in PBMC, as evidenced by Southern blotting (2). However, the genetic stability of HTLV requires that clonal expansion be the norm. In this study the HTLV-I proviral integration sites have been amplified from PBMC DNA from a number of asymptomatic patients and TSP/HAM patients. All samples showed evidence of oligoclonal expansion of between >5 and 20 HTLV-I-bearing cells.

MATERIALS AND METHODS

Primers. Oligonucleotides were synthesized on an Applied Biosystems model 380 DNA synthesizer by phosphoramidite chemistry. After detritylation and alkaline deprotection, crude material was used for PCR. For amplification of the integration sites the primers were BIO1, 5′ TCATGATCAATGGGACGATCA CATG; BIO2, 5′ CTGTTCTGCGCCGTTACAGATCGA (positive strand, 8898→8921); BIO3, 5′ CCTTTCATTCACGACTGACTGCCG (positive strand, 8938→8961); BIO4, 5′ TCATGATCAATGGGACGATCA; and BIO5, 5′ TGG CTCGGAGCCAGCGACAGCCCAT (positive strand, 8995→9019, probe). For amplification of the 3′ *pol* plus 5′ *env* region the primers were HSP1, 5′ GGA **CTCGAG**TTAATAGCCGCCAGTGGAAA (positive strand, 5025→5044); HSP2, 5′ GGAC**CTCGAG**TGAGAGTACAGCAGCTGGG (negative strand, 5290→5271); and HSPS, 5′ CATGGGTAAGTTTCTCGCCACTTTG (positive strand, 5201→5225, probe). All coordinates are those of the λATK-1 sequence (18). The BIO1 sequence is artificial. The sequence of BIO4 lacks the terminal CATG sequence of BIO1 (underlined above). All HTLV-I primers mapped to conserved regions.

Amplification of integration sites. Three micrograms of total DNA was digested with 10 U of *Nla*III in 1× *Nla*III buffer for 3 h at 37°C. Digestion was controlled by gel electrophoresis. DNA was phenol-chloroform extracted and ethanol precipitated. One microgram of digested DNA was ligated with 10 pmol of BIO1 primer with 20 U of T4 DNA ligase in 40 μl for 16 h at 15°C. This was

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followed by phenol-chloroform extraction and precipitation. Ligated DNA was amplified for 100 cycles with the BIO2 primer alone. A solution containing 1× *Taq* buffer (10 mM Tris HCl [pH 8.3], 50 mM KCl, 2.5 mM $MgCl_2$), 40 pmol of BIO2, 200 μM each deoxynucleoside triphosphate, and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus) in a final volume of 100 μl was used. Thermal cycling was done once at 95°C for 5 min and 100 times at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s); thermal cycling was followed by a final elongation step for 10 min at 72°C. Ten microliters of this linear PCR reaction was used in a classical PCR amplification with the BIO3 and BIO4 primer pair. Amplification conditions were as before with 40 pmol of each primer, again in a final volume of 100 μl. Thermal cycling was done once at 95°C for 5 min and 35 times at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; thermal cycling was followed by a final elongation step for 10 min at 72°C. For amplification of the 3′ *pol* plus 5′ *env* fragment a solution containing 1 μg of DNA, 40 pmol of primers, and 2.5 U of *Taq* was used. Cycling was done once at 95°C for 5 min and 30 times at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; cycling was followed by an elongation step for 10 min at 72°C. These *env* primers carried 9 bases at the 5′ end which encoded *Xho*I restriction sites (shown above in boldface) and clamps. As usual, efforts to avoid contamination were made (25). All *Nla*III digestions, ligations, and first-round PCR were carried out in laboratories which had never handled an HTLV-I plasmid. Second-round and fluorescent amplifications were performed in yet another laboratory. No contamination was detected.

