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University of Alberta

Functional Studies of HIV-1 Vpr Protein and Development of hu-PBL-SCID/beige Mouse Model for the Studies of HIV-1 Infection *in vivo*

by

Chengsheng Zhang ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**

Department of Medical Microbiology and Immunology

Edmonton, Alberta

Spring, 1998

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled " **Functional Studies of HIV-1 Vpr Protein and Development of hu-PBL-SCID/beige Mouse Model for the Studies of HIV-1 Infection *in vivo*** " submitted by **Chengsheng Zhang** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY.**

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April 21 , 1998

Abstract

Infection of human $CD4^+$ lymphocytes with human immunodeficiency virus type 1 (HIV-1) often leads to cell death and persistent infection rarely occurs in tissue culture. In this study, several chronically infected cell lines were established. Mutations with HIV-1vpr gene occurred and no Vpr expression was detected in all the chronically infected cell lines, suggesting that Vpr may play a critical role in preventing the establishment of persistently infected cell lines.

A conditional expression system has been used to characterize the effects of HIV-1 Vpr, HIV-2/SIV Vpr and Vpx in the fission yeast *Schizosaccharomyces pombe* (*S. pombe*). Vpr or Vpx could inhibit cell proliferation and induce cell cycle arrest at G1/S and G2/M phases in *S. pombe*. In addition, Vpr or Vpx were found to be cytotoxic in *S. pombe*. HIV-1 Vpr-induced growth inhibition and cytotoxicity were mapped to the C-terminal region of Vpr.

Further studies showed that p34/cdc2 kinase activity was apparently suppressed in yeast cells expressing Vpr or Vpx, indicating that Vpr and Vpx may induce cell cycle arrest through a p34/cdc2 pathway. We have also shown that three p34/cdc2 mutants including cdc2-3w, cdc2-1w, and cdc2-1w, Δmik1 were susceptible to Vpr-induced cell cycle arrest, suggesting that Vpr might not act directly on the Cdc25, Wee1 or Mik1. HIV-1 NCp7 was able to slow down the cell cycle progression in *S. pombe*. Two Vpr-resistant mutant yeast strains were generated by EMS mutagenesis. We have attempted to identify Vpr-interacting proteins by the screening of yeast genomic DNA libraries. However, we were unable to identify any target protein.

We have developed a hu-PBL-SCID/beige mouse model. High levels of human immunoglobulins and lymphocytes were detected in the reconstituted mice. These mice could be infected by cell-free HIV-1 viruses. Reversal of CD4/CD8 ratio was observed in most of the HIV-1 infected mice. In addition, this mouse model was used to study anti-HIV-1 protective immunity *in vivo* by which we have provided evidence suggesting that some multiply exposed, HIV-1 seronegative individuals have protective immunity to HIV-1 infection.

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Abbreviations

AIDS: Acquired immunodeficiency syndrome

BSA: Bovine serum albumin

CDC: Cell division cycle

CDK: Cyclin-dependent kinase

CTL: Cytotoxic T lymphocyte

DAB: 3.3'-diaminobenzidinetetrahydrochloride

DMEM: Dulbecco's Modified Eagle's Medium

DNase I : Deoxyribonuclease I

DTT: Dithiothreitol

EBV: Epstein-barr virus

EDTA: Ethylenediamino-tetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

EM: Electron microscopy

EMS: Ethylmethanesulfonate

Env: Envelope

FACS: Fluorescence activated cell sorter

FAIDS: Feline acquired immunodeficiency syndrome

FBS: Fetal bovine serum

Gag: Group specific antigen

gp41: Glycoprotein 41

gp120: Glycoprotein 120

HBSS: Hanks' balanced salt solution

HIV-1: Human immunodeficiency virus type 1

HIV-2: Human immunodeficiency virus type 2

HRP: Horseradish peroxidase

HSP: Heat shock protein

HTLV-1: Human T-cell leukemia virus type 1

IEM: Immuno-electron microscopy

Ig: Immunoglobulin

IFN-γ: Interferon-γ

IL-2: Interleukin 2

i.p: Intraperitoneal

kDa: KiloDalton

KLH: Keyhole limpet hemocynin

LTNP: Long-term non-progressor

LTR: Long terminal repeat

MA: Matrix protein

MM: minimal medium (for yeast culture)

MOI: Multiplicity of infection

NCp7: Nuclear capsid protein 7

Nef: Negative factor

NF-κB: Nuclear factor κB

NK cells: Natural killer cells

NLS: Nuclear localization signal

NP-40: Nonidet P40

PAGE: Polyacrylamide gel electrophoresis

PBL: Peripheral blood lymphocyte

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PHA: Phytohemagglutinin

PI: Propidium iodide

PMA: Phorbol-12-myristate-13-acetate

PMSF:Phenylmethylsufonyl fluoride

PP2A: Protein phosphatase 2A

Rev: Regulator of expression of virion proteins

RIP: Vpr interaction protein

RIP-1: Vpr interaction protein-1

RNase A: Ribonuclease A

RT: Reverse transcriptase

SAIDS: Simian acquired immunodeficiency syndrome

SDS: Sodium dodecyl sulfate

SEM: Scanning electron microscopy

SIV: Simian immunodeficiency virus

S. cerevisiae: Saccharomyces cerevisiae

S. pombe: Schizosaccharomyces pombe

SCID mice: Severe combined immunodeficiency mice

TAR: Transactivation response element

Tat: Trans-activator of transcription

TEM: Transmission electron microscopy

$TCID_{50}$: 50% of tissue culture infective dose

UNG: Uracil DNA glycosylase

Vif: virion infectivity factor

Vpr: Viral protein R

Vpu: viral protein U

Vpx: Viral protein X

Chapter I

Introduction

Human immunodeficiency virus type 1 and type 2 (HIV-1, HIV-2) have been identified to be the etiological agents of human acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983; Gallo et al, 1984; Levy et al., 1984; Popovic et al., 1984). AIDS is characterized by a progressive depletion of the $CD4^+$ helper T lymphocytes, leading to severe immunosuppression and a variety of clinical diseases including neurological diseases, opportunistic infections, and neoplasms (Levy, J.A., 1993a; Phair, 1994). In addition, simian immunodeficiency virus (SIV), the animal counterpart of HIV-1 and HIV-2, has been identified to be the etiological agent of simian acquired immunodeficiency syndrome (SAIDS), which resembles human AIDS, and is an excellent animal model for the studies of HIV infection (Daniel et al., 1985; Desrosiers, 1990; Hirsch et al.,1995; Letvin et al., 1985).

A. HIV-1 and lentiviruses

Extraordinary efforts have been made by the scientific community to study HIV and AIDS since its discovery in the early 1980s. A large volume of knowledge has been accumulated on the studies of HIV structure, life cycle of HIV replication, and pathogenesis of HIV infection (Luciw, 1996). It is conceivable that HIV is the most complex retrovirus identified thus far (Coffin, 1996).

1. Genomic structure of HIV and SIV

HIV-1 is a typical retrovirus consisting of two long terminal repeat (LTR) elements flanking three genes encoding the structural viral proteins, gag, pol, and env genes (Cullen, 1991). Unlike most retroviruses, however, HIV-1, HIV-2, and SIV exhibit

a surprising degree of genomic complexity (Greene, 1991; Haseltine, 1991). In addition to the prototypic gag, pol, and env coding sequence, HIV-1 contains six open reading frames including tat, rev, vpr, vif, nef, and vpu (Subbrammanian and Cohen, 1994; Trono, 1995). The closely related HIV-2 and SIV do not code for Vpu, but instead code for another late protein Vpx, which is not found in HIV-1 (Figure 1.1. Genomic organization of HIV-1, 2, and SIV). Tat is a major transactivator of transcription of the proviral long terminal repeat (LTR), and rev mainly acts posttranscriptionally to ensure the switch from the early to the late phase of viral gene expression (Cullen, 1992; Green, 1993). It has been shown that mutations affecting either tat or rev could severely impair viral replication, indicating that tat and rev are essential for virus replication (Dayton et al., 1986; Fishe et al., 1986; Sodroski et al., 1986). In contrast, mutations affecting other auxiliary genes do not apparently affect the viral replication kinetics *in vitro*, and these proteins have further been shown to be dispensable for virus replication in many tissue culture systems, and are thus commonly called accessory proteins (Cullen and Creene, 1990; Subbramanian and Cohen, 1994; Trono, 1995). Nevertheless, the high degree of conservation of these proteins in HIV and SIV indicates that they may perform crucial functions *in vivo*. Indeed, increasing evidence suggests that these accessory proteins may play important roles in the pathogenesis of HIV-1 infection (Trono, 1995).

2. Life cycle of HIV-1 replication

The life cycle of HIV-1 replication is initiated by the interaction of the viral envelope glycoprotein, gp120, with its receptors on the target cells (Coffin, 1996). The CD4 molecule has long been demonstrated to be the essential and main receptor for HIV infection (Dalgleish, et al., 1984; Klatzmann et al., 1984; Mcdougal et al., 1986). However, the co-receptors, which are also required for HIV infection, were identified last year after a searching for more than a decade. These co-receptors, which mainly

include CCR5, CCR3, and CXCR4, have been found to be the members of the chemokine receptors belonging to a large family of G-protein-coupled receptors with seven-transmembrane domains (Deng et al., 1996; Draggic, et al., 1996; Feng et al., 1996). However, the detailed mechanisms regarding the binding of gp120 and its receptor and co-receptors are largely unknown, and have been actively investigated (Berger, 1997). Fusion between the viral envelope and cellular membrane occurs following the binding of the HIV-1 gp120 and its receptors on the target cells (Berson et al., 1996; Simmons et al.,1996; Wu et al., 1996). After fusion, the viral core disintegrates, and viral RNA is released into the cytoplasm and subsequently transcribed into double-strand DNA by HIV-encoded reverse transcriptase (RT) (Cann and Karn, 1989; Luciw, 1996). The double-stranded viral DNA is transported into the nucleus and integrated into the genome of the host cells (Cann and Karn, 1989; Luciw, 1996). The integrated viral DNA, also called proviral DNA, is transcribed into viral genomic or messenger RNA using the cellular machinery (Coffin, 1996). The HIV mRNA is subsequently translated into viral proteins. The viral proteins, enzymes, and genomic RNA assemble at the host cell plasma membrane to form the viral core (Cann and Karn,1989; Luciw, 1996). Subsequently the viral core acquires its external and transmembrane envelop to form the viral particle and it buds through the host cell membrane (Figure 1.2 Life cycle of HIV-1 replication). It is noteworthy that the life cycles of HIV-2 and SIV, and the usage of receptors and co-receptors by these viruses are similar to that of HIV-1 (Bron, et al., 1997; Chen et al., 1997; Deng et al., 1997; Marcon et al., 1997; Sol et al., 1997).

3. HIV-1 infection *in vivo*

HIV-1 infection can result in different clinical outcomes (Levy, J.A., 1993a). In most cases (typical progressors), HIV-1 infection causes a gradual depletion of $CD4^+$ T cells and subsequent progression to AIDS over a median period of 8 to 10 years after

primary HIV-1 infection (Lifson et al., 1991; Pantaleo et al., 1996; Rutherford et al., 1990). However, some HIV-1 infected individuals (rapid progressors, about 10%) develop full-blown AIDS within 2 to 3 years after primary infection (Phair et al., 1994). In addition, there are some individuals (Long-Term Non-Progressors, LTNP, less than 5%) who remain healthy and asymptomatic, and have normal $CD4^+$ T cell counts for more than 10 years after primary infection (Cao et al., 1995; Levy, J.A., 1993b; Kirchhoff et al., 1995; Pantaleo et al., 1995).

Acute HIV-1 infection: HIV-1 infection usually occurs with an acute, self-limited syndrome which is characterized by fever, rash, diarrhea, generalized lymphadenopathy. This syndrome typically resolves within 1-2 weeks (Clark et al., 1991; Daar et al., 1991; Levy, J.A., 1993a). Seroconversion (the development of detectable antibodies to HIV-1) can appear weeks to months after the initial infection (Pantaleo et al., 1993). During acute HIV infections, there is a burst of viremia and a decline in $CD4^+$ T cells (Daar et al., 1991; Clark et al., 1991, Pantaleo et al., 1993). Subsequently most of the infected individuals remain clinically quiescent for a long period of time (over 8-10 years) before the onset of AIDS. Earlier studies proposed that there was a very low viral replication during the " clinical latency " (Levy, J.A., 1993a). However, recent studies have demonstrated that HIV-1 replication and turnover are very high during this period (Ho et al., 1995; Wei et al., 1995). In addition, other studies have shown that there is active and continuous HIV-1 replication in the lymphoid organs during the long period of clinical latency (Pantaleo et al., 1993; Pantaleo et al., 1996). Taken together, these studies suggest that HIV-1 infection remains active and progressive throughout the course of infection.

Persistent HIV-1 infection and Long-Term Non-Progressors: While the majority of individuals develop AIDS over 8-10 years after primary HIV-1 infection,

there is a small group of HIV-1 infected people (LTNPs) who show no symptoms of HIV-1 infection, and have normal level of $CD4^+$ T cell counts for a long period of time after infection (Buchbinder et al., 1994; Cao et al., 1995; Kirchhoff et al., 1995; Levy, J.A., 1993b; Pantaleo et al., 1995). A number of studies have demonstrated that the LTNPs have low levels of viral load, normal structure of lymphoid tissues, and normal immune functions (Buchbinder et al., 1994; Cao et al., 1995; Pantaleo et al., 1995; Sheppard et al., 1993). However, it is not clear whether the LTNPs have had a typical primary infection as described above.

High-risk, multiply exposed, HIV-1 seronegative individuals: In addition to the studies of HIV-1 infected LTNPs, much attention has also been paid to the investigation of the individuals who have been at high risk, multiply exposed, but remain HIV-1 seronegative (Haynes et al., 1996; Shearer et al., 1996). These individuals may include intravenous drug users having the history of needle sharing; individuals having unprotected sexual intercourse with HIV-1 infected partners; recipients of HIV-contaminated blood or blood products; prostitutes; and newborn infants of HIV-1 infected mothers. A number of cohort studies have suggested that some of the high-risk, multiply exposed individuals seem to be resistant to HIV-1 infection *in vivo* (Detels et al., 1994; Fowke et al., 1996; Paxton et al., 1996; Taylor, 1994a). It has been proposed that many factors, including the host genetic, virological, and immunological factors, may contribute to the resistance to HIV-1 infection in this group of individuals (Fauci, 1993; Fauci, 1996; Haynes et al., 1996; Taylor, 1994b). However, so far the mechanisms responsible for the resistance are not completely understood. It has been demonstrated that HIV-1-specific, cell-mediated immune responses were detected in some of the high-risk, multiply exposed, HIV-1 seronegative individuals (Clerici et al., 1992; Langlade-Demoyen et al., 1994; Rowland-Jones et al., 1993; Rowland-Jones; et al., 1995). In addition, a number of

recent studies have suggested that the mutations of the co-receptor CCR5 may contribute to the resistance to HIV-1 infection in some multiply-exposed individuals (Huang et al., 1996; Liu et al., 1996; Samson et al., 1996). We have been interested in the studies of the multiply exposed, HIV-seronegative individuals and have examined the possible role of anti-HIV immune response in these individuals. Interestingly, by using the hu-PBL-SCID/beige mouse model, we have provided some data indicating that anti-HIV-1 protective immunity does exist *in vivo* in some of the multiply exposed, HIV-1 seronegative individuals (Zhang et al., 1996; Chapter V).

Taken together, HIV-1 infection *in vivo* is a very complex and dynamic process which can result in a variety of clinical courses including acute and latent infections. While a tremendous knowledge of HIV-1 infection *in vivo* has been accumulated from the studies of different HIV-1 infected individuals in the last decade, a number of key issues regarding the pathogenesis of HIV-1 infection are still far from being fully understood. For instance, it is unknown how HIV-1 infection eventually leads to the depletion of $CD4^+$ T cells and causes immunodeficiency in most of the HIV-1 infected individuals. In addition, it is still a mystery why HIV replication is controlled and there are no clinical symptoms in some HIV-1 infected LTNPs. Furthermore, it is not totally clear why some of the high-risk, multiply exposed individuals remain HIV-1 seronegative despite their frequent exposure to HIV-1 infected subjects.

4. HIV-1 infection of human $CD4^+$ cells *in vitro*

Similar to HIV-1 infection *in vivo*, HIV-1 is also able to infect a variety of human $CD4^+$ cell lines and peripheral blood lymphocytes (PBLs), and can induce either acute or persistent infection in the tissue culture systems (Levy, J.A., 1993a). Therefore, we think that the tissue culture system can be used as a model to study the molecular

mechanisms of acute and persistent HIV-1 infections, and thereby provide valuble information for the understanding of HIV-1 infection *in vivo*.

Tropism of HIV-1: HIV-1 isolates vary in their ability to infect primary $CD4^+$ T cells, established T-cell lines, and cells of the monocyte/macrophages lineage (Broder and Berger, 1995; Levy, J.A., 1993a). Based on its ability to replicate in human immortalized T cell lines and primary monocyte/macrophages, HIV-1 isolates have been divided into three types: T cell-tropic viruses (T-tropic), which replicate preferentially in immortalized T cell lines, but not in primary monocyte and macrophage; macrophage-tropic viruses (M-tropic), which replicate predominantly in monocyte and macrophages, but not in T cell lines; dual-tropic viruses, which replicate efficiently in both T cell lines and macrophages (Cheng-Mayer, et al., 1988; Fenyo et al., 1988; Border and Berger et al., 1995). It has been demonstrated that the third variable (V3) region of gp120 of the viral envelope glycoprotein determines the viral tropism (Cheng-Mayey et al., 1990; Chesebro et al., 1992; O'Brien et al., 1990; Willey et al., 1994). On the other hand, HIV-1 tropism has also been found to correlate with the co-receptor usage by different HIV-1 strains (Berger, 1997; Dittmar et al., 1997; Zhang et al., 1996). T-tropic strains have been shown to use CXCR4 (or fusin) which is expressed abundantly on the T cell lines; M-tropic viruses use CCR5 or/and CCR3 which are differentially expressed on primary T cells, monocyte, and macrophages; whereas dual-tropic viruses can use either CXCR4, CCR5, or CCR3 (Bluel et al., 1997; Choe et al., 1996; Doranz et al., 1996; Simmons et al., 1996; Zhang et al, 1996). In our current study, two laboratory-adapted HIV-1 strains have been used. One is HIV_{NL4-3}, which is a T-tropic virus and able to replicate in T cell lines and primary T lymphocytes, but not in macrophages. The second strain is HIV_{NLAD8}, which is a M-tropic virus and able to replicate in macrophages and primary T cells, but not in T cell lines (Adachi et al., 1986). It has been shown that HIV_{NL4-3}

use the co-receptor of CXCR4, while HIV_{NLAD8} predominantly use CCR5 and CCR3 but not CXCR4 (Zhang et al., 1996; He et al., 1997).

Interestingly, the phenotypes of HIV-1 tropism also exist *in vivo* , and the change in co-receptor use are suggested to be correlated with the disease progression during HIV-1 infection. It was shown that early during the course of HIV-1 infection, the majority of HIV-1 isolates were M-tropic, whereas a shift of HIV variants from M-tropic to T-tropic was observed during disease progression of AIDS (Connor and Ho, 1994a; Connor and Ho., 1994b; Connor et al., 1997; Zhu et al., 1993). Therefore, the alternation of viral tropism may play an important role in the pathogenesis of HIV infection.

Acute HIV-1 infection: Infection of human $CD4^+$ T cell lines and primary lymphocytes with HIV-1 in tissue culture often results in acute infection with marked cytopathic effects and cell death (Somasundaran and Robinson, 1988). The molecular mechanisms responsible for cell death are not completely understood. HIV-1 induced syncytia formation has been suggested to play a role in HIV-mediated cytopathicity in some human $CD4^+$ T cell lines *in vitro.* For instance, it has been suggested that uninfected $CD4^+$ T cells can be killed indirectly by fusion with the infected $CD4^+$ T cells in the tissue culture systems (Lifson et al., 1986a and 1986b). In addition, other mechanisms have been proposed to explain HIV-1 induced cell killing in various kinds of cultured cells (Somasundaran and Robinson, 1987). These mechanisms include toxic proteins made by HIV-1, destruction of the cell membrane by large quantities of budding virions, and accumulation of high level of unintegrated viral DNA (Hofmann et al., 1990; Linette et al., 1988; Pang et al., 1990; Stevenson et al., 1988). Furthermore, a number of studies have suggested that programmed cell death or apoptosis may also play an important role in HIV-induced cell death (Groux et al.,

1992; Laurent-Crawford et al., 1991; Lu et al., 1995; Savarino et al., 1997). However, it is not clear exactly how HIV-1 infection can induce apoptosis. HIV-1 Tat protein has been demonstrated to induce apoptosis in human T cells (Li et al., 1995). Recent studies have also shown that HIV-1 Vpr protein can induce apoptosis in human T cell lines and primary lymphocytes (Ayyavoo et al., 1997a; Stewart et al., 1997; L-J. Chang et al., unpublished data).

Persistent HIV-1 infection: While HIV-1 infection in human T cell lines often causes acute infection, persistent HIV-1 infection in some T cell lines has been reported (Banerjee et al., 1992; Folks et al., 1987; Poli et al., 1990). Most of these persistently infected cell lines produce low levels of HIV-1 in culture systems. We have also studied acute and persistent HIV-1 infections in tissue culture. Interestingly, we have successfully established several chronically infected cell lines that continuously produce high titers of HIV-1 (Chang and Zhang, unpublished data, Chapter II). It is not completely clear why HIV-1 induces acute infection and cell killing in most circumstances and causes persistent infection under some conditions. In particular, it is unknown what conditions are required for the establishment of persistent infection. A number of studies have suggested that the mutations of HIV-1 accessory proteins including Vpr, Vif, Nef, or Vpu may play important roles in determining the fate of HIV-1 infected cells in tissue culture (Kishi et al., 1992; Nakaya et al., 1994; Nishino et al., 1994). Further studies have indicated that HIV-1 Vpr can prevent the establishment of the chronically infected cell lines, suggesting that lack of functional Vpr is required for the establishment of persistently infected cell lines (Planelles et al., 1995; Rogel et al.,1995). We have investigated this suggestion by examining the possible role of Vpr in chronically infected cell lines (Chapter II).

B. HIV-1 Vpr

HIV-1 viral protein R (Vpr) is a 96 amino acid, 14 kDa virion-associated protein (Cohen et al., 1990b; Yuan et al., 1990). The vpr gene is highly conserved within the genome of the primate lentiviruses including HIV-1, HIV-2, and SIV isolates (Sharp et al., 1996), indicating that Vpr may play a role in viral replication. In addition, HIV-2 and some of the SIV isolates contain a unique 12-16 kDa Vpx protein which is not present in HIV-1 (Yu et al., 1988). It has been shown that Vpr and Vpx share a strong homology (Tristem et al., 1992). Based on the sequence similarity between these two genes, Vpr and Vpx have been proposed to be evolutionarily related, probably arisen through gene duplication (Tristem et al., 1992).

1. Possible roles of Vpr in viral infection and replication

Several possible roles have been suggested for Vpr in the regulation of HIV infection and replication, including facilitation of nuclear import of the preintegration complex, trans-activation of HIV-1 LTR, facilitation of infection of macrophages and irradiated cells, activation of latent viral infection, and prevention of the establishment of chronic infection.

Virion-association of Vpr: Both Vpr and Vpx have been demonstrated to be incorporated into the viral particles in HIV-1, HIV-2, and SIV (Cohen et al., 1990b; Yu et al., 1988; Yu et al., 1990). It has been shown that the p6 Gag protein is necessary for the incorporation of Vpr into nascent viral particles, and absence or truncation of p6 prevents Vpr incorporation into the virion (Lavallee et al., 1994; Lu et al., 1993; Paxton et al., 1993). However, it is uncertain which domain of Vpr is required for its virion incorporation. Some of the previous studies have suggested that the basic residue-rich region at the C-terminal of Vpr is required for incorporation (Paxton et al., 1993; Yao et al., 1995). Other studies show that the alpha-helical

region at the N-terminal of Vpr is required for the virion incorporation (Mahalingam et al., 1995a and 1995d ; Mahalingam et al., 1997; Marzio et al., 1995). The virion association function of Vpr is highly indicative of its participation in the early events during viral replication (Cohen et al., 1990b).

Nuclear localization of Vpr and its related functions: HIV-1 Vpr is localized predominantly in the nucleus although it does not possess a classical nuclear localization signal sequence (Lu et al., 1993; Marzio et al., 1995; Mahalingam et al., 1995b; Yao et al., 1995). By interacting with the matrix (MA) protein, Vpr may play a critical role in the translocation of viral preintegration complex to the nucleus in the infected cells (Heinzinger et al., 1994). The nuclear localization of Vpr may also play important roles in other aspects of viral replication, such as reverse transcription, and stabilization of RNA-DNA or DNA-DNA structures (Cullen, 1992). It has been proposed that Vpr may translocate into the nucleus by specific interactions with cellular proteins (Refaeli et al., 1995; Zhao et al., 1994a). However, the detailed mechanism by which Vpr is translocated into the nucleus is not clear.

Effect of Vpr on the transcription of LTR: Transfection of Vpr into cells containing a CAT reporter construct linked to the HIV long terminal repeat (LTR) and other promoters increases transcription three- to ten-fold (Cohen, et al., 1990a). In addition, HIV-1 Vpr interacts with the cellular transcription factor Sp1 to trans-activate HIV-1 LTR-directed transcription (Wang, et al., 1995). These studies suggest that Vpr may play a role in the transactivation of HIV-1 LTR in the early tat-independent phase of HIV transcription (Cohen, et al., 1990a, Wang et al., 1995).

Effect of Vpr on viral infection and replication: While Vpr is dispensable for viral replication in T cell lines and activated PBL, it is required for efficient viral

replication in primary monocyte and macrophages (Balliet et al., 1994; Ogawa et al., 1989). HIV-2/SIV Vpx is also required for efficient viral replication in primary lymphocytes and macrophages (Guyader et al., 1989; Kappes et al., 1991; Kawamura et al., 1994; Park et al., 1995). Previous studies have shown that expression of HIV-1 Vpr can enhance viral replication *in vitro* (Cohen et al., 1990a; Ogawa et al., 1989). In addition, purified Vpr protein from the plasma of HIV-1 infected individuals or recombinant Vpr have been shown to stimulate the viral production of latent infections in chronically infected cell lines or primary PBL from asymptomatic HIV-1 infected patients (Ayyavoo et al., 1997b ; Levy, D.N. et al., 1994a and 1994b; Levy et al., 1995). Furthermore, other studies have shown that inhibition of HIV-1 Vpr expression by using anti-sense oligonucleotide can suppress HIV replication in monocytic cells (Balotta et al., 1993). All these studies suggest that Vpr plays a role in viral replication.

2. Vpr and cell cycle progression

An interesting and puzzling discovery is that Vpr can perturb the host cell cycle progression. However, the biological significance and the molecular mechanisms of Vpr-induced cell cycle arrest remain unknown.

Cell cycle regulation in eukaryotic cells: The cell cycle describes the progression of a cell from a resting (non-cycling) state (G0) to grow and divide into two daughter cells (Murray and Hunt, 1993). There are four stages through which the cell cycle progresses, including the G1 phase, where the RNAs and proteins are synthesized; the S phase, where the DNA replication occurs; the G2 and M phases where the DNA and proteins are divided into two daughter cells (Murray and Hunt, 1993).

Physiologically, cell cycle progression is controlled by different checkpoints that control the order and timing of cell cycle transitions and ensure that critical events such as DNA replication and chromosome segregation are completed in fidelity (Murray, 1992; Nurse, 1994). There are at least two tightly controlled cell cycle checkpoints in eukaryotes, G1/S and G2/M (King et al., 1994; Nurse, 1994; Sherr et al., 1994). It is believed that cell cycle progression is driven by sequential expression of diverse cyclins and the resultant activation of cyclin-dependent kinases (CDKs) (Grana and Reddy, 1995; Lees, 1995; Morgan, 1995). In mammalian cells, different cyclin/CDK complexes are assembled and activated at specific points of the cell cycle (Grana and Reddy, 1995; Lees, 1995). In this respect, the G1 checkpoint is mainly controlled by cyclin D-CDK4 and -CDK6 complexes, and cyclin E-CDK2 complexes, whereas the G2/M transition and mitosis are controlled by cyclin B complexes (Grana and Reddy, 1995). In addition to the binding of cyclins, CDK activity is modulated by positive and negative regulatory factors (Dunphy, 1994; Lees, 1995).

The basic cellular machinery regulating cell cycle progression is highly conserved in all eukaryotes (Bernander., 1994; Murray and Hunt, 1993). In fact, a tremendous amount of information regarding cell cycle progression has been obtained by the studies of the cell cycle of yeast cells (Moreno et al., 1991; Murray and Hunt, 1993). For example, one of the major breakthroughs in our understanding of cell cycle regulation was the discovery of the cdc2 gene and its product p34/cdc2 kinase in *Schizosaccharomyces pombe* (Nurse and Bissett, 1981; Murray., 1989). In *Saccharomyces cerevisiae*, a homologous gene termed cdc28 performs similar functions (Reed et al., 1985; Wittenberg et al., 1988). The human homologue of cdc2, designated as $cdc2^{Hs}$ (also known as cdk1), was cloned based on studies demonstrating that human cdk1 could complement cdc2 or cdc28 yeast mutants (Lee and Nurse, 1987). It has been shown that p34/cdc2 kinase is required for both G1/S and G2/M transitions in *S. pombe*

(Atherton-Fessler et al., 1993; Moreno et al., 1989). However, although Cdc2 (Cdk1) is required for G2/M transition in mammalian cells, G1/S progression is predominantly controlled by Cdk2 (Grana and Reddy, 1995).

It has been demonstrated that the p34/cdc2 protein kinase plays a crucial role in cell cycle regulation in eukaryotic cells (Norbury and Nurse, 1989; Moreno et al., 1989; Stern and Nurse, 1996). In the normal cycle of yeast and mammalian cells, the timing of mitosis is determined by the inhibitory phosphorylation of Cdc2 (Dunphy, 1994). In the fission yeast *S. pombe*, phosphorylation of p34/cdc2 occurs at Tyr-15 and is catalyzed by the kinases Wee1 and Mik1, with Wee1 being the most active (Gould and Nurse, 1989; Lundgren et al., 1991). Cdc25, a protein phosphatase, activates the p34/cdc2 kinase by the dephosphorylation of p34/cdc2 at Tyr-15 (Gautier et a., 1991; Lee et al., 1992; Russell and Nurse, 1986; Strausfeld et al., 1991). In higher eukaryotes, in addition to Tyr-15, p34/cdc2 is also phosphorylated at Thr-14, and dephosphorylation of both residues by Cdc25 is required for the activation of p34/cdc2 kinase (Krek et al., 1991; Norbury et al, 1991, Solomon, et al., 1992). On the other hand, it has been shown that the phosphorylation of p34/cdc2 on Thr-167 in *S. pombe* (Thr-161 in higher eukaryotes) is required for the activation of p34/cdc2 kinase activity (Desai et al., 1992; Ducommun et al., 1991; Gould et al., 1991; Solomon et al., 1992).

