The Genetic and Phenotypic Characterization of Serogroup 2 Simian AIDS Type D Retroviruses

By

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CERTIFICATE OF APPROVAL

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Chance only favours intervention for minds which are prepared for discoveries by patient study and persevering efforts.

LOUIS PASTEUR

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Your name belongs on the title page along with mine.

II. TABLE of ABBREVIATIONS

Viral Genome and Proteins:

Abbreviation		Definition
LTR		Long Terminal Repeat; U3-R-U5 in the provirus
ψ		Psi site; for encapsidation of the viral RNA
pbs		Primer binding site; initiates minus strand DNA synthesis
gag		Group specific antigen gene; encodes viral core proteins
	MA	Matrix protein
	CA	Capsid protein
	NC	Nucleocapsid protein
prt		Protease gene
	dUTPase	Deoxyuridine-triphosphatase
pol		Polymerase gene
	RT	Reverse Transcriptase
	IN	Integrase
env		Envelope glycoprotein gene
	SU	Surface or gp70 domain
	TM	Transmembrane or gp22/20 domain
ppt		Polypurine tract; initiates positive strand DNA synthesis
Pr		Designates a precursor protein
pp		Designates a phosphoprotein
gp		Designates a glycoprotein

Protein "motifs":

PY	PPPY amino acid motif found in the gag phosphoprotein
L	Late domain: associated with virion release
MHR	Major homology region found in CA
Cys-His/ CCHC	Zinc finger motif; Cys-X_2-Cys-X_4-His-X_4-Cys found in NC
HHCC	Zinc finger-like motif; H-X_{3-7}-H-X_{23-32}-C-X_2-C found IN
DD35E	Central core domain of IN; D-X_{39-58}-D-X_{35}-E
ISP	Immunosuppressive peptide found in the env TM
MSD	Membrane spanning domain found in the env TM

Viruses:

MMTV	Mouse mammary tumor virus
ASLV	Avian sarcoma and leukosis virus
MuLV	Murine leukemia virus
RSV	Rous sarcoma virus
HTLV-1 and -II	Human T-lymphotropic virus -I and -II
STLV-1	Simian T-lymphotropic virus -I
BLV	Bovine leukemia virus
MPMV	Mason-Pfizer monkey virus
Po-1-Lu (old world)	Endogenous type D virus of the spectacled langur
SMRV (new world)	Squirrel monkey retrovirus; endogenous type D virus
HIV	Human immunodeficiency virus
SIV	Simian immunodeficiency virus
BaEV	Baboon endogenous virus
RD114	A feline endogenous virus
SRV	Simian retrovirus
SRV-1-5	Simian retrovirus-serogroups 1-5

D1/CYC/NE	SRV-1/ from a cyclopis macaque/ at New England
D1/RHE/CA	SRV-1/ from a rhesus macaque/ at California
D2/CEL/OR	SRV-2/ from a Celebes macaque/ at Oregon
D2/RHE/OR	SRV-2/ from a rhesus macaque/ at Oregon
D2/RHE/OR/V1	SRV-2/ from a rhesus macaque/ at Oregon/ variant 1
D2/MNE/WA	SRV-2/ from a pig-tailed macaque/ at Washington
D4/CYN/CA	SRV-4/ from a cynomolgus macaque/ from Berkeley, CA
D5/RHE/OR	SRV-5/ from a rhesus macaque/ at Oregon

Clones:

pV1	Full-length D2/RHE/OR/V1 in pBR322
11B-1	Full-length D2/RHE/OR/V1 in lambda
18B-1	Full-length D2/RHE/OR in lambda
p52R3V1	Chimeric clone, 5' region from D2/RHE/OR, 3' from D2/RHE/OR/V1
p5V132R	Chimeric clone, 5' region from D2/RHE/OR/V1, 3' from D2/RHE/OR
pJ450	5' Bam HI fragment of D2/RHE/OR
p2R7a	3' Bam HI fragment of D2/RHE/OR

Macaque species:

CEL	Celebes	*Macaca nigra*
CYC	Taiwan	*Macaca cyclopis*
CYN	Cynomolgus (Crab-eating)	*Macaca fascicularis*
JPN	Japanese	*Macaca fuscata*
MNE	Pig-tailed	*Macaca nemestrina*
RHE	Rhesus	*Macaca mulatta*

Biomedical Research Facilities:

RPRC	Regional Primate Research Center
CA	California (RPRC)
NE	New England (RPRC)
OR	Oregon (RPRC)
WA	Washington (RPRC)

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IV. ABSTRACT

What is the first business of one who studies philosophy?
To part with self-conceit.
For it is impossible for anyone to begin to learn what he thinks he already knows. EPICTETUS

Type D retroviruses are recognized as the etiologic agent of spontaneous simian AIDS (SAIDS) which endemically infects Asian macaques housed in various biomedical research colonies and severely compromises the use of these species as research animals. Five neutralization groups of type D simian retroviruses (SRV-1-5) have been identified. SRV-2 is the only serogroup to have a known reservoir in wild populations of macaques, and is the predominant etiologic agent of SAIDS at several primate research facilities. Immunodeficiency and retroperitoneal fibromatosis have been associated with SRV-2 infection of several macaque species. Four different serogroup 2 SRVs have been characterized at the Oregon RPRC; each isolate is characterized by unique restriction site polymorphisms. Two closely related serogroup 2 SRV isolates, D2/RHE/OR and D2/RHE/OR/V1, have distinct differences in pathogenic outcome and in vitro tropism. D2/RHE/OR/V1 was isolated from a severely ill rhesus macaque originating from group-housed animals endemically infected with D2/RHE/OR, whereas D2/RHE/OR has been associated with a less severe pathological outcome in rhesus macaques. Cell-free isolates of D2/RHE/OR readily infect human B-lineage cells (Raji), but do not grow as well in some T lineage cells (Hut 78 and MT-4) in vitro. In contrast, cell-free D2/RHE/OR/V1 infects both human Raji cells and T-cells (Hut 78, CEM-SS, MT-4 and SupT-1) in vitro.

Genetic and infectivity analyses were designed 1) to characterize the genetic variability of these two serogroup 2 polymorphic isolates, 2) to determine the relatedness of these two isolates to other characterized SRVs, and 3) to elucidate the viral genetic domains that are responsible for the apparent differences in pathogenesis and in vitro cell tropism.

Infectious full-length molecular clones of D2/RHE/OR and D2/RHE/OR/V1 were isolated and sequenced. D2/RHE/OR and D2/RHE/OR/V1 are 99.3% similar at the amino acid level, exhibiting

only 17 residue differences, ten of which are located in the envelope glycoprotein. Three distinct phylogenetic clusters were derived from comparison of the *env* or *gag* genes among all cloned SRVs. SRV-1, -3 and -4 comprise one cluster, the members of SRV-2 constitute a second cluster with D2/RHE/OR and D2/RHE/OR/V1 demonstrating a greater similarity to each other than to D2/CEL/OR. SRV-5 is the sole member of the third cluster. In vitro T-cell experiments with the two cloned viruses and their reciprocal chimeras demonstrate that viral determinants necessary for entry and the spread of infection are located in both the 5' and 3' domains of the viral genome.

V. INTRODUCTION

V.1 GENERAL RETROVIROLOGY

Make everything as simple as possible, but not simpler. ALBERT EINSTEIN

V.1.A. Classification. Retroviruses are enveloped animal viruses which enter their target cells by fusion with a host cell membrane. These viruses infect a wide variety of hosts including humans, cats, chickens, mice, horses, goats, sheep and non-human primates. Transmission of retroviruses can occur horizontally via exogenous infection or vertically by integration of the viral genome into host germ-line cells. The nucleocapsid core of the retrovirus contains capsid structural proteins, two single positive strands of genomic RNA, reverse transcriptase and tRNA which augments the synthesis of minus strand DNA during replication in a new target cell. The species of tRNA paired with the genome is dependent on the family of retrovirus and is usually a $tRNA^{pro}$ or $tRNA^{lys}$ (Varmus, 1988). All retroviruses contain at least three open reading frames for the *gag*, *pol* and *env* genes which encode the structural core proteins, reverse transcriptase/endonuclease/integrase and envelope glycoprotein, respectively (Figure 1) (Coffin *et al.* 1997). Seven subgroups of retroviruses have been identified based on their respective morphologies, pathogenicities and replication strategies. Mouse Mammary Tumor Virus (MMTV) is the sole member of the mammalian B-type virus subgroup which is identified by a characteristic eccentric spherical core. MMTV can induce tumors by exogenous (secreted in the milk) as well as endogenous (integrated in host DNA) virus (Fields and Knipe, 1991).

Two subgroups of retroviruses have a C-type morphology, the avian sarcoma and leukosis viral group (ASLV; ex: Rous sarcoma virus), and the murine leukemia-related viral group (MuLV; ex: Moloney murine leukemia virus). Reverse transcriptase in avian type C viruses requires Mg^{+2}, whereas in mammalian type C viruses it requires Mn^{+2}. Type C retroviruses may encode viral oncogenes which effect the growth properties of the target cell. For example, Rous sarcoma virus (RSV) encodes the *src* oncogene. Some of the species infected by type C viruses are avian,

baboons, Old World monkeys, great apes, cats, fish, and mice. The mature virion of C-type retroviruses contains a central spherical core.

The human T-cell leukemia-bovine leukemia subgroup of retroviruses consists of the bovine leukemia virus (BLV) and the closely related isolates of human T-cell lymphotropic virus, types -I and II (HTLV-I and HTLV-II), and simian T-cell lymphotropic virus, type-I (STLV-I). HTLV-BLV viruses are not generally associated with an immunosuppressive disease, but rather with the appearance of leukemias or lymphomas (Evans and Kaslow, 1997; Coffin *et al.* 1997). HTLV-I is the prototype human retrovirus and was originally associated with T-cell leukemia-lymphomas and spinal spastic paresis. HTLV-I was isolated primarily from endemically infected populations of Japan, but has also been identified in Caribbean populations. HTLV-II is a less characterized member of this subfamily and has been associated with T-cell hairy leukemia (Evans and Kaslow, 1997; Coffin *et al.* 1997). These T-cell lymphotropic viruses are unusual in that they contain at least two viral non-structural proteins required for viral gene expression and activation of quiescent host T cells (Coffin *et al.* 1997). These additional genes distinguish the HTLV-BLV genome as complex, in contrast to retroviruses in other subfamilies which encode only *gag*, *pol* and *env* genes and are considered to have simple genomes (Figure 1).

Mason-Pfizer Monkey virus (MPMV) is the prototype member of the subgroup of type D retroviruses, which consists of five different serogroups, with MPMV belonging to serogroup 3. The nomenclature of exogenous type D retroviruses is patterned after the influenza virus convention and signify <type D and serogroup>/<species isolated from>/<location of primate center>; for example, D2/RHE/OR belongs to serogroup 2/isolated from a rhesus macaque at the Oregon Regional Primate Research Center. Interestingly, the SRVs are the only subfamily with multiple isolates which are limited to exogenous infection of primarily macaque (genus *Macaca*) monkeys (Marx and Lowenstine, 1987). Two endogenous type D isolates have been identified in the Spectacled langur (*Presbytis obscurus*), an Old World monkey, and the Squirrel monkey (*Saimiri sciureus*), a New World monkey (Fine and Schochetman, 1978; Fine and Schochetman, 1978; Gardner *et al.* 1988; Marx and Lowenstine, 1987). The type D genome encodes a viral protease gene (*prt*) in a separate reading

frame, which distinguishes these viruses from other retroviruses with the classic *gag-pol-env* genomic organization. In addition, D-type viruses are distinguished from primate lentiviruses in that they encode a dUTPase in the 5' portion of the *prt* gene (Elder *et al.* 1992). All of the exogenous type D isolates contain a rod shaped nucleoid in the mature virion (Desrosiers, 1988).

Lentiviruses are a subgroup of retrovirus which are not directly implicated in any malignancies, but instead induce a protracted immunodeficiency disease course which is characterized by a long latency period. Lentiviral infections have been observed to cause a latent infection of monocyte and macrophage cells with subsequent ability to spread to and infect other cell-types, such as CD4+ lymphocytes (Gendelman *et al.* 1985). As with Type C viruses, many species are infected by the lentivirus group, including sheep (Visna-Maedi Virus), goats (Caprine Arthritis-Encephalitis Virus), horses (Equine Infectious Anemia Virus), cattle (Bovine Immunodeficiency Virus), non-human primates (Simian Immunodeficiency Virus) and most notably humans (HIV-1 and HIV-2) (Coffin *et al.* 1997; Narayan and Clements, 1990). In addition to the *gag*, *pol* and *env* genes, five or six additional small open reading frames have been identified in the genomes of these complex viruses (Desrosiers, 1988). Interestingly, an SIV gene, *vpx,* is not present in HIV-l but is encoded by HIV-2. SIV_{mac} and HIV-l have an overall degree of homology of 40%, whereas the degree of homology between SIV_{mac} and HIV-2 is 75% (Desrosiers, 1988). The presence of the vpx gene in HIV-2 and SIV_{mac} further confirms the close relatedness between these two viruses (Sharp *et al.* 1995; Gao *et al.* 1994). A bar shaped nucleoid core is characteristic of this group.

The initial identification of the last subgroup of retrovirus, spumavirus, came from the observation of a vacuolated or foamy appearance in the cytoplasm of cells cultured from apparently healthy monkeys. In addition to monkeys, spumaviruses have been recovered from humans, cattle and cats (Fields and Knipe, 1991). Recently, human foamy virus (HFV) has been demonstrated to contain elements during replication which are reminiscent of hepadnavirus replication; thus, at this point in time HFV classification is not clearly distinguished as either a retrovirus or hepadnavirus (Yu, S.F. *et al.* 1996).

V.1.B. Replication. Between virion fusion with the target membrane and the initiation of reverse transcription is the uncoating stage. Information regarding virion uncoating is limited; recently however, compelling evidence has demonstrated the presence of cyclophilin A (CyPA) in HIV particles, but not in SIV (Franke *et al.* 1994; Thali *et al.* 1994). The use of cyclosporine A (an inhibitor of CyPA binding) or mutagenesis of the gag protein result in decreased replicative capacity of the incoming virion. Characterization of the block in replication has demonstrated a marked decrease or absence in the synthesis of early DNA products by reverse transcription, suggesting that the block to replication is at virion uncoating (Braaten *et al.* 1996a). The identification of some HIV (clade O) and SIV strains, and of an isolate of $MuLV_{4070A}$, which do not require CyPA for infectivity, suggests that some viruses contain a functional domain in the gag protein that can function independently of CyPA during viral replication (Braaten *et al.* 1996b).

The hallmark event of the retrovirus life cycle is the synthesis of double stranded DNA from the two single strands of genomic RNA, followed by integration into the host cell chromosome as a provirus (Figure 2) (Varmus, 1988). Reverse transcription does not require specific host cell factors but probably occurs when the RNA genome is still associated with the nucleocapsid protein complex in the cytoplasm (Fujiwara and Mizuuchi, 1988). Reverse transcription of the RNA genome of the retrovirus is a unique and elegant process and is carried out by the viral pol gene product, reverse transcriptase (RT). Five conserved amino acid motifs are present in the RT domain (Figure 16: Paper 2: Marracci *et al.*, in preparation) (Poch *et al.* 1989). Four amino acids are invariant and 18 are strictly conserved between the first four motifs among the RNA dependent RNA and DNA polymerases represented in different RNA virus families. The conserved Asp of motif A and the first Asp of the two found in motif C are critical for the function of the reverse transcriptase as demonstrated by mutational analyses (Poch *et al.* 1989). An apparent paradox exists in that the genomic RNA is shorter than the final double stranded DNA product. Genomic RNA is organized as: 5' R-U5-ψ-gag-pol-env-ppt-U3-R 3', with R-U5 and U3-R representing incomplete long terminal repeats (LTRs); intact LTRs are necessary for viral mRNA expression. Minus (-) DNA strand synthesis is initiated at the 5' end of the genome using a tRNA as the primer (Figure 3). Synthesis proceeds right to left (in the

usual map convention) a short distance to the end of the 5' R region (strong stop DNA). After RnaseH digestion and removal of the RNA portion of the heteroduplex, the nascent (-) DNA molecule undergoes an interstrand transfer to the 3' R region of the second genomic RNA molecule. Negative strand DNA synthesis proceeds concomitant with RnaseH degradation of the RNA template. The polypurine tract (ppt) which is resistant to RnaseH serves as the primer for synthesis of positive (+) strand DNA. After a second intrastrand transfer, the (+) and (-) DNA strands serve as templates for the reciprocal strand and the genesis of a single homoduplex DNA molecule with reconstituted LTRs, each containing copies of the U3-R-U5 regions (Figure 3) (Panganiban and Fiore, 1988; Gilboa *et al.* 1979; Temin 1981).

A linear viral DNA molecule with blunt ends or a dinucleotide recess of its 3' ends is found in the nucleoprotein preintegration complex in the cytoplasm. However, after nuclear localization of the preintegration complex, circular DNA forms have also been identified which contain 1 or 2 LTRs along with the linear form (Fujiwara and Mizuuchi, 1988). The current model for integration strongly suggests that the linear DNA molecule with the 3' recessed ends is the immediate precursor for integration into the host chromosome (Craigie *et al.* 1990; Fujiwara and Mizuuchi, 1988). Integration proceeds under the auspices of the viral protein, integrase (IN). The IN protein consists of three identifiable domains: the N-terminal region which contains the HHCC ($HX_{3-7}HX_{23-32}CX_2C$) zinc finger-like motif, the central core domain which contains the conserved DD35E ($DX_{39-58}DX_{35}E$) motif and which retains the enzymatic activity of IN, and finally the C terminal domain, which has DNA binding properties (Figure 15; Paper 2: Marracci *et al.*, in preparation). IN is part of a superfamily of polynucleotidyl transferases which also includes RNAseH and Mu transposase (Shibagaki *et al.* 1997; Rice *et al.* 1996). With few exceptions, most C-type retroviruses require dividing cells in order to synthesize and integrate viral DNA into the host chromosome (Miller *et al.* 1990; Springett *et al.* 1989). However, integration of HIV, a lentivirus, has been demonstrated in metabolically active but nondividing cells. An ATP source is necessary for transport of the preintegration complex into the nucleus (Bukrinsky *et al.* 1992).

Integration requires four distinct steps beginning with the already described reverse transcription of genomic RNA and synthesis of a linear duplex DNA molecule. Second, post integration sequence analyses have determined that 1-2 bases have been removed from the 3' ends of the provirus (Craigie *et al.* 1990; Katz *et al.* 1990). The 3' dinucleotide recess is considered a product of the *pol* gene; mutations in the *pol* gene can prevent formation of the 3' recessed ends without loss of DNA synthesis ability, suggesting that the 3' recessed ends are formed by enzymatic activity rather than incomplete DNA synthesis (Fujiwara and Craigie, 1989; Fujiwara and Mizuuchi, 1988). A striking conservation of nucleotide sequence is found at the ends of all proviral DNA and is characterized by 5'TG...CA3' terminal dinucleotides. These dinucleotide boundaries have also been observed in Yeast Ty, drosophila Copia, and bacterial transposable elements (Craigie *et al.* 1990; Katz *et al.* 1990). Third, a 4-6 base pair stretch of flanking chromosomal DNA has been duplicated during integration of the provirus (Fujiwara and Craigie, 1989; Craigie *et al.* 1990; Fujiwara and Mizuuchi, 1988; Katz *et al.* 1990). The current model which explains this observation is introduction of a staggered cut into the target DNA, resulting in a 5' overhang. The chromosomal $5'PO_4$ can then join via a cellular repair process to the 3'OH of the recessed viral termini along with the excision of the mismatched 5' dinucleotides. The integration reaction resembles a topoisomerase directed break and join reaction as an extrinsic source of energy is unnecessary for integration of the provirus (Fujiwara and Mizuuchi, 1988). Finally, if replication proceeds normally, each infectious virion produces one provirus (Hu and Temin, 1990; Panganiban and Fiore, 1988).

V.1.C. Viral gene expression. After integration, transcription of viral mRNA is directed by the LTRs located at the terminal ends of the proviral genome. The LTR contains directions for the promotion, initiation and polyadenylation of viral transcripts (Varmus, 1982; Narayan and Clements, 1990; Varmus, 1988). Specific transcription initiation sequences are found in the U3 region of the LTR that can be recognized efficiently by host cell machinery. TATA and CCAAT sequences are present in well characterized sites upstream of the cap site (Fields and Knipe, 1991; Sonigo *et al.* 1986; Varmus, 1982). Enhancers can increase the transcriptional activity of the viral promoter. The number or type of enhancers present may affect the virulence or target cell specificity of the virus.

Recognition sites may be present in the viral LTR for other cellular transcription factors such as Sp-1 or NF-κB, which would restrict viral expression to particular cell types (Narayan and Clements, 1990). Regions of the LTR may have a specific negative effect on viral expression (ex: HIV), or exert a negative response in some cell types and a positive effect in others (ex: MuLV) (Fields and Knipe, 1991).

Host cell machinery recognizes the polyadenylation (AAUAAA) signal within the viral transcript (Fields and Knipe, 1991). The full-length polyadenylated transcript serves not only as mRNA for expression of the gag and pol proteins, but as genomic RNA which is packaged into virus particles (Varmus, 1988). Two possible mechanisms have been proposed in order to express the gag and pol proteins from one mRNA. The first method exists in the murine leukemia virus in which the *gag* and *pol* genes are in frame with each other with an interjected stop codon. This stop codon is suppressed by the misincorporation of a Glu residue thereby allowing read-through into the downstream sequence (Fields and Knipe, 1991). In other systems, a stop codon is between two genes which are not in frame with each other, and ribosome slippage to the -1 frame occurs, resulting in an insertion of an amino acid at the location of the stop codon and continued synthesis of the polyprotein product (Fields and Knipe, 1991; Sonigo *et al.* 1986). The envelope glycoprotein is translated from a subgenomic transcript, generated by a cell-mediated splicing event, on ribosomes associated with the endoplasmic reticulum (Fields and Knipe, 1991; Hunter and Swanstrom, 1990). The envelope precursor polypeptide is glycosylated in the ER and after proper folding and oligomerization is transported to the Golgi network for further post-translational modifications. A cellular endopeptidase in the Golgi apparatus cleaves the glycoprotein precursor and generates the SU and TM domains; without cleavage infectivity is drastically reduced (Barr, 1991; McCune *et al.* 1988; Westervelt *et al.* 1992; Brody *et al.* 1992; Freed and Myers, 1992; Sommerfelt *et al.* 1992a; Rein *et al.* 1994).

V.1.D. Assembly. Assembly of the virus particle takes place in the cytoplasm or at the plasma membrane concurrent with budding. Assembly at the plasma membrane is a characteristic of the Lentivirinae and Type C Oncovirinae families. Similar to intracisternal A-type particles, type B

and D viruses assemble their nucleocapsids in the cytoplasm, and later associate with the plasma membrane allowing for release of infectious virus particles (Rhee *et al.* 1990a).

The release of infectious progeny virus is dependent on the appropriate interaction between the matrix (MA) protein and the host plasma membrane. Myristylation of the N-terminal glycine of the MA facilitates the association between the plasma membrane and matrix protein (Rhee and Hunter, 1987; Rein *et al.* 1986; Hansen *et al.* 1990; Weaver and Panganiban, 1990). The importance of the myristylation event has been demonstrated for Type D MPMV, Type C Moloney murine leukemia virus, spleen necrosis virus, and HIV; the lack of the myristoyl group results in an aborted infection (Rhee and Hunter, 1987; Hansen *et al.* 1990; Weaver and Panganiban, 1990; Göttlinger *et al.* 1989). In contrast, the matrix protein of the equine infectious anemia virus (EIAV) has been shown to contain an N-terminal block to Edman degradation, and a myristoyl group is not present (Henderson *et al.* 1987). Furthermore, bovine immunodeficiency virus (BIV) has an N-terminal Met present in the MA protein with no fatty-acid modification, supporting the hypothesis that myristylation of the MA is necessary but perhaps not sufficient for membrane targeting and that additional MA domains play a role (Tobin *et al.* 1994).

V.1.E. Generation of diversity. The evolution of a viral genome is defined by the diversity of nucleotide sequence observed in recovered isolates from an individual or a population over time. The existence of distinct yet closely related genomes within a viral population leads to an overall increased fitness level of the population (Holland *et al.* 1992; Coffin, 1992; Nowak *et al.* 1991; Wong-Staal, 1991). Increased fitness can be defined as a phenotypic change resulting in an increased ability of a viral genome to infect, replicate and spread within a particular environment. Phenotypic changes affecting the fitness of a viral genome can be characterized by alterations in host range, antigenic configuration, replication rates, and pathogenesis (Nowak *et al.* 1991; Goudsmit *et al.* 1991; Vaishnav and Wong-Staal, 1991; Katz and Skalka, 1990). Furthermore, the divergence of the "filial" viral genome from the "parental" genome, following the acquisition of point mutations, insertions or deletions, leads to the phenotypic variations that yield increased fitness (Coffin, 1992; Zhang and Temin, 1993; Goodenow *et al.* 1989; Katz and Skalka, 1990). The selective forces that determine

fitness during viral infection depends upon the species infected, immune response, availability of permissive target cells, or the chemotherapeutic environment (Holland *et al.* 1992; Nowak *et al.* 1991; Goudsmit *et al.* 1991; Vaishnav and Wong-Staal, 1991; Katz and Skalka, 1990). The success of the fluid nature of the RNA genome is suggested by the fact that families of "riboelements" have adapted to diverse cellular organizations, including bacteria, yeast, and eukaryotic species.

Diversity in the retroviral genome is generated by three basic mechanisms. These mechanisms are recombination, misincorporation and rearrangements within the viral genome; different mechanisms are at work which create genomic variations at distinct stages of the viral life cycle (Zhang and Temin, 1993; Vaishnav and Wong-Staal, 1991; Katz and Skalka, 1990). Early stages of the viral life cycle are characterized by reverse transcription of the RNA genome followed by integration of the provirus, whereas late stages consist of viral mRNA expression by cellular RNA polymerase II, post-translational processing, splicing, and assembly. For example, recombination during synthesis of DNA between two related viral genomes occurs at an early stage, whereas transduction of a host gene/oncogene is a late stage mechanism in the generation of diversity. Furthermore, the mutations which result from the various mechanisms have their effects at different stages in the life cycle of the retrovirus. The retrovirus is able to capitalize on the infidelity of the reverse transcriptase and the diploid nature of the RNA genome in order to increase the fitness of the population by evolving more rapidly in order to meet the shifting demands of its microenvironment. Interestingly, in addition to mutations which increase fitness by altering a protein product, other mutations affect untranslated regions of the viral genome (Coffin, 1992; Katz and Skalka, 1990). In some cases, expression of the retroviral genome in previously nonpermissive target cells resulted from mutational events in the long terminal repeat (LTR) or in the 5' leader section of the genomic transcript (Loh *et al.* 1988; Katz and Skalka, 1990; Coffin, 1992) (Linney *et al.* 1984; Fan *et al.* 1986). For example, in the Murine leukemia virus (MuLV), a single point-mutation in the 5' intergenic region permits replication in normally nonpermissive embryonal carcinoma cells (Yamauchi *et al.* 1995; Kempler *et al.* 1993; Loh *et al.* 1988).

Two mechanisms of recombination, homologous and non-homologous (or illegitimate), can utilize reverse transcriptase to generate diversity in the provirus. Recombination during early stages of the viral life cycle is a product of the innate ability of reverse transcriptase to switch templates during the synthesis of DNA from the genomic RNA template in order to synthesize intact LTR structures (Panganiban and Fiore, 1988). Two models of homologous recombination have been proposed, forced copy choice and strand displacement; both are characterized by template switching during the generation of a recombinant genome. The forced copy choice mechanism of recombination is distinguished by recombination during (-) strand DNA synthesis and generation of a novel genome, which is dependent upon the infecting virus being a heterozygote. Two similar proviruses within the same cell or encapsidation of a transcript from an endogenous provirus can serve to create a heterologous viral particle (Golovkina *et al.* 1994; Yin and Hu, 1997). Due to the presence of breaks in the RNA template and possible non-homologous domains, the agility of reverse transcriptase in template switching is mandatory to synthesize a contiguous minus strand of DNA. After plus strand synthesis, a single DNA homoduplex is synthesized (Hu and Temin, 1990).

The strand displacement model of homologous recombination is proposed to occur during plus DNA strand synthesis. This model is characterized by an H structure as a recombination intermediate, and assumes that plus strand synthesis occurs at many places along the minus DNA template by using RNA primers generated by the incomplete digestion of Rnase H. A nascent "incoming" strand of DNA can displace the tail of a second strand of DNA; the displaced tail then aligns itself with the alternate template thereby forming a hybrid provirus. This model is dependent upon the synthesis of two minus strands of DNA from both RNA genomes and the eventual synthesis of two double stranded DNA molecules with heteroduplex regions (Katz and Skalka, 1990). The model also includes the possibility that the 3' end of the nascent plus strand DNA switches templates in a similar manner, and utilizes the second template for completing the synthesis of the positive DNA strand (Coffin, 1992; Katz and Skalka, 1990). In summary, both models of homologous recombination depend upon the heterozygosity of the viral genome. The forced copy choice model of recombination occurs at the level of minus DNA synthesis and results in synthesis of a single

homoduplex molecule; in contrast, the strand displacement model takes place at the level of plus DNA synthesis and results in the generation of two double stranded DNA molecules with heteroduplex regions (Hu and Temin, 1990; Coffin, 1992; Katz and Skalka, 1990).

The non-homologous or illegitimate model of recombination does not depend upon a recombination event to occur, although very small (5-8bp) regions of identity have been observed at potential recombination junctions in genomic products. Non-homologous recombination often results in the acquisition of cellular DNA, for example a cellular oncogene, which then could affect the pathogenic potential of the virion progeny. This model assumes that the provirus integrates within a cellular proto-oncogene, and a certain level of read-through transcription occurs in order to generate viral-host chimeric transcripts that retain a viral packaging signal. Incorrect splicing of the chimeric transcript results in loss of viral sequences. Alternatively, a cellular transcript could be erroneously packaged by the virus. During synthesis of minus strand DNA, the reverse transcriptase jumps to a nonhomologous site and continues synthesis generating a single DNA homoduplex. Hu *et al.* clearly demonstrated with the use of a recombinant virus which had LTRs from MuLV and spleen necrosis virus (SNV), that the initial strong stop (-) DNA strand could find an alternate site in which to continue (-) strand synthesis in the presence of nonhomologous "R" regions during reverse transcription (Yin *et al.* 1997). Most interesting were the observations that even with deletions of the CCAAT and TATA motifs in the resulting U3 region of the 5' LTR, a low level of viral mRNA expression was retained, and that one nucleotide was sufficient for "homology" and (-) strand transfer (Yin *et al.* 1997). In addition, a weak-stop DNA molecule was also functional in the strand transfer reaction and able to induce the completion of (-) strand synthesis on the new template (Yin *et al.* 1997). Due to the deletion of some viral sequences, a replication-defective virus may be the result of nonhomologous recombination. The presence of a replication-competent or helper-virus in subsequent rounds of infection is necessary for transmission of the defective virion (Zhang and Temin, 1993). Zhang and Temin clearly demonstrated the ability of a retrovirus to capture new sequences by nonhomologous recombination in an experimental system that was dependent upon only one round of replication; these investigations determined the rate of nonhomologous

recombination to be 5×10^{-5} per round of replication which is approximately 0.1 to 1% the rate of homologous recombination (Zhang and Temin, 1993).

Diversity is also created by misincorporation of nucleotides during replication, and is a direct result of the inability of the reverse transcriptase to proofread and edit the nascent DNA strand, RNA polymerase II is also unable to edit the mRNA product. The frequency of misincorporation of nucleotides by reverse transcriptase is 10^{-4} to 10^{-5}/basepair/replication cycle (Zhang and Temin, 1993; Katz and Skalka, 1990). In addition, a high degree of preferential misincorporation exists as illustrated by the strong predilection for G to A transitions which explains the observation that tryptophan frequently changes to a stop codon, TGG to TAG, TGA or TAA (Goodenow *et al.* 1989).

Finally, rearrangements within a viral genome may result in defective replication. Reverse transcription (an early replication event) across direct repeats in the viral genome is central for the generation of deletions or duplications. Template slippage or mispriming at direct repeats generates small deletions, whereas homologous recombination between direct repeats results in the generation of large deletions. The deletion of direct repeats is the direct consequence of homologous recombination and (-) strand DNA transfer. The (-) DNA strand may perform an intramolecular or intermolecular transfer, with synthesis continued at the homologous site from the 3' direct repeat; alternatively, DNA synthesis can continue from the homologous site of the 5' direct repeat. Hu *et al.* demonstrated the preferred use of intramolecular strand transfers in generating the deletion of a direct repeat (Hu *et al.* 1997). In addition, aberrant endonuclease activity by the IN protein may truncate the LTR.

In conclusion, several different mechanisms contribute to the creation of genomic diversity which results in the generation of a population of distinct yet related genomes defined as an isolate, strain, or quasi-species. The models proposed consist of recombination events between the RNA molecules of a heterozygous virus particle, misincorporation of nucleotides by reverse transcriptase or RNA pol II, and the generation of deletions or duplications in the double stranded DNA product. No single genome present in an existing isolate may actually match a derived "consensus" sequence. Genotypes from the same individual are more related to one another than to genotypes recovered

from another individual (Vaishnav and Wong-Staal, 1991). As new genomes with advantageous mutations arise and dominate the population, pre-existing members are not lost, but can constantly serve as a genomic pool from which new variants can originate in response to different selective pressures (Holland *et al.* 1992; Coffin, 1992; Wong-Staal, 1991; Katz and Skalka, 1990). During the initial course of infection by an immunosuppressive virus, rapidly replicating genomes are eliminated by the healthy immune response, thereby placing selective pressure on the appearance of more slowly replicating genomes which can persistently infect the host by evading the immune response mounted against the prior strains. At this stage of infection, the more slowly replicating genome is the most fit relative to its environment, a healthy immune system (Vaishnav and Wong-Staal, 1991). As the immune system deteriorates with a concomitant decrease in selective pressure, new viral genomes with an increased replicative capacity are not being cleared, and may eventually become dominant in the population (Nowak *et al.* 1991; Vaishnav and Wong-Staal, 1991).

V.2 MOLECULAR CHARACTERISTICS OF A PROTOTYPE TYPE D RETROVIRUS

It is not the possession of knowledge that makes the man of science
but his persistent and relentless critical quest for truth. KARL POPPER

Four small reading frames, *gag*, *prt*, *pol* and *env* are present within the genome of MPMV (Figure 1). The genome is organized in the following sequential order: 5' LTR-pbs-φ-*gag-prt-pol-env*-ppt-LTR 3', which includes three *cis*-acting sites beginning with the primer binding site (pbs) which is recognized by a $tRNA^{lys}$ during minus strand DNA synthesis. The pbs also determines the 3' boundary of the 5' LTR (Sonigo *et al.* 1986). The φ site is the packaging signal for the genomic RNA and is located downstream of the pbs and continues into the *gag* gene (Bryant *et al.* 1986a). The polypurine tract (ppt) marks the 5' boundary of the 3' LTR and is necessary during replication for the initiation of plus strand DNA synthesis (Sonigo *et al.* 1986).

V.2.A. The long terminal repeat regions. The LTR contains sequences specific for the promotion, initiation and polyadenylation of the viral transcript. The LTR is divided into three domains in the following order: U3-R-U5. The U3 region contains the viral promoter sequences, a TATA box homolog represented by the sequence TATATAAG, which is preceded by a CAT sequence, GCAT (Figure 13; Paper 2: Marracci *et al.*, in preparation). MPMV is similar to the type B virus, MMTV, in that the polyadenylation signal, ATTAA, resides in the U3 region of the LTR rather than the R domain. The LTR of MPMV contains a strong transcriptional promoter, but activity is not controlled by virally encoded transacting factors (Thielan *et al.* 1987).

V.2.B. The gag proteins. Three gag polyprotein precursors can be identified in an infected cell and are designated, Pr78, Pr95 and Pr180 (Bradac and Hunter, 1984). Pr78 encodes the precursor gag polyprotein with Pr95 encoding the gag-prt and Pr180 the gag-prt-pol protein precursors. After processing by the retroviral protease, the Pr78 is converted into the following mature gag proteins: p10, pp24/pp16, p12, p27, p14, and p4 (Vile *et al.* 1992).

The p10, or matrix protein (MA), is at the N-terminus of the precursor and has been well characterized at both the molecular and functional levels. The MA serves as a bridge between the nucleocapsid core of the virus and the plasma membrane. Random mutagenesis of the MA domain

has led to the identification of a role for the MA in three critical steps of viral replication and type D morphogenesis: 1) the formation of a stable gag precursor capable of intracytoplasmic capsid assembly 2) transport of the capsids to the plasma membrane and 3) budding and release of the virion from the membrane (Rhee and Hunter, 1991). The modification of the N-terminal glycine residue of MA with the attachment of a myristoyl group by cellular enzymes is necessary for the transport and association of the assembled nucleocapsid within the plasma membrane. However, myristylation is not necessary for the intracytoplasmic assembly of the capsid itself (Rhee and Hunter, 1987; Rhee and Hunter, 1990b). Myristylation of the MA protein is not sufficient for the transport and targeting of the MA to the plasma membrane. Interestingly, cellular proteins that are modified by myristic acid have been identified, which are retained in the cytoplasm. The MA contains a dominant signal that determines cytoplasmic localization during the capsid assembly process; a single amino acid mutation converting an Arg to a Trp residue at position 55 (R55W) allows capsid assembly to occur at the plasma membrane as with type C viruses but with absence of infectivity (Rhee and Hunter, 1990a). The gag precursor has been demonstrated to be the sole requirement for capsid assembly in the cytoplasm of infected cells and the presence of the env glycoprotein is not necessary for targeting and release from the plasma membrane (Rhee *et al.* 1990a; Sommerfelt *et al.* 1993; Sakalian *et al.* 1996). However, without the presence of the envelope moiety, the virus is rendered noninfectious. Heteronuclear nuclear magnetic resonance studies have demonstrated that the matrix protein consists of four α-helical domains (Conte *et al.* 1997). The MA is highly basic and contains two regions of positive charge in the N-terminal domain (A and B helix; C-D loop) and a third region, the C-D helix, also includes the R55W point mutation. Although the primary sequence homology of MA between MPMV and HIV, HTLV, BLV and SIV is low, the helical structure is conserved which suggests a similarity of function (Conte *et al.* 1997). Interestingly, two distinct mutants of MPMV MA abrogate cleavage of the gp22 transmembrane (TM) envelope glycoprotein domain to gp20 by the viral protease, thus reducing infectivity by >90% (Brody *et al.* 1992). The current model of assembly proposes that the MA carries a dominant signal which localizes the gag polyprotein precursor to the cytoplasm where assembly of the virus particle ensues. After assembly and encapsidation of the

genomic RNA, an intrinsic capsid signal directs transport of the immature virion to the plasma membrane independent of the presence of envelope glycoproteins. At the time of budding and release of the virus particle, interaction between the gag precursor polyprotein and the TM of the envelope is necessary for final proteolysis of the TM and subsequent viral infectivity. The phosphoprotein, pp24/pp16 has a conserved PPPY motif at its C-terminus (Figure 14; Paper 2: Marracci *et al.*, in preparation). Experiments with MPMV mutants have demonstrated that pp24/pp16 has a role in gag precursor stability, inactivation of the viral protease and end-stage release, but not in maturation of the virus particle (Yasuda and Hunter, 1998). Phosphorylation occurs on a serine residue and takes place while the phosphoprotein is still part of the gag precursor molecule (Bradac and Hunter, 1984).

Type B and D retroviruses each code for an acidic protein located between the matrix and major capsid protein domains. The MPMV protein, designated p12 and the p3 of MMTV, have roles in the intracellular assembly of these two viruses. Interestingly, type C retroviruses which assemble at the plasma membrane do not encode a p12 homolog. Genetic studies in which the p12 domain was compromised had two outcomes dependent upon the level of gag polyprotein synthesis. At high levels of expression, p12 was not required for assembly; however, at lower levels the stability of the intracellular capsid array was assisted by p12. Furthermore, the p12 domain was necessary for the release of infectious virions (Sommerfelt *et al.* 1992b). Sommerfelt *et al.* described p12 as a "*cis*-acting catalytic domain" with a role in the assembly process (Sommerfelt *et al.* 1992b).

The major capsid protein (CA) is designated p27 and is the largest of the gag polyprotein constituents. As with the conservation of the matrix protein structure among different retroviral families, an extremely conserved site is identified within the CA protein and is designated the major homology region (MHR). Of the 20 amino acids which define the MHR, six are identical or with only a single conservative amino acid substitution observed among various types B, C, D and lentivirus isolates. The MHRs between SIV and MPMV are 100% conserved, suggesting that the MHR has an important role in the life cycle of the retrovirus among different virus families (Strambio-de-Castillia and Hunter, 1992). Mutations in the MHR have demonstrated a range of outcomes; mutants mostly

retain the ability to assemble in the cytoplasm but with reduced or total absence of infectivity. However, some mutants unable to assemble were also observed (Strambio-de-Castillia and Hunter, 1992).

MPMV p14 is highly basic and is the major component of the ribonucleoprotein complex. p14 has been designated the nucleocapsid (NC) protein (Rhee and Hunter, 1987). The function of the NC is thought to be in the binding and encapsidation of the viral genomic RNA. The NC contains a highly conserved Cys-His motif which is present in plant as well as human viruses, suggesting that the Cys-His box has a critical function during viral replication. The Cys-His box has the consensus amino acid sequence: $CysX_2CysX_4HisX_4Cys$ in which X is any amino acid, and from the initial Cys an aromatic residue is located at the first or second, and ninth positions, with a conserved Gly at the seventh position. When two Cys-His boxes are present, the first or proximal motif is very conserved whereas the distal box is degenerate. MPMV has two Cys-His motifs in NC consistent with the above description (Figure 14; Paper 2: Marracci *et al.*, in preparation). The presence of two dissimilar Cys-His motifs has been demonstrated to be more important than the actual order of the motifs in Rous sarcoma virus (RSV). In addition, when two proximal-type motifs were present the RNA content in the released virions and their corresponding infectivity were markedly reduced (Bowles *et al.* 1993).

A frame-shift mechanism is expected to allow for translation of the protease and polymerase gene transcripts. Evidence from studies with SRV-1 suggests that an RNA structure is present which allows for slippage during translation such that the reading frame is misplaced by -1. The structure proposed is that of a pseudo-knot (2 double stranded stems and two connecting loops), that is preceded by a seven nucleotide slip site (X XXY YYN, triplets indicate the initial reading frame) and a seven nucleotide spacer region. Biochemical and mutational analyses have confirmed the presence of a structure consistent with that of a pseudo-knot which exhibited a frameshifting efficiency of 23% in a rabbit reticulocyte lysate translation assay (Fehrmann *et al.* 1997; ten Dam *et al.* 1994; ten Dam *et al.* 1995). Sequence analyses of an endogenous retrovirus, which forms intracisternal A-type particles in rodent cells, have identified a 156 nucleotide region that overlaps the

gag-prt orfs and shares homology with a prototypical pseudo-knot element. This region is predicted to function in the frameshifting event that allows expression of the *prt* gene (Fehrmann *et al.* 1997).

V.2.C. The protease of MPMV. The *prt* reading frame of MPMV overlaps the 3' end of *gag* by 55 codons and the 5' end of *pol* by 5 codons. Translation of the gag-prt polyprotein, Pr95, is considered to occur by a frameshift event which occurs at the end of gag. Pr95 is represented at levels approximately 10% to those of Pr78, suggesting that the frame shift mechanism is quite efficient. The *prt* gene product has two functional domains: a deoxyuridine-triphosphatase (dUTPase) at the N-terminus and an aspartyl protease at the C-terminus (Sommerfelt *et al.* 1992a). The N-terminal end of the protease of SRV-1 is reported to be similar to other proteins encoding dUTPase activity (McGeoch, 1990). Furthermore, biochemical analyses have demonstrated the presence of dUTPase activity in the corresponding region of MPMV and SRV-1 (Elder *et al.* 1992). The prt precursor of progeny virions is autocatalytically cleaved late in the assembly as evidenced by the inactivity of the prt while A-type particles remain in the cytoplasm (Sommerfelt *et al.* 1992a). The carboxy-terminal portion of the viral protease has significant homology to other retroviral and cellular aspartyl proteases including the presence of the Asp-Thr-Gly active site between amino acid positions 188-190 (Figure 15; Paper 2: Marracci *et al.*, in preparation) (Thayer *et al.* 1987; Shiigi *et al.* 1985; Toh *et al.* 1985; Seiki *et al.* 1983; Schwartz *et al.* 1983; Power *et al.* 1986).

V.2.D. The reverse transcriptase and integrase proteins. The *pol* gene encodes amino acid sequences which share homology with the reverse transcriptase, endonuclease and integrase of *pol* genes from other subfamilies. A second frameshift at the overlap between *prt* and *pol* in the Pr180 transcript is responsible for the expression of reverse transcriptase/integrase. The Pr180 polyprotein precursor is present at levels approximately 1% to those of Pr78 (10% of the level of Pr95), suggesting that the frameshift mechanism is as efficient at this site as at the *gag-prt* overlap.

V.2.E. The envelope glycoprotein. The fourth reading frame encodes a glycosylated precursor of 586 amino acids, which corresponds to the envelope precursor, gPr86, and is translated from a spliced subgenomic mRNA on membrane bound ribosomes (Sonigo *et al.* 1986; Hunter and Swanstrom, 1990). The gPr86 is post-translationally processed in the lumen of the endoplasmic

reticulum (ER); high mannose-containing side chains are added to the Asn-X-Ser/Thr N-linked glycosylation motifs. Glycosylation of the envelope peptide functions to drive the proper folding of the protein required for transport out of the ER, recognition and binding of the cell surface receptor, and protection of the peptide structure from proteases (Bradac and Hunter, 1986; Hunter and Swanstrom, 1990). Protection from cellular proteases can decrease the immunogenicity of the envelope peptides by masking epitopes which could interact with cells of the immune system (Bradac and Hunter, 1986; Hunter and Swanstrom, 1990). After transport to the Golgi apparatus, high-mannose containing side chains are further modified by the addition of N-acetyl-glucosamine, galactose and fucose molecules. High mannose groups are not consistently modified as demonstrated by the sensitivity of the TM peptide to digestion by endoglycosidase H (Bradac and Hunter, 1986; Hunter and Swanstrom, 1990). Following treatment of MPMV infected cells with tunicamycin, noninfectious virions are produced, suggesting that glycosylation of the envelope glycoprotein is important for the generation of infectious virions (Bradac and Hunter, 1986).

