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Induction of Simian Acquired Immune Deficiency Syndrome (SAIDS) with a Molecular Clone of a Type D SAIDS Retrovirus

GISELA HEIDECKER,[1] NICHOLAS W. LERCHE,[2] LINDA J. LOWENSTINE,[3] ANDREW A. LACKNER,[2] KENT G. OSBORN,[2†] MURRAY B. GARDNER,[3] AND PRESTON A. MARX[2*]

Laboratory of Viral Carcinogenesis, Section of Genetics, National Cancer Institute, Frederick, Maryland 21701,[1] and California Primate Research Center[2] and Medical Pathology,[3] University of California, Davis, California 95616

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We have isolated a molecular clone of the full-length integrated provirus of simian acquired immune deficiency syndrome retrovirus serotype 1 (SRV-1) from a fatal case of simian acquired immune deficiency syndrome in a juvenile rhesus macaque. An integrated SRV-1 provirus was cloned, sequenced, and found to contain four large open reading frames encoding *gag*-precursor protein, protease, polymerase, and envelope. The proviral clone was transfected into D17 canine osteosarcoma cells and found to produce infectious virus. A comparison of the sequences of this clone with a noninfectious clone showed 20 differences, resulting in 10 amino acid changes. Also, a cluster of exchanges, short insertions, and deletions in the 5′ leader sequences resulted in extension of the $tRNA^{Lys}$ primer-binding site from 14 to 19 nucleotides. Virus isolated from transfected cells was shown to be infectious and pathogenic, resulting in disease that followed the same time course and mortality as disease induced by uncloned, in vitro cultivated virus isolated from diseased animals. These results unequivocally show that a type D retrovirus (SRV-1) causes a fatal immunosuppressive syndrome in rhesus monkeys.

A type D retrovirus, simian acquired immune deficiency syndrome (SAIDS) retrovirus serotype 1 (SRV-1), has consistently been associated with SAIDS occurring spontaneously in rhesus monkeys (*Macaca mulatta*) at the California Primate Research Center (19, 20, 21). This fatal immunosuppressive disease was experimentally transmitted to rhesus monkeys with tissue culture-derived virus. Animals with terminal disease had a variety of clinical signs of SAIDS, including wasting, chronic diarrhea, noma, disseminated cytomegalovirus infections, and bacterial pneumonia (19, 20, 21, 26). At the cellular level, a markedly diminished response of peripheral blood mononuclear cells to mitogens, as well as anemia and lymphopenia, was reported (22, 25). The disease was fatal in about half of the cases (four of nine), although inapparent carriers which are virus positive but apparently free of the disease have also been identified (16).

New England, Washington, and Oregon Regional Primate Centers have also reported SAIDS outbreaks in their colonies, affecting not only rhesus monkeys (*M. mulatta*), but also Taiwanese rock macaques (*M. cyclopis*), crab-eating macaques (*M. fascicularis*), and pigtailed macaques (*M. nemestrina*) (7, 19, 31). In each case, it was found that the disease was strongly linked to infection with type D retroviruses. In addition to immune suppression, the animals at the Washington and Oregon Primate Centers, which are infected with a distinct SAIDS retrovirus serogroup (SAIDS retrovirus type 2 [SRV-2]) (19; P. A. Marx, unpublished data), have developed retroperitoneal fibromatosis, an aggressive proliferation of fibrous tissue in the abdominal cavity (10, 31). The viruses isolated at each of the four centers were shown to be related to Mason-Pfizer monkey virus (MPMV) by competitive radioimmune assays with the *gag*-derived p27 polypeptide (19, 20, 31) and by nucleic acid hybridizations (2, 7, 31). However, radioimmune assays done with envelope glycoprotein gp70 revealed that each isolate was distinct from MPMV (1). Neutralization assays also showed that MPMV, SRV-1, and SRV-2 belong to distinct serogroups (5, 19).

