

ability of the pU3R-III plasmid to direct CAT gene expression was also seen in another lymphocyte line, Jurkat L8460D, infected in vitro with HTLV-III (14) (Table 1). As the majority of transiently expressing DNA is extrachromosomal, factors present in HTLV-III-infected H9 cells that modulate gene expression by the HTLV-III LTR must act in *trans*. We conclude that factors associated with HTLV-III viral infection *trans*-activate transcription directed by the HTLV-III LTR.

To examine whether the *trans*-acting factors present in HTLV-III-infected cells could stimulate CAT gene expression directed by other HTLV-BLV LTR's, we introduced plasmids that contain the U3 region of HTLV-I (pU3R-I), HTLV-II (pU3-II), and BLV (pBLV-CAT) (4, 5) into HTLV-III-infected and uninfected H9 cells. In all cases there was no significant difference in the level of CAT activity in infected and uninfected cells (Table 1). We conclude that the viral *trans*-acting factors in HTLV-III-infected H9 cells do not transcriptionally activate the LTR's of HTLV-I, HTLV-II, or BLV.

The magnitude of the transcriptional activation of the HTLV-III LTR in the infected H9 and Jurkat cell lines is noteworthy. Relative to the activity of the SV40 promoter, the level of CAT activity directed by the HTLV-III LTR in the infected cells was about five to ten times the maximum level of CAT activity directed by the HTLV-I or HTLV-II LTR's and about 50 to 100 times that observed for the BLV LTR in the appropriate infected cell type (4, 5). Efficient *trans*-activation of the viral LTR may be related to the high levels of virus production observed in some HTLV-III-infected cell lines (3). High levels of virus production may be responsible, at least in part, for the ability of HTLV-III to establish a chronic viremia, which is unusual for the other HTLV's or BLV (1, 15).

Transcriptional *trans*-activation of the LTR in infected cells places HTLV-III in the retroviral family that includes HTLV-I, HTLV-II, and BLV, in accord with demonstrated similarities among the genomes and proteins of these viruses (3, 8, 9). The ability of these viruses to alter the transcriptional environment of the host cell suggests that they might exert their phenotypic effects via transcriptional regulation of specific host cellular genes. The specific activation of LTR sequences of the infecting virus suggests that sequences recognized by HTLV-III-associated *trans*-acting factors differ from those recognized by fac-

tors present in HTLV-I- or HTLV-II-infected cells. The cellular genes regulated by such factors might also differ, leading to cell death consequent to infection by HTLV-III and to cell proliferation following infection by HTLV-I or HTLV-II.

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## Sequence Homology and Morphologic Similarity of HTLV-III and Visna Virus, a Pathogenic Lentivirus

**Abstract.** A study was conducted of the genetic relation between human T-cell lymphotropic retroviruses and visna virus. The human T-cell lymphotropic viruses include those associated with T-cell malignancies (HTLV-I and HTLV-II) as well as the etiologic agent of the acquired immune deficiency syndrome (HTLV-III). Visna virus, a slowly replicating and pathogenic but nononcogenic retrovirus of sheep, is a member of the subfamily Lentivirinae. Results obtained by molecular hybridization and heteroduplex analysis indicated that a greater extent of nucleotide sequence homology exists between HTLV-III and visna virus than between HTLV-III and any of the other viruses. The homology observed under conditions of low stringency spanned the entire genome, but was strongest in the gag/pol region. The morphogenesis and fine structure of HTLV-III and visna virus also demonstrated striking similarities. The data provide strong evidence for a close taxonomic and thus evolutionary relation between HTLV-III and the Lentivirinae subfamily.

The family Retroviridae consists of animal viruses that contain an RNA-dependent DNA polymerase (or reverse transcriptase). These viruses replicate by way of DNA intermediates and often

integrate into the host genome and can, in some instances, be transmitted through the germ line. Their gross morphogenesis is similar in that an encapsidated viral RNA genome is released

from infected cells by budding from the plasma membrane; however, they also show fine morphological as well as biochemical differences that enable one to differentiate between individual members of this family.