Once in the Golgi compartment, a cellular endopeptidase potentiates the cleavage between the gp70 surface (SU) and the gp22 transmembrane (TM) domains, leaving a noncovalent association between the SU and TM domains (Brody *et al.* 1992; Sommerfelt *et al.* 1992a; Brody and Hunter, 1992; Hunter and Swanstrom, 1990). The putative consensus sequence for the endopeptidase is a multiple basic amino acid motif, Arg/Lys-X-Arg/Lys/Arg-Arg, which corresponds to the Lys-Ala-Lys-Arg sequence found in MPMV (Schwartz *et al.* 1983; Hosaka *et al.* 1991; McCune *et al.* 1988; Brody *et al.* 1992). A Ca^{2+} -dependent serine protease member of the mammalian subtilisin family may be responsible for the catalysis at the carboxy terminal side of the Arg (Barr, 1991; Hosaka *et al.* 1991; Brody *et al.* 1992; Freed and Myers, 1992; Hunter and Swanstrom, 1990). The SU is the larger domain and is highly glycosylated; recognition of the viral receptor on the target cell is its primary function (Bradac and Hunter, 1986; Hunter and Swanstrom, 1990). The TM domain is smaller and anchors the viral envelope glycoprotein to the plasma membrane, and principally functions in the facilitation of fusion and virus entry into the target host cell (Sonigo *et al.* 1986; Bradac and Hunter, 1986; Hunter and Swanstrom, 1990; Chambers *et al.* 1990). Longitudinal analyses of

viral isolates recovered from single individuals have demonstrated a large degree of heterogeneity in the envelope proteins (Pang *et al.* 1992; Fisher *et al.* 1988; Sánchez-Palomino *et al.* 1993; Lane *et al.* 1995). Equine infectious anemia virus is a classic example of the development of envelope heterogeneity following a neutralizing immune response in order to escape detection and clearance. Despite the high degree of heterogeneity of envelope glycoproteins, certain functions must be conserved such as receptor recognition, fusion ability, cleavage between the surface and transmembrane domains, and anchoring of the TM within the plasma membrane (Doms *et al.* 1990).

The envelope precursors of all retroviruses have four conserved hydrophobic domains. The N-terminal domain of MPMV contains a 25 amino acid residue signal peptide within a larger leader sequence. The signal peptide enables the nascent polypeptide to be inserted into the endoplasmic reticulum for further translation and processing. The signal sequence is cleaved co-translationally following the Gly/Asp bond by a cellular signal peptidase (Hunter and Swanstrom, 1990). The membrane spanning domain (MSD) of the transmembrane envelope subunit is at the C-terminus and functions to stop the translocation of the polypeptide into the lumen of the ER and anchors the peptide in the membrane (Hunter and Swanstrom, 1990). If translation is terminated prior to the anchoring of the TM subunit in the membrane, only soluble glycoprotein forms are synthesized.

The third highly conserved region is the fusion domain at the N terminus of the TM. The fusion domain is distinguished by its hydrophobicity and is followed by a Lys-Arg rich domain which is characterized by heptad repeats with a sequence periodicity of (a-b-c-d-e-f-g) in which no helix breakers such as Pro are located (Brody *et al.* 1992; Freed and Myers, 1992; Hunter and Swanstrom, 1990; Chambers *et al.* 1990). The residues located at a and d contain bulky and hydrophobic or neutral amino acids. The heptad repeats are thought to generate a structure consisting of α-helices coiled upon themselves that allows interaction between the hydrophobic faces. These supersecondary structures can function as hydrophobic spikes which destabilize the target membrane by the formation of hydrophobic channels, thus aiding the hydrophobic N-terminus of the TM protein in fusion and virus entry. Additionally, the external domain of the TM peptide is important in forming an oligomeric structure of envelope molecules while in the ER (Doms *et al.* 1990). Oligomerization

of the envelope precursor is not only be necessary for transport out of the ER, but perhaps also for sequestration of the fusion domain and prevention of its exposure to intracellular membranes (Hunter and Swanstrom, 1990; Doms *et al.* 1990; Einfeld and Hunter, 1988). Finally, a highly conserved stretch of hydrophobic amino acids present in a number of retroviruses has been defined as the immunosuppressive peptide (ISP)(Cianciolo *et al.* 1985; Cianciolo *et al.* 1984). Particularly important is the presence of the ISP in retroviruses which are oncogenic, such as Moloney-murine leukemia virus (MoMuLV), RSV, feline leukemia virus (FeLV) and reticuloendotheliosis-associated virus (Rev-A) (Carpenter *et al.* 1978; Wilhelmsen *et al.* 1984; Carpenter *et al.* 1977; Rup *et al.* 1979; Brody and Hunter, 1992). The immunosuppressive peptide, p15E in MoMuLV, has been demonstrated to inhibit the in vitro transformation of lymphocytes due to a decrease in IL-2 production (Cianciolo *et al.* 1984). MuLV p15E is able to suppress human monocyte function in vitro and the ability of murine macrophages to accumulate at inflammatory foci in vivo (Cianciolo *et al.* 1984). The p15E of FeLV has been demonstrated to inhibit the lymphocyte blastogenic response to mitogen and alloantigen stimulus, as well as inhibiting the Con A transformation of human lymphocytes in vitro (Cianciolo *et al.* 1985). The ISP of MPMV is nearly identical to the ISPs of these very different virus isolates, and is identical to the ISPs of SRV-1 and two members of SRV-2 (Figure 17; Paper 2: Marracci *et al.*, in preparation). The ISP domain is located within the heptad repeat domain of the TM and therefore may also have an important role in the structure or function of this glycoprotein. Mutation analyses of MPMV has determined that an 11 amino acid deletion at the N-terminus of the ISP results in the normal processing of the gPr86 precursor, but released virions are noninfectious due to the loss of the gp70 SU domain from the virion surface (Brody and Hunter, 1992; Brody *et al.* 1994a). The role of this peptide in type D induced immunosuppression is unknown; the high degree of conservation between the different members of the SRV family suggests that the ISP domain is vital to the integrity of the envelope structure and the subsequent production of infectious virions.

Binding of the SU domain to the cell surface receptor defines the host range at the level of entry. Retroviruses enter via a non-pH dependent mechanism of virus-cell membrane fusion followed by release of the nucleocapsid into the cytoplasm (Hunter and Swanstrom, 1990; Kielian and

of incompletely spliced RNA (Kjems *et al.* 1991; Roodman *et al.* 1991; Bray *et al.* 1994). The 3' untranslated region between the end of the *env* gene and 3' LTR of MPMV contains a 219 nucleotide sequence which can function as a Rev-response-like element in replication of a REV(-)RRE(-) HIV mutant. The RRE-like RNA domain of MPMV is designated the constitutive transport element (CTE) (Bray *et al.* 1994). This element maps to positions 7620-7859 of SRV-1 and 7607-7844 of MPMV, and is predicted to contain extensive secondary structure essential for its function (Tabernero *et al.* 1996). The recognition of an RRE-like element in MPMV and SRV-1 suggests that a cellular Rev-like protein is capable of binding this RNA sequence and permitting the export of unspliced transcripts from the nucleus (Zolotukhin *et al.* 1994; Bray *et al.* 1994). RNA helicase A and factor(s) required for export of mRNA, specifically the TAP protein, have also been identified as putative binding proteins of the CTE (Grüter *et al.* 1998; Pasquinelli *et al.* 1997; Tang *et al.* 1997). Moreover, the relative ability of a virus to productively replicate in a specific cell or tissue type is implied by the availability of these Rev-like factors (Trono and Baltimore, 1990; Dayton *et al.* 1993).

V.3 A SHORT HISTORY OF TYPE D SIMIAN RETROVIRUSES

Let science neither be a crown to put proudly on your head nor an axe to chop wood. TALMUD

Type D simian retrovirus (SRV) infection is the predominant cause of Simian Acquired Immunodeficiency Syndrome (SAIDS) in biomedical research colonies in the United States. Type D retroviruses are endemic to cynomolgus (*Macaca fasicularis*) macaques in Indonesia (Desrosiers, 1988, Thouless *et al.* 1988). Alternatively, the lentivirus, simian immunodeficiency virus (SIV), is endemic to African animals such as the African green, mandrill and sooty mangabey monkeys. Unlike SIV, SRVs cause disease in their natural host; in contrast, SIV induces immunodeficiency disease when transmitted to macaque species. Type D viruses have similar yet distinct disease manifestations from those of SIV and are classified as an independent family of immunodeficiency disease viruses that have evolved separately. The fact that SRV is endemic to macaque species gives us a unique opportunity to study the mechanism of immunodeficiency caused by a virus in a natural host.

Nearly 30 years have passed since the first type-D retrovirus, Mason-Pfizer monkey virus (MPMV) was isolated from an eight year old female rhesus macaque (*Macaca mulatta*) with a breast carcinoma (Jensen *et al.* 1970; Chopra and Mason, 1970). Virus isolation was facilitated by co-cultivation of tumor tissue with monkey embryo cell cultures (Fine and Schochetman, 1978). Exogenous transmission was demonstrated by directly infecting monkey embryo cells with cell-free culture fluid from the original tumor. The in vitro host range of MPMV is restricted to primate and human cell cultures (Fine and Schochetman, 1978). Attempts to verify the oncogenic nature of this virus by inoculating rhesus macaques were unsuccessful (Fine *et al.* 1975; Fine *et al.* 1972). Numerous reasons can account for these observations. For example MPMV may not be tumorigenic; alternatively, a requirement for a long latent period may be necessary. Although MPMV did not induce a breast carcinoma in these monkeys, inoculated neonatal rhesus macaques did develop an immunosuppressive disease characterized by severe lymphadenopathy, weight loss, thymic atrophy and opportunistic infections (Fine and Schochetman, 1978; Bryant *et al.* 1986a; Fine *et al.* 1975).

Since that time four additional serogroups of SRV have been recovered from animals with varying degrees of SAIDS. Neutralizing antibodies recognizing the gp70 (SU) of the envelope glycoprotein can discriminate between the different SRV isolates which has lead to the identification of five different serogroups; MPMV is a member of serogroup 3 (Marx and Lowenstine, 1987; Bryant *et al.* 1986b; Marx *et al.* 1985; Stromberg *et al.* 1984).

As early as 1976, various species of the genus *Macaca* have exhibited symptoms of spontaneous immunosuppressive disease at different regional primate research centers (RPRCs) across the country (Table 2) (Henrickson *et al.* 1983; Letvin *et al.* 1983; Gardner *et al.* 1988). Not until 1983 was the first retrovirus isolate identified at the New England RPRC from animals with SAIDS; the isolate was designated D1/CYC/NE (Daniel *et al.* 1984). Peripheral blood lymphocytes isolated from macaques exhibiting symptoms of SAIDS were cocultivated with Raji (human B-lymphoblastoid cell-line) cells and monitored for infection by syncytia formation and electron microscopy. Passage of filtered medium from infected cultures to uninfected Raji cells demonstrated the exogenous nature of the virus and the ability of cell-free virus to infect permissive cells in vitro. Electron microscopy revealed that this SRV-1 isolate (SRV-n, where n is the serogroup) was morphologically a type D retrovirus because it possessed a bar shaped nucleoid and intracytoplasmic A-type particles were present. Transmission studies were conducted by infecting rhesus macaques with tissue culture fluids derived from cocultivation of *Macaca cyclopis* PBLs infected with SRV-1, and Raji cells, or from a thymus explant of a rhesus macaque experimentally inoculated with an infected tissue homogenate (Letvin *et al.* 1984). The infected animals did not exhibit the same degree of immunosuppression originally described at the New England center; in contrast, the immunosuppression was transient and mild (Table 2) (Letvin *et al.* 1983). Additional characterization of five isolates from three different macaque species (*Macaca mulatta*, *Macaca cyclopis* and *Macaca fascicularis*) by restriction endonuclease analysis demonstrated the replicative intermediate genomes to be invariant at 30 different sites, suggesting that one viral isolate had infected the different species at the New England colony (Desrosiers *et al.* 1985).

Concurrently, a severe SAIDS epidemic was being described at the California RPRC (Henrickson *et al.* 1983). One of the outdoor corrals had a 40% mortality rate from SAIDS, which was a mortality eight times greater than other similar corrals. The profile of clinical manifestations in the afflicted macaques included chronic wasting, malignancies, opportunistic infections, decreased response to mitogen stimulation, and no encephalopathies (Henrickson *et al.* 1983). The etiologic agent was ascertained to be of an infectious nature and subsequent isolation by cocultivation of infected blood samples with rhesus monkey kidney cells revealed the presence of a type D retrovirus (Gravell *et al.* 1984a; Lerche *et al.* 1987). Transmission studies were undertaken to verify that the virus isolate designated D1/RHE/CA was the etiologic agent of SAIDS found in the California corral. Transmission of SAIDS was demonstrated to occur with blood or filtered plasma, inoculation of macaques with infected cell-free tissue culture fluids, and by transmission of a molecular clone of D1/RHE/CA, thereby confirming the etiologic role of the D1/RHE/CA virus isolate in SAIDS (Gravell *et al.* 1984b; Marx *et al.* 1984; Heidecker *et al.* 1987). Although the restriction endonuclease pattern was almost identical to that of D1/CYC/NE, the D1/RHE/CA isolate was observed to infect both T and B lineage cells in vitro, and was associated with increased virulence (Table 2) (Power *et al.* 1986; Gardner *et al.* 1988). Immunohistochemistry, electron microscopy, in situ RNA hybridization and Southern analysis techniques were employed to determine the distribution of the D1/RHE/CA retrovirus in infected macaque tissues. The retrovirus predominantly targeted the salivary and germinal centers of lymphoid organs. Interestingly, the lack of viral antigen in the central nervous system was not precluded by the absence of viral genomic sequence in these cells, which suggests a latent infection or possibly an as yet unidentified block to viral replication in neural tissue (Lackner *et al.* 1988).

Concomitant with the characterization of a type D retrovirus as the etiologic agent of SAIDS at the New England and California RPRCs, the Washington RPRC recovered an isolate from retroperitoneal fibromatosis (RF) tissue of a pig-tailed macaque (*Macaca nemestrina*) (Table 2) (Stromberg *et al.* 1984). This isolate belonged to a second serogroup and was designated D2/MNE/WA. RF is unique to SRV-2 infection and develops in 35% of SRV-2 infected Celebes

(*Macaca nigra*), cynomolgus and pig-tailed macaques (Marx and Lowenstine, 1987; Giddens, Jr. *et al.* 1985; Stromberg *et al.* 1984; Marx *et al.* 1985). The combined presence of 1) reverse transcriptase activity after in vitro propagation of infected RF tissue explants, 2) intracisternal A-type particles and 3) free virus particles with typical D-like morphology demonstrated that the D2/MNE/WA isolate was similar to D1/CYC/NE and D1/RHE/CA (Stromberg *et al.* 1984). Transmission studies utilizing uncloned D2/MNE/WA isolates have induced immunodeficiency and RF in cynomolgus and pig-tailed macaques, verifying the etiologic nature of this isolate (Giddens, Jr. *et al.* 1985; Benveniste *et al.* 1985).

Another member of serogroup 2 was subsequently identified at the California RPRC from the peripheral blood mononuclear cells (PBMC) of Oregon RPRC Celebes macaques with SAIDS (Marx *et al.* 1985). D2/CEL/OR is associated with severe SAIDS and RF in Celebes macaques (Table 2) (Marx *et al.* 1985). Furthermore, rhesus macaques infected with D2/CEL/OR seroconvert and become aviremic without manifesting any measurable symptoms of disease (Wilson *et al.* 1986b). Subsequent to the initial isolation of D2/CEL/OR at the California RPRC, three additional serogroup 2 isolates have now been identified at the Oregon RPRC. Although the four isolates are related by serology, each is characterized by novel restriction site polymorphisms. D2/RHE/OR, originally isolated from infected PBMC of a rhesus macaque following cocultivation with Raji cells, is associated with a mild immunodeficiency disease in rhesus macaques and is thought to be similar to the Washington RPRC isolate recovered from a pig-tailed macaque and described above (Bryant *et al.* 1986b; Hefti *et al.* 1983; Stromberg *et al.* 1984). However, D2/RHE/OR is recovered from Japanese macaques (*Macaca fuscata*) with severe and fatal immunodeficiency disease. Another polymorphic variant, D2/RHE/OR/V1, has been recovered from a severely ill rhesus macaque originating from group-housed animals endemically infected with D2/RHE/OR (Table 2). A fourth polymorphic variant, D2/CYN/OR/V2, has been recovered from cynomolgus macaques with severe immunodeficiency disease. The pattern of disease progression is not only dependent on the species infected but also on the infecting virus (Table 2). Thus, the differences in disease associated with the serogroup 2

family is intriguing given that the molecular clones of D2/RHE/OR and D2/RHE/OR/V1 share greater than 95% similarity with the molecular clone of D2/CEL/OR.

Two additional serogroups have been recovered from infected animals. D4/CYN/CA is the sole isolate of serogroup 4 and was recovered from a group of cynomolgus macaques in Berkeley, California. Serogroup 5 is also represented by a single isolate, D5/RHE/OR, and was recovered from rhesus macaques imported from the People's Republic of China to the Oregon RPRC. When D5/RHE/OR is transmitted into juvenile rhesus macaques of Indian origin, a severe immunodeficiency disease develops (Marracci *et al.* 1995).

Genetic relatedness of the SRV family has been suggested by immunologic analyses demonstrating that antigenic cross-reactivity exists between different serogroup core proteins (p27 CA and p10 MA; Stromberg *et al.* 1984; Marx *et al.* 1985). Moreover, immunological cross-reactivity does not exist among the SRVs and human immunodeficiency virus (HIV) as determined by immunoblot and immunofluorescence assays using human anti-HIV sera against purified viral proteins or in vitro infected cells (Bryant *et al.* 1985). In addition to immunologic methods, genetic relatedness of different SRVs has been demonstrated by Southern blot analysis of proviral DNA and protein sequence analysis. Similarity exists among the sequences of SRV-1, SRV-2 and MPMV proviruses but not with HIV (Bryant *et al.* 1986b; Bryant *et al.* 1985; Desrosiers *et al.* 1985; Marx *et al.* 1985). Upon comparison of D1/CYC/NE and MPMV, 46% of the tested restriction endonuclease sites were conserved (Desrosiers *et al.* 1985). Furthermore, cross-hybridization of their respective genomes indicated a close genetic similarity. However, weaker hybridization was observed at the *pol-env* region compared to the *gag* gene, 3' end and LTRs (Desrosiers *et al.* 1985). Under low stringency hybridization conditions, the SRV-2 sequences were detected using MPMV or SRV-1 probes (Bryant *et al.* 1986b). More extensive genetic analysis has been accomplished with the advent of complete nucleotide sequence analyses of MPMV, D1/RHE/CA and D2/CEL/OR (Thayer *et al.* 1987; Power *et al.* 1986; Sonigo *et al.* 1986). Thayer *et al.* confirmed prior observations of relatedness by direct sequence comparisons of molecular clones of SRV-1 (D1/RHE/CA), MPMV and SRV-2 (D2/CEL/OR) (Thayer *et al.* 1987). Fewer than 6% amino acid residue differences are

predicted between MPMV and SRV-1 within the *gag-prt-pol* and C-terminus of the *env* genes. The proteins of D2/RHE/OR/V1 are more closely related to D2/RHE/OR and D2/CEL/OR than to SRV-1 and MPMV (Table 2). SRV-1 and MPMV are 83% similar at the N-terminus of the *env* gene (analysis not shown). In contrast, comparison of the env gp70 among the SRV-2 isolates results in greater than 97% similarity and less than 61% similarity to the env gp70 of SRV-1 and MPMV (Table 2). Genomic organization of this family of retroviruses is conserved with a typical proviral arrangement of 5'-LTR-*gag-prt-pol-env*-LTR-3'. A separate *prt* reading frame is unique to this family of retrovirus. N-terminal protein sequence analysis has further demonstrated the distinct yet close relationship of MPMV core proteins to those of D2/MNE/WA (Henderson *et al.* 1985).

Serogroup 2 SRVs are the most frequent isolates found in U.S. macaque colonies, regardless of species. Recently, serogroup 2 SRVs have been isolated from cynomolgus macaques obtained from wild Indonesian populations (Thouless *et al.* 1988). In addition, an infectious SRV from Ethiopian baboons (*Papio cynocephalus*; SRV-Pc) has been isolated and partially characterized; sera from these baboons exhibited some cross-reactivity to SRV-2 antigens (Benveniste *et al.* 1993; Grant *et al.* 1995). To date, studies in India, the source of founder animals for most of the rhesus macaques held in biomedical research colonies, have failed to identify serologic evidence of type D SRV in wild Indian populations of rhesus and bonnet macaques (Marx, unpublished data). Thus, most rhesus macaques appear to harbor SRV-2s originating from Indonesian species. Natural reservoirs for serogroups 1, 3, 4 and 5 SRV have not been identified. Interestingly, all SRVs isolated from humans or human cell lines that have been reported to date are members of the SRV-1 serogroup.

V.4 VIRUS INDUCED IMMUNODEFICIENCY DISEASE AND EXPERIMENTAL RATIONALE

There are no such things as incurables; there are only things for which man has not found a cure. BERNARD BARUCH

V.4.A. Virus induced immunodeficiency disease. Both SIV and SRV are exogenous lymphotropic retroviruses which cause immunosuppression when transmitted to naive macaque species (Marx *et al.* 1984; Heidecker *et al.* 1987; Wilson *et al.* 1986b; Gravell *et al.* 1984a; Letvin *et al.* 1985; Letvin *et al.* 1983; Feichtinger *et al.* 1992; Kestler *et al.* 1990; Gravell *et al.* 1984a; Johnson and Hirsch, 1991; Gardner *et al.* 1988; Gravell *et al.* 1984b). SIV and SRV do not encode oncogenes nor are they oncogenic (Gardner *et al.* 1988). The immunological manifestations of SIV and SRV induced disease in rhesus macaques are similar. Both SIV and SRV are characterized by lymphadenopathy with initial follicular hyperplasia followed by involution, thymic atrophy, paracortical and germinal center depletion of lymphoid tissue, and a decreased response of B and T cells to pokeweed mitogen. Late stage disease is characterized by opportunistic infections, particularly chronic and recurrent diarrheal infections which are recalcitrant to therapy, and eventual death (Arthur *et al.* 1986; Henrickson *et al.* 1983; Osborn *et al.* 1984; Johnson and Hirsch, 1991; Gardner *et al.* 1988). Similarly, HIV infection is characterized by collapse of the follicular dendritic cell matrix and depletion of the paracortical and germinal centers of the lymphoid tissue. The resulting spillover of virus and CD4+ T lymphocytes into the peripheral blood effectively increases the measurable virus load and the level of circulating infected cells (Embretson *et al.* 1993; Pantaleo *et al.* 1993).

Four potential outcomes to infection are possible with these viruses. Approximately one third of the animals will develop persistent viremia with no antibody response. An acute course of disease is usually fatal within a few months post inoculation (Johnson and Hirsch, 1991). One third to half of the animals survive one to two years with a high antibody response and persistent viremia. A subacute infection is due to the total absence or inefficiency of neutralizing antibody. These animals will eventually develop immunodeficiency and die (Johnson and Hirsch, 1991). A small portion of the infected animals develop a very high antibody response with no viremia although viral genomes are detectable by PCR methods (Johnson and Hirsch, 1991). The fourth group of animals develop a

persistent viremia, whether antibodies are present or not, yet they stay healthy and can live ten years or longer after infection.

The profile of bacterial, parasitic and viral opportunistic infections which afflict SIV and SRV infected animals is very similar. Opportunistic infections secondary to viral induced immunosuppression are characterized by the presence of cytomegalovirus, adenovirus, Candida albicans, cryptosporidium, Klebsiella spp., Corynebacterium renale, Streptococcus pneumonia and viridans, Staphylococcus aureus and epidermidis, Escherichia coli and others (Gravell *et al.* 1984b; Henrickson *et al.* 1983; Osborn *et al.* 1984). Interestingly, RF has not been observed in SIV infected rhesus macaques, although their immunosuppression is generally of a greater severity than that which occurs with SRV (Gilboa *et al.* 1979; Narayan and Clements, 1990; and unpublished data). SIV infected macaques often develop Pneumocystis carinii pneumonia, but this opportunist has yet to be observed in SRV infected macaques (Gardner, 1993; Gardner *et al.* 1988).

Encephalopathies and B cell lymphomas have not been observed in SRV infected animals; however they are pathologically significant in SIV infections (Bryant *et al.* 1986b; Gardner, 1993; Gardner *et al.* 1988). Moreover, the appearance of macrophage tropic SIV isolates correlates with primary neurologic and pneumonic disease (Sharma *et al.* 1992b; Desrosiers *et al.* 1991b; Kestler *et al.* 1990b). SIV is tropic for macrophages and T lymphocytes carrying the viral CD4 receptor and appropriate chemokine coreceptor (Edinger *et al.* 1997; Chen *et al.* 1997). The brain, lungs, and gastrointestinal tract of SIV infected animals are common targets for pathology due to the macrophage tropic properties of SIV (Johnson and Hirsch, 1991). SRV is found in vivo in T and B lymphocytes, and in mesenchymal, epithelial, neural and salivary tissues (Lackner *et al.* 1988; Pilcher *et al.* 1994; Gardner *et al.* 1988). SRV clearly has a broader tissue host range, suggesting that the as yet unknown receptor is wide spread on a variety of cell types.

Immunologically, differences exist between SIV and SRV infections of macaques. SRV is characterized by hypogammaglobulinemia whereas hypergammaglobulinemia is a property of SIV infection; however, in macaques infected with particularly virulent strains of SIV, hypogammaglobulinemia has been observed (Gravell *et al.* 1984b; Gardner *et al.* 1988). Most

notable is the apparent ability of the humoral and cell-mediated immune response to the envelope glycoprotein of SRV to prevent infection (Gardner *et al.* 1988). The presence of neutralizing antibody is correlated with resistance to SRV infection (Wilson *et al.* 1986a; Shiigi *et al.* 1986; Gardner, 1993; Gardner *et al.* 1988). The hallmark of SIV infection is the inverted CD4/CD8 ratio of circulating T lymphocytes; in contrast, SRV infected animals develop a general depletion of both T and B lymphocyte populations, not solely a CD4+-lymphocyte depletion (Gravell *et al.* 1984b; Arthur *et al.* 1986; Osborn *et al.* 1984; Gardner *et al.* 1988).

V.4.B. Determinants of tropism. The ability of a virus to infect a particular cell or tissue type generally plays a major role in pathogenesis. Infection of the receptor bearing cell is often responsible for the pathology observed in the host as typified by Epstein-Barr, hepatitis, polio, and papilloma virus infections of B cells, liver cells, nerves, and dermal cells, respectively (Mims, 1989). In HIV and SIV infections, a decrease in CD4+ T-lymphocytes is the harbinger of opportunistic infections and more severe disease, while infection of macrophages correlates with the presence of dementia and encephalopathy, pneumonitis and dermatitis (Cheng-Mayer *et al.* 1988; Fenyö *et al.* 1988; Connor and Ho, 1994; Schuitemaker *et al.* 1992; Desrosiers *et al.* 1991; Kestler *et al.* 1990; Mori *et al.* 1992; Sharma *et al.* 1992; Anderson *et al.* 1993; O'Brien, 1992). Tropism can be affected by factors which contribute to infectivity, the rate of replication or the magnitude of released viral particles (York-Higgins *et al.* 1990).

Both the virus and target cell contribute factors which influence the ability of a virus to induce a productive infection. The infectivity of a target cell is primarily determined at the level of entry via a receptor on the plasma membrane, which is recognized by the envelope glycoprotein of the virus. After entry of the virus, replicative capacity and magnitude of virion production is determined by such factors as promoter/enhancer elements resident in the retroviral LTR, transport of viral mRNA from the nucleus to sites of protein synthesis in the cytoplasm, assembly, and release of infectious virion particles.

LTR and 5' untranslated region determinants. Two distinct domains have been characterized which restrict the ability of Moloney murine leukemia virus (M-MuLV) to productively infect F9

embryonal carcinoma (EC) cells, an undifferentiated stem-cell line. In contrast, productive infection is not restricted in NIH 3T3 fibroblasts, a differentiated cell line. Linney *et al.* demonstrated with the use of chimeric LTR constructs, that the enhancer element of a polyoma virus mutant (PyF101) is able to replace the M-MuLV U3 enhancer and to induce a productive infection in F9 EC cells (Linney *et al.* 1984). Interestingly, a second domain spanning the tRNA primer binding site (pbs) of M-MuLV has been demonstrated to inhibit productive infection in F9 EC cells (Loh *et al.* 1988). A protein which binds to the DNA sequence overlapping the M-MuLV pbs has been identified in F9 EC cells; this protein factor is present in decreased amounts in differentiated cell lines, suggesting that its limited presence releases the restriction to M-MuLV infection (Yamauchi *et al.* 1995).

Gag gene. A biphasic determination of susceptibility to MuLV infection depends on the presence of specific genetic loci in the target cell as well as the appropriate viral receptor. The first phase is receptor recognition, that is the presence of an ecotropic, xenotropic or amphotropic receptor molecule on the target cell. The second phase determines the replicative capacity after the virus enters the target cell. Susceptibility or resistance to MuLV strains is determined by the *Fv1* genetic locus (Goff and Lobel, 1987). N-tropic MuLV can infect mice homozygous for $Fv1^{nn}$, but this locus is not permissive to B-tropic viruses. In contrast, B-tropic MuLV can infect mice homozygous for $Fv1^{bb}$, whereas N-tropic viruses cannot. Cells homozygous for the n or b alleles at the *Fv1* locus are permissive to infection by NB-tropic MuLV. The heterozygosity of these viruses releases them from the restricted replication that is imposed by the *Fv1* locus. Restriction at the level of post-penetration is due to the gag proteins and acts at a level(s) prior to integration of the provirus; mutations in the *gag* gene can override the restriction to infection imposed by the *Fv1* locus (Goff and Lobel, 1987; Kozak and Chakraborti, 1996; Ou *et al.* 1983; DesGroseillers and Jolicoeur, 1983). The *Fv1* locus has recently been cloned and has been demonstrated to be homologous to an endogenous retroviral *gag* gene (Best *et al.* 1996). The current model proposes that the endogenous *gag* gene somehow blocks transport of the preintegration complex to the nucleus (Goff, 1996).

Envelope glycoproteins. A body of literature exists which characterizes the infection of various primary human cells and transformed cell lines by HIV and SIV. The tropic phenotype of HIV

and SIV, as determined by their respective abilities to infect these various cell types, falls into one of two categories: 1) macrophage tropic (M-tropic) isolates, which are able to infect primary CD4+ lymphocytes and macrophages but not transformed CD4+ cell lines in vitro and 2) T lymphocyte tropic (T-tropic) isolates, which are able to infect CD4+ T lymphocytes and transformed T-cell lines in vitro but not primary macrophages (Hwang *et al.* 1991). Recently a primary isolate of HIV has been identified which infects both primary macrophages and transformed T-cell lines in vitro, and induces formation of syncytia; this isolate has been defined as dual-tropic (Collman *et al.* 1992). The general properties of macrophage tropic viruses include the inability to induce syncytia and down-regulate the CD4 receptor molecule, and resistance to serum neutralization (Cheng-Mayer *et al.* 1990). The principal cellular surface molecule recognized by HIV and SIV is the CD4 protein on the helper subset of T lymphocytes and macrophages (Maddon *et al.* 1986; Dalgleish *et al.* 1984; Klatzmann *et al.* 1984). The CD4 binding site was mapped to the SU domain of the envelope glycoprotein. The importance of the HIV gp120 domain in receptor recognition and tropism was demonstrated by a single amino acid substitution that resulted in abrogating the ability of the virus to bind CD4, and rendering the virus non-infectious (Cordonnier *et al.* 1989). Construction of reciprocal chimeric genomes between two HIV molecular clones with different replicative capacities in T cell lines in vitro demonstrated that the in vitro phenotype segregated with the *env* gene (York-Higgins *et al.* 1990). Viral determinants of cell tropism were elucidated by construction of reciprocal *env* gene chimeric viruses between the M- and T-tropic molecular clones HIV_{SF162} and HIV_{SF2}, respectively. These data demonstrated that the *env* gene was a primary factor in dictating host cell tropism (Cheng-Mayer *et al.* 1990). Moreover, M-tropic HIV_{SF162} can be transfected into the human T-cell line Hut 78 with subsequent release of infectious viral particles, suggesting that the block to infection is at a postbinding and entry step. Overriding an entry level block to infection has also been demonstrated by transfection of additional monocytoid and transformed T-cell lines with "non-infectious" HIV clone DNA (Cann *et al.* 1990; Moriuchi *et al.* 1997; Morris *et al.* 1994).

The V3 loop, a highly variable region within the envelope glycoprotein, is central to the macrophage versus T lymphocyte tropic phenotype of the viral genome (Cheng-Mayer *et al.* 1991;

Cheng-Mayer *et al.* 1990; Cann *et al.* 1992; Hwang *et al.* 1991). The construction of reciprocal chimeric viral genomes between M- and T-tropic HIV clones demonstrated that a 159 amino acid region within the V3 loop encoded the determinant(s) for infection of macrophages (Shioda *et al.* 1991). The T-tropic 321 amino acid domain overlapped the M-tropic region. The T-tropic domain mapped to the V1, V2 and V4 hypervariable regions as well as to the V3 loop and CD4 receptor binding domains in the envelope glycoprotein (Shioda *et al.* 1991). Cann et al demonstrated that the presence of the V3 region was sufficient for T-cell tropism using a battery of human cell lines as well as human PBLs (Cann *et al.* 1992). Westervelt et al demonstrated that a region N-terminal to the V3 loop was deemed necessary for efficient replication in macrophages (Westervelt *et al.* 1992). In contrast, Hwang et al mapped the region necessary to confer macrophage tropism to a T tropic HIV isolate to 20 amino acids from within the V3 region of a macrophage tropic isolate (Hwang *et al.* 1991). Recent infection analyses have demonstrated that the V1 and V2 domains of SU are necessary for efficient spread of the viral infection in macrophages (Toohey *et al.* 1995). These data suggest that the macrophage tropic phenotype is multifactorial, and that the complete collection of determinants is required for infectivity and efficient replicative capacity within the target cell. These studies also involved different HIV isolates, which suggests that the necessity of the V3 domain is not specific to a particular isolate of HIV but is a widespread phenomenon (Cann *et al.* 1992; Cheng-Mayer *et al.* 1990).

Determinants of SIV tropism are similar yet distinct to those of HIV. SIV_{mac239} is a T-lymphocyte tropic, pathogenic molecular clone of simian immunodeficiency virus. Infection of macaque species with SIV_{mac239} leads to the development of AIDS. Neurologic and pneumonic sequelae are observed in some monkeys after SIV_{mac239} infection, and are a consequence of the infection of macrophages or microglial cells (Lane *et al.* 1995; Kodama *et al.* 1993; Sharma *et al.* 1992; Desrosiers *et al.* 1991). Variants of SIV_{mac239} which replicated 100-1000 fold greater in alveolar macrophages were found to have 3-9 amino acid changes in the envelope glycoprotein gene (Mori *et al.* 1993). Construction of chimeric viral genomes demonstrated that five of these amino acid changes were biologically relevant <u>in vitro</u>, and were spread over both the SU and TM envelope

domains; none of these changes were localized to the HIV-1 V3 homolog (Mori *et al.* 1993; Mori *et al.* 1992). Amino acid substitutions in the SIV V1 domain have been correlated with neurovirulence and immune escape mutants (Rudensey *et al.* 1998; Lane *et al.* 1995). Amino acid residue differences identified by Mori et al are similar to those found in M-tropic SIV recovered from microglial cells or brain tissue after infection of rhesus macaques with SIV_{mac251} or SIV_{mac239} , respectively (Anderson *et al.* 1993; Lane *et al.* 1995). Interestingly, diminished replication of SIV_{mac239} in macrophages <u>in vitro</u> was not due to a block at entry since new viral DNA was efficiently synthesized (Mori *et al.* 1993).

The influence of the V3 domain is at a post-CD4-binding step since the V3 loop does not bind to CD4 and antibody bound to V3 does not interfere with CD4 binding by the virus. In addition, a virus can bind to its CD4 receptor yet infection does not ensue (Page *et al.* 1992). Virus-cell fusion is considered to be dependent on the presence of an auxiliary membrane protein in order to facilitate virus entry into the cell (Hunter and Swanstrom, 1990). Originally, a cell surface protease was proposed to be required for processing sequence specific sites within the V3 loop prior to the virus-cell fusion event (Clements *et al.* 1991; McClure *et al.* 1990; Hattori *et al.* 1989; Hwang *et al.* 1991, Callebaut *et al.* 1993). HIV-1 tropism would then depend not only on the correct recognition sequence within the V3 loop, but also on the presence of cell-surface proteases in order for a target cell to be permissive for entry of HIV-1. Further infection analyses demonstrated that the CD26 protease, originally identified by Callebaut et al, did not confer permissivity upon HL-60 or NIH 3T3 cells for macrophage tropic HIV isolates; furthermore, other cell types which expressed CD26 were not infected by macrophage tropic HIV (Alkhatib *et al.* 1996a). Therefore, the CD26 protease was postulated to be coincidentally co-expressed with another cofactor necessary for entry/fusion or to have an indirect role in permissivity (Alkhatib *et al.* 1996a).

In contrast, another line of investigation led to the observation that autologous CD8+ T-lymphocytes of some healthy, HIV infected patients inhibited the replication of HIV in CD8-depleted peripheral mononuclear cells (PMC; Walker *et al.* 1986). Moreover, this "suppressor factor" was dose dependent and filtered CD8+ culture medium suppressed HIV replication in CD8+-depleted PMC

cultures. The identification of CD8+ T cell suppressive factors such as the β-chemokines known as RANTES (regulated-upon-activation, normal T expressed and secreted), MIP-1α and MIP-1β (macrophage inflammatory proteins-1α and β) eventually led to conclusive identification of the HIV-1 coreceptors (Cocchi *et al.* 1995; Alkhatib *et al.* 1996b; Deng *et al.* 1996; Feng *et al.* 1996). Chemokines are segregated into two classes and defined as β, if the first two cysteine residues are adjacent, and α if they are separated by a single residue, C-X-C. In a series of elegant experiments, Feng et al was able to demonstrate that a CXC-like receptor (originally designated fusin) was a cofactor in T-tropic HIV-1 entry (Feng *et al.* 1996). Three lines of evidence secured the role of the putative coreceptor in membrane fusion with HIV: 1) without the presence of fusin on the target cell surface, no fusion was detected by T tropic isolates of HIV-1, 2) the expression of recombinant CD4 and fusin in an otherwise nonpermissive cell line allowed for fusion with a T-tropic HIV-1 isolate and 3) the use of anti-fusin antibody effectively blocked infection (Feng *et al.* 1996). The natural ligand of the fusin receptor was identified as the C-X-C chemokine stromal cell-derived factor-1 (SDF-1); HIV-1 infection of fusin/CXCR4 expressing cells was inhibited in the presence of SDF-1 (Oberlin *et al.* 1996; Bleul *et al.* 1996). Close on the heels of this breakthrough was the identification of the CCR5 chemokine receptor as the cofactor necessary for entry by macrophage tropic HIV-1 isolates (Deng *et al.* 1996; Alkhatib *et al.* 1996b; Dragic *et al.* 1996). Interestingly, macrophage-tropic isolates were able to enter and replicate in T cell lines which express the CCR5 and CD4 receptors, thereby blurring the distinction between T-cell and macrophage tropic isolates (Dragic *et al.* 1996). Additionally, the utilization of chemokine coreceptors in viral entry has been demonstrated for several T-cell and macrophage tropic isolates of SIV (Edinger *et al.* 1997; Chen *et al.* 1997). Interestingly, several SIV isolates are able to utilize both human and rhesus CCR5, but not human CXCR4, 1 or 2 and CCR1, 2, 3, or 4 for viral entry; furthermore, rhesus homologs of both CXCR4 and CCR5 coreceptors supported fusion/entry by HIV-1 (Edinger *et al.* 1997; Chen *et al.* 1997; Broder and Collman, 1997). An overall positive (basic) net charge of the HIV-1 V3 loop facilitated usage of the CXCR4 coreceptor and replication in T-lymphocytes, whereas an overall neutral or negative charge (acidic) facilitated utilization of the CCR5 coreceptor and HIV-1 replication in macrophages (Fouchier

et al. 1992; Cocchi *et al.* 1996). Seven additional human coreceptors have been identified; the natural ligand of some of these receptor molecules is not yet known so they remain "orphan receptors" (Broder and Collman, 1997).

In addition to the role of the V3 loop in viral entry, the N-terminal region of the envelope glycoprotein TM facilitates virus-cell fusion and release of the NC into the cytoplasm. The efficiency of this process determines the ability of the virus to actually enter the target cell regardless of the ability of the virus to bind a surface receptor (Johnson and Cann, 1992). Infection of Hut 78 cells by SIV resulted in the emergence of a truncated TM concomitant with a productive infection; reversion to the full-length TM took place after infection of rhesus macaques with virus that encoded the truncated form of *env* (Johnston *et al.* 1998; Kodama *et al.* 1989). Truncation of the TM cytoplasmic domain has increased the fusogenic capacity of MPMV, MuLV, SIV_{mac239}, and SIV_{mne}, perhaps by altering the conformation of the exposed fusion domain on the surface of the plasma membrane (Spies *et al.* 1994; Rein *et al.* 1994; Zingler and Littman, 1993; Kodama *et al.* 1989; Kimata and Overbaugh, 1997; Brody *et al.* 1994b).

In addition to the role of the *env* gene in determining entry/fusion into a target cell, viral and host cell factors influence the replicative capacity at post-penetration levels of viral replication. Post-entry determinants of tropism can be characterized by permissiveness for transcription by the viral LTR, export of viral genomic mRNA and post-translational processing of viral proteins. Passage of HIV isolates through different cell types in vitro results in the emergence of virus progeny with increased replication kinetics in the same cell type and a broadened host range (Cheng-Mayer *et al.* 1991; DiFronzo *et al.* 1997; Morris *et al.* 1994). Alteration of the replicative phenotype may be due to altered patterns of glycosylation, which is a host cell specific process (Cheng-Mayer *et al.* 1991). Interestingly, the pathogenesis of influenza virus can increase in the presence of a secondary bacterial infection; bacteria can produce a protease which can effectively process the influenza hemagglutinin molecule and alter the native virulence of the virus into an extremely severe form (Mims, 1989).

Export of unspliced mRNA. The ability to transport unspliced genomic mRNA from the nucleus to the cytoplasm of the host cell is imperative for productive retroviral replication. In HIV-1, this function is performed by the joint action of the Rev gene product and the Rev response element (RRE; Kjems *et al.* 1991; Zolotukhin *et al.* 1994; Malim and Cullen, 1991; Malim *et al.* 1989; Bray *et al.* 1994). Within the 3' untranslated regions of Rous sarcoma virus (RSV), SRV-1 and MPMV resides an element which confers Rev independent replication to HIV. The 219 bp region of MPMV is designated the constitutive transport element (CTE) (Zolotukhin *et al.* 1994; Ogert *et al.* 1996; Bray *et al.* 1994). The CTE of SRV-1 has also been demonstrated to support replication in Rev (-)/RRE(-) mutant HIV genomes, although with attenuated replicative capacity (Zolotukhin *et al.* 1994). These results suggest that the type D retroviruses depend upon a putative cellular factor that substitutes for the Rev protein function in order to export full-length viral genomes from the nucleus to the cytoplasm. The necessity of a cellular cofactor is supported by the work of Trono and Baltimore which demonstrated that the block to murine cell infection by HIV was relieved by fusion of the murine cell with a human cell (Trono and Baltimore, 1990). Expression of human CD4 and CCR5 on murine cells permits entry and synthesis of HIV-1 DNA, but not the release of progeny virions, confirming the necessity of additional human factor(s) for a productive infection (Browning *et al.* 1997). Recent observations by Pasquinelli *et al.* have demonstrated that CTE containing RNA competes for a host factor(s) necessary for nuclear export; the identity of this factor has been suggested by Tang *et al.* to be an RNA helicase and by Grüter et al to be the yeast Mex67p human homolog, TAP (Tang *et al.* 1997; Pasquinelli *et al.* 1997; Grüter *et al.* 1998). Dayton *et al.* observed that mutations in the RRE of HIV-1 differentially affected the ability of the virus to replicate in various host cells, suggesting that the RRE is a cis-acting factor which determines viral tropism by affecting the replicative capacity of the virus in various host cells (Dayton *et al.* 1993).

V.4.C. In vitro correlates of pathogenesis. Both host and viral factors play a role in the severity of the immunosuppressive disease which develops after infection with any of the profiled lentiviruses or type D simian retroviruses. Oral, mucosal, or intravenous routes of infection affect pathogenicity by selecting for the entry of particular viral isolates which can replicate and spread within

a specific environment. The ability of the infecting inoculum to infect versus immunize the host may determine viral pathogenicity. Host determinants such as age at time of infection, induction of a beneficial versus detrimental immune response, and host species also affect the clinical outcome. Finally, the range of target cells in which a virus can enter and productively replicate is limited by whether a specific host protein(s) is(are) present, and viral tissue tropism determines the subsequent clinical sequelae. Examination of target cell tropism, replicative capacity, and cytopathogenicity in vitro have allowed the correlation of the biological phenotype of the virus with clinical disease and prognosis.

