Genetic Variation in HTLV-III/LAV Over Time in Patients with AIDS or at Risk for AIDS

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In a study of genetic variation in the AIDS virus, HTLV-III/LAV, sequential virus isolates from persistently infected individuals were examined by Southern blot genomic analysis, molecular cloning, and nucleotide sequencing. Four to six virus isolates were obtained from each of three individuals over a 1-year or 2-year period. Changes were detected throughout the viral genomes and consisted of isolated and clustered nucleotide point mutations as well as short deletions or insertions. Results from genomic restriction mapping and nucleotide sequence comparisons indicated that viruses isolated sequentially had evolved in parallel from a common progenitor virus. The rate of evolution of HTLV-III/LAV was estimated to be at least 10^{-3} nucleotide substitutions per site per year for the *env* gene and 10^{-4} for the *gag* gene, values a millionfold greater than for most DNA genomes. Despite this relatively rapid rate of sequence divergence, virus isolates from any one patient were all much more related to each other than to viruses from other individuals. In view of the substantial heterogeneity among most independent HTLV-III/LAV isolates, the repeated isolation from a given individual of only highly related viruses raises the possibility that some type of interference mechanism may prevent simultaneous infection by more than one major genotypic form of the virus.

GENOMIC HETEROGENEITY HAS been firmly established as a prominent characteristic of the AIDS virus, HTLV-III/LAV (*1–9*). Furthermore, the gene encoding the extracellular envelope glycoprotein of this virus is relatively hypervariable in comparison with the remainder of the viral genome (*3–5, 9*). These findings, along with analogous data for equine infectious anemia virus and visna virus, two lentiviruses in which variation in the envelope region correlates with demonstrable changes in antigenicity (*10*), suggest that genetic variation in HTLV-III/LAV might affect viral pathogenesis, perhaps allowing the virus to escape host immune defenses. Variation within the *env* gene, as well as other genes, might also give rise to viruses with altered virulence, tissue tropism, or drug sensitivity.

We recently compared the nucleotide and deduced amino acid sequences of the *env* genes of five independent isolates of HTLV-III/LAV (*9*). The results indicated that (i) within the extracellular *env* gene there are localized regions of hypervariability interspersed with regions of strong conservation; (ii) genetic changes among different viruses result largely from duplications, insertions, or deletions of short stretches of nucleotides as well as from an accumulation of nucleotide point mutations; and (iii) hypervariable *env* regions, and some constant regions, have properties predictive of antigenicity based on analysis of predicted secondary protein structure, hydrophilicity, and glycosylation pattern (*9*).

To define further the extent, rate, and nature of genetic variation of HTLV-III/LAV in vivo we have studied sequential virus isolates from three persistently infected patients with AIDS or risk factors for AIDS. The isolates were obtained on four to six occasions over a 1- or 2-year period. The first patient (coded WMJ) was a Haitian child with AIDS who was born in Miami, Florida, in 1982 and infected perinatally by her HTLV-III/LAV–positive mother. The second patient (coded WMF) was a 34-year-old Haitian woman with AIDS-related complex (ARC) who was also from Miami and whose only known risk factor for HTLV-III/LAV infection was heterosexual exposure. The third patient (coded RJS) was a 31-year-old American homosexual man from California who, despite chronic HTLV-III/LAV infection, remained clinically well for 5 years but eventually developed evidence of T-cell dysfunction [Walter Reed stage V (*11*)]. He reported having sexual encounters with at least 1000 different men between 1980 and 1985. These three subjects thus represented a broad clinical spectrum of HTLV-III/LAV–related disease (AIDS, ARC, and clinically normal virus carrier), different modes of virus transmission (perinatal, heterosexual, and homosexual), and widely different numbers of exposures to the AIDS virus (presumably one for WMJ, limited encounters for WMF, and very large numbers for RJS). All virus isolations were made from the patients' peripheral blood mononuclear cells (PBMC) that were cocultivated with normal PBMC and, in most instances, transmitted to immortalized T-cell lines.

Figure 1 shows that, for patient WMJ, the Sst I, Eco RI, and Pst I restriction enzyme patterns for each of the five isolates were identical and that the Bgl II and Pvu II patterns were almost identical. With Hind III, the isolates were clearly distinct but still highly related; for example, the loss of a single Hind III site in WMJ-1 led to the Hind III pattern observed in WMJ-2 (see Fig. 2). In contrast, the enzyme cleavage patterns of the five WMJ isolates, which as a group were quite similar to each other, differed markedly from viruses of patients WMF and RJS as well as from all other patients' viruses tested (*1, 2, 8*).

The observation that serial virus isolates from a single patient (WMJ) were more highly related to each other than to viruses from other patients was also true for isolates from WMF and RJS. For example, the four isolates from WMF were identical in Sst I and Eco RI patterns and nearly identical when analyzed with Pvu II and Pst I. Moreover, the differences with Hind III could be explained simply by either the loss or gain of single restriction sites. For RJS, all six isolates were identical with Sst I and Eco RI and nearly identical with Bgl II, Pvu II, and Pst I.