At present, the family Retroviridae is composed of three subfamilies: Oncovirinae, Spumivirinae, and Lentivirinae (1). Of these, only the Oncovirinae and Lentivirinae produce pathologic disorders germane to this study. The oncoviruses, which can be further subdivided into interstitial type A, and types B, C, and D, and which include certain tumor-causing viruses, are the only retroviruses that can be transmitted both as exogenous infectious viruses and as endogenous genetic elements. Lentiviruses, in contrast, are exogenous viruses which, to date, are only known to infect ungulate (hoofed) mammals, in particular, domestic sheep (2), goats (3), cattle (4), and horses (5). The lentiviruses cause persistent but debilitating infections, replicate at a slow but progressive rate, and have been demonstrated to have pathogenic potential in vitro, causing syncytia, cell lysis, and death of susceptible cells during virus replication (4-6).

Several isolates of human retroviruses, known collectively as human T-cell leukemia (lymphotropic) viruses (HTLV), have been obtained from patients with certain T-cell malignancies (7). Initially, two major types (HTLV-I and HTLV-II) were identified. HTLV-I is strongly associated with adult T-cell leukemia (7, 8) while HTLV-II was first detected and isolated from cultured cells from a patient with hairy cell leukemia (9). Another

type, HTLV-III, is believed to be the etiologic agent of the acquired immune deficiency syndrome (AIDS) (10, 11).

Particles of HTLV-I and -II observed by thin-section electron microscopy have been likened to oncoviruses of type C morphology (8). HTLV-III, however, is morphologically distinct from HTLV-I and -II and type C viruses (10-15). Whereas HTLV-III, during maturation of its extracellular particles, shows the formation of bar-shaped nucleoids, HTLV-I, -II, and type C viruses show round, central, variably electron-dense cores. Differences between HTLV types I and II and type C viruses are found in the thickness and spacing of the electron-dense core material in both budding and mature particles and the diameter of mature particles. Morphologically, HTLV-III resembles visna and equine infectious anemia viruses, both members of the lentivirus family, more closely than HTLV-I or -II or type C viruses (15-18). Other workers (14) have pointed out a resemblance between mature virions of lymphadenopathy associated virus (LAV), which may be similar or identical to HTLV-III, and both equine infectious anemia virus (EIAV) and type D retroviruses; however, we emphasize that there is a clear distinction in morphogenesis between HTLV-III and type D viruses. It has also been reported that the core protein of LAV was precipitated by sera from horses infected with EIAV (14, 19). This observation, along with the demonstrated cytopathic effect of HTLV-III in vitro (10), further suggested the possibility of a taxonomic link to pathogenic lentiviruses.

Analysis of the structural and evolutionary relation between HTLV-III and other retroviruses has been facilitated by the ability to isolate and amplify their genomes by molecular cloning techniques (20-22). In the present study, we used thin-section electron microscopy, molecular hybridization, and heteroduplex nucleotide sequence analysis to detect structural similarities and identify regions of homology between HTLV-III and visna virus.

HTLV-III was grown in an established human T-cell line (H9) (10-12), and, after being washed and pelleted by centrifugation, the infected cells were fixed in glutaraldehyde and embedded in epoxy resins for electron microscopy. Visna virus, strain 1514, was propagated in sheep choroid plexus cells. The infected cells were harvested 5 days after cytopathic effects were observed and similarly processed for electron microscopy. Examination of thin sections revealed striking similarities between HTLV-III and visna virus at all stages of maturation (Fig. 1, A-J). Buds (120 to 140 nm in diameter) with crescent or semicircular cores appeared at the cell membranes in both cultures. The double-membrane envelope and electron-dense laminar core were separated by a lighter electron-dense intermediate layer (Fig. 1, A, B, C, and G). The electron-lucent region between the core and the double-membrane envelope that is typical of type C viruses was not evident in these viruses (18). Free, extracellular immature particles were observed in both cultures (Fig. 1, C and H). These had a similar distribution of virus-specific layers as budding virus and frequently had an electron-lucent center, which was also seen in some budding forms. In the mature extracellular particles (90 to 130 nm in diameter), the cores condensed, often forming a bar-shaped nucleoid (Fig. 1, D and I) which, in cross sections, appeared smaller, circular, and most often eccentrically located (Fig. 1, E and J).