Viruses recovered early in HIV and SIV infection predominantly replicate in macrophages and primary CD4+ lymphocytes in vitro. As disease progresses, viruses recovered from peripheral blood primarily replicated in transformed T-cell lines in vitro (Cheng-Mayer *et al.* 1990; Hwang *et al.* 1991). Virus rescued from advanced stages of disease are also characterized by increased cytopathicity and replication kinetics and a broader host range in a variety of human cell lines in vitro (Tersmette *et al.* 1989; Cheng-Mayer *et al.* 1988; Fenyö *et al.* 1988). Restriction endonuclease and Southern analyses of proviral genomes indicate that genomic heterogeneity exists among sequential viral isolates from an individual, but this difference is not as great as the polymorphism which is found in genomes isolated from different individuals (Cheng-Mayer *et al.* 1988). Macrophage tropic isolates may be important for viral persistence, movement of the virus from the blood into different tissue compartments, and appearance of clinical disease manifestations in which the infected macrophage is the predominant cell type in the tissue. Virus induced encephalopathy and pneumonic disease are examples of pathology due to the presence of virus-infected tissue-macrophages. The SIV infected rhesus macaque model clearly demonstrates that macrophage tropic isolates are not necessary for induction of AIDS and death, but macrophage tropism can influence the course of the disease and its clinical presentation (Desrosiers *et al.* 1991; Sharma *et al.* 1992; Kestler *et al.* 1990).

The observations of Schuitemaker et al demonstrate that HIV recovered from asymptomatic individuals is primarily non-syncytia inducing (NSI) in primary T lymphocytes, is not transmitted to T cell lines and has the slow/low replication kinetics associated with macrophage tropic isolates in vitro

(Fenyö *et al.* 1988). Predominately monocytotropic virus isolates are recovered from peripheral blood of individuals during the asymptomatic period and close to the time of seroconversion (Schuitemaker *et al.* 1992). The rescue of isolates from the peripheral blood with broadened host range as defined by their ability to infect T cell lines in vitro and their syncytia inducing (SI) phenotype correlated with the decline of the CD4+ population of T lymphocytes in vivo as disease progressed (Fenyö *et al.* 1988; Connor and Ho, 1994; Schuitemaker *et al.* 1992). However, monocytotropic virus can still be recovered in late stages of disease as demonstrated by the rescue of isolates from tissues such as the lung in which the macrophage is the primary cell infected (Schuitemaker *et al.* 1992).

Additionally, Connor and Ho demonstrated that during the asymptomatic phase of HIV infection, isolates with different in vitro phenotypes were recovered from a patient (Connor and Ho, 1994). Initially, virus isolates with increased replication kinetics in macrophages were observed, followed by the appearance of isolates with an SI phenotype able to replicate at high levels in both macrophages and CD4+ T lymphocytes. The more slowly replicating NSI isolates were recoverable at all time points after infection suggesting that a continuum of viral phenotypes is present in an HIV infected individual beginning with the asymptomatic stage. The increased replicative capacity is considered to contribute to disease progression by increasing the viral load, or by giving rise to variants with a higher degree of cytopathicity. These results are consistent with those of Tersmette et al who previously observed a temporal relationship between the first appearance of a rapidly replicating SI phenotype during the asymptomatic phase and the advent of clinical symptoms after 9-15 months (Tersmette *et al.* 1989).

Kestler *et al.* used the highly T-lymphotropic SIV_{mac239} clone to infect 11 rhesus macaque animals. One animal developed SAIDS complicated by giant cell pneumonia, a recurrent cutaneous rash, and granulomatous encephalomyelitis (Kestler *et al.* 1990). This rhesus monkey died five months after intravenous inoculation with little or no antibody response. Lung lavage from this animal displayed numerous SIV-infected macrophages. The recovered virus was able to efficiently replicate in primary alveolar macrophage cultures in vitro. The rhesus monkeys which were able to mount a strong immune response have remained alive, although they are persistently infected as

demonstrated by the recovery of SIV from their peripheral blood mononuclear cells. The remaining animals succumbed at various time points after infection with various symptoms, including emaciation, opportunistic infections, lymphoid hyperplasia, thymic atrophy, and generalized lymphoid depletion (Kestler *et al.* 1990). Serial in vivo passage of lymphocyte tropic SIV_{mac239} in rhesus macaques resulted in the recovery of a macrophage tropic strain SIV_{mac239}/R71, which was found specifically in microglial cells in vivo (Sharma *et al.* 1992b). In addition, SIV viral gene products were detected in macaque brain endothelial cells (MBEC) in vivo and SIV_{mac239}/17E-Br (recovered after intracerebral inoculation with SIV_{mac239}/R71) was able to replicate in MBEC in vitro, thus strengthening the correlation between in vitro tropism and pathogenic outcome (Mankowski *et al.* 1994). In an independent study, serial passage of SIV-infected microglial cells in rhesus macaques resulted in the development of encephalitis. Subsequent sequence analyses of *env* DNA isolated from infected microglial cells demonstrated the presence of a homogeneous population of *env* genotypes, strikingly similar to the *env* genes characterized from macrophage tropic SIV clones (Mori *et al.* 1992; Anderson *et al.* 1993; Lane *et al.* 1995). These experiments fulfil Koch's postulates and confirm SIV to be the etiologic agent of simian AIDS. In addition, the presence of a macrophage tropic isolate in the inoculum is not necessary for the induction of SAIDS (Sharma *et al.* 1992b; Sharma *et al.* 1992b; Kestler *et al.* 1990b; Desrosiers *et al.* 1991b). In addition, these data show a strong correlation between the emergence of a macrophage tropic isolate with the appearance of pneumonic and neurologic disease.

Determinants of neuropathogenesis versus leukemogenesis have been defined in MuLV. The generation of chimeric viral genomes between the neuropathogenic PVC-211 and leukemogenic Friend MuLVs demonstrated that clinical pathogenesis was multifactorial; both the SU of the envelope gene and two distinct regions of the 5' end of the PVC-211 MuLV genome were required for neuropathogenesis in rats (Masuda *et al.* 1993). Moreover, in vitro infection of brain capillary endothelial cells (BCEC), the primary target of CNS infection, was directly correlated with the pathogenic outcome induced by this virus. Moreover, the presence of the PyF101 enhancer induced a different disease outcome in inoculated NIH Swiss mice dependent upon the location of the

polyoma virus enhancer relative to the native enhancer element in the M-MuLV genome (Fan *et al.* 1986). The M-MuLV-PyF101 and PVC-211-Fr MuLV models of tropism and pathogenesis demonstrate a role for the use of reciprocal chimeric viral genomes for in vitro infection analyses designed to characterize potential viral genetic determinants of tropism and the multiple genetic determinants that mitigate productive infection and pathogenesis in vitro and in vivo (Yamauchi *et al.* 1995; Linney *et al.* 1984; Masuda *et al.* 1993; Loh *et al.* 1988).

V.4.D. Experimental Rationale. Restriction endonuclease analyses have led to the identification of at least four distinct serogroup 2 SRV isolates. These isolates include D2/CEL/OR from a Celebes macaque (*Macaca nigra*), D2/RHE/OR from a rhesus macaque (*Macaca mulatta*), D2/RHE/OR/V1, a variant recovered from a rhesus macaque endemically infected with D2/RHE/OR, and D2/CYN/OR/V2 from a cynomolgus macaque (*Macaca fasicularis*). The pathogenic outcome is dependent on both the species of the infected animal and on the genotype of the infecting virus (Table 1). For example, D2/CEL/OR is unable to induce disease in rhesus macaques, but D2/RHE/OR is able to productively infect Celebes macaques. Moreover, when Japanese macaques (*Macaca fuscata*) are inoculated with D2/RHE/OR, a severe immunodeficiency disease results, whereas the same virus in a rhesus macaque results in a less virulent outcome.

The serogroup 2 SRV isolates D2/RHE/OR and D2/RHE/OR/V1 exhibit divergent host ranges in vitro. Cell-free isolates of D2/RHE/OR which induce a less severe disease in rhesus macaques readily infect only Raji cells and grow less well in some T lineage cells after cell-free inoculation in vitro. In contrast, cell-free D2/RHE/OR/V1 recovered from rhesus macaques with a more severe disease is capable of readily infecting both human T-(Hut 78) and B-lineage cells in vitro. A pattern of increased range of host cell tropism in vitro is also observed with D1/RHE/CA and D5/RHE/OR after recovery from animals with more severe disease. The in vitro tropism serves as a marker for the altered pathogenic outcome observed in vivo.

In summary, differential tropism for lymphocytes and macrophages in vitro have been used as phenotypic markers for divergent pathogenic outcomes of lentiviruses and some oncoviruses. Modification of the envelope glycoprotein gene has been the primary determinant for the observed

divergence in tropism by different viral isolates. The construction of chimeric viral genomes has been a useful tool in mapping genetic determinants of tropism such as receptor recognition and fusion ability. Since the retroviral envelope glycoprotein gene is a major determinant of tropism at the level of attachment to and entry into the host cell, my doctoral research sought 1) to characterize the genetic variability of two serogroup 2 polymorphic isolates, D2/RHE/OR and D2/RHE/OR/V1, which were isolated from infected rhesus macaques exhibiting quite different pathological outcomes, 2) to determine the relatedness of these two isolates to other characterized SRVs, and 3) to elucidate the viral genetic domains that are responsible for the apparent differences in pathogenesis and in vitro cell tropism with the use of reciprocal chimeric viruses.

Figure 1. Simple and complex organization of retroviral genomes. Simple genomes are represented by avian sarcoma leukemia (ASLV) and Mason-Pfizer monkey (MPMV) viruses, with *gag, pol* and *env* open reading frames. In addition, MPMV has a separate reading frame for the protease gene, *pro*, which also contains the dUTPase gene. Complex genomes are distinguished by human T-cell leukemia virus-I (HTLV-I) and Rous sarcoma virus (RSV), which have reading frames for auxiliary proteins and an oncogene, respectively.

Simple and Complex Organization Of Retroviral Genomes

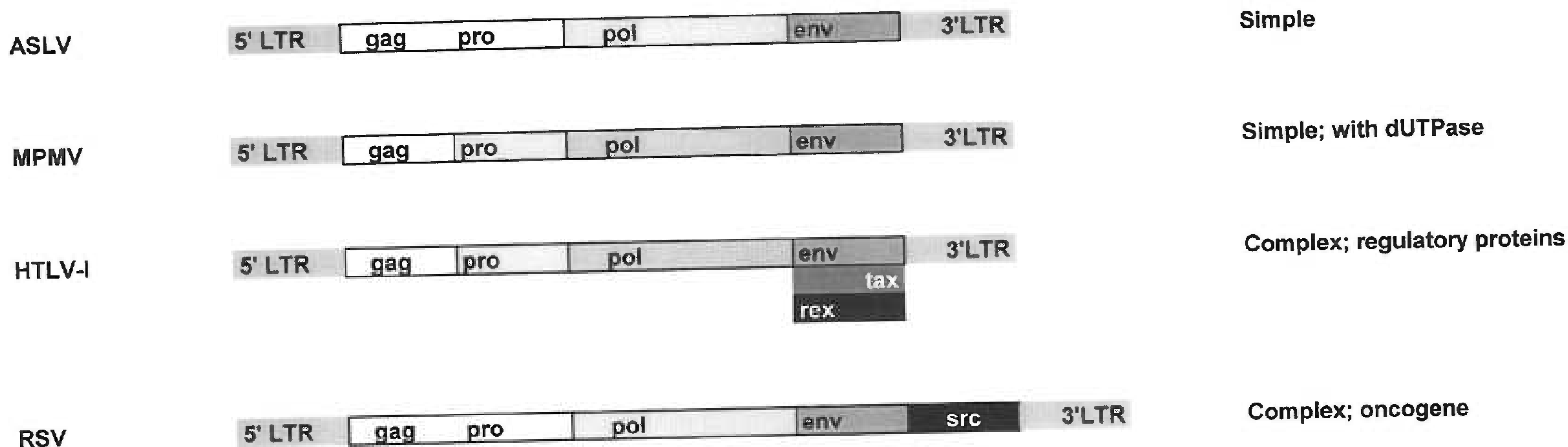


Figure 2. Diagram of the retroviral life cycle. Retroviral replication is initiated at the plasma membrane when virus-cell fusion allows delivery of the nucleocapsid into the host cell cytoplasm. Synthesis of a double stranded DNA molecule proceeds via reverse transcription, and is followed by transport of the preintegration complex into the nucleus where integration of the provirus into the host chromosome takes place. Expression and transport of the viral RNA from the nucleus to the cytoplasm is followed by protein synthesis, assembly and egress via budding at the plasma membrane. [Reprinted with permission from Cold Spring Harbor Laboratory Press.]

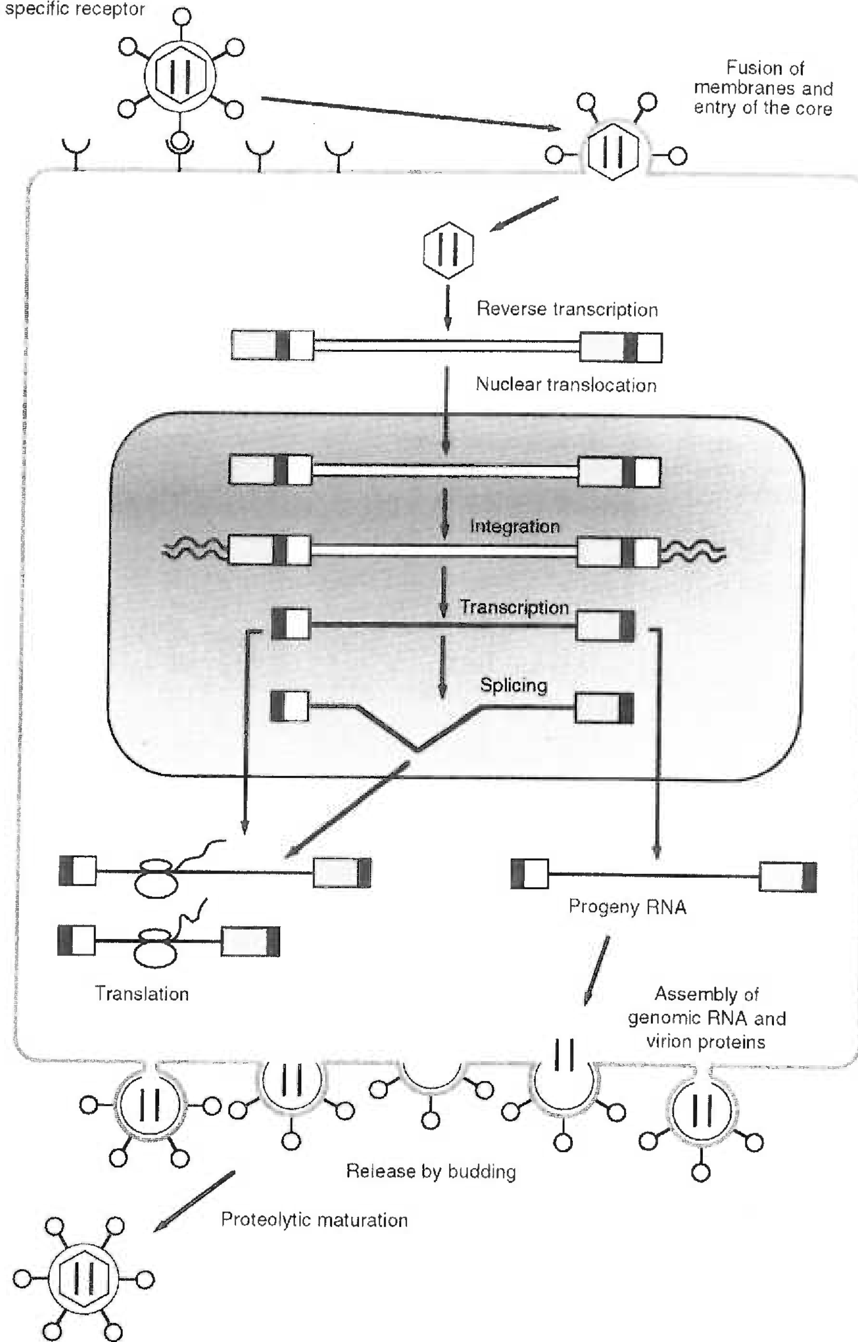
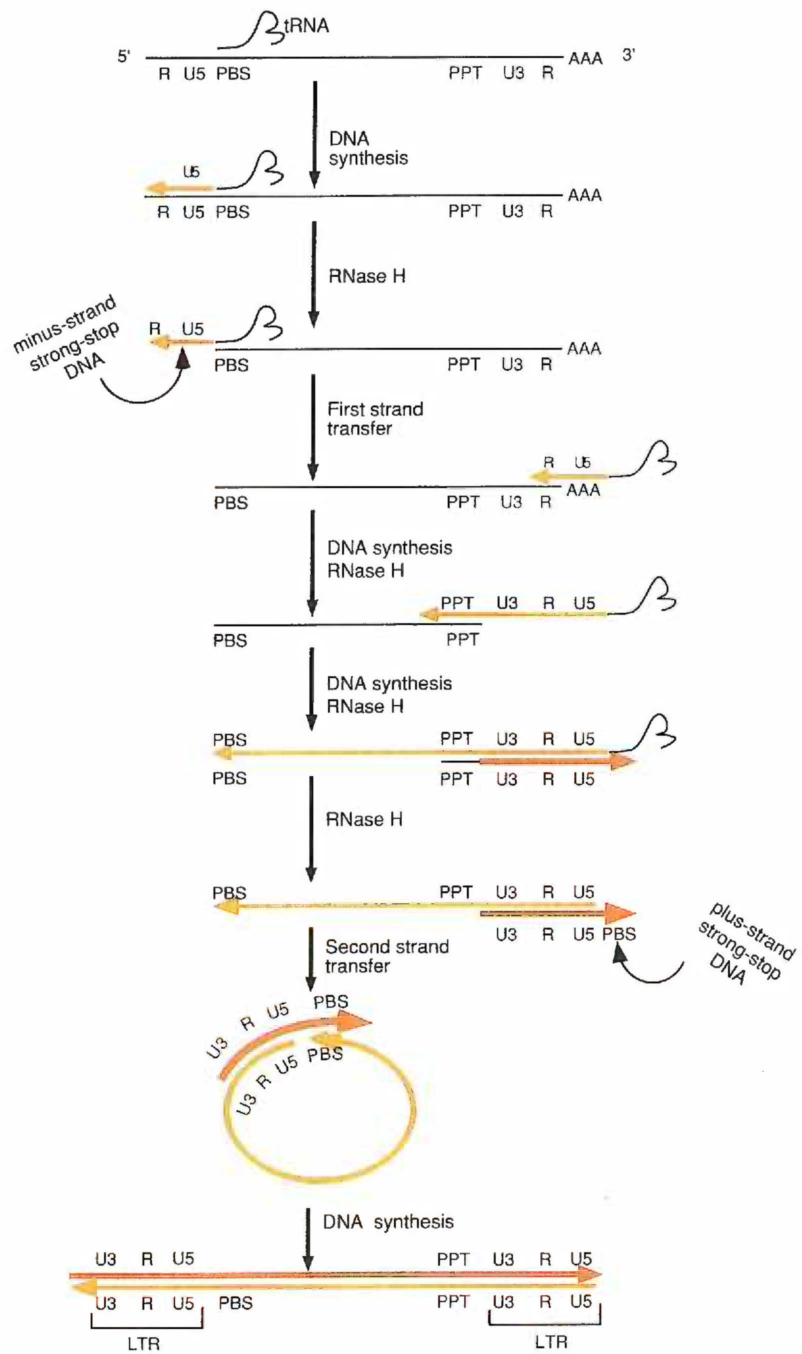

Adsorption to specific receptor
Fusion of
membranes and
entry of the core
Reverse transcription
Nuclear translocation
Integration
Transcription
Splicing
Progeny RNA
Translation
Assembly of
genomic RNA and
virion proteins
Release by budding
Proteolytic maturation

Figure 3. Reverse transcription and reconstruction of retroviral LTRs. Synthesis of strong stop minus DNA is initiated at the primer binding site (pbs) with $tRNA^{lys}$ as the primer. The growing strand is transferred to the R region at the 3' end of the second genomic RNA molecule and minus strand DNA synthesis continues. Positive strand DNA is initiated from the polypurine tract. An intrastrand DNA transfer occurs which allows continued synthesis of the minus and positive strand DNA molecules using the reciprocal strand as the template. Upon completion of reverse transcription, the LTR regions will have been reconstituted. [Reprinted with permission from Cold Spring Harbor Laboratory Press.]



tRNA
5'
AAA
3'
R U5 PBS
PPT U3 R
DNA synthesis
RNase H
minus-strand strong-stop DNA
First strand transfer
DNA synthesis RNase H
RNase H
plus-strand strong-stop DNA
Second strand transfer
DNA synthesis
LTR
LTR

Table 1. Simian retrovirus induced immunodeficiency disease. The variable severities of immunodeficiency following infection by a specific SRV are indicated for each species of macaque. Low: immunodeficiency that is characterized by its transience. Neutropenia, lymphadenopathy, splenomegaly, thymic atrophy and seroconversion with clearance of viremia are also exhibited. Medium: a prolonged disease course, characterized by viremia, enterocolitis and increased mortality over that observed in low. High: a shortened disease course (six months to 2.5 years) characterized by increased involution and loss of the T- and B-cell areas of the lymph nodes, a broader spectrum of opportunistic infections (viral, bacterial and protozoal) and a high rate of mortality. Retroperitoneal fibromatosis (RF) is a mesenchymal proliferative disorder (MPD) and occurs in SRV-2 infected cynomolgus, Celebes and pig-tailed macaques. CYC (*M. cyclopis*), RHE (*M. mulatta*), CYN (*M. fascicularis*), CEL (*M. nigra*), PTM (*M. nemestrina*), JPN (*M. fuscata*).

IMMUNODEFICIENCY ASSOCIATED WITH EACH OF THE KNOWN TYPE-D SRVs

	MACAQUE SPECIES					
VIRUS	CYC	RHE	CYN	CEL	PTM	JPN
D1/CYC/NE	LOW	LOW	LOW			
D1/RHE/CA		HIGH				
D2/CEL/OR				HIGH/RF		
D2/MNE/WA			MED/RF		HIGH/RF	
D2/RHE/OR		LOW				HIGH
D2/RHE/OR/V1		HIGH				?
MPMV(D3)		MED				
D4/CYN/CA			LOW			
D5/RHE/OR		HIGH				

Table 2. Percentage similarity of SRV 1-3 amino acids to D2/RHE/OR/V1. The different protein domains of D2/RHE/OR/V1 were compared to the corresponding domains of D2/RHE/OR, D2/CEL/OR, MPMV and D1/RHE/CA. The percentage similarity was calculated by dividing the number of identical amino acids by the total number of amino acids found in the domain of interest.

PERCENTAGE SIMILARITY AMONG PROTEINS OF OTHER SEROGROUP SRVs TO D2/RHE/OR/V1

	GAG	PRT	POL	ENV gp70	ENV gp20
D2/RHE/OR/V1	100	100	100	100	100
D2/RHE/OR	99.5	99.7	99.7	98.4	97.9
D2/CEL/OR	96.3	98.4	97.3	96.3	97.9
MPMV	83.6	83.8	84.1	61.0	91.8
D1/RHE/CA	83.6	82.8	82.7	59.7	83.9

VI. RESULTS

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Simian AIDS Type D Serogroup 2 Retrovirus:

Isolation of an Infectious Molecular Clone and Sequence Analyses

of its Envelope Glycoprotein Gene and 3' Long Terminal Repeat

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ABSTRACT

We describe the molecular cloning of a serogroup 2 simian retrovirus (SRV; D2/RHE/OR), and present the sequence of its envelope (env) glycoprotein gene and 3' long terminal repeat (LTR) region. This report documents the first infectious molecular clone of a serogroup 2 SRV and provides env sequence verification of genetic diversity among serogroup 2 SRV isolates.

Type D simian retroviruses (SRVs) from macaques comprise five classical neutralization serogroups. Serogroup 2 SRVs are the predominant etiological agents of Simian AIDS in Asian macaque colonies at several Regional Primate Research Centers (RPRC), and have been associated with severe immunodeficiency and retroperitoneal fibromatosis (RF) in Celebes (Macaca nigra) , pigtailed (Macaca nemestrina), and cynomolgus (Macaca fascicularis) macaques, severe immunodeficiency, anemia, and thrombocytopenia in Japanese macaques (Macaca fuscata), and immunodeficiency of variable severity in rhesus macaques (Macaca mulatta) (Tsai *et al.* 1990; Tsai *et al.* 1986; Shiigi *et al.* 1985; Giddens, Jr. *et al.* 1985; Gardner and Luciw, 1989; Axthelm *et al.* 1987). D2/CEL/OR, the first molecularly-cloned serogroup 2 SRV, was originally isolated at the Oregon RPRC from a Celebes black macaque afflicted with Simian AIDS and retroperitoneal fibromatosis (Marx *et al.* 1985). D2/RHE/OR and D2/PTM/WA, two additional members of serogroup 2 simian retroviruses, were isolated from rhesus and pig-tailed macaques at the Oregon and Washington RPRCs, respectively, and are believed to represent identical biotypes based on restriction enzyme mapping (Stromberg *et al.* 1984; Hallick *et al.* 1987; Bryant *et al.* 1986). D2/RHE/OR induces mild immunodeficiency in rhesus macaques. Novel serogroup 2 variants, originally identified by restriction site polymorphisms, have been recovered from infected macaques. These variants, designated D2/RHE/OR/V1 and D2/CYN/OR/V2, contain mutations in their envelope glycoprotein genes relative to D2/RHE/OR, target different immune cells *in vitro* , and are associated with severe immunodeficiency (Marracci *et al.* 1992; Kelley *et al.* 1989).

We sought to molecularly clone the D2/RHE/OR genome, in order to gain further insights into the genetic diversity of the serogroup 2 SRV envelope glycoproteins that may be important determinants of pathogenicity in Asian macaques. We now describe the isolation of an infectious molecular clone of a second serogroup 2 SRV, D2/RHE/OR, and report the sequence of its envelope (env) glycoprotein gene and 3' long terminal repeat region (LTR). In support of prior neutralization and restriction mapping analyses (Werner *et al.* 1990; Hu *et al.* 1989; Axthelm *et al.*

1987), this report provides the first env sequence verification of genetic diversity among serogroup 2 SRV isolates.

The D2/RHE/OR and D2/CEL/OR env genes and 3' LTRs are highly conserved and provides evidence for genetic diversity among serogroup 2 SRVs. Genomic DNA was extracted from Raji cells (ATCC CCL-86) cocultivated with peripheral blood mononuclear cells obtained from an infected rhesus macaque (ORPRC animal 305-13005). This animal was shown by restriction endonuclease mapping analysis to contain a virus isolate characteristic of D2/RHE/OR (Kelley *et al.* 1989; Axthelm *et al.* 1987). Genomic DNA was partially digested with Sau 3A-1, size-fractionated on 10-40% sucrose gradients to enrich for 9 -22 Kb DNA fragments, and fragments subsequently ligated to EMBL3 arms and packaged. Clone 2R-18B1 contained restriction endonuclease patterns equivalent to those observed for genomic Southern analyses; two overlapping restriction fragments (2.6 kb Eco RI - Sph I and 5.0 kb Bam HI restriction fragments), containing the entire D2/RHE/OR env gene and 3' LTR, were subcloned and sequenced. Like D2/CEL/OR (Thayer *et al.* 1987) isolated from a Celebes macaque, the D2/RHE/OR env gene encodes a deduced glycoprotein of 574 amino acids (Figure 4). Both serogroup 2 envelope glycoproteins contain 22 conserved cysteine residues and 11 potential Asn-X-Ser(Thr) glycosylation sites. The D2/RHE/OR env glycoprotein contains conserved dibasic amino acid proteolysis sites (residues 381-382; arginine - arginine site) determining the cleavage site between the extrinsic glycoprotein gp70 and membrane anchor gp20 (Stromberg *et al.* 1984; Hunter and Swanstrom, 1990), and a highly conserved stretch of hydrophobic amino acids (residues 395-417) believed to serve as the transmembrane portion of gp20.

The env genes of D2/RHE/OR and D2/CEL/OR are 96.2% similar overall at the amino acid level, and 95.8% and 96.8% similar within the gp70 and gp20 regions, respectively (Figure 5). The D2/RHE/OR env glycoprotein is 67.2% and 67.4% similar to the serogroup 1 (D1/RHE/CA) and 3 (MPMV) SRV envelope glycoproteins, respectively (Sonigo *et al.* 1986; Power *et al.* 1986).

There are 22 amino acid substitutions between the D2/RHE/OR and D2/CEL/OR glycoprotein sequences; five substitutions are alanine to threonine, representing nonpolar to uncharged polar residue conversions, at positions 51, 59, 109, 311, and 404. Other notable modifications in the D2/RHE/OR envelope sequence include the substitution of a histidine for a helix-destabilizing proline at position 160, and a glycine to arginine (uncharged polar to basic) conversion at position 519. Interestingly, there is also a cluster of amino acid conversions between residues 281 and 317 in the gp70 region of the D2/RHE/OR envelope glycoprotein. This region, heavily targeted for residue conversions in other serogroup 2 simian retrovirus envelope gene variants (Marracci *et al.* 1992), is bounded by a proline-rich sequence (residues 237-280), indicative of the presence of a potential β-turn structure, and also by the end of the gp70 molecule. This region also contains several Asn-X-Ser(Thr) glycosylation sites, and is presumably exposed at the cell surface.

The nucleotide sequence of the D2/RHE/OR 3' LTR, and an alignment with 3' LTR sequences from D2/CEL/OR and representative members of serogroup 1 and 3 SRVs (D1/RHE/CA and MPMV, respectively) are shown in Figure 6. Based on analogies with other retrovirus systems, the 5' boundary of the rightward (3') LTR is a polypurine track which serves to initiate plus-strand DNA synthesis (Varmus and Swanstrom, 1984). The polypurine track in the D2/RHE/OR 3' LTR (Figure 6) is identical to corresponding sequences in D2/CEL/OR. Interestingly, the corresponding regions in the D1/RHE/CA and MPMV 3' LTRs are interspersed with several T residues. The D2/RHE/OR 3' LTR sequence, like its D2/CEL/OR counterpart, is composed of 367 bases, and is 96.2%, 73.3%, and 76.0% conserved with the D2/CEL/OR, D1/RHE/CA, and MPMV 3' LTR sequences, respectively. The D2/RHE/OR 3' LTR contains the U3 enhancer region (TGTCC; position 201 - 205) and U5 enhancer region (GGACA; position 542-546), which are precisely conserved in both sequence and location with corresponding D2/CEL/OR, D1/RHE/CA, and MPMV sequences.

D2/RHE/OR molecular clone generates infectious virus from cocultivated Raji cells. In order to verify that the D2/RHE/OR molecular clone was capable of synthesizing infectious virus, we transfected 2R18B1 lambda DNA into D-17 canine cells (ATCC CCL 183), and cocultivated the transfectants with permissive Raji cells to promote virus rescue. Since D-17 cells lack type D virus receptors and were incapable of promoting virus spread, the transfected D-17 cells were cocultivated with permissive Raji cells (1×10^5 cells) to amplify potentially low levels of released virus, and cocultivation was allowed to proceed for 4 days. The Raji cells were subsequently removed from the coculture and propagated independently in fresh RPMI medium. Concomitant with the isolation of the cocultivated Raji cells, the recovered medium was filtered (0.2 μm filter), and introduced onto fresh, uninfected Raji cells. Both the original and medium-inoculated Raji cultures were monitored daily for syncitia formation, a morphological indicator of type D SRV infection. At day 22 post-transfection, syncitia were visible in the original and medium-inoculated Raji cultures. As a negative control, D-17 cells were also subjected to mock transfection without DNA, and subsequently cocultivated.

We performed an immunofluorescence assay (IFA) on both the original cocultivated and medium-inoculated Raji cells, at 29 days post-transfection. Cells were incubated for 30 min on ice with 25 μl of a 1 μg/ml solution of monoclonal IgG2a or the mouse F2-1 anti-gp20 monoclonal antibody (Kwang *et al.* 1987) (gift of Dr. Niels Pederson, University of California at Davis), followed by a secondary incubation with a 1:20 dilution of FITC-F(ab')$_2$-goat anti-mouse IgG (H & L chain specificity; Tago). Fluorescent-activated cell sorting (FACS) analyses were conducted on an EPICS C flow cytometer and confirmed that both Raji cultures were 99.6 - 100% infected (Figure 7). All IgG2a control incubations, and anti-gp20 incubations using uninfected Raji cells and Raji cells cocultivated with D-17 cells subjected to mock transfection, were negative. IFA and FACS analysis using polyclonal anti-SRV1, anti-SRV2, and anti-SRV5 antibodies were next utilized to confirm serogroup-specificity of the transfected virus. In this analysis, both the original cocultivated and medium-inoculated Raji cultures were avidly recognized by the anti-SRV2 serum,

and were clearly not recognized by anti-SRV5 serum (unpublished observations). Both the experimental Raji cultures, as well as the SRV2 positive control culture, were partially recognized by the anti-SRV1 serum (unpublished observations), which is not surprising in light of the close sequence similarity (67.2%) between the SRV1 and SRV 2 env genes.

Additionally, electron microscopy showed the budding and release of type D virus particles from cocultivated Raji cells (unpublished observations). The morphology of the released viruses are typical of type D retroviruses, which contain an electron-dense nucleoid that is eccentrically located.

Molecular analyses confirm that the 2R18B1 clone is integrated as proviral DNA and that the transfected simian retrovirus is genetically equivalent to the natural D2/RHE/OR virus. PCR was conducted using SRV2env1 and SRV2env2 primers, both 30-mers with high specificity for the SRV serogroup 2 env sequences (Pilcher *et al.* 1994). This primer pair will not produce an amplified fragment from genomic DNAs obtained from serogroups 1, 3, 4, or 5- infected cells or tissues. For our transfection and cocultivation analyses, both the experimental Raji DNA and SRV serogroup 2 positive control DNA yielded identical 1050 bp env PCR fragments (unpublished observations).

Genomic DNAs from the experimental Raji cultures, and from positive and negative control cultures, were digested with combinations of restriction enzymes (Figure 8). This restriction digest pattern matched the pattern using genomic DNA from a positive control D2/RHE/OR-infected Raji cell line, and is identical to the restriction pattern observed using the 2R-18B1 molecular clone (unpublished observations). As expected, no fragments from uninfected Raji cells, or from Raji cells cocultivated with D-17 cells subjected to mock transfection, were recognized with the SRV-2 hybridization probe (Figure 8). Thus, the PCR and restriction endonuclease analyses of genomic DNAs from the experimental Raji cultures provides evidence that the 2R18B1 molecular clone is

integrated as proviral DNA , and that the transfected simian retrovirus, at the level of limited restriction site analysis, is genetically equivalent to the input clone.

Implications for cell tropism and pathogenesis. It is recognized that the envelope glycoprotein interacts with host cell receptors to initiate virus adsorption and penetration; this interaction serves as a primary determinant of the cell and tissue tropism of the infecting retrovirus (Hunter and Swanstrom, 1990). With the simian and human immunodeficiency viruses (SIV and HIV, respectively), envelope glycoprotein sequence variability can exist between individual isolates at different times during the progression of the disease (Wong-Staal, 1991; Vaishnav and Wong-Staal, 1991), and in some cases results in differential cell tropism (e.g.: lymphocyte vs. macrophage tropism). Although no study comparing the cell tropism of the D2/RHE/OR and D2/CEL/OR viruses has been previously published, these serogroup 2 virus isolates develop distinct patterns of disease. Interestingly, D1/RHE/NE appears to replicate only in African Burkitt's lymphoma and pre-B cell lines but not Epstein-Barr virus- (EBV) transformed normal B lymphocytes, whereas D1/RHE/CA has broad cell tropism for both B and T lymphocytes and monocytes, including EBV-transformed normal B cells (Yetz and Letvin, 1987; van Kuyk *et al.* 1991; Maul *et al.* 1988). Changes in the microconformation of the external envelope glycoprotein may potentially change the affinity or range of host cell receptors recognized by the serogroup 2 retroviruses. These potential structural differences between the D2/RHE/OR and D2/CEL/OR envelope glycoproteins may result in divergent cell or tissue tropism that ultimately affect the pathogenic potential of the different serogroup 2 retroviruses.

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Figure 4. Nucleotide sequence of the D2/RHE/OR envelope gene and 3' LTR. The deduced amino acid sequence of the D2/RHE/OR env gene is shown. Potential Asn-X-Ser (Thr) glycosylation sites are illustrated in bold letters. Cysteine residues, which may serve as potential sites of disulfide bridging, are denoted by the symbol ^ placed above the residue.

10 20 30 40 50 60 70 80 90

ATG ACT GTT AAG GAC ATC CCG TTT TGG AGA GTA CTG CTG ATT TTC CAA ACT GCT CGA GTT TAT GCC GGT TTC GGT GAC CCG CGC GAG GCT ATT ACT ATA

Met Thr Val Lys Asp Ile Pro Phe Trp Arg Val Leu Leu Ile Phe Gln Thr Ala Arg Val Tyr Ala Gly Phe Gly Asp Pro Arg Glu Ala Ile Thr Ile

100 110 120 130 140 150 160 170 180 190

ATA CAT CAA CAA CAC GGT AAG CCT TGT GAT TGT GCG GGA GGA TAT GTC ATC ACT GCA CCT ACA GTC TAT CTT GCA ACT GTT TCT TGT TCT TCT CAC ACT

Ile His Gln Gln His Gly Lys Pro Cys Asp Cys Ala Gly Gly Tyr Val Ile Thr Ala Pro Thr Val Tyr Leu Ala Thr Val Ser Cys Ser Ser His Thr

200 210 220 230 240 250 260 270 280 290

GCA TAT CAG CCA AGT GAC TCC CTT AAG TGG CGG TGT GTC TCT AAC CCG ACA TTA GCC AAT GGA GAA AAT ATA GGG AAT TGT CCT TGC CAG ACA TTC AAA

Ala Tyr Gln Pro Ser Asp Ser Leu Lys Trp Arg Cys Val Ser Asn Pro Thr Leu Ala Asn Gly Glu Asn Ile Gly Asn Cys Pro Cys Gln Thr Phe Lys

300 310 320 330 340 350 360 370 380 390

GAA TCT GTA CAT AGC TCT TGT TAC ACC ACC TAT CAG GAA TGT TTT TTT GGT AAT AAG ACT TAC TAC ACC GCC ATT TTG GCC AGT AAT AGA GCC CCT ACT

Glu Ser Val His Ser Ser Cys Tyr Thr Thr Tyr Gln Glu Cys Phe Phe Gly **Asn Lys Thr** Tyr Tyr Thr Ala Ile Leu Ala Ser Asn Arg Ala Pro Thr

400 410 420 430 440 450 460 470 480 490

ATA GGA ACT AGC AAT GTC CCC ACA GTT TTG GGA AAC ACC CAC AAT CTA CTG TCA GCT GGA TGC ACC GGG ACT GTG GGT CAG CAC ATT TGC TGG AAT CCT

Ile Gly Thr Ser Asn Val Pro Thr Val Leu Gly Asn Thr His Asn Leu Leu Ser Ala Gly Cys Thr Gly Thr Val Gly Gln His Ile Cys Trp Asn Pro

500 510 520 530 540 550 560 570 580 590

AAA GCT CCC GTC CAT ATC TCC GAT GGA GGA GGA CCA CAA GAT AAG GCT CGA GAA ATT GCA GTA CAA AAA AGG CTT GAG GAA ATA CAT AGG TCC TTA TTT

Lys Ala Pro Val His Ile Ser Asp Gly Gly Gly Pro Gln Asp Lys Ala Arg Glu Ile Ala Val Gln Lys Arg Leu Glu Glu Ile His Arg Ser Leu Phe

600 610 620 630 640 650 660 670 680 690

CCC GAA TTA CGA TAC CAC CCC TTA GCT TTG CCT AAG GCC CGT GGT AAA GAA AAG ATC GAT GCT CAA ACC TTT AAT CTT CTT ACT GCT ACA TAT AGT TTG

Pro Glu Leu Arg Tyr His Pro Leu Ala Leu Pro Lys Ala Arg Gly Lys Glu Lys Ile Asp Ala Gln Thr Phe Asn Leu Leu Thr Ala Thr Tyr Ser Leu

700 710 720 730 740 750 760 770 780 790

CTT AAT AAG TCT AAC CCT AAT TTG GCC AAC GAG TGC TGG CTA TGC TTA CCA TCT GGA AAT CCC GTC CCG CTT GCC ATA CCT AGC AAT GAC TCA TTT CTT

Leu **Asn Lys Ser** Asn Pro Asn Leu Ala Asn Glu Cys Trp Leu Cys Leu Pro Ser Gly Asn Pro Val Pro Leu Ala Ile Pro Ser **Asn Asp Ser** Phe Leu

800 810 820 830 840 850 860 870 880 890

GGT TCT AAT CTT TCT TGC CCT ATT ATT CCT CCC TTG TTG GTA CAA CCT CTT GAA TTT ATT AAT CTT ATT AAT GCC TCT TGC CTT TAT TCC CCT TCT CAG

Gly Ser **Asn Leu Ser** Cys Pro Ile Ile Pro Pro Leu Leu Val Gln Pro Leu Glu Phe Ile Asn Leu Ile **Asn Ala Ser** Cys Leu Tyr Ser Pro Ser Gln

900 910 920 930 940 950 960 970 980 990

AAT AAC TCA TTT GAT GTT GAT GTA GGC TTA GTT GAA TTT ACC AAC TGC TCT ACC ACT CTT AAT ATT TCT CAC TCC TTA TGT GCC CCT AAT AGC TCA GTT

Asn Asn Ser Phe Asp Val Asp Val Gly Leu Val Glu Phe Thr **Asn Cys Ser** Thr Thr Leu **Asn Ile Ser** His Ser Leu Cys Ala Pro **Asn Ser Ser** Val

1000 1010 1020 1030 1040 1050 1060 1070 1080

TTT GTG TGC GGT AAT AAC AAG GCC TAC ACG TAC TTA CCC ACA AAC TGG ACA GGA ACT TGC GTA TTA GCT ACC CTT CTG CCA GAT ATA GAT ATT GTC CCT

Phe Val Cys Gly Asn Asn Lys Ala Tyr Thr Tyr Leu Pro Thr **Asn Trp Thr** Gly Thr Cys Val Leu Ala Thr Leu Leu Pro Asp Ile Asp Ile Val Pro

GP70 <-- --> GP20

1090 1100 1110 1120 1130 1140 |1150 1160 . 1170 1180

GGT GAT GCA CCT GTG CCT GTT CCA GCT ATA GAT CAT TAT CTA CAT AGA GCT AGG AGA GCA GTA CAG TTT ATC CCC TTA CTT GTT GGG TTA GGC ATA ACC

Gly Asp Ala Pro Val Pro Val Pro Ala Ile Asp His Tyr Leu His Arg Ala Arg Arg Ala Val Gln Phe Ile Pro Leu Leu Val Gly Leu Gly Ile Thr

1190 1200 1210 1220 1230 1240 1250 1260 1270 1280

ACT GCC GTC TCA ACT GGG ACT ACT GGT CTA GGG TAT TCT ATT ACC CAG TAC ACC AAA CTA TCT CGT CAA CTA ATC TCA GAC GTA CAG GCT ATC TCA AGC

Thr Ala Val Ser Thr Gly Thr Thr Gly Leu Gly Tyr Ser Ile Thr Gln Tyr Thr Lys Leu Ser Arg Gln Leu Ile Ser Asp Val Gln Ala Ile Ser Ser

1290 1300 1310 1320 1330 1340 1350 1360 1370 1380

ACT ATT CAA GAT TTA CAA GAC CAA GTA GAC TCT CTA GCA GAA GTG GTG CTT CAA AAT AGA AGA GGA TTA GAT CTA TTT ACC GCA GAG CAG GGA GGG ATT

Thr Ile Gln Asp Leu Gln Asp Gln Val Asp Ser Leu Ala Glu Val Val Leu Gln Asn Arg Arg Gly Leu Asp Leu Phe Thr Ala Glu Gln Gly Gly Ile

1390 1400 1410 1420 1430 1440 1450 1460 1470 1480

TGT TTA GCC CTA CAG GAA AAG TGC TGT TTC TAT GCC AAC AAA TCT GGA ATC GTC AGA GAT AAG ATC AAA CGT CTC CAA GAA GAC CTG GAA AAA CGA CGT

Cys Leu Ala Leu Gln Glu Lys Cys Cys Phe Tyr Ala **Asn Lys Ser** Gly Ile Val Arg Asp Lys Ile Lys Arg Leu Gln Glu Asp Leu Glu Lys Arg Arg

1490 1500 1510 1520 1530 1540 1550 1560 1570 1580

AAA GAA ATC ATT GAC AAT CCA TTT TGG ACT GGT CTA CAT GGA CTC CTC CCC TAT TTG CTA CCT TTG CTA CGA CCT TTG CTT TGC TTG TTA CTA TTA ATT

Lys Glu Ile Ile Asp Asn Pro Phe Trp Thr Gly Leu His Gly Leu Leu Pro Tyr Leu Leu Pro Leu Leu Arg Pro Leu Leu Cys Leu Leu Leu Leu Ile

1590 1600 1610 1620 1630 1640 1650 1660 1670 1680

ACC TTT GGA CCC TTA ATT TTC AAT AAG ATC ATA GCA TTT GTT AAA CAA CAA ATG GAT GCC ATC CAA GCT AAG CCT ATT CAG GTC CAT TAC CAC CGC CTT

Thr Phe Gly Pro Leu Ile Phe Asn Lys Ile Ile Ala Phe Val Lys Gln Gln Met Asp Ala Ile Gln Ala Lys Pro Ile Gln Val His Tyr His Arg Leu

1690 1700 1710 1720 1730 1740 1750 1760 1770 1780

GAG CAA GAA GAC AAT GGT GGT GTA TAC TTA CGT GTA TCG TAG ACC ACC GCC CCT GTG CGC TAA ACT GGT CAG CCA ATG ACG GGT AAG AGA GTG TCG TTT

Glu Gln Glu Asp Asn Gly Gly Val Tyr Leu Arg Val Ser ***

1790 1800 1810 1820 1830 1840 1850 1860 1870 1880

CTC ACT AAC CTA AGA CAG GAG GGT CGT CTT AGC TAC TAC CTT TTC CTA TGA CGG GTA CCA GTG AAG AAA CTG TAT CAC TCC AAC CTA AGA CAG GCG CAG