For most virus isolates shown in Fig. 1, the sizes of the restriction fragments generated by any given enzyme added up to 9 kilobases, the approximate genomic size of HTLV-III/LAV (*1, 12, 13*). However, for certain virus isolates from patients WMF and RJS, some of the restriction enzymes generated fragments that added up to more than one genomic equivalent (for example, Bgl II, Pvu II, and Pst I digestions of RJS-4; the Hind III patterns of RJS-2, RJS-5, and RJS-6; the Bgl II pattern of WMF-1; and the Pst I pattern of WMF-3). These particular isolates were therefore composed of a mixture of more than one highly related, yet genotypically distinct, virus populations. That the restriction digestions did, in fact, reflect accurately the presence of more than one predominant viral genotype and were not reflecting incomplete digestions was shown by the following: (i) several different enzymes generated bands whose additive size in each case was greater than 9 kb; (ii)

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Fig. 1. Southern blot hybridization analysis of sequential HTLV-III/LAV isolates. Virus was isolated from patients' peripheral blood mononuclear cells and propagated in tissue culture. High molecular weight DNA was prepared and Southern transfers performed as described (*26, 27*). WMJ isolates 1, 2, 3, 4, and 5 were obtained from blood samples at intervals of 3, 4, 1, and 4 months, respectively. The last of these samples was obtained 1 month before the patient's death at 3 years of age. WMF isolates 1, 2, 3, and 4 were obtained at intervals of 6, 10, and 6 months, respectively, and RJS isolates 1, 2, 3, 4, 5, and 6 at intervals of 3, 4, 5, 7, and 4 months, respectively. Neither WMF nor RJS had developed AIDS. For each set of isolates from each patient, a panel of six restriction enzymes was used to map the viral genomes. The number of each virus isolate is indicated above the panels and the restriction enzymes are shown below each panel. Of note, the restriction enzymes Hind III, Bgl II, Eco RI, Pvu II, and Pst I were used in combination with Sst I, an enzyme known to cut the viral long terminal repeat. Thus, all viral bands generated represent internal viral fragments. Viral DNA's of patient RJS were also digested with the five restriction enzymes alone (without the addition of Sst I) because of the extra Sst I site present in the 3′ half of all RJS isolates that was not present in WMJ or WMF isolates.

repeated digestions of these same DNA's with excess enzyme gave identical results; and (iii) molecular clones of viral DNA obtained from several of these DNA's corresponded to the different viral patterns apparent on the blot hybridizations (*14*).

Although more than one predominant viral genotype was present in some virus isolates, all of the genotypically distinct viral forms in any one patient were still very similar to each other as opposed to independent virus isolates from different individuals that were distinct. For example, although two viral forms could be distinguished by the Bgl II digestion of WMF-1, these two viral forms were identical in their Sst I, Hind III, Eco RI, Pvu II, and Pst I patterns. Similarly, the two viral forms distinguishable by Hind III in RJS-2, RJS-5, and RJS-6 were identical in Sst I, Eco RI, Pvu II, and Pst I patterns. By way of contrast, the WMF viruses and the RJS viruses, as groups, differed from each other dramatically in Sst I, Hind III, Bgl II, Pvu II, and Pst I patterns. Thus, within any given individual, even one presumably exposed to numerous different AIDS viruses [see also (*2*)], only one or a very limited number of virus strains, as defined by restriction enzyme analysis, could be detected by virus isolation and DNA blot hybridization. It is possible, of course, that these patients were actually infected with many different genotypic viral forms but, because of their unequal growth characteristics, most were not evident after cultivation in vitro. We cannot rule out this possibility, but it is of note that recent work involving Southern blot hybridization analyses of un-