Molecular clones of HTLV-I, -II, -III, and visna virus (20-22) were used in heteroduplex mapping studies to determine the relative conservation and location of any sequence homology. We used the technique of thermal melt analysis, whereby the relative stringency of spreads is varied by increasing or decreasing the concentrations of formamide and salt in the spreading solution [see (23) and legend to Fig. 2]. The analysis was performed at  $T_m - 52^\circ\text{C}$  and  $T_m - 45^\circ\text{C}$  (with 20 and 30 percent formamide, respectively, in hyperphases containing 0.2M TES, pH 8.5). The relative conservation of sequences was ex-

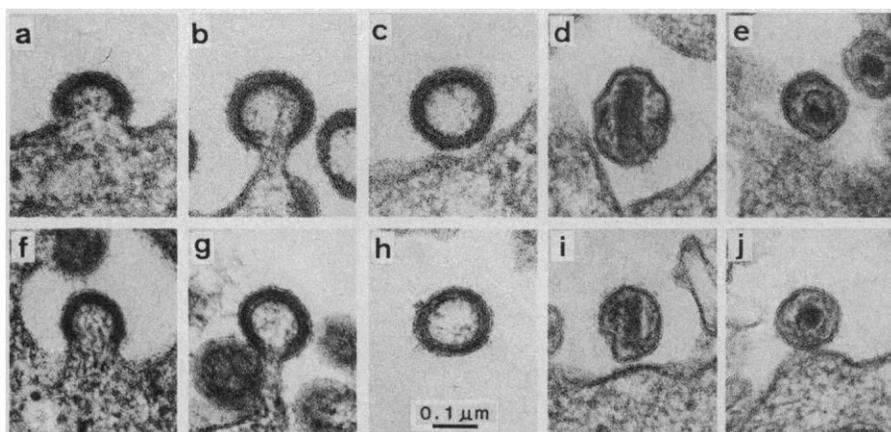


Fig. 1. Electron microscopy ( $\times 100,000$ ) of thin sections of cells infected with HTLV-III and visna virus (strain 1514). Cell pellets were fixed in 1.25 percent buffered glutaraldehyde and then in 1 percent osmium tetroxide, dehydrated in graded alcohols, and embedded in epoxy resins. Thin sections were cut and stained with uranyl acetate and lead citrate. (A to E) HTLV-III-infected H9 human lymphocytes. (F to J) Visna virus-infected sheep choroid plexus cells. (A, B, F, and G) Bud formation of virus particles at the plasma membrane. (C and H) Free immature, extracellular virus particles. (D and I) Free mature, extracellular virus particles with bar-shaped nucleoid. (E and J) Free mature extracellular virus particles with condensed circular, eccentric cores.

pressed as a percentage, with the amount of duplexed DNA in the heteroduplex being used as the numerator and 9.0 kilobases (kb) as the denominator [9.0 kb being the size of a cloned HTLV-III genome containing one long terminal repeat (LTR) sequence as determined separately in gel electrophoresis experiments].

All heteroduplex analyses were performed with inserts in bacteriophage  $\lambda$  DNA cloned in the opposite orientation of the plus strand of the  $\lambda$  DNA arms. This configuration allowed the highly homologous  $\lambda$  DNA arms to drive the reaction and bring in close proximity the cloned inserts for base pairing. In heteroduplexes of HTLV-I and HTLV-III (Fig. 2A) or HTLV-I and visna virus (Fig. 2B), in spreads from 20 percent formamide, less than 6 percent of the HTLV-I genome was homologous to sequences in HTLV-III, and only 3 percent of the HTLV-I genome formed a duplex with visna virus. Most of the homologous sequences were within the 5' one-third of the inserts (*gag* or *gag/pol* gene region), and were limited to two or three small regions of approximately 0.1 to 0.3 kb each. When the stringency was increased to 30 percent formamide, less than 3 percent of the HTLV-I genome was homologous to HTLV-III and none of the genome was homologous to visna virus (data not shown). Heteroduplexes between HTLV-II and HTLV-III revealed no regions of identity in spreads from either 20 to 30 percent formamide (data not shown). In contrast, heteroduplexes formed between  $\lambda$  clones of HTLV-III and visna virus (Fig. 2C) revealed a striking amount of homology. In spreads from 20 percent formamide, approximately 35 percent of the HTLV-III genome was duplexed with visna virus, with the homology dispersed over the entire genome. In spreads from 30 percent formamide, 15 percent of the HTLV-III genome was duplexed with visna virus, with the conserved sequences occurring largely, but not exclusively, in the *gag/pol* region (data not shown). To verify that the conditions (20 percent formamide, 0.2M TES, pH 8.5) for heteroduplexing were not so mild as to allow nonspecific hybridization over short stretches of DNA, we hybridized to the  $\lambda$  visna clone a clone of HTLV-III that was in the reverse orientation of the clone used in Fig. 2C. No regions of hybridization were observed in any of these heteroduplexes (Fig. 2D).