Since results of transmission studies in rhesus monkeys with type D retroviruses have suggested that some type D viral isolates found at the various primate centers may be less pathogenic than others (17), we have sought to determine if this fatal immunodeficiency disease could be induced with molecularly cloned virus. The first clone of SRV-1 obtained was not infectious (27; M. Bryant, unpublished data), so additional clones were derived and tested for this study. We here report that a full-length molecular clone of SRV-1 was transfected into heterologous cells to produce infectious virus. Inoculation of this molecularly cloned SRV-1 resulted in fatal SAIDS in three of six animals. The other three surviving animals in the study developed a milder form of the disease. In this paper, we describe the molecular cloning, present a brief summary of the DNA sequence of this pathogenic clone, and report the induction of a fatal immunosuppressive disease in rhesus monkeys with the molecularly cloned virus. The complete nucleotide sequence of this pathogenic clone is filed with the National Biomedical Resource Foundation, Washington, D.C.

MATERIALS AND METHODS

Molecular cloning of SRV-1. High-molecular-weight DNA of chronically SRV-1-infected ATCC HTB-82 cells, a human rhabdomyosarcoma cell line, was partially digested with restriction endonuclease *Mbo*I. Fragments 20 kilobases in length were isolated by sucrose gradient centrifugation and ligated into lambda EMBL3 arms (9), as described by Maniatis et al. (18). Recombinant bacteriophage (1.5×10^6 PFU) was screened with ^{32}P-labeled cloned MPMV DNA (3, 4). Four positive clones were plaque purified and restriction mapped by standard protocol (18).

DNA sequencing. The proviral DNA of clone λ GH2 was completely sequenced by using the dideoxy chain terminator method (29) and M13mp subclones as sequencing templates

* Corresponding author.
† Present address: Zoo Hospital, San Diego Zoo, San Diego, CA 92112.

TABLE 1. Induction of SAIDS in juvenile rhesus monkeys with molecularly cloned SRV-1[a]

Rhesus monkey number and group	Age (mo)	Dose (i.v.)[b]	Viremia duration	Clinical outcome
Group 1				
21914	9	1.5×10^5	Persistent until death	Dead with SAIDS at 3 mo p.i., generalized lymphadenopathy, splenomegaly, weight loss, anemia, lymphopenia, lymphoid depletion, marrow hyperplasia, septicemia
21913	9	1.5×10^5	Transient	Clinically normal at 2 yr p.i., transient clinical signs
Group 2				
21954	12	1.6×10^6	Persistent	Alive with SRC at 2 yr p.i., generalized lymphadenopathy, splenomegaly, recurrent diarrhea
21964	11	1.6×10^6	Persistent until death	Dead from SAIDS at 2 mo p.i., generalized lymphadenopathy, splenomegaly, anemia, lymphoid depletion, marrow hyperplasia, disseminated cytomegalovirus infections
Group 3				
21915	14	8.2×10^4	Persistent	Alive with SRC at 2 yr p.i., generalized lymphadenopathy, splenomegaly, transient neutropenia, recurrent diarrhea
21931	17	8.2×10^4	Persistent until death	Dead from SAIDS at 3 mo p.i., generalized lymphadenopathy, splenomegaly, weight loss, anemia, lymphoid depletion, marrow hyperplasia, septicemia, persistent diarrhea

[a] All became positive for SRV-1 antibody at ≥1:100 dilution by enzyme-linked immunosorbent assay p.i. (20).
[b] Titers of SRV-1 are SIU/ml in Raji cells (7).

(23, 24). The subclones contained fragments obtained by partially digesting gel-purified DNA with a combination of restriction enzymes, each of which generates blunt ends. Other templates were nested sets of deletions on the primer proximal side of larger inserts obtained as described previously (33). DNA sequence data were analyzed by computer with standard programs (14, 15).

DNA transfection. λ GH2 DNA (23 μg) and linearized pSV2neo (2 μg) (30) were transfected in 10^5 exponentially growing D17 canine osteosarcoma cells by the calcium phosphate coprecipitation protocol (11). At 48 h after transfection, the cells were transferred to medium containing 200 μg of G418 per ml (30). Individual colonies became visible after 7 to 10 days and were cloned out and expanded.

Animals. Six healthy rhesus monkeys 9 to 17 months old were chosen from the SAIDS-free colony at the California Primate Research Center. All were born at the California Primate Research Center and were maintained on standard laboratory monkey chow (Ralston Purina Co.) and water ad libitum. They were confirmed free of SRV-1 antibodies by enzyme-linked immunosorbent assay (19) (Table 1), and preexisting type D viremia was excluded by cocultivating their peripheral blood mononuclear cells with Raji cells as described elsewhere (14a). The six monkeys were randomly divided into three pairs, and each pair was housed separately in a HEPA-filtered isolator.