Cloning and sequencing. PCR products were blunt end cloned into M13mp18 replicative-form DNA as previously described (23). Clones were screened in situ with either a ^{32}P-labelled BIO5 or HSPS oligonucleotide probe. Hybridization was carried out in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 42°C, and filters were washed in 2× SSC–0.1% sodium dodecyl sulfate at 42°C. Single-stranded templates were sequenced with fluorescent dideoxynucleotides. The products were resolved on an Applied Biosystems model 370A DNA sequencer with model 373A software.

Fluorescent amplification and resolution. Fluorescent BIO5 (FluoBIO5) was synthesized by coupling the FAM fluorophore to an aminolink II group at the 5′ end of BIO5, an HTLV-I-specific oligonucleotide. Thus, FluoBIO5 is FAM–aminolink–5′ TGGCTCGGAGCCAGCGACAGCCCAT. It was purified first by ethanol precipitation followed by reverse-phase high-pressure liquid chromatography. Approximately 2 to 3 μl of PCR product, amplified by BIO3 and BIO4, was amplified under standard conditions in only 20 μl overlaid with 40 μl of mineral oil. Between three and eight linear cycles with FluoBIO5 at a final concentration of 0.1 μM were sufficient. Cycling was done as described above. Fluorescence-labelled PCR products were resolved on the Applied Biosystems sequencer. Raw data were analyzed with the Immunoscope 2.10 software package (14). Length standards were 96, 140, 157, 289, and 307 bases long.

RESULTS

Two asymptomatic HTLV-I carriers from Guadeloupe were studied in particular detail. Patient 19 (P19) was a 54-year-old male with an HTLV proviral load of approximately one copy per 75 PBMC, while patient 20 (P20) was a 46-year-old female whose proviral load was close to one copy per 16 PBMC (27, 28). In order to assess the degree of clonality of cells harboring HTLV-I proviruses, the 3′ long terminal repeat and flanking cellular sequences were amplified by PCR via oligonucleotide linker ligation (Fig. 1). High-molecular-weight DNA was digested with *Nla*III and an oligonucleotide (BIO1) ligated to the 3′ protruding ends. DNA was first amplified from the 3′ long terminal repeat into the flanking cellular sequences in 100 cycles with an HTLV-I positive-strand-specific primer (BIO2). This was followed by 35 cycles of normal PCR with an HTLV positive-strand-specific primer (BIO3) and a primer (BIO4) complementary to BIO1 yet lacking the terminal palindromic sequence CATG defined by the *Nla*III restriction site. PCR products were cloned into M13mp18 replicative-form DNA, and >100 clones from each patient were sequenced.

Figure 2 shows the length variation of the flanking cellular sequences derived from the two patients. A plateau was frequently, but not always, synonymous with a cluster or clone of identical cellular flanking sequences. Among the 102 cellular flanking sequences established for P19 nine clones accounted for 94 (Fig. 2, inset). Six remaining sequences were unique. Two additional sequences formed an interesting pair. The cellular flanking sequences from P19's DNA were as follows: sequence 198 (P19-198), GGTGTGGGTCACAGACCCAAG GTGCTGACTTGGAAGGAGGGACAGTTTAGGGAAG

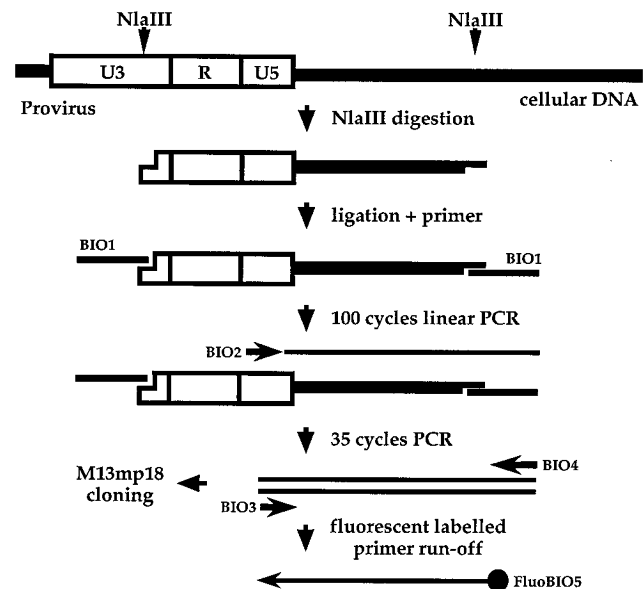

FIG. 1. Linker ligation PCR protocol used to amplify HTLV-I integration sites.

