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Genomic Diversity of Human T-Lymphotropic Virus Type III (HTLV-III)

Abstract. *The DNA genomes of human T-lymphotropic virus type III (HTLV-III) isolated from 18 individuals with AIDS or who were at risk for AIDS were evaluated for evidence of variation. Although all of the 18 viral DNA's hybridized throughout their entire genomes to a full-length cloned probe of the original HTLV-III isolate, each of the 18 isolates showed a different restriction enzyme pattern. The number of restriction site differences between isolates ranged from only 1 site in 23 to at least 16 sites in 31. No particular viral genotype was associated with a particular disease state and 2 of the 18 patients had evidence of concurrent infection by more than one viral genotype. Propagation of three different viral isolates in vitro for up to 9 months did not lead to detectable changes in their restriction patterns. These findings indicate that different isolates of HTLV-III comprise a spectrum of highly related but distinguishable viruses and have important implications regarding the pathogenicity of HTLV-III and attempts to develop effective diagnostic, therapeutic, and preventive measures for this virus.*

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Human T-lymphotropic virus type-III (HTLV-III), also referred to as lymphadenopathy-associated virus (LAV) and AIDS-related virus (ARV), is the etiologic agent of the acquired immune deficiency syndrome (AIDS) and related immunological disorders (*1, 2*). Diversity, or heterogeneity, in the genomes of different isolates of HTLV-III was first noted by our laboratory in the course of our initial characterization of the virus (*3*) and subsequently by the comparison of the nucleotide sequences of four different AIDS virus isolates (*4–6*). The latter studies also suggested that the extent of divergence between isolates could be minor (1 to 2 percent among BH-10, BH-5/8, and LAV-1A) or more extensive (7 percent between BH-10 and ARV-2) but, because so few isolates had been examined, the extent of variation among AIDS viruses was unclear. Thus, to gain more information on the extent and nature of genomic variation in the HTLV-III group of viruses, we analyzed by Southern hybridization the viral DNA from 17 consecutively studied patients with either AIDS or AIDS-related complex (ARC) and one subject at risk for AIDS but with no apparent clinical disease.

Virus from nine patients with AIDS or ARC and one healthy subject was transmitted to established neoplastic T-cell lines (H4, H9, or JM) or to normal peripheral blood lymphocytes (PBL) (*1, 7*). Primary tissues (lymph node or brain) from the eight AIDS or ARC patients were examined directly (Table 1). DNA from cultured cells or primary tissues was digested with a panel of restriction enzymes and analyzed by Southern hybridization to 9-kb cloned HTLV-III probe (BH-10) (*3*). DNA bands constituting an entire genomic complement were detected by this probe for each of the viruses under high stringency hybridization and wash conditions indicating that a high degree of homology exists among all of the isolates (Fig. 1). Despite this overall conservation in sequence homology, a striking degree of restriction enzyme site polymorphism was found among the isolates. For example, Sst I generated three predominant genotypic patterns: a 9.0-kb fragment (isolates RH, RF, and TM), 5.5- and 3.5-kb fragments (isolates HW, JS, and SC), and 5.5-, 1.8-, and 1.7-kb fragments (isolates MN and SL). The cell line H9/HTLV-III_B (Fig. 1, lane IIIB), which was derived from the HT line that was infected by virus from several different patients (*8*), contains both the 9.0-kb and (5.5 + 3.5)-kb Sst I genotypes. Although all 18 isolates that we examined contained one of the three Sst I patterns described above, digestion of these isolates with three other enzymes (Bgl II, Hind III, and Pst I) yielded overall restriction patterns different for each of the viruses. It should be noted that the interpretation of restriction site differences between HTLV-III isolates is complicated by the simulta-