1890 1900 1910 1920 1930 1940 1950 1960 1970 1980

TTT CCG AGG GGA TTC TTT TGA AAA AAT AAA AAA GGG GGA CCT GTC CGG AGC CGT GCA GCC CGG ACG ACG TTC TGG CTT CTG GCG ATT GCA CTC CAT AGT

1990 2000 2010 2020 2030 2040 2050 2060 2070

AGT GGA TGC AAG ATG GCG CAC TTC CTG GTT CTC TTT CAC CCT GTT TTC CCG CCG GCG CCA ATA TTT CCC GCC TTA GAC TAC GTG GCA TTT CCT GAC TTA

2080 2090

GCT ACT GAG CAT GC

Figure 5. Comparison of the envelope glycoprotein of D2/RHE/OR to other SRVs. The envelope glycoproteins of other characterized SRVs was compared to that of D2/RHE/OR. The D2/RHE/OR envelope gene is the reference (top) sequence. Amino acid residue changes in the D2/CEL/OR, D1/RHE/CA, and Mason-Pfizer monkey virus (MPMV) envelope sequences are denoted in lower case letters. Vertical lines, with additional sequence, denote insertions relative to the reference sequence. Hyphens represent gaps introduced in amino acid sequence to maximize alignment with the reference sequence.

```
                   5     10    15     20     25    30     35     40    45     50     55    60     65     70    75     80     85    90
                          *            *            *             *            *            *             *            *            *
D2/RHE/OR     HTV KDI PFW RVL LIF QTA RVY AGF GDP REA ITI IHQ QHG KPC DCA GGY VIT APT VYL ATV SCS SHT AYQ PSD SLK WRC VSN PTL ANG ENI
D2/CEL/OR       l                                       m                     na          a
D1/RHE/CA      nf nhh f   slv i s  if q q             lle  q  k               ss p   ns  t      ty    s vtn      q    t    t  sp th
                       i
                       |
MPMV           nf -ny h   slv ils  is q q             lae  q  k               ss p i ns  t      t     s vtn      q    t    t psn th

                  95    100   105    110    115   120    125    130   135    140    145   150    155    160   165    170    175   180
                          *            *            *             *            *            *             *            *            *
D2/RHE/OR     GNC PCQ TFK ESV HSS CYT TYQ ECF FGN KTY YTA ILA SNR APT IGT SNV PTV LGN THN LLS AGC TGT VGQ HIC WNP KAP VHI SDG GGP QDK
D2/CEL/OR           k                 a                                                             n     p
                   ns
                   |
                 sq|                                                                           p
                  ||                                                                           |
D1/RHE/CA      s      sy- d    at   n h   q t i        l tmi rdk  s s s d g     i     nq   ii     enk k   vv   s qps    m
                 ge     y                                                                      p
                  |     |                                                                      |
MPMV           s    n  is d    a    n h   q n i        l tit gd   t a   d g         t s    it     n k k   vv   s rps

                 185    190   195    200    205   210    215    220   225    230    235   240    245    250   255    260    265   270
                          *            *            *             *            *            *             *            *            *
D2/RHE/OR     ARE IAV QKR LEE IHR SLF PEL RYH PLA LPK ARG KEK IDA QTF NLL TAT YSL LNK SNP NLA NEC WLC LPS GNP VPL AIP SND SFL GSN LSC
D2/CEL/OR                       k                                                                            i
                                                                                 v                              ydntscs     c
                                                                                 |                                |         |
D1/RHE/CA     v    i  n k f   l k         s         e            h   d   atv h     s qr  q  ed       r   d       l  nst f f n   c
                                                                                                                             ac
                                                                                                                             |
                                                                                                                          lcsn
                                                                                                                            ||
MPMV            d  iv n k f   l           s         e            h l d   atv h     a  q  s  ed       q   d       l  y   t   snh -

                 275    280   285    290    295   300    305    310   315    320    325   330    335    340   345    350    355   360
                          *            *            *             *            *            *             *            *            *
D2/RHE/OR     PII PPL LVQ PLE FIN LIN ASC LYS PSQ NNS FDV DVG LVE FTN CST TLN ISH SLC APN SSV FVC GNN KAY TYL PTN WTG TCV LAT LLP DID
D2/CEL/OR                        m           f   f                   a                                            s
                                                                           skp
                                                                            |
D1/RHE/CA     l t   f      --  -  fth sv   a dy         i      ag       s yi s p                                        s
                                                                  syynv
                                                                    |
MPMV           lt   f      --  -  ftd sn   a hy         i      as          ask  p n                                      s

                 365    370   375    380    385   390    395    400   405    410    415   420    425    430   435    440    445   450
                          *            *            *             *            *            *             *            *            *
D2/RHE/OR     IVP GDA PVP VPA IDH YLH RAR RAV QFI PLL VGL GIT TAV STG TTG LGY SIT QYT KLS RQL ISD VQA ISS TIQ DLQ DQV DSL AEV VLQ NRR
D2/CEL/OR                                                             a
D1/RHE/CA      i   se     i       f g  pk   i       v i               a    v             h
MPMV           i   se     i       f g k k   i       f                aa    v             h

                 455    460   465    470    475   480    485    490   495    500    505   510    515    520   525    530    535   540
                          *            *            *             *            *            *             *            *            *
D2/RHE/OR     GLD LFT AEQ GGI CLA LQE KCC FYA NKS GIV RDK IKR LQE DLE KRR KEI IDN PFW TGL HGL LPY LLP LLR PLL CLL LLI TFG PLI FNK IIA
D2/CEL/OR          l                                                                                   g   f                       t
D1/RHE/CA                                                   n   d         ql           f         vm    g         vl  s    i     lmt
MPMV                                                        n   d     r  rql          sf   f     vm    g     s   vl  s    i     lmt

                 545    550   555    560    565   570   574
                          *            *            *
D2/RHE/OR     FVK QQM DAI QAK PIQ VHY HRL EQE DNG GVY LRV S
D2/CEL/OR           i
D1/RHE/CA      i  h i  es                      h   s   nl t
MPMV           i  h i  es                      s   s   tl t
```

Figure 6. Comparison of the D2/RHE/OR 3' LTR to other SRVs. The displayed D2/RHE/OR reference (top) sequence begins with the TAG terminator of the envelope gene and proceeds to the end of the 3' LTR (U5 region at position 542-546). Changes in the corresponding D2/CEL/OR, D1/RHE/CA, and MPMV sequences are denoted in lower case letters. Vertical lines, with additional sequence, denote insertions relative to the reference sequence. Hyphens represent gaps introduced in the compared sequence to maximize alignment with the reference sequence. The polypurine track (bases 179 -198), denoting the 5' boundary of the rightward (3') LTR, and the U3 enhancer region (bases 201 -205) are overlined in the figure. The D2/CEL/OR molecular clone described by Thayer et. al. (Thayer *et al.* 1987) is permuted, and does not contain the complete 3' LTR. Only two additional bases beyond the polypurine track in the D2/CEL/OR molecular clone were directly sequenced. The remainder of the published D2/CEL/OR 3' LTR are predicted nucleotides inferred by direct sequencing of the leftward (5') LTR, and depicted differences between the D2/RHE/OR and D2/CEL/OR 3' LTR sequences are based on these inferences.

```
                       10           20           30            40           50           60            70           80
                        *            *            *             *            *            *             *            *
D2/RHE/OR   TAG ACC ACC GCC CCT GTG CGC TAA ACT GGT CAG CCA ATG ACG GGT AAG AGA GTG TCG TTT CTC ACT AAC CTA AGA CAG GAG

D2/CEL/OR

D1/RHE/CA               t        c  a       g     a                                 a a     t

MPMV            g       t           a             a                                 a a


                       90           100          110           120          130          140           150          160
                        *            *            *             *            *            *             *            *
D2/RHE/OR   GGT CGT CTT AGC TAC TAC CTT TTC CTA TGA CGG GTA CCA GTG AAG AAA CTG TAT CAC TCC AAC CTA AGA CAG GCG CAG TTT

D2/CEL/OR         a

D1/RHE/CA     c      ag          g    a a    a  a          aa       ta      a                                      c
                                                                 ac
                                                                  |
MPMV          c      aa          g    a a    a             at               -                                      cc


                      170           180          190           200          210          220           230          240
                        *            *            *             *            *            *             *            *
                                   -----------------------------------
D2/RHE/OR   CCG AGG GGA TTC TTT TGA AAA AAT AAA AAA GGG GGA CCT GTC CGG AGC CGT GCA GCC CGG ACG ACG TTC TGG CTT CTG GCG

D2/CEL/OR             g                                     *

                   atttgtc                                                                                      ttt
                     |                                                                                           |
D1/RHE/CA             t c g      at  t  t   tt          t                         t          t   t   ct      c       t

                    at          tttt                                                                              t
                     |             |                                                                              |
MPMV                   t g         t t t     t           t    a                    t          t   t  ct      c      tt


                  250          260           270           280          290          300           310         320
                   *            *             *             *            *            *             *           *
D2/RHE/OR   ATT GCA CTC CAT AGT AGT GGA TGC AAG ATG GCG CAC TTC CTG GTT CTC TTT CAC CCT GTT TTC CCG CCG GCG CCA ATA TTT

D1/RHE/CA   c a   t   a  -g ct   -- a    t              a                 t c c tgt gt   c                   g    g

MPMV        c c tag          -  ta    a  tt             t t               t c c gt   t ac                    g    g


                  330          340          350          360           370
                   *            *            *            *             *
D2/RHE/OR   CCC GCC TTA GAC TAC GTG GCA TTT CCT GAC TTA GCT ACT GAG CAT G

                 t        t
                 |        |
D1/RHE/CA                g            -       c agt  ct   a gt

                 t        t
                 |        |
MPMV                   g  g            -         tg   ct
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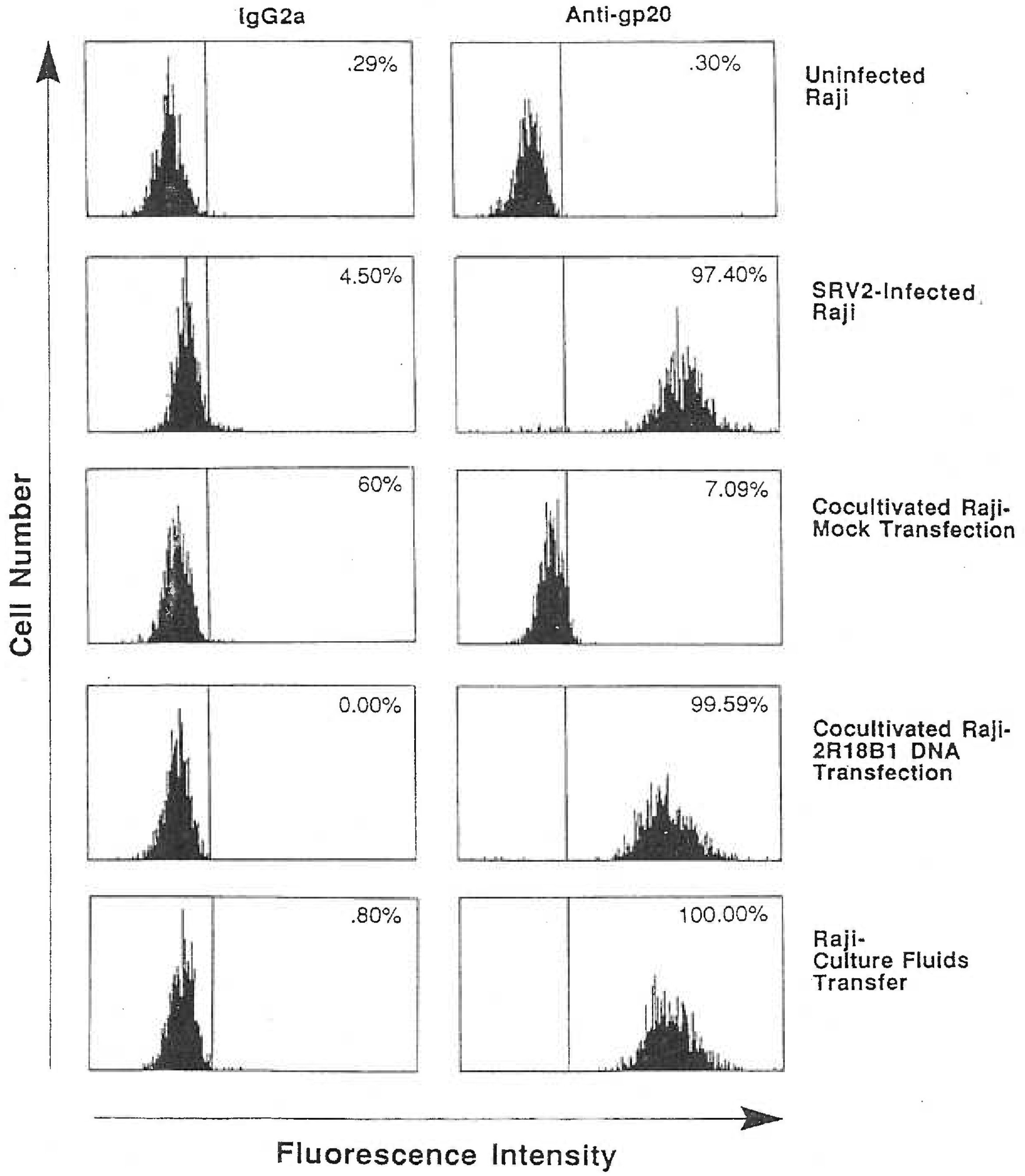
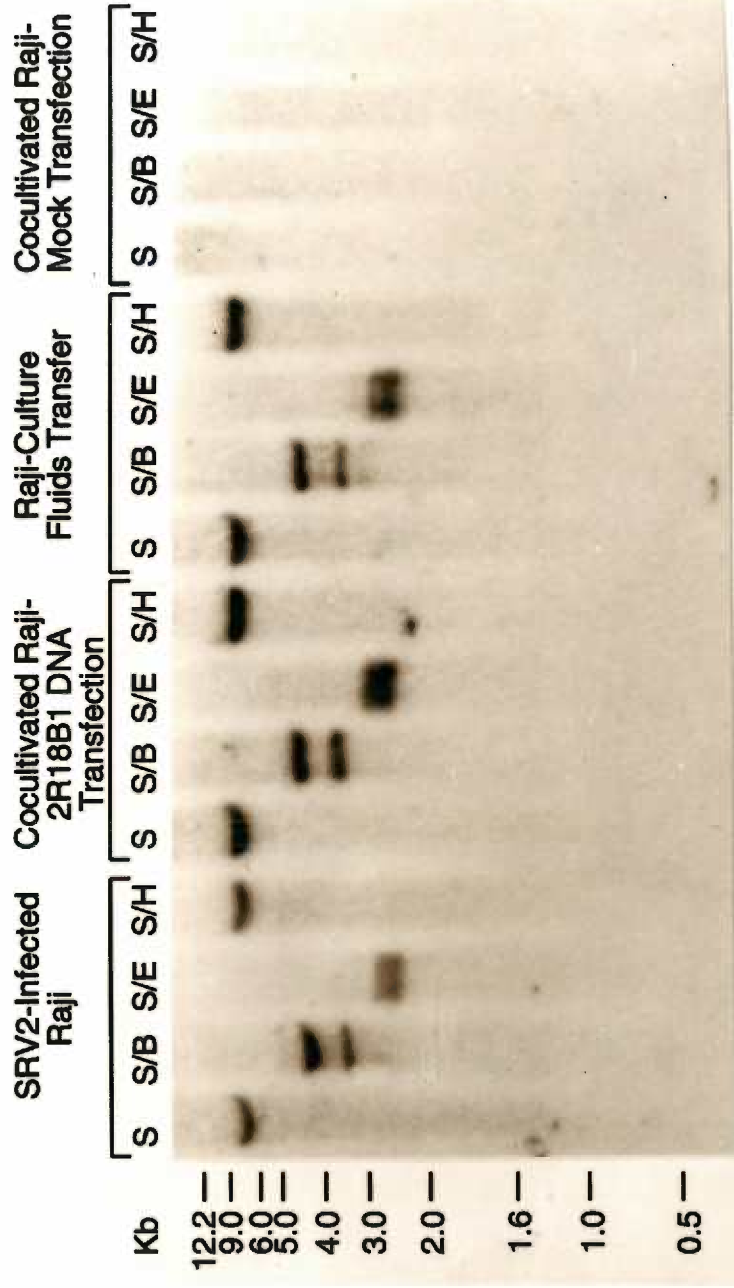
Figure 7. FACS analyses of D2/RHE/OR infected Raji cells. Immunofluorescent and FACS analyses, using negative control IgG2a (left panels) and type D SRV env anti-gp20 (right panels) monoclonal antibodies, were conducted on five experimental and control Raji cell lines, including 1) uninfected Raji cells, 2) positive-control SRV 2-infected Raji cells, 3) Raji cells previously cocultivated with mock-transfected D-17 cells, 4) Raji cells previously cocultivated with D-17 cells transfected with the 2R18B1 molecular clone, and 5) Raji cells inoculated with medium obtained from cocultivated Raji cells described in number 4 above. Cell lines 1 - 5 are denoted in order from top to bottom in Figure 4. The percentage of positively-stained cells, recognized above threshold values, are shown in the upper right hand corner of each histogram.



Monoclonal Antibody
IgG2a
Anti-gp20
.29%
.30%
Uninfected Raji
4.50%
97.40%
SRV2-Infected Raji
60%
7.09%
Cocultivated Raji-Mock Transfection
0.00%
99.59%
Cocultivated Raji-2R18B1 DNA Transfection
.80%
100.00%
Raji-Culture Fluids Transfer
Cell Number
Fluorescence Intensity

Figure 8. Southern analyses of genomic DNA from D2/RHE/OR infected cells. Genomic DNA obtained from different experimental and control Raji cell lines, including 1) positive-control SRV 2-infected Raji cells, 2) Raji cells cocultivated with D-17 cells transfected with the 2R18B1 molecular clone, and 3) Raji cells inoculated with medium obtained from Raji cocultivated cell line described in number 2 above, and 4) Raji cells cocultivated with mock-transfected D17 cells were analyzed by restriction endonuclease digest and Southern blot. Twenty mg of genomic DNA was digested with Sph I (S), or with Sph I in combination with Bam HI (B), Eco RI (E), or Hind III (H), and subjected to agarose gel electrophoresis and transfer to nylon membranes. Blots were hybridized with ^{32}P-labeled D2/CEL/OR full-length genomic probe p8A-1, washed at high stringency, and developed for autoradiography. Molecular weight markers are 1 kb ladder (BRL).


SRV2-Infected Raji
Cocultivated Raji- 2R18B1 DNA Transfection
Raji-Culture Fluids Transfer
Cocultivated Raji- Mock Transfection
S S/B S/E S/H
S S/B S/E S/H
S S/B S/E S/H
S S/B S/E S/H
Kb
12.2
9.0
6.0
5.0
4.0
3.0
2.0
1.6
1.0
0.5

VI.2 Manuscript prepared for submission to Virology

Molecular Cloning and Cell-Specific Growth Characterization of Polymorphic Variants of Type D Serogroup 2 Simian Retroviruses

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ABSTRACT

Simian retroviruses (SRVs), the etiologic agent of spontaneous Simian Acquired Immunodeficiency Syndrome (SAIDS), endemically infects large percentages of Asian macaques housed in biomedical research colonies, and severely compromises the effective use of these species as a viable research animal. We recently described the molecular cloning of a serogroup 2 SRV, D2/RHE/OR, which causes mild immunosuppression in rhesus macaques. A restriction site variant, D2/RHE/OR/V1, has also been recovered from severely ill animals endemically infected with D2/RHE/OR. We now report the complete nucleotide sequences of D2/RHE/OR and D2/RHE/OR/V1. Both infectious molecular clones retain the genetic structure typical of type D SRVs (5' LTR-*gag*-*prt*-*pol*-*env*-3'LTR) and encode identically-sized 8105 base pair (bp) proviruses. D2/RHE/OR and D2/RHE/OR/V1 are 99.3% similar at the amino acid level, exhibiting only 17 residue differences, ten of which are located in the envelope (env) glycoproteins. The molecular clones and reciprocal chimeric viruses were used to assess the contribution of different genetic domains to virus infectivity in a T-cell infection assay. These experiments indicate that D2/RHE/OR has a reduced ability to infect T-cell lines, especially Hut-78 and MT-4 cells, and that the envelope gene is not the sole determinant of tropism.

INTRODUCTION

Nearly 30 years have passed since the first type D retrovirus was originally isolated and characterized from a breast carcinoma of an eight year old female rhesus macaque (*Macaca mulatta*) (Jensen *et al.* 1970; Chopra and Mason, 1970). Several unsuccessful attempts had been made to verify the oncogenic nature of this recovered virus, identified as Mason-Pfizer monkey virus (MPMV), by inoculating rhesus macaques (Fine *et al.* 1975; Fine *et al.* 1972). Although MPMV did not induce breast carcinomas in these animals, inoculated neonatal rhesus macaques did develop an immunosuppressive disease characterized by severe lymphadenopathy, weight loss, thymic atrophy and opportunistic infections (Bryant *et al.* 1986a; Henrickson *et al.* 1983; Arthur *et al.* 1986; Fine *et al.* 1975). Since the initial discovery of MPMV, four additional SRV serogroups have been recovered from multiple macaque species exhibiting varying degrees and symptoms of spontaneous immunosuppressive disease (Heidecker *et al.* 1987; Maul *et al.* 1986; Benveniste *et al.* 1985; Letvin *et al.* 1984; Shiigi *et al.* 1986).

The five serogroups of type D SRVs are distinguished by neutralization assays. Serogroup 1 SRVs (SRV-1) were characterized at the New England and California Regional Primate Research Centers (RPRCs). The D1/CYC/NE isolate was recovered from a Taiwanese rock macaque (*Macaca cyclopis*) (Daniel *et al.* 1984). The California isolate, D1/RHE/CA, was recovered from a rhesus macaque (*Macaca mulatta*) and is associated with more severe immunodeficiency than the D1/CYC/NE isolate (Marx *et al.* 1984; Henrickson *et al.* 1983; London *et al.* 1983). Serogroup 2 viruses have been identified at the Washington (D2/MNE/WA) and Oregon (D2/CEL/OR, D2/RHE/OR) RPRCs, and has been associated with retroperitoneal fibromatosis (RF) in some macaque species (Celebes, pig-tailed and cynomolgus) in addition to SAIDS (Giddens, Jr. *et al.* 1985; Shiigi *et al.* 1986; Benveniste *et al.* 1985; Stromberg *et al.* 1984; Marx *et al.* 1985). The original MPMV and D3/RHE/WI, isolated at the Wisconsin RPRC, belong to serogroup 3 (Sonigo *et al.* 1986). Single members of serogroups 4 (D4/CYN/CA) and 5 (D5/RHE/OR) were recovered from a group of

cynomolgus macaques in Berkeley, California and from rhesus macaques imported from the People's Republic of China to the Oregon RPRC, respectively (M.A. Axthelm, personal communication).

Four different serogroup 2 SRVs have been characterized at the Oregon RPRC. Although they are related by serology, each of the four isolates is characterized by unique restriction site polymorphisms. Besides the Celebes (D2/CEL/OR) and rhesus (D2/RHE/OR) isolates described above, two additional serogroup 2 SRVs have also been identified at the Oregon RPRC. One variant, D2/RHE/OR/V1, was isolated from a severely ill rhesus macaque originating from group-housed animals endemically infected with D2/RHE/OR. Transmission studies performed at the Oregon RPRC have demonstrated that rhesus macaques infected with the Celebes isolate (D2/CEL/OR) seroconvert and become aviremic without manifesting any measurable symptoms of disease (Marx *et al.* 1985). D2/RHE/OR is associated with a mild immunodeficiency disease in rhesus macaques and is thought to be similar to the Washington RPRC isolate, D2/MNE/WA, recovered from a pig-tailed macaque (*Macaca nemestrina*) (Henderson *et al.* 1985; Grant *et al.* 1995a; Grant *et al.* 1995b). However, D2/RHE/OR is recovered from Japanese macaques (*Macaca fuscata*) with severe and fatal immunodeficiency disease. A second variant, D2/CYN/OR/V2, was recovered from cynomolgus macaques (*Macaca fascicularis*) with severe immunodeficiency disease.

The differences in pathogenic outcome associated with the serogroup 2 SRV family is intriguing given the high degree of genetic similarity between the different isolates (Marx *et al.* 1985; Bryant *et al.* 1986; Thayer *et al.* 1987). Small alterations in nucleotide sequence identified among viruses with a high degree of similarity have been demonstrated to invoke significant differences in the biological behavior of the virus. A domain spanning the tRNA primer binding site of Moloney-murine leukemia virus (M-MuLV) has been demonstrated to inhibit productive infection in F9 embryonal carcinoma cells but not differentiated NIH 3T3 cells (Loh *et al.* 1988). Differences in *gag* gene sequence can release MuLV from restricted replication that is imposed by the *Fv1* locus in certain target cells (Goff and Lobel, 1987; Kozak and Chakraborti, 1996; Ou *et al.* 1983; DesGroseillers and Jolicoeur, 1983). Alterations in the amino acid sequence of the HIV env

glycoproteins, which lead to an overall positive (basic) net charge of the V3-loop, can facilitate usage of the CXCR4 coreceptor and replication in T-lymphocytes (Fouchier *et al.* 1992; Cocchi *et al.* 1996). SRV-1 from the New England RPRC was demonstrated to infect human B-cells (Raji); in contrast, SRV-1 from the California RPRC readily infected both human B- and T-cell lines in vitro (Maul *et al.* 1988; Yetz and Letvin, 1987). To better characterize the serogroup 2 SRVs isolated at the ORPRC, we have isolated and sequenced full-length infectious molecular clones of D2/RHE/OR and D2/RHE/OR/V1. In addition, reciprocal chimeric viruses were constructed to analyze the contributions made by genomic determinants to in vitro infectivity of lymphoid cell lines that may be responsible for the variations observed in pathological outcome.

RESULTS

Library construction and the isolation of a full-length infectious D2/RHE/OR/V1 molecular clone. The D2/RHE/OR/V1 molecular clone was derived from a genomic DNA library generated from infected Raji cell DNA. Infectivity was determined by transfection of canine D-17 cells followed by co-cultivation with permissive Raji cells to rescue and amplify the virus population (see Materials and Methods). Productive infection was demonstrated by the presence of syncytia, viral envelope gp20 expression, electron microscopic visualization of virions, and the transmission of infection by cell-free virus from transfected cell culture fluid (Figure 9). All infection criteria were met. In addition, using Southern analyses, identical restriction fragment patterns were observed using DNA from experimental infected Raji cultures and from cells infected with a biological D2/RHE/OR/V1 isolate, confirming the D2/RHE/OR/V1 identity of the molecular clone (Figure 10).

In addition, the full-length D2/RHE/OR/V1 genome has been subcloned into the plasmid pBR322 using Hind III restriction sites which are located in the human flanking DNA. The D2/RHE/OR/V1 plasmid clone, designated pV1, has been demonstrated to be equivalent to the parental lambda clone by restriction endonuclease digestion, Southern and infection analyses. The pV1 molecular clone was then utilized as a template for sequence analyses and for the generation of infectious virus used in studies reported here.

Nucleotide sequences of the D2/RHE/OR and D2/RHE/OR/V1 proviruses. D2/RHE/OR/V1 was first isolated from a rhesus macaque endemically infected with D2/RHE/OR and afflicted with severe immunodeficiency. Sequencing the complete genomes of D2/RHE/OR and D2/RHE/OR/V1 was performed to further characterize the genetic variability of these two serogroup 2 polymorphic isolates and identify the genetic domain(s) which may be responsible for different pathological outcomes in infected rhesus macaques.

The D2/RHE/OR and D2/RHE/OR/V1 viruses retain the genetic structure of type D SRVs (5' LTR-*gag-prt-pol-env*-3'LTR) and encode identically-sized 8105 bp proviruses (Figures 11 and 12). The *gag, prt, pol*, and *env* genes encode the viral core proteins, viral protease, reverse transcriptase/ endonuclease/integrase, and envelope glycoproteins, respectively. As in other molecularly-cloned type D SRVs (Thayer *et al.* 1987; Sonigo *et al.* 1986; Power *et al.* 1986), the *gag*, *prt*, and *pol* genes contained in both D2/RHE/OR and D2/RHE/OR/V1 are over-lapping and are predicted to generate the Pr78, Pr95 and Pr180 precursor polyproteins, respectively (Bradac and Hunter, 1984). Sequence variability between D2/RHE/OR and D2/RHE/OR/V1 is restricted to nucleotide substitutions. All reported nucleotide sequence or deduced amino acid residues will be relative to that of D2/RHE/OR/V1 and referenced first in all ensuing comparison statements unless otherwise noted.

Long terminal repeat (LTR) and intergenic spacer regions. Retroviral LTRs contain critical sequences necessary for the integration, synthesis, and expression of viral DNA (Vaishnav and Wong-Staal, 1991; Sonigo *et al.* 1986). Both D2/RHE/OR and D2/RHE/OR/V1 genomes contain identical LTRs of 346 bp. The polypurine tract for initiation of positive strand DNA synthesis and the $tRNA^{lys1,2}$ primer binding site for negative strand DNA synthesis are identical for all molecularly-cloned serogroup 2 SRVs (Figures 13 and 18) (Thayer *et al.* 1987; Maul *et al.* 1986). The D2/RHE/OR and D2/RHE/OR/V1 LTRs are 96.2, 73.3, and 76.0% conserved to corresponding sequences in D2/CEL/OR, D1/RHE/CA, and MPMV, respectively. The U3 (TGTCC) and the U5 (GGACA) inverted repeats found in the LTRs of both D2/RHE/OR and D2/RHE/OR/V1 are precisely conserved in both sequence and location with corresponding D2/CEL/OR, D1/RHE/CA, and MPMV sequences. The identification of the exact transcriptional cap and polyadenylation sites and active promoter and enhancer elements will require direct sequence analyses of the viral transcript and functional assessment of regulatory sequences. Identical candidate TATA boxes have been identified in the 5' LTRs of both D2/RHE/OR and D2/RHE/OR/V1 (TATATA); slight sequence variations in the TATA element have been observed in D1/RHE/CA and in MPMV (TATATAA). Potential polyadenylation

signals (positions 7991 - 7996; Figures 11 and 13) have been identified in D2/RHE/OR and D2/RHE/OR/V1; these signals are identical among the molecularly-cloned SRVs, D2/CEL/OR, D1/RHE/CA, and MPMV. Interestingly, this potential polyadenylation signal (ATTAAA) is different from those (AATAAA) most commonly found in eukaryotic genes and viruses (Proudfoot and Brownlee, 1974).

The *gag* gene. Both D2/RHE/OR and D2/RHE/OR/V1 contain *gag* open reading frames (ORF) of 659 codons, with a predicted translation product of 654 amino acids. The amino-terminal glycine residue present in the penultimate amino acid position of the gag precursor is conserved in all molecularly-cloned SRVs and is required for myristylation and subsequent intracytoplasmic transport of mature viral capsids to the membrane, as a prelude to virion budding and release (Rhee and Hunter, 1987) (Figure 14). The Pr78 gag precursor is proteolytically-processed to form six mature virion core proteins in the following order: NH_2-p10-pp24/pp18-p12-p27-p14-p4 (Henderson *et al.* 1985; Bradac and Hunter, 1984). The D2/RHE/OR and D2/RHE/OR/V1 *gag* genes are highly conserved, exhibiting only three deduced amino acid differences out of 654 residues (Figure 14). These three nonconservative differences are located within the phosphoprotein (pp24/pp18) at positions 127 (Thr to Met), 182 (Glu to Lys) and 205 (Arg to Gly). Amino-terminal protein sequence analysis has determined the first 32 residues of the D2/MNE/WA gag phosphoprotein, and has identified changes at positions 117 (Asp to Gln), 125 (Lys to Ser) and 127 (Thr to Met) (Henderson *et al.* 1985). Interestingly, the Met at 127 is conserved in D2/RHE/OR and D2/CEL/OR (Figure 14). The phosphoprotein of D2/CEL/OR exhibits 11 amino acid differences at positions 121, 123, 126, 146, 147, 149, 161, 167, 173, 187, and 207. Moreover, serogroup 2 SRVs encode a larger phosphoprotein than MPMV, with an amino-terminus starting at gag residue 101; MPMV has an amino-terminus gag residue at position 160 resulting in a phosphoprotein of 18kD (Henderson *et al.* 1985; Sonigo *et al.* 1986). Of the six amino acid differences present in p12 of D2/CEL/OR, three are nonconservative at positions 272 (Arg to Trp), 279 (Ile to Lys), and 293 (Ser to Pro). Interestingly,

although the gag p27 is 100% conserved between D2/RHE/OR and D2/RHE/OR/V1, the D2/MNE/WA p27 has a single nonconserved residue at position 353 (a small, nonpolar Ala to a polar Thr) which is retained in D2/CEL/OR, D1/RHE/CA and MPMV [amino acid residues 351-490 of D2/MNE/WA were deduced from nucleotide sequence analysis] (Grant *et al.* 1995b). A polar Thr and Asn replaces a nonpolar Ala and basic Lys at positions 353 and 427 of p27, respectively, in D2/CEL/OR. Consistent with a previous observation in Rous sarcoma virus, there are two Cys-His motifs (Cys-X_2-Cys-X_4-His-X_4-Cys) in the p14 nucleocapsid with the more distal moiety exhibiting a lesser degree of conservation (Bowles *et al.* 1993). Two nonconservative differences are found in the p14 gag peptide of D2/CEL/OR at positions 535 (Phe to Leu) and 569 (Leu to Ser). The least conserved peptide of the gag polyprotein among all molecularly-cloned SRVs is the pp24/pp18 phosphoprotein; the most highly conserved is the major capsid peptide, p27. Overall, the D2/RHE/OR and D2/RHE/OR/V1 gag polyproteins are 96.2% and 96.3% conserved, respectively, relative to corresponding sequences found in D2/CEL/OR, and 83.6% conserved relative to the gag polyproteins found in both D1/RHE/CA and MPMV.

The *prt* gene. The D2/RHE/OR and D2/RHE/OR/V1 viral protease is encoded by the *prt* open reading frame, which overlaps *gag* by 61 codons and *pol* by 8 codons. The resulting frameshift would produce a polyprotein with a deduced molecular weight of 97 kd, consistent in size with the second gag-associated precursor (Pr 95) observed in MPMV. The D2/RHE/OR and D2/RHE/OR/V1 *prt* genes both encode proteins containing 314 amino acids that are 99.7% conserved at the amino acid level with a single change at position 98 (polar Gln to nonpolar Leu) (Figure 15). Predictably, the D2/RHE/OR/V1 prt product is 98.4% conserved to corresponding amino acid sequences in D2/CEL/OR with only five amino acid differences at positions 117 (Lys to Arg), 135 (Ile to Val), 153 (Asn to Asp), 169 (Asn to His) and 307 (Asn to Asp) (Figure 15). In addition, the D2/RHE/OR/V1 prt is 82.8% and 83.8% conserved to comparable proteins encoded by D1/RHE/CA and MPMV, respectively. The predicted D2/RHE/OR and D2/RHE/OR/V1 proteases are highly-related to other

retroviral and cellular proteases (Toh *et al.* 1985; Seiki *et al.* 1983; Schwartz *et al.* 1983; Power *et al.* 1986; Katz and Skalka, 1990). The amino-terminal end of D1/RHE/CA viral protease is reported to be similar to other proteins encoding deoxyuridine-triphosphatase (dUTPase) enzymatic activity (McGeoch, 1990). Furthermore, biochemical analysis has demonstrated the presence of dUTPase activity in the corresponding region of MPMV and SRV-1 (Elder *et al.* 1992). The carboxy-terminal portion of the viral protease has significant homology to other retroviral and cellular aspartyl proteases (Toh *et al.* 1985; Seiki *et al.* 1983; Schwartz *et al.* 1983; Power *et al.* 1986), including the presence of the Asp-Thr-Gly active site at amino acid positions 188-190 (Figure 15) (Thayer *et al.* 1987; Shiigi *et al.* 1985).

The *pol* gene. A second frameshift within the *prt-pol* overlap would generate a 200 kd polyprotein encoding gag, prt, and pol sequences, in agreement with the observed molecular weight of the third gag-associated precursor (Pr180) observed in MPMV (Bradac and Hunter, 1984). The *pol* genes of all molecularly-cloned type D SRVs encode predicted products exhibiting two functional domains, the reverse transcriptase and endonuclease/integrase domains. Deduced amino acid sequence similarity to the pol of RSV predicts that this protein may be enzymatically processed near amino acid 593, thereby releasing the reverse transcriptase from the endonuclease/integrase domain (Hippenmeyer and Grandgenett, 1984). This putative processing site is conserved among all molecularly-cloned serogroup 2 SRVs (Thayer *et al.* 1987). The product of the D2/RHE/OR/V1 *pol* gene is 99.7% similar to that of D2/RHE/OR with three differences at positions 111 (Leu to Pro), 151 (Met to Ile) and 337 (Leu to Phe); the D2/RHE/OR/V1 pol product is closely-related to corresponding sequences in D2/CEL/OR (97.3%) and less-related to MPMV and D1/RHE/CA (82.7% and 84.1%, respectively) (Figure 16).

The *env* gene. The *env* genes of all three cloned serogroup 2 SRVs (D2/RHE/OR, D2/RHE/OR/V1 and D2/CEL/OR) encode glycoproteins of 574 amino acid residues (Figure 17). All

three envelope glycoproteins contain 22 cysteine residues and 11 conserved Asn-X-Ser(Thr) glycosylation sites. Ten of the potential glycosylation sites are found within the extrinsic gp70 domain and one within the transmembrane gp20 domain (Figure 17). The D2/RHE/OR and D2/RHE/OR/V1 envelope glycoproteins are 98.3% similar overall at the amino acid level, and 98.4% and 97.9% similar within the gp70 and gp20 regions, respectively. The hydrophobic and heptad-repeat domains at the gp20 amino terminus (amino acid residues 410-458) are present and well conserved in all molecularly cloned SRVs, including D2/MNE/WA (Chambers *et al.* 1990; Grant *et al.* 1995b). Ten amino acid residue substitutions are found between the D2/RHE/OR and D2/RHE/OR/V1 glycoproteins occurring at positions 3, 155, 160, 296, 302, 316, 404, 415, 455, and 519. Of the 10 predicted amino acid differences, three appear to be conserved between D2/RHE/OR and D2/CEL/OR and are located at residues 302 (a small, nonpolar Gly to a charged Asp), 316 (a bulkier Ile to a polar Thr), and 415 (a conservative change from Arg to Lys). The immunosuppressive peptide found at residues 447-472 is identical among all molecularly cloned SRVs, with the exception of D2/RHE/OR which varies at position 455 (Leu to Phe) (Figure 17) (Grant *et al.* 1995b; Cianciolo *et al.* 1984). The partial gp70 sequence (residues 144-259) and the complete gp20 domain of D2/MNE/WA have also been compared to the serogroup 2 SRVs found at the Oregon RPRC (Grant *et al.* 1995a; Grant *et al.* 1995b). Interestingly, the D2/MNE/WA isolate shares three amino acid residues (Ala_{404}, Leu_{455} and Gly_{519}) with D2/RHE/OR/V1 within the gp20 domain that are distinct from D2/RHE/OR (Thr_{404}, Phe_{455} and Arg_{519}). Except for a Leu to Phe difference at position 446 (within a heptad-repeat of the gp20 domain), the remaining D2/MNE/WA amino acid differences are conservative with respect to polarity, charge and bulk, when compared to D2/RHE/OR/V1 sequence (Figure 17).

Construction of reciprocal chimeric viruses and analysis of determinants of in vitro tropism. The chimeric viruses, p5'V13'2R and p5'2R3'V1, contain reciprocal combinations of the 5' and 3' halves of D2/RHE/OR and D2/RHE/OR/V1 (Figure 19). These recombinants were constructed

in a pBR322 backbone (see Materials and Methods), and the chimeric nature of these clones was verified by polymerase chain reaction, restriction endonuclease digestion and sequence analyses (data not shown). By utilizing a unique Bam HI restriction endonuclease site in the *pol* gene, we were able to segregate the amino acid differences between the *env* gene in the 3' half and the *gag-prt-pol* genes in the 5' half of both viral genomes. Infectivity for each chimeric virus was determined by transfection of canine D17 cells followed by rescue and amplification in Raji cells, as described above. Criteria for successful infection was defined by syncytia formation in the Raji cell culture, gp20 expression, and the transmission of infection using cell-free virus from filtered (.45 µm) transfection culture fluid (data not shown). All criteria of infection were met and the infectious chimeric viruses were then used to determine the role of the different genetic domains in in vitro cell tropism.

A battery of T cell-lines, CEM-SS, Hut78, MT-4 and SupT-1, in addition to Raji cells (as a positive control), were targets for infection by molecularly cloned viruses D2/RHE/OR and D2/RHE/OR/V1, and the reciprocal chimeric viruses p5'V13'2R and p5'2R3'V1. Equivalent endpoint viral loads were determined by reverse transcriptase assays of cell-free culture medium that were subsequently used to infect fresh cells. Infection was assessed by monitoring env gp20 expression; uninfected cultures for each cell line and an infected Hut78 culture were employed as the negative and positive assay controls, respectively. Immunofluorescence assays for gp20 expression were repeated at weekly intervals following the first positive measurement to verify virus infection. The current infection experiments were designed to continue for up to 60 days.

Three reproducible patterns of infection were observed using cell-free inoculation of the different T- and B-cell lines. The first pattern of gp20 expression is characterized by rapid infection (>50% by day 10-16) and a retention of gp20 expression over time. This pattern of infection kinetics was observed in Raji and CEM-SS cell lines infected with D2/RHE/OR/V1 and D2/RHE/OR, and the chimeric viruses p5'2R3'V1 and p5'V13'2R, and also observed in MT-4 cells infected by

D2/RHE/OR/V1, p5'2R3'V1 and p5'V13'2R (Figure 19). In addition, D2/RHE/OR/V1 and p5V132R infected Raji cells formed numerous large and ballooning syncytia, whereas infection with D2/RHE/OR or p52R3V1 in the same cell line formed very few and small syncytia (data not shown). The syncytia forming capacity of each viral clone was reproducible and appeared to be independent of gp20 expression in the remaining cell lines (data not shown).

The second pattern of infection was characterized by rapid infection (>50% by day 10) of SupT-1 cells, followed by a decrease in the percentage of gp20 expressing cells (from a range of 84-99% at day 10 to 14-45% after 24 days, Figure 19). Moreover, after the first 10 days of infection, the greatest dilution of cell-free culture medium still capable of forming syncytia was unusually high, but deteriorated over the course of the study (data not shown). This pattern of gp20 expression was unique to infection of SupT1 cells and was present following infection by all the virus clones. Due to the kinetics observed in Figure 19, we extended the observation period for infection of SupT-1 cells for an additional week in a subsequent experiment to determine whether the levels remained depressed. We observed a rebound from a range of 20-70% of the population infected at day 24, to 81-97% at day 31, for each of the viruses tested (data not shown). This novel pattern of infection and gp20 expression has not been observed before in our infection assays. Interestingly, the level of syncytia forming units released by infected SupT-1 cultures remained relatively high, even with a depressed gp20 expression value (data not shown).

The third pattern of infection was defined by a delayed onset of viral gp20 expression, with retention of *env* expression in the population over time. This pattern of gp20 expression was observed in MT-4 cells infected with the D2/RHE/OR virus if cells were maintained long enough (37% infected after 38 days and 80% after 45 days in culture; data not shown). A similar delay in gp20 expression was also documented in a subsequent experiment in which D2/RHE/OR gp20 expression in MT-4 cells did not reach 95% until day 24, as much as two to three weeks after all other viruses

(D2/RHE/OR/V1, p5'2R3'V1 and p5'V13'2R) had maximally infected this same cell line. As can be seen in Figure 19, Panel B, infection of the Hut78 cell line by D2/RHE/OR was markedly reduced as defined by gp20 expression, infection of Raji cells with cell-free Hut78 culture medium, and PCR analysis of genomic DNA (data not shown). However, in some experiments after an extended period of culture, the molecular clone of D2/RHE/OR exhibited a delayed onset of positive gp20 expression in Hut78 cells (data not shown). Interestingly, each of these five different cell lines was exposed to the same infectious titer of D2/RHE/OR clone virus, yet infections varied from as few as 10 days to as many as 45 days before reaching near maximum levels of gp20 expression within the population.

DISCUSSION

Serogroup 2 SRVs are the most frequent type D virus isolates found in U.S. macaque colonies, regardless of species. Recently, serogroup 2 SRVs have been isolated from *Macaca fascicularis* obtained from wild Indonesian populations (Thouless *et al.* 1988). Studies in India, the source of founder animals for most of the rhesus macaques held in biomedical research colonies, failed to provide serologic evidence of type D SRV infection in wild populations of rhesus and bonnet macaques. Thus, most rhesus macaques appear to harbor SRVs originating from Indonesian populations. Natural reservoirs for serogroups 1, 3, 4 and 5 SRV have not been identified. The potential role of type D retroviral infection in immunodeficiency in humans is controversial (Morozov *et al.* 1991; Kzhyshkowska *et al.* 1996; Krause *et al.* 1989; Heneine *et al.* 1993). However, putative human type D retroviruses have been isolated from an AIDS patient with an atypical variant of a Burkitt's-type B-cell lymphoma (Bohannon *et al.* 1991) and more recently from the salivary glands of patients with Sjogren's Syndrome (Griffiths *et al.* 1997). Interestingly, all SRVs isolated from humans or human cell lines that have been characterized to date are members of the SRV-1 serogroup.

We have successfully isolated molecular clones of proviral genomes of two serogroup 2 SRVs, D2/RHE/OR and D2/RHE/OR/V1. Restriction endonuclease mapping using a limited battery of enzymes has demonstrated that serogroup 2 SRVs contain conserved restriction endonuclease sites in the *pol* gene (Bam HI) and in the *gag/prt* gene (Eco RI); however, a second Eco RI site present in the *pol* gene in most serogroup 2 SRVs is absent from D2/RHE/OR/V1. To further distinguish between the D2/RHE/OR and D2/RHE/OR/V1 viruses, we completed the sequencing of their respective genomes.

We have identified 17 amino acid residue differences between D2/RHE/OR and D2/RHE/OR/V1 (Figure 20). Ten of these differences are found within the *env* gene. Of these ten, three occurring at positions 302, 316, and 415 are unique to D2/RHE/OR/V1, when compared to

D2/RHE/OR and D2/CEL/OR; the amino acids at positions 302 (small, nonpolar Gly to Asp) and 316 (nonpolar Ile to polar Thr) represent nonconservative amino acid substitutions. In addition, six other residue differences occurring at positions 156 (Asn to Thr), 160 (Pro to His), 296 (Phe to Ser), 404 (Ala to Thr), 455 (Leu to Phe), and 519 (Gly to Arg) are nonconservative changes in D2/RHE/OR relative to D2/RHE/OR/V1 or D2/CEL/OR. Potential microconformational changes induced by nonconservative amino acid changes may affect the ability of the envelope glycoprotein to form multimers in the host cell plasma membrane, alter recognition by the host immune response of the external envelope glycoprotein, or possibly affect the availability and function of the fusion peptide domain (Johnston *et al.* 1993; Gardner and Luciw, 1989). The T-cell activation domain, as defined by env amino acid residues 233-249, is 100% conserved among all SRV-2 isolates including D2/MNE/WA (Malley *et al.* 1991). The immunosuppressive peptide residing between env residues 447-472 is identical among all SRV serogroups with the exception of D2/RHE/OR, which predicts a Phe at position 455 (Cianciolo *et al.* 1984). Unlike lentiviral or type C retroviral immunodysfunction, the immunosuppressive peptide may not serve a major role in type D virus immunosuppression (Sonigo *et al.* 1986). The significance of the Phe at position 455 in immunosuppression or host range in D2/RHE/OR infection is unknown. The fusion domain contains a hydrophobic region at the N-terminus of the gp20 (amino acid residues 383-409) followed by a series of heptad-repeats (amino acids 410-463) and is well conserved in all SRV serogroups (Chambers *et al.* 1990). The remainder of the amino acid differences between D2/RHE/OR and D2/RHE/OR/V1 are dispersed among the three other SRV genes, *gag, prt*, and *pol.*

All amino acid differences between D2/RHE/OR and D2/RHE/OR/V1 found within the *gag* gene reside in the phosphoprotein, pp24/pp18. This peptide is the least conserved of those predicted by the sequences of all molecular clones of SRV-2. D2/CEL/OR and D2/MNE/WA have been associated with retroperitoneal fibromatosis in Celebes and pig-tailed macaques, and have a nonpolar Met at position 127 of the phosphoprotein, identical to the corresponding residue found in D2/RHE/OR

but distinct from the polar Thr present in D2/RHE/OR/V1 (Figure 14) (Giddens, Jr. *et al.* 1985; Shiigi *et al.* 1986; Benveniste *et al.* 1985; Stromberg *et al.* 1984; Marx *et al.* 1985; Henderson *et al.* 1985). The potential structural differences of these phosphoprotein gene products may result in cell or tissue specificity in vivo that may ultimately affect the pathogenic outcome of serogroup 2 SRV infection. Recently, a conserved proline-rich motif, PPPY, has been characterized in the C-terminal domain of the phosphoprotein, pp16, of MPMV (Yasuda and Hunter, 1998). Deletion mutagenesis has suggested that the PPPY motif is necessary for late stage virus release from the infected cell. Additionally, the pp24 and pp16 regions outside of the PPPY motif were demonstrated to be necessary for virus particle infectivity, capsid precursor stability and inhibition of premature viral protease activity (Yasuda and Hunter, 1998). The gag p12 protein is 100% conserved between the molecular clones D2/RHE/OR and D2/RHE/OR/V1, but the corresponding protein in D2/CEL/OR contains three nonconservative changes relative to D2/RHE/OR/V1 at positions 272 (Arg to Trp), 279 (Ile to Lys), and 293 (Ser to Pro) which exactly match the residues found in the molecular clones of D1/RHE/CA and MPMV. Additional studies indicate that the gag p12 domain may assist in MPMV capsid assembly and appears to be required for virion infectivity (Sommerfelt *et al.* 1992). Limited N-terminus amino acid and nucleotide sequence analyses have demonstrated that the D2/MNE/WA virus recovered from *Macaca nemestrina* is very similar yet distinct from the D2/CEL/OR, D2/RHE/OR and D2/RHE/OR/V1 molecular clones within the *gag* and *env* genes (Grant *et al.* 1995a; Grant *et al.* 1995b; Henderson *et al.* 1985). Notably, the matrix protein (MA) is completely conserved between all the serogroup 2 SRVs and retains two non-contiguous regions rich in basic amino acid residues which are conserved between all molecularly-cloned SRVs, suggesting a retention of function during viral replication (Conte *et al.* 1997).

The five motifs found in the dUTPase (DU) domain of the *prt* gene are well conserved among the SRV-2 molecular clones (Data not shown) (McGeoch, 1990). Within motif five, an acidic Asp resides at position 153 in D2/CEL/OR, whereas a polar Asn or Gln is found in D2/RHE/OR and

D2/RHE/OR/V1 or D1/RHE/CA and MPMV, respectively. How this nonconserved amino acid change may affect the function of the dUTPase is not clear. dUTPase functions by decreasing the cellular dUTP levels in actively dividing cells, which may otherwise lead to misincorporation into the newly synthesized proviral genome and development of point mutations or strand breakage (Grafstrom *et al.* 1978). Moreover, feline immunodeficiency virus has been observed to incur a 5-fold increase in error rate over the wild-type virus during replication in primary macrophages if the viral dUTPase is not functional (Lerner *et al.* 1995). Archived RF tumor tissues, fresh tissues and lymphocytes from *Macaca nemestrina* infected with D2/MNE/WA, collected over a period of 11 years, were used to analyze genetic changes within the envelope gp70 domain (nucleotides 144-260, which include an overlapping 3'*orf*) (Grant *et al.* 1995a). Using PCR-sequence analysis techniques, Grant *et al* were able to demonstrate a remarkable stability of the genomic sequences analyzed (Grant *et al.* 1995a). These results were consistent with our observations by Southern analysis which repeatedly demonstrate the same restriction endonuclease patterns for each of the virus isolates so far described (unpublished results). Interestingly, unlike the nonprimate lentiviruses and types B and D retroviruses, primate lentiviruses do not encode a dUTPase (Elder *et al.* 1992; Köppe *et al.* 1994; Shao *et al.* 1997; Wagaman *et al.* 1993; Turelli *et al.* 1996). This high degree of genome conservation suggests that dUTPase activity may impart a stability to the SRV genome which may be absent in the primate lentiviruses and undergoes a higher degree of genetic variability.

The D2/RHE/OR virus was initially observed and isolated from group-housed rhesus macaques exhibiting mild symptoms of SAIDS. A restriction endonuclease site variant, D2/RHE/OR/V1, was recovered and identified from rhesus macaques endemically infected with D2/RHE/OR, but exhibiting more severe symptoms and a higher rate of mortality. Whether the presence of D2/RHE/OR/V1 was secondary to an evolutionary process or the result of an independent virus transmission event has not been determined. However, a correlation existed between the appearance of severe immunodeficiency disease and recovery of the variant virus

genotype. Initial in vitro tropism analyses distinguished a difference in the target cells of D2/RHE/OR and D2/RHE/OR/V1 infection as defined by the ability of cell-free inocula to infect Hut78 cells. Unlike D2/RHE/OR, cell-free D2/RHE/OR/V1 readily infected Hut78 cells in vitro. Moreover, infection of Hut78 cells was established by cell-to-cell transmission of Hut78 cells and rhesus peripheral blood leukocytes (PBLs) infected with D2/RHE/OR or D2/RHE/OR/V1 (M. Axthelm, personal communication). Both D2/RHE/OR and D2/RHE/OR/V1 isolates are able to readily infect Raji cells. Differences between in vitro and in vivo cell tropism have been identified within different isolates of the serogroup 1 SRVs (Yetz and Letvin, 1987; van Kuyk *et al.* 1991; Maul *et al.* 1988; Legrand *et al.* 1985) and may correlate with differences observed in pathogenic outcome following infection with D1/RHE/CA or D1/CYC/NE (Power *et al.* 1986; Marx *et al.* 1984; Letvin *et al.* 1983; Letvin *et al.* 1984; Desrosiers *et al.* 1985; Daniel *et al.* 1984). In vitro correlates of pathogenesis have been strongly associated with *env* gene diversity in both the simian and human immunodeficiency viruses (York-Higgins *et al.* 1990; Cheng-Mayer *et al.* 1990; Cann et al. 1992; Toohey *et al.* 1995; Lane *et al.* 1995; Kodama *et al.* 1993; Mori *et al.* 1993; Sharma *et al.* 1992; Rudensey *et al.* 1998). Specifically, virus obtained at different stages of disease progression may be enriched for an isolate displaying a unique cell tropic, replicative or cytopathic phenotypes in vitro (Anderson *et al.* 1993; Schuitemaker *et al.* 1992; Fenyö *et al.* 1988; Tersmette *et al.* 1989; Cheng-Mayer *et al.* 1988; Åsjö *et al.* 1986). In this report, in vitro tropism has been used as a potential marker for pathogenic potential.

Cell-free infection of T- and B-cell lines has demonstrated that viral determinants of SRV host range may be multifactorial and dependent upon genetic domains contained outside of the *env* gene. Interestingly, p5V132R infected CEM-SS and SupT1 cells appeared to release virus particles in a manner equivalent to D2/RHE/OR/V1, suggesting that this function was dictated by determinants contained in the 5' genomic fragment. Moreover, p52R3V1 infected CEM-SS and SupT1 cells released virus particles congruent with the ability of D2/RHE/OR in the same cell lines, further supporting the hypothesis that virus particle release is determined by the 5' domain of the genome.

Interestingly, the appearance of syncytia correlates solely with the envelope gene expressed; p5V132R and D2/RHE/OR infected cultures exhibit very few and very small syncytia. In contrast, D2/RHE/OR/V1 and p52R3V1 infected cultures display numerous large, ballooning syncytial structures. The relative capacity to induce syncytia may correlate with the efficiency of cell to cell transmission of the virus and spread of infection.

Our data suggest a model in which host range may be determined by the ability of the virus to efficiently release and spread throughout a cell culture via fusion from without and fusion from within mechanisms of transmission. D2/RHE/OR/V1 virus is fully competent and can spread infection throughout a culture either by high titer virion release in SupT1 and CEM-SS infections, or by cell-to-cell spread as observed in the Hut78 and MT4 infections. Therefore, in cell lines in which virus release is hindered, spread of infection may rely more heavily upon cell-to-cell mechanisms, or fusion from within. In contrast, virus infection of cell lines that support high levels of virus release may not be as dependent upon cell-associated mechanisms for spread of infection. Productive infection by D2/RHE/OR seems to be limited to Raji, SupT1 and CEM-SS cells, suggesting that host-specific factors may also play a role in determining tropism. The ability of D2/RHE/OR to productively infect Hut78 cells has been stochastic (2 in 12 infection attempts), and has only succeeded after prolonged culture periods sometimes exceeding 60 days. The role of titer as a factor in the ability to infect Hut78 cells cannot be ruled out. Although equal amounts of reverse transcriptase activity was applied to each culture, the syncytia forming capacity of each inocula was not equivalent (data not shown). In vitro propagation and infection analyses of D2/RHE/OR has revealed a low titer of released virions which seems to be influenced by the specific cell infected. Therefore, host range is affected by both *env* and non-*env* viral determinants; additionally, host factors may be necessary for interaction with both the envelope glycoprotein in facilitation of virus-cell and cell-cell membrane fusion, and the gag pp18 in promotion of viral release and infectivity.

What does this imply for pathogenic mechanisms? The in vitro kinetics of D2/RHE/OR infection, as defined by time required to achieve complete or near-complete virus spread, appears to be dependent on the specific T-cell population infected. In vivo, some macaque T-cell subsets may be less readily infected by D2/RHE/OR; these cells may express a low, almost undetectable level of virus that are 1) still functional and 2) escape removal by host-induced mechanisms. This low level infection may be the result of the delicate balance between progression toward virulent infection and maintenance of host immune functions by which the animal can respond to the opportunistic infections that threaten its survival. In contrast, the increased replicative capacity of D2/RHE/OR/V1 may result in greater dysfunction of the immune system and the appearance of more severe immunodeficiency. Animal studies will be necessary to determine the pathogenicity of these SRV-2 molecular clones.

MATERIALS AND METHODS

Cells and virus. All Raji and T-cell lines (CEM-SS, Hut78, MT-4 and SupT-1) were grown in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum and 1% penicillin, neomycin, streptomycin and L-glutamine. Canine D17 cells were grown in MEM supplemented with 10% heat inactivated fetal calf serum and 1% penicillin, neomycin, streptomycin, L-glutamine and non-essential amino acids (NEAA). Suspension cultures were split-passaged twice weekly at a ratio of 1:10 in 10 ml of fresh media. The D17 cells were split at a ratio of 1:10 one time per week and fed with 10 ml of fresh MEM once per week.

Molecular cloning of the full-length D2/RHE/OR/V1 genome. Genomic DNA was prepared from Raji cells which had been cocultivated with peripheral blood lymphocytes from a virus-infected rhesus macaque (ORPRC animal #305-08544). The DNA exhibited a restriction endonuclease pattern representative of D2/RHE/OR/V1 and was used for construction of genomic DNA libraries (unpublished data).

The library was constructed using partial Sau3A-1 digested DNA fragments (enriched for 9-21Kb fragments) ligated to EMBL3 arms (Stratagene), and packaged with Gigapack II Gold (Stratagene). Using methods previously described in the analysis of D2/RHE/OR (Marracci *et al.* 1995), the library was plated, transferred to nitrocellulose membranes, and screened by high-stringency hybridization with ^{32}P-labeled p8A-1 (recombinant plasmid containing the complete D2/CEL/OR genome). Post-hybridization washes were highly stringent and followed wash parameters previously described in the library screening for D2/RHE/OR (Marracci *et al.* 1995). Positive clones were initially examined for the presence of 5' LTR, 3' LTR, *gag*, and *env* gene sequences by polymerase chain reaction (PCR; see below) to identify candidate clones with the highest likelihood of containing full-length SRV genomes. Phage DNA prepared by the plate lysate procedure (Ausubel *et al.* 1987) were digested with restriction endonucleases to identify full-length

clones with the D2/RHE/OR/V1 restriction map pattern. Three clones were identified as containing the full-length D2/RHE/OR/V1 genome; one clone (11B-V1) was demonstrated to be infectious and was selected for further characterization. The construction of the D2/RHE/OR molecular clone has been described (Marracci *et al.* 1995).

Polymerase chain reaction identification of candidate lambda clones containing potentially full-length D2/RHE/OR/V1 genomes. Phage were eluted from positive plaques (in 100 µl volume) and were subsequently filtered and heated to 100°C for 15 minutes to disrupt capsids; DNA extract was then clarified from debris by brief centrifugation. PCR using phage DNA (3 µl) as template was conducted as previously described (Pilcher *et al.* 1994). PCR was conducted using chimeric virus plasmid DNA (1-3 µl) as template in a core buffer consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 µM each dNTP, 100 pmol of each primer, and 2.5 units Taq DNA polymerase. The reaction mixture was subjected to an initial denaturation at 95°C for 1 minute, followed by 30 cycles of 93°C for 40 seconds, 55°C for 1.5 minute, and 73°C for 55 seconds. A final extension at 70°C for 10 minutes was performed followed by a 4°C soak. The forward and reverse primers used to confirm the presence of the full-length viral genomes were 20-mers (FGM1, [nucleotides 4364-4383]: 5'TTTTGGACTTGAACCATCCA3'; RGM1, [nucleotides 4818-4799]: 5'ATTTTGCCGTGTCTGAGATA3'). These primer sets were based on consensus sequences found within D2/RHE/OR/V1, D2/CEL/OR and D2/RHE/OR.

DNA isolation and sequencing of D2/RHE/OR and D2/RHE/OR/V1 molecular clones. The 15 kb Hind III restriction fragment containing the entire D2/RHE/OR/VI genome, including flanking human sequences, was subcloned into pBR322. The resulting recombinant is designated pV1. Standard methods of transformation into DH5α cells, alkaline lysis, and cesium chloride gradient purification of plasmid DNA (Ausubel *et al.* 1987) were used to prepare plasmid DNA for dideoxynucleotide sequence analysis and for transfections. The D2/RHE/OR and D2/RHE/OR/V1

envelope genes and 3' long terminal repeat (LTR) regions were sequenced using the Sequenase version 2.0 kit (USB). The remainder of the D2/RHE/OR and D2/RHE/OR/V1 genomes were sequenced using the Perkin Elmer/Applied Biosystems automated sequencer (373 DNA Sequencer Stretch; Foster City, CA). Serogroup 2 specific oligonucleotides for primer walking were synthesized at the ORPRC Molecular Biology Core facility. Sequence was confirmed in both directions.

Transfection of D2/RHE/OR/V1 molecular clone. Canine D-17 cells were transfected with 4 µg of 11B-V1 (recombinant lambda clone) DNA or 4 µg of pV1 (recombinant plasmid clone) DNA using the calcium phosphate procedure (Ausubel *et al.* 1987). Mock transfections using calcium phosphate precipitate without DNA were also conducted. After 24 hours, Raji cells (10^5) were added to the D-17 transfectant cultures and co-cultivated for an additional four days. The Raji cells were then harvested and cultured independently. In addition, the culture medium was filtered (0.45 µm) and added to uninfected Raji cells to verify the existence and transmissibility of cell-free infectious virus. The cultures were passaged (1:10 split ratio, twice weekly) until they tested positive for virus production.

Immunofluorescence detection of simian retrovirus antigens expressed in infected cells. For transfection analyses, at 2-4 weeks post-transfection, gp20 immunofluorescence assays (IFA) were performed on uninfected Raji cells, control SRV-2 infected Raji cells, and Raji cells subjected to cocultivation with transfected D-17 cells and virus rescue. In addition, other cell lines, either directly infected with cell-free virus preparations or cocultivated with single cell suspensions of tissues obtained from infected macaques, were subjected to IFA. Cells were fixed in 50% ethanol (190 proof) in Dulbecco's phosphate buffered saline (D-PBS; no Ca++ or Mg++). A mouse anti-gp20 monoclonal antibody isolated from ascites fluid (obtained from Dr. Niels Pederson, University of California at Davis) and goat $F(ab')_2$ anti-mouse IgG (H&L chain) FITC (Tago) were used to detect SRV-infected cells. In addition, serogroup-specificity of the transmitted SRV was determined by

membrane IFA on unfixed cells using negative control monkey serum or monkey antiserum specific for SRV-1, SRV-2, or SRV-5, with subsequent labeling with goat anti-monkey IgG-FITC. The monkey anti-SRV serum recognizes predominantly env- and gag-related antigens (Shiigi *et al.* 1989); thus, in the case of unfixed SRV-infected cells, the major target would be the env-encoded antigen gp70. For infection analyses, gp20 IFAs were performed on uninfected Raji, Hut78, CEM-SS, MT-4, and SupT-1 cells, and on cells infected with molecularly-cloned D2/RHE/OR and D2/RHE/OR/V1 virus, and corresponding chimeric viruses. In all cases, stained cells were then analyzed on an EPICS C flow cytometer (Coulter Electronics).

Restriction Digest and Southern Analysis of Genomic DNA from Infected Raji cells. Genomic DNA was isolated and digested with restriction enzymes (Sph I, Bam HI, Eco RI, and Hind III) to verify the identity of the integrating virus. DNA digestion, transfer of nucleic acids to nylon membranes, prehybridization, hybridization, and post-hybridization washes were conducted using previously described methods (Marracci *et al.* 1995). Hybridizations (>10^6 cpm/ml) were conducted with the full-length SRV-2 (p8A-1; D2/CEL/OR) probe overnight at 37°C. Post-hybridization washes were conducted with 2X SSC/1.0% SDS for 15 minutes at room temperature (2-3 times); 2X SSC/1.0% SDS for 15 minutes at 65°C (once); and 0.2X SSC/1.0% SDS for 15 minutes at 65°C (1-3 times). Autoradiography was conducted overnight with an intensifying screen at -86°C.

Construction of the p5'2R3'V1 Chimera. A serogroup 2 SRV chimera was constructed by utilizing a conserved Bam HI restriction endonuclease site found in the *pol* gene. The chimera contains the 5' half of D2/RHE/OR and 3' half of D2/RHE/OR/V1, relative to the Bam HI site. The pV1 plasmid, containing the full-length D2/RHE/OR/V1 genome, was digested with Bam HI; a 10 Kb fragment containing the *env* gene and all downstream sequence including vector was religated to construct the p3'V1 subgenomic molecular clone. After digestion of p3'V1 and a second recombinant J450 (containing D2/RHE/OR sequence upstream of the unique Bam HI site) with Bam HI and Sal

I, an ~ 10 Kb *env*-containing fragment from p3'V1 was ligated with the 6.6 Kb J450 upstream fragment. Initial screening for full-length clones was conducted by polymerase chain reaction. Additional verification of the chimeric virus clones was achieved by restriction endonuclease digestion and sequence analyses. The infectivity of the verified chimeric virus was then confirmed by transfection onto D17 cells, followed by co-cultivation with permissive Raji cells at 24 hours post-transfection. After 4 days of additional culture, the Raji cells were removed and cultured independently. Concurrently, the culture medium was filtered (.45 µm) and applied to fresh Raji cells, in order to determine the infectious nature of cell-free virions.

Construction of the p5'V13'2R Chimera. The construction of the reciprocal SRV chimera was also completed by ligating the 5' half of D2/RHE/OR/V1 and 3' half of D2/RHE/OR at the unique Bam HI restriction site found in the *pol* gene. In order to utilize this novel Bam HI endonuclease site, the plasmid pBR322 was modified to contain a Bgl II restriction endonuclease site. pBR322 was digested with Sal I and subsequently treated with T4 DNA polymerase to fill-in the ends. After ligation with Bgl II linkers, the concatamerized linkers were removed by digestion with Bgl II; the linearized DNA was subsequently purified and religated. Following transformation in DH5α bacterial cells, a plasmid clone was isolated which contained the Bgl II restriction site adjacent to the reconstructed Sal I restriction site in pBR322, and was designated pBRSaBg. pBRSaBg was digested completely with Bam HI and Bgl II and the resulting ends were dephosphorylated by treatment with calf intestinal phosphatase to prevent recircularization of vector DNA. The pV1 molecular clone, containing the complete D2/RHE/OR/V1 viral genome, was concurrently digested with Bam HI; the resulting 5.5 Kb fragment which contains the complete 5' portion of the genome upstream of the unique Bam HI site was gel-purified and ligated to the prepared plasmid vector. DH5α transformants were screened to isolate a clone(s) with the correct insert orientation. Several clones were isolated; one clone designated pBRSaBg5V1 was selected for further analysis. pBRSaBg5V1 was then digested with Bam HI and ligated with a second Bam HI fragment which contained the 3' portion of the D2/RHE/OR

genome. The verification of this chimera was conducted by restriction endonuclease digestion with Eco RI and Sph I, PCR amplification using primers flanking the Bam HI site, and sequence analysis. Plasmid DNA was prepared and the infectivity of the p5V132R chimera was confirmed by transfection of D17 cells as described above.

Reverse Transcriptase (RT) Assay. D2/RHE/OR, D2/RHE/OR/V1 and chimeric virus Infected Raji cells were cocultivated with uninfected Raji cells daily for up to seven days; culture fluids were collected daily from days 4-7 and an RT assay performed to determine the day of peak RT activity. Supernatants from infected cultures were filtered (.45 µm); 1 ml was used for the RT assay and the remainder was stored at 4°C until use at the completion of the assay. The culture medium containing the peak value of RT activity was used as the inoculum in the infection assay. The virus was pelleted by centrifugation and the supernatant was discarded (TLA-45 rotor in a Beckman Optima™ TL Ultracentrifuge, 4°C, 45,000 RPM, 10 min). Virus pellets were stored at 4°C until all samples could be processed simultaneously. The virus was resuspended in 25 µl of lysis buffer (1% NP40 in TE) and allowed to lyse for 30 min on ice. The reaction mixture was added to the lysed pellets and incubation at 37°C continued for one hour in a shaking water bath [25 µl 4X reaction buffer: (20 mM $MgCl_2$, 200 mM KCl, 24 mM DTT, 300 mM Tris pH 8.0, 0.4 mg/ml BSA; 40 µl prepared RNA template: heated to 42°C for 5 min and cooled slowly to room temperature prior to use (Pharmacia, 1 Unit/ml); ^{3}H-Thymidine 5'Triphosphate (5000 µCi/5000 µl, 2.5 µl/sample dried and resuspended in 10 µl water per sample]. The samples were then precipitated in 2 ml of ice cold 10% TCA (with 0.02 M sodium pyrophosphate) and 10 µl yeast t-RNA/tube (10 mg/ml) for 20 min on ice. The incorporated counts were collected on glass filters and counted in 5 ml scintillation fluid for a duration of 1 minute per sample.

Infection of Raji and T-cell cultures with equivalent multiplicities of infection as determined by RT assay. Filtered viral collections were applied to uninfected Raji or T-cell lines at

equivalent input virus amounts (RT cpm/cell). The final volume of virus inoculum was adjusted by addition of fresh RPMI medium with a final cell number of $1X10^5$. Cultures were analyzed weekly beginning at 10 days post infection; culture fluids were removed, filtered (.45 µm) and stored at -86°C. Cells were retained for an IFA analysis, using anti-gp20 monoclonal antibody to determine the infection status of the cultures. The cultures were then allowed to propagate until all cultures were positive by anti-gp20 IFA analysis or until 60 days had passed.

Nucleotide Sequence Accession Numbers. The D2/RHE/OR envelope and 3' LTR sequences have been previously published by our laboratory (27) and are available at GenBank accession number L38695. The remainder of the D2/RHE/OR genomic sequence, which has not been directly displayed in this manuscript, and the complete D2/RHE/OR/V1 genomic sequence will be deposited in GenBank.

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Figure 9. Detection of env gp20 in D2/RHE/OR/V1 infected Raji cells. FACS analyses, using anti-SRV env gp20 monoclonal antibodies, were conducted on several experimental and control Raji cell lines. The tested cell lines include 1) uninfected Raji cells, 2) positive-control SRV 2-infected Raji cells, Raji cells cocultivated with either 3) mock transfected D-17 cells or 4) transfected with the D2/RHE/OR/V1 lambda clone, and Raji cells inoculated with either 5) filtered culture medium obtained from either the mock-transfected co-cultures or 6) D2/RHE/OR/V1 lambda clone transfected co-cultures. The percentage of positively-stained cells, recognized above threshold values, are shown in the upper right-hand corner of each histogram. FACS analyses conducted with these six cell lines, using negative control IgG2a monoclonal antibodies, were below background (unpublished observations).

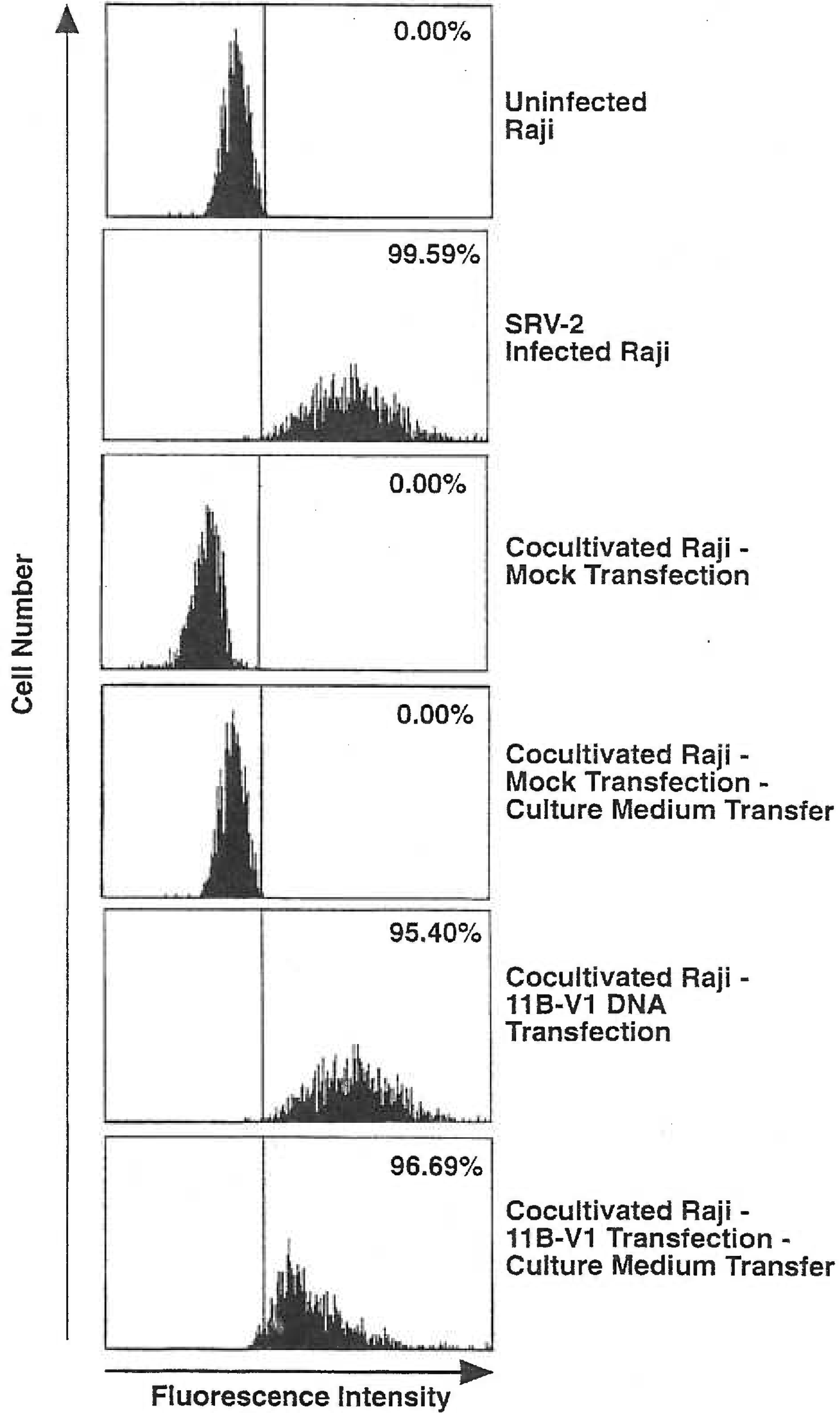
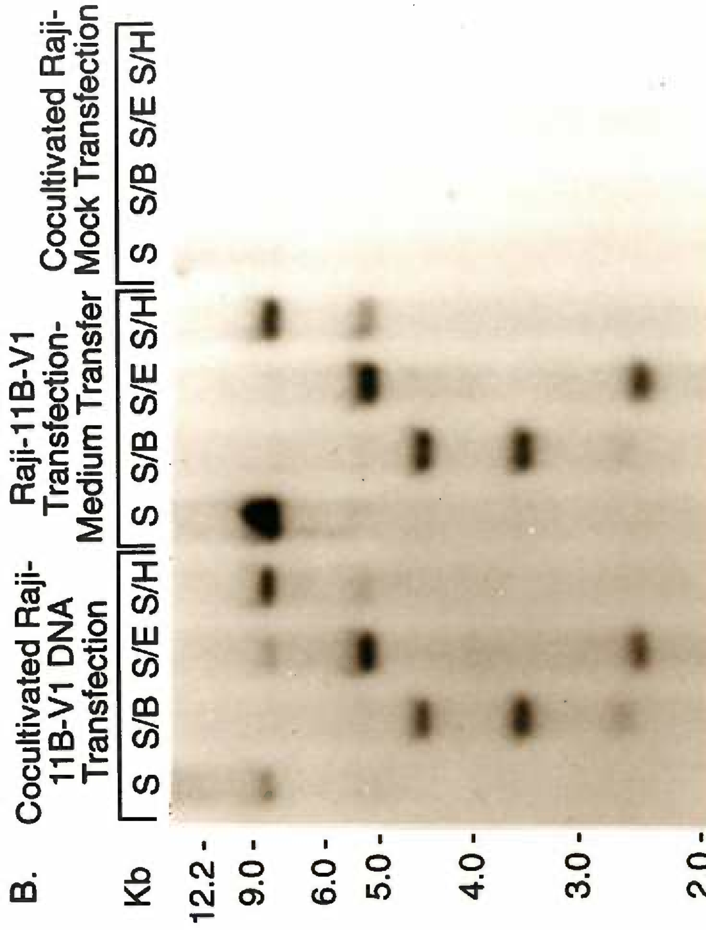

Monoclonal Antibody
Anti-gp20
0.00%
Uninfected Raji
99.59%
SRV-2 Infected Raji
0.00%
Cocultivated Raji - Mock Transfection
0.00%
Cocultivated Raji - Mock Transfection - Culture Medium Transfer
95.40%
Cocultivated Raji - 11B-V1 DNA Transfection
96.69%
Cocultivated Raji - 11B-V1 Transfection - Culture Medium Transfer
Cell Number
Fluorescence Intensity

Figure 10. Southern analyses of genomic DNA from D2/RHE/OR/V1 infected cells. Genomic DNA obtained from different experimental and control Raji cell lines, including positive control D2/RHE/OR/V1-infected Raji cells, Raji cells cocultivated with D-17 cells transfected with the D2/RHE/OR/V1 lambda clone, and Raji cells inoculated with medium obtained from the experimental co-culture. Twenty micrograms of genomic DNA were digested with Sph I (S), or with Sph I in combination with Bam HI (B), Eco RI (E), or Hind III (H) and subjected to agarose gel electrophoresis and transfer to nylon membranes. Blots were hybridized with the ^{32}P-labeled full-length SRV-2 (D2/CEL/OR) probe, washed at high stringency, and developed for autoradiography. The Sph I restriction endonuclease recognizes sites in both the 5' and 3' LTRs. The small Sph I - Bam HI (or Sph I - Sph I) restriction fragment, that is weakly recognized by the SRV-2 probe, may contain D2/RHE/OR/V1 LTR sequence that extends outward from the Sph I restriction site into the flanking genomic region. Molecular weight markers are a 1-Kb DNA ladder (Bethesda Research Laboratories).


B.
Cocultivated Raji-11B-V1 DNA Transfection
Raji-11B-V1 Transfection-Medium Transfer
Cocultivated Raji-Mock Transfection
S S/B S/E S/H
S S/B S/E S/H
S S/B S/E S/H
Kb
12.2
9.0
6.0
5.0
4.0
3.0
2.0

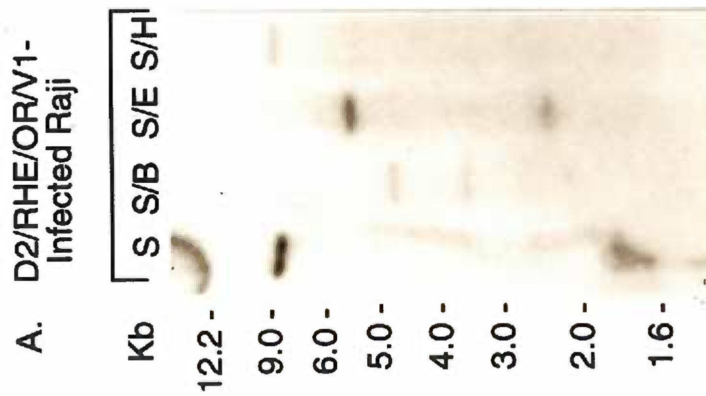

A.
D2/RHE/OR/V1-Infected Raji
S S/B S/E S/H
Kb
12.2
9.0
6.0
5.0
4.0
3.0
2.0
1.6

Figure 11. Nucleotide sequence of the D2/RHE/OR/V1 genome. The predicted amino acid sequences of *gag*, *prt*, *pol*, and *env* are presented. Numbering of nucleotides begins at the 5' boundary of the leftward LTR. The U3 and U5 boundaries, putative TATA box, and the polypurine tract (for initiation of plus-strand DNA synthesis) are all underlined. The sequence complementary to $tRNA^{lys1,2}$ (primer for minus-strand viral DNA synthesis) is over lined. The N-termini of processed *gag* and *env* gene products are also depicted.

