Characterization of a Novel Baboon Virus Closely Resembling Human T-Cell Leukemia Virus

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We report the isolation of a virus from a baboon imported from Kenya and the analysis of the nucleotide sequence of the env gene. Comparison of the complete nucleotide sequence of the env gene of different HTLV-1 strains and the baboon T-cell leukemia virus (designated BTLV) indicated similarities ranging from 92.5 to 97.4%. In contrast, only 89.1% similarity was observed between the BTLV env sequence and that of simian T-cell leukemia virus (PtM3). The sequences corresponding to the glycosylation sites, endoproteolytic processing site, and major immunological determinants were strictly conserved between BTLV and HTLV-1. To characterize the expressed protein we used a vaccinia expression system, which indicated that a protein of 62 kDa is encoded by the envelope gene. The protein acquired mostly high mannose modifications and was localized predominantly in the endoplasmic reticulum. A fraction of the protein was expressed at the cell surface, where it could induce membrane fusion of target cells. The existence of HTLV-1-like viruses in baboons indicates the potential risk of transmission of such virus from these nonhuman primates to humans, thus highlighting the need for specific screening for such viruses during xenotransplantation.

INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1) is a C-type retrovirus associated with adult T-cell leukemia/lymphoma (ATL) (Poiesz et al., 1980). ATL is characterized by the malignant proliferation of CD4+ T lymphocytes with symptoms including hypercalcemia, lymphadenopathy, skin lesions, involvement of the liver or spleen, and immunodeficiency (Stewart et al., 1994). In addition, HTLV-1 infection is implicated in a demyelinating disorder, tropical spastic paraparesis (Furukawa et al., 1992), and other conditions including alveolitis and arthritis. Simian T-cell leukemia virus type 1 (STLV-1) shares many properties with HTLV-1 and is associated with the development of lymphomas in gorillas and macaques, and leukemia in African green monkeys (Homma et al., 1984; Schatzl et al., 1992). HTLV-1 and STLV-1 share common molecular features as well as pathogenic properties. The genomic organization of both viruses is similar, consisting of LTR–gag–pol–env–tax/rex–LTR. Sequence analyses demonstrate that the genomes exhibit an overall similarity of 90–95% (Watanabe et al., 1986).

Although data are available on the nucleotide sequences of envelope genes from several HTLV and STLV isolates, not much information is available on the properties of the proteins from viruses isolated from nonhuman primates. In this report, we describe the isolation of a novel virus from a baboon (Papio anubis) imported from Kenya. The nucleotide sequence of the envelope gene and the properties of the protein encoded by the gene have been determined. The nucleotide sequences have been compared with available sequences and the relatedness between different HTLV and STLV isolates and the possible evolutionary features are discussed.

MATERIALS AND METHODS

Cells, viruses, and expression plasmid

HeLa T4 cells (Maddon et al., 1986) and vaccinia virus vTF7-3 (Fuerst et al., 1986) that synthesizes the T7 RNA polymerase in infected cells were obtained through the AIDS Research and Reference Reagent Program and used for the expression studies. The gene encoding the envelope glycoprotein of baboon T-cell leukemia virus (BTLV) was cloned under the control of the T7 promoter in the expression vector pcDNA-1 Amp (Invitrogen). Antibodies used in this study were 1621 antiserum obtained from the BTLV-infected baboon or a monoclonal antibody to HTLV-1 (0.5 Alpha supernatant) obtained through the AIDS Research and Reference Reagent Program (Mat-sushita et al., 1986).

Cloning of the BTLV envelope gene from genomic DNA

Using primers (STLV-env1 5’ CCGATCCCAAGAA- AAGACACCAC 3’ and STLV-env2 5’ GTTACTATTT- GCAGGGGAGCTG 3’) which are conserved for both HTLV-1 and STLV-1, PCR amplification was carried out...
using 2 units of Vent polymerase (New England Biolabs) with 0.1 \( \mu \)g of the genomic DNA from a cell line developed from the infected baboon and each of the primers at 1 \( \mu \)M in a 100-\( \mu \)l volume containing 10 \( \mu \)l of 10 \times \) Vent buffer and 200 \( \mu \)M each dNTP. The amplification was carried out for 20 cycles, denaturing at 95\(^\circ\)C (20 sec), annealing at 55\(^\circ\)C (20 sec), extension at 72\(^\circ\)C (60 sec), and final incubation at 72\(^\circ\)C for 5 min. After precipitation and removal of excess primers, the fragment was purified and cloned into the eukaryotic expression vector pcDNA-1 Amp (Invitrogen).