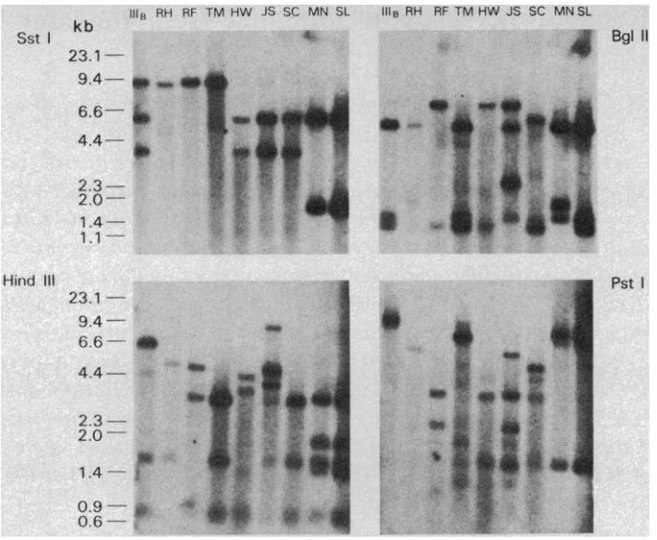

Fig. 1. Heterogeneity of HTLV-III genomes. High-molecular-weight DNA was extracted from virus infected cells (see Table 1) as (*3*). The DNA (5 to 15 μg) was digested with the indicated restriction enzymes according to the manufacturers' recommendations. The DNA was then subjected to electrophoresis through 0.6 cm thick 0.8 percent agarose slab gels and blotted in 10× SSC (standard saline citrate) onto 0.1 μm nitrocellulose filters (Schleicher and Schuell). Hybridizations were performed at 37°C for 18 hours in 2.4× SSC, 40 percent formamide, 10 percent dextran sulfate, 1 mg each, per milliliter, of bovine serum albumin, polyvinylpyrollidone, and Ficoll, and 20 μg of transfer RNA per milliliter. Filters were washed for 2 hours at 65°C in 1× SSC. The probe used was the 9 kb Sst I-Sst I insert from BH-10 (*3*) which contains the entire HTLV-III genome less 180 basepairs of LTR sequences, 3×10^6 dpm/ml (specific activity approximately 2×10^8 dpm/g). Blots were exposed to Kodak XAR-5 film for 2 days.

neous presence of unintegrated linear and circular DNA as well as integrated proviral DNA (*3*); we avoided this problem by using enzymes (Sst I, Bgl II, and Hind III) whose site in the viral LTR is well conserved (*9*) and by identifying internal restriction fragments. In sum, each of the 18 viral genomes could be distinguished from the others by at least one, and usually many, restriction site differences and no genotypic pattern was evident that correlated with a particular disease state such as AIDS, ARC, or asymptomatic carrier.

The results in Fig. 1 suggested that different isolates of HTLV-III represent a spectrum of related viruses. To evaluate further this spectrum of diversity, we compared two closely related isolates, MN and SL, with the more distantly related isolates in the H9/HTLV-III_B producer cell line (Fig. 2). This blot-hybridization shows that MN and SL differ from each other in only 1 of 23 restriction sites (see Bgl II digestion), yet they differ from HTLV-III_B in more than half of the sites tested.

To determine how the differences in restriction patterns of the isolates shown in Figs. 1 and 2 correlate with differences in nucleotide sequence, we have, in other studies, determined the nucleotide sequence of a full-length clone of the isolate RF. This RF clone (HAT-3) differs from the BH-10 clone of the H9/HTLV-III_B line in 14 of 31 restriction sites and in 8.5 percent of base pairs over the entire length of the viral genome (*10*). Similarly, ARV-2 differs from BH-10 in approximately half of its restriction sites and in 6.5 percent of its base pairs, while LAV-1A is very similar to BH-10 in restriction pattern and differs in 1.9 percent of its base pairs. Thus, as expected, the extent of restriction site differences between AIDS virus isolates correlates with their degree of nucleotide sequence divergence, and comparison of restriction patterns provides an important estimate of their overall relatedness. It should be noted, however, that while genomic restriction mapping is useful in evaluating overall similarities between viral isolates, it does not adequately reflect the clustering of nucleotide sequence changes that have been identified in certain regions of the viral genome, especially the envelope (*6, 11*).

The finding of a spectrum of diversity among different HTLV-III isolates probably reflects progressive changes in the viral genome over time. Isolates MN and SL were both isolated at approximately the same time (1984) from patients in the New York–New Jersey area and they are quite similar in restriction pattern. Similarly, HTLV-III_B and LAV were both isolated from patients with contacts in the New York–New Jersey area in 1983

Table 1. Patients evaluated for HTLV-III DNA.

