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Cross-reactivity to human T-lymphotropic virus type III/lymphadenopathy-associated virus and molecular cloning of simian T-cell lymphotropic virus type III from African green monkeys

(simian retrovirus/unintegrated viral DNA/acquired immunodeficiency syndrome/nucleic acid hybridization)

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ABSTRACT **Simian T-lymphotropic retroviruses with structural, antigenic, and cytopathic features similar to the etiologic agent of human acquired immunodeficiency syndrome, human T-lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV), have been isolated from a variety of primate species including African green monkeys (STLV-III$_{AGM}$). This report describes nucleic acid cross-reactivity between STLV-III$_{AGM}$ and HTLV-III/LAV, molecular cloning of the STLV-III$_{AGM}$ genome, and evaluation of its structure and genetic relationship to other retroviruses. Overlapping clones from a cell line infected with virus from a single animal were found to encompass the entire STLV-III$_{AGM}$ genome and exhibit a limited degree of restriction-site variability. Specific hybridizing fragments were detected in DNA from this and other STLV-III$_{AGM}$-infected cell lines. A fraction of viral DNA present in at least two STLV-III$_{AGM}$ lines persists as unintegrated viral DNA, a characteristic of infection with cytopathic retroviruses. Strongest cross-reactivity was detected between HTLV-III/LAV *pol*- and *gag*- genes and STLV-III$_{AGM}$, whereas no cross-reactivity was detected between STLV-III$_{AGM}$ and molecular clones of human T-lymphotropic virus types I and II (HTLV-I and -II), visna virus, bovine leukemia virus, or feline leukemia virus.**

A group of exogenous type C retroviruses has recently been described in a variety of nonhuman primate species. The first one, simian T-lymphotropic virus type I (STLV-I) (1–3), was identified in Japanese macaques by virtue of its immunologic cross-reactivity with the etiologic agent of adult T-cell leukemia, the human T-lymphotropic virus type I, HTLV-I (4). HTLV-I and STLV-I are associated with spontaneous lymphoid malignancies in humans (4) and primates (5, 6), respectively, and share ≈90% nucleic acid homology over the 3′ 3 kilobases (kb) of their genomes (7) as well as the ability to immortalize human T4 lymphocytes *in vitro* (8, 9). Similarly, STLV-III viruses, isolated from Old World primates, such as African green monkeys (10, 11) and other primate species (12–15), were identified by virtue of their antigenic cross-reactivity with human T-lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV) (16–19).† The type III viruses of human and simian origin have similar ultrastructural morphology and Mg^{2+}-dependent reverse transcriptase (11, 13). Likewise, radioimmunoprecipitation analysis of STLV-III-infected cell lines demonstrates proteins of similar size and cross-reactive with the major *gag*-, *pol*-, *env*-, and *3′orf*-encoded proteins of HTLV-III/LAV (10–12, 20). Thus, there is likely to be structural homology between type III viruses at the protein level, which may be useful in development of vaccines. HTLV-III/LAV and STLV-III viruses also share a tropism and are cytopathic for human T4 lymphocytes in culture (11–17), suggesting that study of STLV-III may yield insight into mechanisms of disease induction relevant to human acquired immunodeficiency syndrome (AIDS).

STLV-III was first identified in captive, ill rhesus monkeys (*Macaca mulatta*) in the United States (STLV-III$_{mac}$) (12, 13) and a very similar virus was found in a large proportion of healthy African green monkeys (*Cercopithecus aethiops*) in Africa (STLV-III$_{AGM}$) (10, 11). STLV-III$_{mac}$ has been associated with an AIDS-like syndrome in macaques (21), whereas no disease association has been demonstrated for STLV-III$_{AGM}$ in African green monkeys (10) or in sooty mangabeys (15). A human virus, also without known pathogenicity, has recently been described in healthy individuals residing in West Africa (22, 23). This virus, designated HTLV-IV, is related to HTLV-III/LAV. However, it bears stronger immunological cross-reactivity and ultrastructural similarity to STLV-III$_{AGM}$ (22, 23), suggesting that STLV-III$_{AGM}$ and HTLV-IV originated from a common ancestor in relatively recent evolutionary time.