Induction of SRV-1 infection and SAIDS. Animals received a single intravenous (i.v.) inoculation of SRV-1 derived from the infectious molecular clone transfected into D17 cells. The criteria used to determine if an inoculated monkey developed SAIDS were as previously published (26), specifically, that rhesus monkeys diagnosed with SAIDS must have persistent generalized lymphadenopathy and four or more of the following clinical signs: (i) splenomegaly; (ii) weight loss of >10%; (iii) anemia (<30% packed erythrocyte volume); (iv) neutropenia; (v) persistent lymphopenia; (vi) lymphoid depletion; (vii) marrow hyperplasia; (viii) persistent diarrhea unresponsive to appropriate therapy; and (ix) opportunistic infections, such as generalized cytomegalovirus infections, intestinal cryptosporidiosis, esophageal candidiasis, or multiple bacterial infections unresponsive to appropriate therapy. SAIDS-related complex (SRC) is generalized lymphadenopathy and less than four clinical signs of SAIDS.

After i.v. inoculation of molecularly cloned SRV-1, each animal was examined weekly for signs of SAIDS. Sera were collected immediately before inoculation and every 4 weeks thereafter to test for SRV-1 antibody in an enzyme-linked immunosorbent assay. Viremia with SRV-1 was determined by cocultivation of peripheral blood mononuclear cells with Raji cells in a syncytium induction assay (19). All Raji cell cultures displaying characteristic syncytia were confirmed as type D positive by fixed-cell immunofluorescence with anti-SRV-1 monoclonal antibody against the gp20 envelope polypeptide (19; H. S. Kwang, manuscript in preparation).

RESULTS

Molecular clones of the integrated provirus of SRV-1. SRV-1 isolated from a rhesus monkey with terminal SAIDS was transferred from rhesus kidney cells to heterologous cell line ATCC HTB-82 (a human rhabdomyosarcoma cell line) to avoid difficulties during the molecular cloning of the integrated proviral DNA caused by endogenous rhesus sequences which are related to type D retroviral sequences (6). Although the cells were transferred for several passages after infection before high-molecular-weight DNA was extracted for cloning, we isolated only four clones from 1.5×10^6 PFU, suggesting that there was still less than one integrated provirus per cell. Restriction mapping (Fig. 1) showed that only one recombinant phage contained a full-length integrated provirus, based on the fragments common to all clones and compared with the restriction map of MPMV (3). No polymorphism was found between the sequences which were present in the four clones of integrated provirus and in one additional clone representing an uninte-