To confirm and extend these findings, we performed reciprocal hybridizations using HTLV-III or visna virus as the probes under relaxed conditions ( $T_m$

$-39^\circ\text{C}$ ; see legend to Fig. 3). The cloned viral DNA's studied included HTLV-I, -II, and -III, visna virus, and bovine leukemia virus (BLV), an exogenous virus of cattle associated with leukemia and lymphosarcoma. In its genomic complexity and organization, BLV is similar to both HTLV-I and HTLV-II and it shows a clear but distant relation to these viruses (24). Viral DNA inserts were separated from their cloning vectors by restriction enzyme digestion and gel electrophoresis. Purified inserts were again digested with restriction enzymes to molecularly dissect the genomes, and were subjected to gel electrophoresis

and Southern blotting. A limited restriction enzyme map is shown in Fig. 3A, which depicts the subgenomic fragments of each viral clone and the relative genomic complexity where known. The location and extent of homology between HTLV-III or visna virus and each of these genomes was quantitated visually by autoradiography after hybridization of a  $^{32}\text{P}$ -labeled nick-translated probe.

When visna virus was used as the probe, it hybridized most strongly to itself and to a 4.0-kb fragment in HTLV-III that included most of the *gag* and *pol* genes (Fig. 3B). In the reciprocal blot in which HTLV-III was used as the probe,