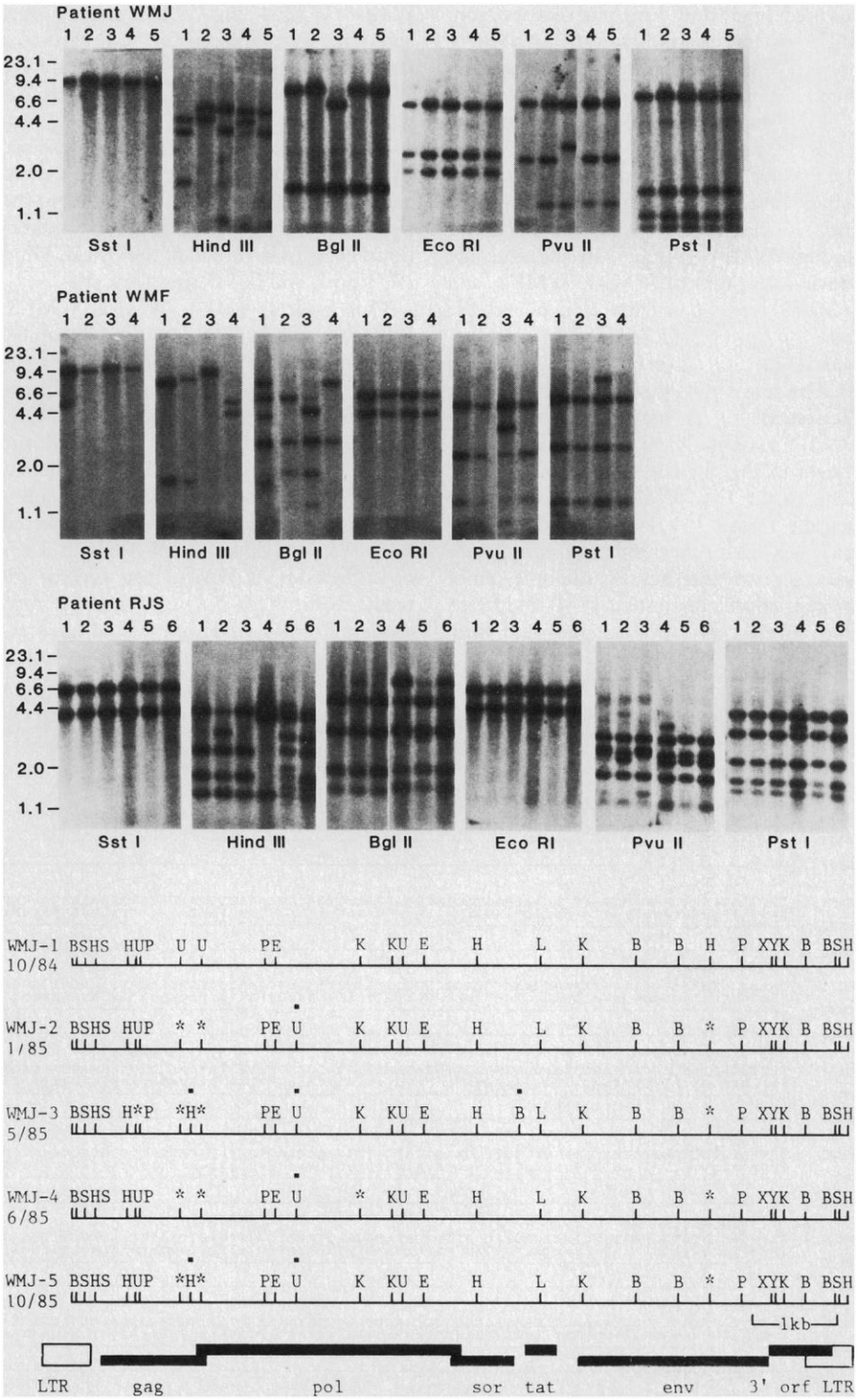
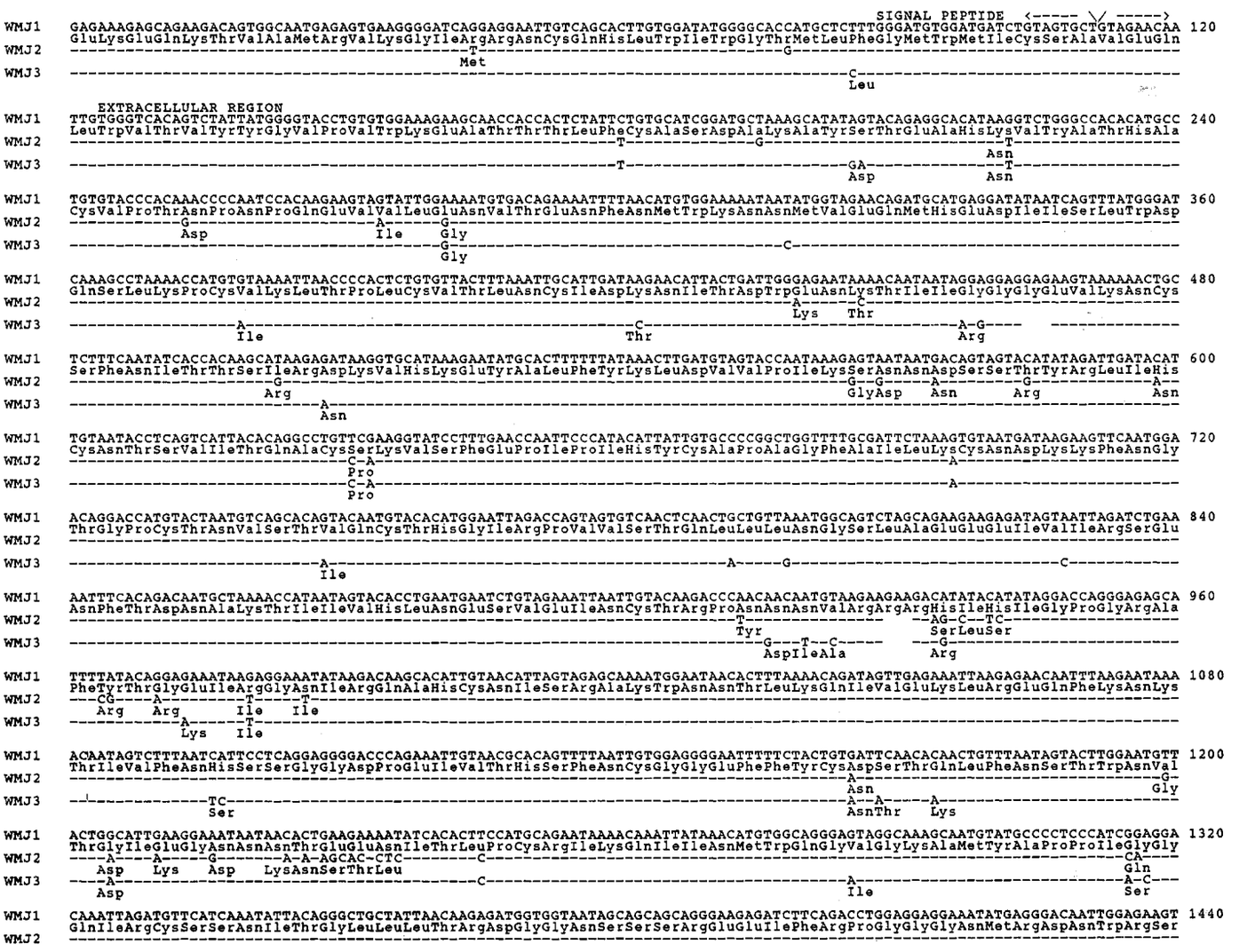


