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Genomic diversity of the acquired immune deficiency syndrome virus HTLV-III: Different viruses exhibit greatest divergence in their envelope genes

(human retroviruses)

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ABSTRACT **Converging lines of research have linked human T-cell lymphotropic virus type III (HTLV-III) to the pathogenesis of the acquired immune deficiency syndrome. A characteristic feature of this virus is its genomic heterogeneity, which occurs to varying degrees in different viral isolates. To define further the nature and extent of these genomic changes, we compared the molecularly cloned genomes of two variant HTLV-III isolates by extensive restriction enzyme mapping and heteroduplex thermal melt analysis. Both viral isolates were found to be highly related to each other throughout their entire genomic complement, yet they differed markedly in their restriction enzyme maps. Electron microscopic heteroduplex analysis revealed several distinct regions of divergence located almost exclusively in the part of the genome that encodes the viral envelope gene. *In vitro* culture of one of these viruses over a period of 3 months did not result in any genomic changes as determined by restriction analysis of viral DNA. These results, as well as the recently published nucleotide sequences of other HTLV-III isolates, indicate that the most substantial variation among HTLV-III isolates is located in the envelope. These findings raise the possibility that viral isolates from different individuals could have important biological differences in their envelope antigens, a consideration relevant to ongoing attempts to develop a vaccine against HTLV-III.**

Human T-cell lymphotropic virus type III (HTLV-III) (1–4), also referred to as lymphoadenopathy-associated virus (LAV) (5), and more recently as acquired immune deficiency syndrome (AIDS)-related virus (ARV) (6), is a human T-cell lymphotropic, cytopathic retrovirus responsible for a variety of immunological abnormalities (7). The clinical sequelae of HTLV-III infection, although not yet entirely defined, comprise a wide spectrum ranging from asymptomatic apparently healthy conditions to full-blown AIDS. The factors that dictate the clinical outcome of HTLV-III infection presumably include a combination of viral, host, and environmental factors, but these are only now beginning to be understood.

We reported previously that diversity, or heterogeneity, in the genomes of different HTLV-III isolates is a characteristic feature of this virus (8, 9). This conclusion was based on detailed restriction enzyme analysis of viral DNA in both fresh uncultured tissues from patients with AIDS and in virally infected cell cultures derived from these tissues. Questions of central importance, therefore, include whether such genomic differences could be responsible for the different clinical phenotypes observed in HTLV-III-infected individuals and whether such changes could alter the antigenic properties of HTLV-III so as to allow this virus to evade the host's immune surveillance mechanisms. There is precedent for both: for example, the U3 long terminal repeat (LTR) enhancer sequences determine the leukemogenic potential of different murine leukemia virus strains since subtle changes in these sequences convert a nonleukemogenic virus into a highly leukemogenic one (10, 11). The other scenario is exemplified by naturally occurring infections with visna virus and equine infectious anemia virus, both of which undergo progressive antigenic variation of their envelope glycoproteins, presumably in response to selective pressures from the host's immune system (12, 13). This spontaneous alteration in envelope antigenicity presents a major obstacle to the development of effective vaccines against diseases caused by these viruses.

To define further the extent and location of the genomic changes in HTLV-III viruses, we molecularly cloned the full-length genome of a provirus from a Haitian man with AIDS (λHAT-3) and compared it by extensive restriction enzyme mapping and heteroduplex analysis to another viral clone (λHXB-2), which was derived from the producer cell line H9/HTLV-IIIb (2, 8). We chose λHAT-3 and λHXB-2 because preliminary analysis had shown that these two viral isolates differed considerably in their restriction enzyme pattern (8). We also addressed the question of whether HTLV-III undergoes genomic variation *in vitro* during long-term tissue culture. The results of this study demonstrate that the two isolates of HTLV-III analyzed possess substantial diversity in their genomes, especially in the region encoding the envelope, and that these genomic changes are not the result of *in vitro* propagation of the virus.

