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Zidovudine Resistance-Associated Mutations and Sequence Diversity in Brain-Derived HIV-1 Reverse Transcriptase

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ZIDOVUDINE RESISTANCE-ASSOCIATED MUTATIONS AND SEQUENCE DIVERSITY IN BRAIN-DERIVED HIV-1 REVERSE TRANSCRIPTASE

BY

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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To my wife Yu-Wei Qian and daughter Han-Yun Liu

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) infects brain cells such as glial cells, resulting in the development of neurological diseases including HIV associated dementia (HIVD). The reverse transcriptase (RT) is essential for viral replication and thus is a target of antiviral agents such as zidovudine (ZDV). ZDV is the only proven treatment for HIVD currently. However, the appearance of ZDV resistance due to zidovudine resistance associated mutations (ZRAMs) in RT has complicated the treatment. Studies of ZDV resistance on blood-derived HIV-1 isolates have shown an association between ZDV resistance and disease progression. However, little is known about ZDV resistance and occurrence of ZRAMs in brain-derived HIV-1 and whether ZDV resistance plays a role in the development of HIVD is unclear.

To determine the frequency of ZRAM and molecular features of viral mutagenesis in brain-derived HIV-1, I PCR-amplified and sequenced codons 1 through 240 of RT coding region for 10 brain-derived HIV-1 isolates from 10 AIDS patients who were classified into demented (HIVD) [N=5] and non-demented (ND) [N=5] groups. ZRAMs were found in HIV-1 RT from only two patients who received ZDV treatment for 35 and 39 months respectively; one in the HIVD group demonstrating two ZRAMs M41L and F215Y, and the other in the ND group displaying only one ZRAM M41L. None of the other 8 samples had ZRAMs despite the duration of ZDV therapy ranging from 2-30 months. However, 32 other amino acid substitutions relative to the B clade RT consensus sequence were identified in brain-derived HIV-1 RT, among which 24 were unrecognized previously in the established databases. These mutations clustered nonrandomly to several regions, similar to the reference sequences in the established databases.

A lower nucleotide mutation frequency was found in brain-derived RT compared to blood-derived RT sequences, probably due to the lower HIV-1 replication rate in the brain relative to the blood. This may provide an explanation for the lower frequency of ZRAMs in brain-derived RT. In addition, ZRAMs found in blood-derived RT but not in brain-derived RT had a higher mutation frequency than the overall amino acid mutation rate. This might have resulted from a higher selective pressure imposed by ZDV in the blood compared to brain, perhaps due to a higher ZDV concentration in blood than.

To examine the patterns of viral mutagenesis, we compared nucleotide substitutions among brain-derived HIV-1 RT to blood-derived RT sequences. The predominant transversion pattern in the brain-derived RT group was A to C or T nucleotide mutations, which accounted for 25% of total nucleotide mutations, 71% of transversions and 56% of the novel amino acid substitutions. In contrast, A to C or T mutations represented only 8% of total nucleotide mutations in blood-derived RT. The higher percentage of A to C or T nucleotide mutations in the brain-derived RT group may indicate a different intracellular nucleotide pool existing in the brain environment. The fact that A to C or T nucleotide mutations accounted for 56% of the novel amino acid substitutions in brain-derived HIV-1 RT may imply a different influence on molecular features of mutagenesis of HIV-1 imposed by the brain environment, compared to the blood environment.

We conclude that ZRAMs are infrequent in brain-derived HIV-1 despite prolonged ZDV therapy and that RT mutagenesis may influence the development of HIV-1 neurotropism.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

HIV-1	the human immunodeficiency virus type 1
NL4-3	recombinant HIV-1 reference strain
RT	reverse transcriptase
env	gene for HIV-1 envelope proteins
gp120	HIV-1 glycosylated envelope protein with a size of 120 kDa
gp41	HIV-1 glycosylated envelope protein with a size of 41 kDa
Tat and Nef	HIV-1 regulatory proteins
CD4	CD4 molecule, a cellular receptor for HIV
GalC	galactosyl ceramide, a glycolipid receptor for HIV-1 in non-$CD4^+$ cells
AIDS	the acquired immunodeficiency syndrome
HIVD	HIV dementia, HIV-1 associated dementia complex or AIDS dementia complex
ND	non demented
CNS	the central nervous system
CSF	cerebralspinal fluid
PBMC	peripheral blood mononuclear cell
ZDV	1, 3'-α-azido-2', 3'-dideoxythymidine (zidovudine)
ZDV-TP	1, 3'-α-azido-2', 3'-dideoxythymidine triphosphate
CI ZDV	continuous intravenous zidovudine therapy
ddC	2', 3'-dideoxycytidine
ddI	2', 3'-dideoxyinosine
D4T	2', 3'-didehydro-2', 3'-dideoxythymidine
3TC	2'-deoxy-3'-thiacytidine
FTC	2'-deoxy-5-fluoro-3'-thiacytidine
ZRAM	zidovudine resistance-associated mutation
M41L	mutation in RT at codon 41 from methionine to leucine
D67N	mutation in RT at codon 67 from aspartic acid to asparagine
K70R	mutation in RT at codon 70 from lysine to arginine
T215F	mutation in RT at codon 215 from threonine to phenylalanine
T215Y	mutation in RT at codon 215 from threonine to tyrosine
K219Q	mutation in RT at codon 219 from lysine to glutamine
A62V	mutation in RT at codon 62 from alanine to valine
V75I	mutation in RT at codon 75 from valine to isoleucine
F77L	mutation in RT at codon 77 from phenylalanine to leucine
F116Y	mutation in RT at codon 116 from phenylalanine to tyrosine
Q151M	mutation in RT at codon 151 from glutamine to methionine
PCR	polymerase chain reaction
A, C, G and T	deoxyadenine, deoxycytidine, deoxyguanine and deoxythymidine

I. INTRODUCTION

I. 1. HIV and AIDS

The human immunodeficiency virus (HIV) is a lentivirus that causes a variety of diseases known as the acquired immunodeficiency syndrome (AIDS) leading to death. 16 million people in the world have been estimated to be infected by HIV (Caldwell and Caldwell, 1996). Due to its rapid rate of spread, its manner of sexual transmission, and its ability to integrate its genetic information into the genome of infected cells, HIV presents a major challenge to researchers, physicians and other health-related workers throughout the world.

AIDS was first identified among homosexual men and intravenous drug abusers in the early 1980s (Gottlieb *et al.*, 1981; Masur *et al.*, 1981; Mildvan *et al.*, 1982). Later, a human retrovirus was confirmed to be the etiologic agent for AIDS in 1984 (Gallo *et al.*, 1984; Levy *et al.*, 1984; Montagnier *et al.*, 1984) and was named the human immunodeficiency virus type I (HIV-1) by the International Committee on Taxonomy of Viruses in 1986 (Coffin *et al.*, 1986). A second human immunodeficiency virus (HIV-2) was isolated in West Africa in 1986 (Clavel *et al.*, 1986).

I. 1. 1. Viral structure and replication

HIV-1 and HIV-2 are members of the lentivirus subgroup of retroviruses (Gonda *et al.*, 1985; Levy *et al.*, 1985). The HIV-1 virion has a cone-shaped core surrounding a ribonucleoprotein complex containing two identical genomic RNA strands with sense polarity, reverse transcriptase (RT), integrase, and two nucleocapsid proteins. Outside the core is the envelope with 72 protruding knobs containing multimers of the envelope

proteins, the gp120 and the gp41 which are glycosylated proteins (gp) with a size of 120 kDa and 41 kDa respectively (Barker *et al.*, 1995).

The HIV-1 genomic RNA is approximately 9.8 kilobases in size and encodes open reading frames for the structural genes, *gag*, *pol* and *env*, and at least six other genes referred to as the regulatory genes, *tat*, *rev* and *nef*, and the accessory genes, *vif*, *vpr* and *vpu*. In the DNA provirus these genes are flanked by identical long terminal repeat sequences (LTRs). The LTR sequences harbor most of the viral transcriptional regulatory elements and the viral replication start site for reverse transcription of the viral RNA genome (Barker *et al.*, 1995).

HIV-1 enters a cell through an interaction of its envelope proteins with the CD4 molecule, which serves as a cellular receptor (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984). After a series of conformational changes occurring in CD4 and gp120 (Poulin *et al.*, 1991; Sattentau and Moore, 1991), fusion takes place resulting in the release of the viral core into the cell (Sinangil *et al.*, 1988). The fusion may involve co-receptors, for example, fusin (Feng *et al.*, 1996) and CC CKR5 (Alkhatib *et al.*, 1996) found in T cells and macrophages respectively .

The discovery of CD4 molecule as the cellular receptor for HIV infection may explain the preferential growth of HIV in $CD4^+$ lymphocytes (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984). However, in non-CD4 cells, other molecules such as a glycolipid, galactosyl ceramide (GalC), mediate the binding of the gp120 to the brain-derived and bowel-derived cells (Harouse *et al.*, 1991; Yahi *et al.*, 1992). Also, a 260-KDa protein on human fetal astrocytes has been found to mediate the binding of recombinant gp120 to the primary cultures of human fetal astrocytes (Ma *et al.*, 1994).

After the core is released into the cytoplasm, reverse transcription initiated by the enzyme reverse transcriptase (RT), produce a double-stranded DNA copy from the viral genomic RNA. Subsequently, the DNA is transported into the nucleus, where random integration of the DNA into chromosomal DNA takes place, catalyzed by the viral integrase (Whitcomb and Hughes, 1992).

The integrated provirus DNA is transcribed into a full-length primary transcript, which is subsequently processed, resulting in the production of several subclasses of singly-spliced and multiply-spliced viral mRNAs. Each subclass gives rise to a particular subset of viral proteins (Peterlin, 1995).

The viral proteins, together with viral genomic RNA, form the viral capsid (Luciw and Shacklett, 1993). With the incorporation of the viral envelope proteins present on the cell surface, the complete virion is generated and exits cells through budding (Gottlinger *et al.*, 1989; Bryant and Ratner, 1990; Smith *et al.*, 1993).

I. 1. 2. HIV infection

HIV-1 is primarily transmitted through blood and genital fluids (Brookmeyer, 1991). The virus has also been isolated from the cerebralspinal fluid (CSF) (Ho *et al.*, 1985) and semen (Tindall *et al.*, 1992). Infected individuals first present a virus-like illness called acute seroconversion. Following the acute HIV-1 infection, the infected individuals enter a so-called "steady state" without symptoms for a period of many months to many years (Ranki *et al.*, 1987; Ou *et al.*, 1988; Imagawa *et al.*, 1989). Two studies (Ho *et al.*, 1995; Wei *et al.*, 1995) using viral RT and protease inhibitors to perturb the steady state, revealed a surprisingly high rate of HIV-1 replication and turnover of infected cells *in vivo*. On

average, 10^8 virions were produced in the plasma every day and 2×10^9 $CD4^+$ cells were produced and destroyed every day.

The findings of the rapid turnover of plasma virions and $CD4^+$ cells in the infected subjects suggest a possible pathogenic pathway of the infection. The stress imposed on the $CD4^+$ cell production system by HIV-1 infection over several years may alone be sufficient to destroy the body's ability to maintain an effective immune system (Coffin, 1995). However, many other pathogenic mechanisms have also been proposed. These mechanisms include side effects such as apoptosis induced by viral gene products; inappropriate expression of cytokines by infected cells, disrupting immune signaling; indirect effects causing lymphoid degeneration; autoimmunity induced by mimicry between viral components and host factors; and the appearance of viral variants that are more virulent and/or are able to evade the immune response (Levy, 1994).

Besides the effect on immune functions, HIV-1 infection is also associated with diseases in the central nervous system (CNS) (Michaels *et al.*, 1988; Price *et al.*, 1988), the gastrointestinal system (Serwadda *et al.*, 1985; Tindall and Cooper, 1991) and other systems (Withrington *et al.*, 1987; Dean *et al.*, 1988; Cohen *et al.*, 1989; Lipshultz *et al.*, 1990). However, it is not yet clear how HIV-1 plays a role in these disorders.

I. 1. 3. HIV-1 infection in the central nervous system (CNS)

Involvement of the central nervous system in HIV-1 infection is common. Autopsy studies have demonstrated CNS pathology in up to 50 to 70% of cases (Glass *et al.*, 1993). Neurological manifestations may occur in both adults and children at any time during the course of HIV-1 infection (Levy *et al.*, 1986; Gabuzda and Hirsch, 1987; Belman *et al.*,

1988) which include both primary HIV-1-induced illnesses such as aseptic meningitis, vacuolar myelopathy, diverse peripheral neuropathies, and HIV dementia (HIVD), and secondary or opportunistic illnesses caused by other viruses, bacteria and parasites (Johnson *et al.*, 1988).

HIVD (also called HIV-1 associated dementia complex or AIDS dementia complex), which affects 15-20% of all patients with AIDS, is one of the most devastating complications of HIV infection, with a mean survival time of 7 months (McArthur *et al.*, 1993). It is characterized by a clinical triad of cognitive impairment, motor dysfunction and behavioral abnormalities, with presenting symptoms such as memory loss, depressive symptoms, apathy, withdrawal from routine activities, and occasionally psychosis (Navia *et al.*, 1986; McArthur, 1987).

Diagnostic criteria recommended by the American Academy of Neurology for HIVD include: 1) HIV-1 seropositivity; 2) history of progressive cognitive and behavioral decline; 3) neurological and/or neuropsychological evaluation consistent with decline from premorbid baseline; and 4) CNS opportunistic processes excluded by computerized topography (CT) or magnetic resonance imaging (MRI) and CSF analysis (Janssen *et al.*, 1991).

While the pathogenic mechanisms of HIVD remain unknown, many hypotheses have been proposed. These hypotheses can be broadly divided into two: direct mechanisms of injury to effector cells of the nervous system such as neurons; and indirect mechanisms in which the main target of virus infection is the microglia, with secondary neurotoxic effects (Gonzalez-Scarano *et al*, 1995).

The hypothesis of direct mechanisms of HIVD is based on observations of *in vitro* HIV-1 infection of neural cells such as glia and neurons, as well as the presence of viral proteins and proviral DNA in neural cells *in vivo* (Cheng-Mayer and Levy, 1990). Using immunohistochemistry, *in situ* hybridization and more recently *in situ* PCR, investigators have shown HIV-1 infection in microglia, astrocytes, oligodendrocytes and rarely neurons (Wiley *et al.*, 1986; Meyenhofer *et al.*, 1987; Pumarola-Sune *et al.*, 1987; Nuovo *et al.*, 1994). The infection can be a productive infection with expression of viral structural proteins such as the gp41, a restricted infection in which only regulatory proteins such as Nef are detectable, or a latent infection with only proviral DNA being detectable.

However, compared to findings in microglia and macrophages, HIV-1 infection of neurons and oligodendrocytes has been found to be infrequent with limited production of infectious virions and without evident cytopathic changes in most cases. Therefore, questions such as to what extent a minimal infection of these neural cells is capable of inducing dysfunction have been raised to challenge the hypotheses of direct mechanisms.

In contrast, the majority of investigators in this field have hypothesized indirect mechanisms based on observations of the diffuse nature of nervous system injuries, the relatively limited evidence of direct infection of effector cells of the nervous system (Price *et al.*, 1988), and infection of microglia and macrophages found in all studies (Koenig, *et al.*, 1986; Wiley *et al.*, 1986; Price *et al.*, 1988). The damage of neurons or astrocytes could be caused by cytotoxic viral gene products and toxic cellular products released from infected microglia and macrophages.

For example, it has been shown that the gp120 is toxic to neurons (Dreyer *et al.*, 1990; Kaiser *et al.*, 1990; Benos *et al.*, 1994) . Involvement of n-methyl-D-aspartate

(NMDA) receptors and NO synthase in this toxicity has been suggested (Lipton *et al.*, 1991; Dawson *et al.*,1993). Tat has also been demonstrated to be toxic to neurons and astrocytes in primary rodent brain cell cultures (Kolson *et al.*, 1993) and when inoculated intracerebrally into mice (Sabatier *et al.*, 1991). That Nef acts as a neurotoxin has also been suggested based on observations that Nef was able to change normal cellular transmembrane conduction, a phenomenon similar to that caused by scorpion toxin peptide, and that a certain extent of amino acid homology has been found between Nef and scorpion toxins (Werner *et al.*, 1991).

With respect to cellular products, cytokines such as tumor necrosis factor alpha (TNF-α) (Gallo *et al.*, 1989; Matsuyama *et al.*, 1991; Nuovo *et al.*, 1993) and arachidonic acid metabolites (Brouwers *et al.*, 1993; Heyes *et al.*, 1991) have been implicated in HIVD pathogenesis since the levels of all these products have been found elevated in either patients with HIVD or the mixed cultures of astrocytes, macrophages and neurons. All these products can be released from macrophage upon its activation.

Power *et al.* (1994) compared sequences of the V3 and its flanking regions in the gp120 from HIVD and non-demented AIDS patients. They found significant diversity at two positions, 305 and 329 between the two clinical groups, indicating that there are distinct HIV sequences associated with the clinical development of HIVD.

Not only are pathogenic mechanisms of neurological disorders including HIVD uncertain, but how the virus enters the brain also remains a mystery. Studies have shown that macrophages, particularly ones found in perivascular areas, are HIV-1 infected (Koenig *et al.*, 1986; Price *et al.*, 1988). Some investigators believe that these cells are peripheral blood macrophages, since studies in animals have demonstrated that some macrophages

continuously circulate in the CNS during life. Based on these findings, many researchers have concluded that the peripheral blood macrophages brought HIV-1 into the brain via the endothelial cell spaces, cause the CNS pathology (Koenig *et al.*, 1986; Price *et al.*, 1988). However, since activated T cells also continuously circulate in the CNS during life as demonstrated by studies with animals, they also could be the initial source of HIV-1 infection (Wekerle *et al.*, 1986; Hickey *et al.*, 1991). In addition, because the capillary endothelium (Owens *et al.*, 1991; Moses *et al.*, 1993) and astrocytes (Meyenhofer *et al.*, 1987; Pumarola-Sune *et al.*, 1987; Nuovo *et al.*, 1994) have been shown to be susceptible to HIV-1, initial entry by direct viral infection of these cells should be considered.

Although the pathogenesis of HIVD is unclear, treatment with zidovudine (ZDV) appears to improve signs and symptoms in adults with HIVD (Schmitt *et al.*, 1988; Portegies *et al.*, 1989; Sidtis *et al.*, 1993) and performance in children with AIDS-related delayed motor and cognitive development (Pizzo *et al.*, 1988). The observation that HIVD is, in part, reversible, provides optimism in considering therapeutic interventions. At present, clinical trials on other nucleoside analogs, calcium channel blockers and inhibitors of TNF-α synthesis are in progress (McArthur J., personal communication).

I. 2. Zidovudine (ZDV) and its viral target

Most steps in the HIV-1 life cycle, from the binding of the virus to target cells to viral budding, could serve as a target for antiviral intervention. Antiviral drugs can exert their effects by interacting with viral receptors, enzymes, structural components, genes or their transcripts, or cellular factors required for viral infection (Mitsuya *et al.*, 1990).

The therapeutic agents could take many different forms such as vaccines which stimulate immune responses directed at viral or cellular proteins; chemical compounds which are enzyme inhibitors; cytokines which inhibit HIV-1 replication directly and/or stimulate cell-mediated immune response destroying HIV-1 infected cells; and genetic antivirals which are transferred as DNA or RNA into cells and act on their intracellular targets either directly or after expression as RNA or proteins (Mitsuya *et al.*, 1990; Dropulic and Jeang, 1994).