```
     U3
1    TGTCCGGAGCCGTGCAGCCCGGACGACGTTCTGGCTTCTGGCGATTGCACTCCATAGTAGTGGATGCAAGATGGCGCACTTCCTGGTTCTCTTTCACCCTGTTTTCCCGCCGGCGCCAAT      L
                                                                                                                                        T
121  ATTTCCCGCCTTAGACTACGTGGCATTTCCTGACTTAGCTACTGAGCATGCGCTCAGTGACGTTGTTCTCCACCCCCGCCGCCTGTCTATATATTGCCTCGCATCACCGCCATTAAACGA      R
                                                                                                       U5
241  GACTTGATCAGAGCCCTGTCTTGTCTCCATTTCTTGTGTCTCTTGTCTCCCCCAATTCCCACCCCCTCATCCAGGTTCTACGTTGCTGATCCCGCGGGTCGGGACACGTGGCGCCCAACG

361  TGGGGCTGGATACGAGGGAATCCTGTGAGGAAGAGAGCGCGTAACACGGCCGGCAGTCAAAGTGAAAGAAAAACCTTCCAGCTGCCGCGGGAACCTGCCGCGTCGGCAATAAAGGTAAGC
                   p10
481  GTTGCGTTCGAATATGGGACAAGAATTAAGCCAACATGAACTTTATGTAGAACAGTTAAAAAAGGCTCTAAAGACACGGGGAGTAAAGGTTAAGGGTAATGATTTACTCAAATTCTTTGA      g
                   MetGlyGlnGluLeuSerGlnHisGluLeuTyrValGluGlnLeuLysLysAlaLeuLysThrArgGlyValLysValLysGlyAsnAspLeuLeuLysPhePheAsp    36      a
                                                                                                                                        g
601  TTTTGTAAAGGATACCTGCCCTTGGTTCCCACAAGAGGGAACAATAGACATAAAAAGATGGCGCAGGGTAGGAGATTGTTTTCAGGATTATTATAACACTTTTGGCCCGGAAAAGATACC
      PheValLysAspThrCysProTrpPheProGlnGluGlyThrIleAspIleLysArgTrpArgArgValGlyAspCysPheGlnAspTyrTyrAsnThrPheGlyProGluLysIlePro    76
                                                                                  pp24
721  AGTAACTGCCTTCTCCTACTGGAATCTTATCAAAGATTTAATAGATAAAAAGGAGGCTGACCCACAAGTAATGGCTGCGGTCACCCAGACAGAAAAAATATTAAAAGTTAGTTCACAAAC
      ValThrAlaPheSerTyrTrpAsnLeuIleLysAspLeuIleAspLysLysGluAlaAspProGlnValMetAlaAlaValThrGlnThrGluLysIleLeuLysValSerSerGlnThr   116

841  AGATCTCAGGGACAAATCACAAAATAAGGAAACGGATCTCATCTCTCTTGAAAGTGATGATGAGGAAGCCAAGGCCCCCTCAGAGAAAATATCAATGCCCAATAAAAGTCCTAAAAAATA
      AspLeuArgAspLysSerGlnAsnLysGluThrAspLeuIleSerLeuGluSerAspAspGluGluAlaLysAlaProSerGluLysIleSerMetProAsnLysSerProLysLysTyr   156
                          pp18
961  TCCAGCTATGTTAACATCACAAAATAATAATATGGACAAAGACCCTGATTCGTCAGAGGTGGATTGGGATGGCTTAGAGGACGAGGCAGCAAGGTATCATAACCCTGATTGGCCTCCTTT
      ProAlaMetLeuThrSerGlnAsnAsnAsnMetAspLysAspProAspSerSerGluValAspTrpAspGlyLeuGluAspGluAlaAlaArgTyrHisAsnProAspTrpProProPhe   196
                                                              p12
1081 CCTCTCTCGTCCACCCCCCTATAATAGGACAACTGCTACAGCCCCTGCTGTGATGGCGGTAGTCAATCCTAAAGAGGAACTTAAAGAAAAAATTTCTCAATTAGAGGAGCAGATAAAATT
      LeuSerArgProProProTyrAsnArgThrThrAlaThrAlaProAlaValMetAlaValValAsnProLysGluGluLeuLysGluLysIleSerGlnLeuGluGluGlnIleLysLeu   236

1201 AGAGGAGTTACACCAGTCCCTAATTATTCGGTTACAAAAACTAAAAACAGGAAGTGAAAGGGTAACTAGTTCAGGCAATATGGAGAGTCACTCTCGTACGCCCAAACGGCCTGGACAGCA
      GluGluLeuHisGlnSerLeuIleIleArgLeuGlnLysLeuLysThrGlySerGluArgValThrSerSerGlyAsnMetGluSerHisSerArgThrProLysArgProGlyGlnHis   276
                                                                          p27
1321 CCTTCCAATAGGAAAATACCTCATTAACAAAAATACAGAAGAATATCCTTCCAAGGATATTTTCCCAGTAACGGAGACTATGGATGGACAAGGTCAGGCCTGGAGACATCATAATGGGTT
      LeuProIleGlyLysTyrLeuIleAsnLysAsnThrGluGluTyrProSerLysAspIlePheProValThrGluThrMetAspGlyGlnGlyGlnAlaTrpArgHisHisAsnGlyPhe   316

1441 TGACTTTACTGTTATAAAAGAACTAAAAACTGCTGTTTCCCAATACGGAGCTACTGCTCCATATACTTTGGCCATAGTAGAATCCATTGCTGATAATTGGCTTACTCCAGCTGACTGGAA
      AspPheThrValIleLysGluLeuLysThrAlaValSerGlnTyrGlyAlaThrAlaProTyrThrLeuAlaIleValGluSerIleAlaAspAsnTrpLeuThrProAlaAspTrpAsn   356

1561 TACGCTCGTTAGAGCAGTCCTCTCAGGGGGAGACCACTTAATTTGGAAGTCTGAATTTTTTGAAAATTGTAGGGACACAGCAAAAAGAAATCAACAGGCAGGAAATGGCTGGGACTTTGA
      ThrLeuValArgAlaValLeuSerGlyGlyAspHisLeuIleTrpLysSerGluPhePheGluAsnCysArgAspThrAlaLysArgAsnGlnGlnAlaGlyAsnGlyTrpAspPheAsp   396

1681 CATGTTAACAGGCTCTGGTAACTATGCCAATACTGATGCACAAATGCAGTATGATCCAGGACTGTTTGCTCAAATTCAGGCAGCCGCCACAAAAGCCTGGAGAAAACTCCCTGTTAAAGG
      MetLeuThrGlySerGlyAsnTyrAlaAsnThrAspAlaGlnMetGlnTyrAspProGlyLeuPheAlaGlnIleGlnAlaAlaAlaThrLysAlaTrpArgLysLeuProValLysGly   436

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1921 TTATGTTAAACAACTAGCCTATGAAAATGCTAACCCAGCCTGTCAAGCAGCAATTCGTCCTTACCGAAAGAAAACAGATCTAACAGGATATATTCGCCTTTGTTCAGATATTGGGCCCTC
      TyrValLysGlnLeuAlaTyrGluAsnAlaAsnProAlaCysGlnAlaAlaIleArgProTyrArgLysLysThrAspLeuThrGlyTyrIleArgLeuCysSerAspIleGlyProSer   516
                          p14
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      TyrGlnGlnGlyLeuAlaMetAlaAlaAlaPheSerGlyGlnThrValLysAspPheLeuAsnAsnLysAsnLysAspArgGlyGlyCysPheLysCysGlyLysArgGlyHisPheAla   556

2161 AAAAGATTGCCGTGATCATTCAAACAAAAATCCAGAATTGAAAGTGCCAGGCTTGTGCCCCAGATGTAAAAGAGGAAAACACTGGGCTAATGAATGCAAATCCAAGACTGATAGTCAAGG
      LysAspCysArgAspHisSerAsnLysAsnProGluLeuLysValProGlyLeuCysProArgCysLysArgGlyLysHisTrpAlaAsnGluCysLysSerLysThrAspSerGlnGly   596
                                                                                                                    SerArg      2      p
                                                  p4                                                                                   r
2281 AAATCCCTTACCACCCCATCAGGGAAACGGGATGAGGGGCCAGCCCCAGGCCCCGAAGCAAGCCTATGGGGCAGTCAGCTTTGTTCCAGCCAGCAGCAACAATCCATTTCAAAACTTAAT      t
      AsnProLeuProProHisGlnGlyAsnGlyMetArgGlyGlnProGlnAlaProLysGlnAlaTyrGlyAlaValSerPheValProAlaSerSerAsnAsnProPheGlnAsnLeuIle   636
     LysSerLeuThrThrProSerGlyLysArgAspGluGlyProAlaProGlyProGluAlaSerLeuTrpGlySerGlnLeuCysSerSerGlnGlnGlnSerIleSerLysLeuAsn      42

2401 AGAGCCTCCCCAGGAAGTGCAGGACTGGACCTCTGTTCCACCACCCACACAGTATTAACCCCTGAAATGGGGCCCCAAACTTTAGCTACTGGAGTATATGGACCTTTGCCCCCTAATACG
      GluProProGlnGluValGlnAspTrpThrSerValProProProThrGlnTyr                                                                    654
     ArgAlaSerProGlySerAlaGlyLeuAspLeuCysSerThrThrHisThrValLeuThrProGluMetGlyProGlnThrLeuAlaThrGlyValTyrGlyProLeuProProAsnThr    82

2521 TTTGGGTTAATACTAGGCAGAGGCAGTACCACAGTAAAAGGTTTACAAATTTACCCAGGAGTCATTGATAATGATTATACAGGGGAATTCAAAATAATGGCCAAGGCTATTTCTAGTATC
     PheGlyLeuIleLeuGlyArgGlySerThrThrValLysGlyLeuGlnIleTyrProGlyValIleAspAsnAspTyrThrGlyGluPheLysIleMetAlaLysAlaIleSerSerIle    122