Isolate	DNA source	Patient diagnosis	Risk factor	Geographic location	Year of isolation
H9/HTLV-III_B	PBL/H9*	AIDS and ARC	Homosexuals	New York and New Jersey	1983
RF	PBL/H4*	AIDS	Haitian	Haiti and Philadelphia	1983
RH	PBL†	Healthy	Homosexual	Washington, D.C.	1984
TM	PBL/N-PBL*	AIDS	Homosexual	Boston	1984
HW	PBL/N-PBL*	AIDS	Heterosexual promiscuity	Europe and Africa	1984
MN	PBL/JM*	ARC	Child whose mother used intravenous drugs	New York and New Jersey	1984
SL	PBL/N-PBL*	ARC	Homosexual	New York and New Jersey	1984
SC	PBL/N-PBL*	ARC	Homosexual	California	1984
JS	PBL/N-PBL*	ARC	Homosexual	California	1984
MJ	PBL/H9*	AIDS	Child of Haitian mother	Haiti and Miami	1985
RJ	PBL/N-PBL*	AIDS	Homosexual	Boston	1985
FO	Lymph node‡	AIDS	Homosexual	New York and New Jersey	1984
KC	Lymph node‡	ARC	Intravenous drug user	New York and New Jersey	1984
JR 1	Brain‡	AIDS	Child whose mother used intravenous drugs	New York and New Jersey	1984
JR 2	Brain‡	AIDS	Homosexual	New York and New Jersey	1984
LS	Brain‡	AIDS	Child whose mother had ARC	New York and New Jersey	1984
JT	Brain‡	AIDS	Homosexual	New York and New Jersey	1984
RC	Brain‡	AIDS	Homosexual	New York and New Jersey	1984
RR	Brain‡	AIDS	Homosexual	New York and New Jersey	1984

*Virus transmitted from patients' PBL into H9, H4, or JM cell lines, or into normal allogeneic PBL. †Virus grown directly in patient's own PBL without transmission. ‡Viral DNA detected and analyzed in uncultured tissue.

and their nucleotide sequences are also quite similar (*5*). Conversely, isolates ARV and RF were obtained from patients from California and Haiti, respectively, and they are considerably more divergent (*5, 6, 10*).

Characterization of viral DNA in cultured and primary cells has allowed us to determine whether more than one viral genotype could be present in a given patient at any one point in time. Of the 18 patients evaluated, two (JS and RJ) had evidence of infection with more than one viral genotype. This is shown for patient JS in Fig. 1, where enzymes that cut in the viral LTR (Bgl II and Hind III) generated bands adding up to more than one 10-kb genomic complement. This finding was confirmed by using other enzymes including double digestions with Sst I, which also cuts within the LTR. Although more than one viral genotype was not found in the other 16 patients, it is possible that they were present in the uncultured tissues but not in amounts sufficient for detection by blot-hybridization [fresh tissue specimens generally contain only very small amounts of viral DNA; see (*3, 11*)]. At the same time, propagation of virus in vitro could provide a selective growth advantage for one form of the virus and that other forms present in vivo could go unrecognized. However, we cultured virus from patient JS by cocultivating his peripheral blood mononuclear cells with phytohemagglutinin (PHA)-stimulated allogeneic lymphocytes (*7*) on four separate occasions over a period of 1 year, and on each occasion two similar viral forms were identified by restriction analysis. This suggests that the virus which grows in culture is representative of predominant forms in vivo but still does not rule out the possibility that some viral forms may not grow readily in vitro.

We have also analyzed the effects of long-term propagation of the virus in vitro on its genomic restriction pattern. Figure 3 demonstrates that the major internal restriction fragments for Sst I, Pvu II, and Hind III (as well as for Bgl II, Eco RI, and Pst I, not shown) were unchanged for H9/HTLV-III$_B$ after 9 months in culture. This cell line has served as the principal source of reagents for characterizing the HTLV-III virus and for detecting antibodies in patient sera directed specifically against it (*1*). In studies of two other viral isolates (RF and MOV), we have observed similar genomic stability after cultivation of infected cell lines in vitro for 3 to 9 months (*6*). In other studies (*3*), we have shown that infection of allogeneic lymphocytes and two different T-cell lines by the same virus isolate does not lead to changes in its genomic restriction pattern. These results thus indicate that the genomic differences described here for the ten cultured isolates are not due to changes introduced by cultivation of these viruses in vitro.