To date, only chemical compounds which are either RT inhibitors or viral protease inhibitors are officially licensed for clinical prescriptions. RT inhibitors can be divided into two classes: nucleoside analogs and non-nucleoside RT inhibitors (NNRTIs).

I. 2. 1. ZDV and its antiviral therapy

ZDV, the abbreviated form for 1, 3'-α-azido-2', 3'-dideoxythymidine, is the first drug approved for clinical use. It is a nucleoside analog, belonging to a broad family of antiretroviral 2', 3'-dideoxynucleosides which includes at least three other licensed drugs for HIV-1 infection, 2', 3'-dideoxyinosine (ddI), 2', 3'-dideoxycytidine (ddC) and 2'. 3'-didehydro-2', 3'-dideoxythymidine (D4T). ZDV undergoes phosphorylation catalyzed by cellular enzymes in the cytoplasm to generate ZDV-5'-triphosphate (ZDV-TP), which competes with deoxythymidine 5'-triphosphate (dTTP), the normal substrate for viral DNA synthesis. The incorporation of ZDV is believed to terminate DNA-chain elongation, however, the exact mechanism of ZDV action is unclear (Mitsuya *et al.*, 1990).

ZDV was first shown to block HIV-1 replication in T cells (Mitsuya *et al.*, 1985; Mitsuya *et al.*, 1986), followed by studies showing that it could also suppress HIV-1

replication in monocytes and macrophages *in vitro* (Perno *et al.*, 1988). Based on the *in vitro* data, in July of 1985, ZDV was first administered to individuals with severe HIV-1 infection (Yarchoan *et al.*, 1986; Yarchoan *et al.*, 1987; Fischi *et al.*, 1987). The clinical trials demonstrated that patients with AIDS underwent immunological, virological, and clinical improvement during the therapy with ZDV. In 1987, ZDV was approved as a prescription drug for HIV patients .

In general, ZDV prolongs the survival, improves the quality of life of individuals with advanced HIV infection, and delays clinical progression in certain asymptomatic individuals with HIV infection (Yarchoan *et al.*, 1987; Fischi *et al.*, 1987; Fischi *et al.*, 1990). It has also been shown that ZDV treatment improved or delayed certain HIV-associated neurological symptoms in both adults and children with HIV infection (Pizzo *et al.*, 1988; Schmitt *et al.*, 1988; Portegies *et al.*, 1989; Sidtis *et al.*, 1993).

I. 2. 2. HIV-1 reverse transcriptase (RT): the target of ZDV therapy

HIV-1 RT is produced by translation of the full-length primary viral transcript into Gag-Pol polyprotein, followed by proteolysis of the Gag-Pol polyprotein by viral protease into Gag proteins and three viral enzymes including RT (Hottiger and Hubscher, 1996). RT has three distinct enzymatic activities: an RNA-dependent DNA polymerase activity involved in the synthesis of the minus strand of the proviral DNA from genomic RNA; a DNA-dependent DNA polymerase activity that catalyzes the synthesis of the plus DNA strand using the minus strand as the template; and an RNase H activity. The RNase H activity is responsible for the degradation of the RNA portion of the RNA-DNA hybrid, generating the RNA primer used for the synthesis of the plus DNA strand. The RNase H is

also involved in the final removal of the tRNA primer served to initiate the minus strand synthesis (Hottiger and Hubscher, 1996).

RT has two subunits: a 66 kDa subunit (p66) and a 51 kDa subunit (p51). The p66 subunit has 560 amino acids, with DNA polymerase activities residing in the amino-terminal 440 amino acids and RNase H in the carboxy-terminal 120 amino acids. The p51 subunit, which is formed following truncation of the carboxy-terminal 120 amino acids by the viral protease, contains only DNA polymerase activities (Hansen *et al.*, 1987; Larder *et al.*, 1987). Although it has been shown that recombinant p66/p66 homodimer has DNA polymerase activities in *in vitro* experiments (Restle *et al.*, 1990; Hottiger *et al.*, 1994), it is the p66/p51 heterodimer that functions *in vivo* (Richter-Cook *et al.*, 1992). Based on three-dimensional structural studies of the p66/p51 heterodimer (Wang *et al.*, 1994), it is believed that p66 and p51 are distinct structurally and functionally and only the p66 subunit in the heterodimer has the DNA polymerase activity (Kohlstaedt *et al.*, 1992), and that RNase H is responsible for the removal of the RNA template (Tanese and Goff, 1988). Biochemical studies together with molecular modelling (Larder *et al.*, 1987; Poch *et al.*, 1989; Delarue *et al.*, 1990; Boyer *et al.*, 1994) suggest that side chains of the amino acids 65-74 of p66 interact with the single-stranded template; side chains of the amino acid residues of 110-117, 160-161, 183-186 and 219-221 in p66 are involved in forming the nucleotide-binding site; the amino acids 230-231 and 183-185 interact with the 3' nucleotides of the primer strand. Site-directed mutagenesis studies revealed that when amino acid residues 183- 186 (Tyr-Met-Asp-Asp or YMDD), which is the most conserved element among RTs of HIV-1 and other lentiviruses (Kohlstaedt *et al.*, 1992; Jacobo-Molina *et al.*, 1993), were mutated, HIV-1 RTs were inactive, indicating that the element is the active site of the enzyme

(Larder *et al.*, 1989b; Boyer *et al.*, 1992). The amino acid near the active site include residues 74, 78, 89, and 151-152, which are involved in gripping the template (Larder *et al.*, 1987; Poch *et al.*, 1989; Delarue *et al.*, 1990; Boyer *et al.*, 1994).

Although HIV-1 RT has DNA polymerase activities and RNase H activity, it lacks a 3'→5'exonuclease proof reading mechanism, resulting in a high mutation rate in HIV-1 replication (Preston *et al.*, 1988). When measured *in vitro*, the misincorporation rate of HIV-1 RT is 1 in 1700 to 1 in 4000 nucleotides which is 10- to 100-fold higher than that of bacterial and mammalian DNA polymerases, or 5 to 10 misincorporations per HIV genome per replication *in vivo* (Preston *et al.*, 1988; Roberts *et al.*, 1988; Preston and Gavey 1992) The high mutation rate contributes to viral sequence polymorphisms which affect many aspects of HIV-1 infection such as cellular tropism, kinetics of viral replication and extent of viral production, viral cytopathicity, serological properties, cellular host range and transmission (Levy, 1994).

I. 3. ZDV resistance

Although ZDV treatment provides a benefit to certain patients, emergence of ZDV-resistant HIV variants has posed a challenge to ZDV treatment. In fact, to date, HIV-1 mutants with partial or complete resistance have been found for all compounds used or seriously considered as therapy for HIV-1 infection (Coffin, 1995). The extraordinary dynamics of HIV-1 replication, the lack of proofreading function by HIV-1 RT, and the chronic administration of antiretroviral agents needed for HIV-1 infection, all contribute to the appearance of the resistant viruses .

I. 3. 1. ZDV resistance associated mutations (ZRAM)

As the use of ZDV became more widespread, Larder, et al (1989a) reported the first HIV-1 strains with reduced sensitivity to ZDV which were isolated from patients treated with ZDV. In this study, susceptibility to ZDV of 46 HIV-1 strains isolated from 33 individuals with AIDS was measured. Fifty percent inhibitory dose (ID_{50}) values of 18 strains isolated from untreated patients ranged between 0.01 to 0.05 μM. In contrast, most of the isolates obtained from patients receiving a 6-month or longer course of therapy demonstrated increased ID_{50} values. Some of them showed high-level resistance with a 100-fold decrease in susceptibility (ID_{50} = 1 to 5 μM). By comparing RT sequences of ZDV-sensitive isolates from untreated patients with those of ZDV-resistant mutants, subsequent studies (Larder and Kellam, 1989; Kellam *et al.*, 1992) identified five amino acid substitutions in the RT [Met^{41}→Leu (M41L), Asp^{67}→Asn (D67N), Lys^{70}→Arg (K70R), Thr^{215}→Phe or Tyr (T215F or T215Y), and Lys^{219}→Gln (K219Q)] which were common to all or many of the resistant strains. They were designated as Zidovudine resistance associated mutations (ZRAMs).

Since then, many other studies have also identified ZDV-resistant HIV strains isolated from the blood and other organs of the body (Mayers *et al.*, 1992; Stefano *et al.*, 1993; Wildemann *et al.*, 1993; Erice and Balfour, 1994; Sei *et al.*, 1995; Stefano *et al.*, 1995). Some of them have found one or several of those five ZRAMs described above, others identified some other mutations (A62V, V75I, F77L, F116Y, and Q151M) in RT region of multidrug-resistant HIV-1 strains resulting from ZDV/ddI combination therapy (Iversen, *et al.*, 1996). The association of these mutations with ZDV resistance was confirmed by site-directed mutagenesis studies in which constructed infectious molecular

clones of HIV-1 containing these mutations were shown to be resistant to ZDV *in vitro* (Larder *et al.*, 1991; Kellam *et al.*, 1992; Iversen, *et al.*, 1996).

I. 3. 2. Methods for measurement of ZDV susceptibility

Laboratory methods used for measurement of ZDV susceptibility in HIV can be generally divided into two classes: phenotypic and genotypic. Phenotypic assays measure 50% inhibitory concentration (IC_{50}) or 90% inhibitory concentration (IC_{90}) to define if an HIV strain is sensitive or resistant to ZDV. Under this category there are two methods which are widely used for resistance studies.

The principally used assay is based on the inhibition of plaque formation by ZDV in monolayers of HeLa $CD4^+$ cells infected with cell-free HIV stocks (Chesebro *et al.*, 1988; Larder *et al.*, 1989a). IC_{50} values are derived from plots of the percentage of plaque reduction versus the concentration of ZDV by comparing the untreated control. However, this method is restricted to syncytium-inducing HIV strains, which account for about 30% of clinical isolates.

The other assay uses PBMCs from healthy donors for viral infection and IC_{50} values are determined by nonlinear regression analysis on viral antigen measurement. This method permits evaluation of 80%-95% of the clinical HIV strains. However, it is a time-consuming, labor-intensive and expensive protocol (Japour *et al.*, 1993).

Genotypic methods are used to identify mutations in the RT-coding region of HIV that have been found to be associated with different degrees of susceptibility to ZDV. These methods include PCR-based assays (Gingeras *et al.*, 1991; Larder *et al.*, 1991; Richman *et*

al., 1991; Jung *et al.*, 1992), RNase A mismatch cleavage techniques (Lopez-Galindez, *et al.*, 1991), and RNA-RNA hybridization systems (Japour *et al.*, 1991).

PCR-based methods are most frequently used by investigators in this field. First, a fragment of the RT-coding region encompassing the regions containing ZRAMs is amplified from HIV sequence, either proviral DNA or cDNA converted from mRNA, following which ZRAMs can be identified by two methods: 1) another PCR reaction is set up with the amplified product serving as the template and with primer pairs specifically designed to amplify either wild-type or mutant sequences at the desired codons (Larder *et al.*, 1991; Richman *et al.*, 1991); 2) alternatively, the PCR-amplified fragment is analyzed by sequencing either directly or after being cloned into a plasmid vector (Mayers *et al.*, 1992).

I. 3. 3. The level of resistance is proportional to the number of ZRAMs

While a single ZRAM renders an HIV strain a certain level of resistance to ZDV, the presence of multiple ZRAMs in clinical isolates has been found to be associated with high-level resistance to ZDV although the levels are different with different ZRAM combinations (Richman *et al.*, 1991). In general, ZRAMs are considered to be markers for predicting ZDV resistance.

It has been found that susceptibility to ZDV was four-fold lower in clones containing a single mutation at codon 41, when compared to the wild type; 16-fold lower in clones with a codon 215 mutation; 31-fold lower with three ZRAMs at codons 67, 70 and 215; 179-fold lower with four mutations at codons 41, 67, 70, and 215 (T→F); 147-fold lower with four mutations at codons 67, 70, 215 (T→F), and 219 (Larder *et al.*, 1991).

In another study, infectious HIV-1 clones were constructed, containing single mutation or different combinations of mutations (Shafer, *et al.*, 1994). IC_{50} value for a construct with single mutation Q151M was increased 30 times compared to the parental wild-type strain NL4-3, 40 times higher for a construct with three mutations, V75I, F77L and Q151M, and 200 fold increased when four amino acids were substituted at codons 75, 77, 116, and 151.

However, the coexistence of ZRAMs does not always result in a higher level of resistance. Instead, observation of ZDV resistance suppression has been made between ZRAMs. The coexistence of mutations K70R and T215Y yielded a virus strain with a lower level of resistance (an IC_{50} value of 0.06 μM) than those of viruses with a single mutation (0.08 μM for K70R alone and 0.16 μM for T215Y alone) (Kellam *et al.*, 1992 and 1994).

I. 3. 4. Temporal appearance of ZRAMs

In general, a single ZRAM may occur at any codon of ZRAMs, followed by a progressive but slow appearance of additional ZRAMs during therapy. In one study, the first ZRAM occurred at codon 70. However, the occurrence is transient and the mutation at codon 70 was soon replaced by the appearance of a mutation at codon 215 (Boucher *et al.*, 1992b). In some cases, the codon 70 ZRAM reappeared later. Then a mutation at codon 41 occurred after the appearance of the mutation at codon 215 but before the reappearance of codon 70 ZRAM (Kellam *et al.*, 1992). However, in another study, a different temporal order of appearance of ZRAMs was observed with a mutation at codon 215 appearing first followed by occurrence of mutations at codons 70, 67, and 219 sequentially (Richman *et al.*, 1991). Shirasaka, et al. (1995) monitored sequentially isolated samples along a time course

of one year from two patients who received combination therapy with ZDV and ddI. They found that Q151M developed first, followed by F116Y and F77L mutations. Ultimately, five mutations A62V, V75I, F77L, F116Y, and Q151M appeared in the same isolates. Although appearance of ZRAMs in temporal patterns is suggested by some studies, different patterns have been found in different studies, and there always were some exceptions in each study.

I. 3. 5. Diverse HIV populations coexist in patients during therapy with ZDV

The coexistence of ZDV-resistant and ZDV-sensitive HIV strains in the same patient has been demonstrated not only by phenotypic but also by genotypic assays (Larder *et al.*, 1989a; Richman *et al.*, 1991). The coexistence of wild-type and mutant sequences at codon 215, as well as the presence of different mutants (T215Y and T215F) in the same specimen have been reported (Richman *et al.*, 1991; Mayers *et al.*, 1992). In addition, different genotypes of HIV have been found in plasma and peripheral blood mononuclear cells (PBMCs) from the same patients sampled at the same time (Kozal *et al.*, 1993; Smith *et al.*, 1993).

I. 3. 6. Clinical significance of ZDV resistance

Richman *et al.* (1990) showed that after 12 months of treatment with ZDV, isolates from 89% of patients with late-stage disease were resistant to ZDV and 33% showed high-level resistance. In contrast, isolates from only 31% of individuals with early-stage disease were ZDV-resistant. Similarly, ZDV-resistant virus was more likely to be isolated from patients with low CD4 cell counts. The estimated rates of resistance after 1-year therapy

with AZT were 27%, 41%, and 89% for CD4 cell counts of >400/mm^3, 100-400/mm^3, <100/mm^3, respectively.

In another study (Tudor-Williams *et al.*, 1992) involving 19 children who received 9-39 months of monotherapy with ZDV, the correlation between *in vitro* susceptibility and clinical outcome was highly significant. IC_{50} values were higher for isolates from 10 children with disease progression than for isolates from 9 children who remained in stable condition. Children who harbored resistant viruses had lower CD4 cell counts and higher concentrations of viral antigen in serum.

Furthermore, Shirasaka, *et al.* (1995), showed a correlation between the development of resistance mutations and viremia level. At the time when the viremia level suddenly increased, a single mutation Q151M was identified. When five resistance mutations developed, including A62V, V75I, F77L, F116Y, and Q151M, the viremia level rose even further.

An early study (Larder *et al.*, 1989a) on ZDV resistance found no clear temporal relationship between the development of partial resistance and disease progression. Instead, it was found that the development of highly resistant strains were not required for disease progression. Another study (Najera *et al.*, 1995) identified ZRAMs in RT-coding regions of HIV-1, isolated from subjects who received no ZDV treatment.

The more recent studies (Richman *et al.*, 1990; Tudor-Williams *et al.*, 1992; Shirasaka, *et al.*, 1995) indicate an association between the resistance and the clinical outcome. However, resistance studies may be complicated by other factors such as the viral load (Kozal *et al.*, 1993), the virulence of viral strain with the SI phenotype (St. Clair *et al.*, 1993), and the host genetic background (Tersmette *et al.*, 1989; Boucher *et al.*, 1992a).

I. 3. 7. Molecular mechanism of ZDV resistance

Investigators have showed that the K_i value of RT for ZDV-TP from a posttherapy strain with resistance mutations at codons 62, 75, 77, 116, and 151 was 35-fold higher than that of RT from a pretherapy strain (Shirasaka, *et al.*, 1995). Analyses of the three-dimensional structure of RT revealed that several of these five mutations are located close to the proposed dNTP-binding site of RT and the first nucleotide position of the single-stranded template, suggesting that the mutations may affect the ability of RT to bind to ZDV.

Similarly, in another study (Larder and Kemp, 1989), the possible mechanism of resistance mutations at codons 67, 70, 215 and 219 has been examined. These four residues are also located in the amino-terminal domain of RT, which is believed to be responsible for nucleotide recognition and polymerase function, suggesting that mutations at these codons might affect the abilities of RT to accommodate the 3'-azido group of ZDV and to recognize the authentic 3'-OH group of dNTP. However, no differences were shown in the susceptibility of RT to ZDV-TP between RT from ZDV resistant HIV-1 strains and RT from an ZDV sensitive isolate. Also, the Michaelis constant (K_m) value for dTTP and inhibition constant (K_i) for ZDV-TP were similar when purified RT from resistant and sensitive isolates was compared. The values of K_m and K_i for purified recombinant RT from resistant and sensitive isolates also did not differ significantly.

Furthermore, others have shown that although two mutated RT enzymes constructed by site-directed mutagenesis showed reduced sensitivity to inhibition by ZDV-TP, recombinant infectious viruses containing the mutated RT displayed hypersensitivity to ZDV instead of resistance, when tested in culture (Larder, *et al.*, 1989b). This study

indicated that the phenotype of RT *in vitro* could not reliably predict the phenotype of HIV-1 containing the RT. Therefore, although some attempts have been made to reveal molecular mechanism of resistance, the mechanism is far more complicated than expected and is still not fully understood.

I. 3. 8. ZDV resistance in HIV-1 derived from the CNS

The majority of resistance studies have been done on HIV-1 isolated from the blood. There is very limited data obtained from studies on CNS-derived HIV-1. One study (Stefano, *et al*, 1993) monitored paired blood and CSF isolates from four patients. ZRAMs identified in CSF isolates were the same as those in blood isolates from three of the patients received 12 to 29 months of ZDV treatment, whereas both CSF- and blood-derived HIV-1 strains from the other patient exposed to ZDV for 27 months remained the wild-type.

