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Genomic diversity of the acquired immunodeficiency syndrome retroviruses is reflected in alteration of its translational products

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ABSTRACT We have isolated retroviruses from six acquired immunodeficiency syndrome (AIDS) and three lymphadenopathy syndrome (LAS) patients by cocultivation of patients' lymphocytes with phytohemagglutinin-stimulated normal T cells. In an effort to address the extent to which these viruses have identical genetic information or there is divergence in their genomic sequences, we have compared the nine retrovirus isolates by the following criteria: (*i*) antigenic cross-reactivity by highly specific and sensitive competition radioimmunoassay for the major internal antigen; (*ii*) restriction site mapping analysis; and (*iii*) immunoblot analysis and metabolic labeling of viral structural proteins and their analysis by polyacrylamide gel electrophoresis. The data indicate that individual retroviruses have significant restriction site polymorphism in their genome even though they were isolated from patients residing at one geographic location. Furthermore, this polymorphism is reflected in the variation of the apparent size of the *gag* and *env* gene-encoded structural proteins. The heterogeneity in AIDS retrovirus-encoded proteins may be due to either substitutions in the primary amino acid sequence of the protein or deletions or additions in the coding regions of proteins. The alterations in viral structural proteins among various AIDS retroviruses could have important implications in antigenic properties and/or pathogenicity in development of the disease, its detection, and ultimately its eradication.

The frequent isolation of T-cell lymphotropic cytopathic retroviruses from patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex (ARC), and the presence of specific antibodies to these viruses in patients and those at risk of the syndrome implicate these viruses to be the etiologic agents in AIDS (1–3). The AIDS retroviruses isolated to date include lymphadenopathy-associated virus (LAV; ref. 4), human T-cell lymphotropic viruses type III (HTLV-III; refs. 1 and 5), AIDS-associated retrovirus (ARV-2; ref. 2), and three isolates derived from blood transfusion-associated AIDS patients (3). The availability of molecular clones of three isolates, LAV (6), HTLV-III (7, 8), and ARV-2 (9), and their primary nucleotide sequence analysis (10–12) have been useful for comparison of these isolates and determine homologous regions in the viral genome. These studies have shown that the restriction map and nucleotide sequences of LAV and HTLV-III are closely related to one another, while ARV-2 is related, but significantly different (13). The heterogeneity in genomic sequences could have important implications if it results in changes of antigenic properties and/or pathogenicity by AIDS retroviruses. In an effort to gain knowledge regarding the variation in genomic structure and to study the effect on the translational products encoded by the viral genome, in the present studies, we have compared nine independent retrovirus isolates from AIDS/lymphadenopathy syndrome (LAS) patients by restriction-site analysis. The translational products encoded by the viruses were characterized by use of specific immunoassays and metabolic labeling experiments.

MATERIALS AND METHODS

Isolation of Retroviruses. The nine patients from whom the viruses were isolated in the present study were all male homosexuals except for patient 9, who was a hemophiliac. The hemophiliac had received factor VIII preparation throughout his lifespan of 16 years. All the patients were from areas around Atlanta, Georgia, and the viruses were isolated during May–July 1984. The lymphotropic retroviruses were isolated from the patient blood samples by described methods (4). Briefly, peripheral blood collected from six AIDS and three LAS patients was subjected to a Ficoll-Hypaque gradient for collection of mononuclear cells. The cells were grown in RPMI 1640 medium (GIBCO) containing 10% fetal calf serum and 10 μg of phytohemagglutinin (GIBCO) per ml for 3 days and were further propagated in RPMI 1640 medium containing T-cell growth factor (GIBCO) and goat antibody to human interferon (Miles) diluted 1:5000. After every 7 days, the culture was tested for virus production by cocultivation with 3-day-old phytohemagglutinin-stimulated normal adult T cells. Cell-free supernatants from the cocultivated cultures were analyzed for retrovirus by reverse transcriptase assay (14). In most cases after 10 days in culture, continuous release of virus was observed. The virus was frozen in seed stocks after two passages in phytohemagglutinin-stimulated normal adult human T cells and was subsequently used for further studies after propagation in primary human T cells.

Competition Radioimmunoassay and Immunoblot Analysis. Competition radioimmunoassay for the M_r 24,000 major internal protein (p24) of LAV/HTLV-III was performed as described (15, 16). Detergent-disrupted retroviral proteins (100 μg) were further dissociated in a buffer containing 1% $NaDodSO_4$ and 1% 2-mercaptoethanol and subjected to polyacrylamide gel electrophoresis (17). The proteins migrated on polyacrylamide gels were transferred to nitrocellulose paper and the AIDS retroviral proteins were identified by described methods (18).