CTC**CATG**, and P19-286, GGTGTGGGTCACAGACCCA AGGTGCTGTGGGATGTT**CATG**. The common sequences are underlined, while the *Nla*III site to which the BIO1 oligonucleotide was ligated is in boldface. While the 5′ 26 bases were identical, there was no homology among the remaining sequences. This was not due to a cloning artifact, as both sequences extended into the 3′ PCR primer and flanking M13 sequences. In all our PCR work involving the amplification of direct repeats, no deletions associated with *Taq* polymerase have been noted (12, 15, 23, 25). This might be indicative of clonal expansion with subsequent rearrangement of flanking cellular DNA in one of the proliferating cells.

Despite a greater proviral burden for P20's DNA (one copy per 16 PBMC), more clones of cellular flanking sequences were found in comparison with those of the P19 DNA sample. Nearly half (50 of 104 [Fig. 2, inset]) of the cellular flanking sequences belonged to 15 clones; the remaining 54 sequences were unique. While the length of the cellular flanking sequences could generally be equated with clonality, this was not invariably the case. Thus, two distinct clones (from P19) gave 7-bp flanking sequences and two clones (from P20) showed different 25-bp flanking sequences, while the length of three other independent sequences (again from P20) was 93 bp. Conversely, rearrangement of cellular DNA postintegration could complicate the picture somewhat. However, in general, a plateau identified a single clone of HTLV-I-harboring T cells.

Analysis of the heptameric repeat sequences flanking the integrated provirus (18) showed that they were approximately 59% A+T rich, a value that closely resembles the A+T content of the human genome. The collection of cellular flanking sequences was screened for possible homologies with those in the GenBank database. Only three significant homologies were noted: P19-91 showed 88% (51 of 58 bases) identity with a human Alu repeat (HSALU1), P20-50 showed 93% (219 of 235) identity with the human β-satellite DNA sequence (HUMBSTAC), and P20-256 showed 93% (52 of 56) identity with an Alu repeat in intron C of the human low-density lipoprotein receptor 1 gene (HUMLDLIVS). Given the abun-