MATERIALS AND METHODS

Virus Isolation. The isolation and propagation of different isolates of HTLV-III, including those isolates used in the present study (H9/HTLV-IIIb and H4/HTLV-III$_{RF}$), have been described in detail (2). The H9/HTLV-IIIb cell line was established by infecting H9 cells with a pool of virus from different patients with AIDS or ARC; the H4/HTLV-III$_{RF}$ line was established by infecting H4 cells with virus from a single patient, a heterosexual Haitian man with AIDS. Both

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Abbreviations: HTLV-III, human T-cell lymphotropic virus type III; AIDS, acquired immune deficiency syndrome; LTR, long terminal repeat; kb, kilobase(s).

cell lines are good producers of HTLV-III virus even after a year of *in vitro* propagation.

Southern Blot Hybridization. High molecular weight DNA was prepared from HTLV-III-infected cells by standard techniques (14) and was analyzed by Southern blot hybridization using for probe a 9-kilobase (kb) cloned HTLV-III viral insert (λBH10i) comprising essentially the entire viral genome (15). Hybridization was carried out under high stringency conditions (2.4× NaCl/Cit/50% formamide, 37°C) and filters were washed in 1× NaCl/Cit/0.1% $NaDodSO_4$ at 65°C (1× NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate).

Molecular Cloning of HTLV-III Viruses. The full-length viral genome of H4/HTLV-III_{RF} was cloned from high molecular weight DNA of this cell line by standard techniques (14). Since *Sst* I was known to cleave the proviral DNA in or near the LTRs, this enzyme was used to prepare the viral insert and phage arms of the cloning vector λgtWes.λB. The clones of HTLV-III from the H9/HTLV-IIIb cell line were prepared in the phage J1λ as described (8, 15).

Heteroduplex Analysis. Electron microscopic heteroduplex analysis of cloned DNAs was performed as described (16, 17). To maintain isodenaturing conditions during the thermomelt analyses, only formamide concentrations in the hyperphase and hypophase were adjusted (17, 18). Formamide concentrations in the hyperphase ranged from 50% to 80% formamide. The effective temperatures (expressed as $t_m - \Delta t$, in which Δt is the difference between t_m and the temperature at which the heteroduplex was mounted for microscopy) were t_m −25°C, t_m −18°C, t_m −11°C, and t_m −4°C, for formamide concentrations of 50%, 60%, 70%, and 80%, respectively. Heteroduplexes were prepared with the cloned inserts still in their vectors in order to distinguish heteroduplexes from homoduplexes.

RESULTS

Molecular Characterization of Variant HTLV-III Genomes. A comparison of the restriction enzyme maps of the two HTLV-III clones representing different isolates of this virus is shown in Fig. 1. λHAT-3 contains the full-length proviral genome of HTLV-III_{RF}, which was described in our original report of the detection and characterization of HTLV-III in AIDS (2). λHXB-2 represents a different HTLV-III isolate derived from the producer cell line H9/HTLV-IIIb. This proviral clone has been studied extensively and was described in detail (8). As demonstrated in this figure, both clones are ≈10 kb long and are bounded on each end by LTR elements. As a result of the cloning strategy, λHAT-3 contains only one LTR (*Sst* I cleaves only in the R region of this virus's LTR; data not shown), whereas λHXB-2 contains the complete 5′ and 3′ LTRs and flanking cellular sequences. Hybridization experiments with the viral inserts showed that the two viruses are highly related to each other throughout the entire length of their genomes (8). Comparison of the restriction enzyme patterns of these proviruses, however, demonstrated that >50% of the enzyme sites mapped differed between the clones: there were 15 sites in common and 16 sites that were different. These differences were present throughout the genome with the exception of the 5′ and 3′ ends, where most restriction sites appeared to be conserved.