DNA sequencing

Both strands of the envelope gene were sequenced following the method recommended by United States Biochemical, using Sequenase Version 2.0 T7 DNA polymerase. Briefly, 5 \( \mu \)g of alkali-denatured DNA was used for annealing with the primers at 65\(^\circ\)C for 2 min and the mix was allowed to cool slowly to 35\(^\circ\)C. To the annealed template–primer complex (10 \( \mu \)l), 0.1 M DTT (1.0 \( \mu \)l), diluted labeling mix (2.0 \( \mu \)l), 5 \( \mu \)Ci of [\( \alpha \)-\( ^35 \)S]-dATP (0.5 \( \mu \)l) and diluted Sequenase Version 2.0 (2.0 \( \mu \)l) were added and incubated for 3 min at room temperature. Meanwhile, 2.5-\( \mu \)l aliquots of termination mix (ddGTP, ddATP, ddTTP, and ddCTP) were transferred to individual tubes and pre-warmed at 37\(^\circ\)C for 1 min. When labeling was complete, aliquots of 3.5 \( \mu \)l each were transferred to tubes containing corresponding termination mixes and incubated further for 5 min at 37\(^\circ\)C. The reaction was stopped and the samples were heated at 80\(^\circ\)C, loaded onto an acrylamide–urea gel, and visualized by autoradiography.

Transfection, radioimmunoprecipitation, and protein analysis

Transfection and protein analyses were done as described previously (Vincent et al., 1993). Briefly, cells (5 \( \times \) 10\(^5\)) were seeded onto 35-mm dishes 1 day prior to transfection. The cells were infected with vaccinia virus vTF7-3 (10 m.o.i.), which expresses the T7 RNA polymerase, and DNA (5 \( \mu \)g) and Lipofectin (10 \( \mu \)g) mix were added to the cells. At 15 hr posttransfection, the cells were starved in medium lacking methionine/cysteine and then labeled with \( ^35 \)S labeling mix containing methionine and cysteine (Amersham) for 30 min. At the end of the labeling period, label was removed and DMEM containing excess cold methionine and cysteine was added and chased for different times as indicated. Cell lysates were prepared in RIPA buffer and proteins were immunoprecipitated with specific antibodies. The immunoprecipitated proteins were extensively washed and analyzed by SDS–PAGE and autoradiography. For experiments involving Endo H treatment, the immunoprecipitated proteins were divided into two equal (100-\( \mu \)l) aliquots. To one aliquot 5 U Endo H (New England Biolabs) in sodium phosphate buffer (pH 5.6) was added and the other was left untreated. Both fractions were incubated at 37\(^\circ\)C for 15 hr and analyzed by SDS–PAGE. For tunicamycin treatment, transfected cells were treated with 5 \( \mu \)g/ml tunicamycin (Boehringer Mannheim) in DMEM for 30 min prior to and during labeling.

Indirect immunofluorescence

Cells were seeded onto coverglasses and infection–transfection was performed as described above. At 6 hr posttransfection, the expression and cellular localization of proteins were analyzed after fixing the transfected cells for 10 min with paraformaldehyde (3.6%) and permeabilizing for 5 min with 1.0% NP-40. Cells were then treated with primary antibodies specific to the envelope protein followed by secondary mouse anti-human antibodies conjugated with FITC. The coverslips containing the cells were extensively washed with PBS, mounted on glass slides, and viewed in a fluorescence microscope.

The nucleotide sequence reported in this paper has been submitted to GenBank under Accession No. U56855.

RESULTS

BTLV-infected baboon serum has strong reactivity to HTLV-1 proteins

As part of a collaborative project between the Yerkes Regional Primate Research Center and the Institute of Primate Research in Nairobi, Kenya, feral and captive nonhuman primates in Kenya were screened for antibody reactivity to HIV-1 and HIV-2. Several animal species tested positive for antibodies to HIV-2 using a commercially available ELISA kit (Genetic Systems HIV-2 EIA): Syke’s monkeys, red tail monkeys, African green monkeys, and baboons, including a captive baboon with a code designation of 1621. Baboon 1621 was further evaluated following importation to the Yerkes Center.