Analysis of AIDS Retroviral Proteins. Four-day-old AIDS retrovirus-infected normal human T cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine (200 μCi/ml; 1 Ci = 37 GBq) in methionine- and cysteine-deficient RPMI 1640 medium containing 2% dialyzed fetal calf serum. The labeled cells were sedimented, washed twice with phosphate-buffered saline, and disrupted with lysis buffer containing 6 μl of aprotinin (Sigma) per ml, 1% $NaDodSO_4$, 0.1 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 50 mM phosphate, and 0.25% Triton X-100. An aliquot (100 μl) of ^{35}S-labeled lysate

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Abbreviations: AIDS, acquired immunodeficiency syndrome; ARC, AIDS-related complex; LAV, lymphadenopathy-associated virus; HTLV-III, human T-cell lymphotropic virus type III; ARV-2, AIDS-associated retrovirus type 2; LAS, lymphadenopathy syndrome.

was incubated with 3 μl of serum from an AIDS patient and the immunoprecipitates were recovered with 200 μl of protein A-Sepharose (Pharmacia). The immunoprecipitated proteins were subjected to NaDodSO$_4$/polyacrylamide gel electrophoretic analysis (17). Fluorographed gels were dried, exposed to Kodak XAR film, and developed after 48 hr.

Restriction Enzyme Analysis. The unintegrated viral DNA was prepared from 3-day-old AIDS retrovirus-infected normal human T cells by the procedure described by Hirt (19). The DNA (5–10 μg) was digested with restriction enzyme *Sac* I (New England Biolabs) and subjected to electrophoresis on 0.8% agarose gel. The DNA fragments were transferred to nitrocellulose paper (Schleicher & Schuell) and hybridized with nick-translated HTLV-III/LAV complete genome probe from AIDS retrovirus isolate 9 (S.G.D., unpublished results). The hybridization was performed at 42°C for 18 hr in a buffer containing 50% formamide, 5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate), 1× Denhardt's solution (0.02% bovine serum albumin/0.02% polyvinylpyrrolidone/0.02% Ficoll), 20 mM NaPO$_4$ (pH 6.5), 100 μg of salmon sperm DNA per ml, and 10% sodium dextran sulfate as described (20). The filters were washed three times at 65°C with 1× SSC, and the blots were exposed to Kodak XAR-5 film for 3 days.

Amino-Terminal Amino Acid Sequence Analysis. Amino acid sequence analysis was performed by automated Edman degradation on a gas-phase microsequencer from Applied Biosystems (Foster City, CA) as described (16, 21).

RESULTS

Comparison of AIDS Retroviruses by Competition Radioimmunoassay. The nine retrovirus isolates from six AIDS and three LAS patients were propagated in phytohemagglutinin-stimulated normal adult T cells. The viruses were designated as AIDS retrovirus numbers 1–9. Initial studies were undertaken to identify whether all the retroviruses isolated are bonafide members of the AIDS virus group. Advantage was taken of the fact that the major internal proteins of the retroviruses are more conserved than other retroviral proteins and that they share group specific antigenic determinants, which can be readily identified (22–25). We therefore tested the nine AIDS retroviruses for their immunologic cross-reactivity in a homologous competition immunoassay for the M_r 24,000 (p24) major internal protein isolated from LAV (15) and HTLV-III (16). The data presented (Fig. 1) represent the results from the competition immunoassay for LAV p24, although the results using HTLV-III p24 assay were indistinguishable from those for LAV p24 (data not shown). All the nine detergent-disrupted retrovirus isolates competed efficiently in the LAV p24 competition immunoassay (Fig. 1), thereby indicating that all these isolates are related to the LAV/HTLV-III group of viruses. The lack of competition by human T-cell leukemia virus type I (HTLV-I) and other known retroviruses (15, 16) established the specificity of this immunoassay. The shift in the competition curve for isolate 8 was due to the low titer of virus production by the cells. The presence of immunologically cross-reacting common antigenic determinants among the p24 of all the nine isolates confirmed the fact that they were AIDS retroviruses.