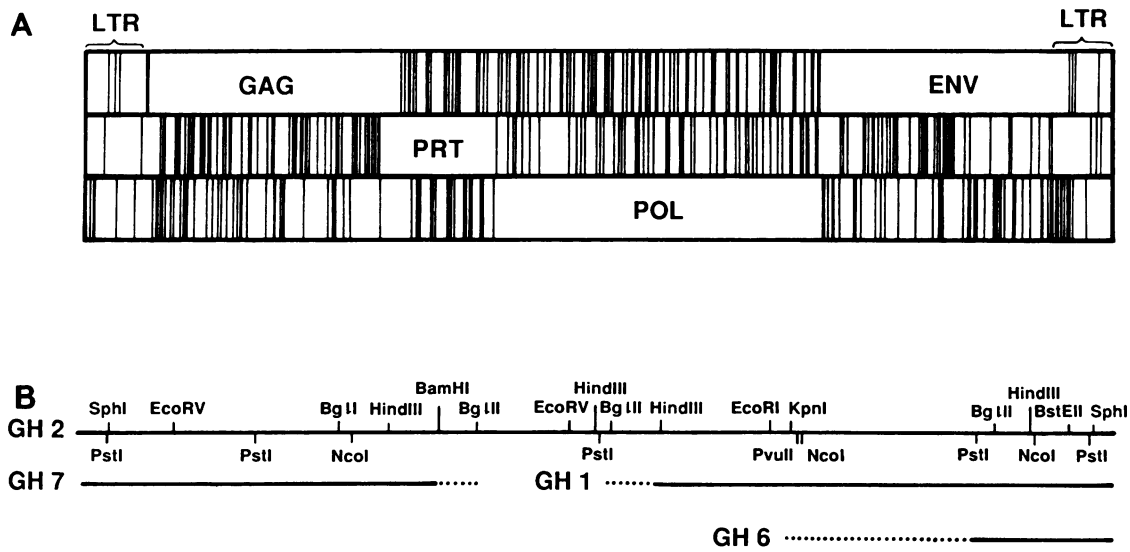

FIG. 1. Molecular cloning of SRV-1 integrated provirus. (A) The open reading frames of lambda GH2 are established by the occurrence of termination codons shown as vertical lines. (B) The truncated proviral clones λ GH1, GH6, and GH7 are shown in relationship to the restriction map of the full-length clone λ GH2.

grated provirus (G. Heidecker, unpublished results). Comparison with the restriction map of MPMV, however, shows variation throughout the virus (8). A published comparison (31a) of the complete nucleotide sequence data confirms that MPMV and SRV-1 differ significantly. The New England type D retrovirus isolate (8) appears to be more similar to SRV-1, in that 22 of 24 restriction sites are identical; however, no nucleotide sequence data are available to confirm the precise relationship of these two type D retroviruses.

The entire sequence of the integrated provirus and a portion of the flanking DNA was determined by the dideoxy chain terminator method on M13mp templates (13, 24, 29). The genome of the integrated provirus of SRV-1 is 8,168 base pairs long and is flanked by 6-base-pair direct repeats (data not shown), probably representing the host sequences duplicated upon integration. As shown previously for the sequence of the unintegrated provirus (27), the integrated provirus of SRV-1 contains four large open reading frames, 663, 314, 866, and 605 codons long, encoding the *gag*, protease, polymerase, and envelope polypeptides, respectively (Fig. 1). A comparison of the sequence of GH2 with that of the noninfectious molecular clone (27; Marx, unpublished data), derived from an unintegrated provirus (27), revealed 20 differences, most of which were simple nucleotide exchanges resulting in 10 amino acid changes (Fig. 2). A cluster of exchanges and small insertions and deletions can be seen in the 5′ leader sequence which extends the $tRNA^{Lys}$ primer-binding site from 14 nucleotides in the noninfectious clone to 19 nucleotides in the infectious GH2 clone (Fig. 2).

Transfection and infectivity of clone lambda GH2. To test the biological activity of the full-length clone of SRV-1, we cotransfected λ GH2 with pSV2neo into the D17 canine osteosarcoma cell line. We chose this heterologous line, rather than a rhesus cell line, to minimize potential recombination of the cloned provirus with endogenous MPMV-related sequences present in the genomes of macaques (5). Such recombination might lead to the reactivation of a defective proviral clone.

We obtained about 400 neomycin-resistant colonies per μg of linearized pSV2neo plasmid DNA. Twenty colonies resulting from transfection with pSV2neo and lambda GH2 were isolated and expanded, along with five colonies from a control experiment in which EMBL4 DNA was cotransfected with pSV2neo alone. The initial transfection plates were then allowed to become confluent and were also analyzed. The supernatant fluid of the neomycin-resistant cultures, both clonal and nonclonal, was tested for the presence of SRV-1 proviral DNA and RNA and virus production by syncytium formation in Raji cells (8, 19). Based on results of the Raji syncytium assay (19), none of the cultures derived from the control experiment produced virus, while 60% of the λ GH2-transfected cultures did. We isolated high-molecular-weight DNA from eight of the transfected cultures and assayed for homologous DNA by Southern blotting (30). Of the eight cultures, two were nonproducing lines; one was negative for integrated proviral DNA, and the other was positive for viral DNA but made no detectable viral RNA, as measured by dot blotting (data not shown). The remaining six cultures contained viral DNA and produced infectious SRV-1.

Inoculation of molecularly cloned SRV-1 into susceptible rhesus monkeys. Molecularly cloned and sequenced SRV-1 (Fig. 2) was inoculated into six juvenile rhesus monkeys ranging in age from 9 to 17 months. Before inoculation, all animals were clinically healthy and seronegative for type D retroviruses (Table 1).