Although the HTLV-III$_B$ restriction patterns did not change noticeably in vitro, what did change in this virus-producing permanent cell line was the number of different viral genotypes present and their state of integration. The disappearance of a subset of Hind III bands (Fig. 3) corresponds to the loss of a specific viral genotype. The appearance of two discrete high-molecular-weight bands in Xba I–digested DNA of late-passage cells, and the disappearance of bands migrating as linear, superhelical, and nicked-circular DNA in the same DNA, reflect a change from predominantly unintegrated viral DNA to predominantly integrated viral DNA. That two discrete high-molecular-weight bands are generated by Xba I, an enzyme that does not cut within the HTLV-III$_B$ genomes, suggests that the cells containing these sequences are proliferating in a clonal fashion. It should be noted that these studies in vitro, involving propagation of a virally infected cell line such as H9/HTLV-III$_B$, do not address the question of the rate and extent of genomic divergence of AIDS viruses since the viral progeny in these lines probably do not progress through a complete life cycle including reverse transcription. It is in this step catalyzed by the RNA-dependent DNA polymerase that changes in the viral genome would be expected to occur most frequently. Studies of viral changes occurring in vivo and after repeated reinfection in vitro will be necessary to examine these issues.

Our results indicate that genomic diversity is a characteristic and prominent feature of the AIDS viruses, that the variation between isolates ranges from slight to rather extensive, and that most patients appear to be infected with only one or two predominant forms of the virus at any one time. Other studies indicate that it is the envelope gene that varies most among different viral isolates (*5, 6, 10*). These findings, along with the demonstration that HTLV-III and visna virus are related in their nucleotide sequence, morphology, cytopathic effects, and propensity to infect brain cells (*11, 12*), point to genomic diversity of HTLV-III as a property likely to be fundamentally important to its biologic activity and

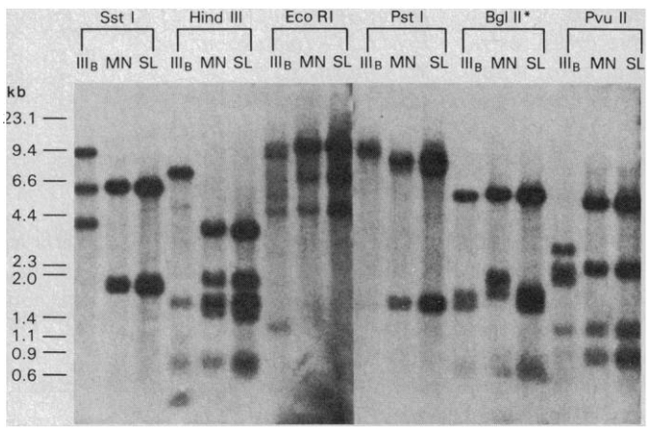

Fig. 2. Comparison of two closely related HTLV-III isolates. DNA from MN and SL (Table 1) was analyzed with six enzymes, with HTLV-III$_B$ used for comparison. Methods were as described in the legend to Fig. 1. As shown, MN and SL differ only in the size of one Bgl II fragment.

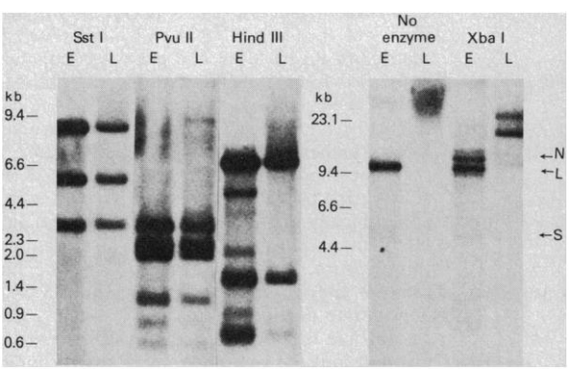

Fig. 3. Comparison of viral DNA in early and late passage H9/HTLV-III$_B$ cells. These cells were harvested on two occasions: 2 months after infection (early, E) and after continuous propagation in culture for an additional 9 months (late, L). Arrows indicate positions of different forms of unintegrated DNA; S. supercoiled; L, linear; N, nicked circular; Sst I, Pvu II, and Hind III cut in the viral LTR and internally. Xba I does not cut within the virus genome. Hybridization conditions were those described in Fig. 1.

pathogenicity. Both visna virus and equine infectious anemia virus (EIAV) are believed to avoid elimination by host immune defense mechanisms by undergoing progressive changes in their envelope proteins during the course of infection (*13*). It is not yet known whether a similar type of immunologic escape occurs with HTLV-III, but the existence of neutralizing antibodies in infected individuals (*14*) and the relatedness of HTLV-III to visna virus and EIAV make this a serious consideration.