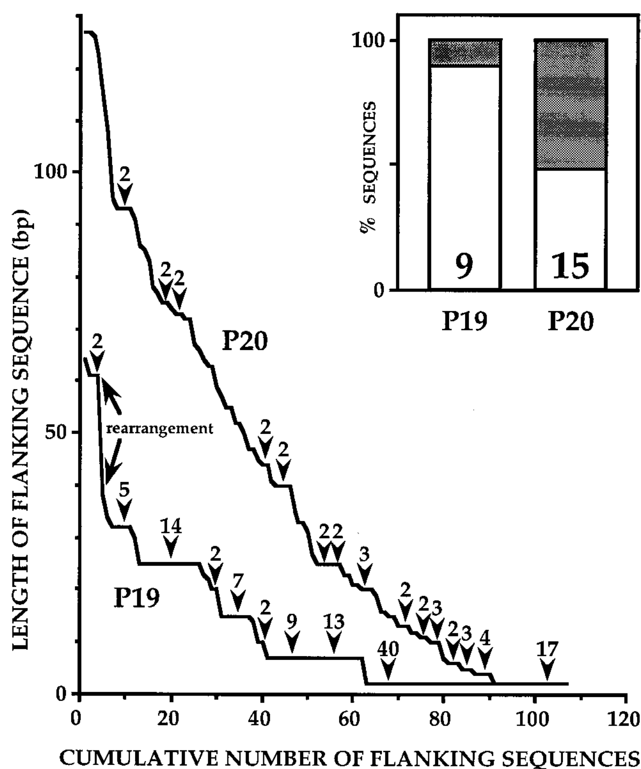

FIG. 2. Length of cellular sequences flanking the 3′ HTLV-I long terminal repeat among approximately 100 clones derived from PBMC of two asymptomatic carriers, P19 and P20. The proviral copy numbers of P19 and P20's DNA were 2,500 and 9,000 copies per 150,000 PBMC, respectively (27, 28). The numbers above arrowheads indicate the number of identical flanking sequences of a given length. The two sequences suggesting rearrangement of flanking cellular sequences in one of the patient's DNA are indicated. The inset shows the proportion of cellular flanking sequences which are parts of clones (shaded) as well as the number of clones identified. In both samples the smallest flanking sequence was the TG dinucleotide. The terminal U5 sequences of an integrated HTLV-I provirus are GTACACA. Thus, integration 5′ to any TG dinucleotide would create an *Nla*III restriction site, i.e., GTACACA·TG. As the frequency of TG in the human genome is 7.4% (21), nearly half of the 17 sequences probably represent independent integration events which were grouped by the choice of *Nla*III. For P19 (Fig. 1) the frequency of this TG species (40 of 102) suggests that a sizable proportion must reflect clonal expansion superimposed upon the background value of ~7%. The *Nla*III digestions were invariably complete. For the P20 data set three sequences were found to have an internal CATG restriction site, i.e., P20's DNA was ~97% digested. P19's DNA was completely digested.

dance of repeated and satellite sequences within the human genome, these findings are not surprising.

To obviate the cloning and sequencing step, fluorescence-labelled products were prepared from PCR material by performing an additional three to eight cycles with the fluorescence-labelled primer, FluoBIO5. The flanking-sequence-length polymorphisms generated by this method could rapidly be resolved with a DNA sequencer. Typical data for integration sites obtained for a third asymptomatic HTLV-I carrier, four TSP/HAM patients, and a single patient with adult T-cell leukemia/lymphoma are shown in Fig. 3. By the appropriate use of length standards and software (14), it was possible to define the lengths of flanking sequences of each clone to within ±1 bp over the 2- to 200-bp range as well as to quantitate each peak. In all cases, between 5 and 20 peaks could be identified. That a number of peaks correspond to flanking cellular sequences of >100 bp suggests that the cloning of the ensemble of HTLV-I flanking cellular sequences resulted in the selection of smaller fragments (i.e., <100 bp [Fig. 2]).

In order to demonstrate that genetic stability could accompany a high proviral burden, a segment of 265 bp spanning the hydrophobic envelope leader sequence was amplified from P19 and P20's DNA and cloned and 100 recombinants were sequenced. This region was chosen because it is one of the regions variable among divergent HTLV-I strains as well as between HTLV-I and simian T-cell leukemia virus (26). So few (three) substitutions were noted (Fig. 4) that they may even be attributable to *Taq* polymerase misincorporation (12).

DISCUSSION

These data show that clonal expansion of HTLV-I-bearing T cells, rather than being an occasional phenomenon in nonmalignant disease (2), was the norm for both symptomatic and asymptomatic carriers. The fluorescence-based analysis was the more powerful, as the cloning step introduced a bias in favor of shorter fragments. However, cloning proved that, in general, a peak corresponded to a clone. This PCR-based method proved to be more sensitive than inverse PCR (data not shown) (21).