Electron Microscopic Heteroduplex Analysis. The genomic organization of λHAT-3 and λHXB-2 were evaluated further by electron microscopic heteroduplex thermal melt analysis. Because the two clones were known to be highly related, we performed this analysis with the entire HTLV-III λ phage clones including phage arms so that homoduplexes and heteroduplexes could be distinguished. In spreads from the lowest stringency, 50% formamide (t_m, −25°C) all heteroduplexes observed showed complete homology over the entire genome (Fig. 2*A*). In spreads from 60% formamide (t_m, −18°C), 46% of the randomly selected heteroduplexes showed a substitution (α) of 0.19 kb, which mapped 2.7 kb from the 3′ terminus (Fig. 2*B*). The same substitution (α) was also found in 90% of the molecules in spreads from 70% formamide (t_m, −11°C). However, at this increased stringency, two new substitutions (β and γ) became apparent in 30% of the molecules. These substitutions mapped 0.4 kb to the 3′ side and 0.5 kb to the 5′ side of the α substitution, respectively (Fig. 2*C*). In spreads from 80% formamide (t_m, −4°C), α, β, and γ substitutions were present in the same location as seen in spreads from 70% formamide, with the α substitution again being the most common. In addition, at this highest stringency level two additional substitutions, δ, located 0.97 kb from the 3′ terminus and ε, mapped 2 kb from the 5′ terminus (Fig. 2*D*), became evident in some molecules. All substitutions were of similar size, ranging from 0.12 kb to 0.25 kb. Additional substitutions were also present at the 5′ and 3′ ends of the inserts in 80% spreads. However, this melting out of the duplexed molecules at their termini did not occur when only the viral inserts were used for heteroduplexing, indicating that they resulted from mechanical stress or

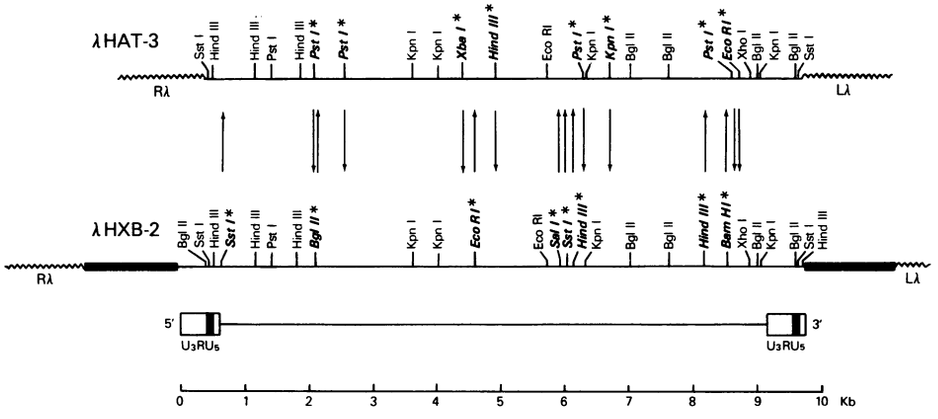

FIG. 1. Comparison of the restriction enzyme maps of two molecularly cloned HTLV-III viruses. λHAT-3, representing a full-length HTLV-III genome derived from a Haitian man with AIDS, lacks the 3′ U5 and 5′ U3 portion of the viral LTR as a result of the cloning strategy. λHXB-2, derived from the H9/HTLV-III cell line, contains a full-length integrated HTLV-III provirus with two complete LTRs (thin lines) as well as flanking cellular sequences (heavy lines) (8). Differences in the restriction enzyme maps of both clones are indicated by arrows pointing to missing restriction enzyme sites and by boldface letters and asterisks.