In another study (Wildemann, *et al*, 1993), RT coding region of HIV-1 isolated from blood and CSF of three patients was sequenced. These patients had progressed to AIDS under long-term ZDV treatment. ZRAMs have been identified in all samples. Positions and frequencies of mutations in CSF-derived RT were comparable to those from blood.

In Stefano et al's study (1995), RT sequences of HIV-1 were analyzed for sequential isolates obtained from blood and CSF of six patients undergoing ZDV therapy for a time period ranging from 1 to 3 years. Isolates from the blood and CSF of one patient showed two identical ZRAMs (at codons 70 and 215). Although isolates from four other patients presented a different pattern of ZRAMs in the two compartments, the percentages of amino acid variations were approximately equal for isolates from the same or different

compartments. HIV from both the blood and CSF of the remaining patient showed the wild type.

These studies indicated that ZRAMs in CSF developed in a manner similar to that for HIV-1 in blood. In addition, many studies demonstrated that features of blood-derived HIV-1 such as cellular tropism were different from those of isolates obtained from brain tissues (Cheng-Mayer *et al.* 1989; Cheng-Mayer and Levy, 1990). Thus, data derived from CSF HIV-1 may not reflect ZRAM development in brain-derived HIV-1.

In the literature, there is only one ZDV resistance study on brain-derived HIV-1 (Sei, *et al.*, 1995). In this study, only amino acid residue at codon 215 in RT coding region was monitored by using selective PCR to amplify proviral DNA isolated from brain tissues (cerebrum and/or cerebellum) and lymphoid tissues (lymph nodes and/or spleen) of 10 patients. Eight were child patients aged 11 months to 12 years. Six patients (aged 11 months to 11 years) received continuous intravenous (CI) ZDV and the remaining four took ZDV orally. By selective PCR amplification, the amino acid residue was identified as one of three existing forms: the wild type, mutant, or a mixture of the wild type and mutant. Among six patients who received CI ZDV, brain-derived HIV from three patients showed a mutant population, whereas their lymphoid tissue counterpart had a mixed population of the wild type and mutant; another patient displayed mixed HIV population in the brain but had the wild type in lymphoid tissues; the remaining two patients had a mixed HIV population in both the brain and lymphoid tissues. In contrast, among four patients who took ZDV orally, two displayed the wild type HIV in their brain but a mixed HIV population in their lymphoid tissues; another one had a mixed HIV population in the brain but mutant in the lymphoid tissues; mixed HIV populations were detected in both the brain and lymphoid

tissues of the remaining patient. In this study, it seems that patients who received CI ZDV had more mutant HIV in their brains than in their lymphoid tissues, meanwhile, patients who took ZDV orally were the opposite, that is, more mutants in the lymphoid tissues than in the brains. However, the appearance of the ZDV resistance mutation in brain-derived HIV versus lymphoid tissue-derived HIV might be also associated with patients' age and clinical status.

I. 4. Rationale and aims of the present study

As discussed above, little is known about the occurrence of ZRAM in brain-derived HIV. Given that HIV-1 primarily replicates in macrophage-like cells in brain (microglia), we expect that the frequency of ZRAM may be low in brain-derived HIV-1 as the viral replication rates in macrophages tend to be lower than in PBMC's or T cell lines. In addition, it is unknown whether the occurrence of ZRAMs is associated with any defined clinical syndrome but their occurrence seems to be associated with disease progression. Hence, it is important to know whether ZRAMs occur in brain-derived HIV-1 and if they are associated with clinical disease such as HIVD because these findings will have direct implications on the treatment of patients with HIV-1 induced neurological disease. Finally, the pattern and rate of HIV-1 mutagenesis in brain in a clinically well-defined population has not been examined. Thus, I have elected to examine the following specific aims in this thesis:

Specific aim 1: To determine the frequency of ZRAM occurrence in brain-derived HIV-1 from clinically well-defined patients treated with ZDV and to examine the

occurrence of ZRAMs in brain-derived HIV-1 in relationship to the clinical phenotype, HIVD.

For this purpose, PCR amplification and sequencing were used on the RT coding region from codon 1 to 240, which covers the previously reported ZRAMs, from brain-derived HIV-1 isolates.

Specific aim 2: To determine the extent to which brain-derived HIV-1 mutagenesis differs from HIV-1 derived from other tissues.

For this purpose, spleen-derived HIV-1 samples were PCR amplified and sequenced for the same RT coding region. These spleen-derived HIV-1 isolates were paired with brain-derived HIV-1 samples, isolated from the same individuals.

I. 5. Hypothesis

My central hypothesis is that although HIV-1 may replicate in brain more slowly than other organs such as blood, because of the cellular environment within the brain which is immunologically privileged, HIV-1 mutagenesis in the brain is distinct and may influence viral neurotropism.

II. MATERIALS AND METHODS

II. 1. Patient selection

Brain tissue (subcortical white matter) samples were acquired from the AIDS Brain Bank at Johns Hopkins University, which contains frozen and fixed brains from over 300 autopsied patients with AIDS who were characterized clinically by the AIDS Neurology Group, prior to death (McArthur, 1987; Glass, *et al.*, 1993). 36 samples from 36 patients (one sample/patient) were selected based on patients' clinical status and duration of ZDV therapy (described below in Section 1 of Result). The patients were defined as nondemented (ND) and demented (HIVD). Diagnostic criteria for HIVD were those recommended by the American Academy of Neurology (Janssen *et al.*, 1991). The severity of HIV dementia was assessed according to Memorial Sloan-Kettering [MSK] dementia rating scale (Price and Brew, 1988).

II. 2. Preparation of cDNA from frozen brain tissue

To avoid selection bias by *in vitro* viral isolation, we used cDNA synthesized from mRNA, obtained directly from the autopsied brains of the patients, to examine the RT region encoding codons from 1 to 240. The mRNA samples were selected because they reflected replicating viruses and they were also used for analyses of brain-derived *env* sequences which were available for comparison of molecular features of mutagenesis between RT and *env* sequences. Total RNA was prepared according to Wesselingh, *et al.* (1993) from the frozen brain tissues. Briefly, brain tissue was homogenized in cold 4.0 M guanidinium thiocyanate, 0.1 M Tris·Cl (pH 7.5), 0.7% β-mercaptoethanol and DNase. Total RNA was extracted by cesium chloride ultracentrifugation. After phenol, chloroform,

and ethanol washes, approximately 5 μg of total RNA was used for complementary DNA (cDNA) synthesis. First-strand cDNA synthesis was primed with oligodeoxythymidine and carried out for 40 min at 42°C in a 20-μl reaction containing 50 mM Tris·Cl (pH 8.3) and 20 units of avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim, Indianapolis, IN). One cDNA sample was synthesized per mRNA sample.

II. 3. Amplification of HIV-1 RT from cDNA by polymerase chain reaction (PCR)

Because sufficient DNA for cloning and sequencing could not be obtained by using one round of PCR, a nested PCR protocol was used (Chesebro *et al.*, 1992). The first PCR was carried out with primers P1 and 2851C (Table 1) (Wildemann *et al.*, 1993) at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 40°C for 1 min and 72°C for 2 min, plus one cycle at 95°C for 1 min, 40°C for 1 min and 72°C for 10 min, in a reaction volume of 25 μl containing 0.2 μM primers, 2 μl of cDNA, 0.625 U of *Taq* polymerase, and buffers. The negative control reaction was set up containing 2 μl of H_2O instead of cDNA. The positive control reaction contained 2 μl of plasmid DNA which is a recombinant chimeric infectious HIV-1 clone, NL4-3, whose 5' half of the genome came from the HIV-1 clone NY5 and 3' half came from the clone BRU. Since this reaction produced no visible band on the agarose gel, a second PCR reaction was carried out with primers P2 and P4 (Table 1) (Wildemann *et al.*, 1993) at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 50°C for 1 min and 72°C for 2 min, plus one cycle at 95°C for 1 min, 50°C for 1 min and 72°C for 10 min in a reaction volume of 25 μl containing 0.2 μM primers and 2 μl of PCR product from the first PCR. The buffers and *Taq* polymerase were the same as those in the first PCR.

Table 1. Primers used in PCR amplification

Primer	Sequence (5'→3')	Orientation	start position away from the first codon
P1	gtacagtattagtaggacct	upper-strand	80 nucleotides upstream
2851C	tgacgtcgactcattgacagtccagct	lower-strand	766 nucleotides downstream
P2	cacctgtcaacataattggaaga	upper-strand	59 nucleotides upstream
P4	actgtccatttatcaggatg	lower-strand	721 nucleotides downstream

II. 4. Purification of PCR amplified DNA fragments

The purification of the amplified DNA was carried out using Prep-A-Gene DNA Purification Kit (Bio-Rad, Richmond, CA, USA) according to the manufacturer's recommendations. The purified DNA was measured on a spectrophotometer at 260 nm and DNA concentration was calculated using the following formula:

μg DNA/μl = OD value × 50 μg ÷ volume (μl) of DNA used for the measurement. (Sambrook *et al.*, 1989)

II. 5. Cloning of PCR products

The Original TA Cloning Kit (Invitrogen Corporation, San Diego, CA) was used for cloning of the PCR products. The cloning was performed by following the instructions provided by the manufacturer with slight modifications.

20 femtomoles of vector and PCR product in 1:3 (vector:PCR product) molar ratio were used for the ligation reaction. The amount of PCR products were estimated using the following formula :

[780 bp (PCR product) × 50 ng (20 fmol of vector) ÷ 3900 bp (vector)] × 3 × 650/bp = 30 ng of PCR product (Mead *et al.*, 1991)

Therefore, 30 ng of each purified PCR product was used for the ligation reaction. The reaction was set up as recommended and incubated at 14°C overnight. Then 1 μl of 0.5 M β-mercaptoethanol and 2 μl of ligation reaction product were mixed with 25 μl of

competent cells and incubated on ice for 30 min. The mixture was then heat-shocked for exact 30 s in a 42°C water bath and subsequently placed on ice for 2 min. 225 µl of SOC medium was added to the mixture and the tube containing the mixture was shaken at 37°C for 1 hr. 25 µl and 200 µl of the mixture were spread onto two LB agar plates respectively, containing 50 mg/ml of ampicillin and X-gal. The plates were incubated at 37°C for at least 18 hr and then were shifted to 4°C for 2-3 hr for color development. Positive colonies were selected for further examination.

II. 6. Detection of PCR product cloned into plasmid

Detection of the positive colonies was carried out by using PCR (Novagen, 1995). 3 ml of LB broth containing 50 mg/ml of ampicillin was inoculated with a single colony on the plates described in Section 5 and incubated at 37°C overnight. 200 µl of the overnight culture was pelleted by spinning down at 4°C at 13,000g for 5 min. The pellet was resuspended in 200 µl of H_2O and boiled for 10 min. The boiled content was spun down at 4°C for 5 min and the supernatant was collected. A PCR reaction was completed including primers P2 and P4, and 2 µl of the supernatant as the template under reaction conditions previously described for the second PCR (Section 3). PCR products (5 µl) were resolved in a 1% agarose gel.

II. 7. Extraction and purification of plasmid DNA

Wizard™ Minipreps DNA Purification System (Promega, Madison, MI, USA) was used for extraction and purification of the plasmid DNA according to their guidelines. Briefly, 1.5 ml of LB broth containing 50 mg/ml of ampicillin was inoculated with a single

positive colony and incubated at 37°C overnight. The overnight culture was pelleted by spinning down at 13,000g for 2 min and resuspended in 200 μl of Cell Resuspension Solution. The bacteria were lysed with 200 μl of Cell Lysis Solution and neutralized with 200 μl of Neutralization Solution. After being spun down for 5 min, the supernatant was transferred to a fresh tube. 1 ml of DNA Purification Resin was added to the supernatant and mixed. Then the slurry was added into the Minicolumn and washed with Column Wash Solution. The resin in the Minicolumn was then dried by spinning for 2 min. 50 μl of TE buffer was added to the Minicolumn and incubated at room temperature for 1 min. The DNA was eluted by spinning the Minicolumn for 20 s.

II. 8. Sequencing of PCR products and plasmids from the clones

Sequencing was performed according to the dsDNA Cycle Sequencing System (GibcoBRL, Burlington, Ontario, Canada). The DNA fragment to be sequenced was 780 bp long. Because only about 400 nucleotides could usually be read in one direction when two gels, 4% and 8% respectively, were used, internal upper- and lower-strand primers were designed so that the whole DNA fragment could be covered and sequenced twice, once in upper-strand orientation and the other in lower-strand orientation. The primers used for sequencing are shown in Table 2. The primers were 5'-end-labeled under reaction conditions as recommended, including T4 polynucleotide kinase and [γ-^{32}P]ATP (3,000 Ci/mmol, 10 mCi/ml, 3.3 μM, NEN).

Table 2. Primers used in sequencing

Primer	Sequence (5'→3')	Orientation	start position away from the first codon
P2	cacctgtcaacataattggaaga	upper-strand	59 nucleotides upstream
2503C	aatccctggtgtctcattgtt	lower-strand	426 nucleotides downstream
2511	caccagggattagatatcagtacaatgtgctt	upper-strand	416 nucleotides downstream
P4	actgtccatttatcaggatg	lower-strand	721 nucleotides downstream

Sequencing reactions were set up according to the guidelines, containing 5'-end-labeled primer, *Taq* DNA polymerase, buffer, purified PCR product or plasmid as DNA template (50 femtomoles), and Termination Mix A, C, G and T respectively. The reactions were carried out at 95°C for 5 min, followed by 20 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 1 min, plus ten cycles at 95°C for 30 s and 72°C for 1 min.

The contents of sequencing reactions were heated at 95°C for 5 min before 4 μl of each reaction was loaded onto 8% and 4% polyacrylamide gels respectively. The gels were run at 2,000 watts for about 2-2.5 hours until the xylene cyanol in the Stop Solution reached the bottom of the glass plates. Then the gel was disassembled and fixed for 20 minutes in 5% acetic acid/7% methanol, followed by rinsing with stilled H_2O. The gel was transferred from the plate to a sheet of filter paper and vacuum dried at 80° C for 1 hour. The dried gel was exposed to Kodak XAR X-ray film at room temperature for an appropriate time (usually 16 hr).

At first, the bands on the sequencing gels for the plasmid inserts were too faint to be read when supercoiled plasmids were used as templates in sequencing reactions. Linearized plasmids digested with a restriction enzyme BamH I were then used for the sequencing reactions and the readability of the sequencing gels was dramatically increased.

II. 9. Sequence analyses

Sequence analyses were carried out by using a computer program called LASERGENE (DNASTAR Inc., Madison, Wisconsin, USA). The sequences were entered and edited in EDITSEQ and alignments were made in MEGALIGN. A Clustal algorithm (Higgins, *et al.*, 1989) was used for the alignment.

Because RT sequences were found highly conserved, we chose an Identity Table for the method parameters. The Identity Table only scores identical matches.

Originally, we included 8 brain-spleen-matched samples to compare the occurrence of ZRAMs, sequence diversity and mutation patterns between the brain-derived sequences and the spleen-derived sequences from the same individuals. However, because of contamination during PCR amplification, the spleen-derived sequences were not available for comparison. Therefore, for comparison, we selected 6 blood-derived RT sequences from another study. There were at least two criteria for selecting sequences from other studies for comparison: first, the strategy of using PCR amplified DNA directly for the sequencing, the same as that in our study, should be applied; second, the "clade" or subgroup of HIV-1 isolates should be the same as ours and the clinical status including duration of AZT treatment should be similar to our sample group. According to these considerations, RT sequences of 6 blood-derived clinical HIV-1 isolates from one study (Najera, *et al.*, 1995) were selected, which met the criteria (Table 3). Corresponding RT nucleotide sequences from 6 non-brain-derived PBMC or T lymphocyte cell line-passaged reference HIV-1 strains (NY5, MN, SF2, CAM1, HAN, LAI) were also included for comparison (Los Alamos National Laboratory, 1995). These reference HIV-1 strains were isolated from North America or Europe, belonging to the same HIV-1 subgroup, B clade subgroup, as our brain-derived samples.

Because 6 blood-derived clinical RT samples were only sequenced for regions from amino acid residues 41 to 108 and 181 to 219, to compare the same regions, sequences of samples in the other two groups (brain-derived HIV-1 RT group and reference HIV-1 RT group) were truncated to match the blood-derived group for sequence analysis.

To compare sequence diversity and mutation patterns between RT and *env* sequences, we used sequence data from a study (Power, *et al.*, 1994) on C2V3 region of *env* gene of brain-derived HIV-1 isolates which were obtained from the same patients as those from whom the brain HIV-1 RT were derived in the present study. For comparison, we also included sequences of C2V3 region of 6 reference HIV-1 strains, which were the same ones used in the RT comparison described above.

II. 10. Statistical Analyses

Statistical analyses were performed using a computer program called Instat2 (GraphPad Software, San Diego, CA). The statistical method selected for the analyses was the Mann-Whitney U-test. The Mann-Whitney U-test is a nonparametric alternative to the t-test. It may be used with ordinal measures and also with data that deviate from the normal and in situations where equal variances are not encountered. It is almost as powerful as the t-test (Norman and Streiner, 1986).

The statistical analyses were performed on mutation patterns including frequencies of total mutations, transversions, transitions, and percentages of transversions in total mutations and A to C or T mutations in total mutations. The analyses compared these mutation patterns among different sample groups including the brain-derived sample group, the blood-derived clinical sample group, and the reference strain group as well as between different genes, i.e., RT versus *env*, in isolates from the same individuals.

Table 3. Comparison of clinical features between blood-derived isolates[1] and brain-derived isolates

Patient No.	Age (year)	Clinical status[2]	Duration of ZDV therapy (month)[3]	Dose of ZDV (mg/day)
Blood-derived				
D12/+58	30		86 (20)	
D17/+20	39		116 (27)	
D25/+24	29		84 (19)	
D29/-2	33		24 (6)	
D31/+24	25		124 (29)	
D35/-2	46		24 (6)	
Mean±SD	33.7±7.6	AIDS defined	17.8±9.9	500[4]
Brain-derived				
2	31		6	500
20	32		2	500
34	30		24	1200
9	28		30	500
19	36		35	600
17	44		39	500
48	28		13	200
64	32		6	500
4	34		3	500
36	34		0	0
Mean±SD	32.9±4.7	AIDS defined	15.8±14.8	500±306
p[5]	0.81		0.77	

1. Blood-derived isolates were selected from Najera *et al.*'s study (1995). These isolates were sampled in Madrid, belonging to HIV-1 B clade, the same clade to which brain-derived isolates belonged.
2. All patients in both clinical groups were AIDS defined.
3. For blood-derived isolates, the numbers without parentheses indicate weeks of treatment and the numbers within parentheses are months of treatment converted from the numbers of weeks of treatment in order to compare with brain-derived isolates.
4. For blood-derived isolates, the doses of ZDV varied with body weight and clinical status but generally were 500 mg/day.
5. Comparison of means (±SD) of ages and duration of ZDV treatment between blood-derived isolates and brain-derived sequences showed no statistically significant difference, as indicated by the p values.