Cdc25, Wee1, and Mik1 are themselves regulated by other protein kinases and phosphatases (Dunphy, 1994). It is thought that the induction of mitosis is facilitated by the activation of Cdc25 and inhibition of Wee1 and Mik1 activity during G2/M transition (Dunphy, 1994). One of the protein kinases, termed as Chk1, has recently been demonstrated to negatively regulate Cdc25 and mediate G2 phase arrest induced by DNA damage in yeast and mammalian cells (Furnari et al., 1997; Peng et al., 1997; Sanchez., et al., 1997). In addition, protein phosphatase 2A (PP2A) has also been

suggested to negatively regulate Cdc25 (Mayer-Jaekel and Hemmings, 1994). However, the detailed mechanisms controlling the Cdc25 and Wee1 and Mik1 remain largely unknown.

In view of the striking degree of homology of mitotic control mechanisms in fission yeast and mammals, it has been proposed that the *S. pombe* checkpoint control will serve as an useful model for investigating the checkpoint mechanisms in more complex organisms (Nurse, 1990 and 1994).

Cell cycle state and HIV-1 replication: Virus replication is the result of a series of two-way communications between the virus and its host cell. Whether or not a cell is permissive to infection and subsequent virus replication, or whether non-productive infection occurs, is determined by the expression of specific factors which are necessary for each stage in the viral life cycle and cell cycle state (Knipe, 1996). It is well known that oncoretroviruses depend on cell proliferation for their replication because the breakdown of the nuclear envelope at mitosis allows the viral preintegration complex to interact with the host cell chromosomes (Coffin, 1996). In contrast, HIV and other lentiviruses can infect resting T lymphocytes and nondividing cells such as terminally differentiated macrophages (Connor et al, 1995; Fletcher et al, 1996; Levy, J.A., 1993a). Vpr, together with the viral matrix (MA) protein, can facilitate the translocation of the preintegration complex into the nucleus in the absence of cell division. Although HIV-1 can enter quiescent lymphocytes with high efficiency, the reverse transcription process is not completed, and the infection does not result in production of progeny virus (Pomerantz et al., 1990; Zack et al., 1990). However, the reverse transcription can go to completion and progeny virus can be produced if a mitogenic signal is applied shortly after infection of the quiescent lymphocytes

(Pomerantz et al., 1990; Zack et al., 1990). Therefore, it is apparent that the HIV-1 life cycle is closely related to cell cycle progression of the host cells.

Effect of Vpr on host cell proliferation and cell cycle progression: A number of studies have demonstrated that HIV-1 Vpr has profound effects on the host cells. One of the early studies has shown that HIV-1 Vpr can inhibit cell growth and induce cell differentiation in several tumor cell lines including rhabdomyosarcoma and osteosarcoma cell lines (Levy, D.N., et al., 1993). In addition, other studies suggest that Vpr can prevent the establishment of persistent HIV-1 infection in T lymphocytes (Mustafa and Robinson, 1993; Planelles et al., 1995; Rogel et al., 1995). Further studies have consistently demonstrated that HIV-1 Vpr can induce cell cycle arrest at G2/M phase in mammalian cells (Bartz et al., 1996; He et al., 1995; Jowett et al., 1995; Re et al., 1995). Interestingly, we and others have also demonstrated that HIV-1 Vpr can induce cell cycle arrest in yeast cells (Macreadie et al., 1995; Zhang et al., 1997; Zhao et al., 1996), indicating that Vpr may act on a highly conserved pathway involved in the cell cycle regulation. While the p34/cdc2 kinase activity has been shown to be apparently suppressed in the cells expressing Vpr, the precise mechanisms by which Vpr inhibits cell growth and arrest the cell cycle have not been elucidated (Jowett et al., 1995; Marizo et al., 1995; Zhang et al., 1997; Zhao et al., 1996). Since Vpr has been shown to have a variety of effects on its host cells, Vpr-induced growth inhibition and cell cycle arrest may mediate some of these effects. Therefore, further study of the effects of Vpr on cell cycle progression and elucidation of the precise molecular mechanisms of Vpr-induced cell cycle arrest will not only advance our understanding of Vpr function on HIV-1 replication and pathogenicity, but also might provide important insights into basic events controlling cell cycle progression.

3. Potential role of Vpr in the pathogenesis of HIV-1 infection *in vivo*

The first evidence for the importance of Vpr *in vivo* came from the studies of vpr-mutant SIVmac239 in the infection of rhesus monkeys (Gibbs et al., 1995; Lang et al., 1993). When a point mutated vpr gene was added, vpr reversion was found in three of five animals. The animals with vpr reversion had high virus loads, whereas neither of the other two animals carrying a vpr mutation developed high virus loads or disease, indicating that Vpr may play important roles in efficient viral replication and the development of disease in SIV-infected monkeys (Lang et al., 1993). In addition, it has been shown that the disease progression was less severe when rhesus monkeys were infected with SIV carrying both Vpr and Vpx mutations, suggesting that Vpr and Vpx may act coordinately and synergistically in SIV-induced pathogenicity (Gibbs et al., 1995).

C. Animal models of AIDS

A number of animal models have been developed and used in the studies of HIV-1 pathogenesis, development of new and more effective therapies, and evaluation of HIV vaccines (Stott and Almond, 1995). These animal models include the simian immunodeficiency virus (SIV)-induced SAIDS in rhesus monkeys, feline immunodeficiency virus (FIV)-induced FAIDS in cat, HIV-1 infected chimpanzee model, and HIV-1 infected SCID-hu and hu-PBL-SCID mice models. Each of these animal models has its own advantages and limitations.

1. SIV-induced simian AIDS model

The SIV-induced SAIDS in rhesus monkeys remains the most popular animal model for AIDS research. As mentioned previously, SIV is genetically closer to HIV than any other lentiviruses, and also uses CD4 receptor and chemokine co-receptors for its infection of the target cells. Most importantly, SIV can induce human AIDS-like

syndrome (SAIDS) in rhesus monkeys (Kestler et al., 1990; Letvin et al., 1985). Previous studies on the SAIDS model have provided valuable information on the understanding of HIV infection in humans. For example, it was first demonstrated in SIV model that Nef plays a crucial role in the pathogenesis of SAIDS *in vivo* (Kestler et al., 1991). In addition, the SIV model has also been shown to be very useful in the studies of AIDS vaccine and pathogenesis of HIV infection (Daniel et al., 1992; Desrosiers, 1990).

However, there are some limitations of the simian model. One of the limitations is that SIV is not HIV, and therefore is not a perfect model for HIV study. In addition, the use of monkeys may be affected by economic and ethical issues. Moreover, it is much less practical to manipulate monkeys than other small animals such as mice.

2. FIV-induced FAIDS model

Feline immunodeficiency virus can induce AIDS-like diseases (FAIDS) in cat and has been proposed to be used as a model for the study of the pathogenesis of HIV infection (Bendinelli et al., 1993). However, it is obvious that FIV is significantly different from HIV (Miyazawa et al., 1994). In addition, FIV does not infect T cells, which may not make it a valid model for the study of HIV pathogenesis, since HIV mainly infects T cells (English et al., 1993).

3. HIV-1 infected chimpanzee model

It was anticipated that chimpanzee would be the best animal model for HIV studies. However, later studies have suggested that this model is not suitable for the study of HIV pathogenesis and anti-HIV drugs because HIV infection of chimpanzee does not induce apparent HIV-related diseases including immunodeficiency (Castro et al., 1989; Castro et al., 1992). In addition, chimpanzees are rare and protected animals, thus

there is much debate regarding the use of chimpanzee for HIV studies. Nevertheless, the chimpanzee model has been shown to be useful for the evaluation of the HIV-1 vaccines against infection (Berman et al., 1990 ; Fultz et al., 1992; Lubeck et al., 1997).

4. HIV-1 murine models

There are several obvious advantages of murine models of HIV infection (Chang et al., 1996). First, the genetics and immunology of laboratory mouse strains have been well characterized; second, these is no apparent limitation to the numbers of the laboratory mice available; third, it is much cheaper and simpler to use mice than to use bigger animals, such as non-human primates. Ideally, an animal model should closely reproduce the salient features of HIV-1 infection in humans: Using the same HIV-1 isolates, within the same human lymphoid organs, and infecting the same $CD4^+$ target cells. The need for a better small animal model for AIDS research has prompted the development of two murine model systems that involve the transplantation of human lymphoid cells or tissues into the SCID mice, namely the SCID-hu mouse and the hu-PBL-SCID mouse (McCune et al., 1988; Mosier et al, 1988).

SCID mice

Severe combined immunodeficiency (SCID) mice (C.B.17-SCID/SCID mice, namely, SCID mice) were first reported in 1983 by Bosma and his colleagues (Bosma et al., 1983a). SCID mutant mice lack functional T and B lymphocytes and serum immunoglobulins (Bosma et al., 1983a), which was attributed to an autosomal recessive mutation that impairs the V-(D)-J recombination process during the development of mature B and T cells (Bosma and Caroll, 1991). Due to their severe immnodeficiency, SCID mice are found to be very sensitive to the infections of

microorganisms. Therefore, SCID mice have to be bred and maintained under a sterile environment (Bosma and Caroll, 1991).

Soon after the discovery of SCID mice, a number of studies were conducted to explore their utilization of these mutant in biomedical research (Bosma et al., 1989; Bosma and Caroll, 1991). SCID mice have been successfully used as a model for studies of lymphohematopoiesis, functions of natural killer cells (NK cells), production of monoclonal antibodies, and transplantation of tumor cells or other xenografts (Bosma et al., 1989). In particular, the successful engraftment of human lymphoid cells or tissues into SCID mice and the subsequent development of the humanized-SCID mice model generated tremendous interest in the model and provided the foundation for most of the current studies of SCID mice (Mosier et al., 1988; McCune et al., 1988; Bosma and Caroll, 1991).

There are several properties of SCID mice which may contribute to the variation of SCID mice colonies and to inconsistencies in some studies. First, about 15-20% of young adult SCID mice have been reported to have detectable functional T and B cells (Bosma et al., 1983b; Carroll et al., 1989; Carroll et al., 1988). This feature is referred to as leaky phenotype and is believed to affect the reconstitutional efficiency of human PBL into the SCID mice (Bosma and Carroll, 1991). Since almost all the leaky SCID mice have detectable mouse Ig, serum mouse Ig has become the main parameter for the screening of leaky and non-leaky SCID mice (Bosma and Carrol, 1991; Hesselton et al., 1993). In addition, SCID mice have normal or high natural killer (NK) cell function (Dorshkind et al., 1985), and NK cells play a major role in graft rejection (Dennert et al., 1974). Interestingly, when SCID mice were treated with anti-NK cell antibody (e.g. Anti-asialo GM1 antibody) or radiation to suppress NK cell activity *in vivo*, the

reconstitution efficiency of human PBL in the treated mice was higher than that in the untreated mice (Sandhu et al., 1995; Shpitz et al., 1994).

In order to improve the reconstitution of human lymphocytes into the SCID mice, a new strain of immunodeficient mouse, designated as SCID/beige mouse has been used in our current study (Chapter V). In addition to lacking functional T and B lymphocytes, SCID/beige mice have reduced NK cell activity (Clark et al., 1981; Macdougal et al., 1990), suggesting that SCID/beige mice may be a good recipient of xenografts. Indeed, we have demonstrated that SCID/beige mice are excellent recipients of human tumor engraftments and lymphocytes (Chapter V).

SCID-hu mouse model

The SCID-hu mice model was developed by engraftment of human fetal lymphoid tissues including fetal liver, thymus, and lymph nodes into SCID mice (McCune et al., 1988). Such SCID-hu mice display a transient distribution of human $CD4^+$ and $CD8^+$ T cells, and human IgG in the peripheral blood (McCune et al., 1988). In addition, co-implantation of human fetal thymus and liver (Thy/Liv) into SCID mice have generated long-term human hematopoiesis in the resultant SCID-hu mice (McCune et al., 1991; Namikawa et al., 1990). The development of a normal human immune system in SCID-hu mice made it a potential model for AIDS research. Indeed, a number of studies have demonstrated that the SCID-hu mice can be infected by HIV-1 *in vivo* (Aldrovandi et al., 1993; Bonyhadi et al., 1993; Kaneshima et al., 1991; Kollmann et al., 1994; Namikawa et al., 1988). An additional study has shown that 3'-azido-3'-deoxythymidine (AZT) can inhibit HIV-1 infection in SCID-hu mice, suggesting that the SCID-hu mouse can be potentially used for the evaluation of anti-HIV-1 compounds (McCune et al., 1990). SCID-hu mice have also been widely used in the studies of HIV-1 pathogenesis. It has been shown that HIV-1 infection induces

thymocyte depletion in the transplanted thymus and $CD4^+$ T cell depletion in SCID-hu mice (Aldrovandi et al., 1993; Bonyhadi et al., 1993; Jamieson et al., 1997; Kollmann et al., 1994; Kaneshima et al., 1994; Su et al., 1995). It is noteworthy that most of these studies of HIV-1 infection in SCID-hu mice were carried out by direct injection of HIV-1 into the human implants, which may be different from the systemic infection that occurs in humans. However, a modified SCID-hu mouse model has been shown to support a disseminated HIV-1 infection after peritoneal injection of HIV-1 (Goldstein, 1995; Kollmann et al., 1994). Therefore, the SCID-hu mouse model is a very useful and attractive model for HIV-1 infection. However, because human fetal tissues are required to construct SCID-hu mice, ethical issues and the limited source of fetal tissues are limitations. In addition, it is more difficult to construct SCID-hu than hu-PBL-SCID mice because of the surgical operations necessary for the former. Nevertheless, it is conceivable that the SCID-hu mouse model will continue to play a role in the direct analysis of HIV-1 infection *in vivo*.

hu-PBL-SCID mouse model

Almost at the same time of the successful establishment of the SCID-hu mouse model, another humanized SCID mouse model, designated as the hu-PBL-SCID model, was developed by injection of adult human peripheral blood lymphocytes (PBLs) into SCID mice (Mosier et al., 1988). It was shown that human Ig could be detected at one week after human PBL reconstitution (Mosier et al, 1988). The Ig level ranged from 0.1-1.0 mg/ml (normal range of human serum Ig is 7-24 mg/ml). In addition, it was demonstrated that human T and B lymphocytes survived at least six months within this mouse model (Mosier et al., 1988). Furthermore, when hu-PBL-SCID mice were immunized with tetanus toxoid, a protein antigen to which the PBL donors were known to be immune, a secondary antibody response to tetanus toxiod could be elicited after one to three weeks of immunization (Mosier et al., 1988). The amount of antibody

found in the responding mice ranged from 1-10% of the amount found in immunized humans. In agreement with this report, another study demonstrated that the hu-PBL-SCID mice, immunized with a pneumococcal vaccine, could produce a specific human humoral immune response that allowed protection against Streptococcus pneumoniae infections (Aaberge et al.,1992). While a secondary human humoral response was elicited in the hu-PBL-SCID mice immunized with keyhole limpet hemocyanin (KLH), a primary response to exogenous antigens to which the donor has never been exposed could not be induced in hu-PBL-SCID mice (Markham et al., 1992). All these data indicate that a functional human immune system could be transferred into the SCID mice by the injection of human adult PBLs.

Like SCID-hu mice, hu-PBL-SCID mice have been widely used in the studies of HIV-1 infection *in vivo* (Mosier et al., 1991; Koup et al., 1994; Torbett et al., 1991). HIV-1 infection induces $CD4^+$ T cell depletion in hu-PBL-SCID mice, suggesting that the hu-PBL-SCID mouse model could be used in the studies of HIV-1 pathogenesis (Gulizia et al., 1996; Gulizia et al., 1997; Mosier et al., 1993a; Rizza et al., 1996). Hu-PBL-SCID mice have also been successfully used in the studies of passive immunization with anti-HIV-1 monoclonal antibodies and several neutralizing antibodies have been demonstrated to be effective against HIV-1 infection *in vivo* (Cauduin et al., 1995; Parren et al., 1995; Safrit et al., 1993). These data suggest that the hu-PBL-SCID model may be suitable for the evaluation of passive immunization against HIV-1 infection. Since cell-mediated immunity has been proposed to be crucial in the protection from HIV-1 infection *in vivo* (Clerici et al., 1993; Clerici and Shearer, 1993), it is important to know whether hu-PBL-SCID mice can be used to examine the cellular immunity against HIV-1 infection. Interestingly, it has been shown that hu-PBL-SCID mice reconstituted with PBL from donors vaccinated with vaccinia gp160 and recombinant gp160 were resistant to HIV-1 infection (Mosier et al., 1993b).

Adoptive transfer of cloned human Gag-specific $CD8^+$ cytotoxic T lymphocytes (CTL) protects hu-PBL-SCID mice from HIV-1 infection *in vivo* (Kuyk et al., 1994), suggesting that the hu-PBL-SCID mice model could be useful in the evaluation of anti-HIV cellular immunity *in vivo*. Understanding the protective immunity against HIV-1 infection is not only important for the delineation of the immunopathogenesis of HIV-1 infection, but also crucial for the development of HIV-1 vaccines. As mentioned above, some individuals have remained HIV-1 seronegative and appeared to be resistant to HIV-1 infection even though they have been multiply exposed to HIV-1 infected subjects. It was hypothesized that anti-HIV-1 protective immunity might exist in these high-risk, multiply exposed, and HIV-1 seronegative individuals (Clerici, et al., 1992; Clerici et al., 1994; Fowke et al., 1996). However, there is no direct evidence that anti-HIV-1 protective immunity exists in these individuals.

In summary, hu-PBL-SCID mice have been used in different aspects of HIV-1 infection, including the study of the pathogenesis of acute HIV-1 infection *in vivo*, examination of anti-HIV therapeutic compounds, and evaluation of HIV-1 vaccine candidates. However, it is still controversial whether a primary antibody immune response could be induced in the hu-PBL-SCID mice. Further, it is possible that cellular immune responses may be difficult to induce because T cell activation and memory require cytokines and effectors cells to interact in the milieu of human lymphoid organs which do not exist in hu-PBL-SCID mice.

E. Rational, hypothesis, and objectives of this study

Taken together, a tremendous amount of information has been gained on the studies of HIV and AIDS since the discovery of HIV in 1984. However, one of the critical questions regarding HIV-1 infection remains largely unanswered: how does HIV-1 infection cause the depletion of $CD4^+$ T cells and destroy the normal host immune

function *in vivo* ? HIV-1 acutely infects a variety of human $CD4^+$ T cell lines and induces cytotoxicity in most tissue culture systems. However, we have successfully established several chronically HIV-1 infected cell lines which continuously produce high titers of HIV-1 but without obvious cell death. We reasoned that some molecular mechanisms responsible for such chronic HIV-1 infections could be elucidated by the comparative studies of acutely and chronically infected cell lines *in vitro*. Because previous studies suggested that mutations of HIV-1 accessory proteins may play critical roles in the establishment of the persistent HIV-1 infection *in vitro*, we asked whether similar mutations of HIV-1 accessory proteins might also exist in the chronically infected cell lines. In particular, we focused our investigations on Vpr protein since Vpr has been suggested to prevent the establishment of persistent HIV-1 infection *in vitro* . We propose that the ability of Vpr to prevent the establishment of persistent HIV-1 infection *in vitro* relates to its abilities to inhibit cell growth and induce cell differentiation and arrest cell cycle at G2/M phase. Thus, delineating the molecular mechanisms by which HIV-1 Vpr inhibits cell growth and arrest cell cycle may be very helpful for the understanding of HIV-1 induced cell killing.

The fission yeast *S. pombe*, one of the simplest eukaryotes, is a widely used model system for studies of cell cycle regulation of eukaryotic cells. Since a variety of cell cycle regulatory molecules are conserved in yeast and mammalian cells, we hypothesized that Vpr may also affect cell proliferation and cell cycle progression in *S. pombe*. If so, then *S. pombe* could be used as a model system to study the effects of HIV-1 Vpr on cell growth and cell cycle, and to further investigate the molecular mechanisms by which Vpr inhibits cell growth and induce cell cycle arrest.

Studies of HIV-1 infection *in vivo* have been limited by the absence of a suitable experimental animal model. The development of new and more effective therapies

demands a better understanding of HIV pathogenesis and testable animal models. A relevant animal model for HIV infection would allow evaluation of the pathogenesis of AIDS and analysis of potential antiviral agents or vaccine preparations before the initiation of clinical trials. The increasing demand for such a model has prompted us to develop the hu-PBL-SCID/beige mouse model for the studies of HIV-1 infection *in vivo*.

Objectives of this study:

1. To establish and characterize HIV-1 chronically infected cell lines *in vitro*; in particular, to further examine the potential role of Vpr in the establishment of the chronically infected cell lines (Chapter II).

2. To determine the potential effects of HIV-1 Vpr on cell proliferation and cell cycle progression in *S. pombe* (Chapter III).

3. To investigate the molecular mechanisms by which Vpr inhibits cell growth and induces cell cycle arrest in *S. pombe* (Chapter IV).

4. To develop a hu-PBL-SCID/beige mouse model and explore its potential utilization in the studies of HIV-1 infection *in vivo* and evaluation of anti-HIV-1 protective immunity in high-risk, multiply exposed, and HIV-1 seronegative individuals (Chapter V).

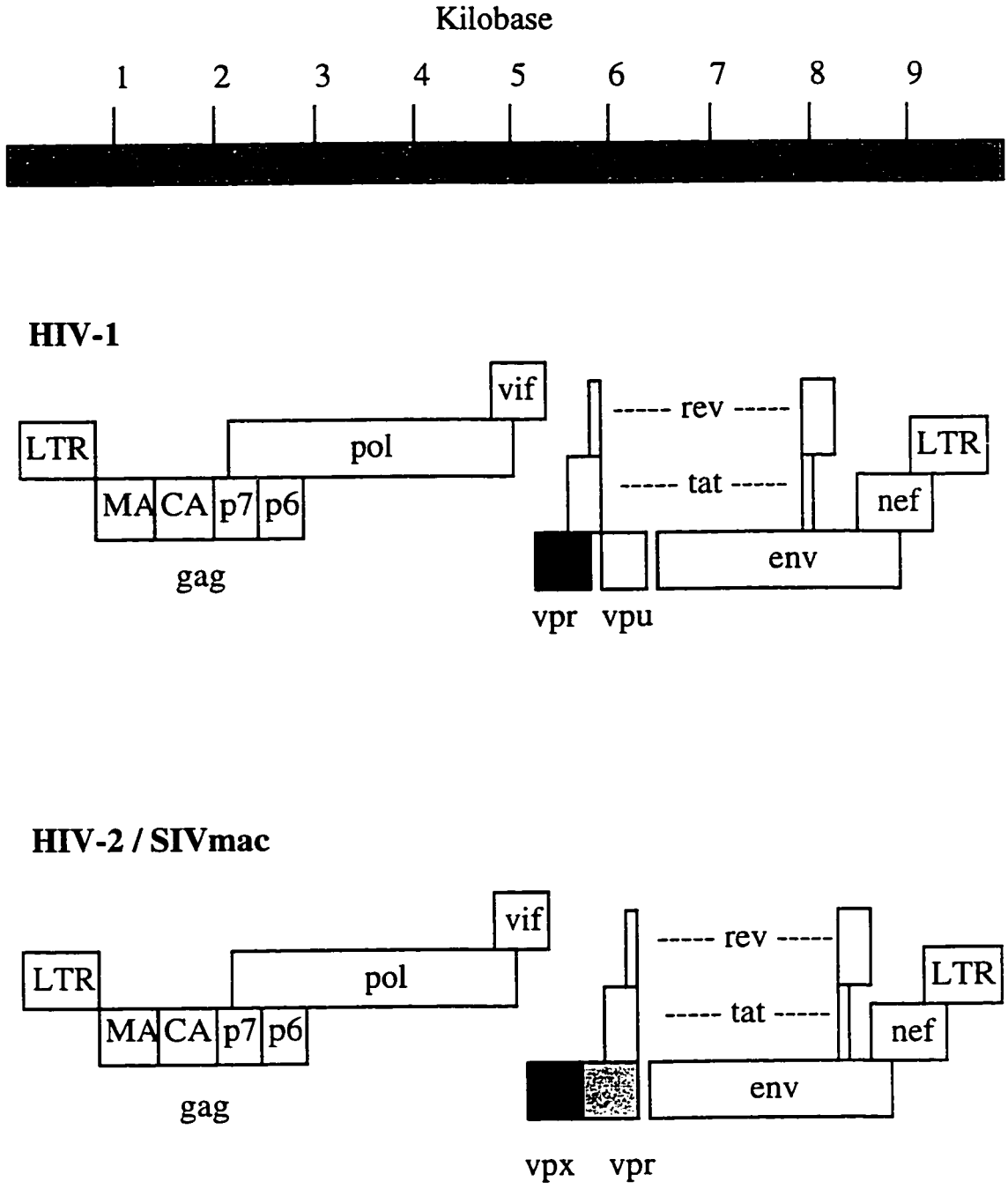


Figure 1.1. The genome structure of human and simian immunodeficiency viruses, HIV-1, and HIV-2/SIVmac

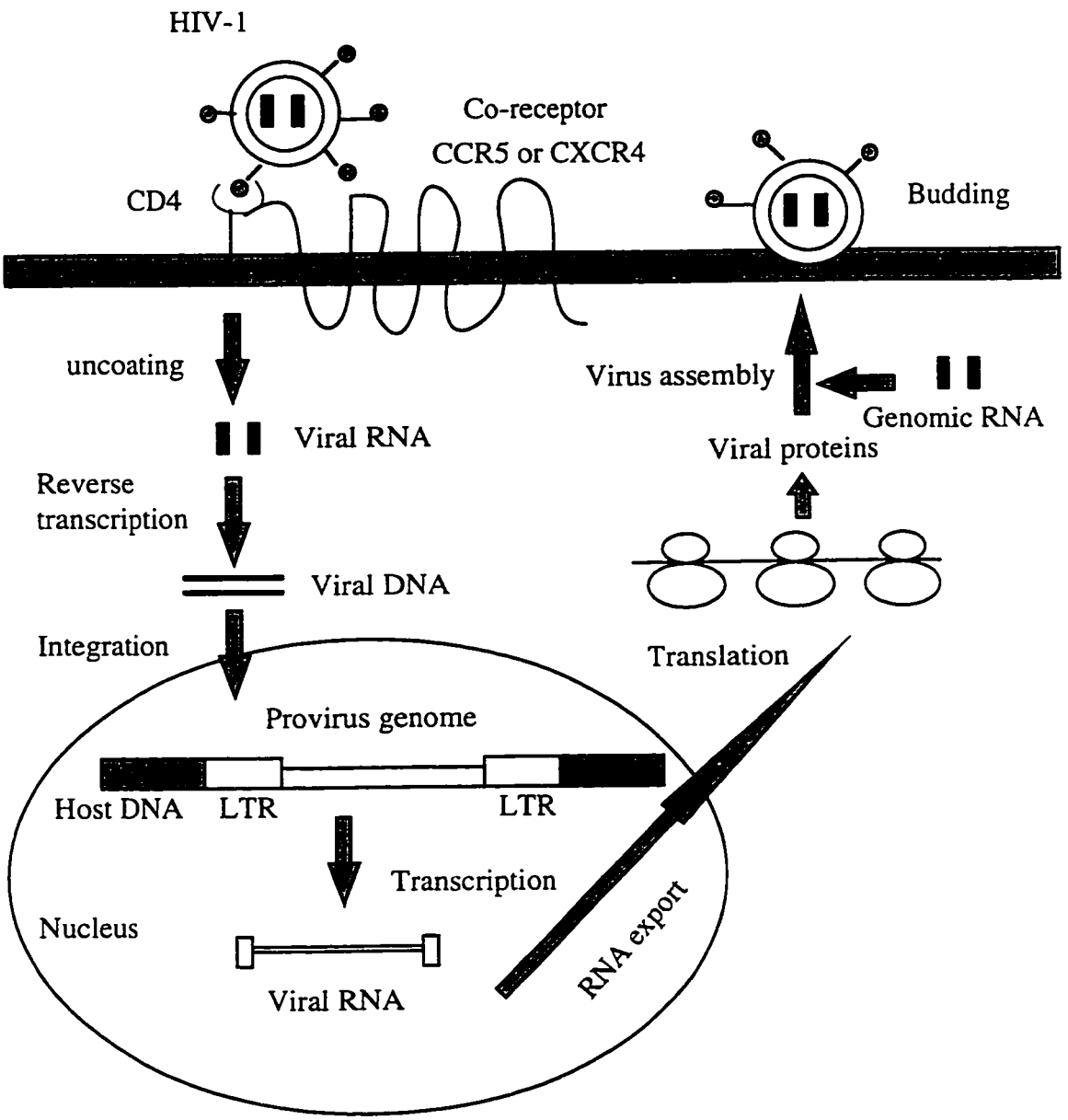


Figure 1.2. Life cycle of HIV-1 replication

Chapter II

Establishment and characterization of HIV-1 chronically infected human CD4+ cell lines *in vitro*

(The data presented in this Chapter have not published previously)

A. Introduction and rationale

As mentioned previously (Chapter I), HIV-1 infection often causes CD4+ T cell depletion and AIDS in most HIV-1 infected individuals. However, a small group of HIV-1 infected individuals show no symptoms of HIV-1 infection and have normal CD4+ T cell counts for a long period of time after infection. Since HIV-1 infects a variety of human CD4+ cell lines, and causes either acute infection with cell death or chronic infection without obvious cell death (Banerjee et al., 1992; Folks et al., 1987; Lifson et al, 1986a; Poli et al., 1990), we reasoned that HIV-1 infected tissue culture systems can be used as *in vitro* model for the studies of the molecular mechanisms of HIV-1 induced CD4+ T cell death or viral persistence. While HIV-1 induced acute infection has been extensively studied in tissue culture, long-term persistent productive infection with HIV-1 has not been well examined.