Fig. 2. Restriction enzyme cleavage patterns of five sequential HTLV-III/LAV isolates from patient WMJ. Virus-infected DNA from WMJ-1 through WMJ-5 (see Fig. 1) was digested with the indicated restriction enzymes, Southern-blotted, and hybridized to subgenomic probes of HTLV-III/LAV (clones λBH5 and λBH8) (*1*) as described. For isolates WMJ-1, WMJ-2, and WMJ-3, the proviral DNA was molecularly cloned into λ phage and the restriction cleavage patterns confirmed. These clones, designated λWMJ-1 (V), λWMJ-2 (III), and λWMJ-3 (III), were subsequently subjected to nucleotide sequence determination (see Fig. 3). Dates of the phlebotomies from which the viruses were derived are indicated. Restriction sites are depicted by letters (B, Bgl II; S, Sst I; H, Hind III; U, Pvu II; P, Pst I; E, Eco RI; K, Kpn I; L, Sal I; X, Xho I; Y, Xba I). Restriction enzyme site differences among the five isolates are shown. Asterisks denote sites present in WMJ-1 but missing in other viruses, whereas solid dots denote sites that are absent in WMJ-1 but present in other viruses. Note that the restriction enzyme differences among the five viruses are not consistent with a direct, sequential evolution of changes from isolates WMJ-1 to WMJ-2, and so on, to WMJ-5 (see text). Beneath the restriction maps is drawn to scale the genomic organization of HTLV-III/LAV (*13*).

cultured brain and lymphoid tissues from AIDS patients also indicates the presence of only one or a very limited number of predominant viral forms in situ (*8*).

To define in greater detail the nature of genetic changes in HTLV-III/LAV that occur in infected patients over time, we used subgenomic probes to map the restriction enzyme cleavage patterns of isolates WMJ-1 through WMJ-5 (Fig. 2). In addition, the proviral genomes of WMJ-1, WMJ-2, and WMJ-3 were molecularly cloned and the nucleotide and deduced amino acid sequences of their *env* genes determined (Fig. 3). The restriction maps in Fig. 2 represent the restriction cleavage patterns of proviral DNA for each of the five WMJ isolates shown in Fig. 1. The results indicate that each of the five WMJ isolates that were obtained over a 1-year period were highly related to each other, differing in only one or a few restriction sites. For example, out of 29 restriction sites tested, WMJ-2 differed from WMJ-1 in only four sites and from WMJ-3, WMJ-4, and WMJ-5 in three, one, and one sites, respectively. Of particular note was the finding that although the five WMJ virus isolates were obtained sequentially, these viruses had apparently not evolved genetically in such a direct fashion, that is, from WMJ-1 to WMJ-2 to WMJ-3, and so on. This could be deduced from the restriction maps by observing the nondirectional changes in certain of the Pvu II, Hind III, Kpn I, and Bgl II sites (Fig. 2).

That isolates WMJ-1, WMJ-2, WMJ-3, WMJ-4, and WMJ-5 had evolved in parallel was confirmed by determining and comparing the *env* nucleotide sequences of molecular clones of proviral DNA from WMJ-1, WMJ-2, and WMJ-3 (Fig. 3). Overall, the *env* of WMJ-1 (clone λWMJ-1-V) differed from WMJ-2 (clone λWMJ-2-III) in 2.8% of nucleotides and that of WMJ-2 differed from WMJ-3 (clone λWMJ-3-III) in 3.1% of nucleotides. If WMJ-3 had evolved directly from WMJ-2, which had in turn evolved from WMJ-1, one would have expected to find approximately 5% to 6% nucleotide differences between WMJ-3 and WMJ-1. This was not the case because WMJ-3 differed from WMJ-1 by only 2.3% of nucleotides. Direct inspection of the nucleotide alignment of WMJ-1, WMJ-2, and WMJ-3 (Fig. 3) also revealed many examples where WMJ-1 was more similar to WMJ-3 than to WMJ-2, and vice versa. Of importance, the restriction patterns of each of the three WMJ proviral clones that were sequenced corresponded exactly (20 out of 20 restriction sites) to the predominant genomic proviral forms for WMJ-1, -2, and -3 present in infected cells and shown in Figs. 1 and 2. Furthermore, between 5 and 20 proviral DNA clones were obtained from the recombinant libraries of each isolate, and, again, the ones chosen for sequence analysis were full-length and representative of the majority. Finally, to be certain that the observed nucleotide differences among the clones were not an artifact due to technical procedures, we sequenced a portion of