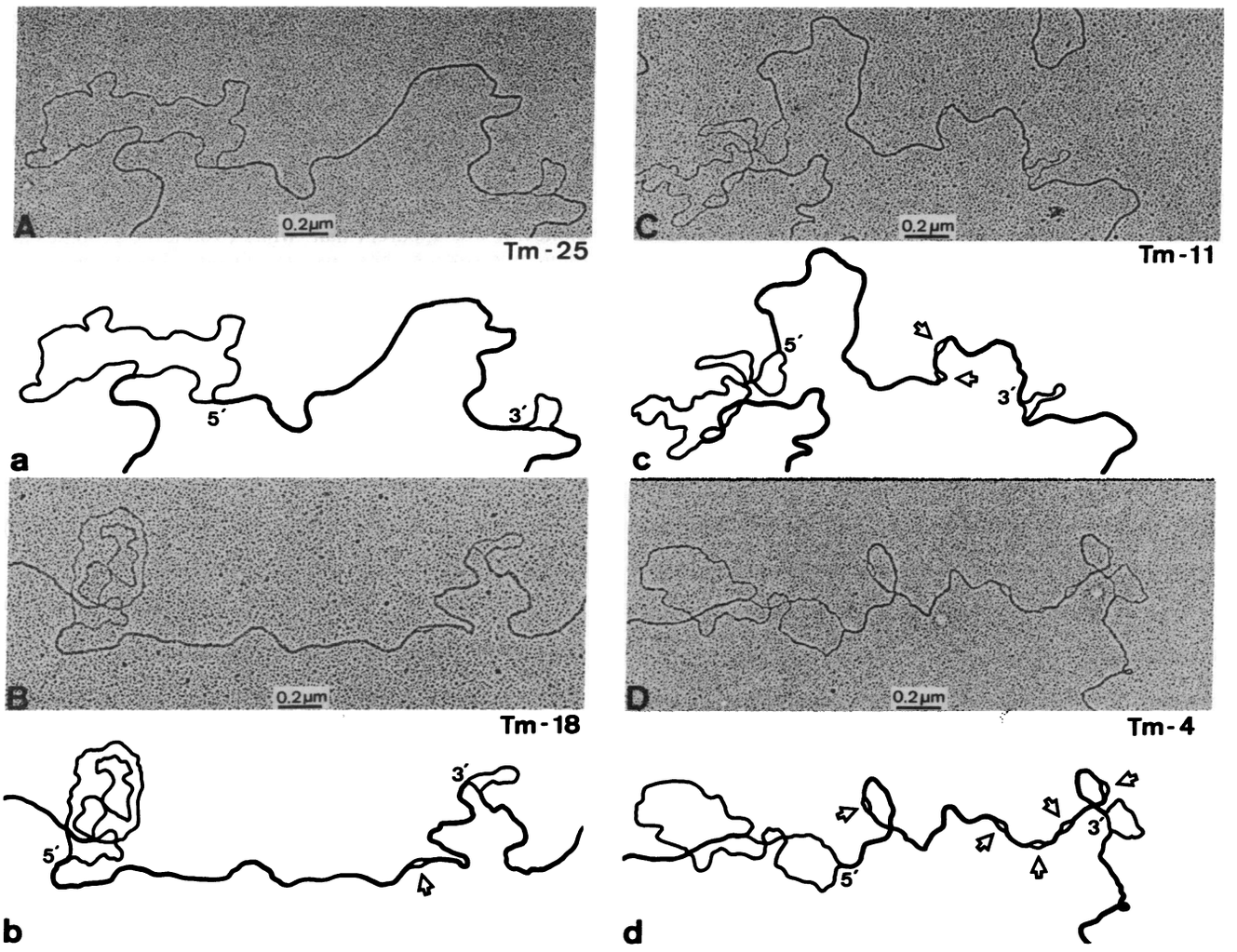

FIG. 2. Heteroduplex analysis of two HTLV-III viruses (λHAT-3 and λHXB-2) at varied stringencies. Heteroduplex analysis was performed with cloned inserts still in their bacteriophage vectors as described (16, 17). Actual heteroduplexes are shown in *A–D* and interpretive drawings are shown in *a–d*, respectively. The 5′ and 3′ ends of the viral inserts are indicated. (*A*) 50% formamide (t_m, −25°C); (*B*) 60% formamide (t_m, −18°C); (*C*) 70% formamide (t_m, −11°C); and (*D*) 80% formamide (t_m, −4°C). Open arrows (*b–d*) indicate substitutions as they appear with increasing stringencies.

shearing caused by the large nonhomologous flanking sequences.