Further investigation into the pathogenesis of STLV-III$_{AGM}$, its relationship to other known retroviruses, and its potential utility in vaccine studies would be advanced by definition of the virus at the genetic level. Toward this end, we investigated the DNA homology between a molecular clone of HTLV-III/LAV and viral sequences present in K2/78, a STLV-III-producing cell line established by cocultivation of uninfected Hut 78 cells with peripheral blood lymphocytes of a healthy seropositive African green monkey (11).

MATERIALS AND METHODS

Cell Lines. Establishment of Hut 78 cell lines infected with STLV-III$_{AGM}$ (11) and type D (New England) retrovirus (24) used in this study have been described. The HTLV-III-infected Molt 3 cell line used was provided by R. C. Gallo.

Hybridization Probes. DNA probes used in this study include BH10, a close to full-length HTLV-III/LAV clone (25), and three subgenomic probes that were derived from

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Abbreviations: STLV-III, simian T-lymphotropic virus type III; HTLV-I, -II, and -III, human T-lymphotropic virus types I, II, and III; LAV, lymphadenopathy-associated virus; AIDS, acquired immunodeficiency syndrome; kb, kilobase(s); LTR, long terminal repeat.

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†The members of a subcommittee empowered by the International Committee on the Taxonomy of Viruses to propose an appropriate name for the retrovirus isolates recently implicated as the causative agents of the acquired immunodeficiency syndrome have proposed the name human immunodeficiency virus, to be abbreviated HIV [(1986) *Science* **232**, 697, and *Nature (London)* **321**, 10].

BH10. The origin of these probes is diagrammed in Fig. 2. They include RH2, a 2-kb *Eco*RI/*Hin*cII fragment from the *pol*$^-$ gene; HD, a 0.5-kb *Hin*dIII fragment derived from the *gag*$^-$ gene; and HC, a 1.5-kb *Hin*dIII/*Sst* I fragment, which includes 500 base pairs of *env*, all of 3′ *orf* and 490 base pairs of the 3′ long terminal repeat (LTR). The STLV-III$_{AGM}$-derived probes are described in *Results*. Cloned DNA probes were gel-purified, ^{32}P nick-translated to a specific activity of 8×10^8 dpm/μg, and used at a concentration of 1×10^6 dpm/ml as described (26).

Filter Hybridization. Restriction enzyme analysis was carried out with 1 μg of cloned DNA or 20 μg of cellular genomic DNA. Digests were separated on 1% agarose gels and transferred to nitrocellulose filters. Hybridizations at low stringency were carried out at 37°C in a mixture containing 25% formamide, 10% dextran sulfate, 0.8 M NaCl, 0.1 M Mops, 0.1% sodium lauroyl sarkosine, denatured herring sperm DNA (100 μg/ml) (pH 7), and 0.6% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll (26). These conditions correspond to t_m −43°C according to the formula $t_m = 81.5 + 16.6 (\log Na^+) + 0.41$ (38% GC in the RH2 fragment) − 0.7 (% formamide) (27). After hybridization, filters were washed at room temperature in four changes of 5× SSC (0.75 M NaCl/0.07 M sodium citrate) plus 0.05% sodium pyrophosphate and 0.1% sodium lauroyl sarkosine. Hybridizations at high stringency (approximate t_m, −23°C) were carried out at 42°C for 20 hr in a similar mixture containing 50% formamide. Filters were washed in 0.2× SSC (0.03 M NaCl/0.003 M sodium citrate) plus 0.05% sodium pyrophosphate and 0.1% sodium lauroyl sarkosine at 50°C. All filters were exposed to Kodak XAR film at −70°C.

Recombinant DNA Library Construction and Screening. K2/78 DNA was submitted to limited *Mbo* I digestion and 8 to 20-kb fragments were selected after centrifugation in a 10–40% sucrose gradient (28). Fractionated eukaryotic DNA was ligated into *Bam*HI-digested bacteriophage λ J1 (26) and λ L47.1 (29) vector arms, packaged *in vitro* (Gigapack from Vector Cloning Systems), and 3×10^6 recombinant phage were plated and screened under low stringency hybridization conditions after transfer to duplicate nitrocellulose filters. Filters were then exposed to Kodak XAR film with an intensifying screen for 6–12 hr at −70°C.