The six rhesus monkeys were divided into three separate transmission groups (Table 1). In the first experiment, transfected SRV-1 from D17 cells was passaged once in rhesus lung fibroblast cells. Since all of the virus used in our previous transmission studies (20, 21) was grown in rhesus cells, we also passaged cloned SRV-1 in rhesus fibroblasts so as not to introduce a new variable in this first transmission study with molecularly cloned virus. Therefore, two animals (21913 and 21914) received a single i.v. inoculation from the same batch of spent medium containing 1.5×10^5 syncytium-inducing units (SIU) (21) of cloned SRV-1 passaged in rhesus lung fibroblast cells. Animal 21914 developed the clinical signs of SAIDS (Table 1), including persistent viremia,

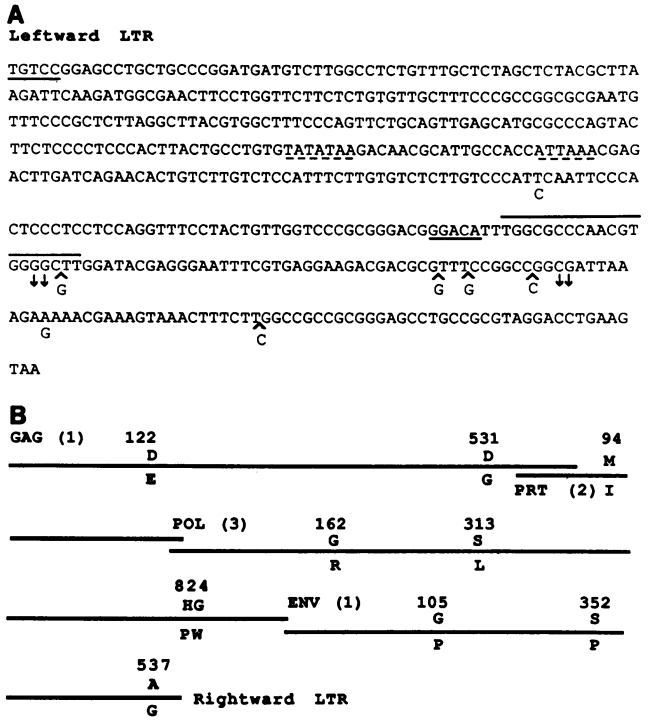

FIG. 2. Comparison of infectious and noninfectious clones of SRV-1. (A) A diagram of the infectious clone, illustrating the differences between the infectious and noninfectious clones. Only the nucleotide sequence of the leftward long terminal repeat is presented. Only amino acid differences between *gag*, *prt*, *pol*, and *env* are shown. Substitutions are indicated by the placement of the substituting base beneath the sequence, insertions are indicated by a carat over the inserted base, and deletions are indicated by arrows below the deleted base. The inverted repeats defining the long terminal repeat are underlined; TATA and ATAA sequences involved in regulation and polyadenylation are shown with dashed lines; the $tRNA^{Lys}$ binding (28) site is overlined. (B) The differences in the noninfectious clone. Numbers in parentheses indicate the reading frame. Amino acids (D, E, M, I, G, R, S, L, H, P, W) (27) above and below the line are from the noninfectious (27) and infectious clones, respectively. Numbers are the amino acid positions. The complete nucleotide sequence of GH2 is filed with the National Biomedical Research Foundation. LTR, Long terminal repeat.