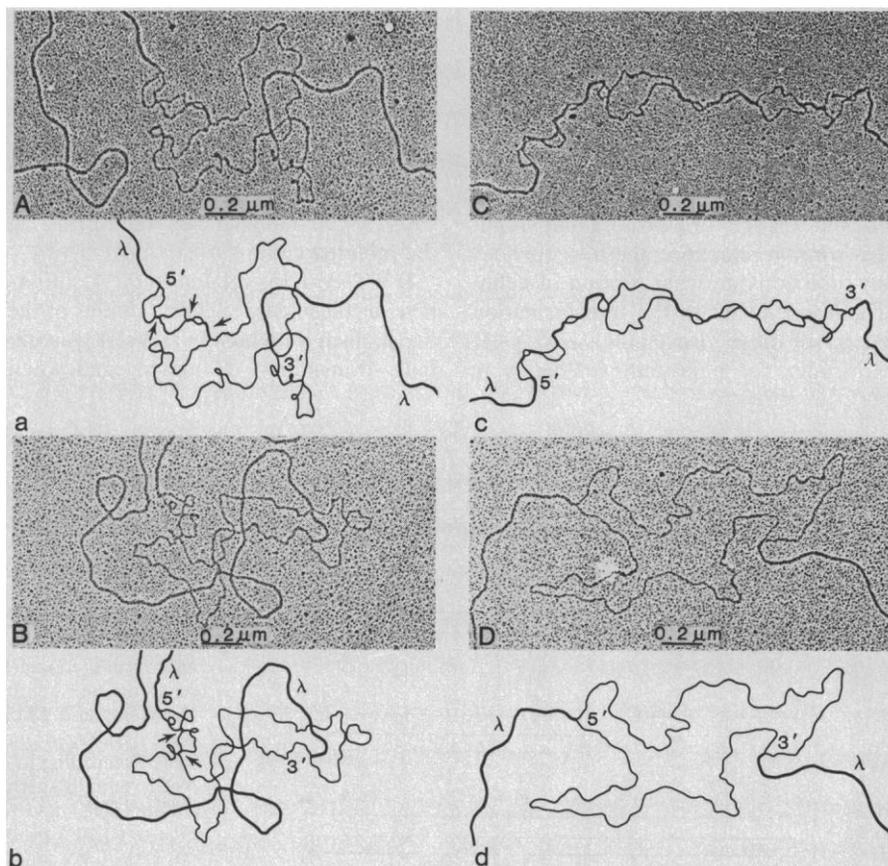


Fig. 2. Heteroduplex analysis of HTLV-I, -II, and -III and visna virus. Heteroduplexes were prepared between HTLV-III and visna virus and each of these to HTLV-I and -II. Heteroduplexes were prepared according to the method of Davis *et al.* (33). When thermal melt analyses were performed, only the formamide concentrations in the hyperphase were adjusted. Spreads typically contained DNA (0.01 to 0.02  $\mu\text{g}$ ), cytochrome C (30  $\mu\text{g}/\text{ml}$ ), 0.2M TES, and 0.02M EDTA, pH 8.5. The concentration of formamide was varied from 20 to 30 percent. Hypophases contained one-tenth the electrolyte and 0.001 percent *n*-octyl  $\beta$ -*d*-glucopyranoside. The stringency of hybridization was calculated from the known guanine plus cytosine (G + C) content of HTLV-I (53.9 percent) derived from Seiki *et al.* (25) and the following relation (34):  $T_m = 81.5 + 16.6 (\log M) + 0.41 (\text{percent G} + \text{C}) - 0.72 (\text{percent formamide})$  in which  $M$  is the monovalent salt molarity and (percent G + C) is the percentage G + C residues in the DNA. The effective temperatures (which are expressed at  $T_m - \Delta T$ , in which  $\Delta T$  is the difference between  $T_m$  and the temperature at which the heteroduplex was mounted for microscopy) for 20 and 30 percent formamide hyperphases were  $T_m - 52^\circ\text{C}$  and  $T_m - 45^\circ\text{C}$ , respectively. All heteroduplex analysis were performed with inserts in bacteriophage  $\lambda$ . (A to D) Actual heteroduplexes. (a to d) Interpretive drawings. (A) Heteroduplex of HTLV-I and HTLV-III. (B) Heteroduplex of HTLV-I and visna virus. (C) Heteroduplex of HTLV-III and visna virus. (D) Heteroduplex of negative control HTLV-III and visna virus in which the HTLV-III insert was in the opposite orientation of the HTLV-III insert in phage  $\lambda$  used in (C). The 5' and 3' ends of the inserts and the  $\lambda$  arms are indicated. Regions of homology in (A) and (B) are noted by solid arrows. All micrographs are at the same magnification.

it hybridized most strongly to itself and a 3.2-kb fragment in visna virus (data not shown). This fragment included most of the visna *gag* and *pol* genes. Under mild washing conditions ( $T_m - 49^\circ\text{C}$ ), low, but specific, hybridization of both the visna and HTLV-III probes was detected with HTLV-I and -II and with BLV, but only when the exposure times were two to three times longer than those used in Fig. 3B (data not shown). As the washing stringencies were increased ( $T_m - 39^\circ\text{C}$  to  $-29^\circ\text{C}$ ), the heterologous hybridization intensities were reduced while the homologous reactions remained undiminished.

The genomes of HTLV-I and -II and BLV are structurally similar in that they have *gag*, *pol*, and *env* genes and a unique segment, termed pX, located between the *env* gene and the 3' LTR (21, 24, 25). The 3' 1-kb coding sequences of the pX regions (also referred to as long open reading frames, or LOR) of HTLV-I and -II are highly related (21) and believed to code for a protein that stimulates viral replication; these sequences may also activate transcription of cellular genes relevant to the transformation activity of these viruses (26). HTLV-III also codes for a protein analogous in

function to that coded for by the LOR of HTLV-I and -II (27). Less is known about the genomic complexity and coding capacity of lentiviruses. Results from our previous study (28), as well as the present data, indicate a low, but significant, hybridization between HTLV-III and HTLV-I and -II. In other studies (22), minimal reactions were seen with type C virus reagents. The strong reactions seen here between visna and HTLV-III (tenfold greater than between HTLV-III and HTLV-I and -II) and the homology among all members of the HTLV family suggest a link between these viruses and lentiviruses.