Restriction Enzyme Analysis. Restriction-site polymorphism provides a measure of differences in the viral genome. Although the above studies have shown that all the nine AIDS retrovirus isolates share common antigenic determinants in their major internal protein, it was of interest to evaluate whether differences exist in various regions of the viral genome. The restriction enzyme analyses of LAV, HTLV-III, and ARV-2 molecular clones (6–9) have shown that the *Sac* I enzyme cleaves the viral genome in the long terminal repeat sequences and that this site is conserved

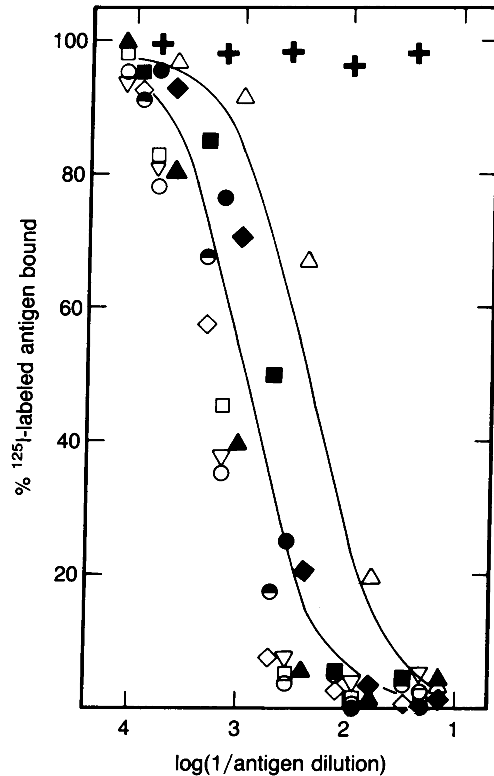

FIG. 1. Homologous competition immunoassay for LAV p24. Detergent-disrupted viral antigens from nine AIDS retroviruses, as well as HTLV-I, were tested at serial dilutions (1:2) for their ability to compete with ^{125}I-labeled LAV p24 in the binding of limiting amounts of rabbit anti-LAV (1:2000) as described in the text. Viruses tested included LAV (▽), AIDS retrovirus isolates 1 (□), 2 (○), 3 (●), 4 (◇), 5 (■), 6 (◓), 7 (◆), 8 (△), 9 (▲), and HTLV-I (+).

among these isolates. The Hirt DNA isolated from nine individual AIDS retrovirus-infected cells was subjected to digestion with the restriction enzyme *Sac* I and the DNA fragments generated were separated by agarose gel electrophoresis and analyzed by Southern blotting (26). Under stringent conditions of hybridization and washing and using complete molecularly cloned genome of one of the isolates (isolate 9) as the probe (S.G.D., unpublished results), we detected significant restriction-site polymorphism among the nine AIDS-retrovirus isolates (Fig. 2). However, the detection of the DNA fragments, even under high stringency of hybridization, also indicates substantial nucleic acid sequence homology among the virus isolates tested. The lack of substantial hybridization for isolates 7 and 8 may be due to a lower yield of Hirt DNA from the cells infected by these viruses. Similar experiments using *Eco*RI, *Hin*dIII, and *Bgl*II

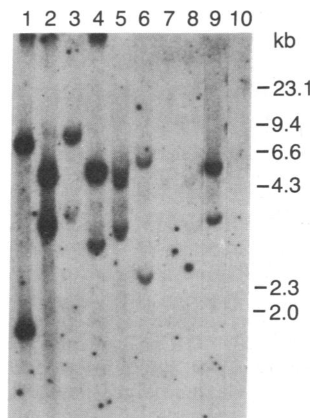

FIG. 2. Restriction enzyme analysis of retroviral unintegrated DNA. Southern blot analysis of the *Sac* I-digested Hirt DNA from nine AIDS retrovirus-infected cells was performed as described, using nick-translated complete genome from molecularly cloned isolate 9 as the probe. Lanes 1–9, AIDS retrovirus isolates 1–9; lane 10, uninfected normal human T cells. The migration of the λ *Hin*dIII-digested markers is indicated. kb, Kilobase(s).

restriction enzymes also confirmed existence of restriction site polymorphism (data not shown), indicating variation in viral genomic sequences among the nine AIDS retrovirus isolates. It was of interest to note that the restriction-site pattern for virus isolates 3 and 5 showed differences in DNA fragments generated by the enzyme *Sac* I, even though these retroviruses were derived from sex partners.