Viral persistence can be generated from different conditions after viral infections. Some animal viruses, such as Sindbis virus, poliovirus, reovirus, and foot-and-mouth disease virus, can be established as persistent infections after acute cytolytic infection (Borzakian et al., 1992; de la Torre et al., 1988; Dermody et al., 1993; Levine et al., 1993). The mechanisms for the establishment of the persistent infection by these

animal viruses are quite complex, including development of attenuated or defective virus strains (Borzakian et al., 1992), the presence of defective interfering virus particles (Huang, 1973; Perreault, 1995), induction of cellular oncogene bcl-2 to block programmed cell death (Levine et al., 1993), and coevolution (mutations) of virus and host cells (de la Torre et al., 1988; Dermody et al., 1993). However, the molecular mechanisms for the establishment of persistent HIV-1 infections are poorly understood. Previous studies suggest that mutations with HIV-1 accessory genes such as vpr, vif, or vpu may be involved in the establishment of chronically infected cell lines *in vitro* (Kishi et al., 1992; Nakaya et al., 1994; Nishino et al., 1991; Nishino et al., 1994). Further studies have demonstrated that HIV-1 Vpr can disturb host cell proliferation and prevent the establishment of chronically infected cell lines (Mustafa and Robinson, 1993; Planelles et al., 1995; Rogel et al., 1995). However, it remains unknown how HIV-1 Vpr affects the host cell growth and prevents the establishment of chronic infection. In addition, it is not clear whether HIV-1 Vpr is cytotoxic or cytostatic in the host cells.

As part of other studies, we have established several chronically infected cell lines that stably produce high titers of HIV-1 without apparent cell death. Here we further characterize these chronically infected cell lines. In particular, since previous studies have suggested that vpr mutations may play a critical role in the establishment of persistently infected cell lines, we have focused on Vpr protein expression and possible vpr gene mutations in the chronically infected cells.

B. Materials and methods

Cell lines and cell culture: Several human $CD4^+$ cell lines with different origins were used in this study (Table 2.1). H9, MT4, C8166, ACH2, and AA2 cell lines were obtained from the NIH AIDS Research and Reference Reagent Program. The MOlt3 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). AA2 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco BRL), 1% glutamine, and 1% penicillin-streptomycin plus 1x nonessential amino acids and 0.1 mM sodium pyruvate (Gibco BRL). The other cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin-streptomycin (Gibco BRL). All the cell cultures were maintained in an incubator with 5% CO_2 at 37^0C.

Preparation of human peripheral blood lymphocytes (PBLs): Human PBLs were isolated from the buffy coats (from the Canadian Red Cross in Edmonton) by gradient centrifugation with Histopaque (Sigma). The PBLs collected from the gradient centrifugation were washed three times with Hanks' balanced salt solution (BRL). The freshly prepared cells were either used immediately for experiments or cryopreserved in liquid nitrogen for later use.

HIV-1 infection of $CD4^+$ cell lines and PBLs: The wild type HIV-1 virus used in this study was a laboratory strain HIV-1_{NL4-3} (T-cell tropic virus), whereas the mutant HIV-1 viruses were either Tat minus or/and with LTR mutations with the backbone of HIV-1_{NL4-3} constructed by Dr. Chang (Chang et al., 1993; Chang and Zhang, 1995). The virus stock for the infection were harvested from the supernatants of transfected HeLa cells or infected MT4 cells as described previously (Chang and Zhang, 1995). The infection was initiated by incubating 2 x 10^6 cells and similar

amounts (as determined by reverse transcriptase activity, see below) of different virus preparations at MOI (multiplicity of infection) of 0.0002, 0.002, 0.02, or 0.2 in 2 ml of culture medium at 37^0C for 3 hr with occasional shaking. The cells were then washed and resuspended in 5 ml fresh medium. For the infection of PBLs with HIV-1, PBLs were first mitogenically stimulated in RPMI 1640 supplemented with 10% fetal bovine serum, 1% glutamine, and 1% penicillin-streptomycin (Gibco BRL), 200 units/ml human IL-2 (Boehringer Mannheim GmbH), plus phytohemagglutinin (PHA, 5μg/ml, Sigma) in a T-25 tissue culture flask for 24 hr at 37^0C, and then infected with HIV-1_{NL4-3} (T cell-tropic) at various MOI as described above. The infected cells were split at a 1:3 ratio every 3-4 days and the virus replication was monitored by RT assay or/and immunohistochemical staining described below.

Reverse transcriptase assay (RT assay): RT assay was performed as described previously (Chang et al., 1993). In brief, 10 μl of the supernatant harvested from the mock or HIV-1 infected cell cultures was added to 50 μl of RT cocktail [60 mM Tris-HCl (pH 7.8), 75 mM KCl, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 1 mM EDTA, 5 μg of poly (rA)/ml, 0.16 μg of oligo (dT) /ml, 8 μl of 0.5 M DTT/ml, and 30 μCi of α-^{32}P-TTP/ml] and incubated at 37^0C for 1 hr. Three microliters of the radioactive products generated from the RT assay were spotted onto a microtiter filter paper and air dried. The filter was washed three times with 2 x SSC (containing 17.53g NaCl and 8.82g sodium citrate/ per liter, pH. 7.0). Then the filter was air dried and quantified by using a Fuji phospho-imager (Fuji Medical Systems, U.S.A., Stamford, CT) or by a scintillation counter (Beckman).

Immunohistochemical staining of HIV-infected cells: To detect the HIV-1 infected cells in the culture, a very sensitive and quantitative immunohistochemical staining was used (Chang and Zhang, 1995). Briefly, HIV-infected cells were washed

with PBS (3 times), attached to the surface of 24-well plate which had been coated with poly-D-lysine (1mg/ml, Sigma) at room temperature for 10 min, and fixed with methanal and aceton (1:1 ratio mix) at room temperature for 2 min. The fixed cells were pretreated with 0.01% H_2O_2 (hydrogen peroxide) at room temperature for 5 min to reduce the endogenous peroxidase activity. The cells were incubated with an HIV-1 seropositive patient serum (from NIH, 1: 2000 dilution in a blocking solution containing 20% FBS, 0.1% Trixon X-100, and 2% dry milk in PBS) at 4^0C overnight with constant shaking. After washing in PBS (four times, 5 min/each), the cells were incubated with normal sheep serum (Amersham, 1: 200 dilution) at room temperature for 30 min to block nonspecific signals. Then the secondary antibody, biotinylated sheep anti-human antibody (Amersham, 1: 2000 dilution), was added to the cells and incubated at room temperature for 1 hour. The cells were washed four times in 0.3% Tween 20 in PBS and incubated in the ABC staining solution (containing avidin and biotinylated horseradish peroxidase, Pierce Chemical Co.) at room temperature for 30 min. After washing four times in PBS-Tween 20, the cells were incubated with DAB solution (3,3'-diaminobenzidinetetrahydrochloride, Sigma) containing 0.3% $NiCl_2$ for 2-3 min at room temperature. The staining reaction was monitored under an inverted microscope and stopped by washing with tap water for 1-2 min. The staining results were scored under an inverted microscope, and the percentage of HIV-infected positive cells determined by taking the average of at least three representative counts of 1,000-10,000 cells.

Transmission, scanning-, and immuno-electron microscopy (All the work after cell fixation was done by Mr. Richard Sherburne in our department): The mock or infected cells were fixed with freshly prepared 2% (v/v) glutaraldehyde, 2% (v/v) formaldehyde in phosphate buffer (PB) at pH 7.3 for 1 hr as described (Armbruster et al, 1982). The samples were then washed in PB and split into three parts in microfuge

tubes for immuno-electron microscopy (IEM), transmission electron microscopy (TEM), and scanning electron microscopy (SEM) sample preparation, respectively. Immuno-gold electron microscopy samples were dehydrated through a graded series of alcohol at low temperature with the use of a Stir-Kool (Thermoelectrics Unlimited, Inc. Wilmington, Delaware); 25% for 30 min at 4^0C, 50% for 30 min at 0^0C, 75% for 30 min at -20^0C, 90% for 1 hr at -30^0C, 100% for 1 hr at -30^0C. The samples were then infiltrated with 25% Lowicryl K4M (JBS Supplies, Pointe Claire, PQ) in 100% ethanol at -30^0C for 30 min, in 50% K4M for 30 min, in 75% K4M for 30 min, and finally 2 changes of 100% K4M for 1 hr each. The microfuge tubes were then suspended in an insulated chamber containing dry ice and equipped with an ultraviolet light source (General Electric 8 W FL8 BLB black light) for K4M polymerization. Samples for SEM and TEM examination were further fixed in 1% (w/v) osmium tetroxide for 1 hr after the glutaradehyde, formaldehyde fixation and PB wash. The cells were washed again with PB and dehydrated in a graded series of ethanol at room temperature. The samples for TEM were transferred to propylene oxide for 30 min while the remaining cells in the petri dish were critical point dried for SEM examination. The TEM samples were then infiltrated with 1:1 mixture of propylene oxide and LX 112 (Ladd Research Industries, Inc. Burlington, VT) and left uncapped for 24 hr. The following day the samples were transferred to pure LX 112 and cured for 24 hr at 60^0C. Images were recorded on Kodak #4489 electron microscope film using a Philips model 410 transmission electron microscope. SEM samples were mounted on standard Cambridge scanning electron microscopy stubs and examined in a Hitachi S4000 field emission scanning electron microscope at an accelerating potential of 2.0 kV. For IEM, the primary antibody was sera from an HIV-1 infected AIDS patient and the secondary antibody were colloidal gold (20 nm)-conjugated goat anti-human IgG (EY Labs, Inc.). Sectioning was performed on a Reichert-Jung Ultracut with a glass knife and the sections were placed on 3 mm 200 mesh formvar coated copper grids. For staining,

the sections were blocked in 1% BSA, transferred to AIDS patient sera, and incubated for 30 min, then blocked with 1% BSA and further incubated with colloidal gold (20 nm)-conjugated goat anti-human IgG, washed in distilled water and examined in the TEM unstained (Roth et al, 1981).

Western blot analysis: The protein lysate was made as described previously (Sambrook et al., 1989). For Western blot analysis, the protein concentration in the samples were quantified with the DC protein assay reagents(Bio-Rad). Ten micrograms of proteins from each sample were applied to a 10-20% gradient Tricine-SDS-PAGE gel (NOVEX, Canada). After electrophoresis, the proteins were transferred to a 0.2 microcon nitrocellulose filter (Schleicher and Schuell, USA), and stained with Ponceau S (Sigma) to label the molecular weight marker. Filters were air-dried and blocked with 10% dried milk in TBS-T (50 mM Tris, pH. 7.5, 150 mM NaCl, 0.3% Tween-20) for 1 hr at room temperature. The filters were then placed into a " seal-a-meal" bag and incubated with the primary antibody (rabbit anti-HIV-1 Vpr, 1:1000 dilution, from NIH) at 4^0C overnight. After washing with TBS-T (four times), the blot was blocked with normal donkey serum at room temperature for 30 min. The blot was incubated with an HRP-conjugated secondary antibody (Donkey anti-rabbit, Amersham, NA 9340, 1:2000 dilution) at room temperature for 1 hr, followed by washing with TBS-T (four times). Finally, the blot was developed using chemiluminescence ECL immuno-detection reagents according to the company's instructions (Amersham Life Science).

RT-PCR: To amplify the vpr gene from the cell-free viruses in the cell cultures, RT-PCR was carried out as described previously (Sambrook et al., 1989; Chang, et al., 1993). Briefly, to collect cell-free viral particles, supernatants of the HIV-1 infected cells (500µl/each) was harvested and centrifuged at a 14,000 rpm for 2 hr in a microcentrifuge at 4^0C. After the centrifugation, 400µl of the supernatant was

discarded. The HIV particles in remaining supernatant were dissociated by vortexing in the presence of 100 µl of 8 M LiCl. The mixture was placed on dry ice for 20 min, transferred to -20^{0}C for 2 hr, and then centrifuged again in the microcentrifuge at 4^{0}C for 20 min. The RNA pellet was rinsed with 70% ethanol, dried briefly under vacuum and resuspended in 20 µl DEPC-treated water. The first strand cDNA was synthesized by reverse transcription reaction as described (Chang et al., 1993). Briefly, the viral RNA was incubated with the cocktail composed of 5 x reverse transcription buffer (Gibco, BRL), dNTPs (10 mM, BRL), DTT (0.1 m M , BRL), RNasin (30 units, BRL), AMV (Avian Myeloblastosis Virus) reverse transcriptase (30 units, BRL), and 3' vpr primer (5'-AACCCGGGTCTAGGATCTACTGGC- 3'), and incubated at 42^{0}C for one hour. The first strand cDNA was further amplified by PCR in the reaction mix consisting of 5 µl of the synthesized first strand DNA, $MgCl_2$ (25 mM , BRL), dNTPs (10 mM, BRL), 5' vpr primer (5'-TTGGATCCACCATGGAACAA-GCCCCAGA-3'), 3' vpr primer (mentioned above), and Taq DNA polymerase (2.5 units, BRL). The PCR was carried out on the PTC-100 Peltier-Effect Cycling (MJ Research, Inc.) for 30 cycles under the following conditions: 95^{0}C for 45 seconds, 55^{0}C for 1 min, and 72^{0}C for 1 min .

DNA sequencing of vpr: The molecular cloning and DNA sequencing in this study was carried out according to the standard protocols described previously (Sambrook et al., 1989). The method used for DNA sequencing was Sanger's dideoxy-mediated chain termination method as described elsewhere (Sambrook et al., 1989). In brief, the vpr fragments generated from the RT-PCR described above were subcloned into the cloning vector of PCR1000 (Invitrogen) and the subsequent sequencing was done by using Sequenase Version 2.0 according to the instructions supplied by the company (United States Biochemical Corp). The primer used for

sequencing was 5' -CAATACGACCACTATAGGG- 3' (T7 promoter sequence within the PCR1000 vector).

C. Results

HIV-1 induces acute infection in a variety of human CD4$^+$ cell lines and PBL *in vitro*

To examine the kinetics and outcomes of HIV-1 infection in tissue culture, a number of human CD4$^+$ cell lines (Table 2.1) and peripheral blood lymphocytes (PBLs) were infected with wild type HIV-1_{NL4-3}. After the infection, the supernatant and infected cells were collected at various time points for RT assay and immunohistochemical staining, respectively. As expected, all of these cell lines and PBLs were highly susceptible to infection by HIV_{NL4-3}. In addition, all the cell lines and PBLs had similar infection kinetics (Figure 2.1 and Figure 2.2). The typical infection with 0.02 MOI usually peaked around day 9 to day 12 post-infection based on the RT assay, which time was consistent with the previous studies (Chang et al., 1993; Chang and Zhang, 1995). The infection kinetics were affected (either accelerated or delayed) by the initial virus input. For example, infection with higher MOI (i.e., 0.2) resulted in an accelerated kinetics, whereas lower MOI (i.e., 0.002 or 0.0002) caused delayed kinetics (Figure 2.1 and Figure 2.2).

We found apparent cell killing in all cultures, including PBL, around day 20 post-infection. At approximately one month post-infection, almost all the infected cells were killed in the cultures and the level of RT activity in the supernatant was close to background (Figure 2.1 and Figure 2.2). In addition, obvious and large syncytia formation was observed in HIV-1 infected H9 and AA2 cell lines (Figure 2.3.C and D), indicating that syncytia formation may contribute to the cell death in H9 and AA2 cells. However, syncytia formation did not form in HIV-1 infected MT4, C8166, Molt3, and PBLs cell cultures (Figure 2.3.A and B), suggesting that HIV-1 infection could kill cells by the mechanisms other than by syncytia formation. Our observations

are consistent with the previous suggestions that HIV-1 infection can induce cell killing by syncytia formation and other mechanisms (Lifson et al., 1986a; Somasundaran and Robinson, 1987).

Establishment of long-term high-titer HIV-1 producer cell lines

While wild type HIV-1 infection of human $CD4^+$ lymphocytes often leads to cell death, persistent HIV-1 infection may occur under some conditions. One of the proposed conditions for the establishment of HIV-1 persistent infection is viral attenuation (Kishi, et al., 1991; Nishino et al, 1994). We have previously established several persistent, high titer producer cell lines, such as MT5-chron. (which was established by infection of MT4 cells with tat-minus HIV-1_{NL4-3}), and Molt3-Mo-MLV-chron. (which was established by infection of Molt3 cells with LTR mutant HIV-1_{NL4-3}, Table 2.2). However, since these chronically infected cell lines were established many passages after the acute infection, it was not clear whether additional viral mutations occurred during the infection.

We have also attempted to establish chronically infected cell lines by the initial infection with HIV_{NL4-3} serially passaged many times *in vitro*. Initially, MT4 cells were infected with wild type HIV_{NL4-3} prepared from HeLa cells. As expected, almost 100% of the cells were killed during this initial infection. To generate potential viral attenuation, cell free viruses were harvested from the supernatant of the infected MT4 cells and used to infect fresh culture of MT4 cells. Potentially attenuated HIV_{NL4-3} was prepared by such serial passage of the viruses *in vitro*. Interestingly, it was found that MT4 cells became chronically infected with passage 20 HIV_{NL4-3} virus. Molt3 and C8166 cells were also chronically infected by the viruses harvested from the chronically infected MT4 cells (Figure 2.4). Therefore, we have established chronically infected cell lines from wild type and LTR or/and tat mutant HIV-1 viruses (Table 2.2). After more than

three years, these chronically infected cell lines continue to stably produce high titers of HIV-1 viruses (Figure 2.5). In addition, almost 100% of the cells in the chronically infected cell cultures are HIV-1 positive (Figure 2.6).

Differential infectivity of wild-type, tat-minus, and tat-plus HIV-1 in lymphoid cell lines and PBLs

To compare the infectivities of tat-minus or tat-plus HIV-1, and wild type (wt) HIV-1, viral stocks were prepared from the supernatant of the wt HIV-1 acutely infected MT4 cells, Tat-minus HIV-1 chronically infected MT4 cells (MT5-chron.), and Tat-plus HIV-1 chronically infected MT4 cells (MT4-chron.), respectively. These viral stocks were titrated by RT assay and subsequently used for the following infections. To set up the infections, 2 x10^6 cells were infected with either wt HIV-1, Tat-minus HIV-1, or Tat-plus HIV-1 at 0.02 MOI, and the infection kinetics monitored by RT assay. Consistently, MT4, Molt3, and C8166 cells were acutely infected by wild type HIV-1 with typical kinetics as shown above (Figure 2.1 and Figure 2.2). In contrast, Tat-minus HIV-1 from MT5-chron. cells could not induce productive infection in these lymphoid cell lines by RT assay. whereas the Tat-plus HIV-1 from the chronically infected MT4-chron. cells could infect these cell lines (Figure 2.7). Neither the Tat-minus nor Tat-plus HIV-1 induced an acute productive infection in human PBL, indicating that host cellular factors may also play an important role in the establishment of productive HIV-1 infections. It was also observed that there were some differences in the ability of Tat-plus HIV-1 to infect lymphoid cell lines. Firstly, the sensitivity to the Tat-plus HIV-1 infection was different in various cell lines, showing that MT4 and C8166 were more sensitive to the infection compared with that of Molt3, H9, and AA2 cells. Secondly, chronic rather than acute infections were established when MT4, Molt3, and C8166 cells were infected with Tat-plus HIV-1 from the chronically infected MT4-chron. cells (Figure 2.7). However, the same Tat-plus HIV-1 induced

acute infection with syncytia formation and cell death in H9 and AA2 cells, suggesting that the development of persistent infection is both virus and host cell dependent .

EM analyses of HIV-1 chronically infected cells

To determine if there are morphological differences between the acutely and chronically infected MT4 cells, we have examined the cells by using the transmission electron microscope (TEM) and the scanning electron microscope (SEM). In addition, HIV-1 antigen was detected using immuno-gold electron microscopy techniques (IEM). The wt HIV-1 infected MT4 cells (Figure 2.8 B, C and D) and Tat-minus HIV-1 infected MT4 cells (Figure 2.8 E-I) are illustrated. The MT4 cells infected wt HIV-1 (Figure 2.8 B) were extensively vacuolized compared with the uninfected MT4 cells (Figure 2.8 A). However, the Tat-minus HIV-1 persistently infected MT4 cells (Figure 2.8 E) exhibited much less severe cytoplasmic vacuolization than the wt HIV-1 infected MT4 cells (Figure 2.8 B). Using immuno-gold labeling techniques, the Tat-minus virions were specifically labeled (Figure 2.8 H). Negative staining was observed when the isotype-matched control antibody was used. Large number of free mature extracellular viral particles were observed by TEM in both wt HIV-1 infected (Figure 2.8C) and Tat-minus HIV-1 infected cells (Figure 2.8F and G). In addition, viral particles were also found on the surface of both wt HIV-1 infected (Figure 2.8 D) and Tat-minus HIV-1 infected cells (Figure 2.8 I) by SEM. Our data demonstrated that the Tat-minus HIV-1 chronically infected cells are less vacuolated than the acutely infected cells as evidenced by the morphological comparison to the uninfected cells. In addition, there were no apparent morphological difference between the wild type and Tat-minus virions produced from these cultures (Figure 2.8C, F, and G).

Absence of Vpr expression in the HIV-1 chronically infected cell cultures

Since HIV-1 chronically infected cell lines could be established by the infection of at least three HIV-1 strains: HIV-1 LTR or tat mutant viruses, and wt HIV-1, we thus reasoned that mutations of other viral genes other than LTR and tat might be essential for the establishment of persistent HIV-1 infection. Previous studies suggested that HIV-1 with mutations in accessory genes such as vpr, vif, or vpu can induce persistent infection in MT4 cells (Nishino et al, 1991; Kishi et al, 1992; Nishino et al, 1994). Other studies suggest that HIV-1 Vpr inhibits cell proliferation and thereby prevents the establishment of chronic infection (Levy, D.N. et al., 1993; Mustafa et al., 1993; Planelles et al, 1995; Rogel et al., 1995). All these studies indicate that HIV-1 Vpr may play an important role in the establishment of persistent HIV-1 infection *in vitro*. To determine whether Vpr expression was altered in the chronically infected cell lines, Western blot analysis was carried out to detect Vpr expression in the acutely infected cells or chronically infected cell lines. Protein samples tested were from uninfected MT4 cells (MT4-mock, Figure 2.9 , lane 1), Tat-minus HIV-1 chronically infected MT4 cells (MT5-chron., lane 2), three Tat-plus HIV-1 chronically infected cell lines (C8166-chron., Molt-3-chron., and MT4-chron., lanes 3-5), and wild type HIV_{NL4-3} acutely infected MT4 cells (MT4-acute, lane 6). As shown in Figure 2.9, Vpr expression was detected in the acutely infected MT4 cells (lane 6), whereas all the four chronically infected cells lacked Vpr expression, indicating that Vpr expression was ablated in the persistently infected cell lines.

Detection of vpr gene mutations in the persistently infected cell cultures

Having demonstrated that Vpr expression was absent in the chronically infected cells, we then asked whether the absence of Vpr expression resulted from vpr gene mutations. To address this issue, the viral RNA was prepared from wt HIV-1 acutely

infected cells and four chronically infected cell cultures, respectively. RT-PCR was carried out as described in the Materials and Methods. The vpr fragments (about 300 bps) have been amplified from wt HIV_{NL4-3} acutely infected MT4 cells (vpr-MT4-acute, Figure 2. 10 , lane 1), Tat-minus HIV-1 chronically infected MT4 cells (vpr-MT5-chron., lane 2), and three Tat-plus HIV-1 chronically infected cell lines (vpr-C8166-chron., vpr-Molt3-chron., and vpr- MT4-chron., lanes 3-5).

For DNA sequencing, the amplified vpr fragments were subcloned into the vector of PCR1000, and DNA sequencing was performed as described in the Materials and Methods. Ten clones (two from each sample) have been sequenced in this study. The vpr clones from the wt HIV-1 acutely infected cultures retain the wt vpr sequence (vpr-wt, Figure 2.11). However, two of the eight vpr clones from the chronically infected cell cultures (vpr-C8166-chron.#1 and vpr-MT4-chron.#2) have been found to contain a novel stop codon at the N-terminus of vpr (^{52}TGG to TGA, Figure 2.11). Since sequence compression occurred at the C-terminal region of vpr in the sequencing reactions with the vpr clones from the chronically infected cell cultures including MT5-chron.#1, MT5-chron.#2, C8166-chron.#2, Molt3-chron.#1, Molt3-chron.#2, and MT4-chron.#1, it is necessary to repeat these sequencing reactions to resolve the compression and determine the sequences.

D. Summary of results

1. Wild type HIV_{NL4-3} infects a variety of human $CD4^+$ cell lines and primary PBLs, and induces acute infection with cell killing.

2. Several chronically infected cell lines, which can produce high titers of HIV-1, have been established in this study.

3. Almost 100% of the cells in the chronically infected cell cultures are infected by HIV-1 as evidenced by the immunohistochemical staining.

4. Marked vacuolization was observed in acutely infected MT4 cells but not in uninfected and chronically infected cells as evidenced by EM examination

5. There was no Vpr expression detected in the chronically infected cells by Western blot analysis.

6. Appearance of a novel stop codon at the N-teminus of vpr was observed in some of the vpr clones from the chronically infected cell cultures.

E. Discussion

In this study, we have examined the acute and chronic HIV-1 infections in tissue culture. In agreement with the previous studies (Adachi et al., 1986), HIV-1_{NL4-3} was shown to acutely infect a variety of human $CD4^+$ cell lines and primary PBL, an outcome often accompanied by cell death. There are several mechanisms which have been proposed to explain the HIV-1 induced cell killing. One of the mechanisms is syncytium formation (formation of multinucleated giant cells), which results from the interactions between the HIV-1 envelope glycoproteins, gp120 and gp41, and its host cell surface receptor, CD4 (Freed and Risser., 1990; Lifson et al., 1986b; Sodroski et al., 1986). In fact, a massive and dramatic syncytium formation was observed in our study when AA2 and H9 cells were infected with HIV-1_{NL4-3} (Figure 2.3), and this infection always results in acute infection with almost 100% of the cell death in the culture. In addition, HIV-1_{NL4-3} could also cause acute infection and cell death without apparent syncytium formation in other cells such as MT4, Molt-3, C8166, and PBL, indicating that mechanisms other than syncytium formation may also contribute to the HIV-1 induced cell death. These mechanisms may include accumulation of unintegrated HIV DNA (Bergeron and Sodroski, 1992; Pang et al., 1990; Shaw et al., 1984, Tang et al., 1992), toxicity of viral proteins (Stevenson et al., 1988; Sabatier et al., 1991), interference of cellular signal transduction (Cohen et al., 1992; Hofmann et al., 1990; Linette et al., 1988), and induction of apoptosis (Groux et al., 1992; Laurent-Crawford et al., 1991; Lu et al., 1995; Savarino et al., 1997).

In addition, we have established and characterized several HIV-1 chronically infected cell lines (Table 2. 2). We have shown that the chronically infected cell lines established in this study produce high titers of HIV-1 (Figure 2.5). Cell lines chronically infected with HIV-1 have been previously established by others in the tissue culture (Folks et al., 1987; Poli et al., 1990). However, these chronically infected cell lines usually

express low levels of viral message and protein (Pomerantz et al.,1990). It is not clear why the chronically infected cell lines established in this study are quite different from those previously established in terms of virus production. Since almost 100% of the cells in the chronically infected cell cultures are infected with HIV-1 as evidenced by the immunohistochemical staining, this may contribute to the high titer of viral production. In addition, we hypothesized that the host cellular factors may also play a role in regulating the viral production in the HIV-1 chronically infected cell lines. To test this hypothesis, we have employed the mRNA differential display technology to isolate cellular genes which may modulate acute infection or/and viral persistence in HIV-1 infected cells (Liang, and Pardee; 1992). However, we were unable to identify any cellular factors in this regard.

We have also compared the acutely and chronically infected MT4 cells by EM examination. Interestingly, apparent cytoplasmic vacuolization was seen in the acutely infected MT4 cells (Figure 2.8B) but not in uninfected and chronically infected MT4 cells (Figure 2.8A and E). Vacuolization is one of the common cytopathological changes induced by bacterial or viral infections, and toxic chemicals such as carbon tetrachloride (Jeong, et al., 1997; Papini, et al., 1997; Sanderson, et al.,1996; Toner et al, 1992). Marked cytoplasmic vacuolization has been reported to occur in HIV-1 infected human microglia and macrophages and this cytopathological change has been suggested to be linked to the HIV-1 induced cell death (Strizki et al., 1996). Therefore, our data indicate that the Tat-minus HIV-1 from the chronically infected MT4 cells could be attenuated for cytopathogenicity.

We have further studied the possible mechanism of the persistent infection in these long-term cultures. It has been suggested that mutations in the HIV-1 accessory genes, particularly vpr, may contribute to the survival of $CD4^+$ cells from an acute HIV-1

infection in tissue culture (Nishino et al., 1991; Kishi et al., 1992; Nishino et al., 1994). Here we have shown that Vpr expression was absent in all the chronically infected cell cultures (Figure 2.9). It is not completely understood why Vpr could not be detected in the chronically infected cell lines. One of the possible explanations is that Vpr could not be detected when Vpr mutated with the appearance of a novel stop codon at the N-terminus of vpr (Figure 2.11). In addition, other studies have shown that the C-terminal domain is essential for the stability of Vpr protein, and the Vpr protein stability can influence the cell cycle effect (Mahalingam et al., 1995; Kewalramani et al., 1996). Indeed, nonsense point mutations at the C-terminal region of vpr gene derived from the chronically infected cell lines have been reported by others (Nishino et al., 1994; Nakaya et al., 1994). Therefore, some of the vpr clones from the chronically infected cell lines may carry mutations at the C-terminal domain, which may render the Vpr protein unstable and subsequently undetectable by Western blot analysis. It will be very informative if we can determine the sequences of the vpr clones from the chronically infected cell lines established in this study. Additional studies have provided direct evidence suggesting that Vpr expression can prevent cell proliferation during chronic infection (Planelles et al., 1995; Rogel et al., 1995). This is consistent with the previous observation showing that HIV-1 Vpr can inhibit cell growth and induce differentiation in some tumor cell lines (Levy, D.N. et al., 1993). More recently, HIV-1 Vpr has also been demonstrated to induce apoptosis in a variety of human cells including lymphocytes (Stewart et al., 1997; Ayyavoo et al., et al 1997a; L-J Chang et al., unpublished data).

Taken together, these studies suggest that HIV-1 Vpr may play an essential role in the establishment of chronically infected cell lines *in vitro*. However, further studies are required to understand the molecular mechanisms by which Vpr inhibits cell growth and affects other cellular functions (Chapter III and IV).

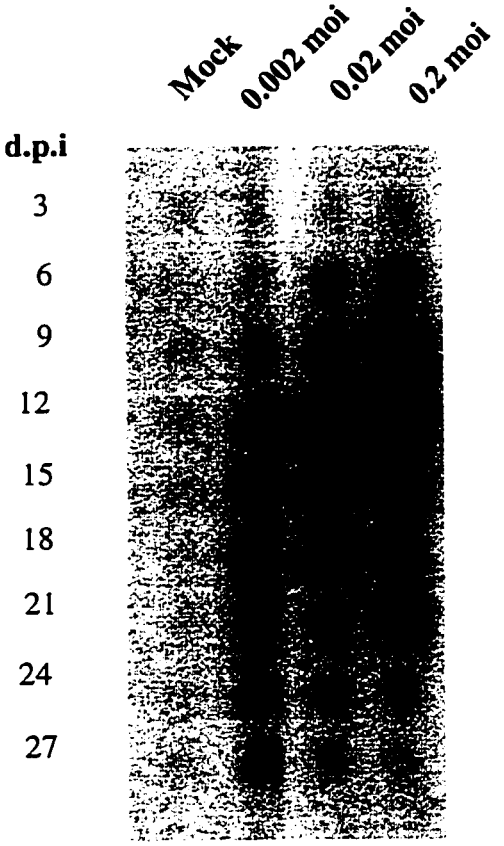


Figure 2.1. Infection kinetics of MT4 cells infected with wild type HIV-1_{NL4-3} in tissue culture.