RESULTS

Detection of HTLV-III/LAV-Related Sequences. Given the demonstrated immunologic cross-reactivity between HTLV-III/LAV and STLV-III, we wished to determine whether HTLV-III/LAV-related sequences could be detected in STLV-III$_{AGM}$-infected cell lines. DNA from one such cell line, K2/78, was digested with various restriction enzymes and probed with BH10, a close to full-length HTLV-III clone (25). Specific hybridizing bands were detected in *Pst* I- and *Hin*dIII-digested DNA at a hybridization stringency of t_m −43°C but not at a t_m of −31°C or higher, corresponding to an approximate sequence homology of 55–70% in the region of cross-reactivity within these fragments. A subgenomic fragment of BH10 from the polymerase region (RH2) hybridized to the same *Pst* I fragment in K2/78 DNA at the same stringency (Fig. 1), and it was used to probe recombinant bacteriophage libraries derived from K2/78.

Cloning of the STLV-III$_{AGM}$ Provirus. Genomic libraries of K2/78 DNA were screened and washed under conditions of low stringency. Six positive clones were obtained following tertiary screening of 3×10^6 recombinant phage. Each clone was analyzed by restriction enzyme mapping and hybridization to BH10 and the subgenomic HTLV-III/LAV probes RH2 (*pol*), HC (3′ *env* LTR), and HD (*gag*). There are no evident restriction-site similarities between BH10 and STLV-III, so alignment to the gene map of HTLV-III/LAV is approximate. As shown in Fig. 2, no one clone contains the entire STLV-III$_{AGM}$ genome predicted by analogy to HTLV-III/LAV. However, restriction enzyme and hybridization analysis reveals overlapping clones colinear with the entire length of HTLV-III/LAV (Fig. 2). The STLV-III$_{AGM}$ clones described represent at least three unique proviruses, as indicated by restriction-site differences in flanking regions. We identify a limited number of polymorphic sites in our clones (encircled sites in Fig. 2) but find a single predominant proviral form in infected cell DNA (data not shown) corresponding to the consensus map at the bottom of Fig. 2.

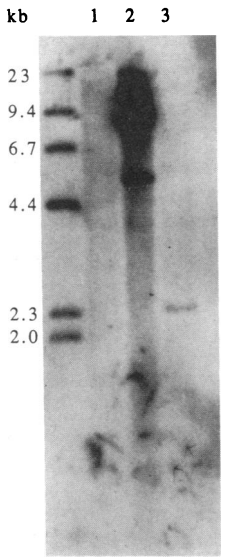

FIG. 1. Nucleic acid homology between HTLV-III/LAV and STLV-III$_{AGM}$. Total cellular DNA (20 μg) from uninfected Hut 78 cells (lane 1), HTLV-III/LAV-infected Molt 3 cells (lane 2), and STLV-III$_{AGM}$-infected Hut 78 cells (lane 3) was digested with *Pst* I, separated on a 1% agarose gel, transferred to nitrocellulose, and hybridized with RH2 under conditions of low stringency.

Variable degrees of hybridization to BH10 were observed in different regions of the STLV-III$_{AGM}$ genome. As shown in Fig. 3 and represented by the shaded bar at the bottom of the figure, the most intense hybridization was observed to the predicted *gag* and *pol* genes and moderate hybridization intensity was observed in the 3′ 1 kb of the genome in a region corresponding to the *3′orf* of HTLV-III/LAV. Weak hybridization was detected in the region that aligns with the *env* gene of HTLV-III/LAV. HC hybridized to the 3′ 1 kb with intensity similar to that seen with BH10 and weakly to the 5′-terminal region of three clones, indicating weak cross-reactivity within LTRs. Furthermore, fragments derived from the predicted terminal regions of proviruses from two overlapping STLV-III$_{AGM}$ clones also hybridize to the terminal region of each of the other provirus clones (Fig. 2), confirming the presence of an ≈10-kb provirus with LTRs. The 5′ LTR probe K2-XbaA hybridized strongly to the 3′ internal *Hin*cII fragment near the 3′ end of the provirus but not to the adjacent fragment extending into the human flanking regions. This suggests that the *Hin*cII site is located close to the 3′ end of the LTR.