Are the 54 and 6 unique cellular flanking sequences identified for P20 and P19's DNA, respectively, indicative of clones? First, there is a problem with sampling associated with sequence analysis. For P20 1 μg of *Nla*III-digested DNA, corresponding to 150,000 cell equivalents or ~9,500 HTLV-I proviral copies, was amplified. The collection of 104 flanking cellular sequences therefore represents ~1% of the total. For P19, 102 of the ~2,000 copies (~5%) were analyzed. Reconstruction experiments using HTLV-I serially diluted plasmid DNA showed that the linker-mediated PCR protocol was able to detect between 50 and 100 copies, indicating that those detected are among the most abundant clones (data not shown). This means that there may be more HTLV-I-expanded clones than were detected. Second, while the fluorescent runoff analysis revealed more examples of clonal expansion because no cloning step was involved (see above), the inherent noise (Fig. 3) precluded interpretation of many of the very small peaks. For these two reasons it is likely that there are a substantial number of low-abundance clones.

In all cases there were >5 discrete integration sites, and very probably more, per sample. As TSP/HAM patients have, in general, a greater proviral load than do asymptomatic carriers (3, 8, 20, 27), it is possible that for some individuals, clonal dominance or selection may occur, which could result in an apparent simplification of HTLV-I integration profiles. In this context it is worth noting that a small deletion in cellular flanking DNA of one HTLV-I-bearing clone suggests that additional mutational events may accompany clonal expansion (32). This might help explain why Furukawa et al. found evidence of a single clone in 30% of TSP/HAM patients studied (2). However, it must also be borne in mind that the sensitivity of Southern blotting is much lower than that of PCR-based techniques.

Clonal expansion of infected cells would help explain the remarkable genetic stability of HTLV-I (3, 19) (Fig. 4), as it would limit the number of rounds of replication involving error-prone reverse transcription. In order to appreciate such genetic stability in the face of an important proviral load, it may be worthwhile to compare HTLV-I with HIV-1. Both viruses replicate preferentially in $CD4^+$ lymphocytes in vivo. In both cases, high proviral loads (>5% of PBMC) correlate with disease (4, 5, 27, 30). Yet if the PCR products corresponding to the HIV-1 envelope signal peptide derived from the PBMC of an AIDS patient and 100 recombinants were sequenced, most