To study the kinetics, MT4 cells were infected with wild type HIV-$1_{NL4-3,}$ and the infection kinetics were monitored by RT assay as described in the Materials and Methods. Similar kinetics were also observed in the other cell lines mentoined in the text.
d.p.i.= days post infection.
moi= multiplicity of infection.

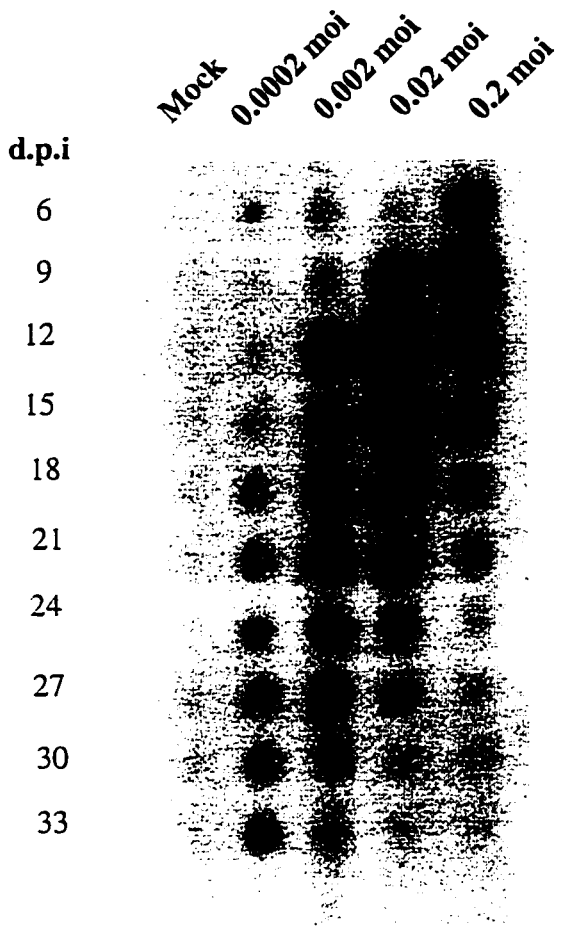


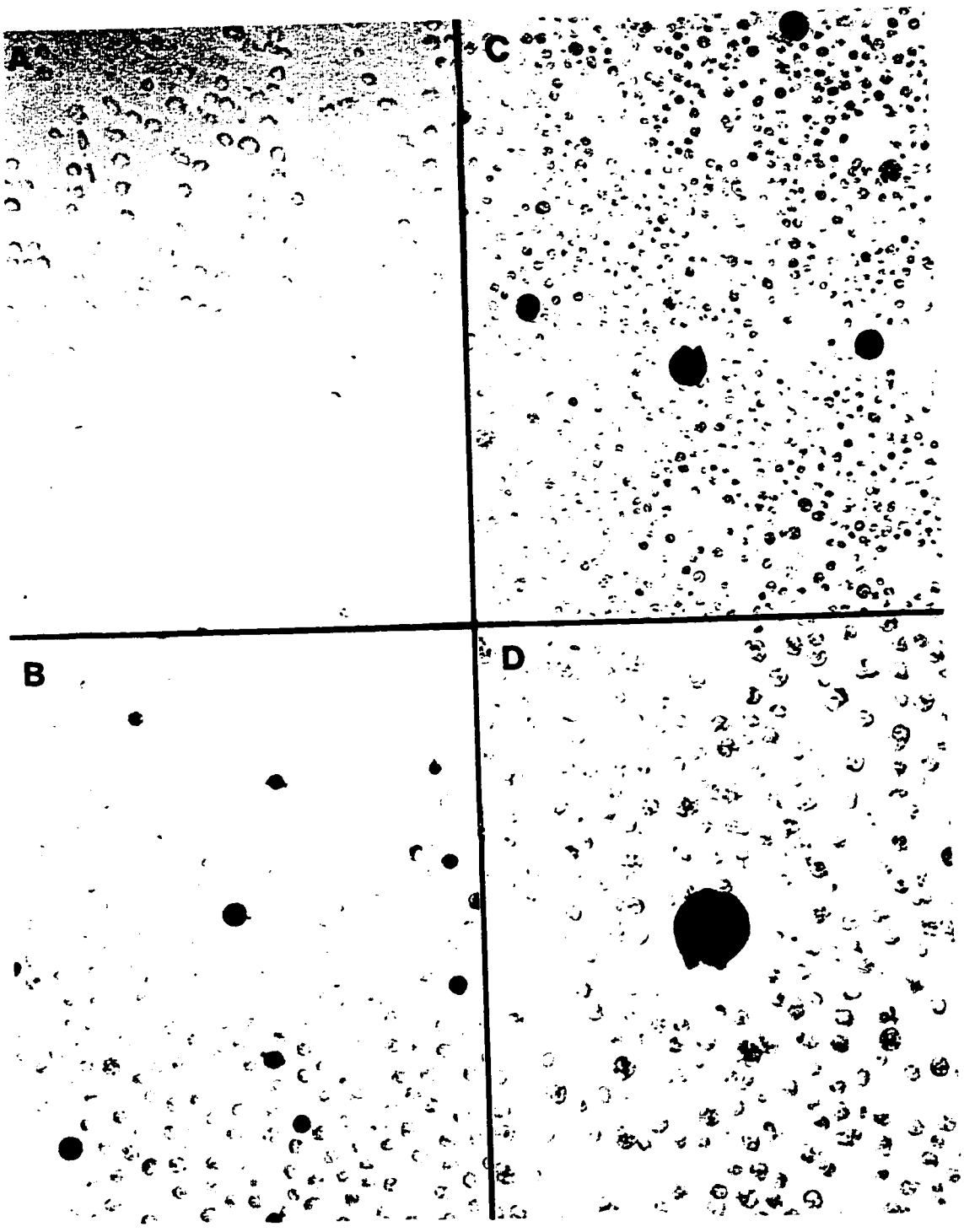
Figure 2.2 . Kinetics of wild type HIV-1 infection of human peripheral blood lymphocyte (PBL) *in vitro*.

Human PBLs were prepared and infected with HIV-1_{NL4-3} as described in the Materials and Methods. The kinetics were monitored by RT assay. Similar results were obtained with macrophage-tropic HIV_{NLAD8}.
d.p.i= days post infection.
moi= multiplicity of infection.

Figure 2.3. HIV-1 induced syncytia formation in the acutely infected H9 cells.

Human PBL (A and B) and H9 cells (C and D) were infected with HIV_{NL4-3} at 0.02 m.o.i as described in the Materials and Methods. The infected cells were examined under an inverted microscope. At 9 days post infection, syncytia formation was observed in HIV-1 infected H9 cells, but not in PBLs. The infected cells were further stained with anti-HIV-1 positive serum and photographed as described in the Materials and Methods.

A. Human PBL: Uninfected cells (x 400).
B. Human PBL: 9 days post infection (x 400).
C. H9 cells: 9 days post infection (x 100).
D. H9 cells: 9 days post infection (x 400).


A
C
B
D

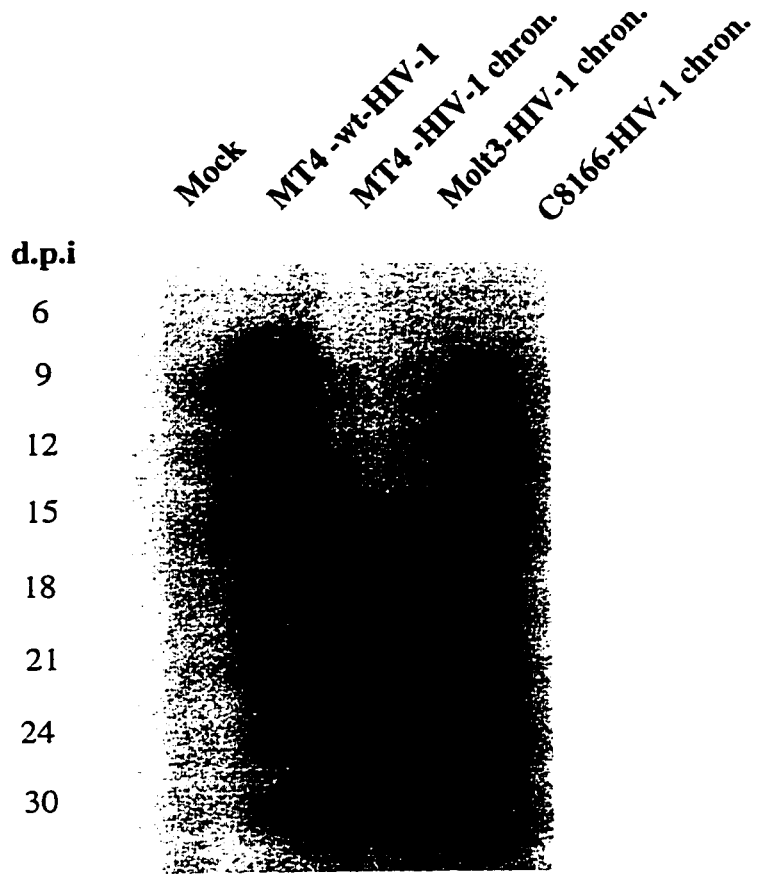


Figure 2.4. Establishment of chronically infected cell lines with serially passaged HIV_{NL4-3} viruses from MT4 cells.

To establish chronically infected cell lines, serial passage of HIV-1 were prepared as described in the text. When MT4 cells were infected with the 20th passage of the virus at 0.02 moi, chronically infected MT4 cell line was established. Subsequently, chronically infected Molt3 and C8166 cell lines were established by infection of supernants (0.02 moi) of the chronically infected MT4 line. The infection kinetics were monitored by RT assay.
d.p.i.= days post infection.

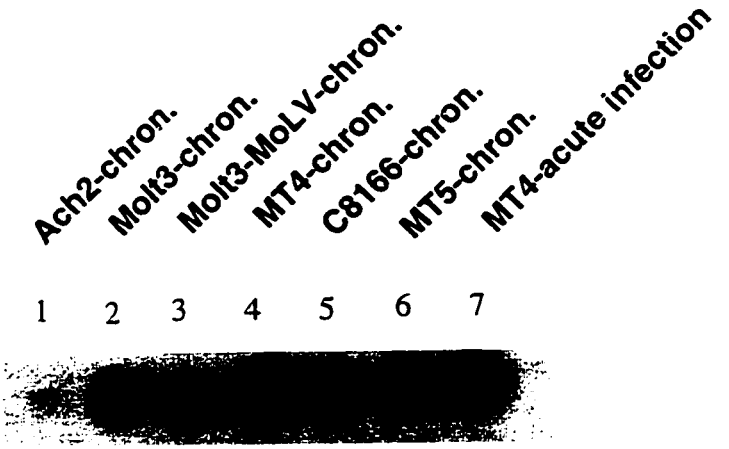


Figure 2.5 High titers of HIV-1 are produced from the chronically infected cell lines.

For the acute infection (lane 7), 2 x 10^6 MT4 cells were infected with HIV_{NL4-3} at 0.02 moi as described in the Materials and Methods. The supernants of the acutely infected MT4 cells were harvested at 9 days post infection for RT assay. For the chronically infected cell lines, 2 x 10^6 cells/each line were cultured and the supernatants were collected just before passage. All the cell cultures had same total cell numbers (1x 10^7 cells in 5 ml) when the supernatants were collected.

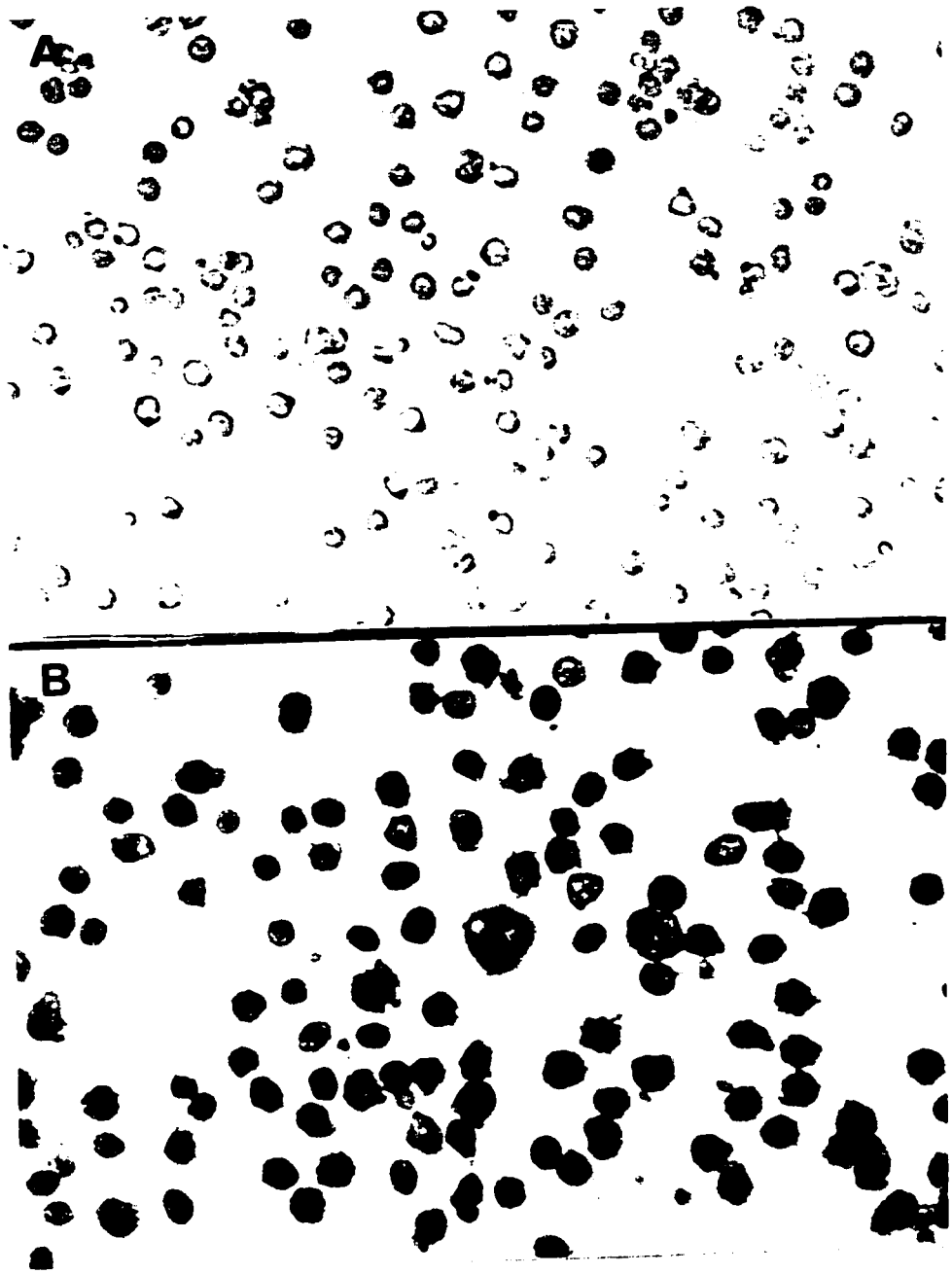
Lane 1: HIV-1 chronically infected Ach2 cells (from NIH).
Lane 2: Tat-plus HIV-1 chronically infected Molt-3 cells
Lane 3: Tat-plus HIV-1 chronically infected Molt-3 cells
Lane 4: Tat-plus HIV-1 chronically infected MT4 cells
Lane 5: Tat-plus HIV-1 chronically infected C8166 cells
Lane 6: Tat-minus HIV-1 chronically infected MT4 cells (MT5-chron.)
Lane 7: Wild type HIV-1 acutely infected MT4 cells.

Figure 2.6. Immunohistochemical staining of HIV-1 chronically infected MT4 cells.

Uninfected or HIV-1 chronically infected MT4 cells were harvested and HIV-1 viral antigens were detected by immunohistochemical staining as described in the Materials and Methods.

A. Uninfected MT4 cell (X400).

B. HIV-1 chronically infected MT4 cells (X400) (after 50 passages over two years).


A
B

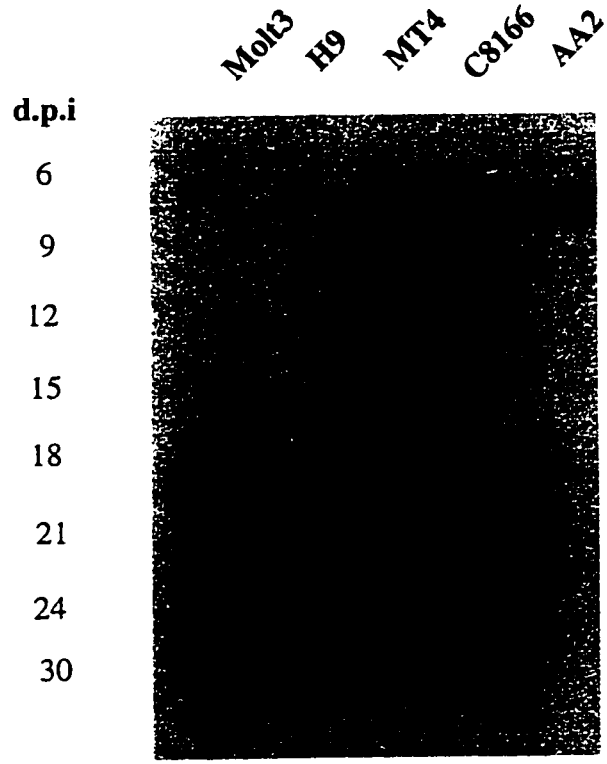


Figure 2.7. Infection of $CD4^+$ cell lines with the HIV-1 viruses produced from the chronically infected MT4 cell lines.

To characterize the infectivity of the HIV-1 produced from the chronically infected MT4 cells, Tat-plus HIV-1 were harvested from the chronically infected MT4. The HIV-1 infection at 0.02 moi was performed as described in the text. Similar results were obtained when MT4 cells were infected with the Tat-plus .HIV-1 from the chronically infected C8166 or Molt3 cells.
d.p.i = days post infection.

Figure 2.8. EM analysis of HIV-1-infected MT4 cells.

Uninfected, acutely, or chronically infected MT4 cells were harvested and prepared for EM examination as described in the Materials and Methods.

A. TEM section of uninfected MT4 cells. bar =1 μm

B. TEM section of wild-type acutely infected MT4 cells, illustrating vacuolization and free extra cellular virus. bar =1 μm

C. TEM section of free mature extra cellular wild type HIV-1 viral particles demonstrating diamond shaped nucleoid core (solid black arrow). bar = 1 μm

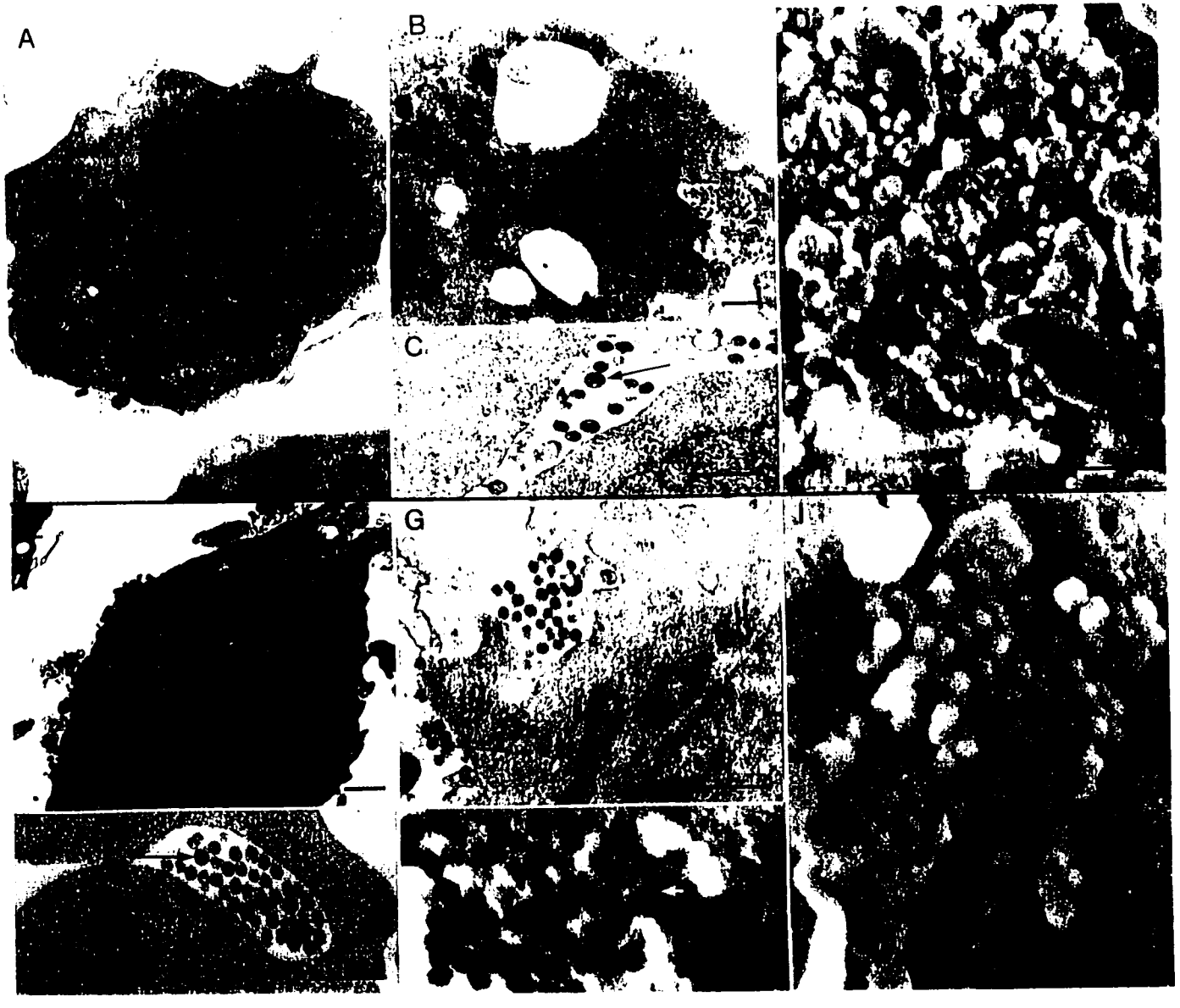
D. SEM image of wild type HIV-1 viral particles on the surface of acutely infected MT4 cells. bar = 500 μm

E. TEM section of tat-minus HIV-1 chronically infected MT4 cells. bar =1 μm

F & G. TEM section of tat-minus HIV-1 chronically infected MT4 cells, indicating bar shaped nucleoid core and condensed circular core (solid arrows). bar = 500 μm

H. Immuno-gold labelled section of tat-minus HIV-1 chronically infected MT4 cells. Positive staining is determined by the presence of gold particles (white arrow). bar = 500 μm

I. SEM image of extracellular viral particles on the surface of tat-minus HIV-1 chronically infected MT4 cells. bar = 500 μm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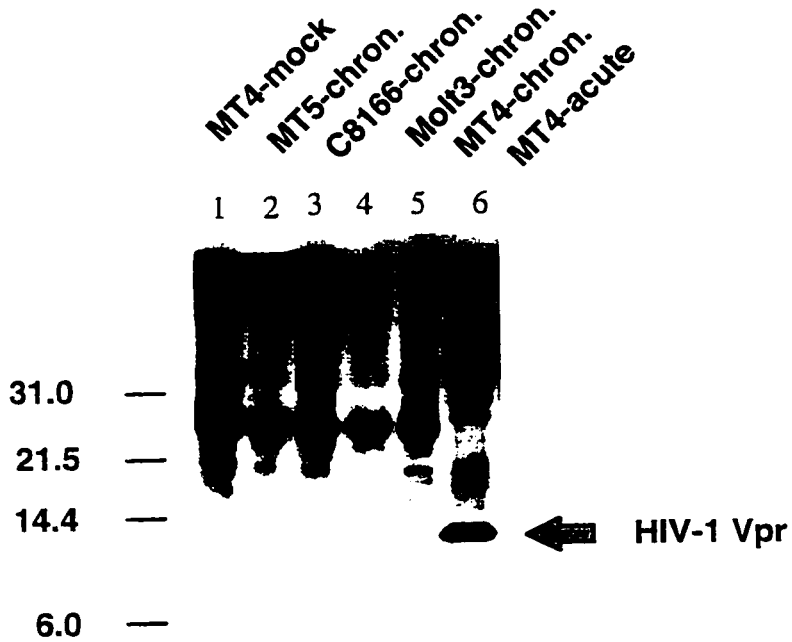


Figure 2.9. Absence of HIV-1 Vpr expression in chronically infected cell lines

To detect Vpr expression in the cell cultures, western blot analysis was carried out as described in the Materials and Methods. Same amount proteins from each sample were loaded for the analysis. Same results were obtained from three times repeats.

Lane 1: MT4-mock infected (negative control).
Lane 2: Tat-minus (MT5-chron.) HIV-1 chronically infected MT4 cells.
Lane 3: Tat-plus (C8166-chron.) HIV-1 chronically infected C8166 cells.
Lane 4: Tat-plus (Molt3-chron.) HIV-1 chronically infected Molt3 cells.
Lane 5: Tat-plus (MT4-chron.) HIV-1 chronically infected MT4 cells.
Lane 6: Wild type (MT4-acute) HIV-1 acutely infected MT4 cells.

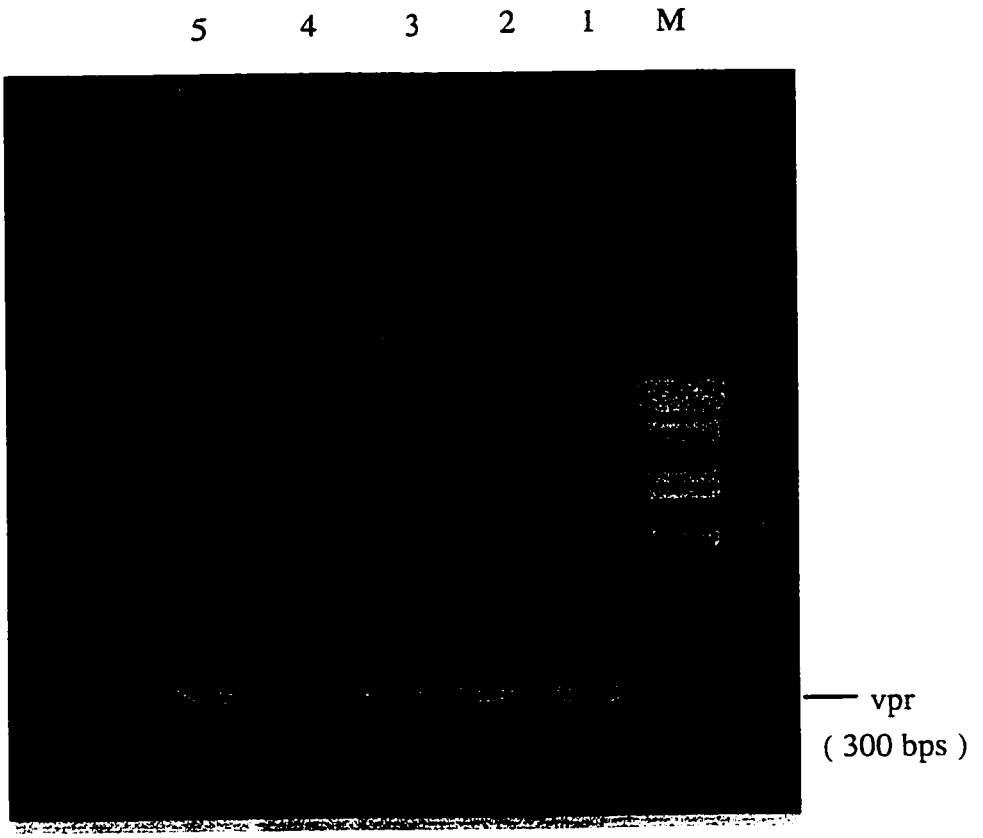


Figure 2. 10. RT-PCR amplification of HIV-1 vpr from acutely and chronically infected cell cultures

PCR was performed as described in the Materials and Methods.

M: DNA molecular weight marker (Lamda DNA-BstE II Digest, NEB).
Lane 1: wild type HIV-1 acutely infected MT4 cells
Lane 2: Tat-minus (MT5-chron.) HIV-1 chronically infected MT4 cells.
Lane 3: Tat-plus (C8166-chron.) HIV-1 chronically infected C8166 cells
Lane 4: Tat-plus (Molt3-chron.) HIV-1 chronically infected MOlt3 infected cells
Lane 5: Tat-plus (MT4-chron.) HIV-1 chronically infected MT4 cells

```
                    1/1                                                        52/18        70/24
                    M   E   Q   A   P   E   D   Q   G   P   Q   R   E   P   Y   N   E   W   T   L   E   L   L   E
vpr-wt              atg gaa caa gcc cca gaa gac caa ggg cca cag agg gag cca tac aat gaa tgg aca cta gag ctt tta gag

vpr-MT4-chron.#2    -------------------------------------------------------------------tga----------------------
vpr-C8166-chron.#1
                    73/25                                                                               148/50
                    E   L   K   S   E   A   V   R   H   F   P   R   I   W   L   H   N   L   G   Q   H   I   Y   E   T   Y
vpr-wt              gaa ctt aag agt gaa gct gtt aga cat ttt cct agg ata tgg ctc cat aac tta gga caa cat atc tat gaa act tac

vpr-MT4-chron.#2    ------------------------------------------------------------------------------------------------------
vpr-C8166-chron.#1
                    151/51                                                                          223/75
                    G   D   T   W   A   G   V   E   A   I   I   R   I   L   Q   Q   Q   L   F   I   H   F   R   I   G
vpr-wt              ggg gat act tgg gca gga gtg gaa gcc ata ata aga att ctg caa ctg caa ctg ttt atc cat ttc aga att ggg

vpr-MT4-chron.#2    ----------------------------------------------------------------------------------------------------
vpr-C8166-chron.#1
                    226/76                                                              288/96
                    C   R   H   S   R   I   G   V   T   R   Q   R   R   A   R   N   G   A   S   R   S
vpr-wt              tgt cga cat agc aga ata ggc gtt act cga cag agg aga gca aga aat gga gcc agt aga tcc tag

vpr-MT4-chron.#2    -----------------------------------------------------------------------------------
vpr-C8166-chron.#1
```

Figure 2.11. Appearance of a novel stop codon (^{52}TGG toTGA) was found in two of the vpr clones (vpr-MT4-chron.#2 and vpr-C8166-chron.#1) from the chronically infected cell cultures. Sequences identical with those of wild type vpr (dashes) and a new stop codon (tga) are indicated.