the *env* gene of two proviral clones from the same library. These two clones (λWMJ-2-III and λWMJ-2-XII), both isolated from a recombinant λ phage library of WMJ-2 DNA, were identical in 1220 out of 1221 nucleotides sequenced. Thus, the sequenced proviral clones were representative of the predominant viral forms in each of the isolates, and the observed sequence differences reflected variation in the HTLV-III/LAV *env* gene.

Since it was shown that WMJ-1, WMJ-2, and WMJ-3 were highly related and had evolved from a common progenitor, and since the date of infection of patient WMJ by HTLV-III/LAV was known, it was possible to determine the approximate rate of genetic evolution for the AIDS virus during a natural infection. Many data (*15*) documenting the rates of evolution of other RNA viruses as well as DNA genomes had led to the conclusion that RNA viruses in general evolve at a rate much greater than DNA genomes. For calculating the rate of genetic change for HTLV-III/LAV, we assumed that WMJ-1, WMJ-2, and WMJ-3 had all evolved from a common progenitor virus sometime within the preceding 5 years. This supposition was based on the following information. (i) Patient WMJ, who had only a single perinatal exposure to HTLV-III/LAV, was 2 years old when isolates 1, 2, and 3 were obtained. (ii) WMJ-1, -2, and -3 were but three of five highly related viruses isolated from this same patient. (iii) All five WMJ viruses were highly related to each other and very different, as a group, from other independent viruses that we had evaluated (including five other isolates from Haitian individuals living in Miami). (iv) Within the hypervariable envelope regions [see (*9*)], the nucleotide sequences of WMJ-1, -2, and -3 were very similar to each other and dramatically different from those of isolates from other patients. These data, taken together, indicate that the five WMJ isolates all evolved from a common progenitor virus. Conceivably, patient WMJ could have been infected perinatally with more than one virus. Even if this were so, however, these viruses would still have had to evolve in the immediate past from a common progenitor virus present in WMJ's mother. A large body of evidence [see (*1, 2, 8, 9*) and herein] indicates that the five WMJ isolates could not have had disparate origins.

Using 1 to 5 years as an estimate of the time since divergence of WMJ-1, WMJ-2, and WMJ-3 from a common point, we calculated the rates of evolution for the *env* and *gag* genes of HTLV-III/LAV as follows:

$$R = D/2T$$

where R is the rate of nucleotide substitutions per site per year and T is the time since divergence of the viruses

$$D = -(3/4) \ln [1 - (4/3)P]$$

where P is the proportion of different nucleotides between the homologous genes. From this equation and the determined nucleotide sequences of WMJ-2 and WMJ-3 [see (*16*)], the rate of evolution (R) for the HTLV-III/LAV *env* gene was estimated to be between 1.58×10^{-2} and 3.17×10^{-3} nucleotide substitutions per site per year for T values of 1 year and 5 years, respectively. Similarly, the rate of genetic mutation for the *gag* p24 and p15 genes was estimated to be between 1.85×10^{-3} and 3.70×10^{-4}.