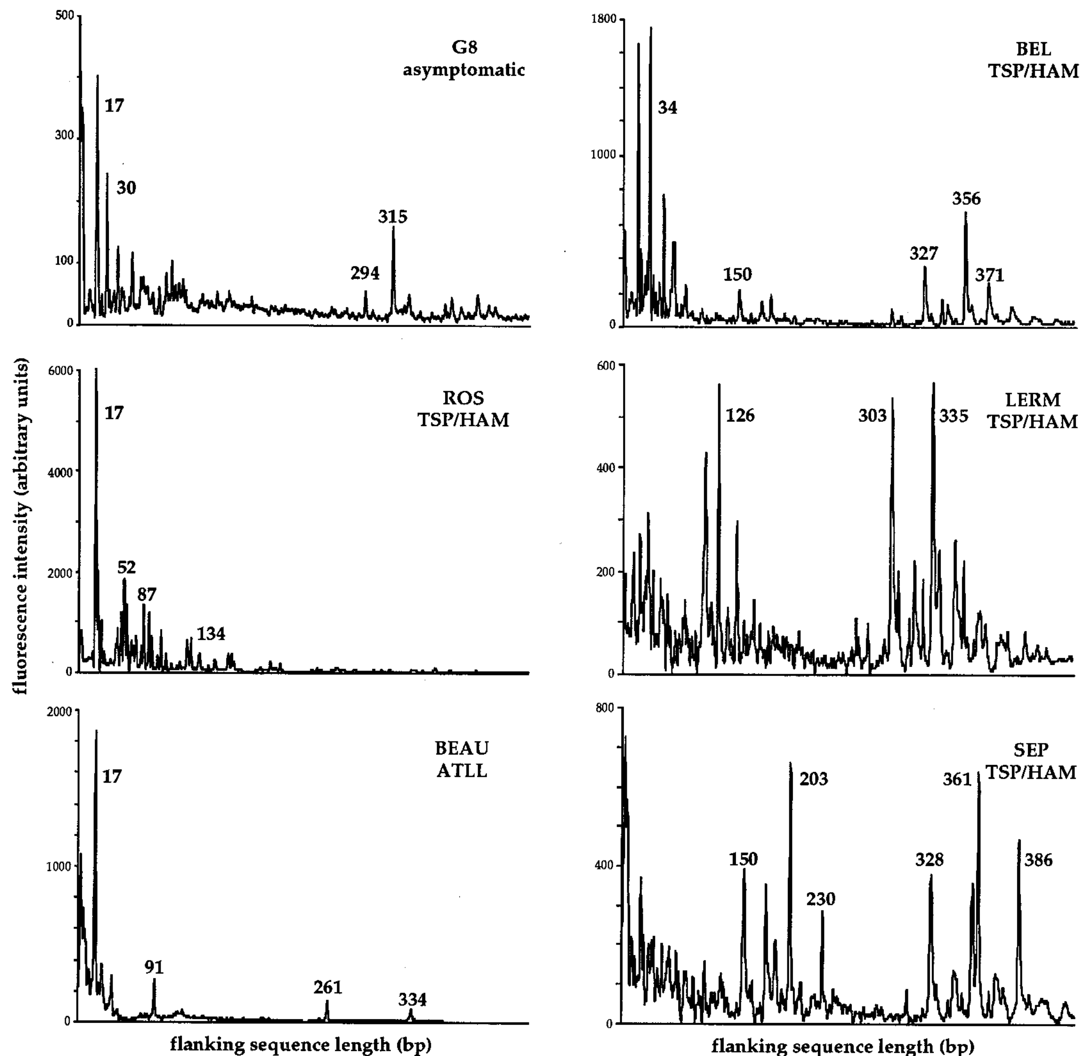

FIG. 3. Fluorescent-primer runoff of PCR-amplified HTLV-I integration sites in six HTLV-I-positive samples. Note that the scales of the ordinates (arbitrary fluorescence intensities) vary. All patients were from Martinique or Guadeloupe in the Caribbean. For nonmalignant disease, oligoclonal expansion, involving 10 to 20 peaks, was evident. As anticipated, the integration site profile was simpler for the sample from the patient with adult T-cell leukemia/lymphoma (ATLL), although minor peaks could be discerned.

probably more than half would be different and reflect internal nucleic acid sequence variation between 3 and 7% (13, 15).

HTLV-I probably establishes high proviral loads via a few rounds of reverse transcription and subsequent cell division, probably via the *trans* effect of p40^{tax} on cellular genes (29). Consequently, little genetic variation would be generated because proviral replication, in concert with cell division, would proceed with faithful cellular DNA replication, which is approximately 10^6 less error prone than reverse transcription. For HIV-1, high proviral loads are ultimately achieved by remorseless rounds of reverse transcription, with extensive genetic variation as an inevitable consequence (24). In fact, this is suggested by the relative genetic organization of the analogous *tax* (HTLV-I) or *tat* (HIV-1) and *rex* (HTLV-I) or *rev* (HIV-1) genes (1). Tax and Tat are the transactivating transcriptional enhancer proteins, while the Rex and Rev proteins regulate the transport of partly spliced mRNAs to the cytoplasm. The HIV *rev* product has a negative effect on Rev expression but relatively little on *tat* and hence on HIV replication, because both *rev*-independent and *rev*-dependent *tat* transcripts (1.8 and 4.5 kb, respectively [17]) exist. Hence, Tat is always produced in an activated T cell, thus ensuring massive virus production. By contrast, both Rex and Tax are derived from the same mRNA, suggesting that *rex* has a negative effect on both Rex and Tax production, which would result in restricted viral replication.