Table 2.1 Human CD4+ cell lines used for HIV-1 infection in this study

Cell lines	Cell type and special features	Source
MT4	Human T cell leukemia cell line, HTLV-1 transformed	NIH
C8166	Human umbilical cord blood lymphocyte cell line, HTLV-1 transformed.	NIH
AA2	Human splenic EBV+ B-lymphoblastiod, CD4+ cell line	NIH
H9	Human T-cell lymphoma cell line	NIH
Molt3	Human acute lymphoblastic leukemia cell line	ATCC

Table 2.2 Establishment of HIV-1 chronically infected cell lines

Cell lines	Initially infected by	Viral titre	Contributors
MT5-chron.	Tat-minus HIV-1	high	L-J Chang
Molt3-Mo-MLV-chron.	LTR mutant HIV-1	high	L-J Chang
MT4-chron.	wt-HIV-1_{NL4-3}	high	C. Zhang & L-J Chang
C8166-chron.	Virus from MT4-chron.	high	C. Zhang & L-J Chang
Molt3-chron.	Virus from MT4-chron.	high	C. Zhang & L-J Chang
Ach2-chron.	wt-HIV-1_{LAV}	low	NIH (T. Folks)

Chapter III

Cell cycle inhibitory effects of HIV and SIV Vpr and Vpx in the fission yeast *Schizosaccharomyces pombe*

(Most of the data presented in this Chapter have been published in an article entitled " Cell cycle inhibitory effects of HIV and SIV Vpr and Vpx in the Yeast *Schizosaccharomyces pombe* " by Chengsheng Zhang, Colin Rasmussen, and Lung-Ji Chang in Virology 230: 103-112, 1997).

A. Introduction and rationale

HIV-1, HIV-2, and SIV contain, in addition to the gag, pol, and env structural genes, small open reading frames encoding the accessory proteins Tat, Rev, Nef, Vpr or/and Vpx, Vpu, and Vif (Luciw, 1996). The vpr and vpx are highly conserved and related genes, with all five groups of primate lentiviruses containing at least one homologue of either vpr or vpx (Franchini and Reitz, 1994; Subbramanian and Cohen, 1994; Tristem et al., 1992). HIV-1, SIVcpz, SIVmad, SIVagm, and SIVsyk contain the vpr gene only, whereas HIV-2, SIVmac, and SIVsm contain both vpr and vpx (Tristem et al., 1992). Since Vpr and Vpx are found in the mature virions, it has been thought that they may play a important role in the early phase of the viral life cycle (Cohen et al., 1990b; Levy, D.N., 1995; Yu et al., 1990). Recent studies suggest that Vpr and the matrix protein of HIV-1 are required for the nuclear migration of the pre-integration complex (Bukrinsky et al., 1993; Heinzinger et al., 1994). Vpr has also been reported to prevent establishment of chronically infected HIV-1 producing cell lines (Planelles et al., 1996; Rogel et al., 1995; Chapter II of this thesis) . This is consistent with an earlier report which showed that HIV-1 Vpr expression could prevent cell division and

induce differentiation of a rhabdomyosarcoma cell line (Levy, D.N. et al., 1993). More recent studies have shown that HIV-1 Vpr can arrest the cell cycle at G2/M phase in mammalian cells (Bartz et al., 1996; He et al., 1995; Jowett et al., 1995; Planelles et al., 1996; Re et al., 1995). HIV-1 Vpr was also reported to cause cell growth arrest of budding yeast *S. cerevisiae* (Macreadia et al., 1995). In addition, HIV-2 and SIV Vpr have been shown to induce cell cycle arrest in mammalian cells, although the effects of HIV-2 and SIV Vpr were not as pronounced as that of HIV-1 Vpr. However, Vpx from HIV-2 and SIV_{mac} did not induce detectable cell cycle arrest in simian CV-1 or human HeLa and 293 cells (Marzio et al., 1995; Planelles et al., 1996).

The fission yeast *Schizosaccharomyces pombe* (*S. pombe*) has been widely used as a model to study the roles of a variety of genes in the control of cell proliferation in eukaryotic cells (Draetta et al., 1987; Nurse, 1994). Therefore we reasoned that if expression of Vpr and Vpx proteins in fission yeast could produce observable phenotypes, it would provide us with an *in vivo* model system with which to study the effects of these proteins on cell function, especially cell proliferation and the molecular mechanisms involved.

In this study, we have examined the effects of HIV-1 Vpr, HIV-2 Vpr/Vpx, and SIV Vpr/Vpx expression on cell cycle progression in *S. pombe*. We found that expression of HIV-1 Vpr in *S. pombe* blocks cell proliferation with cells arrested predominantly in the G1 and G2 phases of the cell cycle. In addition, HIV-Vpr expression was cytotoxic with cell viability rapidly declining in the presence of HIV-1 Vpr. Although Vpr expression induced polyploidy in yeast as in mammalian cells, the cytotoxic effects in yeast contrasted with previous studies in human T cell lines where Vpr expression was found to be cytostatic rather than cytotoxic (Bartz et al.,1996). Furthermore, expression of either HIV-2 Vpr/Vpx or SIV Vpr/Vpx resulted in cell cycle arrest in

yeast although Vpx of HIV-2 and SIV has been reported to be non-cytostatic in mammalian cells (Marzio et al., 1995; Planelles et al., 1996). However, the effects of HIV-2/SIV Vpr and Vpx on cell viability were not as pronounced as with HIV-1 Vpr. Expression of either HIV-1 Tat or Nef proteins produced none of these phenotypes. Moreover, we have further demonstrated that some of the HIV-1 vpr mutants with mutations at the C-terminus of vpr gene lost their effects on cell cycle arrest and cytotoxicity in *S. pombe*. Taken together, our data suggest that these effects on cell proliferation and cytotoxicity are specific to Vpr and Vpx. Thus, our studies indicate that *S. pombe* may be a useful model system with which to study the processes through which Vpr and Vpx affect cellular functions.

B. Materials and Methods

Plasmid constructions

Different primer combinations were used to generate vpr, vpx, tat, and nef fragments for ligation into the pREP41 expression vector (Table 3.1). This expression vector contains the *nmt*1 (Stands for no message with thiamine) promoter which is repressed in the presence of thiamine (Maundrell, 1990). All PCR products were subcloned into pBluescript-KS$^-$ by blunt end ligation. The inserts were then excised by digestion with NdeI and BamHI, and subcloned into pREP41 expression vector. The cloned insert in pREP41 was sequenced to ensure the correct sequence and reading frame.

Culture and transformation of *S. pombe*

The yeast strain used in this study was SP130 (wild type strain, h$^-$, ade6-210, leu1-32; Rasmussen and Rasmussen, 1994). The growth medium was mimimal medium (MM) with or without the supplements of leucine, adenine and uracil according to the selection requirements of each particular strain. Standard culture conditions and techniques were used as described (Moreno et al., 1991). The yeast cells containing pREP41-based expression plasmids were routinely grown in the medium containing 1 μM thiamine-HCl to repress nmt1-dependent transcription (Maundrell, 1990). To induce target protein expression from the nmt1 promoter, cells were washed with sterile water and then reinoculated into MM lacking thiamine-HCl. Transformation of *S. pombe* was carried out by the lithium acetate procedure as described elsewhere (Moreno et al., 1991).

Western blot analysis of Vpr or Vpx protein expression

To detect the target protein expression in the yeast cells, protein extracts were prepared as described previously (Moreno et al., 1991). For Western blot analysis, protein

concentrations of the extracts were quantified using the DC Protein Assay Kit according to the manufacturer's instructions (BIO-RAD). Ten micrograms of proteins from each sample were applied to a 10-20% gradient Tricine-SDS-PAGE gel (NOVEX,Canada). After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell NC). The filters were air-dried and blocked with 10% dried milk in TBS-T (T= 0.3% Tween 20) for 1 hr at room temperature. Filters were then incubated with the primary antibody (rabbit anti-HIV-1 Vpr, rabbit anti-HIV-1 Tat, rabbit anti-SIV Vpr, or rabbit anti-SIV Vpx, all of which antibodies were obtained from NIH, 1:500-1:1000 dilution) at 4^0C overnight. This was followed by an incubation with an HRP-conjugated secondary antibody (Amersham, NA 9340, 1:2000 dilution) at room temperature for 1 hr. After washing the filter, the signal was detected by the ECL Western Blotting Detection Reagents according to the manufacturer's protocols (Amersham Life Science).

Synchronization of yeast cell cultures

To obtain synchronized cell cultures as the control for flow cytometry analysis, the yeast cells were cultured under nitrogen starvation in the nitrogen-limited medium containing 5 mM NH_4Cl (93.5 mM NH_4Cl in the regular MM) at 30^0C for 4 hr, which arrests cells predominantly in G1; or the cells were cultured under glucose starvation in the glucose-limited medium containing 0.5% of glucose (2% glucose in the regular MM), which arrests cells predominantly in G2 (Costello et al., 1986). In addition, to obtain early S phase-arrested cell culture, the yeast cells were treated with 25 mM hydroxyurea for 4 hr as described elsewhere (Muzi-Falconi et al., 1996).

Flow cytometry

The cell samples for flow cytometry were prepared as described previously (Alfa et al., 1992). Briefly, yeast cultures were grown at 30^0C overnight to a density of 1x 10^7/ml.

The cells were collected and fixed with 70% ethanol. For flow cytometric analysis, an aliquot of the fixed cells (about 3 x 10^6) was collected and washed once with 1 ml of 50 mM sodium citrate (pH 7.0). The cells were resuspended in 0.5 ml of 50 mM sodium citrate containing 1 mg/ml RNaseA, and incubated in a 37^{0}C water bath for 2 hr. After incubation, 0.5 ml of 50 mM sodium citrate containing 2 μg/ml propidium iodide (PI) was added to the cells. The DNA content was determined by FACScan analysis and the data was analyzed using the CellFit program (Becton-Dickinson).

Viability assay

To determine the viability of the cells expressing Vpr or Vpx, the yeast cells were grown in thiamine-plus or thiamine-minus medium at 30^{0}C. After different time points (0, 6, 9, 12, 15, 18, and 24 hr), an aliquot of culture was harvested, and approximately 200 cells from each sample were plated onto thiamine-plus plate. The plates were incubated at 30^{0}C for 4-6 days prior to counting colonies.

C. Results

Construction of thiamine-conditional Vpr and Vpx expression plasmids: To engineer the Vpr and Vpx expression vectors, we have amplified the wild type vpr gene of the clone HIV-1_{NL4-3} and the vpr and vpx genes of HIV-2_{ROD} and SIV_{mac239} by PCR. For the construction of mutant Vpr expression vectors, several HIV-1 vpr mutant constructs (generous gifts of Dr. Ling-Jun Zhao, University of Saint-Louis, USA ; Zhao et al., 1994) were used as the templates for PCR amplification to generate mutant vpr fragments for this study (Figure 3.1). It is noteworthy that these vpr mutants were constructed from another HIV-1 strain called HIV-1/89.2 (Zhao et al., 1994). However, the wild type vpr sequence of HIV-1_{NL4-3} is almost identical to the vpr sequence of HIV-1/89.2. For comparative studies, we have cloned wild type vpr from both of these two HIV-1 strains. Subsequently, the PCR fragments were subcloned into a pBluescript-KS^- vector, respectively (Stratagene) by blunt end ligation. Each fragment was then excised by digestion with NdeI and BamHI and cloned into the plasmid pREP41 with 5' of the insert to the thiamine-repressible nmt1 promoter (Figure 3.2). HIV-1 tat and nef were amplified from HIV-1_{NL4-3}. All the recombinant plasmids used in this study were verified by restriction enzyme digestion mapping and DNA sequencing to ensure the correct sequence and reading frame. pREP41 contains the *S. cerevisiae* LEU2 gene which complements the leu1-32 mutation of *S. pombe*. These plasmid were transformed into the *S. pombe* strain SP130 (which carries the leu1-32 mutation), and transformants were selected by their ability to grow on minimal medium lacking leucine. The medium also contained thiamine to prevent expression of Vpr or Vpx in case their presence was lethal to *S. pombe*.

HIV-1 Vpr, HIV-2/SIV Vpr and Vpx arrest *S. pombe* proliferation

To determine if expression of either Vpr or Vpx would affect cell proliferation of *S. pombe*, yeast cells containing the Vpr or Vpx expression plasmids were grown on culture plates with or without thiamine. When grown on the plates plus thiamine (which prevents the protein expression), normal size yeast colonies were formed from yeast cells transformed with either empty pREP41 vector, HIV-1 Tat, HIV-1 Nef, HIV-1 Vpr, HIV-2 Vpr/Vpx, or SIV Vpr/Vpx (Figure 3.3 A). On the plates lacking thiamine (which induces protein expression), control yeast cells transformed with the control plasmids formed normal size colonies, whereas there were no colonies formed by yeast cells expressing HIV-1 Vpr, and much smaller colonies were formed by yeast expressing SIV Vpr or Vpx (Figure 3.3 B). Both of the Vpr constructs from HIV-1_{NL4-3} and HIV-1/89.2 have had identical effects on the cell proliferation. These results suggest that Vpr and Vpx could inhibit cell proliferation in *S. pombe*, and the lack of colony formation in the presence of HIV-1 Vpr suggested that the expression of this protein had a more pronounced effect on cell proliferation.

Because both Vpr and Vpx caused growth arrest, each strain was further characterized to examine the effect on cell proliferation kinetics. Exponentially growing cells cultured in MM plus thiamine were washed in sterile water and re-inoculated into fresh MM +/– thiamine at a density of $1x10^5$ cells/ml. Cell numbers were then determined at various time points. The normal generation time of SP130 cells at 30^0C in MM is about 3 hr. It was found that the generation time of HIV-1 Vpr-, HIV-2 and SIV Vpr- or Vpx-conditional expressing strains grown in the presence of thiamine (i.e., not expressing) was similar to that of the wild-type strain as expected (Figure 3.4). However, in the absence of thiamine, expression of HIV-1 Vpr caused a complete arrest of cell proliferation within 9-12 hr (Figure 3.4A), whereas cells expressing HIV-2 Vpr or Vpx (Figure 3.4B), or SIV Vpr or Vpx (Figure 3.4B), shown a reduced rate of proliferating

starting at 9 hr and eventually cessation of proliferation by 30 hr after switching to conditions that permit expression. The growth kinetics of cells expressing HIV-1 Tat was the same in medium with or without thiamine (Figure 3.4 A), indicating that the effects on cell proliferation were specific to Vpr and Vpx proteins and not due to simply expressing viral proteins.

Western blot analysis of Vpr and Vpx expression

To confirm the correlation between cell growth arrest and Vpr or Vpx expression, yeast protein extracts were prepared at different time points and the expression of HIV-1 Vpr and SIV Vpr and Vpx were detected by western blot as described in the materials and methods. The immunoblot results indicated that Vpr and Vpx expression could be detected within 9 hr after switching cells into the medium lacking thiamine and the levels of expression were constant for at least 24 hr (Figure 3.5). Similar protein expression patterns have been observed in the cells expressing HIV-1 Tat or HIV-2 Vpx. Since the cell proliferation rates showed difference beginning at 9 hr, these results indicate that the arrest of cell proliferation we observed is coincident with Vpr or Vpx expression.

Morphological changes of cells expressing HIV-1 Vpr, HIV-2 and SIV Vpr or Vpx

To study the possible morphological changes of cells expressing Vpr or Vpx, we examined the cells by phase microscopy, fluorescence microscopy, and scanning electron microscopy (SEM, see Chapter II in materials and methods). When examined by phase microscopy, cells expressing HIV-1 Vpr, HIV-2 Vpr or Vpx, and SIV Vpr or Vpx showed an increased cell length (Figure 3.6 A-D). Similar changes have been observed by SEM examination (Figure 3.7 A-D). In addition, when the cells were stained with propidium iodide and examined by fluorescence microscopy, a single

nucleus stained with propidium iodide was observed in each of the arrested cells (Figure 3.6 E-F), indicating that expression of Vpr or Vpx proteins arrests the nuclear division cycle but permits cell growth to continue, which is similar to the phenomenon previously described in some of the cell division cycle (cdc) mutant strains (Nurse et al., 1986).

Expression of HIV-1 Vpr, HIV-2/SIV Vpr and Vpx is cytotoxic in *S. pombe*

Since expression of Vpr and Vpx arrested cell proliferation in *S. pombe*, we were interested in determining whether there were additional effects on cell viability. To address this issue, expression of HIV-1 Vpr, HIV-2 and SIV Vpr or Vpx was induced by growth in MM lacking thiamine for different lengths of time. Approximately 200 cells from each sample were then plated onto MM plus thiamine agar to again repress Vpr or Vpx expression. As a control, cells were treated in the same way except they remained in MM plus thiamine for all parts of the experiments. The number of viable cells was determined by counting the number of colonies formed.

The results show that cells expressing HIV-1 Vpr rapidly lose their viability, with essentially no viable cells remaining after 15 hr in thiamine-free medium (Figure 3.8). This corresponds to about 6 hr of Vpr expression. In contrast, expression of Vpr or Vpx from HIV-2 or SIV showed much less of a cytotoxic effects on *S. pombe* than did expression of HIV-1 Vpr. After 24 hr of incubation, about 50% of the cells expressing HIV-2/SIV Vpr or Vpx were still viable (Figure 3.8). These data indicate that expression of HIV-1 Vpr in *S. pombe* is lethal, while expression of HIV-2 and SIV Vpr or Vpx has less severe, although significant effect on cell survival.

C-terminal region of HIV-1Vpr is related to the cell growth inhibition and cell cycle arrest in *S. pombe*

Several biological functions have been attributed to HIV-1 Vpr, including nuclear localization, viral packaging, and cell cycle arrest. Previous studies have indicated that the cell cycle arrest in mammalian cells and *S. cerevisiae* was largely related to the basic domain at the C-terminal region of HIV-1 Vpr (Macreadie et al., 1995; Marzio et al., 1995). To test whether the cell cycle arrest in our *S. pombe* system is also controlled by the C-terminal region of HIV-1Vpr, a panel of Vpr mutants have been cloned into the pREP41 yeast expression vector as described in the materials and methods. The wild type yeast SP130 were transformed with wild type or different vpr mutant constructs as described above. We found that two of the mutants, vpr-LR-mu and vpr-del 70-96, have lost their effects on the cell cycle arrest, all others still retain the effects of cell cycle arrest (Table 3.2). Both of these two mutants have mutations at the C-terminal region of vpr, indicating that the Vpr-induced cell cycle arrest in *S. pombe* is also related to the C-terminal region of Vpr. It is noteworthy that those two mutants are not cytotoxic when expressed in *S. pombe*, indicating that HIV-1 Vpr-mediated cell cycle arrest and cytotoxicity are closely related to each other.

Vpr or/and Vpx of HIV-1, HIV-2 and SIV arrest cell cycle at G1/S and G2/M phases in *S. pombe*

In order to determine the check point of cell cycle arrest in Vpr- or Vpx-expressing cells, we used flow cytometry to detect the DNA content in control cells and cells expressing Vpr or Vpx. The internal control samples for flow cytometry analysis included cells under nitrogen starvation (MM containing 5 mM NH_4Cl, instead of 93.5 mM in regular MM) which enrich cells predominantly in G1, and glucose starvation (MM containing 0.5% of glucose rather than 2% glucose in the regular MM), which enrich cells predominantly in G2 (Figure 3.9A and B). Control HIV-1 Vpr cells grown

in the presence of thiamine shown the normal distribution of DNA contents with most cells being in the G2 phase of cell cycle (Figure 3.9C). Cells containing the HIV-1 Vpr or Vpx expression vectors and grown in the absence of thiamine for 18 hr had DNA content of both G1 and G2/M blocked cells, indicating that Vpr and Vpx expression blocks cell progression at two distinct points (Figure 3.9 D-F). In addition, cells expressing HIV-1 Vpr also showed signs of extra DNA replication as evidenced by the peak of cells with DNA contents in excess of G2/M peak (Figure 3.9D). This effect was not observed in cells expressing SIV Vpr or Vpx (Figure 3.9E and F). The DNA pattern of cells expressing HIV-2 Vpr or Vpx is similar to that of cells expressing SIV Vpr or Vpx.

Because we observed evidence of yeast cells accumulated not only in G2/M but also in G1 phase of cell cycle, we wanted to determine if Vpr or Vpx expression prevents progression through S phase. To test this hypothesis, we established a culture of cells in which Vpr or Vpx expression had just reached maximal levels (which normally takes 9 to 10 hr under conditions used in this studies), and which had been grown for 4 hr in the presence of hydroxyurea (HU) in order to arrest cells in early S phase. To establish this cell culture, cells containing HIV-1 Vpr or SIV Vpr or Vpx expression vectors were switched to thiamine-minus medium at $t = 0$ hr to permit Vpr or Vpx expression. At t = 6hr, HU was added to the culture. At t = 10hr, cells were washed with sterile water to get rid off HU and inoculated into medium lacking HU and thiamine to release them from S-phase block, but still maintain Vpr or Vpx expression. At t = 20 hr, cell samples were taken and prepared for flow cytometry analysis. To establish that the HU treatment results in cell cycle arrest, a sample of HIV-1 Vpr expressing cells was taken at t =10 hr (i.e., 4 hr after HU treatment), prior to the removal of HU treatment (Figure 3.10A). Our results show that the control cells, in which HIV-1 Vpr expression was repressed, were able to recover from HU treatment and progress through the cell cycle

establishing a normal, predominantly G2/M distribution of cells (compare Figures 3.9C and 3.10B). In contrast, cells allowed to express HIV-1 Vpr or SIV Vpr or Vpx had a significant fraction of the population with DNA contents indicative of cells in G1 or very early S phase (Figure 3.10C-E), suggesting that the presence of Vpr or Vpx attenuates progression through S phase.

D. Summary of results

1. A system in which Vpr or Vpx can be conditionally expressed in *S. pombe* has been successfully established and used in this study.

2. HIV-1 Vpr, HIV-2 and SIV Vpr/Vpx inhibit cell proliferation in *S. pombe*. The effect of HIV-1 Vpr on cell proliferation was more severe than that of HIV-2 and SIV Vpr or Vpx.

3. Expression of Vpr or Vpx induced apparent morphological changes with increased cell length size in *S. pombe*.

4. Expression of HIV-1 Vpr and HIV-2/SIV Vpr or Vpx were cytotoxic in *S. pombe*

5. HIV-1 Vpr-induced cell growth inhibition in *S. pombe* was mapped to the C-terminal region of Vpr.

6. HIV-1 Vpr and HIV-2/SIV Vpr and Vpx could arrest the cell cycle at G1/S and G2/M phases in *S. pombe*.

E. Discussion

In the present study, we have used a thiamine-repressible expression system to study the potential effects of Vpr and other HIV/SIV viral proteins on the fission yeast *S. pombe*. Surprisingly, we have shown that expression of HIV-1 Vpr and HIV-2/SIV Vpr or Vpx in *S. pombe* results in a variety of abnormal changes including inhibition of cell colony formation, increase of the cell size, induction of cytotoxicity, and arrest of cell cycle progression.

Some of our observations are consistent with other studies demonstrating that HIV-1 Vpr could cause cell cycle arrest in mammalian cells, budding yeast *S. cerevisiae*, and fission yeast *S. pombe* (He et al., 1995; Jowett et al., 1995; Macreadie et al., 1995, Re et al., 1995; Rogel et al., 1995; Zhao et al., 1996). In addition, we have extended the studies to include HIV-2 and SIV Vpr and Vpx and demonstrated that all these lentiviral proteins inhibit yeast cell proliferation. Although HIV-2 and SIV Vpr and Vpx cause cell cycle arrest and cytotoxicity in *S. pombe*, they are not as severe as HIV-1 Vpr. Previous studies suggested that the cell cycle arrest effects of Vpr and Vpx could be species-specific and cell-type dependent. Expression of HIV-1 Vpr in HeLa cells, which are of human origin, has a more severe effect than does expression in CV-1 cells, which are of simian origin (Planelles et al., 1996). Accordingly, expression of SIVmac239 Vpr has a less severe effect than HIV-1 Vpr in human 238 cells (Marzio et al., 1995) , and expression of SIVagm Vpr has a more severe effect in CV-1 cells than in HeLa cells (Planelles et al., 1996). Thus, it might initially be surprising that expression of all these proteins should affect cell proliferation in a simpler eukaryote such as *S. pombe*. It should, however, be noted that many of the proteins that regulate cell proliferation in yeast can be functionally complemented by human homologues (Moreno et al, 1991; Nurse, 1994). Thus, the ability of Vpr and Vpx proteins to affect cell proliferation might be mediated via the basic cell cycle control machinery.

Furthermore, the fact that these proteins have different effects depending on cell type helps explain in part why we see some differences in the phenotypes caused by the expression of Vpr or Vpx in *S. pombe*. In addition, the differences of the levels of expression in yeast and mammalian cells may have a significant impact on the differences of apparent phenotypes, especially given that HIV-1 Vpr appears to be lethal when expressed in yeast.

It is not clear why HIV-1 Vpr, HIV-2 and SIV Vpr or Vpx have various effects on the cell cycle progression and viability in *S. pombe*. The levels of Vpr or Vpx expression do not seem to play a main role in this regard since we did not observe apparent differences of Vpr or Vpx expression in the yeast cells within 24 hr (Figure 3.5. Western blot analysis of Vpr or Vpx expression). The stability of the binding-interaction complex between Vpr or Vpx and its target proteins may play an important role in the differences of apparent phenotypes. However, the different effects of HIV-1 Vpr, HIV-2 and SIV Vpr or Vpx on the *S. pombe* cell cycle progression and viability are consistent with the classification of the primate lentiviruses, in which HIV-1 and HIV-2/SIVmac have been divided into two distinct phylogenetic lineages (Franchini and Reitz, 1994). Our findings that the HIV-1 Vpr and HIV-2/SIV Vpr or Vpx differ with regard to how cytotoxic they are in *S. pombe* may be a consequence of differences in their normal functions *in vivo* with regard to their respective roles in the viral life cycle and cellular pathogenesis, as compared to HIV-1 Vpr. These results are also consistent with the recent report that SIVmac239 provirus lacking either vpr or vpx are still capable of causing AIDS in rhesus monkeys, while the vpr/vpx double mutant is severely attenuated in pathogenicity *in vivo* (Gibbs et al, 1995), and with a report of independent functions of HIV-2 and SIV Vpr and Vpx with respect to nuclear import and cell cycle arrest in mammalian cells (Fletcher et al., 1996).

We have also observed apparent changes in cell size and DNA content in yeast cells expressing Vpr or Vpx, which are consistent with the data reported in the human Jurkat cells expressing HIV-1 Vpr (Bartz et al., 1996). Increase in nuclear size and ploidy have been observed in *S. pombe* carrying mutations in the mitotic cdks due to decoupling S phase from mitosis (Broek et al., 1991; Hayles et al., 1994). The elongated cells and S phase staggering in yeast expressing Vpr or Vpx mimic the phenotype of the yeast mitotic cdk mutants (Nurse et al., 1986). In addition, the increased DNA content (> 2N) observed in Vpr expressing cells implies that Vpr may also affect the cellular DNA replication checkpoints. Other studies have shown that overexpression of Cdc18 or Rum1 can result in extra rounds of DNA replication and highly enlarged cells in fission yeast (Moreno and Nurse, 1994; Muzi-Falconi et al., 1996). Therefore, expression of Vpr can mimic the effects induced by overexpression of Cdc18 or Rum1 in fission yeast. More recently, HIV-1 Vpr has been shown to induce cytoskeletal defects in budding yeast *S. Cerevisiae* (Gu et al., 1997). In addition, these data also suggest that Vpr and Vpx may have an effect on cytokinesis.

Although Vpr and Vpx have been shown to block cell cycle in mammalian cells, the cytological consequences of their expression are still uncertain. In contrast to a previous study suggesting that HIV-1 Vpr is primarily cytostatic in human Jurkat T cell line (Bartz et al., 1996), our data demonstrates that HIV-1 Vpr is highly cytotoxic in *S. pombe*, with complete loss of the ability to proliferate within a few hours. Neither HIV-2 and SIV Vpr nor Vpx were this cytotoxic, producing an approximately 50% loss of viability (Figure 3.8). Furthermore, we do not anticipate that this due to residual Vpr since all samples contained maximal levels of Vpr or Vpx proteins at the time they were plated onto the repressing medium. It is not clear what the basis of this difference is, but possible explanations include differences in levels of expression and reversibility of target protein interaction. However, our data regarding to the HIV-1 Vpr cytotoxicity

are consistent with a recent report demonstrating that peptides containing the H(S/F)RIG amino acid sequence motif of HIV-1 Vpr cause cell death in a variety of yeast cells including *S. cerevisiae* and *S. pombe* (Macreadie et al.,1996). Furthermore, HIV-1 Vpr has been demonstrated to induce apoptosis in human fibroblasts, T cells and primary peripheral blood lymphocytes (Stewart et al., 1997; Ayyavoo et al., 1997; Chang et al., unpublished data). Taken together, all these studies suggest that Vpr-induced cytotoxicity may also play a role in the depletion of HIV-1 infected $CD4^+$ T lymphocytes, which normally occurs in HIV-1 infected individuals. In addition, our results demonstrate that expression of Vpr or Vpx in *S. pombe* will provide a useful model system to further understand the roles of these proteins.

In this study, we have also demonstrated that Vpr induced cell cycle arrest in *S. pombe* was largely related to the C-terminal region of Vpr. Our data are consistent with the previous report showing that the C-terminal basic domain of HIV-1 Vpr containing H(S/F) RIG amino acid motifs was related to the cell growth arrest and structural defects in the yeast *S. cerevisias* (Macreadie et al, 1995). Another study also suggested that the C-terminal basic region of Vpr was crucial in Vpr induced cell cycle arrest in mammalian cells, particularly the amino acids of R73 and R80 in the H (S/F) RIG motifs (Marzio et al, 1995). It is noteworthy that there are two repeated H (S/F) RIG motifs in the C-terminal region of HIV-1 Vpr, which is located between the amino acids 70 and 84. Interestingly, one of the Vpr mutants used in our study (Vpr-del 78-87, Table 3.2) still retained the effect on cell cycle arrest even though one of the H (S/F) RIG motifs was deleted. However, the vpr mutant Vpr-del 70-96 in which both of the H (S/F) RIG motifs were deleted lost its effect on cell cycle arrest (Table 3.2). Our data indicate that the two H (S/F) RIG domains may be functionally redundant, and each of them is sufficient for inducing cell cycle arrest. In addition, we also found that another Vpr mutant (Vpr-LR-mu) did not cause cell cycle arrest in *S. pombe*.