STLV-III$_{AGM}$-Related Sequences in Other STLV-III-Infected Cell Lines. An RH2 cross-reactive fragment from clone 2 (K2-XbaB) was used to investigate sequence homology between the K2/78-derived provirus and DNA from cells infected with other simian retroviruses. Cross-reaction at high stringency was detected in *Pst* I-digested DNA from the K2/78 cell line (2.3- and 1.6-kb bands in Fig. 4*A*) and also in Hut 78 cell lines infected with five independent isolates of STLV-III$_{AGM}$ (data not shown). The 2.3-kb band corresponds to that detected in K2/78 DNA probed at low

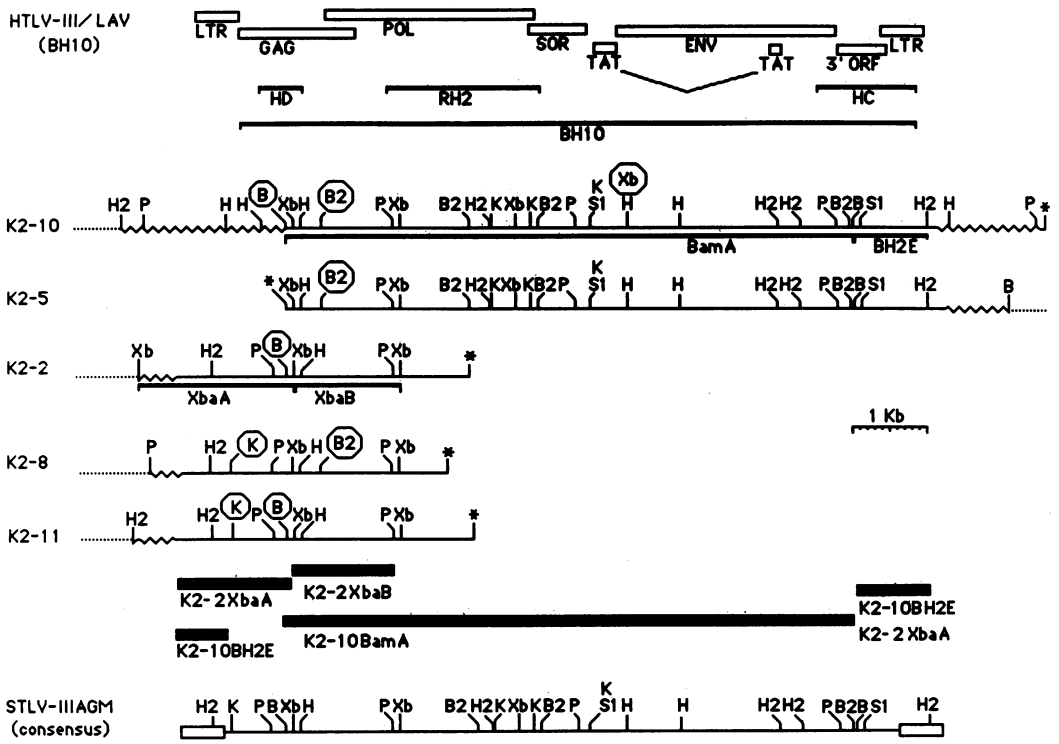

FIG. 2. Restriction endonuclease site map of STLV-III$_{AGM}$ provirus clones from K2/78 cell DNA. Five recombinant clones were analyzed and their inserts were mapped with the enzymes indicated. A sixth clone containing only a very short region of the STLV-III$_{AGM}$ genome was not mapped. A gene map and schematic representation of the HTLV-III/LAV genome is indicated in the upper part of the figure to show the origin of the BH10-derived probes HC, HD, and RH2 (thick lines). Wavy lines indicate regions containing human DNA, and dotted lines indicate extension of the clone beyond the boundaries of the map shown. Restriction enzyme sites not found in all proviruses are circled. Regions of hybridization with STLV-III$_{AGM}$-derived probes are indicated below the STLV-III$_{AGM}$ maps. A consensus map representing the majority of proviruses in this line determined by blot analysis of total genomic DNA (data not shown) and approximate positions of the LTRs is diagrammed at the bottom of the figure. B, *Bam*HI; B2, *Bgl* II; K, *Kpn* I; H, *Hin*dIII; H2, *Hin*cII; P, *Pst* I; SI, *Sst* I; Xb, *Xba* I.

stringency with RH2 (see Fig. 1). In contrast, no hybridization was detected in uninfected Hut 78 cells or in cells infected with simian type D (New England) retrovirus (24). Other regions of the predicted STLV-III$_{AGM}$ provirus were subcloned (Fig. 2) and also found to correspond to exogenous sequences present in STLV-III-infected cells but not in uninfected Hut 78 cells (data not shown). Our results strongly suggest that these clones correspond to the STLV-III$_{AGM}$ genome.

Unintegrated Viral DNA in STLV-III-Infected Cell Lines. Unintegrated viral DNA is a normal intermediate in retrovirus replication, which precedes provirus integration and is usually detectable only 2–10 days after infection *in vitro* (30). Transient high levels of unintegrated viral DNA have been observed in cells infected with cytopathic avian retroviruses (31) and persistent levels of unintegrated viral DNA have been detected in cells infected with the ovine