Baboon 1621 was confirmed to be seropositive to HIV-2 (SIV) as well as HTLV-1 by Western blot (Fig. 1) and commercially available ELISA. The reactivity of the baboon serum to HTLV proteins was evaluated by using HTLV-1-infected cell lysate as the antigen source and then probing the blot strips with sera from an STLV-1-positive sooty mangabey and from the baboon 1621. Sera from the STLV-1-positive sooty mangabey reacted with the envelope (gp46) and with the products of gag polyprotein (CA, p24; MA, p19; and NC, p15) (Fig. 1, lane 2). Sera from baboon 1621 exhibited a similar profile (lane 3). As expected, sera from an uninfected sooty mangabey did not react with any of the HTLV-1 proteins. The proteins were also compared by radioactive labeling and immunoprecipitation to proteins from a cell line developed from baboon 1621 (described below). The sera from baboon 1621 failed to precipitate any proteins of normal PBMC, whereas proteins corresponding in mobility to those
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env gene was amplified using PCR primers as described under Materials and Methods.

Nucleotide sequence of envelope gene of BTLV

The nucleotide sequence of the BTLV env gene and the comparison with other HTLV-1 and STLV-1 isolates is shown in Fig. 2. Of 1464 nucleotides, 1426 nt were found to be identical between HTLV-1 (EL strain) (Paine et al., 1991) and the BTLV isolate, i.e., 97.4% identity at the nucleotide level. Comparison of the complete nucleotide sequence of BTLV with different HTLV isolates revealed that the baboon virus env gene showed striking similarities ranging from 92.5 to 97.4%. In contrast, we found only 89.1% identity (1305 nt identical of 1464 nt) between the baboon virus env gene and that of STLV-1 (PtM3) (Watanabe et al., 1985). When compared to the STLV-1 sequence, changes were clustered at the 5' end of the gene and, to a lesser extent, dispersed throughout the rest of the gene. Most base substitutions, however, were at the second (7.5%) or third (74.0%) position.

FIG. 1. Immunoreactivity of serum from baboon 1621 to HTLV-1 proteins. The Hut-102 cell line (HTLV-1 infected) was used as a source of antigen. Cells (2 x 10⁷) were harvested from culture, washed, and lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.5% sodium deoxycholate, and 1% NP-40). The lysate was cleared by centrifugation and run on a 12% polyacrylamide gel. Following electrophoresis and transfer to a PVDF membrane (Schleicher and Schuell) strips were cut and used for immunodetection with serum samples and a Rad-Free Detection kit (Schleicher and Schuell). The lanes represent results with serum samples from the following animals: (1) an STLV-1-seronegative sooty mangabey monkey, (2) an STLV-1-seropositive sooty mangabey, (3) baboon 1621 infected with BTLV.

shown in the Western blot (Fig. 1) were precipitated from the cell line developed from baboon 1621 (not shown). These results indicated the infection of the baboon 1621 by a virus related to HTLV-1.

Development of a cell line and amplification of the envelope-coding region from genomic DNA

In order to isolate a virus from baboon 1621, PHA/IL-2-stimulated PBMC were cocultured with a number of cell types (normal baboon PBMC, CEMss, CEM x 174, Mol4 Clone 8, U937, and HuT78). All cocultures were found to be negative for the presence of reverse transcriptase (RT) activity except for the CEMss coculture, which showed minimal amounts of RT activity in the supernatant. This culture supernatant was tested for reactivity in an HTLV-I and -II antigen capture assay. Using limiting dilution, cell lines were cloned from this coculture. Twenty-eight separate colonies which grew in the 0.1 cell/well dilution were expanded and tested for the presence of HTLV-I and -II antigen. Three clones were positive (4, 14, and 18), with the most strongly positive culture being No. 4 (termed 1621 clone 4). Genomic DNA was prepared from the 1621 clone 4 cell line and the