III. RESULTS

III. 1. PCR amplification

Among 36 brain-derived samples amplified, 26 were PCR-positive (Figure 1, Table 4). The positive samples had bands with the desired size (780 bp) on agarose gel. However, 5 samples were later found to be contaminated with the positive control, NL4-3 (Section III. 3) and thus they are excluded from the calculation of positive rate of PCR amplification. Therefore, the positive rate of PCR amplification for the brain-derived samples is 68% (21 positive out of 31 amplified). Although all 8 spleen-derived samples were positive, they were all contaminated with NL4-3 (see Section III. 3)

Table 4. Results of PCR amplification and sequencing

		Brain-derived samples	spleen-derived samples
PCR amplified		36	8
Result:	Positive	26 (5 faint samples)	8
Sequenced		21	8
Result:	unique sequences	10	
	contaminated	5	8
	unreadable	6	

III. 2. Purification of PCR amplified DNA fragments

The purified DNA in some samples showed a single band with a size of 780 bp on 1% agarose gel. In the other samples the purified DNA showed two bands with sizes of 780 bp and 850 bp respectively (Figure 2). The amount of the purified DNA for a sample with a band of an average density on agarose gel was 800 ng of DNA, obtained from 10 μl of original PCR reaction. However, 5 samples, although positive, had too little DNA to be purified.

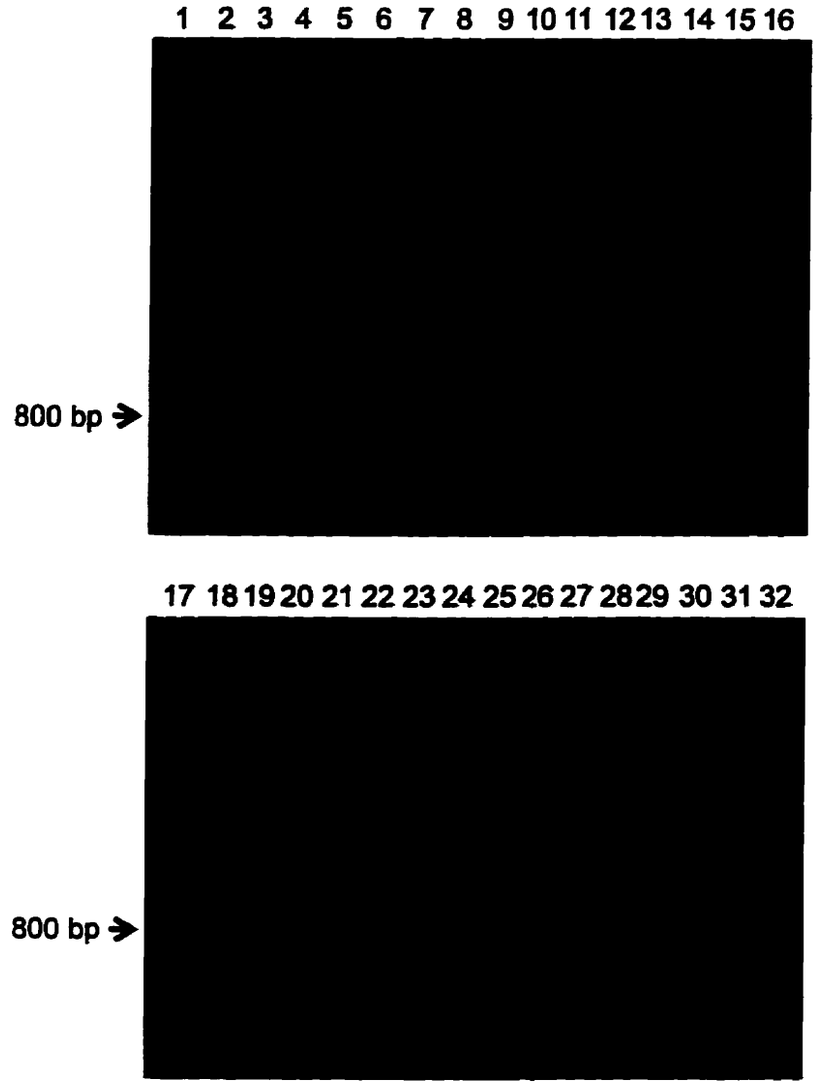

Figure 1. PCR amplification of clinical samples. Electrophoresis performed on 1% agarose gel shows examples of PCR amplification of RT coding region covering codons 1 to 240. A volume of 10 µl, out of 25 µl of each PCR reaction, was loaded on the agarose gel. The expected size of the amplified DNA fragment was 780 bp. Lanes 1 and 17 were 100 bp ladder molecular weight markers. Lanes 16 and 32 were the positive control NL4-3. Lanes 2 and 18 were the negative control in which water was added instead of template DNA. All other lanes were clinical samples.

III. 3. Sequencing of PCR products

21 brain-derived samples and 8 spleen-derived samples were sequenced (Table 4). Six brain-derived samples were unreadable, among which three were due to signal compression in some regions on the sequencing gel. In the compressed regions, usually encompassing 10-15 nucleotides, all four sequencing reactions (A, C, G and T) had bands at the same position with almost the same density and thus no single band could be distinguished as being predominant.

The other three unreadable samples did not show any bands when primer P2 (sense sequencing primer for 5'-end nucleotide sequence) was used. Some mismatches were found within the sequence of primer P2 region in two of these three unreadable samples when sequenced using primer 2503C, the antisense sequencing primer for 5'-end nucleotide. Because these three samples were sequenced once for 5' half using primer 2503C instead twice as for other samples using primers P2 and 2503C, they were not included for subsequent analyses.

All eight spleen-derived samples and five brain-derived samples were found to have sequences either exactly the same as NL4-3, or have only one or two nucleotides different from NL4-3, and thus were considered to be contaminated with NL4-3 (about the criteria for defining the contamination, see Section IV.1.3).

Ten samples showed unique sequences. All samples were brain-derived, five belonging to the HIVD group and the other five being under the ND category. The clinical features of these patients, including age, CD4 cell count, neuropathological findings, and AZT dose and duration did not differ significantly between the HIVD and the ND groups (Table 5).

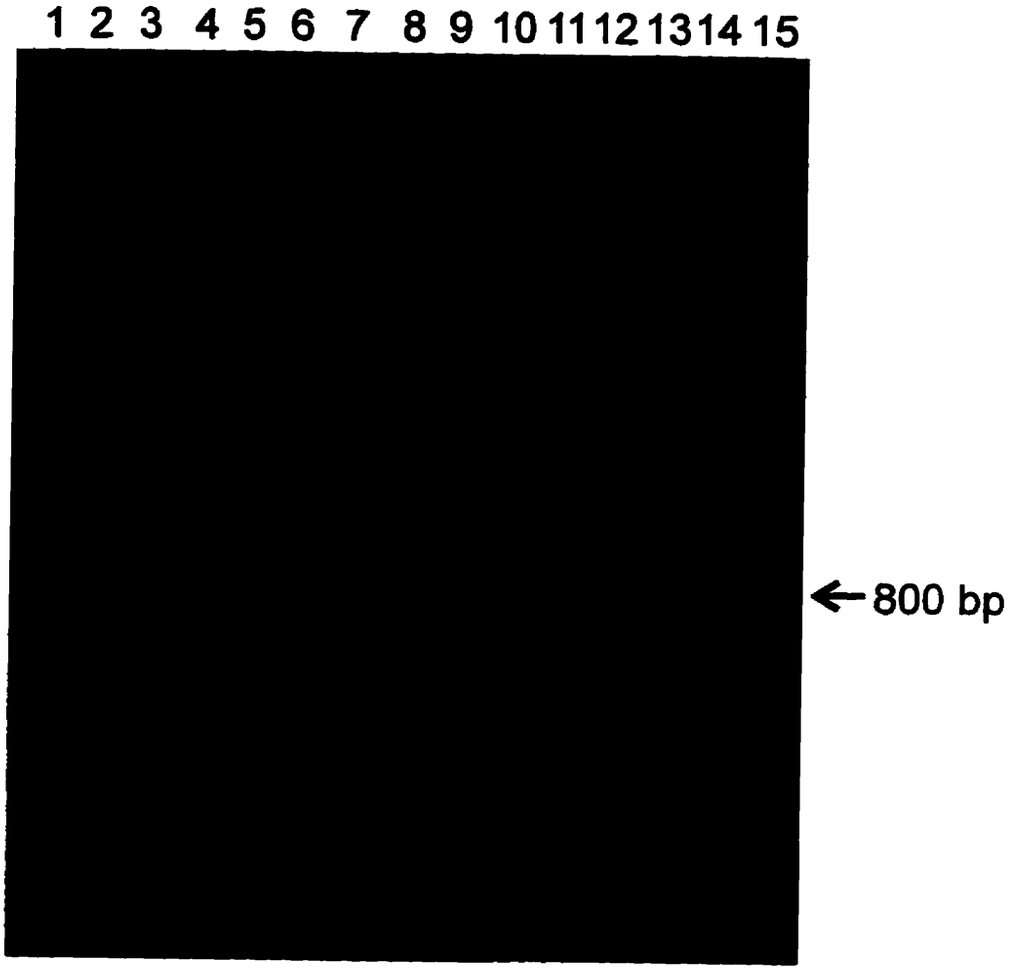

Figure 2. Purification of PCR products. Electrophoresis performed on 1% agarose gel shows examples of purified PCR products. A volume of 5 μl of the purified PCR products (equivalent to 2.5 μl of the original PCR reaction) for each sample was loaded on the agarose gel. Lane 15 was 100 bp ladder molecular weight markers. Lane 14 was NL4-3 control. Some lanes showed double bands of sizes of about 780bp and 850 bp respectively.

III. 4. Cloning of PCR product

To determine if the result of PCR product sequencing reflected the sequences of the majority of the PCR products and to detect ZRAMs which might have been neglected by PCR product sequencing due to being a minority of the PCR products, PCR products of patients 9 and 2 were selected for cloning and subsequent sequencing. PCR products NL4-3 was also used for cloning as a control. The highest cloning efficiencies, which were five to seven times higher than the expected cloning efficiency with the kit (which is 200 colonies per plate using 200 μl of transfected bacterial culture), were observed when unpurified PCR products were used for the cloning (Table 6). They were followed by the cloning efficiencies of freshly purified PCR products, which were 1/4 of the expected cloning efficiency. The lowest cloning efficiencies were observed with purified PCR products after prolonged storage (6 months) were used for the cloning. I used PCR to detect RT inserts (Table 6 and Figure 3).

III. 5. Extraction and purification of plasmid DNA

Plasmids were extracted and purified from overnight cultures inoculated with positive colonies. Approximately 8 to 12 μg of DNA was purified per sample (Figure 4).

Table 5. Clinical features of HIV dementia (HIVD) and nondemented (ND) patients[1]

Clinical group	Mean age (yr) ± SD	Mean CD4 cells (cells/mm^3) ± SD	Mean duration of AZT treatment (mo) ± SD	Mean AZT dose (mg/day) ± SD
HIVD (N=5)	31 ±3.2	15 ±14	19 ±14.6	520 ±408
ND (N=5)	34 ±6.5	47 ±53	12 ±15.7	300 ±173

1. Groups did not differ statistically (p>0.05) for any of the features analyzed (Mann-Whitney U test).

Table 6. Results of Cloning and PCR detection

Sample	Number of colonies		PCR detection positive
	25 μl[1]	200 μl[1]	
9 (purified)[2]	0	1 light blue, 1 blue	1/2
2 (purified)[2]	0	3 light blue, 3 blue, 1 white	5/5
NL4-3 (purified)[3]	3 blue, 18 white	5 blue, ≥ 200 white	2/2
NL4-3 (unpurified)[4]	50 blue, ≥ 100 white	≥ 400 blue, ≥ 1000 white	0/1
negative [5]	0	3 blue	0/2

1. 25 μl and 200 μl of bacterial culture (after transfected and 1 hour-cultivation) were spread onto LB agar plates respectively.

2. Purified PCR products after excessive storage (6 months) were used for cloning.

3. Freshly purified PCR products were used for cloning.

4. Unpurified PCR products were used for cloning.

5. In the negative control, distilled water instead of PCR product was used for ligation.

6. The number on the left of the slash represents the number of RT positive colonies detected by PCR and the number on the right of the slash represents the number of colonies selected for PCR detection.

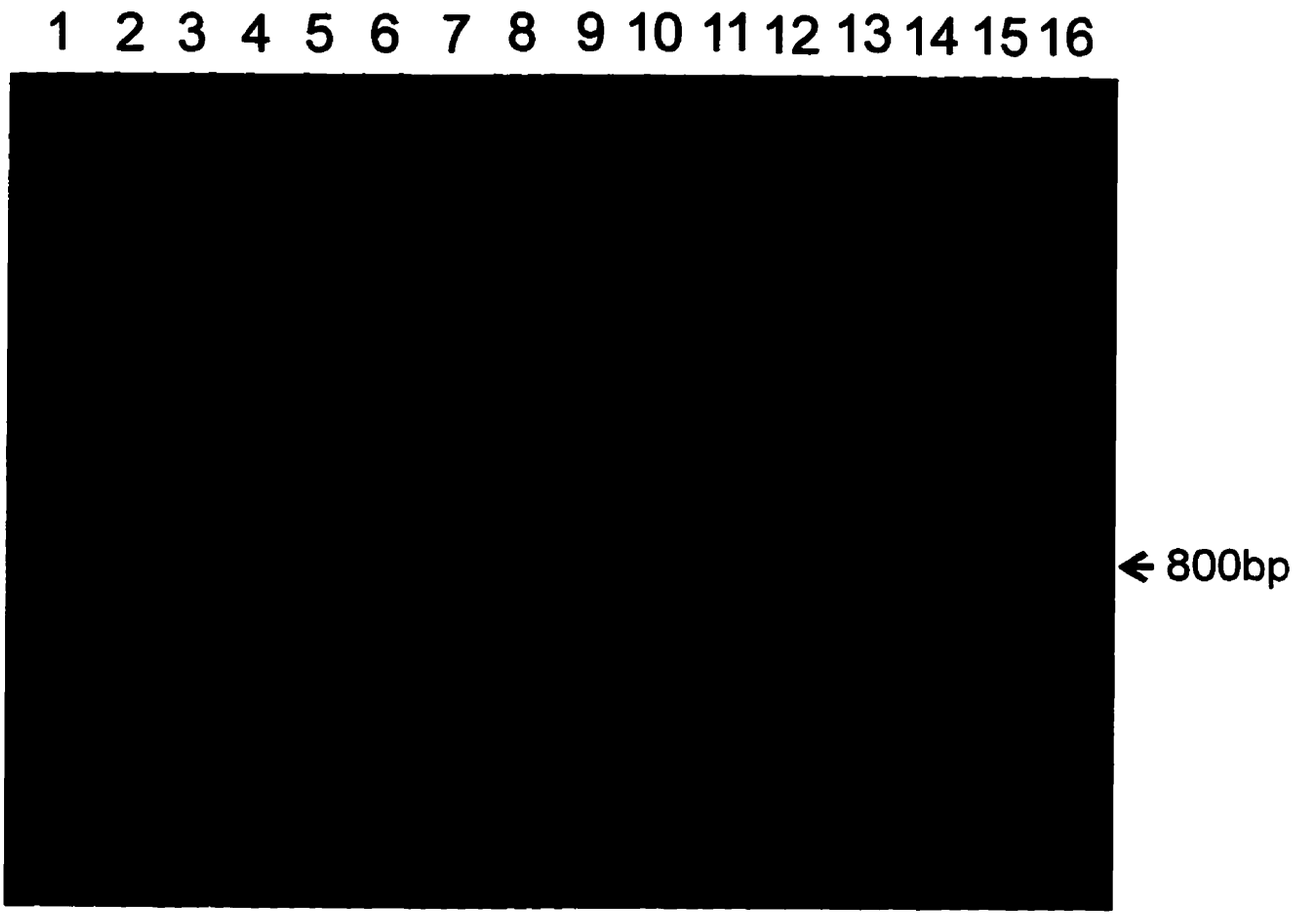

Figure 3. PCR detection of RT inserts in plasmids. A volume of 5 μl out of 25 μl of PCR reaction for each sample was loaded on 1% agarose gel. Lanes 1 and 16 were 100 bp ladder molecular weight markers. Lane 2 was the negative PCR control with water added instead of template DNA. Lanes 3 and 4 were cloning controls with inserts provided with the cloning kit. Lane 15 was the positive PCR control, NL4-3. Lanes 5 and 6 were clones from patient 9. Lanes 7 and 8 were clones for which purified PCR products of NL4-3 were used for cloning. Lane 9 was a clone for which unpurified PCR product of NL4-3 was used for cloning. Lanes 10-14 were clones from patient 2.

III. 6. Sequencing of plasmid inserts

Plasmid inserts from 10 clones were sequenced, one from patient 9, five from patient 2, and the other four from positive controls (NL4-3). The amino acid sequence of plasmid insert from patient 9 is exactly the same as that from direct sequencing of the PCR product. Although the amino acid sequences of 5 plasmid inserts from patient 2 show some differing residues from that of the PCR product, they have over 98% similarity compared to the sequence of the PCR product or to one another (Figure 5). Like the PCR products, the plasmid inserts from patients 2 and 9 have no ZRAMs. In addition, the nucleotide sequences of all four plasmid inserts of NL4-3 show no mutations.

All the plasmid sequencing gels were clearly readable. In general, sequencing gels of the plasmid inserts were much more readable than those of direct sequencing of the PCR products.

III. 7. Sequence analyses for PCR products

III. 7. 1. Diversity of brain-derived HIV-1 RT sequences

By comparing the brain-derived RT amino acid sequences to their consensus (Figure 6), which is identical to the consensus of B clade reference RT sequences (Los Alamos National Laboratory, 1995), 34 residues differing from the consensus, including 2 previously reported ZRAMs and 24 novel mutations, unrecognized previously in established databases (Los Alamos National Laboratory, 1995), were observed among these samples (Figure 6). These differing amino acids were clustered nonrandomly at specific regions, for example, at residues 81-85, 102-106, 122-124, and 211 -214, similar to those found in 6 selected non brain-derived reference RT sequences (Figure 7).