severe anemia, and weight loss and was euthanized with terminal SAIDS at 3 months postinoculation (p.i.). The other paired animal (21913) had transient viremia and transient lymphadenopathy and splenomegaly. It remains alive with no viremia or clinical signs of SAIDS at 2 years p.i. In the second transmission experiment, a pair of animals (21954 and 21964) received a single i.v. inoculation of cloned SRV-1 (passaged in rhesus lung fibroblast cells) that contained about 10-fold more cloned SRV-1 (1.6×10^6 SIU) than in the previous experiment. Of these two, animal 21964 developed severe anemia and generalized lymphadenopathy and died by 2 months p.i. after an acute clinical course. At necropsy, the animal had lymphoid depletion, marrow hyperplasia, and disseminated cytomegalovirus infections. Animal 21954 developed persistent viremia, chronic diarrhea, and generalized lymphadenopathy and splenomegaly but is alive with SRC at 2 years p.i. A third pair of rhesus monkeys received an i.v. inoculation of 8.2×10^4 SIU of cloned SRV-1 obtained directly from the transfected D17 cells. For this transmission experiment, the transfected D17 cells were washed three times with phosphate-buffered saline to remove the neomycin from the culture medium and fresh neomycin-free medium was added. The medium containing released virus (8.2×10^4 SIU) was harvested after 16 h and inoculated i.v. into the remaining pair of juvenile rhesus monkeys (21915 and 21931). Monkey 21915 developed persistent viremia and multiple signs of SAIDS but is alive with viremia and SRC at 2 years p.i. The clinical course of monkey 21931, which was euthanatized with terminal SAIDS at 3 months p.i., included generalized lymphadenopathy, splenomegaly, and viremia, all of which persisted until death. At about 9 weeks, it had anemia, diarrhea, weight loss, and fever which also persisted until death. At necropsy, generalized lymphoid depletion, marrow hyperplasia, bacterial septicemia, and interstitial pneumonia were found.

In conclusion, molecularly cloned SRV-1 induced a broad spectrum of disease ranging from transient viremia to fulminant SAIDS (Table 1) identical to previous transmission studies when either plasma (12) or tissue culture medium containing uncloned SRV-1 was used to transmit the disease (20, 21).

DISCUSSION

We have isolated a molecular clone of the full-length integrated provirus of SRV-1 which produced fatal SAIDS when reinoculated into juvenile rhesus macaques. Clones obtained from unintegrated proviral forms of the same virus isolate which we reported earlier (27) are biologically inactive. Comparison of the two sequences shows only 20 differences at the nucleic acid sequence level, resulting in 10 amino acid exchanges. The high ratio of nonsilent-to-silent exchanges suggests that these two sequences represent two time frames in the evolution of SRV-1 which are not yet separated by selection, which would lower the apparent occurrence of nonsilent mutations. A cluster of deletions or insertions of one nucleotide can be found in the 5′ leader sequence extending the $tRNA^{Lys}$ primer-binding site from 14 nucleotides found in the unintegrated provirus to a sequence of 19 residues, which is in better agreement with those described for other viruses (32). It is unclear at this point whether these nucleotide changes are responsible for the difference in the in vitro infectivity of the clones obtained from the integrated and unintegrated proviral forms. It is also possible that the reversal of the circular permutation necessary to restore the natural configuration of the unintegrated provirus was not successful. Further experiments should elucidate this point.

Most significantly, the infection experiments performed with the virus produced by transfection of lambda GH2 unequivocally demonstrate that this type D virus is able to infect macaques and induce a fatal immunosuppressive syndrome. These results represent conclusive proof of the type D retrovirus etiology of SAIDS in rhesus monkeys at the California Primate Research Center. The disease induced by molecularly cloned SRV-1 is identical to that previously observed in experimental transmission studies with either whole blood-, plasma-, or tissue culture-derived SRV-1 obtained from spontaneous cases of SAIDS (12, 20, 21). With molecularly cloned SRV-1, the entire spectrum of disease from mild signs to fatal SAIDS was observed (Table 1).

Future studies will determine if envelope variation occurs in molecularly cloned SRV-1 after 1 to 2 years of replication in the rhesus monkey host. Other studies will take advantage of this pathogenic clone by the creation of genetic chimeras

to answer questions about the pathogenicity of SRV-1 and closely related type D retroviruses, such as SRV-2 and MPMV. SRV-2 is associated with SAIDS plus an aggressive but nonmetastatic fibrotic tumor termed retroperitoneal fibromatosis. It may be possible to produce recombinants that will identify the role of specific SRV-2 genes that contribute to the development of retroperitoneal fibromatosis. Finally, preliminary evidence indicates that nonpathogenic type D clones can be obtained (Marx, unpublished data). Recombinants of pathogenic and nonpathogenic clones will be used to find the genetic sequences responsible for immunosuppression in rhesus monkeys.

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