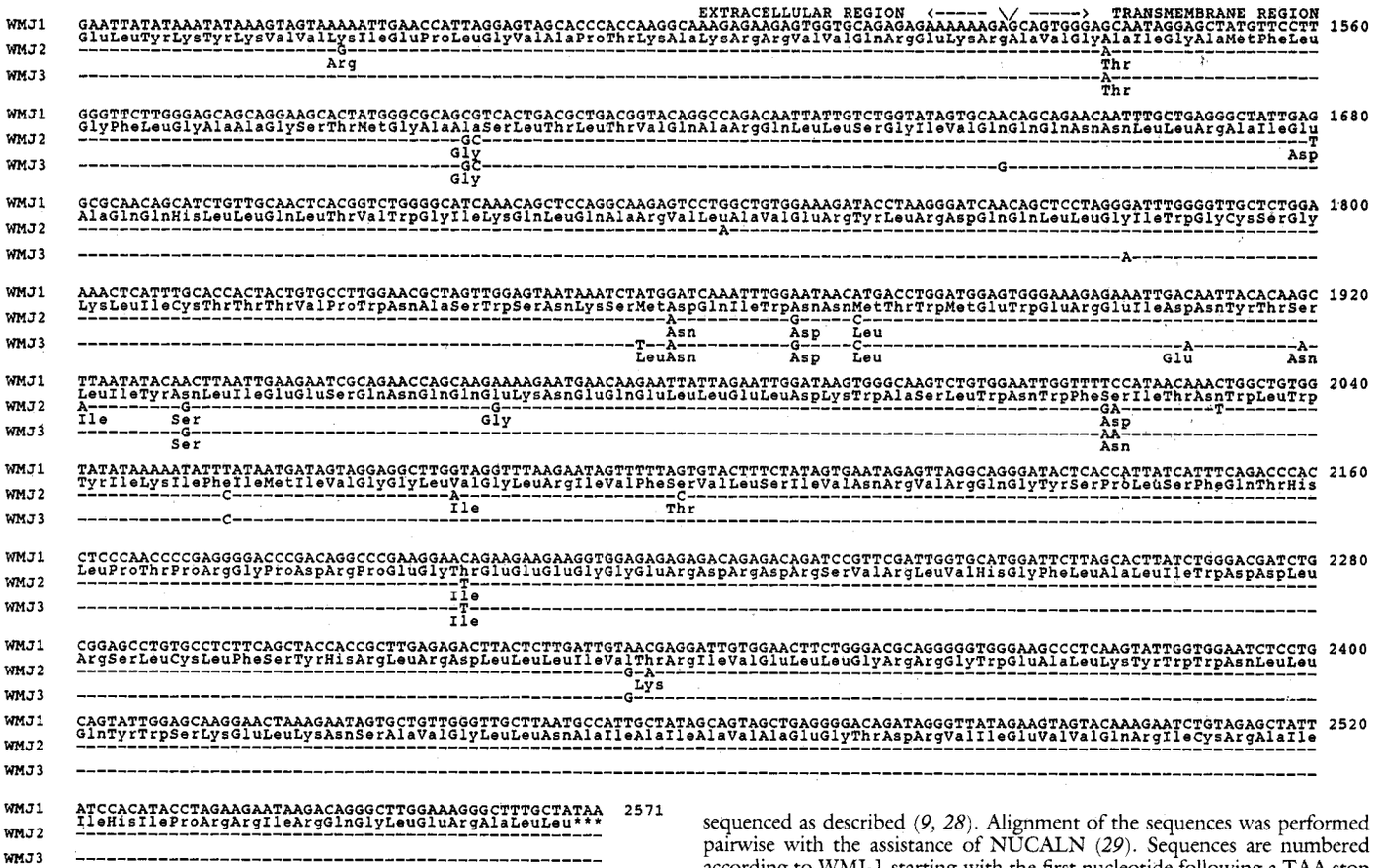


Fig. 3. Nucleotide and amino acid sequence alignment of the entire envelope genes of three sequential virus isolates from patient WMJ. Molecular clones of WMJ-1, WMJ-2, and WMJ-3 [designated λWMJ-1 (V), λWMJ-2 (III), and λWMJ-3 (III), respectively] were obtained and their envelope genes sequenced as described (*9, 28*). Alignment of the sequences was performed pairwise with the assistance of NUCALN (*29*). Sequences are numbered according to WMJ-1 starting with the first nucleotide following a TAA stop codon. Genomic regions corresponding to the signal peptide, the extracellular glycoprotein (gp120), and the membrane-associated glycoprotein (gp41) are shown. Dashes indicate nucleotide identity and spaces indicate deletions in WMJ-2 and WMJ-3 with respect to WMJ-1.

This rate of genetic change for the AIDS virus is more than a millionfold greater than for most DNA genomes (*15, 17–19*) and may even be tenfold greater than for some other RNA viruses including certain retroviruses and influenza A virus (*15, 17, 20*).

We also compared the nature and distribution of nucleotide changes within the *env* genes of sequentially isolated viruses from persistently infected individuals with those in independent virus isolates from different patients (*9, 21*). The changes in the *env* genes of WMJ-1, WMJ-2, and WMJ-3 were almost exclusively nucleotide point mutations. This corroborates our earlier findings with independent virus isolates that single and clustered base pair substitutions, presumably resulting from errors in transcription, are an important source of variation in HTLV-III/LAV (*9*). In addition to point mutations, however, there were also three small (3 bp) deletions in WMJ-2 and WMJ-3 compared to WMJ-1 (Fig. 3). Their presence underscores the importance of nucleotide deletions or insertions as well as substitutions as a mechanism for genomic change in the AIDS virus, a conclusion again based on the analysis of independent HTLV-III/LAV isolates (*9*). The distribution of nucleotide substitutions and deletions or insertions in the three WMJ isolates was not uniform throughout the *env* gene. Instead, such changes were clustered, coinciding with hypervariable regions previously identified in five independent AIDS virus isolates (see Fig. 4).

These analyses allowed us to address a number of important issues concerning the biology of HTLV-III/LAV. The first of these relates to the origin of the genomic variation described here and elsewhere (*1–9*). It is possible that the genomic differences previously identified in independent AIDS virus isolates resulted from artifacts due to the growth of virus in vitro rather than from variation that arose in vivo. Our finding that viruses isolated repeatedly and over a prolonged period from a given patient are similar to each other as a group but different from viruses isolated from other patients indicates that most, if not all, of the genotypic differences are indeed the result of mutations that have occurred in vivo. This conclusion is further supported by the find-