Interestingly, it has been shown that Vpr-LR-mu mutant could not bind to a 180 kDa cellular protein, whereas the wild type Vpr, Vpr-m, and Vpr-del-78-87 could bind to this cellular protein, indicating that this LR domain may play a role in the interaction between Vpr and the cellular factors (Zhao et al., 1994a; Zhao et al., 1994b).

In mammalian cells, HIV-1 Vpr and SIV Vpr have been shown to arrest the cell cycle at G_2/M phase (Bartz et al., 1996; He et al., 1995; Jowett et al., 1995; Re et al.,1995). In our study, we have shown that HIV-1 Vpr, HIV-2 Vpr or Vpx, and SIV Vpr or Vpx arrested cell proliferation both at G1/S and G2/M phases in *S. pombe* . The differences in mammalian and yeast cells may be attributed to the p34/cdc2 kinase. In mammalian cells, the direct homologue of p34/cdc2, $cdc2^{Hs}$ (also known as cdk1), regulates the transition from G2 to M phase, while G1/S progression is predominantly controlled by cdk2. However, in *S. pombe*, p34/cdc2 regulates both cell cycle transitions (Atherton-Fessler et al., 1993; Moreno et al., 1991). Thus, while the observed cell cycle arrest phenotypes in the yeast may not be identical to that observed in mammalian cells, there are clear similarities of growth arrest phenotypes caused by HIV-1 Vpr expression in yeast and mammalian cells. Specifically, the observation that yeast and mammalians cell both arrest in G_2 indicates that Vpr may interact with conserved target(s) in yeast and mammalian cells. In fact, previous studies have shown that p34/cdc2 kinase was observed to be dramatically inactivated in human and in yeast cells expressing HIV-1 Vpr (He et al., 1995; Re et al., 1995; Zhao et al., 1996). In addition, previous study has indicated that Vpr could not bind directly to p34/cdc2, indicating that Vpr may affect p34/cdc2 kinase activity by its interaction with the regulators of p34/cdc2. Further studies are required to address the possible molecular mechanisms by which Vpr inhibit the p34/cdc2 kinase activity and induce cell cycle arrest in *S.pombe* (see Chapter IV).

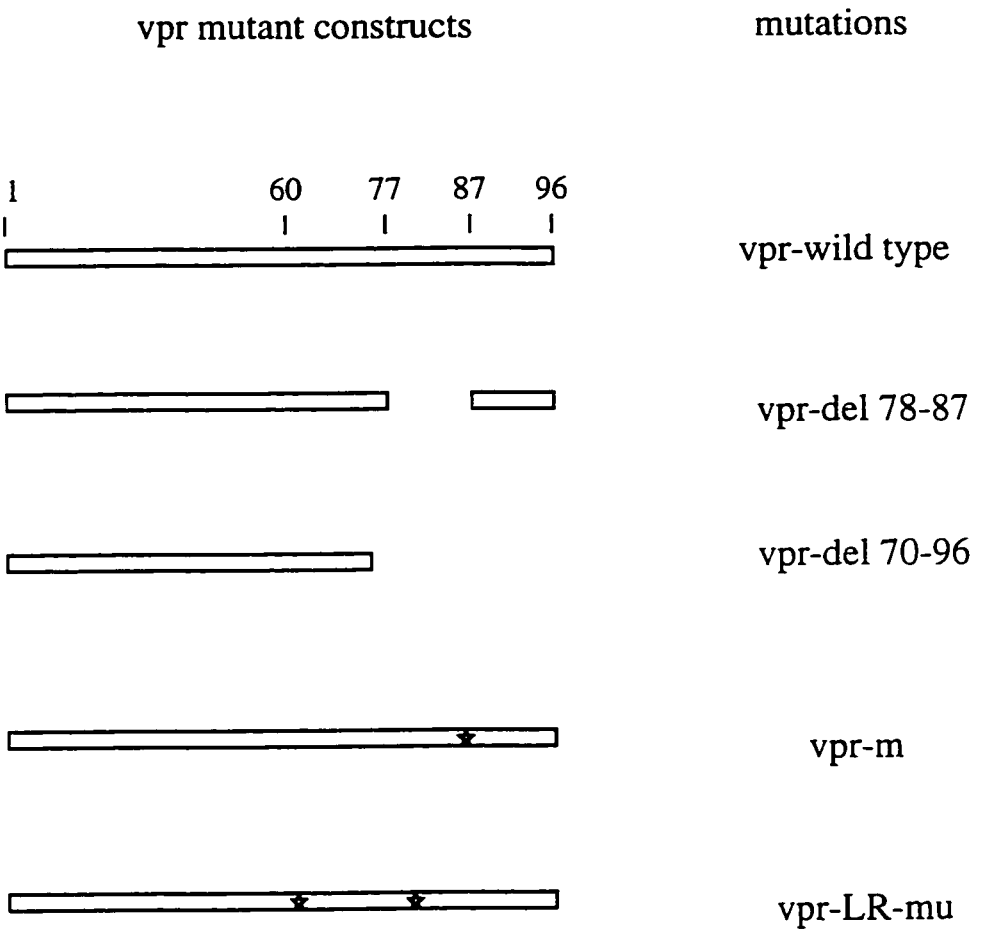


Figure 3.1 Schematic diagram of mutant vpr constructs

vpr-del 78-87: Deletion of amino acid 78 to 87 (10 aa deletion)

vpr-del 70-96: Deletion of amino acid 70 to 96 (26 aa deletion)

vpr-m: Point mutations with ^{87}RRTR to SATS

vpr-LR-mu: Point mutations with
^{60}LIRILQQLLFIHFRI to HARAHQQAAFNHFRN

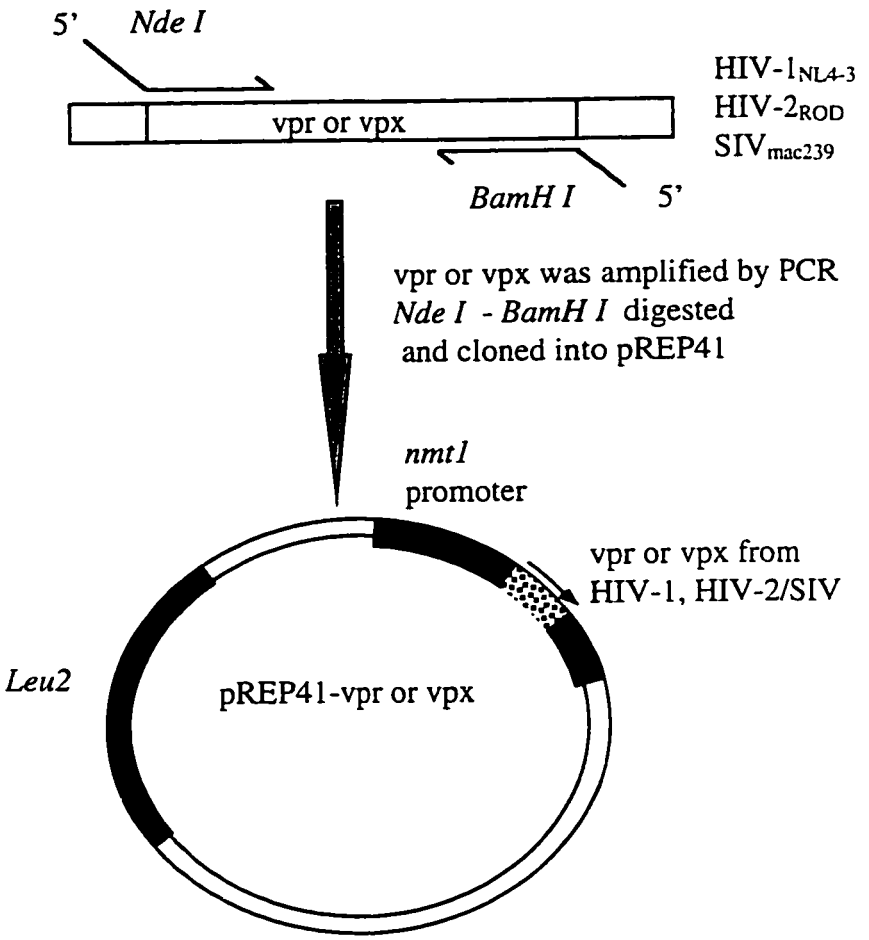


Figure 3.2. Construction of thiamine-conditional plasmid for the expression of Vpr or Vpx in fission yeast.

HIV-1 vpr , HIV-2 and SIV vpx and vpx was amplified by PCR and cloned into pREP41 vector, which carries a thiamine-repressible promoter nmt1. The constructed plasmids were transformed into *S. pombe* 130 (SP130). Vpr or Vpx expression can be induced in the absence of thiamine or stopped in the presence of thiamine.

Figure 3.3. Expression of Vpr or Vpx inhibits cell growth in *S. pombe*.

S. pombe transformed with pREP41 empty vector, pREP41-HIV-1 Vpr, pREP41-SIV Vpr, or pREp41-SIV Vpx were plated onto MM agar with or without thiamime and incubated the plates at 30^0C for 3-6 days. The plates were labeled as follows:

A. Cells grown in the presence of thiamine
1. pREP41-SIV Vpr
2. pREP41 empty vector
3. pREP41-HIV-1 Vpr
4. pREP41-SIV Vpx

B. Cells grown in the absence of thiamine
1. pREP41-SIV Vpr
2. pREP41 empty vector
3. pREP41-HIV-1 Vpr
4. pREP41-SIV Vpx

C. Cells grown in the presence of thiamine
pREP41-SIV Vpr: Normal colony formation

D. Cells grown in the absence of thiamine
pREP41-SIV Vpr: Tiny colony formation

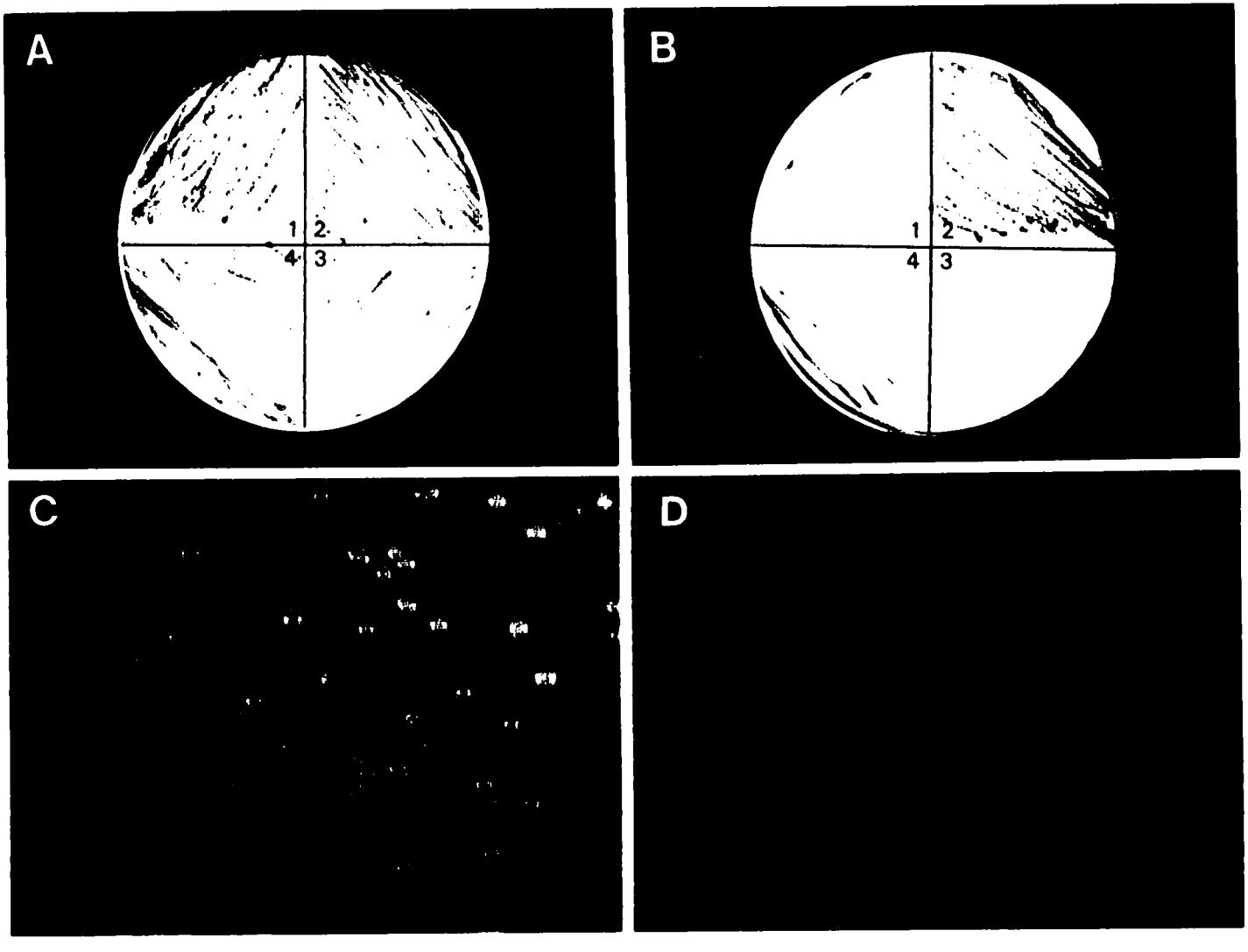

A
1
2
4
3
B
1
2
4
3
C
D

Figure 3.4. Growth kinetics of cells expressing Vpr or Vpx.

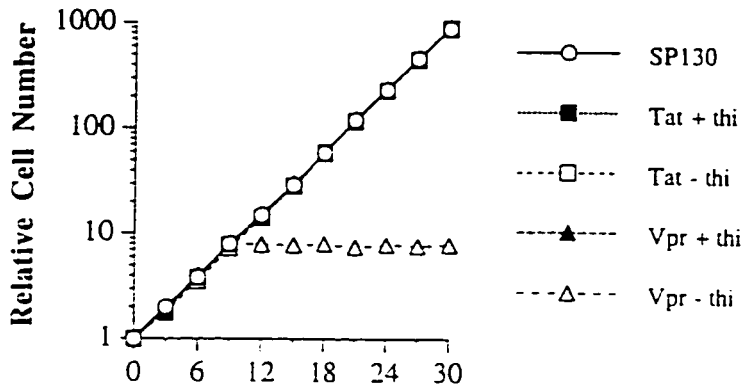
Cells were grown in liquid MM with or without thiamine at at 30^0C. An aliquot of the culture was collected every 3 hr and cell density was determined. The initial cell inoculum was 1x 10^5 cells/ml.

A. Growth kinetics of SP130 cells alone, cells containing the Tat expression plasmid (+/-thiamine), and cells containing the HIV-1 Vpr expression plasmid (+/- thiamine).

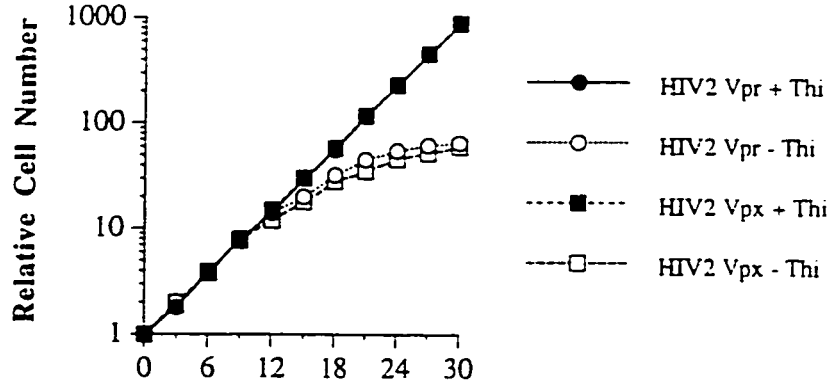
B. Growth kinetics of cells containing HIV-2 Vpr or Vpx expression plasmids (+/- thiamine).

C. Growth kinetics of cells containing the SIV Vpr or Vpx expression plasmids (+/- thiamine).

A.


Relative Cell Number
1000
100
10
1
0
6
12
18
24
30
SP130
Tat + thi
Tat - thi
Vpr + thi
Vpr - thi

B.


Relative Cell Number
1000
100
10
1
0
6
12
18
24
30
HIV2 Vpr + Thi
HIV2 Vpr - Thi
HIV2 Vpx + Thi
HIV2 Vpx - Thi

C.

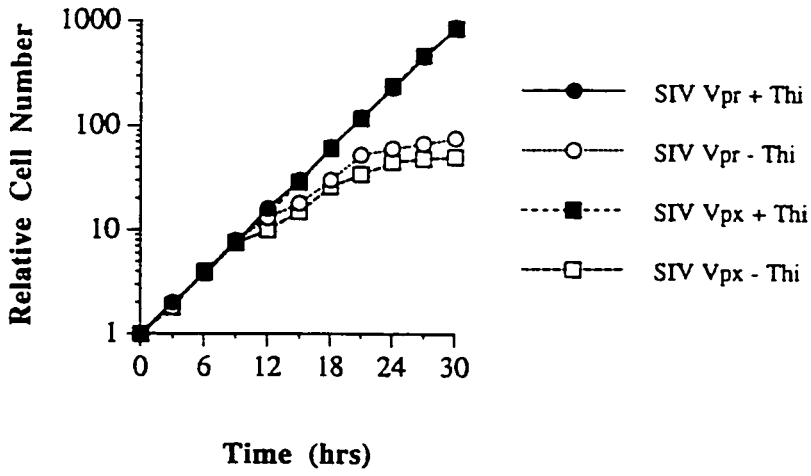

Relative Cell Number
1000
100
10
1
0
6
12
18
24
30
SIV Vpr + Thi
SIV Vpr - Thi
SIV Vpx + Thi
SIV Vpx - Thi
Time (hrs)

Figure 3.5. Western blot analysis of Vpr or Vpx expression in *S. pombe*.

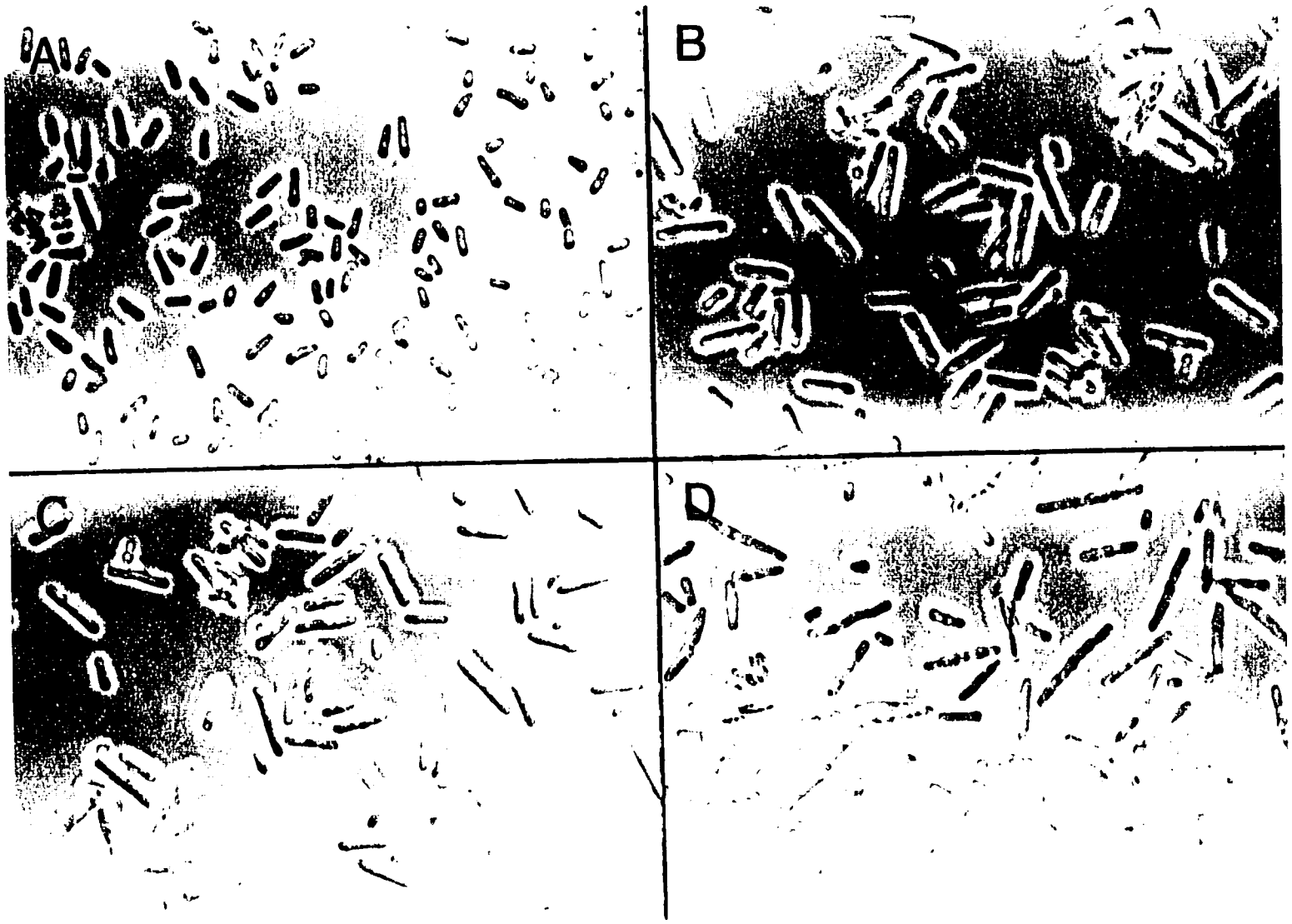
Cells grown in MM after removal of thiamine were harvested at different time points as indicated. Vpr or Vpx expression appeared 9 h after induction. Similar induction kinetics were observed for pREP-HIV-1-Tat transformed *S. pombe* and there is no Vpr, Vpx or Tat protein expression in the presence of thiamine.

Figure 3.6. Morphology of cells expressing Vpr or Vpx.

Exponentially growing Cells grown in MM + thiamine were used to inoculate MM with or without thiamine. Cells were incubated for a further 18 hr at 30^0C, and then collected and fixed for microscopic examination. In addition, an aliquot of each samples was stained with propidium iodide (PI) and examined by fluorescence microscopy. The samples were as follows:

A. pREP41-HIV-1 Vpr + thiamine (x200).
HIV-1 Tat, SIV Vpr and Vpx have an identical phenotypes in the medium containing thiamine (repressing conditions).

B. pREP41-HIV-1 Vpr - thiamine (x200).
C. pREP41-SIV Vpr - thiamine (x200).
D. pREP41-SIV Vpx - thiamine (x200).
E. PI staining: pREP41-HIV-1 Vpr + thiamine (x1000).
F. PI staining: pREP41-HIV-1 Vpr - thiamine (x1000).
G. PI staining: pREP41-SIV Vpr - thiamine (x1000).
H. PI staining: pREP41-SIV Vpx - thiamine (x1000).


A
B
C
D

G
E
H
F

Figure 3.7. SEM analysis of cells expressing Vpr or Vpx.

Exponentially growing Cells grown in MM + thiamine were used to inoculate MM with or without thiamine. Cells were incubated for a further 18 hr at 30^0C, and then collected and fixed for SEM examination as described in the Materials and Methods in Chapter II. The samples were as follows:

A. pREP41-HIV-1 Vpr transformed cells + thiamine (x15,000). HIV-1 Tat, SIV Vpr and Vpx have an identical phenotypes in the medium containing thiamine (repressing conditions).

B. pREP41-HIV-1 Vpr transformed cells - thiamine (x15,000).

C. pREP41-SIV Vpr transformed cells - thiamine (x15,000).

D. pREP41-SIV Vpx transformed cells - thiamine (x15,000).

B
0983 2.0 kV X1.50K 20.0µm

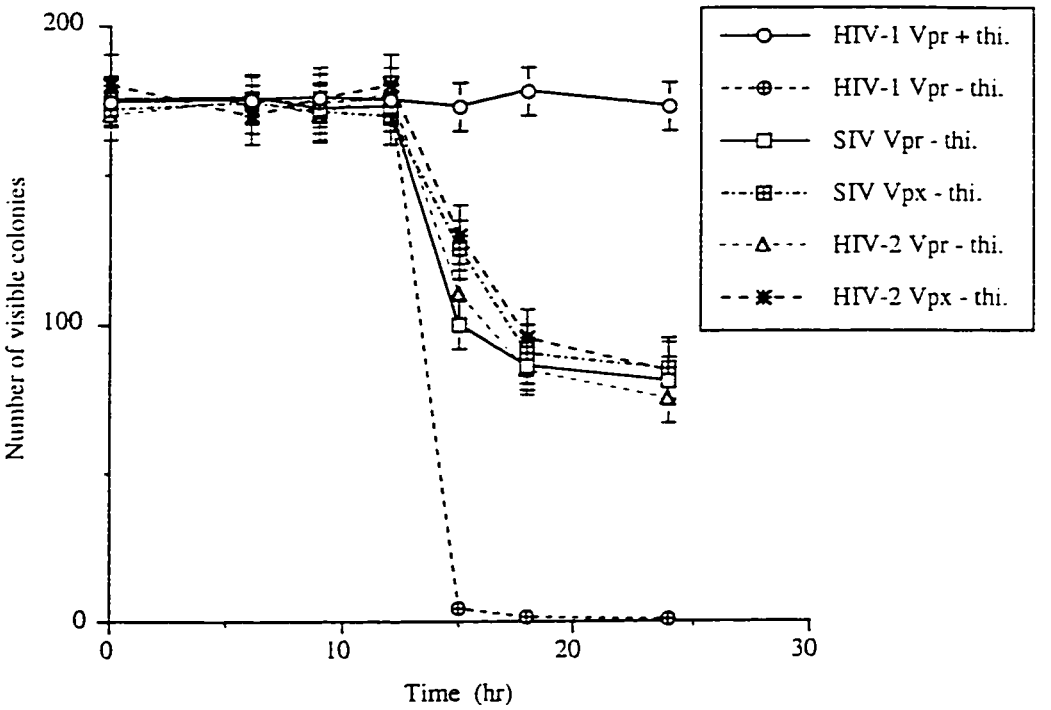


Figure 3.8. Viability of cells expressing Vpr or Vpx

Cells were grown in MM with or without thiamine at 30^0C. An aliquot of cells was collected and approximately 200 cells were plated onto MM agar containing thiamine agar plate. Visible colonies were counted after 3-6 days. The data shown in the graph represented the results obtained from three different experiments (mean ± SD).

Figure 3.9. Flow cytometric analysis of Vpr and Vpx expressing cells.

Cells were grown as indicated below prior to preparation for flow cytometry as described under Materials and Methods. The position of the 1N (G1 DNA content) and 2N (G2 DNA content) peaks are shown by the gray bars. Controls for 1N and 2N peaks are shown in parts A and B. The control for exponentially growing S. pombe (plasmid containing cells grown in the presence of thiamine) is shown in C. Experimental cultures (D-F) were washed with sterile water then placed into thiamine-free MM for 18 hr prior to preparation for flow cytometry.

A. Cells with pREP41-HIV-1 Vpr grown nitrogen limiting medium + thiamine (enriches for G1 cells).
B. Cells with pREP41-HIV-1 Vpr grown in glucose limiting medium + thiamine (enriches for G2 cells).
C. Cells with pREP41-HIV-1 Vpr + thiamine.
D. Cells with pREP41- HIV-1 Vpr - thiamine.
D. Cells with pREP41- SIV Vpr - thiamine.
E. Cells with pREP41- SIV Vpx - thiamine.

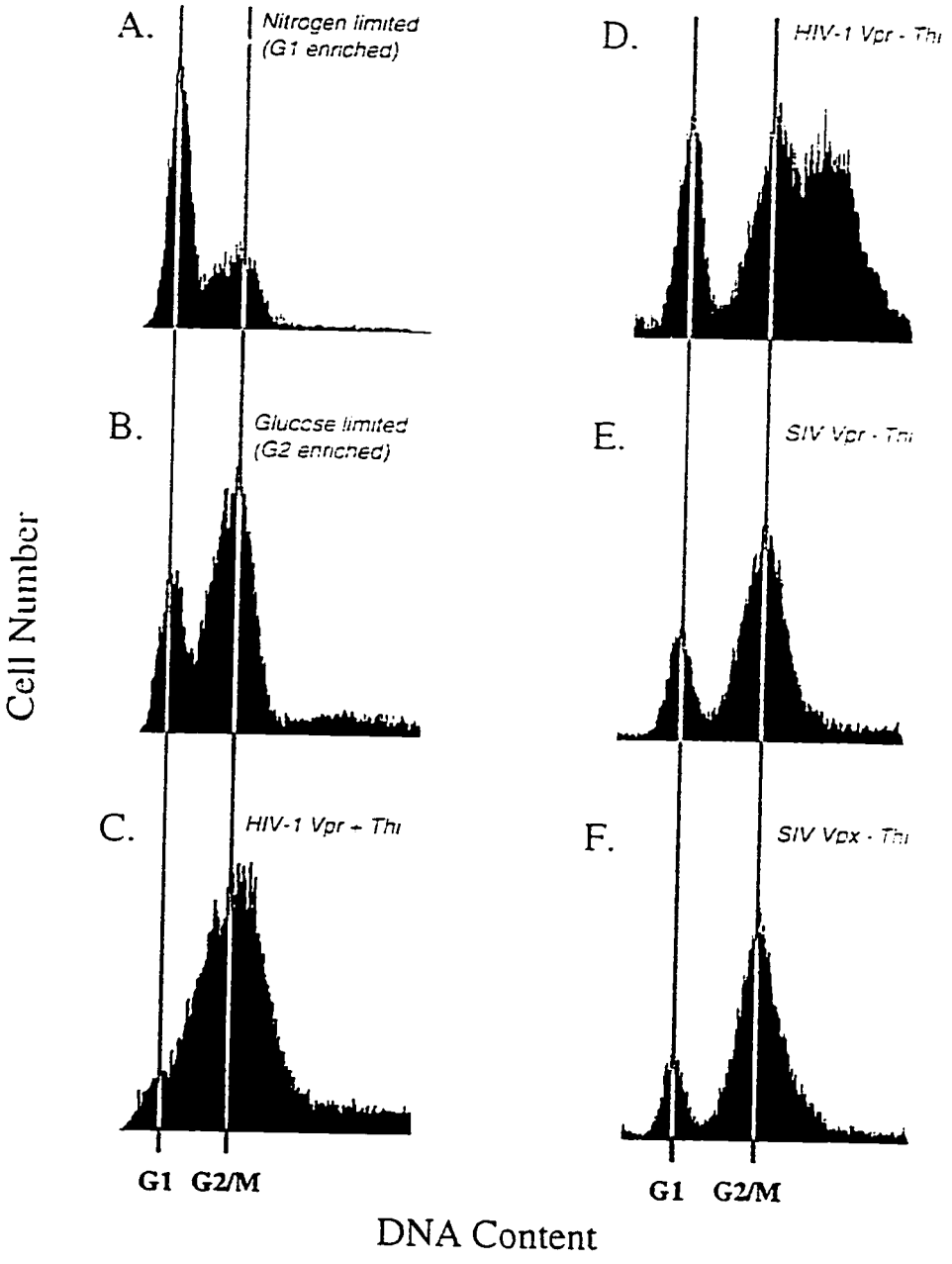

A.
Nitrogen limited
(G1 enriched)
B.
Glucose limited
(G2 enriched)
C.
HIV-1 Vpr + Thi
D.
HIV-1 Vpr - Thi
E.
SIV Vpr - Thi
F.
SIV Vpx - Thi
Cell Number
G1
G2/M
G1
G2/M
DNA Content

Figure 3.10. Effect of Vpr or Vpx expression on cell cycle progression after hydroxyurea synchronization.