Another question is why some individuals infected with HTLV-III develop AIDS, others ARC, and still others no disease at all (*15*). Viral genotype, host immune response, and other factors are likely to be determinants of clinical outcome. From our analysis of the 18 HTLV-III isolates, we were unable to identify any disease-specific restriction pattern. In fact, each of the 18 viral isolates was different from the next and none were identical to HTLV-III$_B$, LAV, or ARV. Further nucleotide sequence analyses and deletion mutant studies (*16*) will be needed to identify regions of the HTLV-III genome responsible for the virus's biologic effects and for correlating viral genotype with clinical outcome.

Finally, it is of interest that in most patients only one predominant form of the AIDS virus was identified. If this is not the result of selective pressures introduced by cultivation in vitro, it suggests that some sort of interference process may occur, since these patients, especially the hemophiliacs, homosexuals, and intravenous drug addicts, are subject to repeated exposures to genotypically diverse viruses. The data would also then suggest that if genotypic variation is being generated in vivo as occurs with visna virus and EIAV (*13*), then either it is a rather slow process or the preexisting viral strains are largely eliminated during the disease course. Analyses of viral isolates from the same patient at different time points and from donor-recipient pairs of individuals would help to address these questions.

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Gram-Positive Bacteria: Possible Photosynthetic Ancestry

Abstract. *A 16S ribosomal RNA gene has been sequenced from* Heliobacterium chlorum, *the recently discovered photosynthetic bacterium that contains a novel form of chlorophyll. Comparisons with other 16S ribosomal RNA sequences show that the organism belongs to the Gram-positive bacteria (one of ten eubacterial "phyla")—more precisely to the so-called low G + C (G, guanine; C, cytosine) subdivision thereof. This brings to five the number of such phyla that contain photosynthetic species, the other four being the purple bacteria and relatives, the green sulfur bacteria, the green nonsulfur bacteria, and the cyanobacteria. The finding suggests that Gram-positive bacteria may be of photosynthetic ancestry, and it strengthens the case for a common photosynthetic ancestry for all eubacteria.*

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The role of photosynthetic bacteria in evolution, although poorly understood, is clearly central. Cyanobacteria and their endosymbiotic descendants, the chloroplasts, are responsible for the oxygen atmosphere surrounding this planet, for the evolution of plant life, and so indirectly, for animal life as well. In some earlier era devoid of oxygen, other photosynthetic bacteria may have had a comparable impact on the evolutionary course and the physical state of the planet. The role of photosynthetic bacteria in the origin of cellular life is not known. Although we are only now beginning to understand the natural relationships among bacteria, the biologist in the past has proposed theories regarding them, particularly theories regarding the relationship of photosynthetic bacteria to bacterial evolution. Oparin long ago argued (*1*) that the primitive oceans were a "soup" of energy-rich biochemicals, and consequently, the first organisms arising therein were nonphotosynthetic, fermentative heterotrophs—there being no need initially for biological systems to generate their own energy-rich compounds.

Fig. 1 (Opposite page). Sequence of *Heliobacterium chlorum* 16*S* rRNA (hc) aligned with three other eubacterial 16*S* rRNA's—(bs) *Bacillus subtilis* (*14*), (an) *Anacystis nidulans* (*15*), and (ec) *Escherichia coli* (*16*). A gene for rRNA was selected by plaque hybridization procedures from a library of *H. chlorum* DNA in lambda phage produced by shotgun cloning of a Sau 3A partial restriction digest (*17*). The gene was subcloned into the phage M13 system as two Eco RI restriction fragments (*18*). [An Eco RI site is located in the sequence in the vicinity of position 680.] The sequence was determined by the dideoxy method, using both the normal primer for the M13 system and a variety of primers produced (for the most part by the University of Illinois DNA Synthesis Facility) specifically for use in sequencing eubacterial 16*S* ribosomal RNA's (*19*). The oligonucleotide catalog for the 16*S* rRNA itself was determined as described (*20*). The starting material for these studies was a frozen cell pellet of *H. chlorum* cells harvested anaerobically in early log phase (*13*). Sequences were aligned by the method indicated in (*21*). Numbering accords with that for the *E. coli* sequence (*16*), each line being 100 positions long by this measure. Our *H. chlorum* sequence begins at *E. coli* position 15 because the original clone (Sau 3A site) began at this point. The 16*S* rRNA oligonucleotides are shown by overlining the appropriate portions of the corresponding gene sequence. Discrepancies between the rRNA catalog and the sequence (discussed in text) are indicated by lowercase symbols for the bases inserted into the overlined segments (to indicate the catalog sequences). An oligonucleotide predicted by the sequence but not found in the catalog is indicated by xxx... in the overlining.