On the basis of their morphology, the viruses now classified as Lentivirinae are indistinguishable from HTLV-III. There are also other features that the lentiviruses have in common with HTLV-III, such as their cytopathic (cell-killing) effects in vitro and their ability to produce persistent debilitating diseases in vivo. In contrast, HTLV-I and -II cause T-cell malignancies, immortalizing the infected cell.

If HTLV-III is related to the Lentivirinae, as these data suggest, studies of the mechanism by which HTLV-III was initially transmitted to humans and when

and where this transmission occurred may prove interesting. Humans have long been closely associated with domestic ungulates, and the transmission of certain lentiviruses to human cells in vitro was recently demonstrated experimentally (29).

Lentiviruses persist in their ungulate hosts by various mechanisms. Although they induce the formation of binding antibodies to all of their respective polypeptides, they vary greatly with respect to the induction of neutralizing antibodies to envelope proteins. For example, visna and EIAV readily induce neutralizing antibodies; however, by a process called "antigenic drift," these viruses mutate rapidly in the *env* gene, thus allowing variants to escape the immune system and induce a new cycle of disease (30). Caprine arthritis encephalitis virus, in contrast, does not induce the formation of neutralizing antibodies during natural or experimental infections (31). Isolates of HTLV-III also show heterogeneity in the *env* gene region, as indicated by restriction enzyme and heteroduplex analyses (22). This further similarity between HTLV-III and lentiviruses indicates that development of a vaccine to prevent AIDS may prove to