Cells were grown in the presence or absence of thiamine and synchronized in S phase with hydroxyurea (HU) as described under Materials and Methods. Cell cycle progression following removal of hydroxyurea was monitored by flow cytometry analysis. As described, the timing of HU removal and shifting to thiamine-free medium were coordinated so that the onset of expression of the Vpr or Vpx proteins (which takes approximately 9 hr) preceded removal of HU by 1 hr. Cells were incubated a further 10 hr following removal of HU.

A. Cells with pREP41-HIV-1 Vpr grown in HU + thiamine. (early S phase arrest).
B. Cells with pREP41-HIV-1 Vpr + thiamine. 10 hr after removal of HU (Control for ability to recover from Hu treatment and resume cell cycle progression).
C. Cells with pREP41-HIV-1 Vpr - thiamine, 10 hr after removal of HU.
D. Cells with pREP41- SIV Vpr - thiamine, 10 hr after removal of HU.
E. Cells with pREP41-SIV Vpx - thiamine, 10 hr after removal of HU.

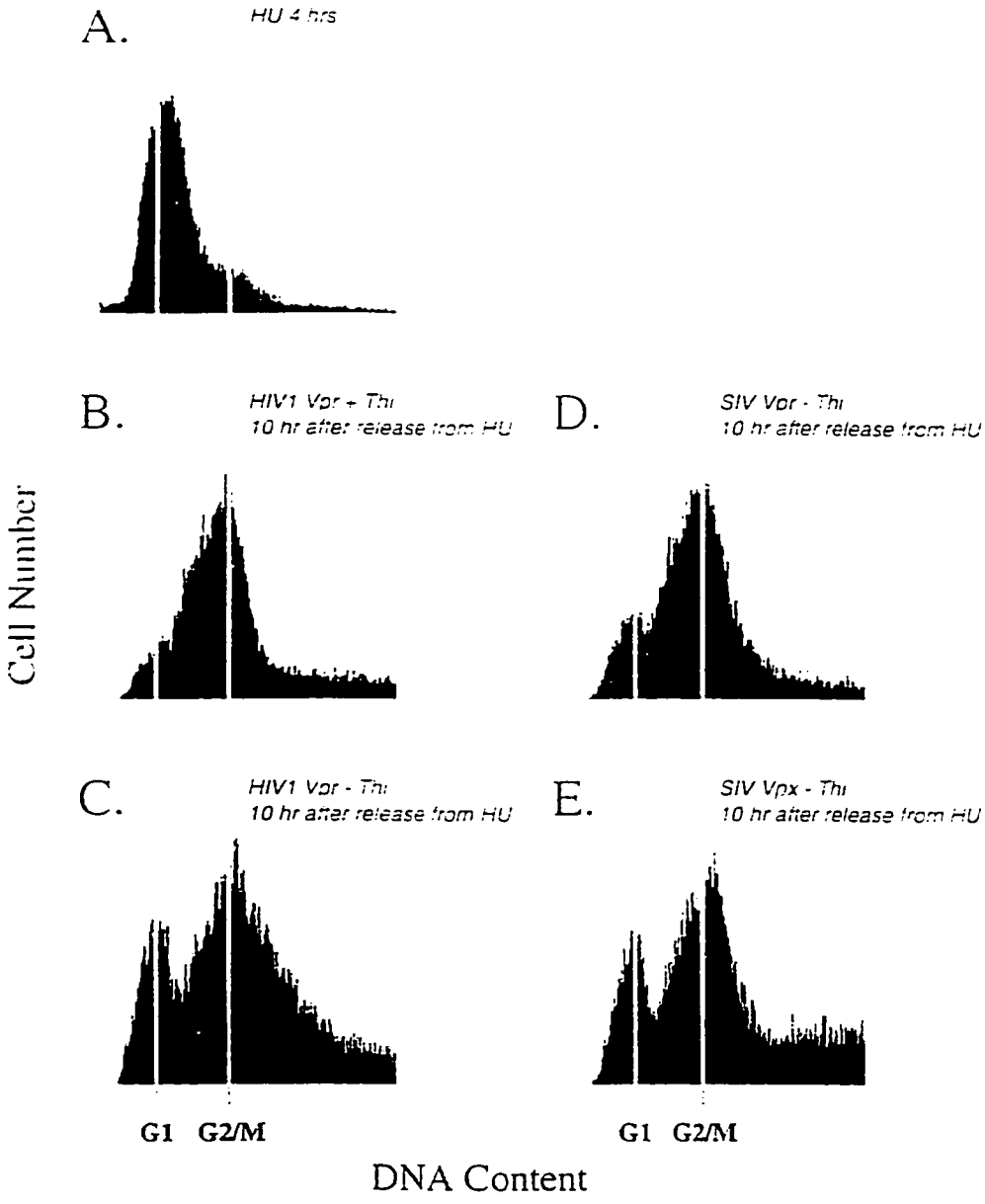

A.
HU 4 hrs
B.
HIV1 Vpr + Thi
10 hr after release from HU
D.
SIV Vpr - Thi
10 hr after release from HU
Cell Number
C.
HIV1 Vpr - Thi
10 hr after release from HU
E.
SIV Vpx - Thi
10 hr after release from HU
G1 G2/M
G1 G2/M
DNA Content

Table 3.1. Primers used for PCR and DNA sequence in this study
(All are listed in the 5' to 3' direction)

HIV-1_{NL4-3} Vpr

5' primer -GAGGACATATGGAACAAGCCCCAGA-

3' primer -CCCGGATCCTAGGATCTACTGGCTCC-

3' primer [vpr-3' truncation (for construction of vpr-del 70-96 mutant)]

-CCCGGATCCAAACAGCAGTTGTTGCAGAAT-

HIV-1_{NL4-3} Tat

5' primer -CTCTCTCATATGGAGCCAGTAGATCCT-

3' primer -AAACGGATCCCTAATCGTACGGATCTGT-

HIV-1 $_{NL4-3}$ Nef

5' primer -CCCAGGACCATATGGGTGGCAAGTGGTCA-

3' primer -CCCAACCGGATCCACACACTACTTGAAGCA- 3'

SIV_{mac239} Vpr

5' primer -CAGAGGACATATGGAAGAAAGACCTCC-

3'primer -CCCGGATCCCATGCTTCTAGAGGGCGG-

SIV_{mac239} Vpx

5' primer -CAGAGGACATATGTCAG ATCCCAGGGA-

3' primer -CCCGGATCCTTATGCTAGTCCTGGAGG-

HIV-2_{ROD} Vpr

5' primer -CTCTCTCATATGGCTGAAGCACCAACA-

3' primer -AAACGGATCCTTATTGCATGTTTCTAGG-

HIV-2_{ROD} Vpx

5' primer -CTCTCTCATATGACAGACCCCAGAGAG-

3' primer -AAACGGATCCTTAGACCAGACCTGGAGG-

Table 3.2 Effects of vpr mutants on cell proliferation of *S. pombe*

vpr constructs	Inhibition of cell growth
pREP41-vpr-wt	+
pREP41-vpr-del 78-87	+
pREP41-vpr-del 70-96	–
pREP41-vpr-m	+
pREP41-vpr-LR-mu	–

" + " indicates that cell growth was inhibited as shown in Figure 3.3 and 3.4.

" - " indicates that cell growth was not affected.

" vpr constructs ": see figure 3.1 for more detail information.

Chapter IV

Studies of the molecular mechanisms by which HIV-1 Vpr inhibits cell growth and arrests cell cycle in *Schizosaccharomyces pombe*

(Some of the data presented in this Chapter have been published in an article entitled " Cell cycle inhibitory effects of HIV and SIV Vpr and Vpx in the Yeast *Schizosaccharomyces pombe* " by Chengsheng Zhang, Colin Rasmussen, and Lung-Ji Chang in Virology 230: 103-112, 1997).

A. Introduction and rationale

While HIV-1 Vpr has been demonstrated to inhibit cell growth and arrest cell cycle progression at G2/M phase in mammalian cells, the molecular mechanisms by which Vpr induces cell cycle arrest remain unknown. Having demonstrated that HIV-1 Vpr could induce similar cellular changes including cell cycle arrest at G2/M phase in *S. pombe*, we reasoned that the conditional protein expression system in *S. pombe* could serve as an excellent model to study the effects of Vpr on cell cycle progression and the possible molecular mechanisms involved.

Cell cycle progression is tightly regulated by a variety of cyclin-dependent kinases in eukaryotic cells (Grana and Reddy, 1995). p34/cdc2, one of the highly conserved protein kinases in eukaryotic cells, is essential for the cell cycle transitions from G1 to S and G2 to M phases in *S. pombe* (Moreno et al., 1989). In addition, p34/cdc2 kinase activity is apparently suppressed in mammalian cells expressing HIV-1Vpr (He et al., 1995; Re et al., 1995). Therefore, we reasoned that HIV-1 Vpr may also arrest cell cycle via the suppression of p34/cdc2 kinase activity in *S. pombe*.

On the other hand, the kinase activity of p34/cdc2 has been shown to be positively regulated by the protein phosphatase Cdc25 which can dephosphorylate and activate p34/cdc2, and be negatively regulated by protein kinases Wee1 and Mik1 which phosphorylate and inactivate p34/cdc2 (Figure 4.1). We thought that Vpr may affect p34/cdc2 kinase activity by interacting with these positive or negative regulators of p34/cdc2 kinase. To test whether Vpr acts directly on these p34/cdc2 regulators, three p34/cdc2 mutants, which fail to respond to either Cdc25, Wee1, or Mik1, respectively, have been transformed with Vpr and the cell cycle progression has been examined thereafter.

In addition, previous studies have shown that Vpr-induced cell cycle arrest could be relieved by the treatment of the Vpr-expressing cells with a protein phosphatase inhibitor okadaic acid, indicating that protein phosphatases, especially protein phosphatase type 2A (PP2A), may play a role in Vpr-induced cell cycle arrest (Re et al., 1995). In fact, increasing evidence has suggested that PP2A may play an important role in the regulation of cell cycle progression and cytokinesis in eukaryotic cells (Mayer-Jaekel and Hemmings, 1994; Yanagida et al., 1992; Wera et al, 1995; Figure 4.1). In the fission yeast *S. pombe*, knock-out of PP2A function resulted in the premature entry into mitosis with decreased cell length and hypersensitivity to okadaic acid treatment; whereas over-expression of PP2A induced cell cycle arrest with increased cell length (Kinoshita et al., 1990; Kinoshita et al., 1993). In addition, HIV-1 Vpr also induced cell cycle arrest with increased cell length (Figure 3.6 and 3.7 in Chapter III), which is similar to the phenotype induced by PP2A over-expression mentioned above. Interestingly, a recent study has showed that NCp7 (one of the HIV-1 nuclear capsid proteins) can potently activate PP2A activity *in vitro* (Tung et al., 1997). In addition, this study has also shown that HIV-1 Vpr could physically bind to NCp7 and form a stable complex, and this complex is a more

potent activator of PP2A than NCp7 alone. Therefore, we hypothesized that NCp7 may affect cell cycle progression in *S. pombe* if PP2A plays a role in Vpr-induced cell cycle arrest as suggested previously. To address this issue, we have cloned HIV-1 NCp7 into the pREP41 expression vector and examined the possible effects of NCp7 on cell growth using the same approaches as we used on the study of Vpr in Chapter III.

Since HIV-1 Vpr can completely inhibit colony formation of wild type *S. pombe*, theoretically it is possible to rescue this phenotype by co-expression of a Vpr-suppresser in the cells expressing Vpr. In this study, we have attempted to take advantage of some classical genetic approaches described in the Materials and Methods to identify Vpr-suppressers in *S. pombe*. Generally, spontaneous mutations in *S. pombe* occurs with a low frequency. A number of mutagenic agents including chemical and physical mutagens have been used to increase the frequency of mutation (Moreno et al., 1991). We have employed a powerful and popular approach by treatment of the yeast cells with the chemical mutagen ethylmethanesulfonate (EMS) to generate mutant strains. EMS is less harmful to humans than most other chemical mutagens such as N-methyl-N'-nitro-N-nitrosoguanidine (NTG) (Moreno et al., 1991). It preferentially induces point mutations from GC to AT (Sega, 1984). In addition, the number of mutations induced per surviving cell is high, making EMS very efficient at inducing genomic alterations (Moreno et al., 1991). We have generated some Vpr-resistant mutant strains by EMS mutagenesis. Subsequently, we have constructed and screened the genomic DNA libraries from mutant or wild type yeast cells to identify Vpr-interacting proteins.

B. Materials and methods

Plasmid construction :

To construct NCp7 expression vector, we amplified the NCp7 gene from HIV-1_{NL4-3} by PCR using the following primers: 5' -ATGCATATGATACAGAAAGGCAA-TTTTAGG -3' (5' Primer) and 5' -TCCGGATCCTTAAAGAAAATTCCCTGG-CCTTCC -3' (3' Primer). The PCR fragments were subcloned into pBluescript-KS^- by blunt end ligation. The NCp7 insert was then excised by digestion with NdeI and BamHI and subcloned into pREP41 expression vector. The cloned insert in pREP41 was sequenced to ensure the correct sequence and reading frame.

Yeast strains, culture and transformation of *S. pombe* :

The yeast strains used in this study included wild type strains SP130 (h^-, ade6-210, leu1-32) and Q360 (h^+, ada6-216, ura4D18), and three p34/cdc2 mutant strains. one of the mutants is cdc2-3w (h^- , leu1-32) which fails to respond to Cdc25; the second mutant is cdc2-1w (h^-, leu1-32) which fails to respond to Wee1; and the third one is cdc2-1w, Δmik1(h^-, leu1-32) which fails to respond to Wee1 and Mik1 (Rasmussen and Rasmussen, 1994). The culture and transformation of the yeast cells were performed as described in Chapter III.

p34/cdc2 histone H1 kinase assay

This assay is based on the finding that p13 (also referred to as suc1) can specifically bind to p34/cdc2 protein kinase complex from various sources, including *S. pombe*, Xenopus, and HeLa cells (Booher et al., 1989; Brizuela et al; 1987; Brizuela et al., 1989; Dunphy et al., 1988; Rasmussen and Rasmussen; 1994). In this study, p13 which was coated on the plate can bind p34/cdc2 in the protein extract, thus the H1 kinase activity measured by this assay mainly represents the kinase activity of

p34/cdc2. The yeast protein extract was made as described above. Briefly, the cells were lysed using glass beads in a lysis buffer composed of 25 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 15 mM EGTA, 0.1% Trixon X-100, 0.1 mM NaF, 60 mM ß-glcycero-phosphate, 15 mM p-nitrophenylphosphate, 0.1 mM Na orthovanadate, 0.1 mM PMSF, 1 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, 1 µg/ml aprotinin, 10 µg/ml TPCK. For H1 histone kinase assay, the assay plates (Immulon-2, Fisher Scientific) were coated with a solution of 5 µg p13 (p13 is a yeast protein which can specifically bind to p34/cdc2 kinase. Rasmussen and Rasmussen, 1994) in 100 µl, 50 mM sodium carbonate buffer, pH 9.6, and then incubated at room temperature overnight. The wells were washed once with PBS, then blocked with PBS containing 3% BSA for 1 hr at room temperature. To prepare samples for assay, 200 µg of total protein was mixed with an equal volume of PBS containing 1% BSA. The final volume was approximately 100-250 µl. The assay samples were added to the p13-coated microwells and incubated at room temperature with slow constant shaking for at least 5 hr. The wells were washed three times with PBS + 0.2% Trixon X-100, then washed once with kinase assay buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, plus protease and phosphatase inhibitors as in the extraction buffer). To start the assay, 100 µl of assay buffer containing 8.3 µg histone H1, 10 µM ATP, 2.5 µCi [γ-^{32}p]ATP was added to each well and incubated for 30 min at 30^{0}C. The reaction was stopped by adding 35 µl 2 x SDS-PAGE sample buffer. Fifteen microliters were spotted onto 3MM paper, incubated for 10 min in the buffer consisting of 10% TCA and 40 mM sodium pyrophosphate (NaPPi), and then washed three times with 5% TCA, followed by a brief rinse in 95% ethanol. The filters were air dried, and quantified by liquid scintillation counter (LSC).

Ethylmethanesulfonate (EMS) mutagenesis

Ethylmethanesulfonate (EMS) mutagenesis was performed as described elsewhere (Alfa et al., 1993). Briefly, SP130 yeast cells carrying pREP41-HIV-1 Vpr plasmid were grown in culture medium lacking leucine, and in the presence of thiamine at 30^0C overnight to a cell density at 6×10^5/ml (exponential growth cells). To do EMS mutagenesis, the cells were washed with sterile water and cell density was adjusted to 1×10^8/ml. One ml aliquots of the cell suspension were transferred to two 15 ml-sterile tubes. One sample was used for EMS mutagenesis and the other used for a control. EMS (Sigma, final concentration 2%, v/v) was added to one of the tubes. The cells were incubated with a slow, constant shaking for 3 hr at room temperature. After incubation, the cells were washed with sterile water to get rid of EMS. The cells with or without EMS treatment were then plated on thiamine-minus culture plates (under a culture condition allowing Vpr expression) and incubated at 30^0C for 4-6 days.

Western blot analysis of Vpr expression

Western blot analysis of Vpr expression was carried out as described in Chapter III.

Screening of Vpr- resistant mutant yeast strains

To identify Vpr-resistant mutant yeast strains after EMS mutagenesis, an immuno-logical screening method was used as described previously (Tomlinson et al., 1992) with some modifications. In brief, the yeast colonies were transferred to a nitrocellulose filter and soaked in 67 mM potassium phosphate buffer (pH 7.5) containing 20 mM EDTA and 20 mM DTT for 5 min at room temperature. The filter was treated with 500 unites/ml of lyticase (Sigma) in 67 mM potassium phosphate buffer (pH 7.5) at 37^0C for 30 min and soaked the filter in 5% SDS for 30 min at 37^0C. The filter was rinsed with 100 mM Tris-HCl (pH 8.0), plus 2 mM $MgCl_2$,

followed by the treatment with DNase I (BRL)in 100 mM Tris-HCl, and 2 mM $MgCl_2$. The filter was incubated with anti-HIV-1 Vpr antibody and subsequently the HRP-conjugated second antibody, and the signal was detected by ECL Western blotting system as described in the section of Western blot analysis.

Diploid construction

To determine the phenotype of the Vpr-resistant mutant yeast strains, a diploid was constructed as described previously (Alfa et al., 1993). Briefly, two haploid yeast strains with opposite sex, such as SP130 (h^-) and Q360 (h^+) were streaked on MM culture plate plus uracil, adenine, and leucine, respectively, and the plates were incubated at 30^0C overnight. A mating mix was set up between SP130 and Q360 in MM medium. A loopful of the mating mix was streaked on an MM plate without adenine, but with phloxine B (a vital stain, also called Magdala Red, Sigma, final concentration 20 μg/ml) and incubated at 30^0C for 4-6 days. For the construction of a stable diploid, a red colony was picked up and restreaked on a new MM plate with phloxine B, and incubated at 30^0C for 4-6 days. The red colony had been restreaked for several passages until all colonies on the plate were in bright red color. The diploid strains have also been constructed from Vpr-resistant mutant cells (h^-) and Q360 (h^+) in this study.

Preparation of yeast genomic DNA

The yeast genomic DNA was prepared as described (Alfa et al., 1993). Wild type SP130 or Vpr-resistant mutant strains were cultured in MM as described above. The cells were grown in 50 ml of MM plus leucine, adenine, and uracil at 30^0C overnight to a density at 5×10^7/ml. The cells were collected by centrifugation at 3000 rpm for 5 min at room temperature and washed with 50 ml of SP1 buffer (pH 5.6) composed of 1.2 M D-sorbitol (Sigma), 50 mM sodium citrate, 50 mM sodium phosphate

($Na_2HPO_4.7H_2O$), and 40 mM EDTA. The cell pellet was resuspended in 10 ml SP2 buffer (1.2 M D-sorbitol, 50 mM sodium citrate, 50 mM sodium phosphate $Na_2HPO_4.7H_2O$) containing 20 mg/ml of Novozym 234 (Sigma) and incubated in a 37^{0}C water bath for 60 min. The digestion of yeast cell wall was monitored by observing under a microscope. The digested cells were collected, and resuspended in 7.5 ml of 5 x TE buffer (50 mM Tris-HCl, pH 7.5, and 5 mM EDTA) and 0.75 ml of 10% SDS. The lysed sample was incubated with 2.5 ml of 5 M potassium acetate for 30 min on ice. The cell lysate was centrifuged at 5000 rpm for 15 min at 4^{0}C and, the supernatant was transferred to a new tube and incubated with 10 ml ice-cold isopropanol for 5 min on ice. The pellet was resuspended in 3 ml of 5 x TE containing 20 µg/ml RNaseA and incubated at 37^{0}C for 60 min, and then 0.1 ml of 10% SDS and 50 µg /ml proteinase K were added to the sample and incubated at 50^{0}C for 30 min. An equal volume of phenol was added to the sample and mixed gently. The genomic DNA was precipitated by adding 1/10 volume of 5 M NaCl and 2.5 volumes of 100% ethanol. The DNA pellet was washed with 70% ethanol and air-dried, and resupended in 200 µl sterile double distilled water.

Construction of genomic DNA library

The yeast genomic DNA from wild type SP130 and the Vpr-resistant strains were prepared, respectively as described above. The genomic DNA libraries were constructed as described elsewhere (Beach et al., 1982; Write et al., 1986) with some modifications. In brief, the genomic DNA was partially digested with Asa1 to generate 2 - 3 Kb of DNA fragments. The partially cut DNA inserts were cleaned and ligated into the yeast genomic library expression vector pCA-1. To amplify the genomic DNA library, the ligation mix was transformed into DH5α by electroporation as described previously (Sambrook et al., 1989). Fourteen colonies from the transformation plates were picked up randomly and grown in liquid culture. The

plasmid DNA from the fourteen samples were isolated and digested with BamH1 to check the genomic DNA insert. The genomic DNA library was amplified and purified by large-scale preparation of DNA with cesium chloride gradient centrifugation (Sambrook et al., 1989).

C. Results

Suppression of p34/cdc2 kinase activity in yeast expressing HIV-1 Vpr, SIV Vpr or Vpx

The primary regulators of G1/S and G2/M progression in eukaryotic cells are the cyclin-dependent kinases (cdks) of which p34/cdc2 of *S. pombe* is the prototype (Broek et al., 1991; Nurse, 1994). In mammalian cells this enzyme is apparently inactivated by the expression of HIV-1 Vpr (He et al., 1995; Re et al., 1995). Since p34/cdc2 is required for both the G1/S and G2/M transitions in *S. pombe*, we asked whether p34/cdc2 kinase activity was inhibited by either Vpr or Vpx expression. To test this, histone H1 kinase assays were carried out to measure the p34/cdc2 kinase activity in control cells and cells expressing HIV-1 Vpr or SIV Vpr or Vpx as described under materials and methods. It was found that the expression of HIV-1 Vpr or SIV Vpr or Vpx caused similar decreases in p34/cdc2 kinase activity (Figure 4.2). In this study, we have not measured the p34/cdc2 protein present in the kinase reactions. It is possible that the decreased p34/cdc2 kinase activity was resulted from the decreased binding of the protein to the wells. However, other studies in which the p34/cdc2 protein have been measured by Western blot and demonstrated that Vpr expression did not apparently affect the binding of p34/cdc2 to p13 or other anti-p34/cdc2 antibodies (He et al., 1995; Re et al., 1995; Zhao et al., 1996). Therefore, the decreased p34/cdc2 kinase activity detected in this study is unlikely to be caused by the decreased binding of p34/cdc2 to the wells. Nevertheless, it would be very informative if we had confirmed the presence of p34/cdc2 protein when we measured its kinase activity. These results suggest that the cell cycle arrest observed in *S. pombe* may be associated with a loss of p34/cdc2 kinase activity.

Three p34/cdc2 mutant yeast strains are sensitive to Vpr-induced inhibition of cell proliferation

The activity of p34/cdc2 in S. *pombe* is positively regulated by the protein phosphatase, Cdc25, and negatively regulated by the protein kinases of Wee1 and Mik1 (Figure 4.1). Since we observed a dramatic decrease in p34/cdc2 kinase activity in cells expressing Vpr or Vpx, we wondered whether mutants of p34/cdc2 which fail to respond correctly to either Cdc25 or Wee1/Mik1 pathways might be resistant to HIV-1 Vpr induced cell cycle arrest. To test this hypothesis, the HIV-1 Vpr expressing plasmid was transformed into three p34/cdc2 mutant strains, one (cdc2-3w) which fails to respond to Cdc25, and one (cdc2-1w) which fails to respond to Wee1, and the third (cdc2-1w, Δmik1) which fails to respond to Wee1 and Mik1. The results showed that these three mutants, like wild type strain SP130 with a normal cdc2 gene, were growth arrested by HIV-1 Vpr expression (Figure 4.3). Similar results were also observed by transformation of these cdc2 mutants with HIV-2 and SIV Vpr or Vpx, indicating that Vpr or Vpx does not act directly through any of these cdc2 regulators.

HIV-1 NCp7 could slow down the cell cycle progression in *S. pombe*

Previous studies have suggested that PP2A may play a role in Vpr-induced cell cycle arrest (Re et al., 1995). In additoin, a recent study has showed that NCp7 can potently activate PP2A activity *in vitro* (Tung et al., 1997). Therefore, we hypothesized that NCp7 may affect cell cycle progression in *S. pombe* if PP2A plays a role in Vpr-induced cell cycle arrest as suggested previously. To determine whether expression of HIV-1 NCp7 affects cell proliferation in *S. pombe*, yeast cells were transformed with the expression plasmid pREP41 containing the NCp7, and grown on MM medium with or without thiamine. When grown on MM + thiamine (which prevents NCp7 expression), normal size yeast colonies were formed, whereas there

were smaller colonies formed from cells expressing NCp7 on plates lacking thiamine. This data indicated that NCp7 could affect cell proliferation in *S. pombe*.

Next, the effects of NCp7 on cell proliferation kinetics were examined. As described in Chapter III, exponentially growing cells cultured in MM + thiamine were washed in sterile water and inoculated into fresh MM +/- thiamine at a density of 1 x 10^5 cells/ml. Cell numbers were then determined at various time points. As mentioned previously, the normal generation time of SP130 cells at 30^0C in MM is about 3 hr. The generation time of HIV-1 Vpr-, and NCp7-conditionally expressing cells grown in the presence of thiamine (no protein expression) was similar to that of the wild type cells as expected (Figure 4.4). However, in the absence of thiamine, expression of HIV-1 Vpr consistently caused a complete arrest of cell proliferation within 9 -10 hr (Figure 3.4 and Figure 4.4). In contrast, cells expressing HIV-1 NCp7 showed a reduced rate of proliferation ($P< 0.05$, t-test) starting at 18 hr (Figure 4.4) and the cells continued to proliferate at a slower rate up to 24 hr after induction. In addition, we have also examined the viability of the cells expressing NCp7 as described for HIV-1 Vpr in Chapter III. However, the expression of NCp7 did not apparently affect the cell viability. We have not confirmed the NCp7 expression by Western blot analysis because we do not have the anti-NCp7 antibody.

Generation of Vpr-resistant mutant yeast strains by EMS mutagenesis

To identify Vpr-suppressers in *S. pombe*, we have carried out EMS-induced mutagenesis to generate Vpr-resistant mutant yeast strains as described in the Materials and Methods. The wild type SP130 cells carrying pREP41-HIV-1 Vpr were treated with EMS and plated on the culture plates without thiamine (under a condition allowing Vpr expression). Since there was no colony formation when SP130 yeast cells expressing HIV-Vpr were plated on MM plate without thiamine

(Figure 3.3), only Vpr-resistant cells could survive and form colonies under the culture conditions inducing Vpr expression. We have obtained about 1000 " Vpr-resistant " colonies by EMS mutagenesis. To confirm whether HIV-1 Vpr was expressed in these colonies, we used an immunological method to screen Vpr-positive colonies (Figure 4.5). Nine of the colonies (about 0.9%) were found to be Vpr-positive by the first round of screening. To eliminate the possible false positive colonies, cell cultures from each of the nine colonies were set up and protein extracts were prepared, and Western blot analysis of Vpr expression was performed as described above. Five of the nine cultures remained Vpr-positive by western blot analysis (Figure 4.6). The four Vpr-negative colonies were not further studied. Interestingly, two of the positive samples had a "larger Vpr " than the wild type Vpr (Lane 1 and 2 in Figure 4.6). To further confirm the nature of Vpr-resistance of the five Vpr-resistant colonies, cells from the five positive colonies were cultured in medium with leucine to eliminate pREP41-HIV-1 Vpr plasmids (i.e., grow the transformed cells in the medium without selection which will result in the elimination of the plasmids inside the cells). After elimination of the plasmids from the cells, there was no colony formation when these cells were plated onto the plate without leucine, whereas there was normal colony formation when the same cells were plated onto the plates in the presence of leucine, confirming the pREP41-HIV-1 plasmids were completely eliminated from these cells. Having confirmed that there were no plasmids remaining inside these Vpr-resistant cells, we re-transformed pREP41-HIV-1 Vpr plasmids into the five cell cultures, respectively. We found that two of the five mutant strains were shown to be resistant to Vpr expression based on the assays of colony formation and Western blot analysis, whereas the other three were sensitive to Vpr-induced cell cycle arrest, suggesting some of the mutations induced by EMS may occur in the plasmid DNA, not in the host genome. Therefore, we have generated two

mutant yeast strains which have been shown to be consistently resistant to Vpr-induced cell cycle arrest.