Expression of the BTLV envelope glycoprotein

In order to characterize the protein encoded by the BTLV envelope gene, we performed transient transfection and immunoprecipitation using the vaccinia T7 expression system. A monoclonal antibody for HTLV-1, human antiserum to HTLV-1, and sera from baboon 1621 all specifically immunoprecipitated a glycoprotein with
FIG. 3. Expression of the BTLV envelope glycoprotein. HeLa T4 cells were infected with vTF7-3 vaccinia virus at m.o.i. of 10 and transfected with plasmid DNA (3 μg) encoding the BTLV envelope protein. After 15 hr cells were labeled for 30 min with 100 μCi of [35S]methionine and cysteine labeling mix. (A) In one set, tunicamycin (5 μg) was added (+) 30 min prior to the labeling and during labeling and other set was labeled without tunicamycin (−). The cells were then washed, lysed, and immunoprecipitated with 1621 antiserum and the proteins were analyzed using 8% SDS-PAGE and autoradiography. a and b represent the envelope precursor proteins which are glycosylated and unglycosylated, respectively. (B) Transfection and labeling were done as above. After the 30-min pulse, the label was removed and medium containing unlabeled amino acids was added and incubated for up to 5 hr. The immunoprecipitated proteins were divided into two equal portions. To one portion 5 units of Endo H was added (+) and the other portion was left untreated (−). Both aliquots were incubated at 37°C for 15 hr and analyzed by SDS-PAGE. a and b denote the glycosylated and deglycosylated proteins, respectively.

FIG. 2. Comparison of env coding sequences of BTLV, HTLV-1, and STLV-1. Genomic DNA was prepared from the 1621 clone 4 cell line and used as template for PCR amplification of the Env coding region. The env gene of BTLV was sequenced using Sequenase version 2 (United States Biochemicals) and the nucleotide sequences were compared with the available sequences of HTLV strains (RH/K30, MT2, and EL) and STLV isolates (PM3 and PPA) using the GCG program. Note that only 522 nucleotides of STLV-1 from PPA were available for comparison (Koralnik et al., 1994). The deduced amino acid sequence of BTLV is presented at the bottom (BTLV AA). The Accession Nos. used in the comparison are RH/K30 (L03561), MT2 (M37747), EL (M67514), PM3 (M11373), and PPA (U03160).

a molecular weight of approximately 62 kDa (data not shown). The core molecular mass and nature of the modification on the protein were analyzed using tunicamycin treatment of the transfected cells and endoglycosidase H treatment of the immunoprecipitated proteins. The transfected cells were treated with tunicamycin for 30 min prior to 35S labeling and analyzed by immunoprecipitation (Fig. 3A). In the absence of tunicamycin, the envelope precursor was found as a polyprotein of 62 kDa. A series of bands of faster electrophoretic mobility was also observed, which may represent incompletely glycosylated forms of the protein. When glycosylation was inhibited, a protein band of approximately 46 kDa was observed representing the unglycosylated form of the precursor glycoprotein. A small fraction of the glycosylated protein was also observed during the tunicamycin treatment, possibly due to the limited time of exposure to the drug prior to labeling.

The nature of the modification of the protein was analyzed by labeling for 30 min and chasing in the presence of unlabeled amino acids, and then digesting the immunoprecipitated proteins using endoglycosidase H followed by SDS-PAGE. As shown in Fig. 3B, proteins synthesized during the 30-min pulse and subsequently digested with Endo H had a molecular weight of approximately 44 kDa. Even after chases up to 5 hr, the precursor protein did not exhibit forms which were resistant to Endo H. This result indicates that the BTLV envelope glycoprotein predominantly acquires high mannose modifications, occurring in the endoplasmic reticulum. In addition, we consistently found that the size of the unglycosylated proteins made during tunicamycin treatment was at least 2 kDa smaller than the deglycosylated proteins observed after Endo H digestion. With HTLV-1, a similar molecular weight difference between tunicamycin and Endo H-treated proteins was observed (Paine et al., 1994). It is possible that the higher apparent molecular weight of the Endo H-treated proteins could be due to the residual N-acetyl glucosamine moieties remaining after Endo H digestion. The proteins were found to be relatively stable with a half-life of nearly 9 hr (data not shown).