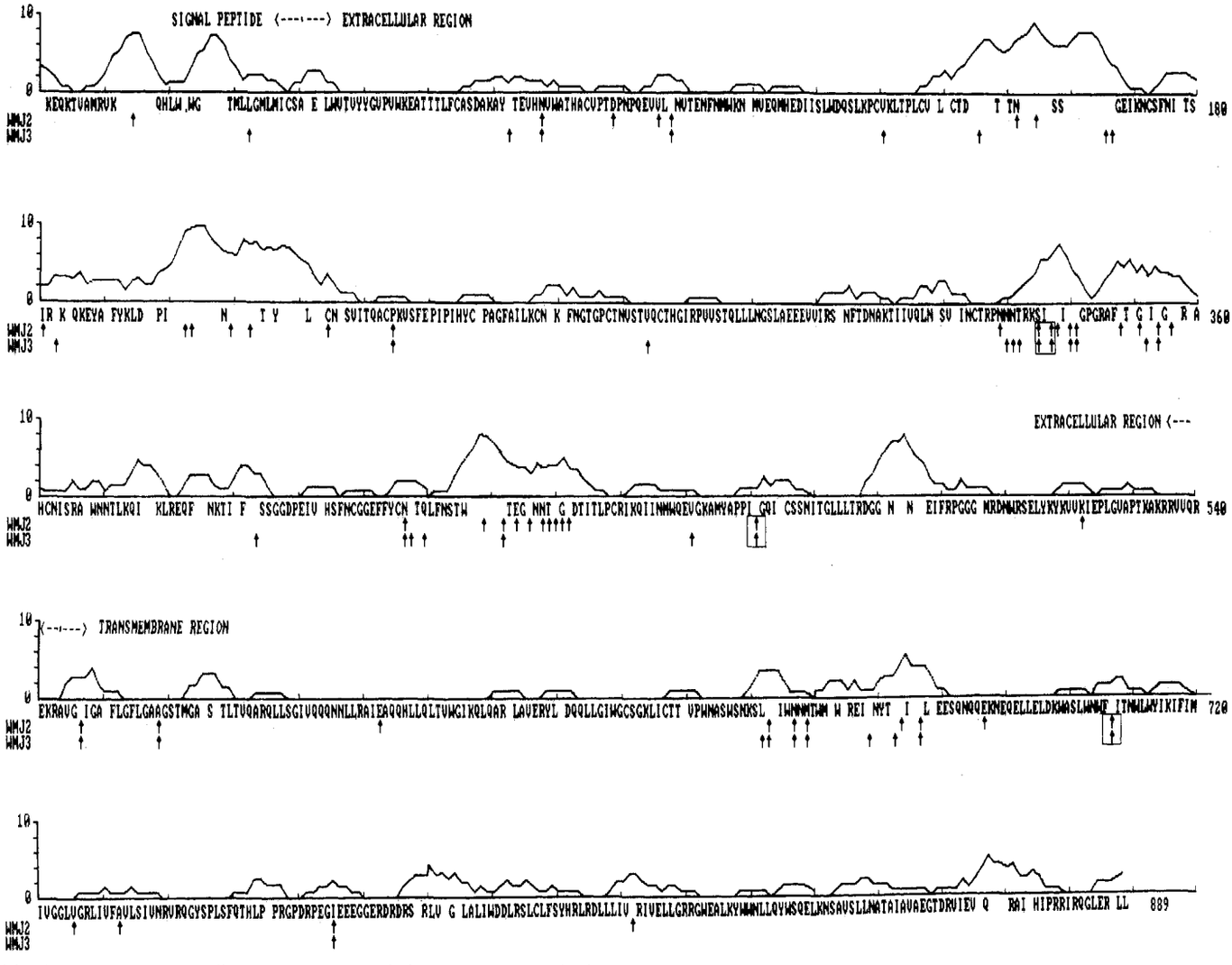


Fig. 4. Computer graphic illustrating relative variation, or divergence, in the envelope genes of five independent AIDS virus isolates and the position of amino acid differences among WMJ-1, WMJ-2, and WMJ-3. The five independent isolates were HTLV-IIIb (BH-10), LAV (LAV-la), ARV (ARV-2), HTLV-III$_{RF}$ (HAT-3), and HTLV-III$_{WMJ-1}$ (WMJ-1) [see (*9*)]. Their relative degrees of variation were calculated as described (*21*) and plotted on the ordinate from 0 (minimum divergence) to 10 (maximum divergence). Beneath the abscissa are single letter codes for conserved amino acids as defined by identity in five out of five or four out of five of the sequences. Arrows beneath this relative consensus sequence indicate the position of amino acid changes in WMJ-2 and WMJ-3 compared to WMJ-1. Double arrows represent identical amino acid substitutions in WMJ-2 and WMJ-3 compared to WMJ-1 while boxed double arrows indicate different amino acid substitutions. Numbering of amino acids is based on the envelope sequence as shown on the abscissa. Note that most of the amino acid differences among the three WMJ isolates are confined to regions of either high or intermediate envelope variability.

ing that different virus isolates obtained from a single specimen of blood (but cultured separately with the use of different, HLA-unrelated donor lymphocytes for virus amplification) have similar genomic restriction cleavage patterns (*14*).