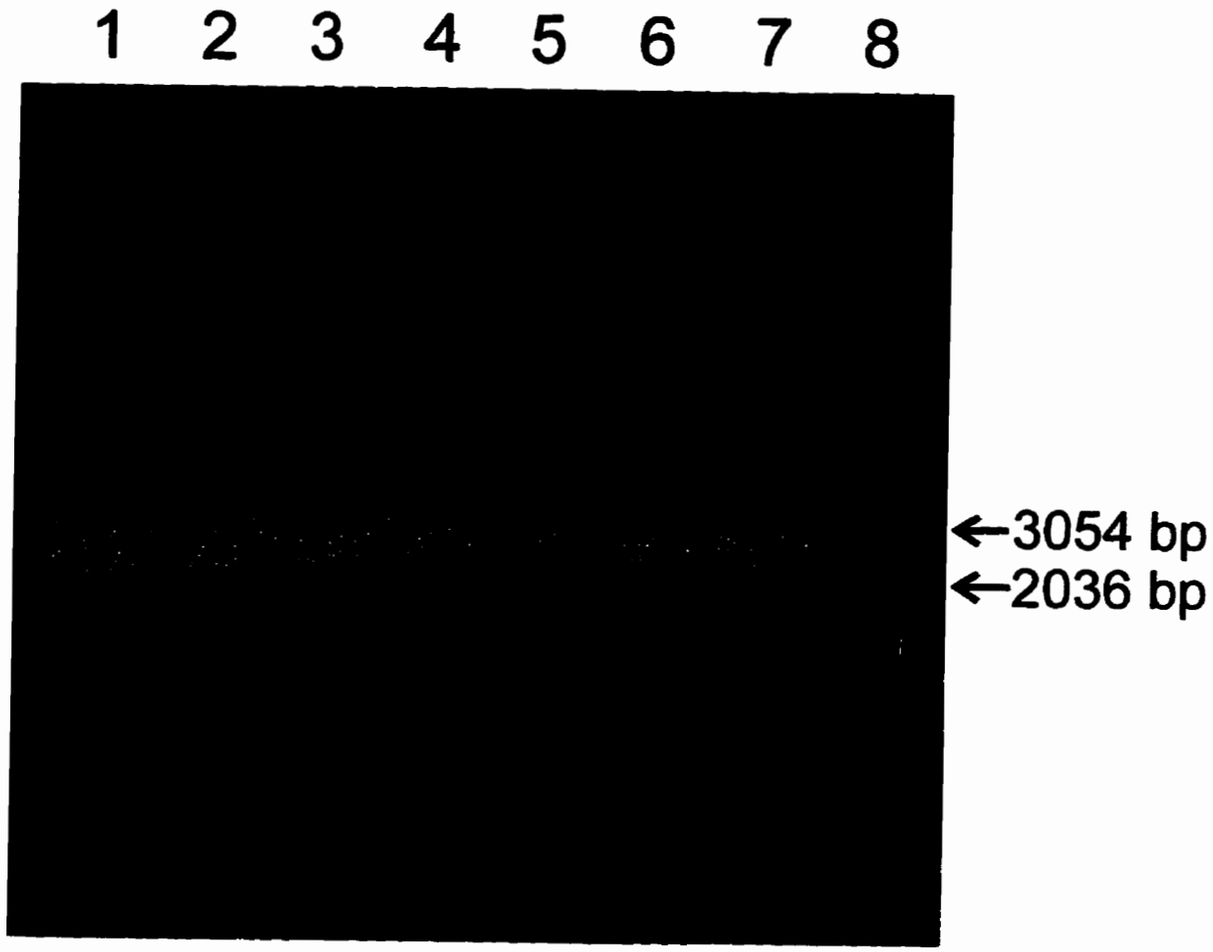

Figure 4. Purification of plasmids with RT inserts. A volume of 10 μl of each sample was loaded on the agarose gel. Lane 8 is a 1 Kb ladder molecular weight marker. Lane 7 is the control, NL4-3. Lane 1 is the clone from patient 9. Lanes 2-6 are clones from patient 2.

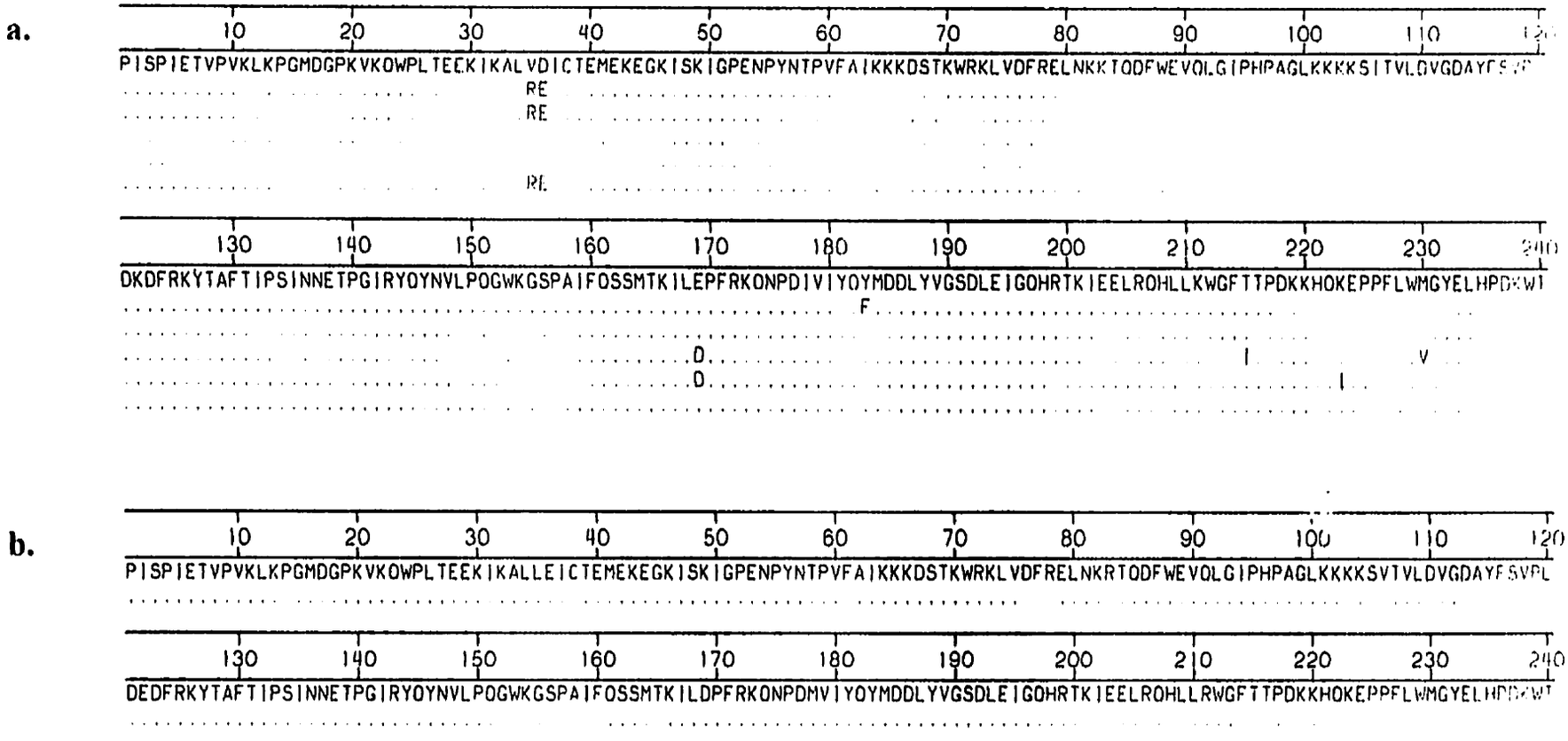

Figure 5. Sequences of clones of brain-derived HIV-1 RT. Sequences displayed are from PCR products, serving as template sequences. Dots and letters below the sequences represent sequences of clones with dots representing amino acid residues which are the same as those of PCR product at the specific codons and letters showing differing amino acid residues. a) sequences from patient 2; b) sequences from patient 9.

However, the frequency and position of these differing residues was not associated with a specific clinical group (ND and HIVD) or the duration of ZDV therapy (Figure 6). The nucleotide mutation frequency for the brain-derived HIV-1 RT sequences was 1.87±0.69/100 nucleotides.

To monitor misincorporations and/or frameshifts introduced into the sequences during PCR amplification, reference HIV-1 strain, NL4-3, was amplified and sequenced together with the samples. No misincorporations or frameshifts have been found in NL4-3, which was sequenced twice. However, sequences of 13 samples (8 spleen-derived and 5 brain-derived) were found to have only one or two nucleotides differing from NL4-3 or have exactly the same nucleotide sequence as NL4-3 and thus they were considered to be contaminated with NL4-3. By including these contaminated samples and NL4-3 controls, the overall PCR-induced mutation rate in this study is estimated to be about 1 error per 1000 bases (0.1/100 nucleotides).

III. 7. 2. ZRAM in brain-derived HIV-1 RT

Previously reported ZRAMs were identified in only two patients (patients 19 and 17) who had been treated with ZDV for 35 months and 39 months respectively (residues in circles in Figure 6). Patient 19 (HIVD) demonstrated two ZRAMs, M41L (Figure 8) and F215Y(Figures 9), and patient 17 (ND) displayed only one ZRAM M41L (although the mutation band could be distinguished on the autoradiography film of sequencing gel, the result could not be reproduced onto paper or photo because the contrast of the figure would be very poor due to a heavy background). None of the other samples had previously reported ZRAMs despite the duration of ZDV therapy ranging from 2-30 months (Figure 6).

There were no significant differences in ZRAM occurrence between the HIVD and the ND groups. In addition, patient 34 was also treated with ddI for 6 months but did not display previously reported ddI resistance associated mutations (Figure 6).

In contrast, five out of six blood-derived RT sequences selected from another study (Najera, *et al.*, 1995) showed ZRAMs although their mean duration of ZDV treatment was not significantly different from that of the patients in this study (Table 7).

III. 7. 3. Mutation Patterns in brain-derived HIV-1 RT

III. 7. 3. 1. Comparison of RT sequences among sample groups

The mean nucleotide mutation frequency (/100 nucleotides) of the brain-derived HIV-1 RT (1.87±0.69) was comparable to that of the reference RT group (Los Alamos National Laboratory, 1995) (1.23±0.52) (p=0.18), but was much lower than that of the blood-derived RT (Najera *et al.*, 1995) (4.67±2.68) (p=0.0017) (Figure 10, Table 8). A similar pattern was observed with transitions (purine to purine substitutions or pyrimidine to pyrimidine substitutions) when RT sequences of these three groups were compared (1.37±0.49, 1.30±0.61, and 3.48±2.62 for brain-derived, reference, and blood-derived respectively) (Figure 10, Table 8) (p=0.64 for comparison between brain-derived and reference, and p=0.0017 for comparison between brain-derived and blood-derived).

However, when frequencies of transversions (purine to pyrimidine substitutions or pyrimidine to purine substitutions) were compared, brain-derived RT group (0.49±0.21) was comparable not only to the reference group (0.12±0.14) (p=0.12) but also to the blood-derived group (1.19±1.03) (p=0.18) (Table 8, Figure 10). This pattern also persisted when

percentages of transversions in total nucleotide substitutions for these three groups were considered (24.1±18.6 vs 6.5±10.7 for brain-derived vs reference, p=0.073 and 24.1±18.6 vs 26.3±22.7 for brain-derived vs blood-derived, p=0.87) (Table 8, Figures 11).

The predominant transversion in the brain-derived RT group was nucleotide mutations from A to C or T, which accounted for 25% of total nucleotide substitutions (particularly, A to C mutations accounted for 18% of total nucleotide substitutions), 71% of transversions (Table 9a), 40% of the amino acid substitutions and 56% of the amino acid substitutions previously unrecognized in established databases (the residues with squares in Figure 6). In contrast, A to C or T mutations only occupied 7% of total nucleotide mutations in the reference RT group (Table 9b) and 8% in the blood-derived RT group (Table 9c). Although the trend that the brain-derived RT group had a higher percentage of A to C or T mutations, was obvious, the differences among groups were not statistically significant (p=0.073 for brain-derived vs reference and p=0.18 for brain-derived vs blood-derived) (Table 8, Figure 11).

III. 7. 3. 2. Comparisons between RT and *env*

Compared to brain-derived RT, brain-derived *env* (Power *et al.*, 1994) showed higher frequencies in total nucleotide mutations, transitions and transversions (all p<0.0001) (Figure 10, Table 8). However, brain-derived *env* was quite similar to brain-derived RT in the percentage of A to C or T mutations (21.8±5.6 vs 23.1±17.3) (Figure 11, Table 8).

When reference RT and reference *env* (Los Alamos National Laboratory, 1995) were compared, similar patterns were observed except for comparison of percentage of A to

C or T mutations, with the reference RT group showing a lower percentage (9.3±10.6 vs 18.3±10.2) (Figure 11, Table 8).

Mutation frequencies in total mutations, transitions and transversions, and percentages of transversions and A to C or T mutations were found to be quite similar between the brain-derived *env* and reference *env* groups (Tables 8, 9d and 9e, Figures 10 and 11).

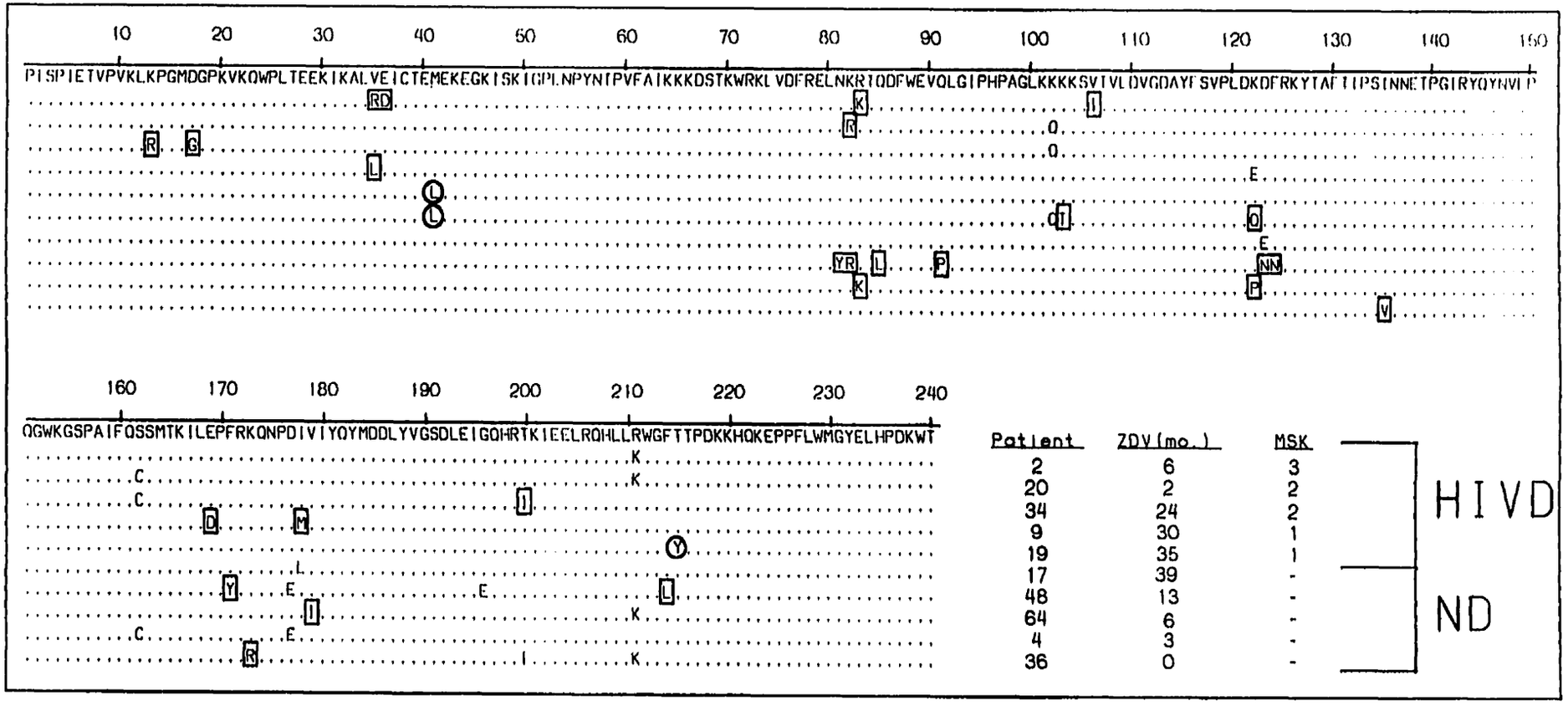

Figure 6. Sequences of brain-derived HIV-1 RT and some clinical features of patients. The sequence displayed is brain-derived HIV-1 RT consensus sequence, which is identical to the consensus sequence of the B clade HIV-1 strains in the database. The numbers above the consensus sequence are amino acid codons. The dots represent amino acid residues which are the same as consensus sequence at each codon. The letters with squares are amino acid residues which are not found in the established databases. The letters with circles represent ZRAMs.

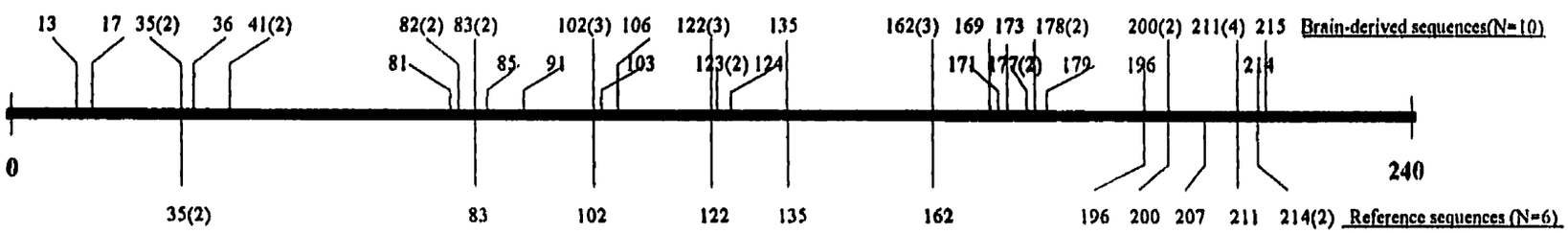

Figure 7. Sequence diversity in brain-derived and reference HIV-1 RT. The thick horizontal line represents the RT coding region from codon 1 to 240. The short vertical line together with the numbers indicate the positions of mutations from the consensus sequence. The numbers in the parentheses show the number of mutations at the specific codons found in the respective sample groups. The mutations numbered above the horizontal line are those found in brain-derived HIV-1 RT and those below the line belong to the reference group.

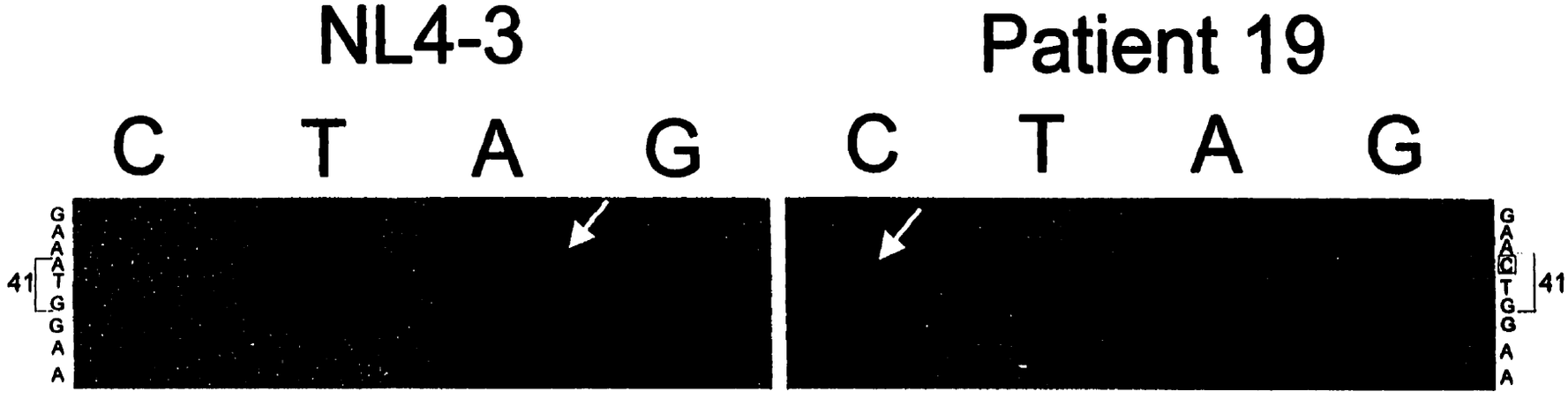

Figure 8. ZRAM at codon 41. A nucleotide mutation at codon 41 identified in the sequence from patient 19. The arrows in the blowups indicate the nucleotides mutating from A to C. The arrows point to the nucleotide mutation from A to C, resulting in the corresponding amino acid residue changing from methionine to leucine (ZRAM).