The intracellular localization of the proteins was further analyzed using indirect immunofluorescence staining. In permeabilized cells, the BTLV envelope was found in the perinuclear region as well as in a reticular staining pattern throughout the cell (Fig. 4A). In addition, a low level of expression was observed at the cell surface by staining of unpermeabilized cells (Fig. 4B). Control cells similarly processed were negative for envelope-specific staining both within the cell (Fig. 4C) and on the cell surface (Fig. 4D).

Induction of syncytia by the BTLV envelope protein

HTLV induces syncytium formation upon infection of susceptible cells. In an attempt to determine if BTLV exhibited similar fusion properties, we analyzed the syncytia-forming potential of the BTLV Env protein using a coculture method. When HeLa T4 cells expressing BTLV Env were cocultured with XC cells, an RSV-transformed rat cell line which is susceptible to cell fusion by HTLV-1, generation of syncytia containing 15–20 nuclei was observed (Fig. 5A). The ability of the BTLV envelope protein to induce syncytia formation indicated that the protein was proteolytically processed and appropriately expressed at the cell surface for subsequent interaction with its receptors. For comparison, we also cocultured XC cells and the cell line developed from baboon 1621 from which BTLV was isolated. This coculture also resulted in the induction of syncytia with XC cells (Fig. 5B), suggesting that expression of viral proteins is main-
FIG. 4. Localization of the BTLV envelope glycoprotein in transfected cells. HeLa T4 cells were transfected as described before. After 6 hr one batch of cells was left unpermeabilized (B, surface) and other batch was permeabilized (A, internal) using 1.0% NP-40. Washed cells were incubated with 1:500 antiserum and subsequently incubated with FITC-conjugated secondary antibodies. (C) (without primary ab) and (D) (vaccinia-infected untransfected cells stained with both primary and secondary antibodies) are controls.

DISCUSSION

Previous studies have indicated that African STLV-1 isolates exhibit a very close relationship among themselves and with HTLV-1 strain EL, isolated from an African ATL patient (Paine et al., 1991; Song et al., 1994). The sequence of the envelope gene obtained from BTLV is also similar to those of HTLV-1 EL. However, the amino acid sequence is more closely related to HTLV-1 strain MT2, which was isolated from Japanese ATL patients. The HTLV strains EL and MT2 differ in 30 nucleotides and 3 amino acids. Paine et al. (1991) compared different HTLV-1 isolates and found fewer nucleotide and amino acid differences in gp41 between isolates obtained from the same geographic region. STLV-1 strains from Africa exhibited a closer genetic relatedness among themselves and tend to segregate with HTLV-1 strain EL from equatorial Zaire. Sequence analyses of LTR, pol, and env regions and restriction fragment length polymorphism of LTR suggested that there could be five different HTLV-1 genotypes from different geographic regions (Komurian et al., 1991; Ureta Vidal et al., 1994). Koralnik et al. (1994) identified seven clades of STLV based on comparing 25 STLV-1 sequences from 12 nonhuman primate species. However, there are examples of STLV-1 sequences from a single host species that fit into genetically distinct clades. We compared a segment between nucleotides 867 and 1388 of BTLV env, which has been used in most of the published comparisons among different isolates (Koralnik et al., 1994; Ibrahim et al., 1995), with the corresponding nucleotides of different STLV-1 and HTLV-1 strains using the GCG program. Among HTLV-1 isolates the similarities ranged between 93.9 and 97.9%, the highest similarity being with H15 from Ivory Coast and lowest similarity to HZ17 from Zaire. Compared to the partial env sequences of STLV clade S6 isolates from Africa, the BTLV sequence exhibited similarities ranging from 94.6 to 96.1%. STLV-1 isolates from clade S7 (East Africa) also exhibited 96.0% similarity to BTLV. An isolate from a different species of baboon, PPA-5X28, showed the highest similarity (97.5%) to BTLV. In contrast, STLV-1 isolates from India or Indonesia (clade S1) were found to show only 86.4 to 90.2% similarity to BTLV. A phylogenetic analysis based on the comparison of the 522-nucleotide
FIG. 5. Membrane fusion induced by the BTLV envelope protein. Cells (HeLa T4) were transfected as described under Materials and Methods. At 6 hr posttransfection, the transfected cells were detached from the plates by versene treatment and pelleted by centrifugation. XC cells, similarly processed, were mixed with HeLa T4 cells and the mixture of both cells was plated onto fresh dishes and cocultured for 15–20 hr. The cells were then observed under the microscope for the presence of multinucleated cells. XC cells were also cocultured with 1621 clone 4 cells (a cell line developed from BTLV infected baboon). (A) HeLa T4 cells expressing BTLV Env, cocultured with XC cells; (B) XC cells cocultured with 1621 clone 4 cells; (C) coculture of vaccinia-infected HeLa T4 and XC cells; and (D) HeLa T4 cells infected by vaccinia virus, vTF7-3.