Metabolic Labeling and Immunoprecipitation Analysis of AIDS Retroviral Proteins. The polymorphism in the restriction sites of the viral genome may indicate changes in the translational products encoded by the virus. To evaluate such changes, one of the approaches is to metabolically label the viral proteins by growing the virus-infected cells in presence of [^{35}S]methionine and [^{35}S]cysteine and testing the cell lysates with serum from a patient with known high-titered antibodies to AIDS retroviral proteins. Using this approach, we and others have identified three glycosylated proteins with molecular weights around 160,000, 120,000, and 41,000 (gp41) and four nonglycosylated proteins with molecular weights around 55,000 (p55), 24,000 (p24), 18,000 (p18), and 15,000 (p15) for AIDS retroviruses (refs. 27 and 28; S.G.D., unpublished results). Similar experiments were performed by labeling the 4-day-old normal human T cells infected with each of the nine retrovirus isolates individually, and the labeled lysates were tested by precipitation with antibodies to AIDS virus from patients' antiserum. Under the labeling conditions used, the M_r 55,000 *gag* gene precursor (p55), gp41, and p24 could be readily visualized (Fig. 3*A*), although the intensities of some of the protein bands were not equal for all the isolates because of differences in the productivity of individual cell lines. In this experiment, significant heterogeneity was observed in the apparent molecular weight of p55, indicating that the variation in genomic sequences does result in alteration of *gag* gene-encoded polyprotein. Furthermore, isolates 3 and 5, which were obtained from sex partners, also showed variation in *gag* gene-encoded polyprotein. These results along with the variation in restriction sites in the genome of isolates 3 and 5 may indicate that the alteration in genome may take place after transmission of the virus from one partner to the other. However, the possibility of either of the partners getting exposed to retrovirus from another source cannot be excluded. The gp41 from the nine isolates also showed an alteration in the apparent molecular weight of the protein (Fig. 3*B*). These results provide evidence for significant heterogeneity in the proteins encoded by the nine AIDS retroviruses.

Immunoblot Analysis of AIDS Retroviral Structural Proteins. The heterogeneity in the AIDS retroviral structural proteins could be further visualized by immunoblot analysis of the density gradient-banded AIDS retrovirus isolates. For this purpose, the proteins from nine individual isolates as well as other known retroviruses including HTLV-I and HTLV-II were subjected to NaDodSO$_4$/PAGE followed by immunoblot analysis. The blots were incubated with antibodies to AIDS retrovirus from the patient's antiserum who had high-titered antibodies to viral structural proteins. The proteins on the blots recognized by these antibodies were visualized by using horseradish peroxidase-conjugated goat anti-human IgG and the peroxidase substrate. The specific proteins, including gp41, p24, and p18, could be readily identified for each of the nine AIDS retrovirus isolates (Fig. 4), whereas the blots of other known retroviral proteins tested, including HTLV-I and HTLV-II, lacked detectable reactivity with the AIDS patient antiserum (data not shown). The AIDS retroviral proteins showed significant variation in apparent molecular weight, especially in p18 derived from the *gag* gene-encoded polyprotein (Fig. 4). These results corroborate those observed by metabolic labeling and immunoprecipitation analysis shown above.

Amino-Terminal Amino Acid Sequence Analysis. To best identify specific changes in the viral genome, it may be necessary to molecularly clone each of the AIDS viral genomes and determine the primary nucleotide sequence. While these studies would require significant time to perform, we have attempted amino-terminal amino acid sequence analysis of p24 from isolate 9 (Fig. 5). The p24 was purified by using described methods (15, 16) and was subjected to amino acid sequence determination. In the first 30 amino-terminal amino acids sequenced, we could identify one change, from isoleucine to leucine in position 6, compared to

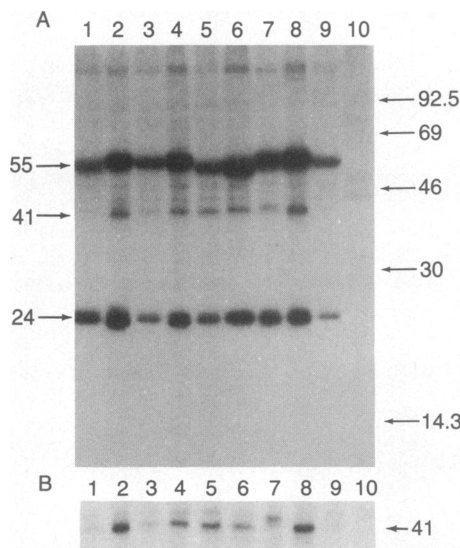

FIG. 3. NaDodSO$_4$/PAGE analysis of ^{35}S-labeled proteins from AIDS retroviruses. AIDS retrovirus-infected cells were labeled with [^{35}S]methionine and [^{35}S]cysteine as described in the text. The labeled cell lysate was incubated with antiserum from an AIDS patient, and the immunoprecipitates were recovered by protein A-Sepharose and subjected to NaDodSO$_4$/PAGE analysis on 12% (*A*) or 7.5% (*B*) polyacrylamide gel. Lanes 1–9, lysate from AIDS retrovirus 1–9 infected cells; lane 10, uninfected normal human T cells.