2641 ATCACTATTCCTCAAGGAGAACGGATTGCACAACTAATCTTGTTACCCTTACTCCGAACAGCTCATAAAATACAACACCCTTATAGAGGAAACAAAAATTTCGGGTCCTCAGATATTTTT
     IleThrIleProGlnGlyGluArgIleAlaGlnLeuIleLeuLeuProLeuLeuArgThrAlaHisLysIleGlnHisProTyrArgGlyAsnLysAsnPheGlySerSerAspIlePhe    162

2761 TGGGTACAGCCAATAACTAATCAAAAACCCTCCTTAGTACTGTGGCTAGACGGTAAAGCATTTACAGGGTTAATAGATACTGGAGCGGATGTCACTATTATCAAACAAGAAGATTGGCCA
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2881 TCCCATTGGCCTACCACAGAAACCTTGACTAATCTGAGAGGAATAGGACAAAGTAATAACCCCAGGCAAAGTTCAAAATACCTTACTTGGAAAGACAAAGAAAATAACTCAGGCCTTATC
     SerHisTrpProThrThrGluThrLeuThrAsnLeuArgGlyIleGlyGlnSerAsnAsnProArgGlnSerSerLysTyrLeuThrTrpLysAspLysGluAsnAsnSerGlyLeuIle    242

3001 AAGCCATTTGTCATCCCTAACTTACCGGTTAATCTTTGGGGCCGAGATTTGCTTTCCCAAATGAAAATTATGATGTGCAGTCCTAATGATATTGTCACTGCTCAAATGTTAGCTCAAGGA
     LysProPheValIleProAsnLeuProValAsnLeuTrpGlyArgAspLeuLeuSerGlnMetLysIleMetMetCysSerProAsnAspIleValThrAlaGlnMetLeuAlaGlnGly    282

3121 TACAGCCCAGGAAAAGGACTAGGAAAAAGAGAAGATGGAATCTTACAACCCATCCCAAATTCAGGACAACTTAACAGAAAAGGATTTGGAAATTTTTAGCTGCGGCCGTTGACATACTTG
     TyrSerProGlyLysGlyLeuGlyLysArgGluAspGlyIleLeuGlnProIleProAsnSerGlyGlnLeuAsnArgLysGlyPheGlyAsnPhe                          314
                                                                           GlnLysArgIleTrpLysPheLeuAlaAlaAlaValAspIleLeu     15      p
                                                                                                                                        o
3241 CACCCCAGAGGTATGCTGACCCCATTACATGGAAGTCAGATGAGCCTGTTTGGGTTGATCAATGGCCCCTAACTCAAGAAAAACTTGCTGCTGCCCAACAGTTAGTGCAAGAACAATTAC      l
    AlaProGlnArgTyrAlaAspProIleThrTrpLysSerAspGluProValTrpValAspGlnTrpProLeuThrGlnGluLysLeuAlaAlaAlaGlnGlnLeuValGlnGluGlnLeu      55

3361 AGGCAGGGCATATTATAGAAAGTAATTCTCCCTGGAATACACCTATATTTGTCATAAAAAAGAAGTCTGGTAAATGGAGGCTTTTGCAAGATTTAAGGGCGGTAAATGCCACCATGGTAT
    GlnAlaGlyHisIleIleGluSerAsnSerProTrpAsnThrProIlePheValIleLysLysLysSerGlyLysTrpArgLeuLeuGlnAspLeuArgAlaValAsnAlaThrMetVal     95

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    LeuMetGlyAlaLeuGlnProGlyLeuProSerProValAlaIleLeuGlnGlyTyrPheLysIleValIleAspLeuLysAspCysPhePheThrIleProLeuGlnProValAspGln     135

3601 AGCGATTTGCTTTTAGTCTCCCGTCTACCAACTTTAAACAACCAATGAAACGTTATCAATGGAAAGTGTTGCCTCAAGGCATGGCCAATAGTCCTACCTTGTGTCAAAAATATGTAGCTG
    LysArgPheAlaPheSerLeuProSerThrAsnPheLysGlnProMetLysArgTyrGlnTrpLysValLeuProGlnGlyMetAlaAsnSerProThrLeuCysGlnLysTyrValAla     175

3721 CTGCTATAGAGCCAATCAGAAAATCTTGGGCACAAATGTACATTATACACTATATGGATGACATTCTAATAGCAGGAAAGATTGGCGAACAAGTTTTGCAGTGTTTTGCTCAACTTAAAC
    AlaAlaIleGluProIleArgLysSerTrpAlaGlnMetTyrIleIleHisTyrMetAspAspIleLeuIleAlaGlyLysIleGlyGluGlnValLeuGlnCysPheAlaGlnLeuLys     215

3841 AAGCATTGACAACTACTGGGTTACAAATAGCTCCAGAAAAGGTACAGCTACAAGATCCATATACCTACCTTGGGTTTCAACTTAATGGTCCCAAAATTACCAATCATAAGGCAGTTATTC
    GlnAlaLeuThrThrThrGlyLeuGlnIleAlaProGluLysValGlnLeuGlnAspProTyrThrTyrLeuGlyPheGlnLeuAsnGlyProLysIleThrAsnHisLysAlaValIle     255

3961 GTAGAGATAAGTTACAAACCCTTAATGATTTTCAAAAACTCTTGGGAGATATCAATTGGCTTAGACCCTATTTACACCTCACTACAGGAGATCTAAAACCTCTTTTTGATATCCTAAAAG
    ArgArgAspLysLeuGlnThrLeuAsnAspPheGlnLysLeuLeuGlyAspIleAsnTrpLeuArgProTyrLeuHisLeuThrThrGlyAspLeuLysProLeuPheAspIleLeuLys     295

4081 GGGATTCTAATCCTAACTCACCCAGATTTTTATCTGAAGCAGCCCTTACGTCTCTTAAAAAGGTAGAAACAGCTATTGCTGAACAATTTGTTACACAAATAGATTATACACAGCCATTGA
    GlyAspSerAsnProAsnSerProArgPheLeuSerGluAlaAlaLeuThrSerLeuLysLysValGluThrAlaIleAlaGluGlnPheValThrGlnIleAspTyrThrGlnProLeu     335

4201 CCCTTTTAATTTTTAATACTACACTGACACCTACTGGCCTCTTCTGGCAAAATAATCCTGTCATGTGGATTCACTTGCCTGCATCGCCAAAAAAGGTATTGTTCCCCTATTATGATGCTA
    ThrLeuLeuIlePheAsnThrThrLeuThrProThrGlyLeuPheTrpGlnAsnAsnProValMetTrpIleHisLeuProAlaSerProLysLysValLeuPheProTyrTyrAspAla     375

4321 TAGCAGATCTTATTATCCTTGGAAGGGACAACAGTAAAAAATATTTTGGACTTGAACCATCTACTATTATACAACCCTATTCTAAATCTCAAATCCATTGGTTAATGCAAAACACAGAAA
    IleAlaAspLeuIleIleLeuGlyArgAspAsnSerLysLysTyrPheGlyLeuGluProSerThrIleIleGlnProTyrSerLysSerGlnIleHisTrpLeuMetGlnAsnThrGlu     415

4441 CATGGCCAATTGCCTGCGCTTCCTATGCAGGCAACATTGATAACCATTATCCACCCAATAAACTTATTCAATTTTGCAAACTCCATGCAGTTGTTTTTCCCCGAATCATTAGTAAAACTC
    ThrTrpProIleAlaCysAlaSerTyrAlaGlyAsnIleAspAsnHisTyrProProAsnLysLeuIleGlnPheCysLysLeuHisAlaValValPheProArgIleIleSerLysThr     455

4561 CTCTAGACAATGCCTTACTGGTATTCACTGATGGATCCTCTACCGGCATAGCCGCATACACTTTTGAAAAGACCACTGTCAAGTTTAAAACCTCCCATACGTCAGCCCAATTAGTAGAAT
    ProLeuAspAsnAlaLeuLeuValPheThrAspGlySerSerThrGlyIleAlaAlaTyrThrPheGluLysThrThrValLysPheLysThrSerHisThrSerAlaGlnLeuValGlu     495
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4681  TACAAGCCCTAATTGCAGTGTTATCGGCCTTTCCTCAACGGGCCCTCAATATTTATACAGATAGTGCCTACTTAGCTCATTCTATACCTCTACTTGAGACAGTATCGCAAATCAAACATA
      LeuGlnAlaLeuIleAlaValLeuSerAlaPheProGlnArgAlaLeuAsnIleTyrThrAspSerAlaTyrLeuAlaHisSerIleProLeuLeuGluThrValSerGlnIleLysHis        535

4801  TCTCAGACACAGCAAAATTATTTTTACAGTGCCAACAATTAATATACAACAGGTCTATACCTTTTTATTTAGGACATATCAGGGCCCATTCAGGATTACCAGGACCTTTATCTCAAGGTA
      IleSerAspThrAlaLysLeuPheLeuGlnCysGlnGlnLeuIleTyrAsnArgSerIleProPheTyrLeuGlyHisIleArgAlaHisSerGlyLeuProGlyProLeuSerGlnGly        575

4921  ATCACATAACTGATTTGGCAACCAAAGTTGTCGCCACCACTCTTACCACAAATTTAACAGAGGCACAAGCTGCTCATGCCTTACATCACTTAAACGCTCAGTCCCTAAGGTTAATGTTTA
      AsnHisIleThrAspLeuAlaThrLysValValAlaThrThrLeuThrThrAsnLeuThrGluAlaGlnAlaAlaHisAlaLeuHisHisLeuAsnAlaGlnSerLeuArgLeuMetPhe        615

5041  AAATTACTAGAGAACAGGCTAGACAAATTGTTAAACAGTGTCCCACATGTGTAACATACTTGCCAATTCCTCATTTTGGAGTTAATCCAAAAGGATTGATACCCAATATGCTATGGCAAA
      LysIleThrArgGluGlnAlaArgGlnIleValLysGlnCysProThrCysValThrTyrLeuProIleProHisPheGlyValAsnProLysGlyLeuIleProAsnMetLeuTrpGln        655

5161  TGGATGTCACTCATTATTCTGAATTTGGTAAATTAAAATATGTTCATGTGTCCATAGATACATTTAGTGGATTTCTTGTAGCCACCTTACAAACAGGAGAAGCTACCAAACATGTTATTG
      MetAspValThrHisTyrSerGluPheGlyLysLeuLysTyrValHisValSerIleAspThrPheSerGlyPheLeuValAlaThrLeuGlnThrGlyGluAlaThrLysHisValIle        695

5281  CCCATCTACTACATTGCTTCTCTATTATTGGTCAACCTATTCACATTAAAACAGACAATGGCCCTGGATACACCTCATCTAGCTTTCGTGCCTTCTGTTCCAAATTACACATCAAACACA
      AlaHisLeuLeuHisCysPheSerIleIleGlyGlnProIleHisIleLysThrAspAsnGlyProGlyTyrThrSerSerSerPheArgAlaPheCysSerLysLeuHisIleLysHis        735

5401  CCTTTGGTATTCCTTACAATCCTCAGGGTCAAGGTATTGTCGAAAGAGCACATCTCTCCTTAAAAAATACTCTTGAAAAAATAAAAAAGGGGGAATGGTACCCCACACAGGGGTCTCCCA
      ThrPheGlyIleProTyrAsnProGlnGlyGlnGlyIleValGluArgAlaHisLeuSerLeuLysAsnThrLeuGluLysIleLysLysGlyGluTrpTyrProThrGlnGlySerPro        775

5521  GGAATATTCTCAATCATGCACTCTTTATTTTAAATTTTTTAAACCTTGATGCTCAAAATAAATCAGCCGCTGACCGATTTTGGCATACTAACTCTAAAAGAGAATATGCCATGGTCAAGT
      ArgAsnIleLeuAsnHisAlaLeuPheIleLeuAsnPheLeuAsnLeuAspAlaGlnAsnLysSerAlaAlaAspArgPheTrpHisThrAsnSerLysArgGluTyrAlaMetValLys        815

5641  GGAAAGACCCATTAGACAATTCGTGGCATGGGCCTGATCCCGTATTAATCTGGGGCAGAGGTTCAGTCTGCGTATACTCTCAAACTCATGATGCAGCCAGATGGCTACCAGAACGGTTGG
      TrpLysAspProLeuAspAsnSerTrpHisGlyProAspProValLeuIleTrpGlyArgGlySerValCysValTyrSerGlnThrHisAspAlaAlaArgTrpLeuProGluArgLeu        855

5761  TAAGGCAAGTGTCTAATGTTACTCAATCCAGGGAGTGATTGTCTCCCTGAGATCACTCCTTTTCTTTGCTCATAGAGATGACTCTCAAGGACATCCCGTTTTGGAGAGTACTGCTGATTT
      ValArgGlnValSerAsnValThrGlnSerArgGlu                                              gp70
                                                    PheSerLeuLeuIleGluMetThrLeuLysAspIleProPheTrpArgValLeuLeuIle         20   e
                                                                                                                                        n
5881  TCCAAACTGCTCGAGTTTATGCCGGTTTCGGTGACCCGCGCGAGGCTATTACTATAATACATCAACAACACGGTAAGCCTTGTGATTGTGCGGGAGGATATGTCATCACTGCACCTACAG        v
      PheGlnThrAlaArgValTyrAlaGlyPheGlyAspProArgGluAlaIleThrIleIleHisGlnGlnHisGlyLysProCysAspCysAlaGlyGlyTyrValIleThrAlaProThr         60

6001  TCTATCTTGCAACTGTTTCTTGTTCTTCTCACACTGCATATCAGCCAAGTGACTCCCTTAAGTGGCGGTGTGTCTCTAACCCGACATTAGCCAATGGAGAAAATATAGGGAATTGTCCTT
      ValTyrLeuAlaThrValSerCysSerSerHisThrAlaTyrGlnProSerAspSerLeuLysTrpArgCysValSerAsnProThrLeuAlaAsnGlyGluAsnIleGlyAsnCysPro        100

6121  GCCAGACATTCAAAGAATCTGTACATAGCTCTTGTTACACCACCTATCAGGAATGTTTTTTGGTAATAAGACTTACTACACCGCCATTTTGGCCAGTAATAGAGCCCCTACTATAGGAA
      CysGlnThrPheLysGluSerValHisSerSerCysTyrThrThrTyrGlnGluCysPhePheGlyAsnLysThrTyrTyrThrAlaIleLeuAlaSerAsnArgAlaProThrIleGly        140

6241  CTAGCAATGTCCCCACAGTTTTGGGAAACACCCACAATCTACTGTCAGCTGGATGCACCGGAAACGTGGGTCAGCCCATTTGCTGGAATCCTAAAGCTCCCGTCCATATCTCCGATGGAG
      ThrSerAsnValProThrValLeuGlyAsnThrHisAsnLeuLeuSerAlaGlyCysThrGlyAsnValGlyGlnProIleCysTrpAsnProLysAlaProValHisIleSerAspGly        180

6361  GAGGACCACAAGATAAGGCTCGAGAAATTGCAGTACAAAAAAGGCTTGAGGAAATACATAGGTCCTTATTTCCCGAATTACGATACCACCCCTTAGCTTTGCCTAAGGCCCGTGGTAAAG
      GlyGlyProGlnAspLysAlaArgGluIleAlaValGlnLysArgLeuGluGluIleHisArgSerLeuPheProGluLeuArgTyrHisProLeuAlaLeuProLysAlaArgGlyLys        220

6481  AAAAGATCGATGCTCAAACCTTTAATCTTCTTACTGCTACATATAGTTTGCTTAATAAGTCTAACCCTAATTTGGCCAACGAGTGCTGGCTATGCTTACCATCTGGAAATCCCGTCCCGC
      GluLysIleAspAlaGlnThrPheAsnLeuLeuThrAlaThrTyrSerLeuLeuAsnLysSerAsnProAsnLeuAlaAsnGluCysTrpLeuCysLeuProSerGlyAsnProValPro        260

6601  TTGCCATACCTAGCAATGACTCATTTCTTGGTTCTAATCTTTCTTGCCCTATTATTCCTCCCTTGTTGGTACAACCTCTTGAATTTATTAATCTTATTAATGCCTCTTGCCTTTATTCCC
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6721  CTTTTCAGAATAACTCATTTGGTGTTGATGTAGGCTTAGTTGAATTTACCAACTGCTCTACCATTCTTAATATTTCTCACTCCTTATGTGCCCCTAATAGCTCAGTTTTTGTGTGCGGTA
      ProPheGlnAsnAsnSerPheGlyValAspValGlyLeuValGluPheThrAsnCysSerThrIleLeuAsnIleSerHisSerLeuCysAlaProAsnSerSerValPheValCysGly        340

6841  ATAACAAGGCCTACACGTACTTACCCACAAACTGGACAGGAACTTGCGTATTAGCTACCCTTCTGCCAGATATAGATATTGTCCCTGGTGATGCACCTGTGCCTGTTCCAGCTATAGATC
      AsnAsnLysAlaTyrThrTyrLeuProThrAsnTrpThrGlyThrCysValLeuAlaThrLeuLeuProAspIleAspIleValProGlyAspAlaProValProValProAlaIleAsp        380
                           gp20
6961  ATTATCTACATAGAGCTAGGAGAGCAGTACAGTTTATCCCCTTACTTGTTGGGTTAGGCATAACCACTGCCGTCTCAACTGGGACTGCTGGTCTAGGGTATTCTATTACCCAGTACACCA
      HisTyrLeuHisArgAlaArgArgAlaValGlnPheIleProLeuLeuValGlyLeuGlyIleThrThrAlaValSerThrGlyThrAlaGlyLeuGlyTyrSerIleThrGlnTyrThr        420

7081  GACTATCTCGTCAACTAATCTCAGACGTACAGGCTATCTCAAGCACTATTCAAGATTTACAAGACCAAGTAGACTCTCTAGCAGAAGTGGTGCTTCAAAATAGAAGAGGATTAGATCTAC
      ArgLeuSerArgGlnLeuIleSerAspValGlnAlaIleSerSerThrIleGlnAspLeuGlnAspGlnValAspSerLeuAlaGluValValLeuGlnAsnArgArgGlyLeuAspLeu        460

7201  TTACCGCAGAGCAGGGAGGGATTTGTTTAGCCCTACAGGAAAAGTGCTGTTTCTATGCCAACAAATCTGGAATCGTCAGAGATAAGATCAAACGTCTCCAAGAAGACCTGGAAAAACGAC
      LeuThrAlaGluGlnGlyGlyIleCysLeuAlaLeuGlnGluLysCysCysPheTyrAlaAsnLysSerGlyIleValArgAspLysIleLysArgLeuGlnGluAspLeuGluLysArg        500

7321  GTAAAGAAATCATTGACAATCCATTTTGGACTGGTCTACATGGACTCCTCCCCTATTTGCTACCTTTGCTAGGACCTTTGCTTTGCTTGTTACTATTAATTACCTTTGGACCCTTAATTT
      ArgLysGluIleIleAspAsnProPheTrpThrGlyLeuHisGlyLeuLeuProTyrLeuLeuProLeuLeuGlyProLeuLeuCysLeuLeuLeuLeuIleThrPheGlyProLeuIle        540

7441  TCAATAAGATCATAGCATTTGTTAAACAACAAATGGATGCCATCCAAGCTAAGCCTATTCAGGTCCATTACCACCGCCTTGAGCAAGAAGACAATGGTGGTGTATACTTACGTGTATCGT
      PheAsnLysIleIleAlaPheValLysGlnGlnMetAspAlaIleGlnAlaLysProIleGlnValHisTyrHisArgLeuGluGlnGluAspAsnGlyGlyValTyrLeuArgValSer        580

7561  AGACCACCGCCCCTGTGCGCTAAACTGGTCAGCCAATGACGGGTAAGAGAGTGTCGTTTCTCACTAACCTAAGACAGGAGGGTCGTCTTAGCTACTACCTTTTCCTATGACGGGTACCAG
                                                                                        U3
7681  TGAAGAAACTGTATCACTCCAACCTAAGACAGGCGCAGTTTCCGAGGGGATTCTTTTGAAAAAATAAAAAAGGGGGACCTGTCCGGAGCCGTGCAGCCCGGACGACGTTCTGGCTTCTGG

7801  CGATTGCACTCCATAGTAGTGGATGCAAGATGGCGCACTTCCTGGTTCTCTTTCACCCTGTTTTCCCGCCGGCGCCAATATTTCCCGCCTTAGACTACGTGGCATTTCCTGACTTAGCTA        L
                                                                                                                                        T
7921  CTGAGCATGCGCTCAGTGACGTTGTTCTCCACCCCCGCCGCCTGTCTATATATTGCCTCGCATCACCGCCATTAAACGAGACTTGATCAGAGCCCTGTCTTGTCTCCATTTCTTGTGTCT        R
                                                                                  U5
8041  CTTGTCTCCCCCAATTCCCACCCCCTCATCCAGGTTCTACGTTGCTGATCCCGCGGGTCGGGACA
```

Figure 12. Nucleotide sequence of the D2/RHE/OR genome. The predicted amino acid sequences of *gag*, *prt*, *pol*, and *env* are presented. Numbering of nucleotides begins at the 5' boundary of the leftward LTR. The U3 and U5 boundaries, putative TATA box, and the polypurine tract (for initiation of plus-strand DNA synthesis) are all underlined. The sequence complementary to $tRNA^{lys1,2}$ (primer for minus-strand viral DNA synthesis) is over lined. The N-termini of processed *gag* and *env* gene products are also depicted.

U3
1 TGTCCGGAGCCGTGCAGCCCGGACGACGTTCTGGCTTCTGGCGATTGCACTCCATAGTAGTGGATGCAAGATGGCGCACTTCCTGGTTCTCTTTCACCCTGTTTTCCCGCCGGCGCCAAT

121 ATTTCCCGCCTTAGACTACGTGGCATTTCCTGACTTAGCTACTGAGCATGCGCTCAGTGACGTTGTTCTCCACCCCCGCCGCCTGTCTATATATTGCCTCGCATCACCGCCATTAAACGA
U5
241 GACTTGATCAGAGCCCTGTCTTGTCTCCATTTCTTGTGTCTCTTGTCTCCCCCAATTCCCACCCCCTCATCCAGGTTCTACGTTGCTGATCCCGCGGGTCGGGACACGTGGCGCCCAACG

361 TGGGGCTGGATACGAGGGAATCCTGTGAGGAAGAGAGCGCGTAACACGGCCGGCAGTCAAAGTGAAAGAAAAACCTTCCAGCTGCCGCGGGAACCTGCCGCGTCGGCAATAAAGGTAAGC

LTR

p10
481 GTTGCGTTCGAATATGGGACAAGAATTAAGCCAACATGAACTTTATGTAGAACAGTTAAAAAAGGCTCTAAAGACACGGGGAGTAAAGGTTAAGGGTAATGATTTACTCAAATTCTTTGA
MetGlyGlnGluLeuSerGlnHisGluLeuTyrValGluGlnLeuLysLysAlaLeuLysThrArgGlyValLysValLysGlyAsnAspLeuLeuLysPhePheAsp 36

601 TTTTGTAAAGGATACCTGCCCTTGGTTCCCACAAGAGGGAACAATAGACATAAAAAGATGGCGCAGGGTAGGAGATTGTTTTCAGGATTATTATAACACTTTTGGCCCGGAAAAGATACC
PheValLysAspThrCysProTrpPheProGlnGluGlyThrIleAspIleLysArgTrpArgArgValGlyAspCysPheGlnAspTyrTyrAsnThrPheGlyProGluLysIlePro 76
pp24
721 AGTAACTGCCTTCTCCTACTGGAATCTTATCAAAGATTTAATAGATAAAAAGGAGGCTGACCCACAAGTAATGGCTGCGGTCACCCAGACAGAAAAAATATTAAAAGTTAGTTCACAAAC
ValThrAlaPheSerTyrTrpAsnLeuIleLysAspLeuIleAspLysLysGluAlaAspProGlnValMetAlaAlaValThrGlnThrGluLysIleLeuLysValSerSerGlnThr 116

841 AGATCTCAGGGACAAATCACAAAATAAGGAAATGGATCTCATCTCTCTTGAAAGTGATGATGAGGAAGCCAAGGCCCCCTCAGAGAAAATATCAATGCCCAATAAAAGTCCTAAAAAATA
AspLeuArgAspLysSerGlnAsnLysGluMetAspLeuIleSerLeuGluSerAspAspGluGluAlaLysAlaProSerGluLysIleSerMetProAsnLysSerProLysLysTyr 156
pp18
961 TCCAGCTATGTTAACATCACAAAATAATAATATGGACAAAGACCCTGATTCGTCAGAGGTGGATTGGGATGGCTTAAAGGACGAGGCAGCAAGGTATCATAACCCTGATTGGCCTCCTTT
ProAlaMetLeuThrSerGlnAsnAsnAsnMetAspLysAspProAspSerSerGluValAspTrpAspGlyLeuLysAspGluAlaAlaArgTyrHisAsnProAspTrpProProPhe 196
p12
1081 CCTCTCTCGTCCACCCCCCTATAATGGGACAACTGCTACAGCCCCTGCTGTGATGGCGGTAGTCAATCCTAAAGAGGAACTTAAAGAAAAAATTTCTCAATTAGAGGAGCAGATAAAATT
LeuSerArgProProProTyrAsnGlyThrThrAlaThrAlaProAlaValMetAlaValValAsnProLysGluGluLeuLysGluLysIleSerGlnLeuGluGluGlnIleLysLeu 236

1201 AGAGGAGTTACACCAGTCCCTAATTATTCGGTTACAAAAACTAAAAACAGGAAGTGAGAGGGTAACTAGTTCAGGCAATATGGAGAGTCACTCTCGTACGCCCAAACGGCCTGGACAGCA
GluGluLeuHisGlnSerLeuIleIleArgLeuGlnLysLeuLysThrGlySerGluArgValThrSerSerGlyAsnMetGluSerHisSerArgThrProLysArgProGlyGlnHis 276
p27
1321 CCTTCCAATAGGAAAATACCTCATTAACAAAAATACAGAAGAATATCCTTCCAAGGATATTTTCCCAGTAACGGAGACTATGGATGGACAAGGTCAGGCCTGGAGACATCATAATGGGTT
LeuProIleGlyLysTyrLeuIleAsnLysAsnThrGluGluTyrProSerLysAspIlePheProValThrGluThrMetAspGlyGlnGlyGlnAlaTrpArgHisHisAsnGlyPhe 316

1441 TGACTTTACTGTTATAAAAGAACTAAAAACTGCTGTTTCCCAATACGGAGCTACTGCTCCATATACTTTGGCCATAGTAGAATCCATTGCTGATAATTGGCTTACTCCAGCTGACTGGAA
AspPheThrValIleLysGluLeuLysThrAlaValSerGlnTyrGlyAlaThrAlaProTyrThrLeuAlaIleValGluSerIleAlaAspAsnTrpLeuThrProAlaAspTrpAsn 356

1561 TACGCTCGTTAGAGCAGTCCTCTCAGGGGGAGACCACTTAATTTGGAAGTCTGAATTTTTTGAAAATTGTAGGGACACAGCAAAAAGAAATCAACAGGCAGGAAATGGCTGGGACTTTGA
ThrLeuValArgAlaValLeuSerGlyGlyAspHisLeuIleTrpLysSerGluPhePheGluAsnCysArgAspThrAlaLysArgAsnGlnGlnAlaGlyAsnGlyTrpAspPheAsp 396

1681 CATGTTAACAGGCTCTGGTAACTATGCCAATACTGATGCACAAATGCAGTATGATCCAGGACTGTTTGCTCAAATTCAGGCAGCCGCCACAAAAGCCTGGAGAAAACTCCCTGTTAAAGG
MetLeuThrGlySerGlyAsnTyrAlaAsnThrAspAlaGlnMetGlnTyrAspProGlyLeuPheAlaGlnIleGlnAlaAlaAlaThrLysAlaTrpArgLysLeuProValLysGly 436

1801 GGACCCAGGTGCTTCACTCACCGGGGTCAAACAAGGACCTGACGAGCCATTCGCAGATTTTGTACATAGACTCATAACAACAGCTGGCAGAATTTTCGGAAACGCCGAGGCAGGTGTAGA
AspProGlyAlaSerLeuThrGlyValLysGlnGlyProAspGluProPheAlaAspPheValHisArgLeuIleThrThrAlaGlyArgIlePheGlyAsnAlaGluAlaGlyValAsp 476

1921 TTATGTTAAACAACTAGCCTATGAAAATGCTAACCCAGCCTGTCAAGCAGCAATTCGTCCTTACCGAAAGAAAACAGATCTAACAGGATATATTCGCCTTTGTTCAGATATTGGGCCCTC
TyrValLysGlnLeuAlaTyrGluAsnAlaAsnProAlaCysGlnAlaAlaIleArgProTyrArgLysLysThrAspLeuThrGlyTyrIleArgLeuCysSerAspIleGlyProSer 516
p14
2041 TTATCAGCAGGGTTTGGCAATGGCTGCCGCCTTTAGCGGACAGACCGTAAAAGATTTCTTAACAATAAAAACAAAGATAGAGGAGGGTGCTTTAAGTGTGGCAAAAGAGGACATTTTGC
TyrGlnGlnGlyLeuAlaMetAlaAlaAlaPheSerGlyGlnThrValLysAspPheLeuAsnAsnLysAsnLysAspArgGlyGlyCysPheLysCysGlyLysArgGlyHisPheAla 556

gag

2161 AAAAGATTGCCGTGATCATTCAAACAAAAATCCAGAATTGAAAGTGCCAGGCTTGTGCCCCAGATGTAAAAGAGGAAAACACTGGGCTAATGAATGCAAATCCAAGACTGATAGTCAAGG
LysAspCysArgAspHisSerAsnLysAsnProGluLeuLysValProGlyLeuCysProArgCysLysArgGlyLysHisTrpAlaAsnGluCysLysSerLysThrAspSerGlnGly 596
SerArg 2
p4
2281 AAATCCCTTACCACCCCATCAGGGAAACGGGATGAGGGGCCAGCCCCAGGCCCCGAAGCAAGCCTATGGGGCAGTCAGCTTTGTTCCAGCCAGCAGCAACAATCCATTTCAAAACTTAAT
AsnProLeuProProHisGlnGlyAsnGlyMetArgGlyGlnProGlnAlaProLysGlnAlaTyrGlyAlaValSerPheValProAlaSerSerAsnAsnProPheGlnAsnLeuIle 636
LysSerLeuThrThrProSerGlyLysArgAspGluGlyProAlaProGlyProGluAlaSerLeuTrpGlySerGlnLeuCysSerSerGlnGlnGlnGlnSerIleSerLysLeuAsn 42

2401 AGAGCCTCCCCAGGAAGTGCAGGACTGGACCTCTGTTCCACCACCCACACAGTATTAACCCCTGAAATGGGGCCCCAAACTTTAGCTACTGGAGTATATGGACCTTTGCCCCCTAATACG
GluProProGlnGluValGlnAspTrpThrSerValProProProThrGlnTyr 654
ArgAlaSerProGlySerAlaGlyLeuAspLeuCysSerThrThrHisThrValLeuThrProGluMetGlyProGlnThrLeuAlaThrGlyValTyrGlyProLeuProProAsnThr 82

2521 TTTGGGTTAATACTAGGCAGAGGCAGTACCACAGTAAAAGGTTTACTAATTTACCCAGGAGTCATTGATAATGATTATACAGGGGAATTCAAAATAATGGCCAAGGCTATTTCTAGTATC
PheGlyLeuIleLeuGlyArgGlySerThrThrValLysGlyLeuLeuIleTyrProGlyValIleAspAsnAspTyrThrGlyGluPheLysIleMetAlaLysAlaIleSerSerIle 122

2641 ATCACTATTCCTCAAGGAGAACGGATTGCACAACTAATCTTGTTACCCTTACTCCGAACAGCTCATAAAATACAACACCCTTATAGAGGAAACAAAAATTTCGGGTCCTCAGATATTTTT
IleThrIleProGlnGlyGluArgIleAlaGlnLeuIleLeuLeuProLeuLeuArgThrAlaHisLysIleGlnHisProTyrArgGlyAsnLysAsnPheGlySerSerAspIlePhe 162

2761 TGGGTACAGCCAATAACTAATCAAAAACCCTCCTTAGTACTGTGGCTAGACGGTAAAGCATTTACAGGGTTAATAGATACTGGAGCGGATGTCACTATTATCAAACAAGAAGATTGGCCA
TrpValGlnProIleThrAsnGlnLysProSerLeuValLeuTrpLeuAspGlyLysAlaPheThrGlyLeuIleAspThrGlyAlaAspValThrIleIleLysGlnGluAspTrpPro 202

2881 TCCCATTGGCCTACCACAGAAACCTTGACTAATCTGAGAGGAATAGGACAAAGTAATAACCCCAGGCAAAGTTCAAAATACCTTACTTGGAAAGACAAAGAAAATAACTCAGGCCTTATC
SerHisTrpProThrThrGluThrLeuThrAsnLeuArgGlyIleGlyGlnSerAsnAsnProArgGlnSerSerLysTyrLeuThrTrpLysAspLysGluAsnAsnSerGlyLeuIle 242

3001 AAGCCATTTGTCATCCCTAACTTACCGGTTAATCTTTGGGGCCGAGATTTGCTTTCCCAAATGAAAATTATGATGTGCAGTCCTAATGATATTGTCACTGCTCAAATGTTAGCTCAAGGA
LysProPheValIleProAsnLeuProValAsnLeuTrpGlyArgAspLeuLeuSerGlnMetLysIleMetMetCysSerProAsnAspIleValThrAlaGlnMetLeuAlaGlnGly 282

3121 TACAGCCCAGGAAAAGGACTAGGAAAAAGAGAAGATGGAATCTTACAACCCATCCCAAATTCAGGACAACTTAACAGAAAAGGATTTGGAAATTTTTAGCTGCGGCCGTTGACATACTTG
TyrSerProGlyLysGlyLeuGlyLysArgGluAspGlyIleLeuGlnProIleProAsnSerGlyGlnLeuAsnArgLysGlyPheGlyAsnPhe 314
GlnLysArgIleTrpLysPheLeuAlaAlaAlaValAspIleLeu 15

prt

3241 CACCCCAGAGGTATGCTGACCCCATTACATGGAAGTCAGATGAGCCTGTTTGGGTTGATCAATGGCCCCTAACTCAAGAAAAACTTGCTGCTGCCCAACAGTTAGTGCAAGAACAATTAC
AlaProGlnArgTyrAlaAspProIleThrTrpLysSerAspGluProValTrpValAspGlnTrpProLeuThrGlnGluLysLeuAlaAlaAlaGlnGlnLeuValGlnGluGlnLeu 55

3361 AGGCAGGGCATATTATAGAAGTAATTCTCCCTGGAATACACCTATATTTGTCATAAAAAAGAAGTCTGGTAAATGGAGGCTTTTGCAAGATTTAAGGGCGGTAAATGCCACCATGGTAT
GlnAlaGlyHisIleIleGluSerAsnSerProTrpAsnThrProIlePheValIleLysLysLysSerGlyLysTrpArgLeuLeuGlnAspLeuArgAlaValAsnAlaThrMetVal 95

3481 TAATGGGAGCTCTCCAACCTGGGCTGCCCTCACCAGTGGCTATTCCTCAGGGATATTTTAAAATAGTCATTGATCTTAAAGATTGTTTTTTTACTATCCCCCTTCAGCCCGTTGATCAAA
LeuMetGlyAlaLeuGlnProGlyLeuProSerProValAlaIleProGlnGlyTyrPheLysIleValIleAspLeuLysAspCysPhePheThrIleProLeuGlnProValAspGln 135

3601 AGCGATTTGCTTTTAGTCTCCCGTCTACCAACTTTAAACAACCAATAAAACGTTATCAATGGAAAGTGTTGCCTCAAGGCATGGCCAATAGTCCTACCTTGTGTCAAAAATATGTAGCTG
LysArgPheAlaPheSerLeuProSerThrAsnPheLysGlnProIleLysArgTyrGlnTrpLysValLeuProGlnGlyMetAlaAsnSerProThrLeuCysGlnLysTyrValAla 175

3721 CTGCTATAGAGCCAATCAGAAAATCTTGGGCACAAATGTACATTATACACTATATGGATGACATTCTAATAGCAGGAAAGATTGGCGAACAAGTTTTGCAGTGTTTTGCTCAACTTAAAC
AlaAlaIleGluProIleArgLysSerTrpAlaGlnMetTyrIleIleHisTyrMetAspAspIleLeuIleAlaGlyLysIleGlyGluGlnValLeuGlnCysPheAlaGlnLeuLys 215

3841 AAGCATTGACAACTACTGGGTTACAAATAGCTCCAGAAAAGGTACAGCTACAAGATCCATATACCTACCTTGGGTTTCAACTTAATGGTCCCAAAATTACCAATCATAAGGCAGTTATTC
GlnAlaLeuThrThrThrGlyLeuGlnIleAlaProGluLysValGlnLeuGlnAspProTyrThrTyrLeuGlyPheGlnLeuAsnGlyProLysIleThrAsnHisLysAlaValIle 255

3961 GTAGAGATAAGTTACAAACCCTTAATGATTTTCAAAAACTCTTGGGAGATATCAATTGGCTTAGACCCTATTTACACCTCACTACAGGAGATCTAAAACCTCTTTTTGATATCCTAAAAG
ArgArgAspLysLeuGlnThrLeuAsnAspPheGlnLysLeuLeuGlyAspIleAsnTrpLeuArgProTyrLeuHisLeuThrThrGlyAspLeuLysProLeuPheAspIleLeuLys 295

4081 AAAGGGGATTCTAATCCTAACTCACCCAGATTTTTATCTGAAGCAGCCCTTACGTCTCTTAAAAAGGTAGAAACAGCTATTGCTGAACAATTTGTTACACAAATAGATTATACACAGCCA
LysGlyAspSerAsnProAsnSerProArgPheLeuSerGluAlaAlaLeuThrSerLeuLysLysValGluThrAlaIleAlaGluGlnPheValThrGlnIleAspTyrThrGlnPro 334

4201 TTGACCTTTTTAATTTTTAATACTACACTGACACCTACTGGCCTCTTCTGGCAAAATAATCCTGTCATGTGGATTCACTTGCCTGCATCGCCAAAAAAGGTATTGTTCCCCTATTATGAT
LeuThrPheLeuIlePheAsnThrThrLeuThrProThrGlyLeuPheTrpGlnAsnAsnProValMetTrpIleHisLeuProAlaSerProLysLysValLeuPheProTyrTyrAsp 374

4321 GCTATAGCAGATCTTATTATCCTTGGAAGGGACAACAGTAAAAAATATTTTGGACTTGAACCATCTACTATTATACAACCCTATTCTAAATCTCAAATCCATTGGTTAATGCAAAACACA
AlaIleAlaAspLeuIleIleLeuGlyArgAspAsnSerLysLysTyrPheGlyLeuGluProSerThrIleIleGlnProTyrSerLysSerGlnIleHisTrpLeuMetGlnAsnThr 414

4441 GAAACATGGCCAATTGCCTGCGCTTCCTATGCAGGCAACATTGATAACCATTATCCACCCAATAAACTTATTCAATTTTGCAAACTCCATGCAGTTGTTTTTCCCCGAATCATTAGTAAA
GluThrTrpProIleAlaCysAlaSerTyrAlaGlyAsnIleAspAsnHisTyrProProAsnLysLeuIleGlnPheCysLysLeuHisAlaValValPheProArgIleIleSerLys 454

4561 ACTCCTCTAGACAATGCCTTACTGGTATTCACTGATGGATCCTCTACCGGCATAGCCGCATACACTTTTGAAAAGACCACTGTCAAGTTTAAAACCTCCCATACGTCAGCCCAATTAGTA
ThrProLeuAspAsnAlaLeuLeuValPheThrAspGlySerSerThrGlyIleAlaAlaTyrThrPheGluLysThrThrValLysPheLysThrSerHisThrSerAlaGlnLeuVal 494

pol

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4681  GAATTACAAGCCCTAATTGCAGTGTTATCGGCCTTTCCTCAACGGGCCCTCAATATTTATACAGATAGTGCCTACTTAGCTCATTCTATACCTCTACTTGAGACAGTATCGCAAATCAAA
      GluLeuGlnAlaLeuIleAlaValLeuSerAlaPheProGlnArgAlaLeuAsnIleTyrThrAspSerAlaTyrLeuAlaHisSerIleProLeuLeuGluThrValSerGlnIleLys      534

4801  CATATCTCAGACACAGCAAAATTATTTTTACAGTGCCAACAATTAATATACAACAGGTCTATACCTTTTTATTTAGGACATATCAGGGCCCATTCAGGATTACCAGGACCTCTATCTCAA
      HisIleSerAspThrAlaLysLeuPheLeuGlnCysGlnGlnLeuIleTyrAsnArgSerIleProPheTyrLeuGlyHisIleArgAlaHisSerGlyLeuProGlyProLeuSerGln      574

4801  TCTCAGACACAGCAAAATTATTTTTACAGTGCCAACAATTAATATACAACAGGTCTATACCTTTTTATTTAGGACATATCAGGGCCCATTCAGGATTACCAGGACCTTTATCTCAAGGTA
     IleSerAspThrAlaLysLeuPheLeuGlnCysGlnGlnLeuIleTyrAsnArgSerIleProPheTyrLeuGlyHisIleArgAlaHisSerGlyLeuProGlyProLeuSerGlnGly      575

4921  ATCACATAACTGATTTGGCAACCAAAGTTGTCGCCACCACTCTTACCACAAATTTAACAGAGGCACAAGCTGCTCATGCCTTACATCACTTAAACGCTCAGTCCCTAAGGTTAATGTTTA
     AsnHisIleThrAspLeuAlaThrLysValValAlaThrThrLeuThrThrAsnLeuThrGluAlaGlnAlaAlaHisAlaLeuHisHisLeuAsnAlaGlnSerLeuArgLeuMetPhe      615

5041  AAATTACTAGAGAACAGGCTAGACAAATTGTTAAACAGTGTCCCACATGTGTAACATACTTGCCAATTCCTCATTTTGGAGTTAATCCAAAAGGATTGATACCCAATATGCTATGGCAAA
     LysIleThrArgGluGlnAlaArgGlnIleValLysGlnCysProThrCysValThrTyrLeuProIleProHisPheGlyValAsnProLysGlyLeuIleProAsnMetLeuTrpGln      655

5161  TGGATGTCACTCATTATTCTGAATTCGGTAAATTAAAATATGTTCATGTGTCCATAGATACATTTAGTGGATTTCTTGTAGCCACCTTACAAACAGGAGAAGCTACCAAACATGTTATTG
     MetAspValThrHisTyrSerGluPheGlyLysLeuLysTyrValHisValSerIleAspThrPheSerGlyPheLeuValAlaThrLeuGlnThrGlyGluAlaThrLysHisValIle      695

5281  CCCATCTACTACATTGCTTCTCTATTATTGGTCAACCTATTCACATTAAAACAGACAATGGCCCTGGATACACCTCATCTAGCTTTCGTGCCTTCTGTTCCAAATTACACATCAAACACA
     AlaHisLeuLeuHisCysPheSerIleIleGlyGlnProIleHisIleLysThrAspAsnGlyProGlyTyrThrSerSerSerPheArgAlaPheCysSerLysLeuHisIleLysHis      735

5401  CCTTTGGTATTCCTTACAATCCTCAGGGTCAAGGTATTGTCGAAAGAGCACATCTCTCCTTAAAAAATACTCTTGAAAAAATAAAAAAGGGGGAATGGTACCCCACACAGGGGTCTCCCA
     ThrPheGlyIleProTyrAsnProGlnGlyGlnGlyIleValGluArgAlaHisLeuSerLeuLysAsnThrLeuGluLysIleLysLysGlyGluTrpTyrProThrGlnGlySerPro      775

5521  GGAATATTCTCAATCATGCACTCTTTATTTTAAATTTTTAAACCTTGATGCTCAAAATAAATCAGCCGCTGACCGATTTTGGCATACTAACTCTAAAAGAGAATATGCCATGGTCAAGT
     ArgAsnIleLeuAsnHisAlaLeuPheIleLeuAsnPheLeuAsnLeuAspAlaGlnAsnLysSerAlaAlaAspArgPheTrpHisThrAsnSerLysArgGluTyrAlaMetValLys      815

5641  GGAAAGACCCATTAGACAATTCGTGGCATGGGCCTGATCCCGTATTAATCTGGGGCAGAGGTTCAGTCTGCGTATACTCTCAAACTCATGATGCAGCCAGATGGCTACCAGAACGGTTGG
     TrpLysAspProLeuAspAsnSerTrpHisGlyProAspProValLeuIleTrpGlyArgGlySerValCysValTyrSerGlnThrHisAspAlaAlaArgTrpLeuProGluArgLeu      855

5761  TAAGGCAAGTGTCTAATGTTACTCAATCCAGGGAGTGATTGTCTCCCTGAGATCACTCCTTTTCTTTGCTCATAGAGATGACTGTTAAGGACATCCCGTTTTGGAGAGTACTGCTGATTT
     ValArgGlnValSerAsnValThrGlnSerArgGlu                                        gp70
                                                                     PheSerLeuLeuIleGluMetThrValLysAspIleProPheTrpArgValLeuLeuIle      20     e
                                                                                                                                              n
5881  TCCAAACTGCTCGAGTTTATGCCGGTTTCGGTGACCCGCGCGAGGCTATTACTATAATACATCAACAACACGGTAAGCCTTGTGATTGTGCGGGAGGATATGTCATCACTGCACCTACAG              v
     PheGlnThrAlaArgValTyrAlaGlyPheGlyAspProArgGluAlaIleThrIleIleHisGlnGlnHisGlyLysProCysAspCysAlaGlyGlyTyrValIleThrAlaProThr      60

6001  TCTATCTTGCAACTGTTTCTTGTTCTTCTCACACTGCATATCAGCCAAGTGACTCCCTTAAGTGGCGGTGTGTCTCTAACCCGACATTAGCCAATGGAGAAAATATAGGGAATTGTCCTT
     ValTyrLeuAlaThrValSerCysSerSerHisThrAlaTyrGlnProSerAspSerLeuLysTrpArgCysValSerAsnProThrLeuAlaAsnGlyGluAsnIleGlyAsnCysPro      100

6121  GCCAGACATTCAAAGAATCTGTACATAGCTCTTGTTACACCACCTATCAGGAATGTTTTTTTGGTAATAAGACTTACTACACCGCCATTTTGGCCAGTAATAGAGCCCCTACTATAGGAA
     CysGlnThrPheLysGluSerValHisSerSerCysTyrThrThrTyrGlnGluCysPhePheGlyAsnLysThrTyrTyrThrAlaIleLeuAlaSerAsnArgAlaProThrIleGly      140

6241  CTAGCAATGTCCCCACAGTTTTGGGAAACACCCACAATCTACTGTCAGCTGGATGCACCGGGACTGTGGGTCAGCACATTTGCTGGAATCCTAAAGCTCCCGTCCATATCTCCGATGGAG
     ThrSerAsnValProThrValLeuGlyAsnThrHisAsnLeuLeuSerAlaGlyCysThrGlyThrValGlyGlnHisIleCysTrpAsnProLysAlaProValHisIleSerAspGly      179

6361  GAGGACCACAAGATAAGGCTCGAGAAATTGCAGTACAAAAAAGGCTTGAGGAAATACATAGGTCCTTATTTCCCGAATTACGATACCACCCCTTAGCTTTGCCTAAGGCCCGTGGTAAAG
     GlyGlyProGlnAspLysAlaArgGluIleAlaValGlnLysArgLeuGluGluIleHisArgSerLeuPheProGluLeuArgTyrHisProLeuAlaLeuProLysAlaArgGlyLys      220

6481  AAAAGATCGATGCTCAAACCTTTAATCTTCTTACTGCTACATATAGTTTGCTTAATAAGTCTAACCCTAATTTGGCCAACGAGTGCTGGCTATGCTTACCATCTGGAAATCCCGTCCCGC
     GluLysIleAspAlaGlnThrPheAsnLeuLeuThrAlaThrTyrSerLeuLeuAsnLysSerAsnProAsnLeuAlaAsnGluCysTrpLeuCysLeuProSerGlyAsnProValPro      260

6601  TTGCCATACCTAGCAATGACTCATTTCTTGGTTCTAATCTTTCTTGCCCTATTATTCCTCCCTTGTTGGTACAACCTCTTGAATTTATTAATCTTATTAATGCCTCTTGCCTTTATTCCC
     LeuAlaIleProSerAsnAspSerPheLeuGlySerAsnLeuSerCysProIleIleProProLeuLeuValGlnProLeuGluPheIleAsnLeuIleAsnAlaSerCysLeuTyrSer      300

6721  CTTCTCAGAATAACTCATTTGATGTTGATGTAGGCTTAGTTGAATTTACCAACTGCTCTACCACTCTTAATATTTCTCACTCCTTATGTGCCCCTAATAGCTCAGTTTTTGTGTGCGGTA
     ProSerGlnAsnAsnSerPheAspValAspValGlyLeuValGluPheThrAsnCysSerThrThrLeuAsnIleSerHisSerLeuCysAlaProAsnSerSerValPheValCysGly      340

6841  ATAACAAGGCCTACACGTACTTACCCACAAACTGGACAGGAACTTGCGTATTAGCTACCCTTCTGCCAGATATAGATATTGTCCCTGGTGATGCACCTGTGCCTGTTCCAGCTATAGATC
     AsnAsnLysAlaTyrThrTyrLeuProThrAsnTrpThrGlyThrCysValLeuAlaThrLeuLeuProAspIleAspIleValProGlyAspAlaProValProValProAlaIleAsp      380
                      gp20
6961  ATTATCTACATAGAGCTAGGAGAGCAGTACAGTTTATCCCCTTACTTGTTGGGTTAGGCATAACCACTGCCGTCTCAACTGGGACTACTGGTCTAGGGTATTCTATTACCCAGTACACCA
     HisTyrLeuHisArgAlaArgArgAlaValGlnPheIleProLeuLeuValGlyLeuGlyIleThrThrAlaValSerThrGlyThrThrGlyLeuGlyTyrSerIleThrGlnTyrThr      420

7081  AACTATCTCGTCAACTAATCTCAGACGTACAGGCTATCTCAAGCACTATTCAAGATTTACAAGACCAAGTAGACTCTCTAGCAGAAGTGGTGCTTCAAAATAGAAGAGGATTAGATCTAT
     LysLeuSerArgGlnLeuIleSerAspValGlnAlaIleSerSerThrIleGlnAspLeuGlnAspGlnValAspSerLeuAlaGluValValLeuGlnAsnArgArgGlyLeuAspLeu      460

7201  TTACCGCAGAGCAGGGAGGGATTTGTTTAGCCCTACAGGAAAAGTGCTGTTTCTATGCCAACAAATCTGGAATCGTCAGAGATAAGATCAAACGTCTCCAAGAAGACCTGGAAAAACGAC
     PheThrAlaGluGlnGlyGlyIleCysLeuAlaLeuGlnGluLysCysCysPheTyrAlaAsnLysSerGlyIleValArgAspLysIleLysArgLeuGlnGluAspLeuGluLysArg      500

7321  GTAAAGAAATCATTGACAATCCATTTTGGACTGGTCTACATGGACTCCTCCCCTATTTGCTACCTTTGCTACGACCTTTGCTTTGCTTGTTACTATTAATTACCTTTGGACCCTTAATTT
     ArgLysGluIleIleAspAsnProPheTrpThrGlyLeuHisGlyLeuLeuProTyrLeuLeuProLeuLeuArgProLeuLeuCysLeuLeuLeuLeuIleThrPheGlyProLeuIle      540

7441  TCAATAAGATCATAGCATTTGTTAAACAACAAATGGATGCCATCCAAGCTAAGCCTATTCAGGTCCATTACCACCGCCTTGAGCAAGAAGACAATGGTGGTGTATACTTACGTGTATCGT
     PheAsnLysIleIleAlaPheValLysGlnGlnMetAspAlaIleGlnAlaLysProIleGlnValHisTyrHisArgLeuGluGlnGluAspAsnGlyGlyValTyrLeuArgValSer      580

7561  AGACCACCGCCCCTGTGCGCTAAACTGGTCAGCCAATGACGGGTAAGAGAGTGTCGTTTCTCACTAACCTAAGACAGGAGGGTCGTCTTAGCTACTACCTTTTCCTATGACGGGTACCAG
                                                                                              U3
7681  TGAAGAAACTGTATCACTCCAACCTAAGACAGGCGCAGTTTCCGAGGGGATTCTTTTGAAAAAATAAAAAAGGGGGACCTGTCCGGAGCCGTGCAGCCCGGACGACGTTCTGGCTTCTGG

7801  CGATTGCACTCCATAGTAGTGGATGCAAGATGGCGCACTTCCTGGTTCTCTTTCACCCTGTTTTCCCGCCGGCGCCAATATTTCCCGCCTTAGACTACGTGGCATTTCCTGACTTAGCTA              L
                                                                                                                                              T
7921  CTGAGCATGCGCTCAGTGACGTTGTTCTCCACCCCCGCCGCCTGTCTATATATTGCCTCGCATCACCGCCATTAAACGAGACTTGATCAGAGCCCTGTCTTGTCTCCATTTCTTGTGTCT              R
                                                                              U5
8041  CTTGTCTCCCCCAATTCCCACCCCCTCATCCAGGTTCTACGTTGCTGATCCCGCGGGTCGGGACA
```