A scheme summarizing the heteroduplex analyses is shown in Fig. 3. This figure also illustrates the location of the substitutions with respect to the different open reading frames in the genome of HTLV-III as determined by nucleotide sequence analysis (19). In contrast to the observed restriction enzyme site changes that are scattered variously over the length of nearly the entire genome, these heteroduplex data indicate that most of the genomic changes cluster in the region of the viral genome encoding the envelope glycoprotein.

Analysis of the H4/HTLV-III$_{RF}$ Cell Line. Having characterized the HTLV-III$_{RF}$ proviral clone λHAT-3 in detail, we asked the following questions: (*i*) Is there only one predominant viral form in the H4/HTLV-III$_{RF}$ cell line and is λHAT-3 representative of it? (*ii*) Does the restriction pattern of the viral genome change detectably during *in vitro* propagation of the virus? (*iii*) What is the nature of viral DNA integration in this infected cell line?

In Fig. 4, a Southern blot analysis of DNA from H4/HTLV-III$_{RF}$ cell line harvested on separate occasions 3 months apart is depicted. As shown, the restriction map deduced from the λHAT-3 clone corresponds exactly to that of the viral DNA in H4/HTLV-III$_{RF}$ cells. There was no evidence from this analysis for more than one predominant viral form in this cell line, because all restriction fragments could be accounted for by a single genomic equivalent represented by λHAT-3. Moreover, two other recombinant clones isolated from the same library as λHAT-3 were indistinguishable from this clone in their restriction enzyme map (data not shown). In addition, this restriction pattern of the HTLV-III$_{RF}$ virus did not change detectably during 3 months of *in vitro* culture.

The nature of viral DNA integration in the H4/HTLV-III$_{RF}$ cell line was evaluated by using restriction enzymes known to cleave the viral genome only once or not at all. For example, *Bam*HI, which does not cut the viral genome, generated a high molecular weight smear indicative of polyclonally integrated DNA plus a predominant band of ≈10 kb representing unintegrated linear viral DNA. *Xba* I, which cleaves the viral DNA once, generated three bands: the linearized circular form of the virus (10 kb) and the two halves of the unintegrated linear form (4.5 and 5.5 kb). The greater intensity of the 10-kb band compared to the sum of the other two smaller bands is due to the presence of more viral DNA in the circular compared to the linear form in this cell line. (This relative abundance of circular DNA is not apparent in the *Bam*HI digest because circular double-stranded DNA that is not nicked is not efficiently transferred and detected by Southern blot techniques.) Thus, HTLV-III$_{RF}$, like other