lentivirus visna (32), the human AIDS virus (33), and the feline AIDS virus (34), leading to the suggestion that persistence of unintegrated viral DNA is indicative of cytopathic retrovirus infection. We therefore examined the state of virus integration in cells infected for >6 months with STLV-III$_{AGM}$, which is cytopathic for human T lymphocytes *in vitro* (11). As shown in Fig. 4*B*, at least two of five isolates of STLV-III$_{AGM}$ have persistent levels of 10-kb unintegrated linear viral DNA, in addition to a high molecular weight smear of integrated proviral DNA.

Homology Between STLV-III and HTLV-III/LAV and Other Type C Retroviruses. Homology of STLV-III$_{AGM}$ to other retroviruses was studied by clone on clone hybridization using STLV-III$_{AGM}$-derived probes. As shown in Fig. 5, we detected homology between the STLV-III$_{AGM}$ K2-XbaB fragment and the *pol* region of HTLV-III/LAV at approximate t_m −36°C or lower (63–77% homology over this region of the genome). In contrast, no cross-reactivity was detected to HTLV-I, HTLV-II, visna virus (Fig. 5), or bovine leukemia virus and feline leukemia virus (data not shown) at stringencies as low as approximate t_m −42°C. The same result was obtained using a STLV-III$_{AGM}$ *gag* probe and combined probes encompassing the entire genome (data not shown). In addition, the HTLV-III/LAV *pol* probe RH2, which hybridizes to STLV-III$_{AGM}$ at t_m −36°C, did not hybridize to HTLV-I, -II, or visna virus at this same stringency. Therefore, STLV-III$_{AGM}$ and HTLV-III/LAV are more closely related than either is to the other retroviruses examined.

DISCUSSION

We have extended parallels previously drawn between the human AIDS virus and STLV-III by demonstrating that they share detectable homology and a unique genetic relationship. The strongest homology detected extends to regions in *pol*, *gag*, and *3'orf*. Regions in which we observed nucleic acid homology are also those that presumably encode viral proteins previously shown to be cross-reactive with antisera to HTLV-III/LAV viral proteins (10–13). In contrast, we find no demonstrable homology between STLV-III and other viruses examined notably visna, indicating that STLV-III and HTLV-III/LAV likely diverged later in evolutionary time than did HTLV-III/LAV and visna. Our results indicate that when comparing viruses that have previously been shown to share a structural or immunologic relationship, STLV-III is the closest known relative of HTLV-III/LAV. The degree of homology is clearly less between STLV-III$_{AGM}$ and HTLV-III/LAV than it is between HTLV-I and STLV-I (90% homology over the 3' third of the genome) (7). The moderate