HTLV-I proviral loads in TSP/HAM may approach 10% of PBMC (8, 9, 20), or on the order of 3×10^9 copies (the proviral burden in secondary lymphoid organs is unknown). If it is assumed that there are perhaps only 10 clones expanding as a consequence of HTLV-I integration, then it would be possible to attain such a proviral load in only 28 divisions. Even if the proviral burden were 10-fold greater, the number of divisions needed would be only 31. As only a small proportion of HTLV-I carriers develop the disease after 30 to 40 years, clonal expansion must clearly be actively held in check. The observed proviral load must represent the steady-state value, with clonal expansion being counterbalanced by loss of HTLV-bearing T cells, perhaps due to intense anti-HTLV-I cytotoxic T-cell responses (7). That the proviral load is generally greater

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                5035                                                                  5095
P19C       TTATAGCCGC CAGTGGAAAG GACCACAGGA GGCTCTCCAA GAAGCTGCCG GCGCTGCTCT CATCCCGGTA
P19-52     .......... .......... .......... .......... .......... .......... ..........
P20C       .......... .......... .......... .......... .......... .......... ..........
P20-111    .......... .......... .......... .......... .......... .......... ..........
P20-115    .......... .......... .......... .......... .......... .......... ..........
λATK-1     .......... .......... .......... .......... .......... .......... ..........

                                                                                      5165
P19C       AGCGCTAGTT CTGCCCAGTG GATCCCGTGG AGACTCCTCA AGCGAGCTGC ATGCCCAAGA CCCGTCGGAG
P19-52     .......... .......... .......... .......... .......... .......... ..........
P20C       .......... .......... .......... .......... .......... .......... ..........
P20-111    .......... .......... .......... .......... .......... .......... ..........
P20-115    .......... .......... .......... .......... .......... ..A....... ..........
λATK-1     .......... .......... .......... .......... .......... .......... ..........

                                                    ➡ env                             5235
P19C       GCCCCGCCGA TCCCAAAGAA AAAGACCACC AACACCATGG GTAAGTTTCT CGCCACTTTG ATTTTATTCT
P19-52     .......... .......... .......... ..........pol⬅...... .......... ..........
P20C       .......... .......... .......... .......... .......... .A........ ..........
P20-111    .......... .......... .......... .......... .......... .A........ ..........
P20-115    .......... .......... .......... .......... .......... .A........ ..........
λATK-1     .......... .......... .......T.. .......... .......... .......... ..........

                                                                 5290
P19C       TCCAGTTCTG CCCCCTCATC CTCGGTGATT ACAGCCCCAG CTGCTGTACT CTCAC
P19-52     ......G... .......... T......... .......... .......... .....
P20C       .......... .......... T......... .......... .......... .....
P20-111    .......... .....G.... T......... .......... .......... .....
P20-115    .......... .......... T......... .......... .......... .....
λATK-1     .......... .......... .......... .......... .......... .....
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FIG. 4. Highly conserved nucleotide sequences derived from the region encoding the HTLV-I 3′ region of *pol* and 5′ *env*. One hundred clones from each sample (from P19 and P20) were sequenced. P19C and P20C represent the vast majority of sequences derived from samples from P19 and P20. The three variant sequences bearing single base substitutions are also given. The prototype HTLV-I sequence, λATK-1 (18), is given for comparison. The beginnings and ends of the *env* and *pol* open reading frames are indicated.

in TSP/HAM patients than in asymptomatic individuals suggests that over time clonal expansion is the stronger of the two (9, 30).

The finding of a small deletion in cellular flanking DNA of one HTLV-I-bearing clone suggests that additional mutational events may accompany clonal expansion (32), the consequences of which might be important for the subsequent development of malignant disease. Finally, these conclusions suggest that cytostatic drugs, rather than reverse transcriptase inhibitors, might be of some use in the treatment of TSP/HAM.

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