Figure 13. Nucleotide sequence comparison to the 5' LTR of D2/RHE/OR/V1. The 5' LTR and untranslated region of D2/RHE/OR/V1 were compared with the corresponding domains encoded by D2/RHE/OR, D2/CEL/OR, D2/MNE/WA, D1/RHE/CA, and MPMV. Amino acid residue changes are indicated by lower case letters; dots represent identical residues. The U3 and U5 borders of the LTR and the primer binding site are overlined. The TATA and GCAT promoter elements and the polyadenylation signal are underlined.

```
                      U3      10        20        30        40        50        60        70        80        90        100
D2/RHE/OR/V1 5'LTR   TGTCCGGAGCCGTGCAGCCCGGACGACGTTCTGGCTTCTGGCGATTGCACTCCATAGTAGTGGATGCAAGATGGCGCACTTCCTGGTTCTCTTTCACCCT

D2/RHE/OR            ....................................................................................................

D2/CEL/OR            ...........................................g...............a...............a..............tg........

                                                           g
                                                           |
MPMV                 a...cat..tc.a.c.a.t...t..t..ct....c...ttt.c.ctag.....-t..ta..a..tt.........t.t...........tc.c.gt.t.

                                                          ttt
                                                           |
D1/RHE/CA            ...............t.......t..t..ct....c......tc.a..t..a.-gct..--a...t..........a.............tc.ctgtgt.


                            110       120       130       140       150       160       170       180       190       200
D2/RHE/OR/V1 5'LTR   GTTTTCCCGCCGGCGCCAATATTTCCCGCCTTAGACTACGTGGCATTTCCTGACTTAGCTACTGAGCATGCGCTCAGTGACGTTGTTCTCCACCCCCGCC

D2/RHE/OR            ....................................................................................................

D2/CEL/OR            ..........................t...............c.......tcc...................c...................t...

                                               c      c
                                               |      |
MPMV                 ac.............g...g.......t...g.gt.......-c......tg..ct................c....at.t..ccc...-c.a.tt..t

                                               t       t                                                    t
                                               |       |                                                    |
D1/RHE/CA            .c.............g...g............g.........-.....cagt.ct..agt...........c....-..-..c.c.c..c.a.tta.t


                            210       220       230       240       250       260       270       280       290       300
D2/RHE/OR/V1 5'LTR   GCCTGTCTATATATTGCCTCGCATCACCGCCATTAAACGAGACTTGATCAGAGCCCTGTCTTGTCTCCATTTCTTGTGTCTCTTGTCTCCCCCAATTCCC

D2/RHE/OR            ....................................................................................................

D2/CEL/OR            .g.c................................................................................................

MPMV                 ......g......ag..aa.a...tg..a........t..............a.a..............................tc..tt........

D1/RHE/CA            ......g......aga.aa.....tg..a.......................a.a...............................c.at.........


                           310       320       330       340   U5   350 pbs   360       370       380       390       400
D2/RHE/OR/V1 5'LTR   ACCCCCTCATCCAGGTTCTACGTTGCTGATCCCGCGGGTCGGGACACGTGGCGCCCAACGTGGGGCTGGATACGAGGGAATCCTGTGAGGAAGAGAGCGC

D2/RHE/OR            ....................................................................................................

D2/CEL/OR            ....................................................................................................

                                    t
                                    |
MPMV                 ..t.....c.......c...--c..t..................gt................................ttc..........cga...

                                                                                                                   g
                                                                                                                   |
D1/RHE/CA            ..t.....c........tcctac..t..g.........a.......tt..............c.t...............ttc..........cga...


                           410       420       430       440       450       460       470       480       490
D2/RHE/OR/V1 5'LTR   GTAACACGGCCGGCAGTCAAAGTGAAAGAAAAACCTTCCAGCTGCCGCGGGAACCTGCCGCGTCGGCAATAAAGGTAAGCGTTGCGTTCGAAT

D2/RHE/OR            ...............................................................................................

D2/CEL/OR            ....................................................g..............................c..a...

                                  c
                                  |
MPMV                 ..tcgc.......g.t.a..........t...ct..cttg..c...................t..acc.g..a.....t......c...g.

                                                c  gta    tt
                                                |   |      |
D1/RHE/CA            ..ttgc.....c.g.t.a....a..........t....g..c.........g..........a..acc.g..a.....t.g....c...g..
```

Figure 14. Comparison of the gag polyprotein of D2/RHE/OR/V1 to other SRVs. The gag protein of D2/RHE/OR/V1 was compared to the corresponding gene products encoded by D2/RHE/OR, D2/CEL/OR, D2/MNE/WA, D1/RHE/CA, and MPMV. Amino acid residue changes are indicated by lower case letters; dots represent identical residues. The major homology region of the p27 capsid protein and the two Cys-His motifs of the nucleocapsid are underlined. For D2/MNE/WA sequence determined by Grant *et al.* (1995a,b) and Henderson *et al* (1985), 1=D, E, N or Q; 2=I or V and x=could not identify, respectively.

```
                            10        20        30        40        50        60        70        80        90       100
D2/RHE/OR/V1 GAG    MGQELSQHELYVEQLKKALKTRGVKVKGNDLLKFFDFVKDTCPWFPQEGTIDIKRWRRVGDCFQDYYNTFGPEKIPVTAFSYWNLIKDLIDKKEADPQVM
D2/RHE/OR           ....................................................................................................
D2/CEL/OR           ....................................................................................................
D1/RHE/CA           .........r......q..........ya..............................................v............e......vn...
MPMV                .........r......q..........ya..............................................v............e......vn...

                           110       120       130       140       150       160       170       180       190       200
D2/RHE/OR/V1 GAG    AAVTQTEKILKVSSQTDLRDKSQNKETDLISLESDDEEAKAPSEKISMPNKSPKKYPAMLTSQNNNMDKDPDSSEVDWDGLEDEAARYHNPDWPPFLSRP
D2/RHE/OR           ............................m.....................................................k.................
D2/CEL/OR           .....................n.h..dm.................mt.s...........a.....t.....l..............k............
D2/MNE/WA           ......q.................s.m.x...
                                         p                       d   c
                                         |                       |   |
D1/RHE/CA           ...a...e...t..h.e.tt....pdl.....d....g..gs.l.knlst.k..rf.vl..a.tsadpe..np.............k..........t..
                                                             lq
                                                             |
MPMV                ...a...e...sn.....tkt...pdl.....d....g..ss.d.glsst.k..rf.vl..a.tskdpe..np.............k..........t..

                           210       220       230       240       250       260       270       280       290       300
D2/RHE/OR/V1 GAG    PPYNRTTATAPAVMAVVNPKEELKEKISQLEEQIKLEELHQSLIIRLQKLKTGSERVTSSGNMESHSRTPKRPGQHLPIGKYLINKNTEEYPSKDIFPVT
D2/RHE/OR           ....g...............................................................................................
D2/CEL/OR           ......a.............................................n........i........w...c..k.............p.......
D2/MNE/WA           ......................g...q......                                                  ...
D1/RHE/CA           ....ka.ps..t...............a.............a..sk.......n.t...petaggf....hw....i.k..ccasrek..qtp.......
MPMV                ....ka.ps..t...............a.............a..sk.......n.t..hpdtaggl....hw....i.k..ccasrek..qip.......

                           310       320       330       340       350       360       370       380       390       400
D2/RHE/OR/V1 GAG    ETMDGQGQAWRHHNGFDFTVIKELKTAVSQYGATAPYTLAIVESIADNWLTPADWNTLVRAVLSGGDHLIWKSEFFENCRDTAKRNQQAGNGWDFDMLTG
D2/RHE/OR           ....................................................................................................
D2/CEL/OR           ...................................................t................................................
D2/MNE/WA           ....................                              ..t.....i.........................................
D1/RHE/CA           ..v......................a...............v.......t................l..........e......................
MPMV                ..v...............a........a...............v.......t................l...............................

                           410       420       430       440       450       460       470       480       490       500
D2/RHE/OR/V1 GAG    SGNYANTDAQMQYDPGLFAQIQAAATKAWRKLPVKGDPGASLTGVKQGPDEPFADFVHRLITTAGRIFGNAEAGVDYVKQLAYENANPACQAAIRPYRKK
D2/RHE/OR           ....................................................................................................
D2/CEL/OR           .........................n..........................................................................
D2/MNE/WA           ...........................................................................................
D1/RHE/CA           ....ss............................................................s.................................
MPMV                ....ss............................................................s.................................

                           510       520       530       540       550       560       570       580       590       600
D2/RHE/OR/V1 GAG    TDLTGYIRLCSDIGPSYQQGLAMAAAFSGQTVKDFLNNKNKDRGGCFKCGKRGHFAKDCRDHSNKNPELKVPGLCPRCKRGKHWANECKSKTDSQGNPLP
D2/RHE/OR           ....................................................................................................
D2/CEL/OR           .................................l................k................s..................................
D2/MNE/WA                                  ...............x.x...
                                                              c
                                                              |
D1/RHE/CA           ........................................ek.......rk.....n.he.ihn.s.t.a..............................
                                                              c
                                                              |
MPMV                ........................................ek........k.....n.he.ahn.a.p.......................n....i.

                           610       620       630       640       650
D2/RHE/OR/V1 GAG    PHQGNGMRGQPQAPKQAYGAVSFVPASSNNPFQNLIEPPQEVQDWTSVPPPTQY
D2/RHE/OR           ......................................................
D2/CEL/OR           ...........................n..........................
D2/MNE/WA                             .........................p
D1/RHE/CA           ......l....................nk.....s.p.................
MPMV                ......w....................nk.....s.p.................
```

Figure 15. Comparison of the prt of D2/RHE/OR/V1 to other SRVs. The protease of D2/RHE/OR/V1 was compared with corresponding gene products encoded by D2/RHE/OR, D2/CEL/OR, D2/MNE/WA, D1/RHE/CA, and MPMV. Amino acid residue changes are indicated by lower case letters; dots represent identical residues. The five conserved motifs of the dUTPase are underlined, and the putative Tyr at the active site is indicated by an * (amino acid residue 75). The Asp-Thr-Gly active sit of the aspartyl protease domain in the C-terminus of the protease is overlined (amino acid residues 189-190).

```
                            10        20        30        40        50        60        70    *   80        90       100
D2/RHE/OR/V1 PRT    SRKSLTTPSGKRDEGPAPGPEASLWGSQLCSSQQQQSISKLNRASPGSAGLDLCSTTHTVLTPEMGPQTLATGVYGPLPPNTFGLILGRGSTTVKGLQTY
D2/CEL/OR           ....................................................................................................
D2/RHE/OR           .................................................................................................l..
MPMV                ...pn.......v........t............k.p....t..t...........s...........a.s..i...............s.i.m....v.
D1/RHE/CA           ...pt.......t........t....g.......k.p....t..t........s..s...........a.s..i...............s.i.i....v.

                           110       120       130       140       150       160       170       180       190       200
D2/RHE/OR/V1 PRT    PGVIDNDYTGEFKIMAKAISSIITIPQGERIAQLILLPLLRTAHKIQHPYRGNKNFGSSDIFWVQPITNQKPSLVLWLDGKAFTGLIDTGADVTIIKQED
D2/CEL/OR           ................r.................v.................d...............h...............................
D2/RHE/OR           ....................................................................................................
MPMV                ...........i......vnn.v.vs..n..........ie.dn.v.q....qgs......y......c.....t....d.m...............l..
D1/RHE/CA           ...........i......vnn.v.v...n..........ie.dn.v.q....qgs......y......c.....t....d.m...............l..

                           210       220       230       240       250       260       270       280       290       300
D2/RHE/OR/V1 PRT    WPSHWPTTETLTNLRGIGQSNNPRQSSKYLTWKDKENNSGLIKPFVIPNLPVNLWGRDLLSQMKIMMCSPNDIVTAQMLAQGYSPGKGLGKREDGILQPI
D2/CEL/OR           ....................................................................................................
D2/RHE/OR           ....................................................................................................
MPMV                ..pn..i.d..............k........r..........................................................k.n...h..
D1/RHE/CA           ..pn..i.d..............k........r.....................................s....................n.n...h..

                           310
D2/RHE/OR/V1 PRT    PNSGQLNRKGFGNF
D2/CEL/OR           ......d.......
D2/RHE/OR           ..............
MPMV                ..q..s.k......
D1/RHE/CA           ..q..fdk......
```

Figure 16. Comparison of the pol protein of D2/RHE/OR/V1 to other SRVs. The pol protein of D2/RHE/OR/V1 was compared with corresponding gene products encoded by D2/RHE/OR, D2/CEL/OR, D2/MNE/WA, D1/RHE/CA, and MPMV. Amino acid residue changes are indicated by lower case letters; dots represent identical residues. The five conserved domains (A-E) of the reverse transcriptase protein are underlined. The invariant amino acids of each domain are indicated by italics. The HHCC and DD35E motifs of the integrase protein are identified with an underline.

```
                         10        20        30        40        50        60        70        80        90       100
D2/RHE/OR/V1 POL QKRIWKFLAAAVDILAPQRYADPITWKSDEPVWVDQWPLTQEKLAAAQQLVQEQLQAGHIIESNSPWNTPIFVIKKKSGKWRLLQDLRAVNATMVIMGAL
D2/RHE/OR        ....................................................................................................
D2/CEL/OR        .........t..........................................................................................
MPMV             ...f....t..i......qc.e..................nd.............e....t..s....................................
D1/RHE/CA        ..g.....t..i.m....qc.e..................s..............e....t.......................................

                                     A                                           B                             C
                        110       120       130       140       150       160       170       180       190       200
D2/RHE/OR/V1 POL QPGLPSPVAILQGYFKIVIDLKDCFFTIPLQPVDQKRFAFSLPSTNFKQPMKRYQWKVLPQGMANSPTLCQKYVAAAIEPIRKSWAQMYIIHYMDDILIA
D2/RHE/OR        ..........p.......................................i.................................................
D2/CEL/OR        ..........p.....................................................................v...................
MPMV             ..........p...l..i........s...h.s...............e..q.f.....................t..hkv.ha.k..............
D1/RHE/CA        ..........p...l..i........s...h.s...............e..q.f.......r.............t..hkv.ha.k..............

                                         D                E
                        210       220       230       240       250       260       270       280       290       300
D2/RHE/OR/V1 POL GKIGEQVLQCFAQLKQALTTTGLQIAPEKVQLQDPYTYLGFQLNGPKITNHKAVIRRDKLQTLNDFQKLLGDINWLRPYLHLTTGDLKPLFDILKGDSNP
D2/RHE/OR        ....................................................................................................
D2/CEL/OR        ..l.......................................i.......q.................................................
MPMV             ..d.q......d....e..aa..h.................e........q.....k.......................k...........t.....d.
D1/RHE/CA        ..d.q......d....e..ia..h.....i...........e........q.....k.......................k...a.......t.......

                        310       320       330       340       350       360       370       380       390       400
D2/RHE/OR/V1 POL NSPRFLSEAALTSLKKVETAIAEQFVTQIDYTQPLTLLIFNTTLTPTGLFWQNNPVMWIHLPASPKKVLFPYYDAIADLIILGRDNSKKYFGLEPSTIIQ
D2/RHE/OR        ....................................f...............................................................
D2/CEL/OR        ....s......a..q.....................f.....................v..........l..............................
MPMV             ..h.s..ke..a..e............h.n.sl..if.....a.........d..i.............l...............h......i.......
D1/RHE/CA        ..h.s..ke..al.d............h.n.sl..mf.....a............i..v..........l...............h......i...v...

                        410       420       430       440       450       460       470       480       490       500
D2/RHE/OR/V1 POL PYSKSQIHWLMQNTETWPIACASYAGNIDNHYPPNKLIQFCKLHAVVFPRIISKTPLDNALLVFTDGSSTGIAAYTFEKTTVKFKTSHTSAQLVELQALI
D2/RHE/OR        ....................................................................................................
D2/CEL/OR        ..................................................................................r.................
PMV              .......d.......m.......fv.il................tf...q.......n.............m....ltd..i..q.nln...........
D1/RHE/CA        .......d.......m........v.il.................fi..q.......n.............m....lad..i..q.nln...........

                        510       520       530       540       550       560       570       580       590       600
D2/RHE/OR/V1 POL AVLSAFPQRALNIYTDSAYLAHSIPLLETVSQIKHISDTAKLFLQCQQLIYNRSIPFYLGHIRAHSGLPGPLSQGNHITDLATKVVATTLTTNLTEAQAA
D2/RHE/OR        ....................................................................................................
D2/CEL/OR        .......h.....v.................h.........f........................................................t.
MPMV             .......nqp....................a......e....................i..v.........ia...qra.....i..snin...es..n.
D1/RHE/CA        .......nqp....................a......e....................i..v.........iah..qka.....t..snin...es..n.

                           HHCC                                                         DD35E
                        610       620       630       640       650       660       670       680       690       700
D2/RHE/OR/V1 POL HALHHLNAQSLRLMFKITREQARQIVKQCPTCVTYLPIPHFGVNPKGLIPNMLWQMDVTHYSEFGKLKYVHVSIDTFSGFLVATLQTGEATKHVIAHLLH
D2/RHE/OR        ....................................................................................................
D2/CEL/OR        ................................................v...................................................
MPMV             .t.......t.....n.p............i......v..l....r..f...i............n...i...........l.......t.....t....
D1/RHE/CA        .t.......t.k...n.p........r...i.a....v..l....r..l...i............n...i...........l.......t.....t....

                        710       720       730       740       750       760       770       780       790       800
D2/RHE/OR/V1 POL CFSIIGQPIHIKTDNGPGYTSSSFRAFCSKLHIKHTFGIPYNPQGQGIVERAHLSLKNTLEKIKKGEWYPTQGSPRNILNHALFILNFLNLDAQNKSAAD
D2/RHE/OR        ....................................................................................................
D2/CEL/OR        ......................n.............................................................................
MPMV             ......l.kq...........kn.qe...t.q...it....................t.i..........rk.t..................d.......
D1/RHE/CA        ......l.kq...........kn.qe...t.q...vt....................t.i...........k.t..................d..h....

                        810       820       830       840       850       860
D2/RHE/OR/V1 POL RFWHTNSKREYAMVKWKDPLDNSWHGPDPVLIWGRGSVCVYSQTHDAARWLPERLVRQVSNVTQSRE
D2/RHE/OR        ...................................................................
D2/CEL/OR        .....s..k.............t............................................
MPMV             ....n.p.kqf...........t.....................y................nn....
D1/RHE/CA        ....s.prkqf...........t.pw....i.........................k.ip.nn....
```

Figure 17. Comparison of the env glycoprotein of D2/RHE/OR/V1 to other SRVs. The env protein of D2/RHE/OR/V1 was compared with the corresponding gene products encoded by D2/RHE/OR, D2/CEL/OR, D2/MNE/WA, D1/RHE/CA, and MPMV. Amino acid residue changes are indicated by lower case letters; dots represent identical residues. The cleavage site between the envelope gp70 (SU) and gp20 (TM) domains is indicated by an *. The amino acids of the heptad repeat region in the fusion domain are italicized. The immunosuppressive peptide (ISP) domain is from amino acid residues 446-477, and is underlined. The membrane spanning domain (MSD) lies between amino acid residues 518-546. The gp22 processing domain is from amino acid residue 554-566 with the His-Tyr cleavage site italicized. For the D2/MNE/WA sequence determined by Grant *et al.* (1995a,b) and Henderson *et al* (1985), 1=D, E, N or Q; 2=I or V and x=could not identify, respectively.

```
                            10        20        30        40        50        60        70        80        90       100
D2/RHE/OR/V1 ENV    MTLKDIPFWRVLLIFQTARVYAGFGDPREAITIIHQQHGKPCDCAGGYVITAPTVYLATVSCSSHTAYQPSDSLKWRCVSNPTLANGENIGNCPCQTFKE
D2/RHE/OR           ..v.................................................................................................
D2/CEL/OR           ................................m.................na.......a...................................k....
                                                                                                                   ns
                                        t                                                                        sq|
                                        |                                                                         ||
D1/RHE/CA           .nfnh-h..slvi.s.ifq.q.........lle.q.k............ssp..ns.t.....ty...svtn....q...t..t.spth..s....sy-d
                                                                                                                 ge   y
                                                                                                                 |    |
MPMV                .nfnyhfi.slvils.isq.q.........lae.q.k............ssp.ins.t.....t....svtn....q...t..tpsnth..s...n.isd

                           110       120       130       140       150       160       170       180       190       200
D2/RHE/OR/V1 ENV    SVHSSCYTTYQECFFGNKTYYTAILASNRAPTIGTSNVPTVLGNTHNLLSAGCTGNVGQPICWNPKAPVHISDGGGPQDKAREIAVQKRLEEIHRSLFPE
D2/RHE/OR           ........................................................t...h.......................................
D2/CEL/OR           ........a.....................................................................................k.....
D2/MNE/WA                                                      ...............1.2.......g................2..............
                                                                            k
                                                                            |
D1/RHE/CA           ...at..nh..q.ti.....l..tmirdks.ss.dg....i...nq..ii...pe.k..vv...sqps..m.........v...i.n.kf..l.k.....
                                                                         p
                                                                         |
MPMV                ...a...nh..q.ni.....l..titgd.t.a..dg.......ts...it...n.kk..vv...srps..............d.i.n.kf..l.......

                           210       220       230       240       250       260       270       280       290       300
D2/RHE/OR/V1 ENV    LRYHPLALPKARGKEKIDAQTFNLLTATYSLLNKSNPNLANECWLCLPSGNPVPLAIPSNDSFLGSNLSCPIIPPLLVQPLEFINLINASCLYSPFQNNS
D2/RHE/OR           ...............................................................................................s....
D2/CEL/OR           ....................................................i..............................m.......f........
D2/MNE/WA           ......................l...........................t.........
                                                     v                   ydntscs    c
                                                     |                       |      |
D1/RHE/CA           .s.......e.........h..d..atvh....s.qrq..ed.....r..d.....l.nstf.fn..c..l.t..f....--.-.fthsv...ady....
                                                                                ac
                                                                                 |
                                                                              lcsn
                                                                                ||
MPMV                .s.......e.........h.ld..atvh....a.q.s..ed.....q..d.....l.y..t..snh-...lt..f....--.-.ftdsn...ahy....

                           310       320       330       340       350       360       370       380  *    390       400
D2/RHE/OR/V1 ENV    FGVDVGLVEFTNCSTILNISHSLCAPNSSVFVCGNNKAYTYLPTNWTGTCVLATLLPDIDIVPGDAPVPVPAIDHYLHRARRAVQFIPLLVGLGITTAVS
D2/RHE/OR           .d.............t....................................................................................
D2/CEL/OR           .d........a....t...........................s........................................................
D2/MNE/WA                                                                                             ..................
                                     skp
                                      |
D1/RHE/CA           .di....ag.....syi...sp..........................s...........i..se...i.....f.g.pk..i.....vi..........
                                   staskp
                                      |
MPMV                .di....as.....syy.v.n...........................s...........i..se...i.....f.gk.k..i.l...f...........

                           410       420       430       440       450       460       470       480       490       500
D2/RHE/OR/V1 ENV    TGTAGLGYSITQYTRLSRQLISDVQAISSTIQDLQDQVDSLAEVVLQNRRGLDLLTAEQGGICLALQEKCCFYANKSGIVRDKIKRLQEDLEKRRKEIID
D2/RHE/OR           ...t..........k.......................................f.............................................
D2/CEL/OR           ..............k.....................................................................................
D2/MNE/WA           ..............k..............................f..................................k...................
D1/RHE/CA           .......v.l....k..h...................................................................n..d.......ql..
MPMV                ..a....v......k..h...................................................................n..d...r..rql..

                           510       520       530       540       550       560       570
D2/RHE/OR/V1 ENV    NPFWTGLHGLLPYLLPLLGPLLCLLLLITFGPLIFNKIIAFVKQQMDAIQAKPIQVHYHRLEQEDNGGVYLRVS
D2/RHE/OR           ...................r......................................................
D2/CEL/OR           ......................f.................t.....i...........................
D2/MNE/WA           ........................................................i.................
D1/RHE/CA           ......f......vm...........vls...i...lmt.i.h.ies..................h..s..nlt
MPMV                .....sf..f...vm...........vls...i...lmt.i.h.ies..................s..s..tlt
```

Figure 18. Comparison of the 3' LTR of D2/RHE/OR/V1 to other SRVs. Nucleotide sequence comparison of the D2/RHE/OR/V1 3' untranslated region and long terminal repeat (LTR) with the corresponding domains of D2/RHE/OR, D2/CEL/OR, D2/MNE/WA, D1/RHE/CA, and MPMV. Amino acid residue changes are indicated by lower case letters; dots represent identical residues. The 3' untranslated region begins at the stop codon of the *env* gene, and the 5' border of the 3' LTR is indicated by the underlined U3. The polypurine tract (ppt) precedes U3 and is underlined.

```
                            10        20        30        40        50        60        70        80        90       100
D2/RHE/OR/V1 3'LTR  TAGACCACCGCCCCTGTGCGCTAAACTGGTCAGCCAATGACGGGTAAGAGAGTGTCGTTTCTCACTAACCTAAGACAGGAGGGTCGTCTTAGCTACTACC

D2/RHE/OR           ....................................................................................................

D2/CEL/OR           ........................................................................................a...........

MPMV                ...g.....t........a....g.....a........................a.a..........................c....aa.......g..

D1/RHE/CA           .........t......c.a.....g....a........................a.a...t......................c....ag.......g..

                           110       120       130       140       150       160       170       180       190       200
D2/RHE/OR/V1 3'LTR  TTTTCCTATGACGGGTACCAGTGAAGAAACTGTATCACTCCAACCTAAGACAGGCGCAGTTTCCGAGGGGATTCTTTTGAAAAAATAAAAAAGGGGACC

D2/RHE/OR           ....................................................................................................

D2/CEL/OR           .......................................................................g............................

MPMV                .aa...a..........at..........-.............................cc.........tg.......tt.t...t........t...a

D1/RHE/CA           .aa...a.a........aa.....ta...a.............................c..........tc.g....at.t.t..tt.......t....

                     U3    210       220       230       240       250       260       270       280       290       300
D2/RHE/OR/V1 3'LTR  TGTCCGGAGCCGTGCAGCCCGGACGACGTTCTGGCTTCTGGCGATTGCACTCCATAGTAGTGGATGCAAGATGGCGCACTTCCTGGTTCTCTTTCACCCT

D2/RHE/OR           ....................................................................................................

D2/CEL/OR           ...........................................g...............a...............a..............tg........

                                                           g
                                                           |
MPMV                a....cat..tc.a.c.a.t...t..t..ct....c...ttt.c.ctag.....-t..ta..a..tt.........t.t...........tc.c.gt.t.

                                                           ttt
                                                            |
D1/RHE/CA           ...............t.......t..t..ct....c......tc.a..t..a.-gct..--a...t..........a.............tc.ctgtgt.

                           310       320       330       340       350       360       370       380       390       400
D2/RHE/OR/V1 3'LTR  GTTTTCCCGCCGGCGCCAATATTTCCCGCCTTAGACTACGTGGCATTTCCTGACTTAGCTACTGAGCATGCGCTCAGTGACGTTGTTCTCCACCCCCGCC

D2/RHE/OR           ....................................................................................................

D2/CEL/OR           .............................t...............c.......tcc....................c...................t...

                                               c      c
                                               |      |
MPMV                ac..............g...g.......t...g.gt.......-c......tg..ct................c....at.t..ccc...-c.a.tt..t

                                                t      t                                                   t
                                                |      |                                                   |
D1/RHE/CA           .c..............g...g.............g.........-.....cagt.ct..agt...........c....-..-..c.c.c..c.a.tta.t

                           410       420       430       440       450       460       470       480       490       500
D2/RHE/OR/V1 3'LTR  GCCTGTCTATATATTGCCTCGCATCACCGCCATTAAACGAGACTTGATCAGAGCCCTGTCTTGTCTCCATTTCTTGTGTCTCTTGTCTCCCCCAATTCCC

D2/RHE/OR           ....................................................................................................

D2/CEL/OR           .g.c................................................................................................

MPMV                ......g......ag..aa.a...tg..a........t..............a.a................................tc..tt.......

D1/RHE/CA           ......g......aga.aa.....tg..a.......................a.a................................c.at.........

                           510       520       530       540
D2/RHE/OR/V1 3'LTR  ACCCCCTCATCCAGGTTCTACGTTGCTGATCCCGCGGGTCGGGACA

D2/RHE/OR           ..............................................

D2/CEL/OR           ...............................................

                                   t
                                   |
MPMV                .t.....c.......c...--...t.....................

D1/RHE/CA           .t.....c........tcctac..t..g..........a.......
```

Figure 20. Cell-free infection of different human T-cell lines by SRV-2 viral clones. Infection of the T-cell lines CEM-SS, Hut 78, MT-4 and SupT-1, by the parental SRV-2 molecular clones (D2/RHE/OR and D2/RHE/OR/V1) and reciprocal chimeras (p5'2R3'V1 and p5'V13'2R) was performed. Fresh cell-free inocula was normalized for reverse transcriptase activity. Cultures were monitored weekly for the expression (%) of envelope gp20 by immunofluorescent assay and FACS analysis. Mock cultures of all experimental cell lines and an infected Hut cell line were utilized as gp20 immunofluorescence assay controls and were found to be consistently negative and positive, respectively (data not shown). Two independent experiments were conducted with similar results; the data in the figure are derived from a single experiment. The following graphs are representative of the infection kinetics observed in both experiments: A) CEM-SS cell infection, B) Hut78 cell infection, C) MT-4 cell infection, D) SupT-1 cell infection, and E) Raji cell infection. D2/RHE/OR infected MT-4 cells became positive by gp20 analysis after day 31; Hut78 cells were negative for D2/RHE/OR infection up to 60 days after infection (data not shown).

THE RECIPROCAL CHIMERAS

Bam HI
*

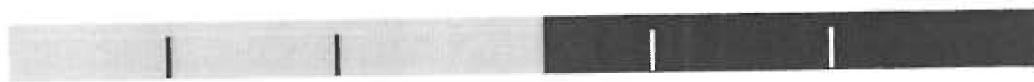
D2/RHE/OR

*

D2/RHE/OR/V1

*

p52R3V1



*

P5V132R

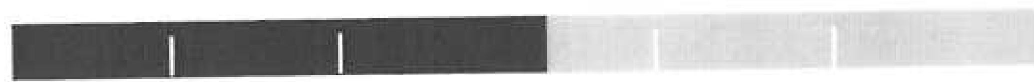
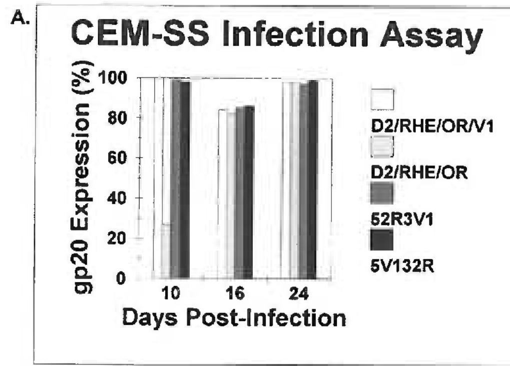
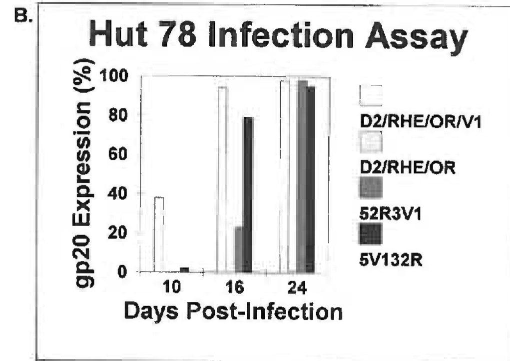


Figure 19. Diagram representing the genomes of the chimeric SRV-2 viruses. The genomes of the parental molecular clones D2/RHE/OR and D2/RHE/OR/V1, and their reciprocal chimeras p52R3V1 and p5V132R are represented with intact LTRs flanking the linearized genome. The unique Bam HI restriction site, which cleaves inside the *pol* gene, was utilized to construct the reciprocal chimeras. Verification of the chimeric structure of each virus was obtained from restriction endonuclease, sequence and PCR analyses. All of the viral inocula used in the infection assays were obtained after cocultivation of infected Raji cells with fresh Raji cells. The black and gray boxes represent the genomic structures of D2/RHE/OR/V1 and D2/RHE/OR, respectively. The relative positions of the *gag, pro, prt* and *env* genes are not drawn to scale.

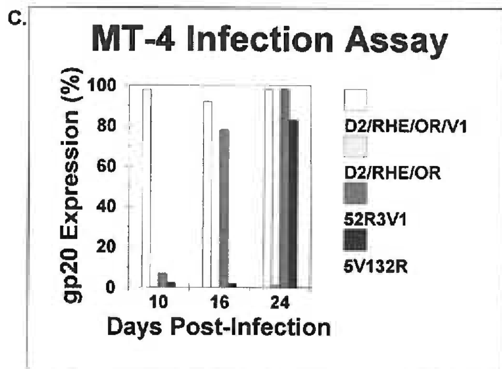
A.



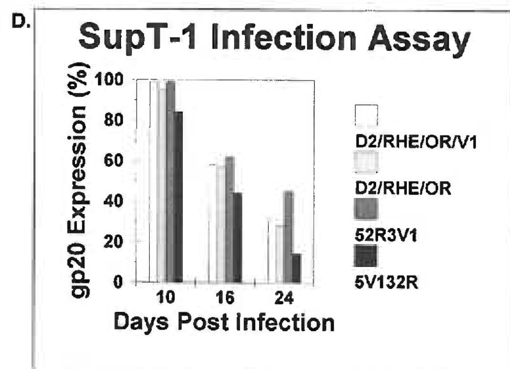
B.



C.



D.



E.

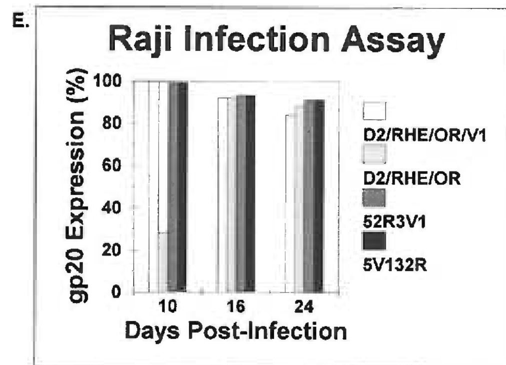
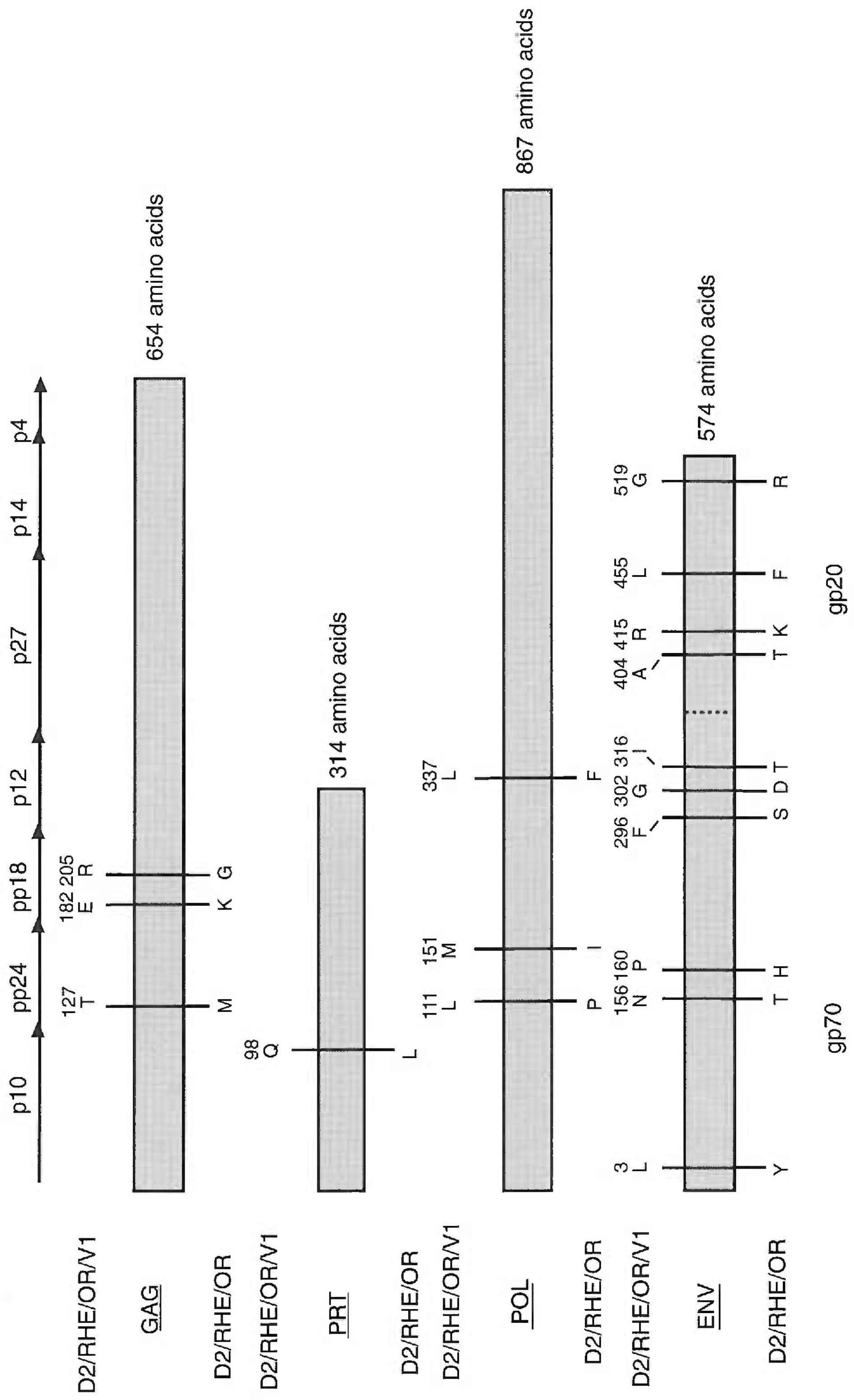


Figure 21. Amino acid residue differences encoded by the D2/RHE/OR and D2/RHE/OR/V1 isolates. Numbers above amino acids denote positions in the coding sequence for each SRV gene. Positions of residue differences are not drawn to scale.


p10
pp24
pp18
p12
p27
p14
p4
D2/RHE/OR/V1
GAG
D2/RHE/OR
654 amino acids
D2/RHE/OR/V1
PRT
D2/RHE/OR
314 amino acids
D2/RHE/OR/V1
POL
D2/RHE/OR
867 amino acids
D2/RHE/OR/V1
ENV
D2/RHE/OR
574 amino acids
gp70
gp20

VII. WORK PERFORMED

I. D2/RHE/OR (lambda clone isolated by R. Kelley in Dr. Hallick's lab)

A. Transfection/infection assays to determine infectivity.

gp70/gp20 IFAs performed by G. Leo, N. Avery and FACS by S. Shiigi

B. Sequencing of the majority of the 5' fragment and *pol* gene portion of 3' fragment

II. D2/RHE/OR/V1

A. Creation of a genomic DNA library, isolation of the envelope gene in lambda and subsequent subcloning into a pBR322 vector.