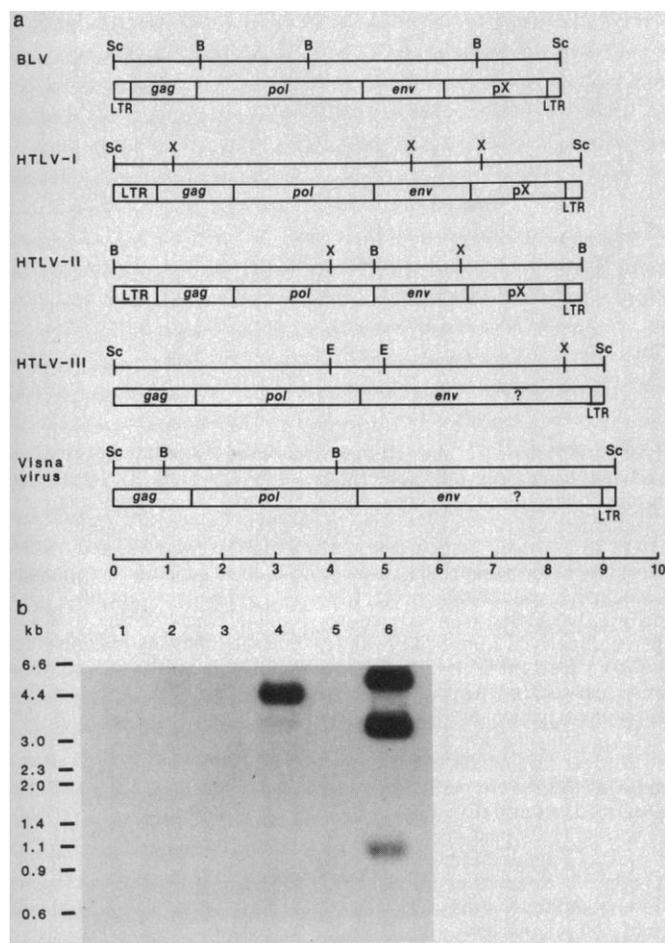


Fig. 3. Related nucleotide sequences detected by  $^{32}\text{P}$ -labeled nick-translated probes (35) in HTLV-I, -II, -III, visna virus, and BLV. Viral DNA inserts were purified free of their cloning vectors (pBR322 or  $\lambda$ WES) by restriction enzyme digestion and gel electrophoresis in low-melt agarose. The purified inserts were again digested with appropriate enzymes to molecularly dissect the genomes, separated on an agarose gel (0.9 percent), and transferred to Gene Screen Plus (New England Nuclear) by the method of Southern (36). The blots were hybridized under nonstringent conditions ( $T_m - 39^\circ\text{C}$ ) as determined according to the equation in Fig. 2 in a solution containing 1M NaCl, salmon sperm DNA (100  $\mu\text{g}/\text{ml}$ ), 0.5 percent sodium dodecyl sulfate (SDS), and 20 percent deionized formamide with a  $^{32}\text{P}$ -labeled nick-translated probe (either HTLV-III or visna virus;  $1 \times 10^7$  cpm/ml) at  $37^\circ\text{C}$  for 36 hours. The filters were then washed initially in  $3 \times \text{SSC}$  ( $1 \times \text{SSC} = 0.15\text{M}$  NaCl and  $0.015\text{M}$  sodium citrate) and 0.5 percent SDS at  $50^\circ\text{C}$  ( $T_m - 49^\circ\text{C}$ ). One microgram of DNA was loaded into each lane except the homologous DNA lane (visna virus), which contained 0.05  $\mu\text{g}$ . (a) Limited restriction enzyme maps show subgenomic fragments and the relative genomic complexity of each virus clone where known. Restriction enzyme digestions were Sac I (Sc), Eco RI (E), Xho (X), and Bam HI (B). The size of cloned inserts are drawn to scale. Restriction enzyme sites are accurate to  $\pm 0.1$  kb. The sequence data for *env* regions of HTLV-III and visna virus are not complete and, therefore, their entire coding capacities are not known. By using one or more of the above enzymes, fragments were obtained of 3.2, 2.0, 1.7, and 1.2 kb for BLV; 4.4, 1.7, 1.3, and 1.1 kb for HTLV-I; 4.0, 2.2, 1.6, and 0.8 kb for HTLV-II; 4.0, 3.3, 1.0, and 0.7 kb for HTLV-III; and 5.1, 3.2, and 0.9 kb for visna virus. (b) The viruses tested were BLV (lane 1), HTLV-I (lane 2), HTLV-II (lane 3), HTLV-III (lane 4), no DNA (lane 5), and visna virus (lane 6). The  $^{32}\text{P}$ -labeled nick-translated probe in (b) was visna virus. Hybridized filters were exposed to x-ray film for 16 hours at  $-70^\circ\text{C}$  before development. Molecular size standards are depicted in kilobases.

be a considerable challenge. A recent study indicating the presence of HTLV-III in brain tissue of AIDS patients (32) further links this virus with lentiviruses (3).

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## HTLV-III Infection in Brains of Children and Adults with AIDS Encephalopathy

**Abstract.** *Unexplained debilitating dementia or encephalopathy occurs frequently in adults and children with the acquired immune deficiency syndrome (AIDS). Brains from 15 individuals with AIDS and encephalopathy were examined by Southern analysis and in situ hybridization for the presence of human T-cell leukemia (lymphotropic) virus type III (HTLV-III), the virus believed to be the causative agent of AIDS. HTLV-III DNA was detected in the brains of five patients, and viral-specific RNA was detected in four of these. In view of these findings and the recent demonstration of morphologic and genetic relatedness between HTLV-III and visna virus, a lentivirus that causes a chronic degenerative neurologic disease in sheep, HTLV-III should be evaluated further as a possible cause of AIDS encephalopathy.*

The acquired immune deficiency syndrome, or AIDS, is frequently complicated by central nervous system (CNS) dysfunction (1-5). In some patients, this is due to well-defined focal lesions in the brain such as those resulting from toxoplasmosis or lymphoma. However, far more common than these focal disturbances is the development of a more generalized encephalopathy that includes dementia as a dominant feature (1, 2). Indeed, many adult AIDS patients eventually develop this encephalopathy which characteristically begins with impaired concentration and mild memory loss and progresses to severe global cognitive impairment. Motor signs, including generalized hyperreflexia and increased tone may accompany the de-

mentia, and some patients develop a spastic-ataxic gait or frank paraparesis. These neurological symptoms and signs usually progress over a course of several weeks to months (1, 2). In children with AIDS, a similar constellation of neurologic abnormalities occurs (3). Although the prevalence of dementia or other unexplained generalized CNS abnormalities in AIDS is uncertain, it is believed to occur to some degree in a substantial number, if not the majority, of patients (1-5).

The histopathological substrate of AIDS encephalopathy has not been defined. Gross cerebral atrophy and scattered microglial nodules consisting of aggregates of microglia and astrocytes are the most common findings in these