Another point of biological importance concerns the molecular basis of genetic variation in HTLV-III/LAV. It is now clear that isolated and clustered nucleotide substitutions as well as deletions, insertions, and duplications are largely responsible for the genetic variation in the *env* gene. The underlying mechanisms responsible for these changes have not been demonstrated, but errors in reverse transcription leading to nucleotide substitutions and copy-choice misreading (*22*) of the viral RNA template seem to be likely sources. Other sources of variation, especially recombination between different viral molecules, could also play a role as in other retroviruses (*22, 23*). This may be especially important in HTLV-III/LAV since genetically distinct viruses can persist in an infected individual for long periods and since the AIDS virus is cytopathic and presumably undergoes large numbers of replicative cycles during the course of infection.

Also important is the rate of evolution of HTLV-III/LAV, as defined as the rate at which viable mutations accumulate in the genome (*15*). Our finding that the rate of genetic change is approximately 10^{-2} to 10^{-3} nucleotide substitutions per site per year for *env* and tenfold less for *gag* raises the possibility that viral properties such as tissue tropism, virulence, rate of replication, sensitivity to antiviral drug therapy, and resistance to immunologic attack may be similarly modified in biologically and clinically important ways.

The observation that viruses isolated sequentially have evolved in parallel implies that a variety of related yet distinct viral forms can persist in individuals for prolonged periods. This is not surprising in view of the complex life cycle of retroviruses that includes a chromosomally integrated proviral DNA stage that has the potential for indefinite persistence. Whether different viruses replicate and express their genomes simultaneously in vivo or whether some are hidden as replication-inactive proviral DNA is not known. Together, the rapid rate of genetic change and the parallel evolution of viral variants support the view that HTLV-III/LAV populations within infected patients may be extremely heterogeneous and that any single isolate from a patient may or may not represent the most abundant or relevant variant at that time. Future studies of viral pathogenesis will need to take these considerations into account.

Our finding, based on restriction enzyme mapping, of either a single variant or several highly related variants in every patient that we have analyzed thus far (total of 35 patients) is remarkable given the fact that many of these individuals were homosexual men from HTLV-III/LAV endemic regions who had hundreds, and in some cases thousands, of different sexual partners. This apparent paradox, that on the one hand extensive genomic heterogeneity of independent AIDS viruses is common yet, on the other hand, each patient appears to be infected with only a limited number of predominant viral forms, has no ready explanation. It is possible that the observation could be due to selective pressures of cultivation in vitro, but the fact that only very similar viruses were cultured from patient RJS over a 2-year period using different, HLA-unrelated donor lymphocytes for virus amplification makes this unlikely. Instead, the finding may imply that once infected with one AIDS virus, individuals are protected from infection by other AIDS viruses. It seems unlikely that such an effect could be the result of classical viral interference mechanisms whereby a cell infected with one strain of virus loses its receptor for that and other closely related viruses (*24*), since many chronically infected patients have normal or near normal numbers of $T4^+$ lymphocytes only a small proportion of which are infected with HTLV-III/LAV at any one time (*1, 25*). It is conceivable that immunologic or nonimmunologic events that occur after the initial infection with HTLV-III/LAV lead to protection from subsequent viral infections. The elucidation of such processes could be important in developing methods for the treatment and prevention of AIDS.

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16. The rate of genetic change for HTLV-III/LAV was calculated as described in the text. This approach was taken from Gojobori and Yokoyama (*17*) and assumes equal rates of substitution among the four types of nucleotides. Since the envelope sequences of WMJ-1, WMJ-2, and WMJ-3 differed from each other by approximately the same degree (see text), and since *gag* p24 and p15 sequences were available only for WMJ-2 and WMJ-3, the rate of genetic change for these last two isolates was determined. The *gag* p24 and p15 sequences of WMJ-2 and WMJ-3 were identical in length but differed in 4 of 1089 nucleotides. The *env* genes of these viruses differed in overall length by 3 nucleotides and throughout the gene by a total of 79 of 2568 nucleotides. The rate of evolution of *env* of HTLV-III/LAV was calculated both with and without the 3-bp deletion included in the calculation; nearly identical results were obtained and the values given in the text include this 3-bp deletion.
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21. A computer program was written that compared the aligned amino acid sequences of BH-10, ARV-2, LAV-1A, WMJ-1, and HAT-3 (*9*) and plotted relative variation, or divergence, as a function of amino acid position within *env*. For this analysis, the aligned amino acid sequences shown in figure 2 of Starcich *et al.* (*9*) were analyzed column by column. Computations of the column scores were based on the number of mismatched pairs with values ranging from 0 (no mismatches, complete homology) to 10 (all mismatches, no homology). The absence of an amino acid (deletion) at any position was considered to be a mismatch. Each column score was averaged over 2 column scores on each side resulting in a window size of 5. This mean value for each amino acid position was plotted from 0 to 10 above the corresponding envelope amino acid position shown on the abscissa.
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30. We thank S. Chastain for preparation of the manuscript. Supported in part by NIH grants AI 23616-01, AI 23854-01, P30 CA 13148, AI 21122, and AI 20736. B.H.H. is a Special Fellow of the Leukemia Society of America. G.M.S. is a Pew Scholar in the Biomedical Sciences.

28 March 1986; accepted 21 May 1986