B. Complete sequencing of the *env* gene and 3' LTR.

C. Initial comparison of D2/RHE/OR, D2/CEL/OR and D2/RHE/OR/V1 *env* genes and 3'LTR.

D. Creation of a second genomic DNA library, screening and isolation of three full-length D2/RHE/OR/V1 clones in lambda.

E. Transfection/infection assays to determine infectivity

gp70/gp20 IFAs performed by G. Leo, N. Avery and FACS by S. Shiigi

F. Subcloning of the full-length genome, and the 5' and 3' subgenomic fragments into pBR322.

G. Various Southern analyses of lambda DNA, full-length plasmid DNA and D2/RHE/OR/V1 infected Raji genomic DNA

H. Transfection/infection analyses of the full-length plasmid clone to verify retention of infectivity.

I. Sequencing of all but the envelope gene and 3'LTR of the new clone.

Env gene sequencing performed by H. Nichols

III. Nucleotide and amino acid analyses:

A. Alignment of all nucleotides and calculations of percentage similarity

B. Alignment of amino acids and calculations of percentage similarity

C. Creation of percentage tables, alignment figures and amino acid bar diagram

IV. Construction of reciprocal chimeric genomes

A. Introduced a Bgl II restriction site into pBR322

B. Isolated fragments, ligated, transformed, and screened bacterial colonies for intact chimeric genomes.

C. Transfection/infection analyses of both chimeric viruses

D. Sequencing, primer design and PCR analyses, and restriction digest analyses to verify that both clones were reciprocal chimeric genomes.

V. Infection Analyses with D2/RHE/OR, D2/RHE/OR/V1 and reciprocal chimeric viral genomes

A. Optimization and determination of peak RT activity from different clone-infected Raji cells

B. RT assays

C. Inoculation and tissue culture propagation of infected cell lines and uninfected cell lines

D. Southern and PCR analyses of genomic DNA from infected Hut78 cells

E. Southern analyses of genomic DNA from D2/RHE/OR/V1 infected Raji cells

F. Comparison and analyses of infections induced by different virus clones in T- and B-cell lines

VI. Clustal analyses of the different serogroup SRV *gag* and *env* genes, and proteins.

SRV-4 and SRV-5 sequences provided by K. Pilcher

VII. Overall analyses of the sequence and infection data and compilation into a second manuscript (to be submitted) including Southern analysis, bar graphs, line graphs, sequence alignments, and clustal tree figures.

VIII. SUMMARY AND DISCUSSION

Why think, try the experiment. John Hunter, in a letter to E. Jenner (1792)

SRV-2 is the most prevalent type D viral serogroup recovered from captive macaque species with SAIDS in the United States. In addition, SRV-2 has been found in wild Indonesian cynomolgus macaques and Ethiopian baboons (Grant *et al.* 1995; Benveniste *et al.* 1993; Thouless *et al.* 1988). A reservoir for SRV-1 and -3 has not yet been identified. Only SRV-2 infected Celebes, cynomolgus and pig-tailed macaques develop retroperitoneal fibromatosis (RF), a mesenchymal proliferative disorder with similarity to human Kaposi's sarcoma. D2/RHE/OR and D2/RHE/OR/V1 were recovered from rhesus macaques which differed in severity of immunodeficiency; however, restriction endonuclease and Southern analyses demonstrated a high degree of genomic similarity between these two viruses. Further genetic and biological analyses of representatives of these two isolates were undertaken to better characterize their genomic differences and to begin to elucidate the viral determinant(s) which may influence pathogenic outcome.

Sequence comparisons among different SRV serogroup clones. The isolation and characterization of molecular clones of SRVs made possible the analysis of nucleotide and amino acid sequences between isolates of the same and different serogroups. The ability to analyze viral sequences at this level of detail allows for a more sophisticated comparison and classification of viral genomes. The addition of D2/RHE/OR and D2/RHE/OR/V1 to the type D molecular toolbox resulted in the initial examination of relationships both among representatives from all five known serogroups, and between isolates of the same serogroup with divergent pathogenic outcomes (Table 1 and Figure 22). Analysis of *env* nucleotide sequences resulted in the derivation of three clusters: SRV-1, 3 and 4 constitute one cluster with SRV-1 and SRV-3 exhibiting greater similarity to each other than to SRV-4. The three cloned members of SRV-2 were in a distinct cluster with D2/RHE/OR/V1 and D2/RHE/OR displaying a greater degree of similarity to each other than to D2/CEL/OR. Finally, SRV-5 is the sole member of the third cluster. Analysis of *gag* nucleotide sequences resulted in the derivation of two distinct clusters: SRV-1, 3 and 5 constituted one cluster with SRV-1 and SRV-3 exhibiting a greater degree of similarity to each other than to SRV-5. Similarly, upon comparison of

the *gag* gene, the cloned members of SRV-2 congregated into a single cluster with D2/RHE/OR and D2/RHE/OR/V1 displaying a greater degree of similarity to each other than to D2/CEL/OR. The *gag* gene of SRV-4 was not available for comparison.

Determination of the genetic domain(s) responsible for divergent in vitro tropism between D2/RHE/OR and D2/RHE/OR/V1. As D2/RHE/OR usually results in a mild immunodeficiency disease in rhesus macaques, the recovery of D2/RHE/OR/V1 from a rhesus macaque endemically infected with D2/RHE/OR and exhibiting severe SAIDS was surprising. Moreover, the in vitro range of tropism was divergent between the two virus isolates in that cell-free D2/RHE/OR/V1 was able to readily infect Hut78 T- and Raji B-lymphoblastoid cell lines. In contrast, cell-free D2/RHE/OR readily infects only the Raji B-cell line. There were few deduced amino acid differences between the molecular clones of D2/RHE/OR and D2/RHE/OR/V1 compared to the phenotypic differences observed in in vitro tropism; therefore elucidation of the genetic domain which may have a role in in vitro tropism and the pathogenic outcome became the next goal. In addition to the parental molecular clones D2/RHE/OR/V1 and D2/RHE/OR, reciprocal chimeric virus genomes were constructed and used to evaluate the roles of the 5' and 3' genetic domains in determining in vitro tropism and replication kinetics (Paper 2: Marracci *et al.*, in preparation).

An expanded repertoire of T lymphoblastoid cell lines (CEM-SS, Hut78, MT-4 and SupT1), in addition to the Raji B-cell line, were utilized in these infection assays (Paper 2: Marracci *et al.*, in preparation). Three patterns of infection kinetics, using envelope gp20 as a marker, were observed (Figure 23). The first pattern is represented by infection in the CEM-SS and Raji cells in which rapid infection and expression of envelope gp20 within 16-24 days post inoculation were observed in all molecular clone viruses (Figure 24). In addition, high-titer virion release was observed in both D2/RHE/OR/V1 and p5V132R infections of CEM-SS and Raji cells (1:256 for p5V132R infected CEM-SS cells and 1:16,384 for D2/RHE/OR/V1 infected Raji cells). In contrast, D2/RHE/OR and p52R3V1 infection of the same cell lines results in an end-point dilution titer of 1:8 or 1:2, respectively; this titer is substantially below that of D2/RHE/OR/V1 and p5V132R infected CEM-SS and SupT1 cells. These observations suggest that the spread of infection via cell-free virus in these particular cell lines may

be more efficient than cell-to-cell transmission. SupT1 infected cells exhibited a different pattern of infection (Figures 23 and 25). These cells were nearly 100% infected by day 10 after inoculation, but experienced a drop in levels of envelope gp20 expression after 2-3 weeks in culture. This pattern of envelope gp20 expression was reproducible for all tested viruses. Moreover, very high levels of virus were released into the culture medium as demonstrated by end-point dilution on Raji cells of 1:32,768 for p5V132R and D2/RHE/OR/V1 infected cells; and 1:256 for D2/RHE/OR and p52R3V1 infected cells. This pattern of SupT1 infection and relative titer of virus released into the culture medium was reproducible for all viruses. These observations are consistent with the suggestion that the spread of infection via cell-free virus is more efficient in these particular cell lines than spread via fusion from within. D2/RHE/OR/V1, p5V132R and p52R3V1 readily infected MT-4 cells, while D2/RHE/OR infected MT-4 cells after a delay in envelope gp20 detection (Figures 23 and 26). Both p5V132R and p52R3V1 readily infected MT-4, but their appearance was delayed relative to that of D2/RHE/OR/V1 (day 16 versus day 10). MT-4 cells infected with both chimeras released a severely diminished titer (1:2) of virions capable of inducing syncytia in Raji cells. In addition, infectious particle release of both D2/RHE/OR and D2/RHE/OR/V1 infected MT-4 cells was depressed, never exceeding 1:4 and 1:16, respectively. These observations suggest that the spread of viral infection via cell-associated mechanisms may be more important in MT-4 cells. The ability of D2/RHE/OR/V1 to efficiently infect MT-4 cells is consistent with the presence of both a functional late domain and efficient envelope expression. The slight delay in the detection of env gp20 from the chimeric virus infected MT-4 cells relative to D2/RHE/OR/V1 is consistent with the decreased efficiency of envelope expression in p5V132R and diminished virion release by p52R3V1, resulting in intermediate infection kinetics between those of D2/RHE/OR and D2/RHE/OR/V1. The data supports the hypothesis that efficient infection of MT-4 cells is dependent upon a different mechanism of viral transmission than that proposed for infection of CEM-SS and SupT1 cells. The mechanism may be dependent upon fusion from within given the low titer of released infectious virions. The ability of D2/RHE/OR to productively infect Hut78 cells has been sporadic (2 in 12 infection attempts), and then has only succeeded after prolonged culture periods sometimes exceeding 60 days (Figures 23 and 26). Moreover, the release

of D2/RHE/OR virus has been only minimally detected on Raji syncytia forming assays and only after prolonged cocultivation with additional fresh Raji cells. The release of virions by D2/RHE/OR/V1, p52R3V1 and p5V132R from infected Hut78 cells was also minimal, with end-point dilution titers reaching no higher than 1:16 (D2/RHE/OR/V1). D2/RHE/OR/V1, which quantitatively infects most cell lines tested by day 10, infects Hut78 cells between 10-16 days after inoculation. Interestingly, in some cases, D2/RHE/OR/V1 did infect Hut78 cells, but only after a 21 day delay relative to D2/RHE/OR/V1 infected Raji cells. Hut78 cell infection by both the p52R3V1 and p5V132R chimeric viruses exhibited a reproducible delay and has never been detected prior to day 16. These data are consistent with original infection analyses performed by cocultivation of Hut78 cells with D2/RHE/OR infected rhesus PBL. The rhesus PBLs may efficiently express the D2/RHE/OR envelope glycoprotein and thus make these cells an effective conduit of infection via fusion from within. Finally, replacement of the 5' or 3' genomic fragments did not create a chimeric virus with expression kinetics similar to D2/RHE/OR/V1 or D2/RHE/OR in all the cell lines tested, which further supports the suggestion that both viral domains in addition to host cell factors are involved in efficient spread of the infection.

In summary, D2/RHE/OR/V1 virus is fully competent and may spread infection throughout a culture either by high titer virion release such as that observed in SupT1 and CEM-SS infections, or by cell-to-cell spread in cell lines such as Hut78 and MT4. However, all of the cells infected by D2/RHE/OR/V1 appeared to release infectious progeny virus much more efficiently than D2/RHE/OR infection of the same cell types. Interestingly, p5V132R infected CEM-SS and SupT1 cells appeared to release virus particles in a manner equivalent to D2/RHE/OR/V1, suggesting that this function was dictated by determinants contained in the 5' genomic fragment. Moreover, p52R3V1 infected CEM-SS and SupT1 cells released virus particles congruent with the ability of D2/RHE/OR in the same cell lines, further supporting the hypothesis that virus particle release is determined by the 5' domain of the genome. In cell lines in which virus release is hindered, spread of infection may rely more heavily upon cell-to-cell mechanisms, or fusion from within. In contrast, virus infection of cell lines that support high levels of virus release may not be as dependent upon cell-associated mechanisms for

spread of infection. Interestingly, the appearance of syncytia correlates solely with the envelope gene expressed; p5V132R and D2/RHE/OR infected cultures exhibit very few and very small syncytia. In contrast, D2/RHE/OR/V1 and p52R3V1 infected cultures display numerous large, ballooning syncytial structures. The relative capacity to induce syncytia correlates with the efficiency of cell-to-cell transmission of the virus and spread of infection. Infection analyses with the parental molecular clones D2/RHE/OR and D2/RHE/OR/V1, and the chimeric clones p52R3V1 and p5V132R, have demonstrated that the *env* gene alone is not the sole determinant of tropism in T-lineage cells. The ability of the chimeric virus p5V132R to productively infect Hut78 cells clearly demonstrates that, in contrast to the original hypothesis, the *env* gene product of D2/RHE/OR is capable of facilitating entry into a Hut78 cell. Furthermore, these infection analyses have revealed that the titer of released virions may be influenced by the specific cell infected as illustrated by D2/RHE/OR/V1 infection of SupT1 and Hut 78 cells. Therefore, host range is affected by both *env* and non-*env* viral determinants; additionally, host factors may be necessary for interaction with both the envelope glycoprotein in facilitation of virus-cell and cell-cell membrane fusion, and the gag phosphoproteins in promotion of viral release and infectivity.

Future Directions: Are the molecular clones D2/RHE/OR and D2/RHE/OR/V1 pathogenic <u>in vivo</u> ? Ultimately, the molecular clones D2/RHE/OR and D2/RHE/OR/V1 need to be used in transmission studies with rhesus macaques in order to demonstrate whether the in vitro tropic and replicative phenotypes correlate with pathogenic outcome. Whether D2/RHE/OR/V1 represents a second independent infection of the D2/RHE/OR infected macaques, or whether it evolved from D2/RHE/OR and became the sole causative determinant of a more fulminant disease, is not known. The latter explanation is supported by the observation that both variants have never been isolated from the same animal. However, only transmission protocols utilizing specific pathogen free animals with cloned virus could completely eliminate the possibility of a dual role of the two viruses. Throughout a transmission study, blood and tissue samples should be collected and analyzed by PCR and sequence methodologies to determine whether novel genotypes have evolved from the input molecular cloned virus, and if so in which tissue. The reciprocal chimeric viruses described in this

work provide the tools necessary to determine whether pathogenic outcome segregates with the *env* domain or the *gag* gene, or whether it requires both. Further subgenomic chimeras and site directed mutagenesis could also be used if initial transmission results justified further investigation and subsequent use of animals in additional transmission studies. Both D2/RHE/OR and D2/RHE/OR/V1 are single molecular clones of viruses with different pathogenic outcomes, and the possibility exists that neither clone may invoke the expected biological phenotype.

What is the role of titer in D2/RHE/OR infection of Hut78 cells? Inoculum used in the T-cell infection assays was equilibrated by reverse transcriptase (RT) activity, but the presence of RT is not a direct measure of viral infectivity. The actual amount of virus capable of inducing syncytia was not equivalent among the different viral inocula, as measured by end point dilution titration on Raji cells. Therefore, viral titer can not be completely ruled out as a determinant in productive infection. To investigate the ability of the D2/RHE/OR virus to infect Hut78 cells with a high titer inoculum, one could take filtered culture medium from D2/RHE/OR infected SupT1 cells, which are known to release virions at a high titer, and infect Hut78 cells with various dilutions of culture medium. Poor binding affinity of the D2/RHE/OR envelope glycoprotein to the Hut78 receptor, decreased SU on the virus particle or a diminished number of host receptors in the plasma membrane may all contribute to the necessity of a higher titer inoculum for productive infection of Hut78 cells by D2/RHE/OR virus.

Can D2/RHE/OR virus generated from Raji cells enter and integrate into the DNA of a Hut78 cell? The presence of proviral DNA suggests that the block to infectivity or delay in envelope gp20 expression is the result of a post-integration step during viral replication. PCR-analyses of genomic DNA from D2/RHE/OR infected Hut78 cells would reveal the presence of the provirus at different time points during the infection. Additionally, restriction endonuclease digests and Southern blot analysis would indicate the presence of a full-length viral genome. Integration would not be expected to be a problem, as the p52R3V1 and p5V132R chimeric viruses readily infected all cell lines tested and expressed envelope gp20, consistent with the integration of the provirus and expression of subgenomic mRNA from the viral LTR. Moreover, the presence of a full-length provirus

has been demonstrated by restriction digest and Southern analysis from a previous infection of Hut78 with D2/RHE/OR. In contrast, the absence of proviral DNA suggests that a step prior to integration is compromised, such as reverse transcription of viral DNA, transport of the pre-integration complex to the nucleus or integration within the host chromosome. Polymerase chain reaction (PCR) analyses can be utilized to detect the presence of newly synthesized DNA in the cytoplasmic fraction (Hirt supernatant). Presence of new DNA would suggest a possible block in transport of the preintegration complex to the nucleus for integration. Finally, detection of the preintegration complex or unintegrated viral DNA in the nucleus would determine whether a block existed in transport to the nucleus.

What is the role of the gag domain in viral infectivity? Recent evidence has demonstrated that the late (L) domains of RSV, HIV-1 and EIAV are interchangeable in their abilities to promote virus budding and release (Garnier *et al.* 1996; Rickles *et al.* 1994; Xiang *et al.* 1996). The late domains of MPMV and RSV both encode a PY motif (Wills *et al.* 1994; Yasuda and Hunter, 1998). The gag pp18 contains the putative L domain of D2/RHE/OR and D2/RHE/OR/V1, with two striking amino acid residue differences flanking the conserved PY motif located in D2/RHE/OR. The Yes-Yap protein interaction is the basis of the current model for the putative host cell factor(s) which may be involved in late stage virion release (Pirozzi *et al.* 1997; Chen *et al.* 1997). The Yes protein is a tyrosine kinase that is membrane bound and contains a region similar to the Src homology domain 3 (SH3) which binds to proteins with a P-X-X-P motif. The Yes oncogene binds to the Yes-associated protein (Yap) which contains a P-X-X-P domain. In addition to this polyproline region, the Yap also has a separate domain of 38-40 conserved amino acids identified by two distant Tryptophan residues. This binding domain has been identified as the WW domain. Moreover, two WW domain binding proteins (WBP-1 and -2) with conserved PY motifs have been demonstrated to be the ligands of Yap. Interestingly, the late domains of HIV and EIAV are dissimilar to the PY motifs of RSV or MPMV, suggesting that more than one type of host protein may be able to function in virion release (Parent *et al.* 1995). Three lines of evidence provided by: 1) the Yes-Yap-WBP-1 and WBP-2 interactions, 2) the presence of PY motifs in the L domains of RSV and MPMV and 3) the rescue of

L domain function with dissimilar sequence motifs, all point to the role of a host protein as a co-factor in virion release at the host cell membrane. The following genetic analyses may elucidate the role of the 5' domain in viral release and spread.

Which domains of gag pp18 are necessary for virion release and infectivity? Construction of reciprocal chimeric genomes between the pp18 domains of D2/RHE/OR and D2/RHE/OR/V1, containing amino acids 174-205 and the flanking PY motif, would determine whether D2/RHE/OR infectivity could be rescued and D2/RHE/OR/V1 infectivity abrogated (Figure 14; Paper 2: Marracci *et al.*, in preparation). A larger or different range of gag pp18 could be investigated if this line of experimentation does not rescue infectivity of D2/RHE/OR. Additional mutational analysis would identify the specific amino acids critical for infectivity of the virion. A productive infection may then be the result of a change in the identity of a specific amino acid of the gag precursor polyprotein, which may be necessary for a specific interaction with another viral or host protein.

Could the presence of a functional L domain in trans rescue the infectivity of D2/RHE/OR? The specific role of the L domain in D2/RHE/OR infectivity can be demonstrated by the rescue of D2/RHE/OR infection with the use of heterologous L domains. A two-step transfection assay may be used to further evaluate the role of the L domain in virion infectivity and release. The first step would be to transfect cells with a D2/RHE/OR/V1 LTR-ψ(-) *gag* gene construct and select clones which express gag p27. Next, these selected clones would be transfected with full-length D2/RHE/OR DNA to rescue infectious virus for use in our T-cell infection assay. An increased titer of released virions would imply that heterologous procapsids are capable of release, and that the D2/RHE/OR gag pp18 is the defective protein. Alternatively, a homologous procapsid with a functional L domain may be necessary for virion release. Additionally, whether the presence of a heterologous L domain can restore infectivity to D2/RHE/OR, could be determined by substituting the PY motif of D2/RHE/OR pp18 with an L domain from a dissimilar virus (ex: EIAV or RSV). High titer virion release and increase in the kinetics of env gp20 expression would imply heterologous complementation of the L domain function of D2/RHE/OR, suggesting the defect in D2/RHE/OR

infection resides in the L domain. The presence of additional pp18 amino acid residues may be necessary for full restoration of infectivity.

Does covalent modification of the virion by the host cell have a role in viral infectivity? Covalent modification of the L domain of HIV with ubiquitin has been demonstrated (Ott *et al.* 1998). The role of ubiquitin is not known, but its presence in HIV, SIV and Moloney-murine leukemia virus is provocative. Although host proteins have not yet been demonstrated to be copackaged with SRV, identification of a covalently bound host protein in the L domain of SRVs may suggest that these viruses have usurped the host cell trafficking machinery to productively infect the target cell. In contrast, the relationship between ubiquitin and proteosomes may suggest that the virus is in a race between successful replication or degradation, and the presence of ubiquitin in a released virus particle may simply signify that the virus won.

Does the gag nucleocapsid or ψ-site have a role in virion particle infectivity? The diminished titer of released virions after infection with D2/RHE/OR was due to at least two factors: 1) a reduced number of virion particles in the media, or 2) a qualitative difference in the ability of the virion to induce an infection. A 5' fragment domain that may influence assembly and thus the release of infectious virions is the gag nucleocapsid (NC). Two CCHC motifs are present in the NC and are thought to recognize the viral encapsidation signal (ψ) necessary for packaging of the genomic RNA into the procapsid. The NC is identical between D2/RHE/OR and D2/RHE/OR/V1, however the ψ-site differs at its 3' end congruent with the changes found within the *gag* pp24/18 gene. Therefore, the NC may not incorporate the genomic RNA of D2/RHE/OR as efficiently which would result in decreased infectivity of the progeny virions. Sequence analysis of the *gag* gene from D2/RHE/OR infected Hut78 cells would determine whether a nucleotide substitution occurred, suggesting a possible complementary mutation to a defective encapsidation signal. If a mutation in the ψ-site were responsible for the differences in infectivity observed, the p52R3V1 chimeric virus would have been expected to exhibit infection kinetics similar to that of D2/RHE/OR in MT-4 or Hut78 cells, but these infection kinetics were not observed. The possibility that the p52R3V1 chimeric virus would have accumulated a genetic change so rapidly as to induce a productive infection within 10-16 days in MT-

4 and Hut78 cells is unlikely. Therefore, the probability of the encapsidation signal having a role in the depressed titer of released virions is low.

Does the level of transcription or RNA stability play a role in the differences between D2/RHE/OR and D2/RHE/OR/V1? The absence of viral *trans*-acting transcription factors suggests that the type D viral promoter is strong. Promoter strength can be influenced by the presence of enhancer elements which act at a distance from the promoter itself. A type D enhancer element may lay within an untranslated region or overlap a protein coding domain. Thus, a change in the nucleotide sequence, such as that found between D2/RHE/OR and D2/RHE/OR/V1 in the domain of pp24 and pp18, may influence promoter strength versus the function of the protein. The isolation of cellular proteins which bind to this DNA domain can identify factors potentially necessary for transcription; the differential presence of these factors in various cell types can lead to the divergent tropism observed between D2/RHE/OR and D2/RHE/OR/V1. Differences in the level of RNA transcribed or available for translation in the cytoplasm could contribute to pathogenesis by affecting the release of infectious virions. The quantitative measurement of newly transcribed RNA from each viral promoter determines the strength of transcription. In addition, determining the stability of the RNA, by measurement of RNA length, demonstrates whether the nucleotide changes influence the replicative capacity of the virus with regard to protein synthesis and packaging of genomic RNA. Mutational analyses of viruses with altered nucleotides that conserve the encoded amino acid would demonstrate whether the pp24/pp18 domain plays a role in replication at the level of RNA.

What is the role of the <u>env</u> gene in viral infectivity? SIV infections of Hut78 cells have demonstrated the acquisition of truncated TMs in order to induce a productive infection (Kodama *et al.* 1989). In addition, truncation of the TM has been shown to alter the conformation of the SU-TM interaction, making the fusion domain more accessible and increasing the fusogenic potential of the membrane bound envelope glycoprotein (Johnston *et al.* 1998; Spies *et al.* 1994; Zingler and Littman, 1993). The virus-cell fusion event may be different than cell-cell fusion, and thus may require a different concentration of envelope glycoproteins for infection of particular cell lines. The ability of a full-length SIV envelope gene is capable of fusion from within between Hut78 cells, yet could not

productively infect Hut78 cells until a premature stop codon had been introduced that resulted in a truncation of the TM domain. The amount of full-length glycoprotein on the cell surface was decreased relative to the amount of truncated glycoprotein; infection of certain cell lines may necessitate a greater amount of glycoprotein on the surface, suggesting an inefficient interaction with host receptors at low concentrations (Johnston *et al.* 1998). D2/RHE/OR is clearly capable of infecting many different cell lines in vitro with differing kinetics of env gp20 expression. Therefore, the following questions propose to elucidate potential mechanisms of delay in expression and virus spread, which might occur after the envelope glycoprotein binds to the host cell receptor.

Did a mutation occur in the viral genome which may have enhanced D2/RHE/OR viral infectivity in Hut78 cells at the time of envelope gp20 expression? Inoculation of fresh Hut78 cells with culture fluids from D2/RHE/OR infected cells may determine whether a change in the viral genome had occurred. Increased kinetics of envelope gp20 expression would imply that the recovered inoculum may somehow be different than the starting inoculum. Sequencing of PCR generated clones of the *env* gene would determine the domain in which the mutation occurred. If no substitutions can be found in the *env* gene, a second site reversion may have occurred in the *gag* gene or elsewhere.

The potential role of an amino acid change in the *env* gene may depend upon the specific domain altered. For example, reversion of Phe455 in the D2/RHE/OR ISP domain to a Leu or another nonpolar amino acid suggests that SU-TM interactions may be more stable and result in enhanced ability of the virus to interact with host cell receptors (Figure 17; Paper 2: Marracci *et al.*, in preparation). The stability of the SU-TM interaction can be determined by analysis of the relative amounts of precursor envelope glycoprotein on the cell membrane and in the virion membrane versus free gp70 in the culture medium (Brody *et al.* 1994a; Brody and Hunter, 1992).

If the basic Arg found in the membrane spanning domain (MSD) is changed to a hydrophobic residue, it may have altered the conformation of the envelope in the membrane (Figure 17; Paper 2: Marracci *et al.*, in preparation). Perhaps the Arg inhibited the formation of a stable oligomer in the

endoplasmic reticulum thus delaying transport to the plasma membrane or increased degradation of viral glycoproteins.

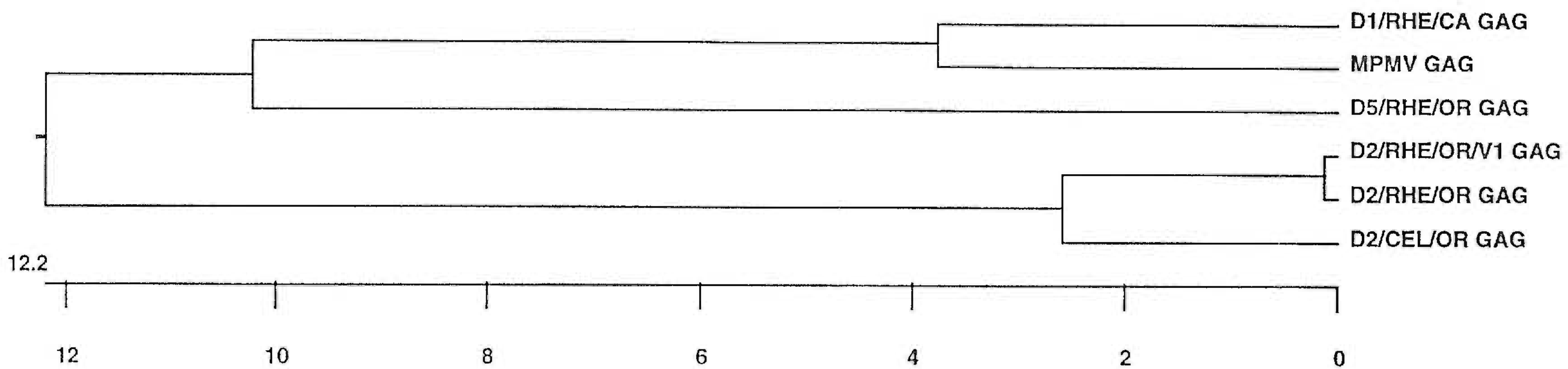
Is the splicing of mRNA different in D2/RHE/OR and D2/RHE/OR/V1 infected Hut78 cells? Perhaps an alternative splice site is utilized which may result in the synthesis of envelope glycoproteins which cannot oligomerize efficiently and are therefore detained in the lumen of the endoplasmic reticulum. Alternatively, formation of heterodimers between the correct envelope precursor and the mutant may either prevent transport to the membrane or reduce efficient receptor binding. Finally, the splice site may be so poorly recognized or utilized by the host cell machinery that very little *env* mRNA is exported for translation. Initially, northern analysis of subgenomic mRNA would elucidate whether D2/RHE/OR *env* mRNA is present at levels equivalent to D2/RHE/OR/V1. The transcription start sites could be determined by using overlapping probes in RNAse protection analyses. N-terminal amino acid sequence analysis would then determine if the envelope glycoprotein generated by D2/RHE/OR was different from D2/RHE/OR/V1, suggesting alternative splicing or signal peptide cleavage as possible mechanisms of the inhibition of expression. The chimeric data do not fully support the alternative splicing hypothesis; if alternative splicing was utilized, one may expect that the p5V132R chimera expression kinetics would be similar to D2/RHE/OR in Hut78 or MT-4 cells, but p5V132R kinetics are intermediate between D2/RHE/OR and D2/RHE/OR/V1. In addition, a *gag* region negative regulator of splicing (*nrs*) has been observed in RSV (Arrigo and Beemon, 1988). Such a mechanism would explain why the p52R3V1 chimera exhibits infection and expression kinetics similar to D2/RHE/OR in MT-4 and Hut78 cells, while the kinetics of p52R3V1 are similar to those of p5V132R. The only difference between D2/RHE/OR and D2/RHE/OR/V1 proximal to a splice acceptor site is the third amino acid codon in the *env* gene. Mutagenesis of this GUC codon to CUC (Val to Leu) may provide insight into the possible role splicing may play in delayed envelope gene expression.

What kinetics of gp20 expression are observed after infection of macaque cells with the different viral clones? By definition, viruses rely upon host cell factors for replication. The same protein may be expressed differently or may support viral replication differently in distinct cell types.

In addition, expression of some cellular factors are regulated by the physiological state of the cell. For example, the stage of differentiation may alter the type or amount of a particular protein present. The same cells from different hosts may vary in ability to support viral replication by the same virus type such that similar cells from different species have altered abilities to support productive virus infections. All the cell lines tested thus far have been of human origin and from different hosts. To better correlate the in vitro infection kinetics of these molecular clones with pathogenesis, determination of infection kinetics in a physiologically relevant in vitro model system will be important. Macaque PBLs from different host animals should be developed and analyzed in similar infection protocols to determine their ability to support productive infections of each of the viral clones.

Conclusion: This body of work represents the first evaluation of all known SRV serogroups at the nucleotide and amino acid level. The examination of these type D genomes has defined the foundation on which subsequent analyses will be built. The isolation and characterization of the molecular clones D2/RHE/OR and D2/RHE/OR/V1, two variants of the same serogroup associated with different pathogenic outcomes, and the construction of their reciprocal chimeric genomes, allowed the first molecular experimentation designed to elucidate which SRV genetic factors determine in vitro tropism and pathogenesis. The observation that the envelope glycoprotein is not the sole determinant of tropism will lead to further studies and identification of additional viral and cellular factors necessary for a productive infection.

Figure 22. Clustal tree based on the nucleotide sequence of *gag* or *env*. The *gag* and *env* genes from molecular clones of representatives of each serogroup of simian retrovirus (SRV) were analyzed for their sequence similarities by the Clustal method of multiple sequence alignment. Serogroups are identified by the D1-5 designation in the virus name, with MPMV belonging to serogroup 3. The figure depicts the results from A) a *gag* gene alignment, and B) an *env* gene alignment. D4/CYN/CA was not available for *gag* gene alignment.


A.
D1/RHE/CA GAG
MPMV GAG
D5/RHE/OR GAG
D2/RHE/OR/V1 GAG
D2/RHE/OR GAG
D2/CEL/OR GAG
12.2
12
10
8
6
4
2
0

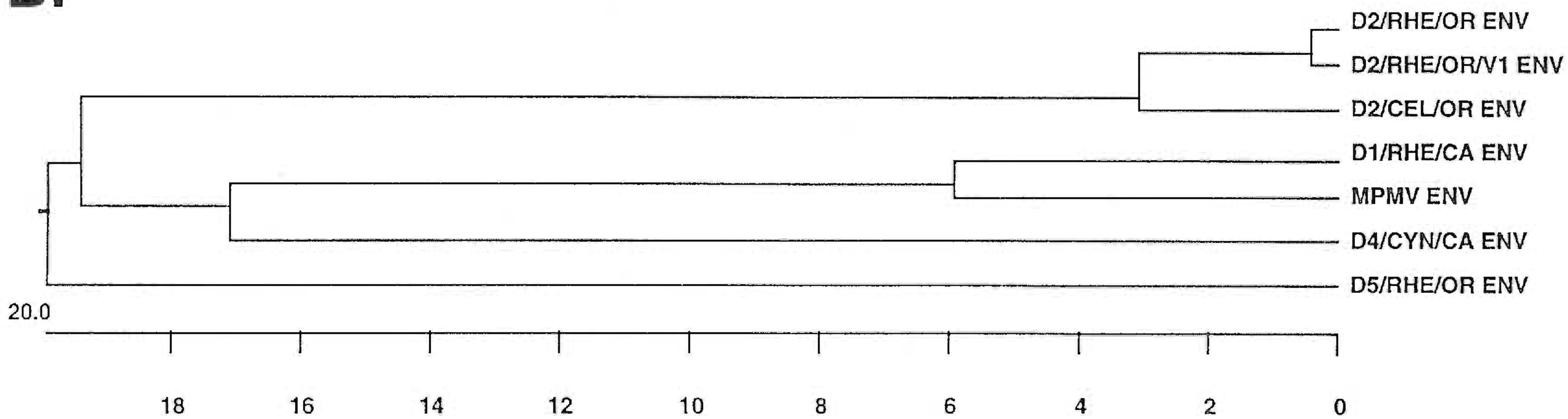
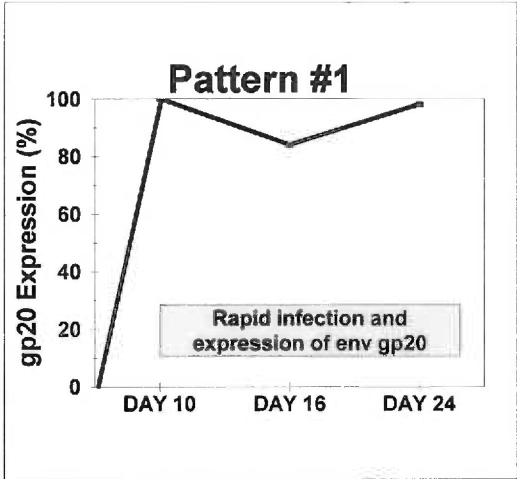
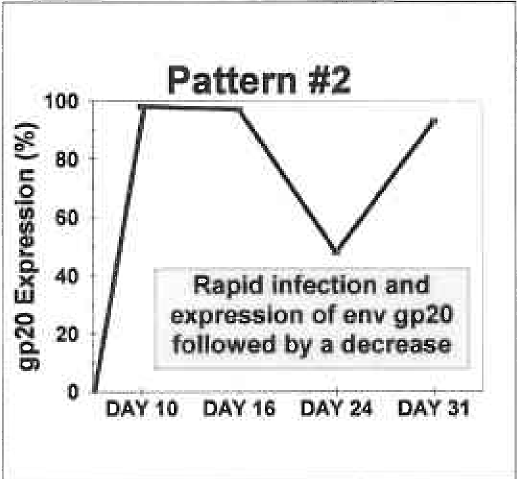

B.
D2/RHE/OR ENV
D2/RHE/OR/V1 ENV
D2/CEL/OR ENV
D1/RHE/CA ENV
MPMV ENV
D4/CYN/CA ENV
D5/RHE/OR ENV
20.0
18
16
14
12
10
8
6
4
2
0

Figure 23. Three models of infection kinetics were observed following infection by D2/RHE/OR and D2/RHE/OR/V1, and their reciprocal chimeras p52R3V1 and p5V132R of a battery of T-cell lines. Top panel, rapid expression of env gp20 after infection; middle panel, rapid expression of env gp20 followed by a decrease and rebound; bottom panel, a delay in env gp20 expression following infection of MT-4 and Hut78 cells by D2/RHE/OR.


Pattern #1
gp20 Expression (%)
100
80
60
40
20
0
Rapid infection and
expression of env gp20
DAY 10
DAY 16
DAY 24


Pattern #2
gp20 Expression (%)
100
80
60
40
20
0
Rapid infection and
expression of env gp20
followed by a decrease
DAY 10
DAY 16
DAY 24
DAY 31

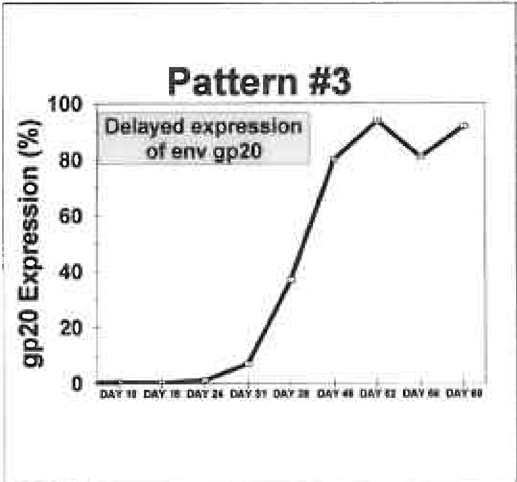
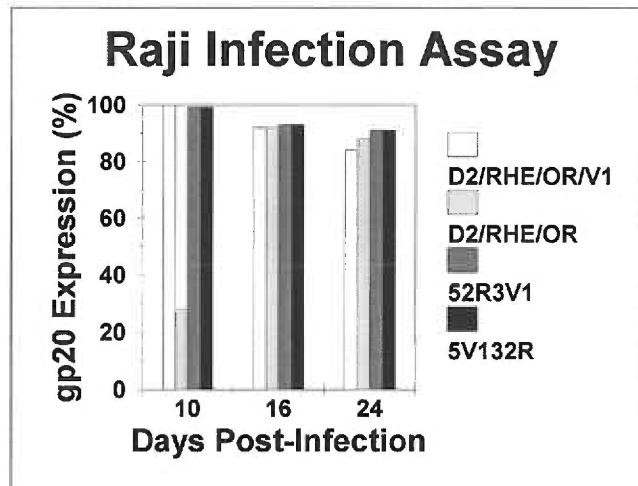

Pattern #3
gp20 Expression (%)
100
80
60
40
20
0
Delayed expression
of env gp20

Figure 24. Infection kinetics representative of pattern 1. Envelope gp20 is detected by day 10 following infection of Raji and CEM-SS cells by D2/RHE/OR, D2/RHE/OR/V1, p52R3V1 and p5V132R.

PATTERN #1 INFECTION KINETICS



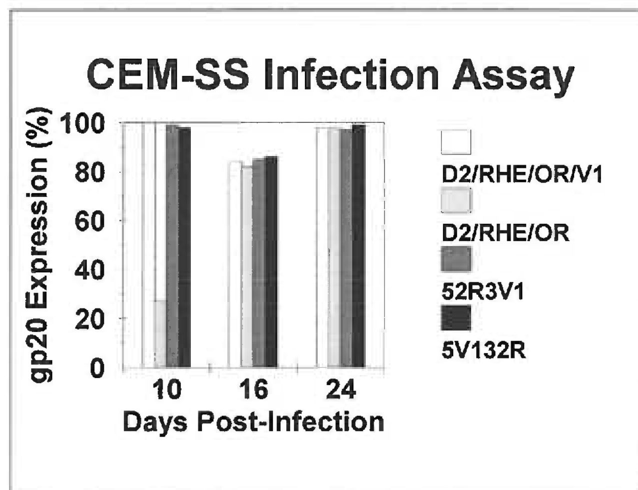
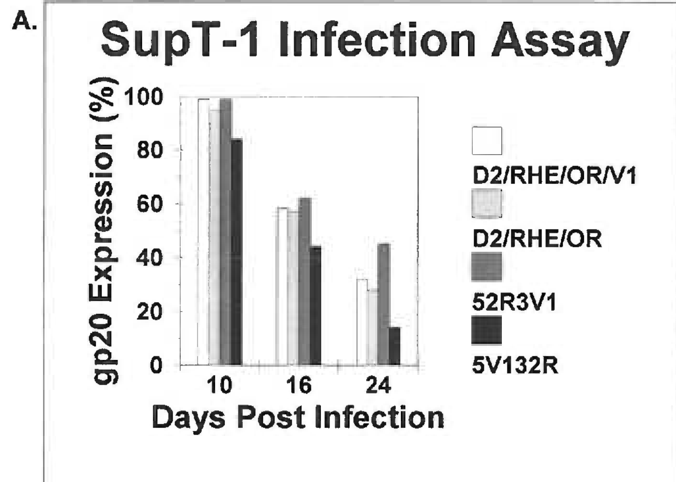


Figure 25. Infection kinetics representative of pattern 2. Following infection of SupT-1 cells with D2/RHE/OR, D2/RHE/OR/V1, p52R3V1 or p5V132R env gp20 is rapidly detected. A drop in the level of envelope gp20 detected is observed from between days 16-24 which is followed by a rebound in expression after continued passage. Panels A) and B) represent data from two independent SupT-1 infection assays.

PATTERN #2 INFECTION KINETICS

A.



B.

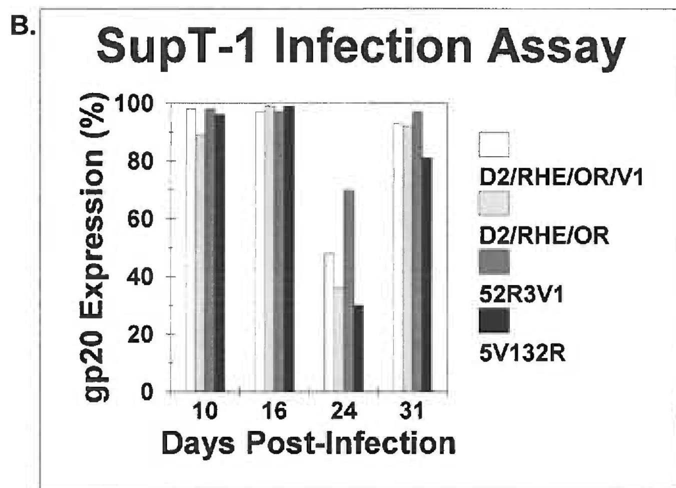
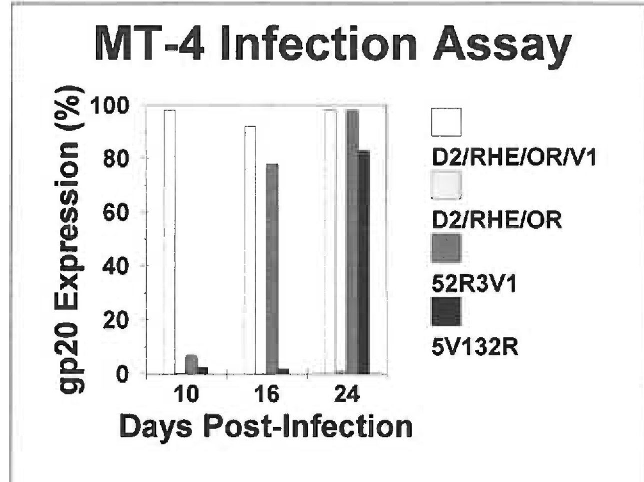
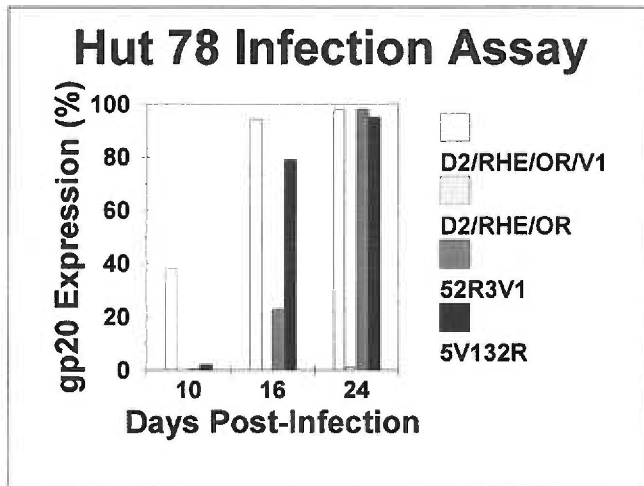


Figure 26. Infection kinetics representative of pattern 3. Infection of MT-4 and Hut 78 cells by D2/RHE/OR results in a marked delay in the ability to detect env gp20. D2/RHE/OR infected MT-4 cells became positive by gp20 analysis after day 31; Hut78 cells were negative for D2/RHE/OR infection up to 60 days after infection (data not shown).

PATTERN #3 INFECTION KINETICS





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By that time you begin to clearly and logically perceive what it is you really want to say. MARK TWAIN

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