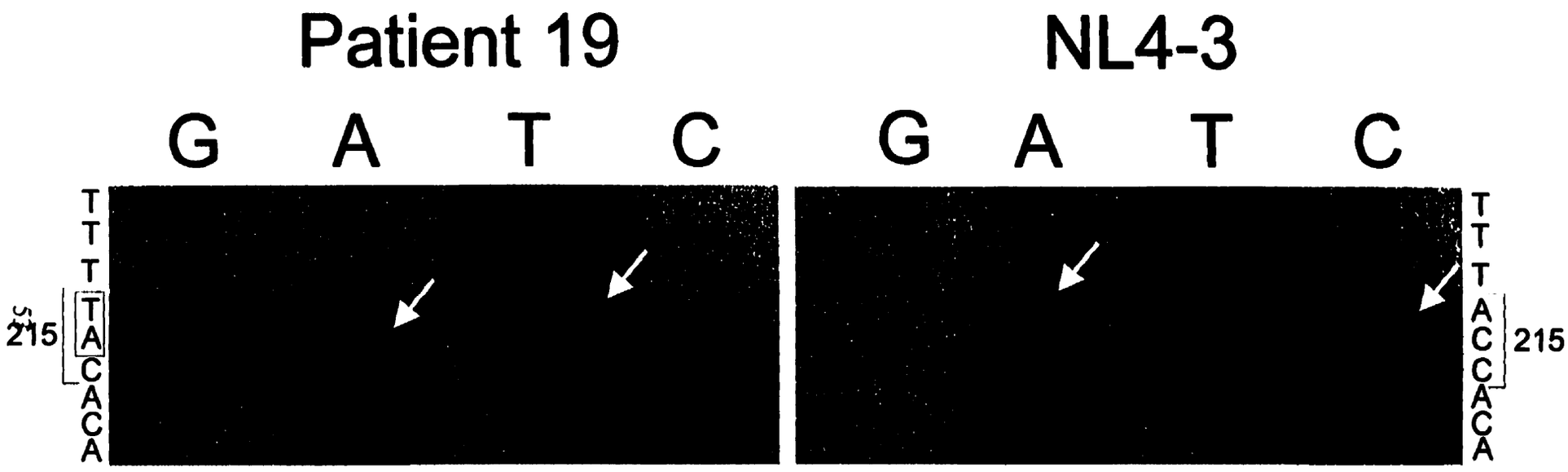

Figure 9. ZRAM at codon 215. Two nucleotide mutations at codon 215 identified in the sequence from patient 19. The arrows in the blowups indicate the nucleotides mutating from A to C. The arrows point to the nucleotides mutating from A an C to T and A respectively, resulting in the corresponding amino acid residue changing from threonine to tyrosine (ZRAM).

Table 7. Comparison of mutation frequency of ZRAMs and the overall amino acid mutation rate in brain- and blood-derived RT.

Group	Mean Amino acid mutation frequency±SD[1]	ZRAM frequency±SD[2]	*p*
Brain-derived RT (N=9)[3]	0.023±0.005	0.067±0.14	0.38
Blood-derived RT (N=6)	0.08±0.015	0.17±0.08	0.051

1. Mutation frequencies have been calculated by dividing the number of amino acid mutations (relative to the consensus sequence) by the total number of amino acid residues compared (codons 41-108 and 181-219) for each sample.

2. ZRAM frequencies have been calculated by dividing the number of ZRAM occurred by the total number of possible positions for ZRAM occurring, which is 5 (codons 41, 67, 70, 215, and 219).

3. Patient 36 did not receive ZDV treatment and thus was excluded from the analysis.

Table 8. Mean frequencies[1] (±SD) of total nucleotide mutations, transitions and transversions, and mean percentages[2] (±SD) of transversions and A to C or T mutations.

Group	Total nucleotide mutation (/100 nucleotides)	Transition (/100 nucleotides)	Transversion (/100 nucleotides)	percentage of transversion	percentage of A to C or T mutation
Brain-derived RT (N=10)	1.87±0.69	1.37±0.49	0.50±0.51	24.1±18.6	23.1±17.3
Reference RT (N=6)	1.40±0.65	1.30±0.61	0.10±0.16	6.5±10.7	6.5±10.7
Blood-derived RT (N=6)	4.67±2.68	3.48±2.62	1.19±1.03	26.3±22.7	9.3±10.6
Brain-derived *env* (N=14)	5.14±1.38	3.07±0.85	2.07±0.57	40.1±3.7	21.8±5.6
Reference *env* (N=6)	5.91±1.04	3.90±1.06	2.00±0.52	34.7±9.9	18.3±10.2

1. The frequencies have been calculated by dividing the number of mutations (total nucleotide mutations, transitions and transversions respectively relative to the consensus sequence) by the total number of nucleotides compared (720) for each sample.

2. The percentages have been calculated by dividing the number of mutations (transversions and A to C or T mutations relative to the consensus sequence) by the number of total nucleotide mutations.

Table 9. Percentage of different types of nucleotide mutations relative to total nucleotide mutations in each sample group.

9a. Brain-derived RT group

		to			
		A	C	G	T
from	A	–	**18**	22	**7**
	C	2	–	0	17
	G	28	0	–	0
	T	0	7	0	–

9b. Reference RT group

		to			
		A	C	G	T
from	A	–	**7**	22	**0**
	C	0	–	0	27
	G	27	0	–	0
	T	0	19	0	–

9c. Blood-derived RT group

		to			
		A	C	G	T
from	A	–	**1**	24	**8**
	C	6	–	1	7
	G	40	2	–	2
	T	2	3	3	–

9d. Brain-derived *env* group

		to			
		A	C	G	T
from	A	–	**17**	20	**5**
	C	6	–	2	6
	G	24	3	–	2
	T	6	10	1	–

9e. Reference *env* group

		to			
		A	C	G	T
from	A	–	**14**	19	**3**
	C	7	–	2	10
	G	28	3	–	3
	T	0	9	3	–

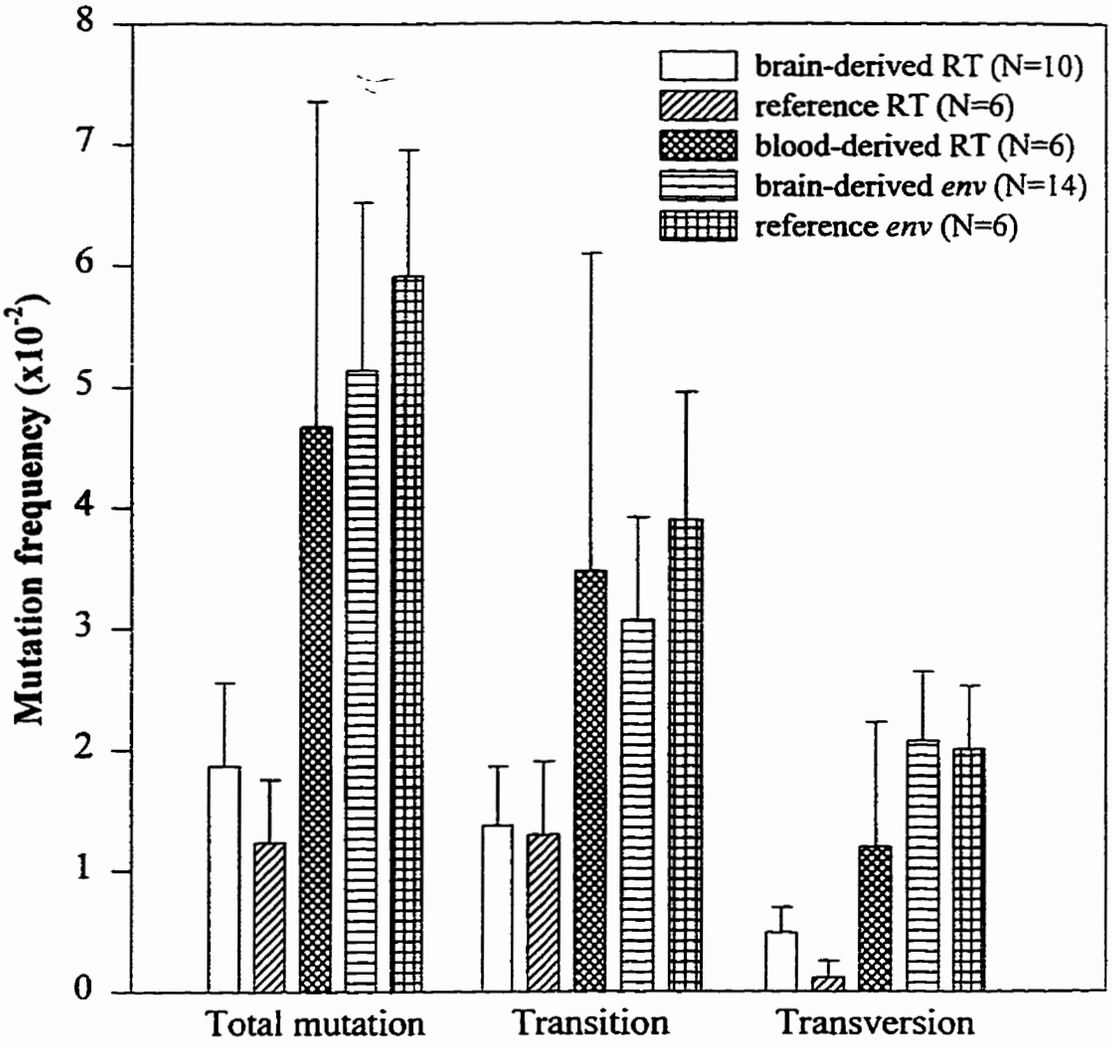

Figure 10. Comparison of mean frequencies (±SD) of total nucleotide mutations, transitions and transversions in RT and *env* sequences. The frequencies have been calculated by dividing the number of mutations (total nucleotide mutations, transitions and transversions respectively relative to the consensus sequence) by the total number of nucleotides (720) compared for each sample.

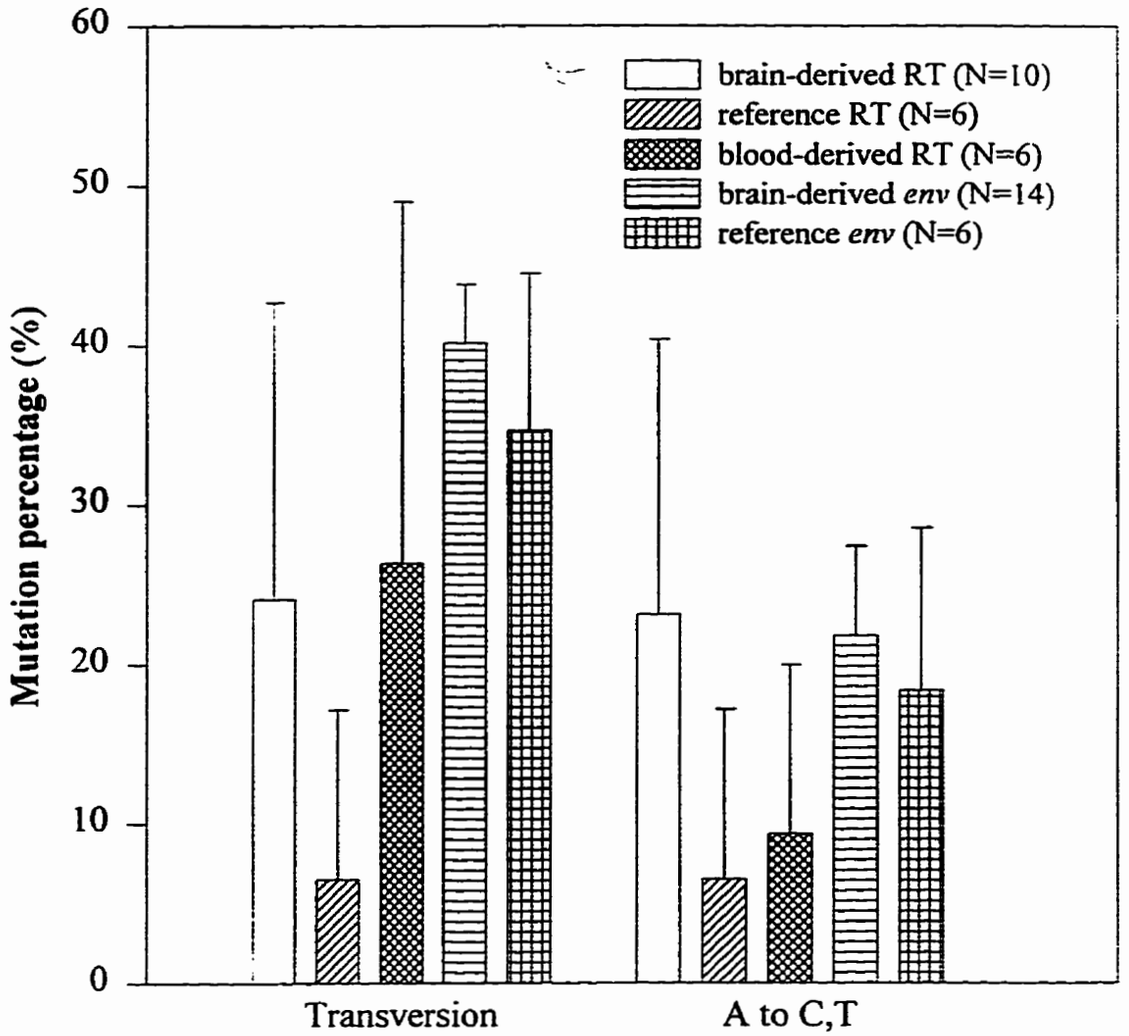

Figure 11. Comparison of mean percentages (±SD) of transversions and A to C or T mutations. The percentages have been calculated by dividing the number of mutations (transversions and A to C or T mutations relative to the consensus sequence) by the number of total nucleotide mutations.

IV. DISCUSSION

IV. 1. Method selection: advantage and disadvantage

IV. 1. 1. Direct PCR amplification versus amplification after passage

The adaptation of clinical HIV-1 isolates to specific cell types during passage has been widely recognized (Meyerhans *et al.*, 1989; Kusumi *et al.*, 1992). HIV-1 quasispecies adapted to cell culture differ from the original population existing in infected individuals in many aspects including infectivity, replication rate, and sequence diversity (Meyerhans *et al.*, 1989; von Briesen *et al.*, 1990; Kusumi *et al.*, 1992). Thus, sequencing data from HIV-1 passaged in cell culture may not truly reflect viral population *in vivo*. Therefore, to avoid bias caused by *in vitro* cultivation, we PCR amplified the RT coding region directly from cDNA which was reverse transcribed from total RNA of clinical isolates.

However, among the *in vivo* viral population amplified, there may have been many defective viruses, which might not contribute to the disease progression (Dimitrov *et al.*, 1993). Thus, although direct amplification may truly reflect viral population *in vivo,* unlike amplification after passage, it cannot differentiate between defective and live viruses.

Isolation of live HIV-1 from brain tissues is technically difficult because biopsied tissues must be used which are not widely available. In addition, culturing of virus from brain tissues may cause a selection bias depending on the cell types used for isolating and propagating the virus. Therefore, direct PCR amplification of clinical brain-derived isolates is a more practical choice for analyzing HIV-1 sequences.

For some samples, the nested PCR produced two bands when the DNA was resolved on agarose gels, with sizes of about 780 bp and 850 bp respectively. 780 bp

matched the desired size of DNA produced by the second round of PCR and 850 bp matched the desired size of DNA produced by the first round of PCR. The production of 850 bp-DNA by the nested PCR was probably due to the presence of primers of the first round of PCR in the second round of PCR. There might be little template cDNA in these samples and thus a considerable amount of the primers of the first round of PCR had not been consumed. These primers were transferred to the second round of PCR together with DNA products of the first round of PCR which was used as the template DNA for the second round of PCR. Some samples had more 850-bp DNA than other samples and for some samples the two bands of the different sizes showed comparable densities. The amount of 850 bp-DNA produced by the nested PCR might depend on the amount of the primers of the first round of PCR transferred to the second round of PCR as well as the efficiency of the second round of PCR. Although there was much more primers of the second round of PCR than the primers of the first round of PCR in the reaction of the second round of PCR, if there were some mismatches between the primers of the second round of PCR and the templates, the production of 780 bp-DNA could be reduced to an amount which was comparable to that of 850 bp-DNA as shown in some samples. Sequencing results supported the conclusion that the production of 850 bp-DNA was due to the presence of the primers of the first round of PCR. Sequencing of DNA purified from cut-out of agarose gel containing both bands, using internal primers, showed a single sequence instead of two different sequences, with 45 extra nucleotides at 3' end and 21 extra nucleotides at 5' end compared to the samples containing a single band of a size of 780 bp on agarose gels. The sequences and sizes of the 3'-end and 5'-end extra nucleotides

exactly matched the sequences and sizes of the regions on the template DNA between the primers of the first and second rounds of PCR.

IV. 1. 2. Direct sequencing of PCR product versus sequencing after cloning

Taq DNA polymerase used to catalyze PCR reaction is an error-prone enzyme due to its lacking of 3'→5' proofreading exonuclease. The rates of misincorporation and frameshift by *Taq* DNA polymerase vary from one misincorporation per 2,400 to 21,000 nucleotides and one frameshift per 22,000 to 110,000 nucleotides synthesized respectively, depending on the reaction conditions used in fidelity assays (Eckert and Kunkel, 1991). The misincorporations and frameshifts are located randomly in amplified products although under certain conditions, the preference of T to C transitions is observed (Eckert and Kunkel, 1991). According to the principle of PCR amplification, as the number of PCR reaction cycles increase, the copy number of PCR amplified products increase exponentially. Since the misincorporations and frameshifts increase with the copy number of PCR products increasing exponentially; new misincorporations and frameshifts may continuously occur during each cycle of PCR amplification and hence the increase rate of misincorporations and frameshifts is greater than that of the copy number of PCR products. Thus, misincorporations of nucleotides and frameshifts during PCR amplification catalyzed by *Taq* DNA polymerase are inevitable, especially for amplifications with many cycles of PCR reaction.

However, sequencing of PCR product detects nucleotides occupying a major percentage at each position in the amplified product pool. Unless misincorporations and frameshifts are introduced into amplified products in very early reaction cycles and thus

having a considerable portion in the pool, they can not be identified on a sequencing gel. To examine if the mutations are brought into sequences during PCR amplification, each sample can be amplified in several separate PCR reactions and the PCR products are subsequently sequenced. Any mutations which are different from the consensus sequence of these PCR products may be considered the mutations brought into the sequence during PCR amplification.

In contrast, if the PCR product is cloned and subsequently the clones are sequenced. each misincorporation and frameshift existing in the clones can be detected on sequencing gel, that do not truly reflect the authentic majority of the product pool. Therefore, we chose direct sequencing of PCR products as the principal method. The consensus sequence of 5 clones for patient 2's sample showed over 99% homology with the sequence of the PCR product (Figure 5), suggesting that the direct sequencing of PCR product did reflect the majority of PCR product pool. However, 1% difference between the consensus sequence of the clones and the direct sequencing does not allow us to conclude the mutation rate by *Taq* enzyme because other factors such as the selection of clones and possibly different cloning efficiency for each clone may also affect the outcome of the consensus sequence.