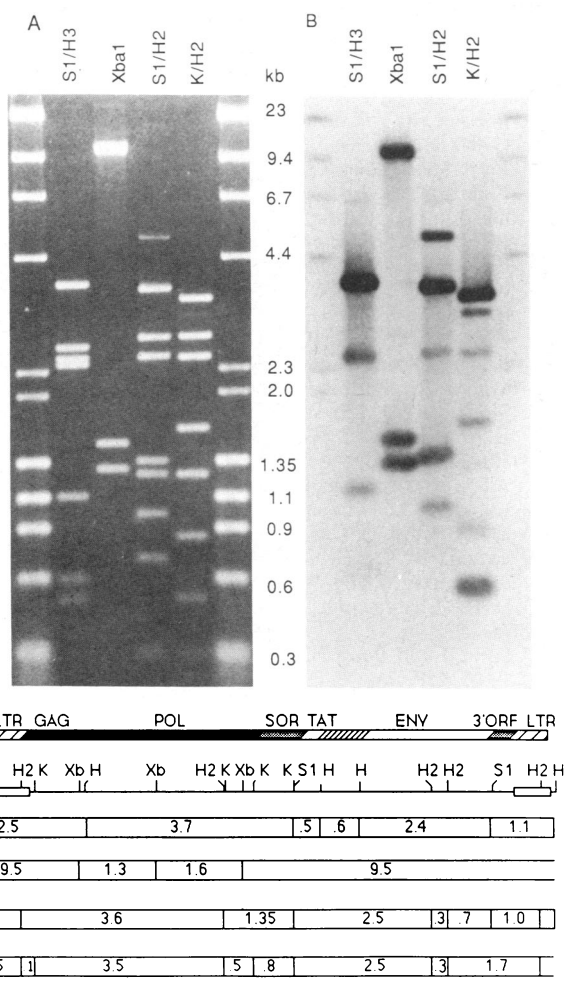

FIG. 3. Homology between HTLV-III/LAV (BH10) and an STLV-III$_{AGM}$ plasmid clone. The STLV-III clone was reconstructed from λ clones (K2-5 and K2-11) diagrammed in Fig. 2 and has a restriction enzyme map identical to the consensus map. An ethidium bromide-stained gel of 1 μg of this clone digested with *Sst* I/*Hin*dIII, *Xba* I, *Sst* I/*Hin*cII, and *Kpn* I/*Hin*cII is represented in *A* and the Southern blot of this gel after hybridization with BH10 is shown in *B*. The variable degree of hybridization across the genome is indicated by the cross-hatched bar at the center of the figure; the darker the cross-hatching, the greater the degree of hybridization. The predicted fragment sizes of each digest are diagrammed at the bottom of the figure. The fragments at 5 kb in the S1/H2 digest and 3.4 kb in the K/H2 digest are the result of partial digestion. The 2.7-kb band that does not hybridize in the S1/H3, S1/H2, and K/H2 digests is the plasmid vector pUC18.

homology between STLV-III$_{AGM}$ and HTLV-III/LAV suggests that neither corresponds to an immediate precursor in recent evolutionary time. However, estimation of evolutionary distance between retroviruses is difficult because of variation in replication rate, immune selection, or potential differences in the fidelity of reverse transcription, all of which affect the stability of the viral genome. These variables may become especially significant when considering HTLV-III/LAV and related viruses because of the considerable genomic diversity observed among different isolates of HTLV-III/LAV (37).