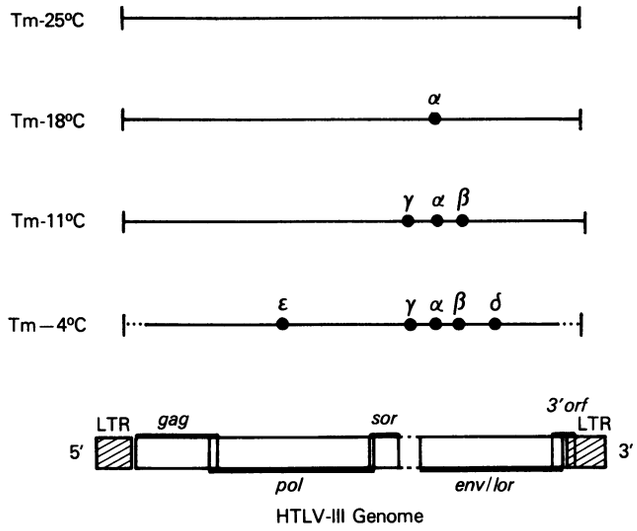

FIG. 3. Schematic diagram demonstrating genome divergence between two HTLV-III variants as determined by heteroduplex thermal melt analysis. Homologous parts of the genomes (solid lines) and substitutions (dots) are drawn to scale and are shown in relation to the coding regions of the HTLV-III genome as determined by nucleotide sequence analysis (19). At least 22 molecules were evaluated at each stringency. At t_m −25°C, no substitutions occurred; at t_m −18°C, a substitution (α) occurred in 46% of the heteroduplexes; and at t_m −11°C, α, β, and γ substitutions occurred in 90%, 30%, and 30% of the heteroduplexes, respectively. At t_m −4°C, α, β, γ, δ, and ε substitutions occurred in 50%, 23%, 37%, 20%, and 13% of the heteroduplex, respectively. Dotted lines at t_m −4°C indicate a melting out of the 3′ and 5′ ends of the viral inserts resulting from mechanical stress or shearing caused by large nonhomologous flanking sequences. *sor*, Short open reading frame; *env/lor*, envelope and *lor* gene; 3′ *orf*, 3′ open reading frame.

HTLV-III viruses (8), persists both as polyclonally integrated provirus as well as unintegrated viral DNA in the infected cell line.

DISCUSSION

We have previously discovered that genomic diversity is a characteristic feature of HTLV-III viruses (8). More recently, we have found that this heterogeneity comprises a spectrum of diversity ranging from viral isolates, which are, by Southern analysis of infected cellular DNA, nearly identical to each other, to others such as HTLV-III$_{RF}$, which are quite different. The recently reported nucleotide sequences of HTLV-III (19), lymphoadenopathy-associated virus (20), and other isolates (21) support this conclusion of a continuum of related viruses.

The goal of the present study was to characterize the extent and nature of this genomic heterogeneity by comparing the cloned viral genomes of two HTLV-III isolates, which appeared to be substantially different from each other. From hybridization and restriction enzyme analyses of these clones, it was apparent that, while overall the genomes are quite homologous, they possess substantial heterogeneity in their restriction maps and, thus, in their nucleic acid sequences. Heteroduplex analysis provided another measure of genomic heterogeneity and this technique revealed several distinct regions of divergence located primarily in the 3′ half of the molecules.

A long open reading frame, designated *env/lor*, has been identified within the part of the viral genome (19) that we found to be most divergent. This gene encodes the precursor for the major envelope glycoprotein of HTLV-III and possibly a second protein analogous to the *lor* protein of other members of the HTLV/BLV family group of viruses (19). Four of the five substitutions that we found in λHAT-3 mapped within the *env/lor* region and the adjacent noncoding region of HTLV-III. Two of these, including the α substitution, were located in the 5′ half of this gene, which encodes the exterior part of the envelope glycoprotein.

We have recently sequenced the *env/lor* region of the λHAT-3 clone; these data corroborate the heteroduplex analysis reported here indicating the presence of clustered nucleotide sequence changes, especially in the NH_2 terminus of the envelope gene of λHAT-3 (unpublished data). Moreover, comparison of the published nucleotide sequence of another isolate of the AIDS virus (21) to λHXB-2 and λHAT-3 also reveals more variability in the exterior envelope coding region than in other parts of the viral genome (22). These findings indicate that certain regions in the extracellular portion of the HTLV-III envelope gene may be quite variable in their nucleotide sequence and could represent "hot spots" of genomic changes.