We also realized that although direct sequencing of PCR products has the advantage of neglecting most of the misincorporations and frameshifts introduced into PCR products during amplification, at the same time, it can not detect authentic minor populations in the product pool. Sequencing of cloned PCR products may identify the authentic minor populations. Thus, we cloned and sequenced some selected samples to identify the minor populations possibly neglected by direct sequencing of PCR products. However, in

principle. we are not able to differentiate between the mutations of the authentic minor populations and the ones incorporated during PCR amplification.

IV. 1. 3. The contamination problem in PCR amplification

Like many other RNA viruses, HIV-1 is highly diverse in its sequence among different strains although it is believed that different HIV-1 strains are derived from a common ancestral strain. It has been estimated that up to 10 base substitutions in the HIV genome occur per replicative cycle. (Preston *et al.*, 1988; Roberts *et al.*, 1988; Los Alamos National Laboratory, 1995). Even for its highly conserved regions, such as RT, there are no two strains which have identical nucleotide sequences. In fact, along the 720-bp region analyzed in the present study, the frequencies of nucleotide mutations for the blood-derived RT and the reference RT groups are 4.67±2.68/100 nucleotides and 1.23±0.52/100 nucleotides respectively (Table 8). In other words, on average, there are 37 and 9 nucleotide mutations in each blood-derived and reference sequences respectively along the 720-bp region.

There is no strict standard of the number of different nucleotides along a region of a certain length between two sequences or strains for defining contamination. However, based on the discussion above of the mutation frequencies among the reference RT and the blood-derived RT groups, if there are only one or two nucleotides differing along the region of 720 bp between two HIV-1 sequences from two different subjects without an obvious relationship (like NL4-3 vs each brain-derived isolate in this study), these two samples would be considered to be contaminated.

There were 13 samples (8 spleen-derived and 5 brain-derived) whose sequences had only one or two nucleotides differing from NL4-3 or had exactly the same nucleotide sequence as NL4-3. Based on the calculation and discussion above, they were considered to be contaminated with NL4-3 (Table 4). By including these contaminated samples and NL4-3 controls, the overall PCR mutation rate in this study is estimated to be about 1 error per 1000 bases (0.1/100 nucleotides).

Because the nucleotide mutation frequency for the brain-derived RT group was 1.87±0.69/100 nucleotides (Table 8), which was much higher than the estimated PCR mutation rate (0.1/100 nucleotides), the mutations found in the brain-derived samples, at least the majority of them, are considered to be authentic, incorporated during *in vivo* viral replication instead of PCR amplification.

In total, 13 samples were found to be contaminated, accounting for about 30% of samples amplified (Table 4). The unusually high contamination rate was probably due to performing PCR in a laboratory where NL4-3 was being widely used as a control at the time, and having not used a separate set of equipment and/or aerosol pipette tips for the amplification of first batch of samples. After the contamination problem was noted, we worked in another laboratory where NL4-3 had never been exposed. However, contamination continued to be detected. This was probably because NL4-3 contaminant had already spread to the reagents being used. In addition, the samples to be amplified had been used many times for another study in a different laboratory where NL4-3 was also being widely used as a control, before the present study. This suspicion was confirmed in at least two samples which were used to amplify another HIV-1 gene before the present study. In

order to prevent contamination occurring during PCR amplification, a separate set of equipment and a separate room, if possible, is preferable.

IV. 1. 4. Cloning efficiency of purified versus unpurified PCR products

The highest cloning efficiency was found using unpurified PCR products, followed by the same samples but using freshly purified PCR products. prolonged storage (6 months) of purified PCR products further reduced cloning efficiency (Table 6). This may be due to some of the 3' A-overhangs of PCR products being lost during purification and storage. The longer the storage period is, the more 3' A-overhangs are lost. 3' A-overhangs have been known to be necessary for the vector-PCR product ligation (Clark, 1988; Mead *et al.*, 1991). Therefore, it is not surprising to find the highest cloning efficiency associated with the samples using unpurified PCR products.

However, with respect to the detection of the desired RT insert, plasmids from most of the colonies which were obtained using the purified DNA for the cloning, contained the desired inserts (Table 6). Meanwhile, all the colonies which were obtained using the unpurified DNA for the cloning did not contain the inserts in their plasmids. This may be due to removal of most of the undesired amplified DNA fragments by the purification. The undesired amplified DNA fragments could also ligate with the vector.

Although the number of colonies selected for PCR detection was low, the results of the cloning efficiency and the detection rate of the desired inserts suggest that using freshly purified DNA for cloning is preferable with the specific kit used.

IV. 2. Sequence diversity

IV. 2. 1. Mutation frequency

By comparing brain-derived RT with blood-derived RT, it was found that the latter had a much higher mutation frequency (1.87±0.69 vs 4.67±2.68, p=0.0017) (Table 8). The rate of divergence from the subgroup consensus sequence can be used as a measure of viral replication among RNA viruses such as HIV-1. (Domingo and Holland, 1994). The lower mutation frequencies in brain-derived RT sequences may suggest lower replication rates of HIV-1 in the brain than in the blood (Valentin *et al.*, 1990). However, an important caveat to this conclusion is that although the brain-derived and blood-derived samples were selected from two clinical groups which were matched for HIV-1 clade, age, clinical status of HIV infection, dose and duration of ZDV, methods of amplification and sequencing, the two groups may have differed by unrecognized factors. The ideal analysis would have been to compare blood and brain samples from the same patients but related blood samples were not available.

The comparison between brain-derived RT and brain-derived *env* sequences from same patients showed that the latter also had a much higher mutation frequency (5.14±1.38) (p<0.0001), which was comparable to that of blood-derived RT (4.67±2.68) (Table 8). It has been documented that different viral proteins or sequences in different loci in HIV-1 genome evolved independently; for example, *rev* versus gp41 and *nef* versus LTR sequences from a same individual (Delassus *et al.*, 1991; Martins *et al.*, 1991). It was believed that the difference in evolution of these sequences was due to different selection pressures. Since neutralizing antibodies for envelope proteins but no neutralizing antibodies

for RT have been detected in the blood of infected patients (Dalgleish *et al.*, 1988; Ho *et al.*, 1988; Broliden *et al.*, 1992; Chiengsong-Popov *et al.*, 1992), the possibility that RT and envelope proteins evolve independently is not surprising.

IV. 2. 2. Mutation patterns

Brain-derived RT showed an obvious trend of mutation from nucleotide A to C when compared to the consensus RT sequence of B clade reference strains (p=0.073) (Tables 8 and 9, Figure 11). The preference of A to C mutations was also observed in brain-derived *env* but not displayed in blood-derived HIV-1 RT (Tables 8 and 9, Figure 11). These mutations did not seem to be introduced into the sequences during PCR amplification: (1) most of these nucleotide mutations were responsible for amino acid mutations and these amino acid mutations were found clustered nonrandomly to several specific sequence regions, similar to those found in reference HIV-1 RT; and (2) none of them appeared at the amino acid residues which were suggested to be necessary or important for the functions of RT by biochemical studies and molecular modelling (Larder *et al.*, 1987; Poch *et al.*, 1989; Delarue *et al.*, 1990; Boyer *et al.*, 1994) (Figures 6 and 7).

Each nucleotide has potential to mutate to any one of other three nucleotides, for example, A may change to C, T or G. Therefore, in total, there are 12 different types of mutations, among which 4 types are transitions (purine nucleotide to purine nucleotide or pyrimidine nucleotide to pyrimidine nucleotide), A to G, G to A, T to C and C to T, and the other 8 types are transversions (purine nucleotide to pyrimidine nucleotide or pyrimidine nucleotide to purine nucleotide). If all mutations occur randomly, each type of mutations would occupy about 8% of total nucleotide mutations and thus, transitions would be half of

transversions. However, in fact, it has been found that retroviruses such as HIV-1, HIV-2, the simian immunodeficiency virus (SIV) and avian myeloblastosis virus (AMV) have more transitions than transversions (Mendelman *et al.*, 1989; Johnson *et al.*, 1991; Gao *et al.*, 1992; Vartanian *et al.*, 1994) With SIV in one study (Johnson *et al.*, 1991), transversions ranged from only 4 to 25% in different SIV clones and most of the clones had less than 10%; no preference of A to C mutations was observed among the transversions; G to A mutations predominated in transitions, in most clones reaching to 50-66% of total nucleotide mutations respectively. This G to A hypermutation was also observed in *env* gene of blood-derived HIV-1 (Vartanian *et al.*, 1994), in some samples reaching to 92 to 100% respectively but no preference of A to C mutations was observed either. It was believed that the G to A hypermutations occurred during proviral synthesis. It has been shown that synthesis of the DNA strand using RNA as the template was several times more accurate than using DNA as the template when HIV-1 RT was used to catalyze the syntheses (Boyer *et al.*, 1992).

Studies of base mispair extension kinetics (Mendelman *et al.*, 1989 and 1990) showed that AMV RT had a higher efficiency of catalyzing chain extension from transition mispairs compared to DNA polymerase α (Pol α) from *Drosophila melanogaster*, when tested using bacteriophage M13 single-stranded DNA as the template. Its standard extension efficiencies for transitions were 10 to 10^5 fold higher than for transversions. If RTs from other retroviruses resemble AMV RT, this finding may provide an explanation for the high percentage of transitions and low percentage of transversions observed in retroviruses. Also, the nature of the nearest neighbor nucleotides have been found to influence mutation specificity. A strong preference for G to A transitions was observed within GpA

dinucleotide in *env* gene of HIV-1. Dislocation of the primer relative to the template was suggested to be the possible mutagenesis mechanism (Vartanian *et al.*, 1991). Further, Vartanian *et al.*'s *in vitro* study (1994) showed that imbalance of intracellular nucleotide pool could also affect mutation specificity. A low concentration of deoxycytidine but a high concentration of deoxythymidine resulted in the preference of G to A mutations in HIV-1 clones.

Although the percentage of transitions was found to be higher than the transversions in brain-derived RT, the percentage of G to A mutations was lower in brain-derived RT than in blood-derived RT but the percentage of A to C transversions was higher in the brain-derived RT than in the blood-derived RT (Tables 8 and 9). Because the sample size was small in the present study, this mutation pattern needs to be confirmed by a further study with more samples. If the mutation pattern persists when more samples are analyzed, what mechanism or mechanisms leading to the mutation pattern would be the next question to be addressed. Because there were no obvious differences in amino acid sequence patterns and thus probably no much differences in the protein structure and function, between brain-derived and blood-derived RTs, it is unlikely that mutation specificity of RT would play a major role in the different preference of mutations found between brain-derived RT and blood-derived RT. Also, because no unique neighbor nucleotide structure pattern could be concluded from either brain-derived RT or blood-derived RT, whether the structure pattern has much influence on the preference of A to C mutations in brain-derived RT is uncertain. The finding of preference of A to C mutations in brain-derived HIV-1 RT may imply a different intracellular nucleotide pool, for example, a high concentration of dGTP but a low concentration of dTTP, existing in brain cells harboring HIV-1.

Also, the rate of A to C mutations may have also been increased by AMV RT, which was used to reverse transcribe the sample RNA into cDNA. In Mendelman *et al.*'s study (1990), AMV RT showed a relatively high standard extension efficiency for C (primer)-T (template DNA) mispairs. A T in the template DNA in their study would be a T in the minus DNA strand in the reverse transcription reaction or an A in genomic RNA. Mispairing a C in the nascent DNA strand in their study or a C in the plus DNA strand in the reverse transcription reaction, with the T would result in a C in the plus DNA strand in subsequent PCR products. In other wards, the whole process would produce an A to C mutation from original RNA to PCR products. The standard extension efficiency for this A to C transversion was comparable to those for transitions except for A to G transitions (100 times lower) but 10 to 5000 times higher than other transversions. The findings appear to be able to explain why there were more A to C mutations in brain-derived RT sequences (AMV RT was used to reverse transcribe brain-derived RNA samples) than blood-derived RT sequences (genomic DNA was used and thus reverse transcription step was omitted). Furthermore, because AMV RT is only 10 times more accurate than HIV-1 RT (Roberts *et al.*, 1988), it appears that a contribution by AMV RT to the observed high A to C mutation rate cannot be ruled out, especially if AMV RT has a comparable standard extension efficiency for C-T mispairs or even a higher one compared to HIV-1 RT. However, what should be kept in mind are the mutation rate of AMV RT (one per 17,000 nucleotides incorporated), the size of the DNA fragment analyzed (720 bps) and how many times AMV RT catalyzed the reverse transcription (twice, one for synthesis of the minus DNA strand and the other for the plus strand). Although AMV RT has a potential to introduce more A to C mutations than other transversions, transitions are still the preferred mutations by AMV

RT. 100 times higher standard extension efficiency for A to G transitions plus equivalent standard extension efficiencies for other transitions reduce further the rate of A to C mutations to probably one per 10^7 nucleotides. Therefore, even if AMV RT made a contribution to the preference of A to C mutations found in brain-derived RT, it would be at an almost negligible level.

IV. 3. ZRAMs in brain-derived RT

IV. 3. 1. Frequency of ZRAMs in brain-derived RT

In the present study, ZRAMs were found in brain-derived HIV-1 RT from only two patients who received ZDV treatment for 35 and 39 months respectively, but not in the 8 other patients exposed to ZDV treatment for 2 to 30 months (Figure 6). The paucity of ZRAMs in brain-derived HIV-1 differed from what was observed in many other studies on blood-derived HIV-1 RT. Five out of the 6 selected HIV-1RT sequences (Najera, et al., 1995), isolated from patients treated with ZDV for 6 to 30 months displayed ZRAMs. The lower frequency in the appearance of ZRAMs in brain-derived HIV-1 may be explained by the lower mutation frequency of brain-derived RT than blood-derived RT.

In addition, an obvious trend was observed in blood-derived RT in that ZRAMs had a higher frequency than the overall amino acid mutation frequency. This was not displayed for ZRAMs in brain-derived RT when compared to the overall amino acid mutation frequency (Table 9). If the phenomenon is true, it may provide another explanation for the lower frequency of ZRAMs in brain-derived RT than in blood-derived RT. The higher ZRAM frequency than the overall amino acid mutation rate found in blood-derived RT, but not in brain-derived RT, might have resulted from a higher selective pressure imposed by

ZDV in the blood rather than in the brain, which might have been due to a higher ZDV concentration in the blood than in the brain (Klecker *et al.*, 1987; Wang *et al.*, 1996).

IV. 3. 2. Association of ZRAMs with clinical status of patients

In the present study, ZRAMs were detected in HIV-1 isolates from one patient in the HIVD group and from another patient in the ND group, indicating no obvious association of ZRAMs with development of HIVD (Figure 6). It seemed that the appearance of ZRAMs in brain-derived HIV-1 only related to the duration of ZDV treatment, as evidenced by occurrence of ZRAMs only in isolates from the two patients treated with ZDV for 35 months or longer.

It has been demonstrated that ZDV treatment prolonged the survival, improved the quality of life of individuals with advanced HIV infection and delayed clinical progression in certain asymptomatic individuals with HIV infection. It has also been shown that ZDV treatment improved or delayed certain HIV-1-associated neurological symptoms in both adults and children with HIV infection. Some investigators suggested that improvement of HIV-1-associated neurological symptoms was due to direct inhibition of HIV-1 replication in the brain by ZDV. In addition, many ZDV resistance studies indicated an association of the appearance of ZRAMs in blood-derived HIV-1 isolates with the later stages of AIDS, lower CD4 cell counts, and higher viremia levels. Based on these facts, we hypothesized that the appearance of ZRAMs in HIV-1 in the brain might be associated with the development of HIVD and we expected occurrence of ZRAMs with higher frequencies in HIV-1 isolates from the HIVD group than from the ND group. However, the results of this small study do not suggest any relationship between the occurrence of ZRAMs and the

development of HIVD in patients treated with ZDV. However, more patients need to be studied to make this conclusion.

If the occurrence of ZRAMs indeed results in the failure of ZDV treatment or makes the treatment less effective, the suggestion that the improvement of HIV-1-associated neurological diseases by ZDV results from direct inhibition of HIV-1 replication in the brain needs to be reconsidered. The association of the appearance of ZRAMs in relation to the duration of ZDV therapy found in the brain-derived HIV-1 isolates is consistent with what has been observed in studies on blood-derived HIV-1 isolates: the longer the ZDV therapy, the more ZRAMs detected (Larder *et al.*, 1989). However, the appearance of ZRAMs in brain-derived HIV-1 occurred much later than in blood-derived HIV-1. This could be explained by the lower mutation rate found in brain-derived HIV-1 RT and possible lower ZDV concentrations in the brain than in the blood. An important clinical implication of these findings is that ZDV treatment may be beneficial in patients with HIVD long after systemic (blood) ZDV resistance has developed.

IV. 3. 3. Discrepancy in ZRAM frequency between this study and another study

Sei, *et al.* (1995) found more frequent occurrence of ZRAMs in brain-derived HIV-1 isolates. At first glance, their results appeared to be inconsistent with our findings. However, by comparing the methods used and the clinical status of the patients, we found some differences between the two studies.

First, Sei *et al.* used selective PCR amplification to detect ZRAMs. Since they did not quantitate the PCR amplification, the percentage occupied by those ZDV resistant HIV-1 in the whole HIV-1 population in the patients was unclear. In other words, whether those

resistant HIV-1 represented the majority in the whole HIV-1 population in the patients was unclear. Due to the high sensitivity of PCR detection, it was very likely that those ZRAM-containing HIV-1 was only a minor population relative to the whole HIV-1 population in the patients but that they were amplified and detected. It is questionable that those ZDV resistant HIV-1 representing a low percentage relative to the whole HIV-1 population could cause the failure of ZDV therapy and thus might be associated with the development of neurological diseases. In contrast, direct sequencing of PCR products used in our study reflected the majority of the whole HIV-1 population in the patients, as evidenced by the sequencing results of the clones (Figure 5).

Secondly, the majority of the patients in their study were children receiving continuous intravenous ZDV treatment. It has been reported that brain structures such as brain-blood barrier structures appeared to be different in children and adults (Nomura *et al.*, 1994). The difference in brain-blood barrier structure in children and the delivering of continuous intravenous ZDV may have resulted in higher ZDV concentrations in the brain of those children compared to the adult patients in our study, who received ZDV orally. Thus, the higher ZDV concentration in the brain of the pediatric patients could favor the more frequent occurrence of ZRAMs in their HIV-1.

IV. 4. Further studies to be considered

IV. 4. 1. ZRAMs and ZDV resistance of viruses

Larder, et al (1989b) reported that two ZRAM-containing RTs, constructed by site-directed mutagenesis showed reduced sensitivity to inhibition by ZDV-TP. However, recombinant infectious viruses containing these mutated RT displayed hypersensitivity to

ZDV, instead of resistance, when tested in culture. This study indicated the discrepancy between the phenotype of the mutated RT and the phenotype of HIV-1 which contained the mutated RT. Thus, although the ZRAMs are considered as indicators of ZDV resistance, but whether all ZRAMs cause the resistance and whether the resistance needs more mutations in RT or even in other HIV-1 genes remain uncertain.

In the present study, although 2 ZRAMs were identified in brain-derived HIV isolates from two patients, whether the viruses containing these ZRAMs are resistant to ZDV and the susceptibility level of these viruses to ZDV have not been examined.