segment of different HTLV-1 and STLV-1 isolates, using the Kimura distance matrix (Kimura, 1980), showed that the BTLV sequence clustered with several of the isolates from Kenya and Tanzania. However, the complete env gene sequence of BTLV shows greatest similarity to that of HTLV-1 EL, which was isolated from Zaire. Since multiple amino acid substitutions have been observed in the amino-terminal region of the envelope gene of STLV-1 (Song et al., 1994), comparison of such complete nucleotide and amino acid sequences will be required to determine the precise relationship of BTLV to other primate T-cell leukemia viruses.

Several studies of retroviruses including HIV-1 indicated that sequence variation in the env gene may profoundly affect virus infectivity, cell tropism, and other biological properties (Li et al., 1987; Szurek et al., 1988; Fenyo et al., 1988). The envelope glycoproteins of all HTLV-1 and STLV-1 viruses contain five potential sites (N-X-S/T) for N-linked glycosylation, which are conserved among strains (Paine et al., 1991). The BTLV sequence also shows strict conservation of these glycosylation sites. In this respect, HTLV-1 and STLV-1 differ from HIV-1 which exhibits variation among strains in glycosylation sites (McNearney et al., 1990). Endoproteolytic processing has been shown to be an important determinant for biological activity of many viral glycoproteins including those of retroviruses. All retroviruses except visna have a highly conserved cleavage site for a trypsin-like protease (Arg-X-Lys/Arg-Arg) just prior to the beginning of the transmembrane subunit (McCune et al., 1986), and this site is recognized by a cellular protease resulting in the generation of the surface and transmembrane components (Barr, 1991; Hallenberger et al., 1992). The BTLV envelope glycoprotein (gp62) has the sequence Arg-Ser-Arg-Arg at aa 308 to 312, which is the probable cleavage site for processing of the precursor (gp62) into the extracellular (gp46) and transmembrane (gp21) components. In our initial labeling and immunoprecipitation studies, no significant amount of gp46 was detected. However, in subsequent labeling studies with [35S]cysteine, we have detected gp46 within the cell and in the extracellular medium (data not shown). Also, the observation of membrane fusion by BTLV Env supports the conclusion that a fraction of the precursor was cleaved.

Since BTLV has the potential to be used for the development of an animal model for HTLV-1, we compared its
sequence with the immunologically characterized regions of HTLV-1. In gp46 of HTLV-1, epitopes involved in generating neutralization and antibody-dependent cellular cytotoxicity (Kuroki et al., 1992), B-cell activity (Baba et al., 1993), and a major T-cell epitope (Baba et al., 1995) have been identified. All of these epitopes are maintained in BTLV. Specific neutralization determinants were identified between aa 88 and 98 which differed between HTLV-I and -II glycoproteins (Palker et al., 1992), and the BTLV sequences correspond to those of HTLV-1 and differ from HTLV-II. Based on sequence homology with the transmembrane proteins of feline and murine leukemia viruses and HIV, a region (amino acids from 377 to 402) in HTLV-1 gp21 was postulated to have immunosuppressive properties (Cianciolo et al., 1984; Ruegg et al., 1989) and this motif is also preserved in the BTLV Env protein.

Recently, HTLV-II has been shown to infect a wide variety of cell types including cells of human, monkey, or porcine origin (Li et al., 1996). Earlier studies employing syncytium formation, vesicular stomatitis virus pseudotyping, and receptor interference (Sommerfelt and Weiss, 1990) indicated that HTLV-I and HTLV-II are likely to employ the same cellular receptor which is encoded by human chromosome 17. BTLV is also likely to interact with the same receptor because of its sequence relatedness. Our results raise the possibility that under certain circumstances, the opportunity may exist for the anesian human T-cell leukemia virus type 1.

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