As an animal model for human AIDS, it will be interesting to determine whether STLV-III has genomic features similar to HTLV-III/LAV; that is, the presence of unique open reading frames such as TAT, SOR, and 3′ orf, some of which are intrinsic to the function of HTLV-III/LAV (38–40). Radioimmunoprecipitation studies (10, 12) and the hybridization results reported here are consistent with a region within STLV-III that is analogous to and cross-reactive with

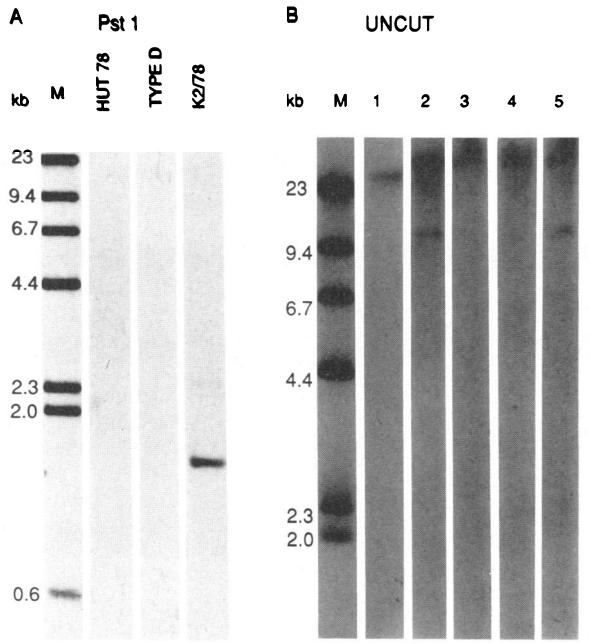

FIG. 4. Analysis of STLV-III$_{AGM}$ in various cell lines by hybridization with a STLV-III$_{AGM}$ *pol* region probe (K2-XbaB) under stringent conditions. (*A*) Genomic DNA (20 μg) from uninfected Hut 78 cells, Hut 78 cells infected with macaque type D (NE) retrovirus, and the STLV-III$_{AGM}$ cell line K2/78. (*B*) DNA (20 μg) from five STLV-III$_{AGM}$ cell lines analyzed without prior digestion.

3′ orf. Molecular clones of STLV-III$_{AGM}$ will allow us to test for analogous genes and to evaluate the function of this virus for such properties as trans-activation (40).

STLV-III isolated from African green monkeys and macaques appear to be closely related viruses. The observation of unintegrated viral DNA in the STLV-III$_{AGM}$ cell line is

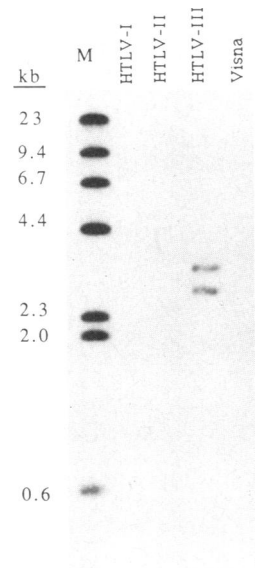

FIG. 5. Sequence homology of STLV-III$_{AGM}$ to other retroviruses. λ clone DNA (1 μg) containing HTLV-I, HTLV-II, HTLV-III/LAV, and visna were hybridized at three levels of stringency with the STLV-III$_{AGM}$ pol probe K2-XbaB. Clones of prototype HTLV-I (UK1) (35), HTLV-II (Mo15) (36), HTLV-III/LAV (BH10) (33), and visna (32) were digested with the following enzymes: *Sma* I (HTLV-I); *Bam*HI (HTLV-II); *Hin*cII (HTLV-III); and *Hin*dIII (visna) and separated on 1% agarose gels. Duplicate filters were prepared and hybridized for 20 hr to nick-translated *Xba* B, in 25% formamide. The approximate hybridization stringency used corresponds to a t_m of −42°C.

consistent with its previously observed cytopathic effect *in vitro* and may indicate a potential for *in vivo* cytopathicity. Apparent differences in disease association have been noted; that is, an association with immunodeficiency disease in macaques but not African green monkeys. This could be the result of subtle genetic differences between the viruses, a species-specific host response, or some combination of both factors. STLV-III_{AGM} clones will be useful as a probe to clone the STLV-III_{mac} genome and explore any differences that may exist at a genetic level. Although our clones are incomplete, reconstruction of a biologically active full-length clone is feasible and useful for the establishment of biologically pure reagents for subsequent inoculation studies essential for elucidating its pathogenesis.

Finally, molecular clones of STLV-III_{AGM} can be used to investigate its genetic relationship to the recently described human retrovirus, HTLV-IV.

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