The biological processes underlying the observed diversity in the HTLV-III genome are only now beginning to be understood. It is evident from this study and others (8, 9) that the observed differences in HTLV-III isolates are not the result of changes occurring in the viral genomes during *in vitro* cultivation, at least not for periods of several months. On the contrary, characterization of HTLV-III sequences in fresh, uncultured tissue specimens (8, 9), in different host cell types (8), and in permanent cell lines over time (Fig. 4;

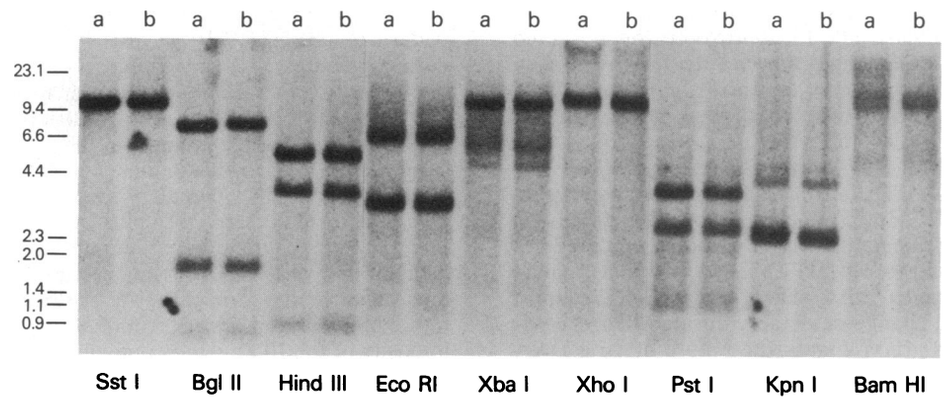

FIG. 4. Southern blot analysis of cell line H4/HTLV-III$_{RF}$. DNA from this cell line was extracted on two different occasions, before (lanes a) and after (lanes b) 3 months of continuous propagation *in vitro*. Ten micrograms of DNA was digested with the restriction enzymes as indicated, electrophoresed on a 0.8% agarose gel, and transferred to nitrocellulose filters. Filters were hybridized to HTLV-III-specific probe (λBH-10i), washed, and autoradiographed as described.

unpublished data) indicate instead that this diversity preexists in the population of individuals infected with the virus. In this light, it is striking that 16 different HTLV-III isolates that we have studied in detail were all distinguishable from each other by restriction enzyme analysis. As would be expected, differences among these isolates ranged from a single restriction site polymorphism to others in which the majority of sites differed (see, for example, Fig. 1). A similar spectrum of genomic diversity ranging from slight to substantial is evident from the comparison of the published nucleotide sequences of HTLV-III, lymphoadenopathy-associated virus, and other isolates (22).

Although it remains to be proven, it is possible that immunologic pressures *in vivo* could select for changes in the HTLV-III genome, especially in the envelope, as occurs with other viruses related to HTLV-III—namely, visna (12) and EIAV (13). Both of these nontransforming retroviruses undergo changes in their envelope glycoproteins during the course of the disease and it has been speculated that these antigenic changes allow these viruses to escape the host's immune surveillance mechanisms. Experimentally, it has been shown that antibodies directed against these envelope glycoproteins at an early time point in the disease do not effectively neutralize subsequent viral isolates from the same animal. It is not unreasonable to expect that HTLV-III could have this property in common with visna, since it is already known that they share a number of other similarities, including cytopathicity (2, 23), tropism both for lymphoid tissues and brain (9, 24), persistence as unintegrated DNA (8, 25), substantial nucleotide sequence homology (26), and nearly identical morphology (26).

The identification of antigenically important determinants in the viral envelope glycoprotein will be necessary to evaluate whether the changes in the nucleotide sequence of the envelope genes of different HTLV-III variants are likely to affect their antigenic properties. If such is the case, attempts to develop effective vaccines may be complicated. Further studies will also need to address the question of whether the various clinical sequelae of HTLV-III infection are the result of differences in the virus, the patients' response to it, or other as yet undefined factors.

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