IV. 4. 2. Novel ZRAMs

Besides five ZRAMs at amino acid residues 41, 67, 70, 215, and 219, found in RT of HIV-1 isolates from patients receiving monotherapy of ZDV by the Larder group, five other mutations at residues 62, 75, 77, 116 and 151 were identified by another group as novel ZRAMs in isolates from patients receiving combination therapy with ZDV and ddI (Iversen *et al.*, 1996). These viral isolates and recombinant infectious viruses containing these novel ZRAMs were resistant to ZDV when tested in cell cultures. It appears that HIV-1 from patients treated differently with antiviral agents could have different mutations in their RT responsible for ZDV resistance.

Brain-derived HIV-1 has many features differing from blood-derived HIV-1, indicating distinct influence on HIV-1 by the brain environment. The present study also showed that brain-derived RT had different mutation frequency and mutation patterns from blood-derived RT. In addition to the two ZRAMs, 24 other mutations which were not documented in the established database (Los Alamos National Laboratory, 1995), were also

identified in the brain-derived HIV-1 isolates. It may be worth testing if any of these mutations are responsible for ZDV resistance.

In addition, some of these 24 novel mutations changed, relative to the consensus RT sequence, from amino acids of one category such as hydrophilic amino acids to another category such as hydrophobic amino acids, or vice versa. For example, RT from patient 34 had two such mutations at codons 17 and 200 from aspartic acid to glycine and from threonine to isoleucine respectively; RT from patient 4 mutated at codon 122 from lysine to proline. Particularly, RT from patient 64 showed 4 such mutations at codons 81, 85, 91 and 124 from lysine to tyrosine, glutamine to leucine, glutamine to proline and phenylalanine to asparagine respectively. Although none of these novel mutations appeared at the amino acid residues which are necessary or important for the enzymatic functions of RT, changes in polarity and space due to these mutations from one amino acid category to another may affect the micro-environment within the three-dimensional structure of RT where functionally necessary or important amino acids are located, that may in turn influence the functional activities of RT. Hence, it may also be worth testing if any of these mutations influences RT enzymatic activities.

IV. 4. 3. Suppression of ZDV resistance

It has been found that some of the mutations in RT, including ZRAMs, could interact with one another when coexisting in a same strain. In some cases the interaction among the mutations could result in considerable increase in ZDV resistance. For example, the coexistence of M41L with T215Y produced a virus strain that was considerably more

resistant than strains with single mutations (an IC_{50} value of 0.6 to 0.7 μM versus 0.04 μM for M41L alone and 0.16 μM for T215Y alone) (Kellam *et al.*, 1992 and 1994).

However, the interaction did not always result in increase in ZDV resistance. Instead, ZDV resistance was suppressed in some cases. For example, appearance of mutation L74V, which alone conferred ddI resistance, in ZDV resistant HIV-1 isolates containing a ZRAM T215Y completely suppressed the ZDV resistance of the isolates. This was confirmed by site-directed mutagenesis studies (St. Clair *et al.*, 1991).

Similar observations of ZDV resistance suppression have also been made with other mutations. Mutation M184V induced an approximately 500-fold increase in the IC_{50} value for antiviral agents 2'-deoxy-3'-thiacytidine (3TC) and 2'-deoxy-5-fluoro-3'-thiacytidine (FTC), a new generation of nucleoside analogue inhibitors (Boucher *et al.*, 1993; Gao *et al.*, 1993; Tisdale *et al.*, 1993). This mutation completely reversed ZDV resistance due to M41L plus T215Y and partially suppressed ZDV resistance rendered by coexistence of four ZRAMs (D67N, K70R, T215F, and L219Q) (Boucher *et al.*, 1993; Tisdale *et al.*, 1993).

In the present study, two ZRAMs together with many other mutations including 24 mutations which were not documented in the established database, were identified in the brain-derived HIV-1 isolates. It may be worth examining if any of these mutations interacts with the ZRAMs, resulting in increase or suppression of ZDV resistance.

It is technically difficult to isolate live HIV-1 from brain tissues. Thus, for three purposes discussed above ([1] to examine if HIV-1 with RT sequences in brain-derived isolates, containing identified ZRAMs, is resistant to ZDV; [2] to examine if there is any interaction between the ZRAMs and other mutations, resulting in increase or suppression in

ZDV resistance; and [3] to identify any possible novel ZRAMs in the brain-derived HIV-1, recombinant infectious viruses can be constructed and tested in cell culture.

IV. 4. 4. HIV-1 RT as a marker of viral evolution

We compared brain-derived HIV-1 RT sequences with blood-derived HIV-1 RT sequences selected from another study and revealed some differences in occurrence of ZRAMs, mutation frequency and mutation patterns between these two groups. However, comparison of RT sequences of HIV-1 isolates from different brain regions or other organs (spleen), in the same individual may be more pertinent. Careful performance of PCR amplification on spleen-derived isolates and subsequent sequencing of PCR products may provide important information on issues.

Studies showed that different HIV-1 strains with different sequences termed quasispecies could be harbored in a same individual (Goodenow *et al.*, 1989). HIV-1 obtained from different organs such as the brain and the blood (Cheng-Mayer *et al.*, 1989), even from different types of cells in the same compartment, for example, T lymphocytes and macrophages in the blood (Schuitemaker *et al.*, 1991), showed different sequences in certain HIV-1 regions.

RT is one of the most conserved genes in HIV-1 (Heringa and Argos, 1993). As shown in the present study, about 98% homology has been observed among brain-derived RT and 95% homology in blood-derived RT (Table 7). Thus, RT is frequently chosen to serve as a good marker for evolutionary analysis (Eickbush, 1994; Heringa and Argos, 1993). Sequence comparison of HIV-1 RT from different organs may provide information on pathways of intra-individual transferring of HIV-1. Since the mechanism by which HIV-

1 enters into the brain is still unknown, comparison of highly conserved RT sequences of HIV-1 isolates from different organs at different time points during the course of disease may be helpful in eliciting pathways and the time of viral entry into the brain.

IV. 5. Conclusion

To determine the frequency of ZRAM and molecular features of mutagenesis in brain-derived HIV-1, sequence from codon 1 through 240 of RT coding region of 10 brain-derived HIV-1 isolates were PCR-amplified and sequenced. Previously described ZRAMs were found in HIV-1 RT from only two patients who received prolonged ZDV treatment. This has an important clinical implication in that ZDV therapy may continue to be beneficial in patients with HIVD long after ZDV resistance has developed in HIV-1 in the blood. The findings in the present study that brain-derived RT sequences had lower nucleotide mutation rates and that ZRAMs in blood-derived HIV-1 but not in brain-derived RT had a higher mutation frequency than the overall amino acid mutation rate may provide an explanation for the few ZRAMs observed in brain-derived HIV-1.

The higher mutation rate in A to C or T mutations found in brain-derived HIV-1 RT and *env* may indicate a different intracellular nucleotide pool existing in the brain environment. The fact that A to C or T nucleotide mutations accounted for 56% of the novel amino acid substitutions in brain-derived HIV-1 RT may imply a different influence on molecular features of mutagenesis of HIV-1 imposed by the brain environment compared to the blood environment.

Since the sample size was small in the present study, the findings need to be confirmed by further studies.

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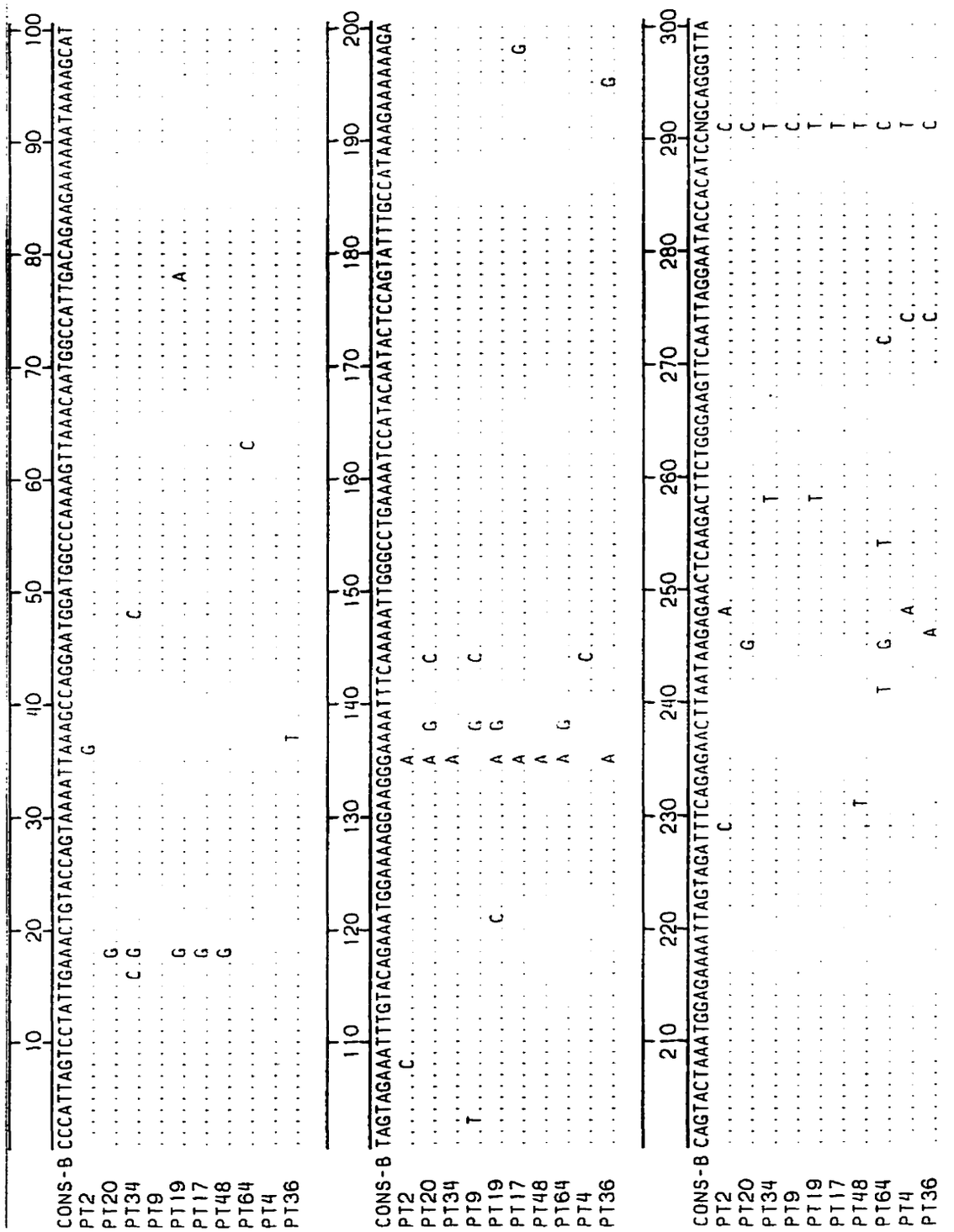
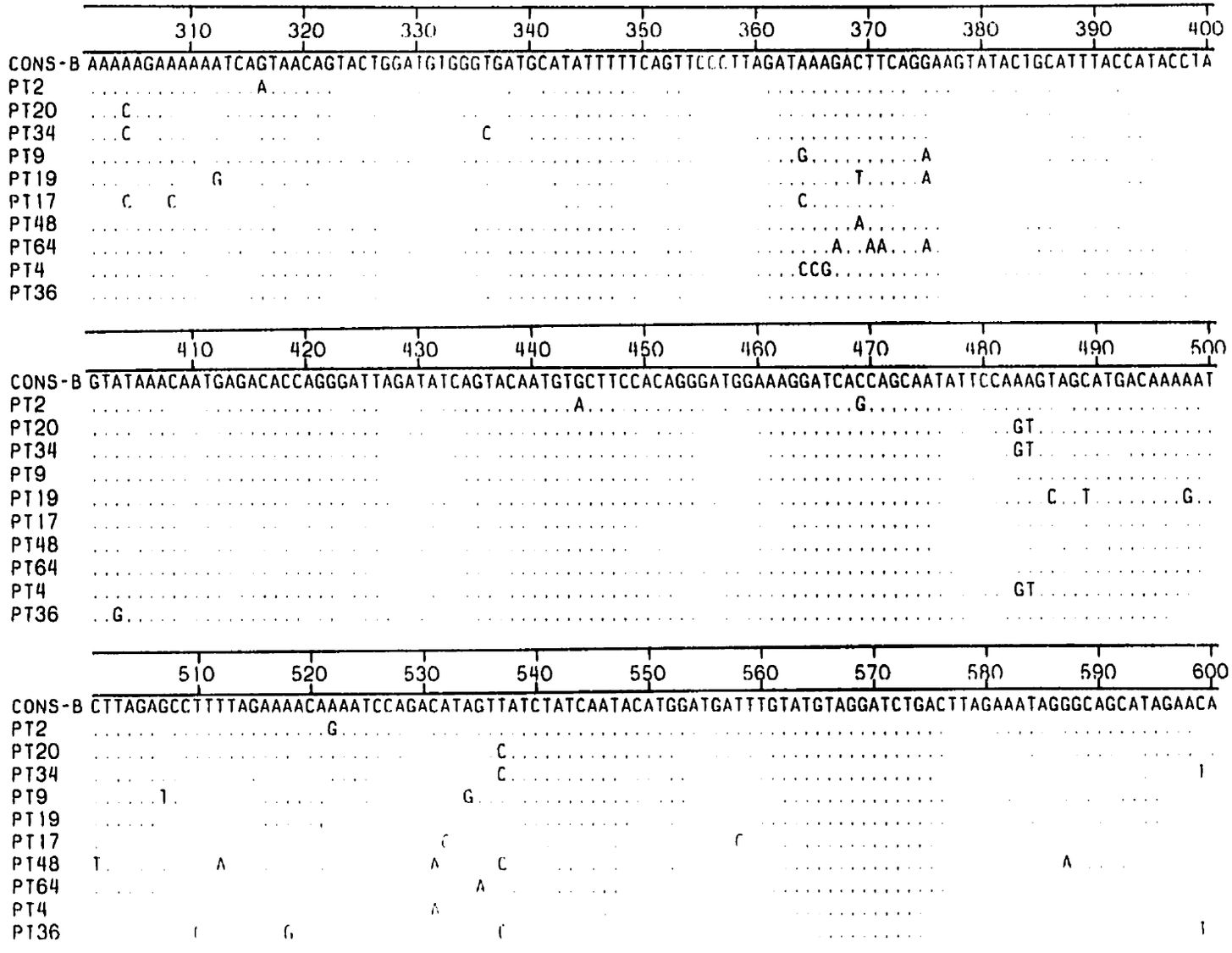
VI. APPENDIX

Brain-derived DNA sequences from PCR-amplified cDNA from 10 patients. aligned with the B clade consensus sequence.

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PT2 A
PT20 C
PT34 C C
PT9 G A
PT19 G T A
PT17 C C C
PT48 A
PT64 A AA A
PT4 CCG
PT36
410 420 430 440 450 460 470 480 490 500
CONS-B GTATAAACAATGAGACACCAGGGATTAGATATCAGTACAATGTGCTTCCACAGGGATGGAAAGGATCACCAGCAATATTCCAAAGTAGCATGACAAAAAT
PT2 A G
PT20 GT
PT34 GT
PT9
PT19 C T G
PT17
PT48
PT64
PT4 GT
PT36 G
510 520 530 540 550 560 570 580 590 600
CONS-B CTTAGAGCCTTTTAGAAAACAAAATCCAGACATAGTTATCTATCAATACATGGATGATTTGTATGTAGGATCTGACTTAGAAATAGGGCAGCATAGAACA
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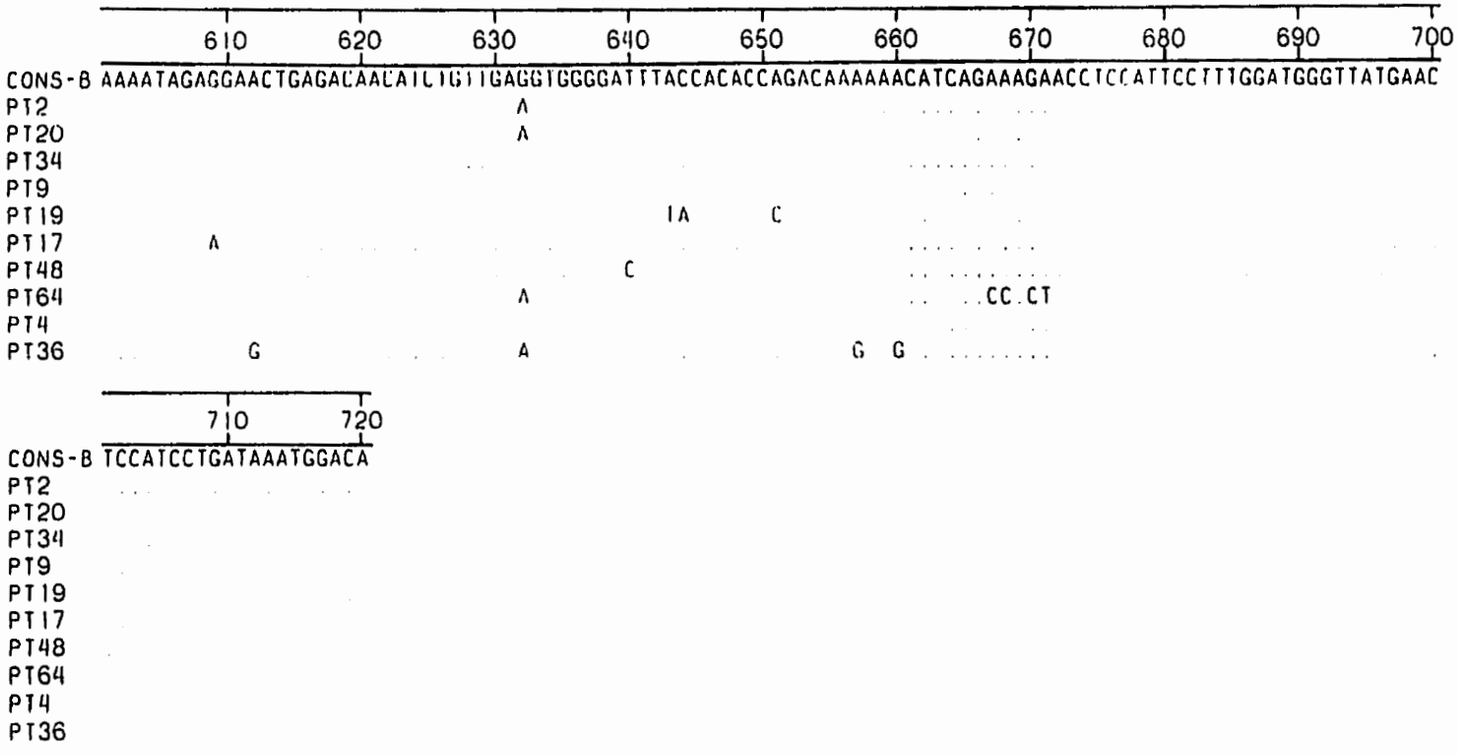
PT20 C
PT34 C T
PT9 T G
PT19
PT17 C C
PT48 T A A C A
PT64 A
PT4 A
PT36 T G C T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PT2 A
PT20 A
PT34
PT9
PT19 IA C
PT17 A
PT48 C
PT64 A CC.CT
PT4
PT36 G A G G
710 720
CONS-B TCCATCCTGATAAATGGACA
PT2
PT20
PT34
PT9
PT19
PT17
PT48
PT64
PT4
PT36