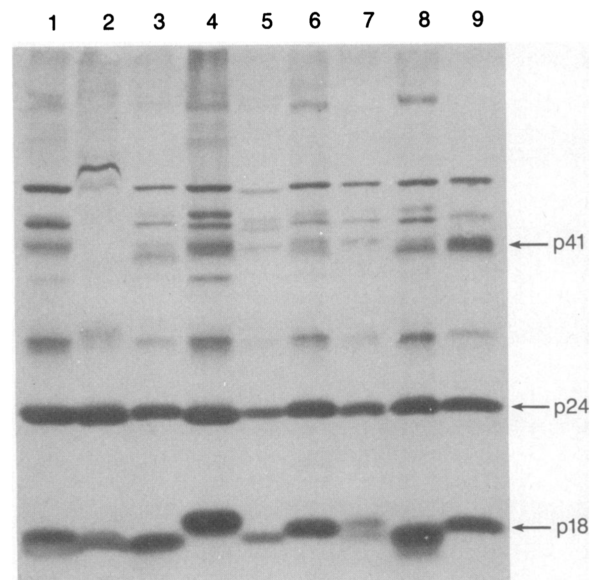

FIG. 4. Immunoblot analysis of AIDS retroviruses. Density gradient-banded retroviruses were disrupted and subjected to immunoblot analysis as described (18). The blots were exposed to antiserum from an AIDS patient (1:2000 dilution), and the immune complexes were visualized with the aid of horseradish peroxidase-conjugated human IgG. Lanes 1–9, AIDS retrovirus isolates 1–9.

					5					10					15
HTLV-III	Pro	Ile	Val	Gln	Asn	Ile	Gln	Gly	Gln	Met	Val	His	Gln	Ala	Ile
CDC-451	Pro	Ile	Val	Gln	Asn	Leu	Gln	Gly	Gln	Met	Val	His	Gln	Ala	Ile

					20					25					30
HTLV-III	Ser	Pro	Arg	Thr	Leu	Asn	Ala	Trp	Val	Lys	Val	Val	Glu	Glu	Lys
CDC-451	Ser	Pro	Arg	Thr	Leu	Asn	Ala	Trp	Val	Lys	Val	Val	Glu	Glu	Lys

FIG. 5. Amino-terminal amino acid sequence analysis of major internal protein from AIDS retrovirus isolate 9. The amino-terminal amino acid sequence of the p24 from isolate 9 was determined as described (16, 21). The sequence was compared with that of HTLV-III p24 (16). The amino acid residues that differ are enclosed in a box.

p24 of LAV/HTLV-III. Although very conservative, it would involve a change of pyrimidine nucleotide cytosine to the purine adenosine in the genomic sequence. The conservation of most of the first 30 amino acid sequences in isolate 9 is not very surprising, since the major internal proteins of the retroviral isolates from various species of animals are also known to have conserved sequences (29, 30).

DISCUSSION

The studies targeted toward developing systems for detection of infection, pathogenesis, and, ultimately, eradication of AIDS by therapeutic agents and vaccine development hinge on our understanding of the variation in the AIDS retroviral genome. There have been several isolates reported to date derived from AIDS/ARC patients (1–5). Viral genomes of three AIDS retroviruses have been studied in detail at the primary nucleotide sequence level (10–12). These studies have demonstrated that at least two of the independent AIDS retroviruses, LAV and HTLV-III, share extensive sequence homology in their genome. In contrast, the third isolate, ARV-2, showed considerable divergence in its sequence compared to LAV/HTLV-III. Studies involving a large number of individual isolates are therefore essential to evaluate the extent of divergence in various AIDS retrovirus isolates and whether this divergence would be reflected in its translation products. In the present studies, we have isolated nine retroviruses from AIDS patients. An attempt was made to analyze these isolates to detect variation in their genome and the proteins encoded by the virus.

All the nine AIDS retroviruses tested in the present studies shared common antigenic determinants in their major internal structural protein, p24. However, the genome of these viruses showed significant polymorphism by restriction enzyme analysis using unintegrated viral DNA. It has been reported recently that substantial heterogeneity exists among individual AIDS retrovirus isolates when analyzed for restriction site variations (31, 33). Our data corroborate these published observations. Whether the changes in the genomic sequence result in the changes of the translational products encoded by the viruses is a very important question. Generally, changes in restriction sites in a genome are a significant measure for divergence in the primary structure of the protein. However, in many instances, (for example, the third base changes in the coding sequence), a change in the restriction sites may not alter the amino acids coded for by the particular sequence. It was therefore of importance to evaluate the proteins encoded by the AIDS retroviruses that exhibit genomic restriction site polymorphism.

The present studies have demonstrated that there are changes in the translational products encoded by the nine individual AIDS retrovirus isolates, as indicated by restriction site polymorphism in the genome. We could detect apparent differences in the molecular weights of the proteins encoded by *gag* genes (precursor p55 and product p18), and *env* genes (gp41). These differences in apparent molecular weight of the proteins may be due to substitution in amino acid sequence or, alternatively, may reflect small deletions or additions in the coding sequences. The nucleotide sequence data from the known AIDS retrovirus isolates have shown that the unmodified precursor product of the *gag* gene contains 512 amino acids in HTLV-III (11) and 500 amino acids in LAV (10), while ARV-2 contains 502 amino acids (12). The difference in these three retroviral *gag* gene precursors is attributed to a deletion of 12 amino acids in LAV and ARV-2 in the p15 part of the polyprotein compared to HTLV-III. These data are in agreement with the heterogeneity of the *gag* gene precursor in the present studies, although more specific evidence is essential to evaluate various additions, deletions, or substitutions in the amino acid sequences of various isolates. The sequencing data also indicated existence of significant amino acid substitutions in the *env* gene sequences among ARV-2 compared to HTLV-III/LAV (10–12). Furthermore, restriction enzyme mapping and heteroduplex analysis of two variant HTLV-III isolates have been shown to exhibit the greatest divergence in their envelope gene (31). Our results indicate that the apparent molecular weight of gp41, one of the products encoded by the *env* gene, does show variation in its mobility. Envelope protein is the first protein exposed to immune surveillance in individuals exposed to retroviruses (34, 35). Furthermore, it is also the protein that plays an important role in recognition of receptor and tropism (36–38). Thus, the variation in *env* gene proteins may have significant implications in pathogenicity of the virus.

AIDS retroviruses have been shown to share antigenic determinants with equine infectious anemia viral (EIAV) proteins (16, 39). Heteroduplex analysis and the morphology of budding particles during replication have shown that HTLV-III is closely related to visna virus (40). More recently, the primary nucleotide sequence analysis of molecularly cloned visna virus has shown the existence of sequence homology with that of LAV/HTLV-III (41). Both EIAV and visna viruses are members of the lentivirus family, whose sequences are known to change considerably during progression of infection (42, 43), presumably to avoid immune surveillance by the host. It is not yet known whether similar mechanisms are operative during AIDS retrovirus infection, but the variation in the genomic structure and the alteration of proteins encoded by the viral genome may be quite important. Our data on the AIDS virus isolation from sex partners that have differences in *Sac* I cleavage pattern and apparent molecular weight differences in the structural proteins suggest that the changes in the genome following transmission may occur *in vivo*. However, it is difficult to exclude the possibility that either one of the partners might have acquired two isolates that were initially different rather than make the assumption that one transmitted it to the other. These observations corroborate the data recently published by Wong-Staal *et al.* (32), which indicate that an individual may have had multiple exposures to various AIDS retroviruses, but that only one predominant form of the virus may replicate in the host. Further studies are necessary to conclusively prove this observation.

Whether certain regions of the viral genome are more conserved than the others during the process of divergence needs to be evaluated, and whether the sequence changes would lead to alteration in antigenic determinants has not yet been elucidated. These studies would be plausible after determination of the primary nucleotide sequences of several AIDS retroviruses and development of specific reagents such as monoclonal antibodies to various epitopes of the structural proteins. The preliminary nucleotide sequence analysis data on the molecularly cloned isolate 9 used in the present studies indicate that there are significant changes in the envelope

glycoprotein gene of the virus. Based on the comparison with sequences of the other known AIDS retroviruses, the amino-terminal region of the envelope gene encoding gp120 showed a much higher number of changes than the gp41, which is more conserved (S.G.D., unpublished results). These data would be useful in identification of the sequences that possess conserved antigenic determinants, their role in pathogenicity, and, ultimately, in design of therapeutics and vaccine.

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