Vpr-resistant mutants carry recessive versus dominant mutation

To further characterize the two newly generated Vpr-resistant yeast strains, and to determine whether these Vpr-resistant mutant strains carry dominant or recessive mutations, diploid cells were constructed from the mutant yeast cells and Q360 cells as described in the Materials and Methods. *S. pombe* is naturally a haploid organism, and diploid zygotes formed from the conjugation of two haploids normally enter meiosis immediately and produce four haploid spores . To construct a stable diploid strain, two haploid strains containing a variety of ade6 mutant alleles have been crossed. One haploid strain is wild type Q360, which is h^+, and contains mutant alleles of ade6-216 and leu1-32; another is Vpr-resistant strain, which is h^-, and carries mutant alleles of ade6-210 and leu1-32. Both Q360 and Vpr-resistant strains could not grow in the absence of adenine. After mating, however, interallelic complementation can occur between one pair of ade6 alleles, ade6-216 and ade6-210. The heterozygous diploid can grow in the absence of adenine and form diploid clones. Other allele combinations do not complement and these diploids require adenine for growth. We have successfully constructed two stable diploid strains from Q360 and two Vpr-resistant strains using interallelic complementation (Figure 4.7). In addition, as a control study, we have also constructed a diploid strain from Q360 and wild type SP130. All of these diploid strains have been shown to be adenine prototrophs and able to sporulate as observed by microscopic examination. In addition, there are no obvious difference in morphology between the parental and diploid cells.

Next, the phenotypes of these diploids were tested by transformation of the diploids with the pREP41-HIV-1 Vpr plasmids. The transformation and cell culture of yeast cells were carried out as described above. While there was normal colony formation from the parental mutant cells expressing Vpr, there was no colony formation from the diploid cells expressing Vpr, indicating that the parental Vpr-resistant mutant yeasts carry recessive mutations versus dominant mutations.

Construction of genomic DNA library

To construct a genomic library, the yeast genomic DNA from wild type SP130 and the Vpr-resistant strains were prepared, respectively, as described in Materials and Methods. The genomic DNA were partially digested with Asa1 to generate 2 - 3 Kb of DNA fragments (Figure 4.8). The partially cut DNA inserts were cleaned and ligated into the yeast genomic library expression vector pCA-1 (Figure 4.9). The ligation mix was then transformed into DH5α by electroporation. Fourteen colonies from the transformation plates were picked up randomly and grown in liquid culture. The plasmid DNA from the fourteen samples were isolated and digested with BamH1. The result of the restriction enzymatic digestion mapping showed that the cloned genomic DNA inserts were approximately within 2-3 Kb (Figure 4.10). The genomic DNA library was amplified and purified by large-scale preparation of DNA with cesium chloride gradient centrifugation (Sambrook et al., 1989). We have constructed three sets of genomic DNA libraries in this study, two of them were from the Vpr-resistant mutants, respectively, another was from the wild type SP130.

Screening of the genomic DNA library

We have hypothesized that Vpr may arrest cell cycle by interacting with cellular factors and subsequently disrupts the normal cellular machinery responsible for the cell cycle progression. In attempt to identify the potential Vpr-interacting proteins,

we have screened the genomic DNA libraries described above. Since the Vpr-resistant mutant strains have recessive mutations, we may be unable to identify the Vpr-suppressers by screening the DNA library made from the Vpr-resistant mutant cells. However, since the cloning vector used for the construction of the genomic DNA library is a multi-copy expressing vector, it is possible that the recessive suppresser may overcome the effect of its wild type counterpart and rescue the Vpr-induced phenotype. Nevertheless, we also screened the genomic DNA library made from wild type yeast cells.

Firstly, we have screened the genomic DNA library made from the mutant cells since the procedure used in this screening was much simpler than the later one used for wild type genomic DNA library (see below). Briefly, wild type SP130 were firstly transformed with pREP41-HIV-1 Vpr plasmids and the transformants were subsequently transformed with the genomic DNA library made from the mutant cells. The transformants bearing two kinds of plasmids were plated onto MM plates lacking leucine and uracil in the absence of thiamine, and incubated at 30^0C for 4-6 days (Figure 4.11). The transformant can grow and form a colony if a Vpr-suppresser is expressed from the transformed mutant gene. We have repeated this screening experiments five times. However, we have not been able to identify any Vpr-suppresser by this approach.

To screen the DNA library constructed from wild type yeast cells, the Vpr-resistant mutant yeast cells were cultured as described in the Materials and Methods. The mutant cells were sequentially co-transformed with Vpr-expressing plasmid pREP41-HIV-1 Vpr and the genomic DNA library from wild type yeast cells (Figure 4. 12). The mutant cells were first transformed with pREP41-HIV-1 Vpr and the transformants were grown in MM medium lacking leucine in the presence of

thiamine. The cells carrying pREP41-HIV-1 Vpr plasmids were then transformed with the genomic DNA library made from wild type cells. The transformants were grown and selected on MM plates lacking leucine and uracil in the presence of thiamine, and incubated at 30^0C for 4-6 days. Theoretically, all the transformants should form colonies under these culture conditions because there was no Vpr expression. Next, a replica plating was prepared from the master plate, and the cells were transferred onto a MM plate lacking leucine and uracil in the absence of thiamine. The transferred colony is unable to grow on the MM plate without thiamine if it contains a wild type gene product which can overcome its mutant counterpart. We have also repeated this sequential transformation five times. However, we have not found any "positive" colony either by this study.

D. Summary of results

1. p34/cdc2 kinase activity was apparently suppressed in yeast cells expressing HIV-1 Vpr, HIV-2 Vpr or Vpx, and SIV Vpr or Vpx, indicating that HIV-1 Vpr may induce cell cycle arrest through p34/cdc2 pathway.

2. We have shown that three p34/cdc2 mutants (cdc2-3w, cdc2-1w and cdc2-1w, Δmik1) which fail to respond either to Cdc25, or Wee1/Mik1, were susceptible to Vpr-induced cell cycle arrest, suggesting that Vpr might not act directly on the Cdc25, Wee1 or Mik1 but may act on other regulator (s) of p34/cdc2 kinase.

3. We have demonstrated that HIV-1 NCp7 was able to slow down cell growth in *S. pombe*.

4. Two Vpr-resistant mutant yeast strains carrying recessive mutations have been generated by EMS mutagenesis in this study.

5. We have attempted to identify Vpr-interacting proteins by screening the genomic DNA libraries made from the mutant or wild type yeast cells. However, we were unable to identify any potential target proteins by this study.

E. Discussion

We have previously demonstrated that expression of Vpr or Vpx result in a variety of abnormal changes in *S. pombe*. In the current study, further investigations have been carried out to understand the molecular mechanisms by which Vpr or Vpx induce these cellular changes. We found that p34/cdc2 kinase activity was significantly reduced in cells expressing Vpr or Vpx. Our data are consistent with other studies in which p34/cdc2 was observed to be dramatically inactivated in human cells and in yeast expressing HIV-1 Vpr (Re et al., 1995; Zhao et al., 1996).

In mammalian cells, HIV-1 Vpr and SIV Vpr have been shown to arrest the cell cycle in G2/Mphase (Bartz et al., 1996; He et al. , 1995; Jowett et al., 1995; Re et al. , 1995). In our study, we have shown that in *S. pombe* HIV-1 Vpr, HIV-2 Vpr or Vpx, and SIV Vpr or Vpx arrested cell proliferation both at G1/S and G2/M phases. Since we have observed a decline in p34/cdc2 kinase activity in yeast cells expressing these various proteins, the differences may be attributed to p34/cdc2 kinase. In mammalian cells, the direct homologue of p34/cdc2, $cdc2^{Hs}$ (also known as cdk1), regulates the transition from G2 to M phase, while G1/S progression is predominantly controlled by cdk2 (Atherton-Fessler et al., 1993). However, in *S. pombe*, p34/cdc2 is required for both G1/S and G2/M cell cycle transitions (Moreno et al., 1991). Our observations would therefore suggest that p34/cdc2, or $cdc2^{Hs}$ in human cells, might be the target, directly or indirectly, of Vpr or Vpx. Thus, while the observed cell cycle arrest phenotypes may not be identical to that observed in mammalian cells, there are clear similarities of growth arrest phenotype caused by Vpr and Vpx expression in yeast and mammalian cells. Specifically, the observation that yeast and mammalian cells both arrest in G_2 with reduced p34/cdc2 kinase activity indicates that Vpr and Vpx interact with conserved target(s) in yeast and mammalian cells.

p34/cdc2 kinase is positively regulated by Cdc25, and negatively regulated by Wee1/Mik1 kinases. Therefore, any suppression of p34/cdc2 kinase activity could result either from the loss of positive regulation of Cdc25 or from the enhancement of the negative regulation of Wee1/Mik1. To test these possibilities, we have taken the advantage of yeast genetic analysis by which the possible effects of Vpr on the cell proliferation of three p34/cdc2 mutant strains have been investigated. One of the mutants is cdc2-3w, which relieves the requirement for Cdc25 but leaves cdc2 responsive to Wee1 and Mik1. Another mutant is cdc2-1w, which fails to respond to Wee1 kinase but leaves cdc2 responsive to Cdc25 and Mik1; the third one is cdc2-1w, Δmik1, which fails to respond to both Wee1 and Mik1 but leaves cdc2 responsive to Cdc25. The cdc2-3w yeast cells may become resistant to Vpr-induced cell cycle arrest if the effect of Vpr is dependent on Cdc25. On the other hand, other two mutants (cdc2-1w and cdc2-1w, Δmik1) may be resistant to Vpr-induced cell cycle arrest if Vpr acts on Wee1/Mik1 mediated pathway. However, surprisingly, our data have shown that all of these three cdc2 mutants were sensitive to Vpr-induced inhibition of cell proliferation, indicating that Vpr may not directly act on Cdc25 and Wee1/Mik1. Instead, Vpr may act on cdc2 regulators other than Cdc25, Wee1/Mik1. Our data seem to be inconsistent with the previous study showing that Cdc25 in Vpr-expressing HeLa cells was in the inactive, unphosphorylated state, and suggesting that the inactivation of Cdc25 could be responsible for the Vpr-induced inactivation of p34/cdc2 kinase (Re et al., 1995). We did not directly examine the activity of Cdc25 in the Vpr-expressing yeast cells in our study, so it is not clear whether the Cdc25 was in the inactive, unphosphorylated state in the Vpr-expressing yeast cells. In addition, it has been shown that the cdc2-3w yeast cells still remain responsive to changes in Cdc25 activity although the cdc2-3w allele bypasses the requirement for Cdc25 (Russell et al., 1986). Hence, it is not conclusive whether or not Vpr acts on Cdc25 mediated pathway in *S. pombe*. There is another yeast mutant, called cdc2-3w

Δcdc25, which is completely deficient in the activity of Cdc25 (Urnari et al., 1997). It will be very informative for further understanding the possible role of Cdc25 in Vpr-induced cell cycle arrest if the effects of Vpr on the cell proliferation of cdc2-3w, Δcdc25 cells could be examined.

After having examined the pathways mediated by Cdc25 and Wee1/Mik1, we reasoned that Vpr may acts through other novel pathways which may also modulate the p34/cdc2 kinase activity. Previous studies have shown that Vpr-induced cell cycle arrest could be relieved by the inhibitor of protein phosphatase type 2A (PP2A), okadaic acid (OA), indicating that PP2A may play a role in Vpr-induced cell cycle arrest. PP2A has been shown to act as an inhibitor of cell entry into mitosis (Kinoshita et al., 1990) and regulate cytokinesis (Wera et al., 1995). However, the possible role of PP2A in Vpr-induced cell cycle is inconclusive. Interestingly, a recent study has demonstrated that the HIV-1 nuclear capsid protein NCp7 is a potent activator of PP2A *in vitro* (Tung et al., 1997). It has also shown that HIV-1 Vpr could physically bind to NCp7 and form a tight complex. The Vpr-NCp7 complex was shown to be more potent than NCp7 or Vpr alone in terms of the activation of PP2A. Therefore, it was hypothesized that NCp7 may mediate the Vpr-induced cell cycle arrest *in vivo* (Tung et al., 1997). However, this hypothesis does not agree with the previous observations demonstrating that Vpr per se is sufficient to induce cell cycle arrest in yeast and mammalian cells. Nevertheless, the examination of the effects of NCp7 on the cell proliferation of *S. pombe* may provide us further information for understanding the possible role of PP2A in Vpr-induced cell cycle arrest. In this study, we have found that the effects of NCp7 on the cell proliferation of *S. pombe* was obviously different from that of HIV-1 Vpr. Instead of causing complete cell cycle arrest in Vpr-expressing cells, NCp7 slowed the cell proliferation in *S. pombe*, suggesting that NCp7 alone is not sufficient to induce cell cycle arrest *in*

vivo. It is noteworthy that a variety of viral proteins including simian virus 40 (SV40) T antigens, and polyomavirus middle and small T antigens have been shown to interact with PP2A, and the interactions between PP2A and these viral antigens usually result in the decrease of PP2A activity and induce cell proliferation (Mayer-Jaekel et al., 1994; Sontag et al., 1993). Vpr may interact with PP2A and change its specificity of substrates, and subsequently induce cell cycle arrest. Further studies including the analysis of PP2A activity and substrate specificity in NCp7- or Vpr-expressing yeast cells may shed more light on understanding the role of PP2A in Vpr-induced cell cycle arrest.

To identify the Vpr interacting proteins in *S. pombe*, a classical genetic strategy, screening the genomic DNA library, has been performed in this study. We have generated two Vpr-resistant mutant yeast strains with recessive mutations. Initially, we sequentially co-transformed wild type yeast cells with the Vpr-expressing plasmid and the genomic DNA library made from the Vpr-resistant mutant yeast cells. We have not been able to identify any Vpr-suppressers which could rescue Vpr-induced cell cycle arrest in wild type yeast cells. One possibility is that a recessive mutation may not overcome the effect of its wild type counterpart. However, it is not clear why we could not isolate any Vpr-interacting proteins by screening the genomic DNA library made from wild type yeast cells.

Taken together, we have demonstrated that Vpr may induce cell cycle arrest in *S. pombe* via the inhibition of p34/cdc2 kinase activity. In addition, we have provided some data indicating that Vpr may not directly act on Cdc25, Wee1 or Mik1. However, the precise mechanism of Vpr-induced cell cycle arrest still remains unknown.

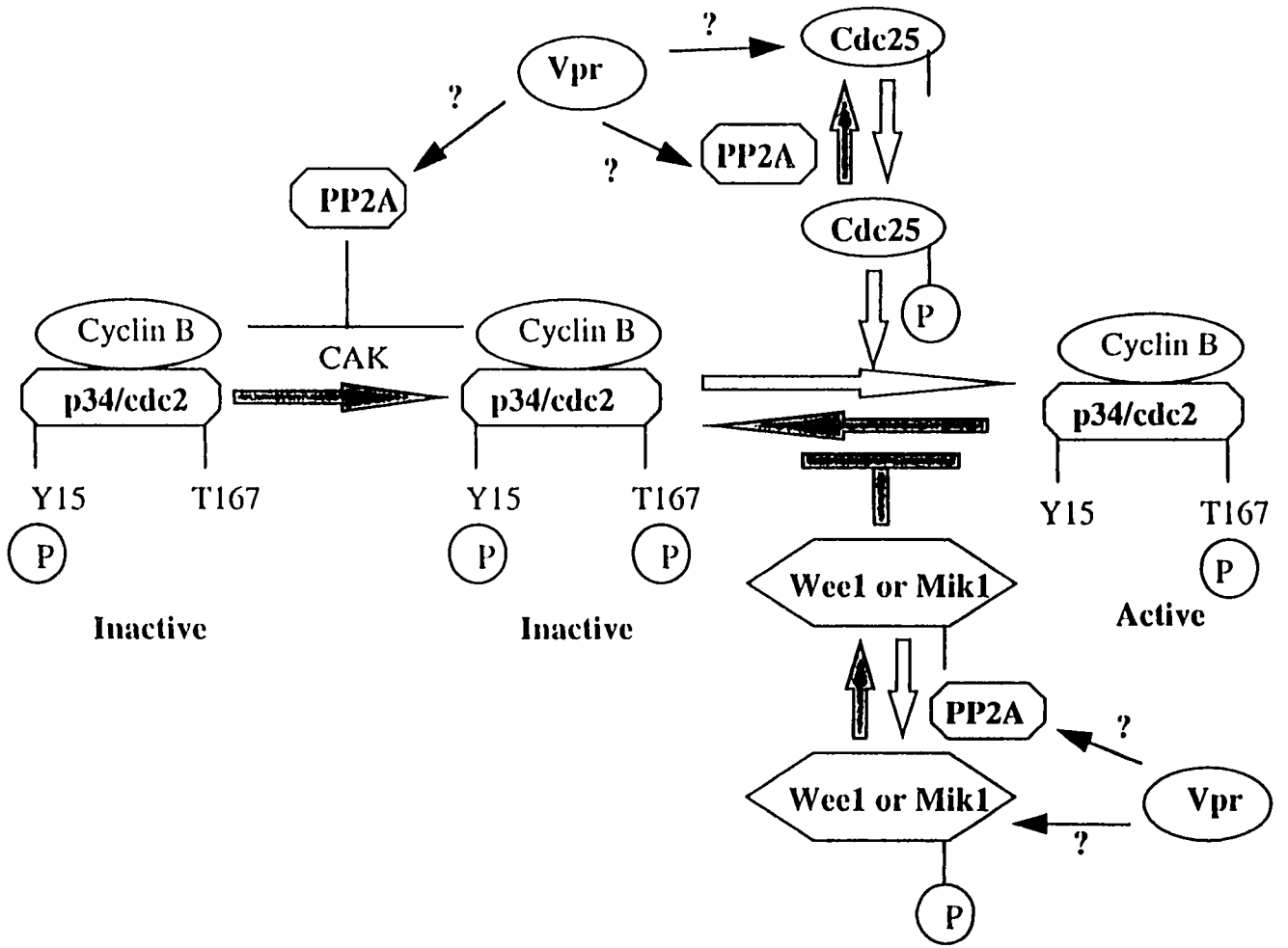


Figure 4.1. Hypothetical model of Vpr-induced cell cycle arrest through various regulators of p34/cdc2 pathway in fission yeast *S. pombe* (see the Chapter I for details).

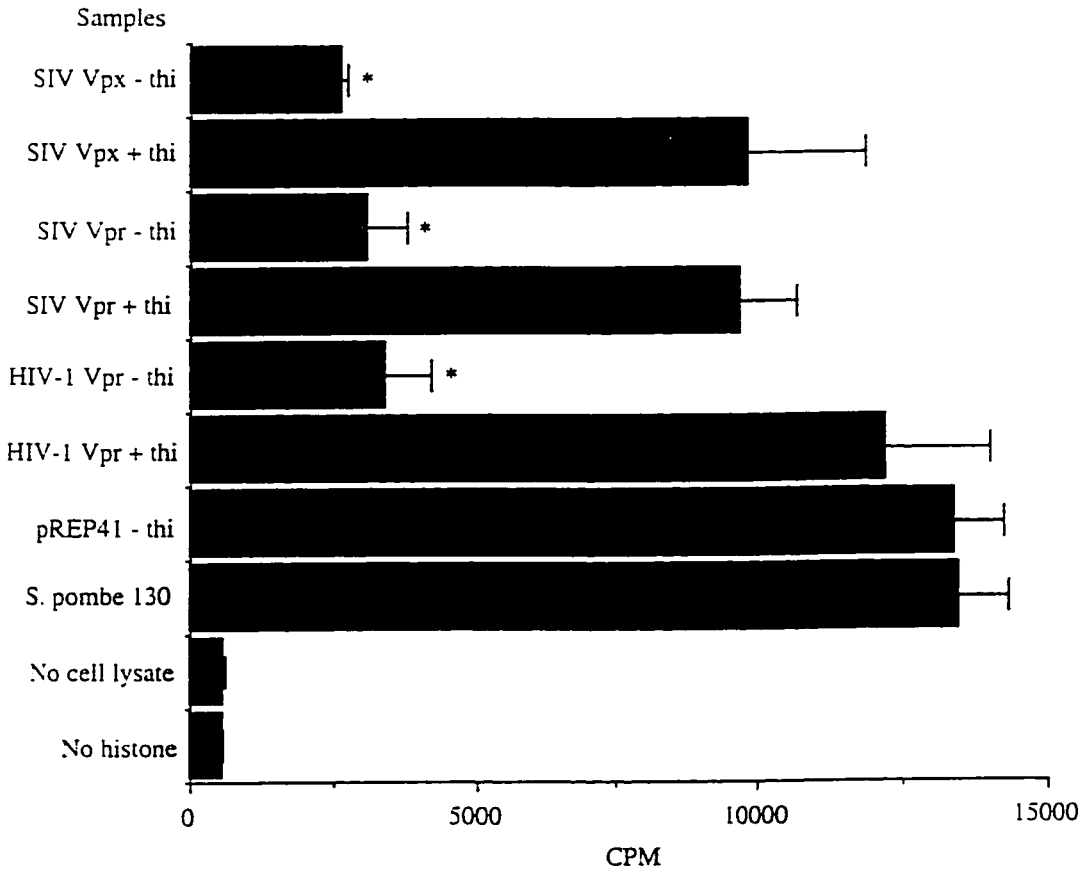


Figure 4.2 Effects of Vpr and Vpx expression on p34/cdc2 kinase activity

Cells extracts were prepared from exponentially growing cells. Samples are indicated along the y-axis. Samples listed as " - thi " were initially grown in medium containing thiamine, harvested by centrifugation, washed in sterile water, then reinoculated in MM lacking thiamine and incubated for 15 hr prior to preparing extracts. H1 kinase assay was performed as described in the Materials and Methods. The data represented the results obtained from three independent kinase assays (mean ± SD).

* p34/cdc2 kinase activity of yeast cells expressing HIV-1 Vpr, SIVVpr or Vpx is significantly different ($P < 0.01$, t-test) from that of control cells without Vpr or Vpx expression.

" + thi " : in the presence of thiamine.
" - thi " : in the absence of thiamine.

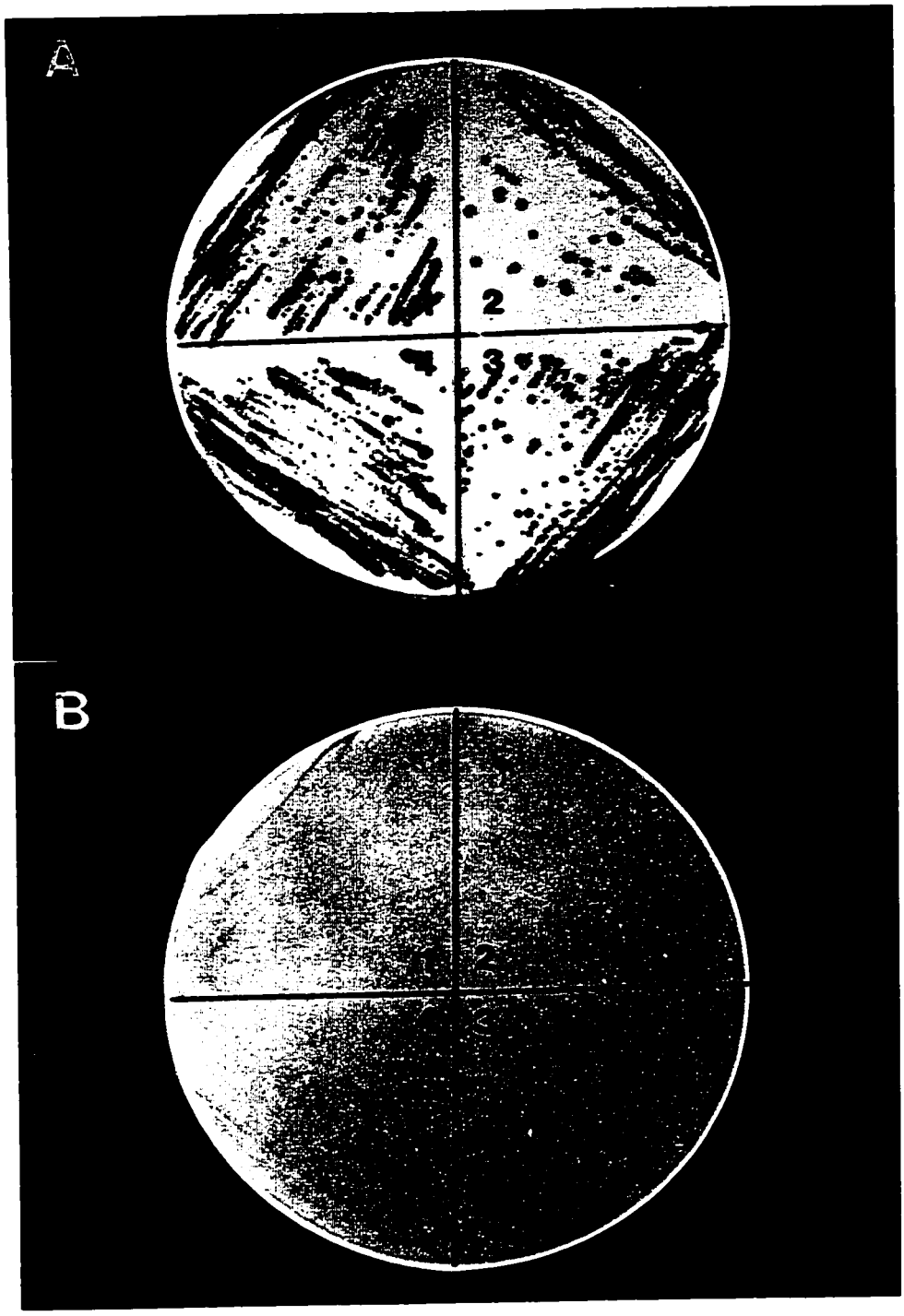
Figure 4.3. Genetic analysis of Vpr-dependent inhibition of yeast cell proliferation.

Strains of *S. pombe* with normal or a mutant cdc2 allele were transformed with the pREP41-HIV-1 Vpr expression plasmid to determine if the cell cycle inhibitory effect was mediated through the known p34/cdc2 regulators, Cdc25, Wee1, or Mik1.

A. Cells grown in the presence of thiamine (repressing conditions).

B. Cells grown in the absence of thiamine (expressing conditions).

1. SP130 (normal cdc2 gene).
2. cdc2-1w (defective response to Wee1).
3. cdc2-3W (defective response to Cdc25).
4. cdc2-1W, Δ mik1 (defective response to Wee1 and deletion of mik1).


A
2
3
B

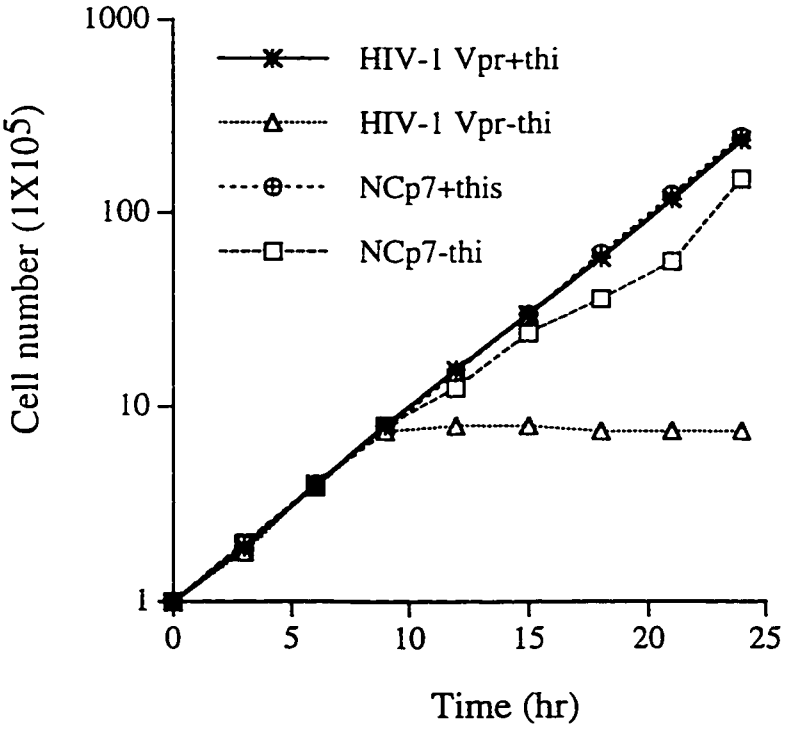


Figure 4.4. Growth kinetics of yeast cells expressing HIV-1 Vpr or NCp7.

Cells were grown in liquid MM with or without thiamine at 30^0C. An aliquot of the cell culture was collected every 3 hr and cell density was determined. The initial inoculum was $1X10^5$ cells/ml. The cells expressing HIV-1 Vpr was arrested at 9 to10 hr after the induction. There is no significant difference in the cell growth rate between the control and NCp7- expressing cells within 15 hr after induction. However, significant difference ($P<0.05$) in the cell growth rate was observed between the control and NCp7- expressing cells at 18, 21, 24 hr after the induction. The data represented the results obtained from three independent experiments.

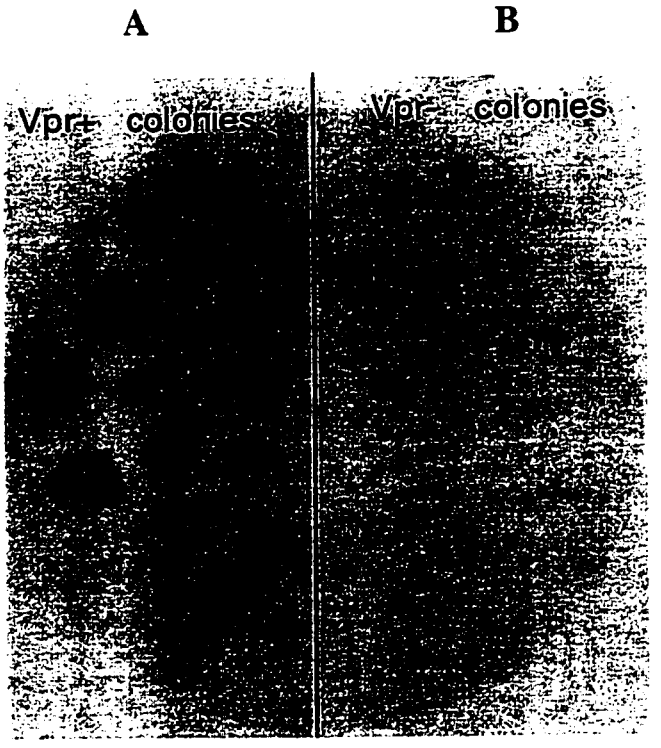


Figure 4.5. Screening of Vpr-positive colonies by immunological detection.

To screen Vpr-expressing colonies, an immunological method was used and performed as described in the Materials and Methods.

A. Yeast colonies expressing Vpr (positive staining).

B. Yeast colonies without Vpr expression (negative staining).

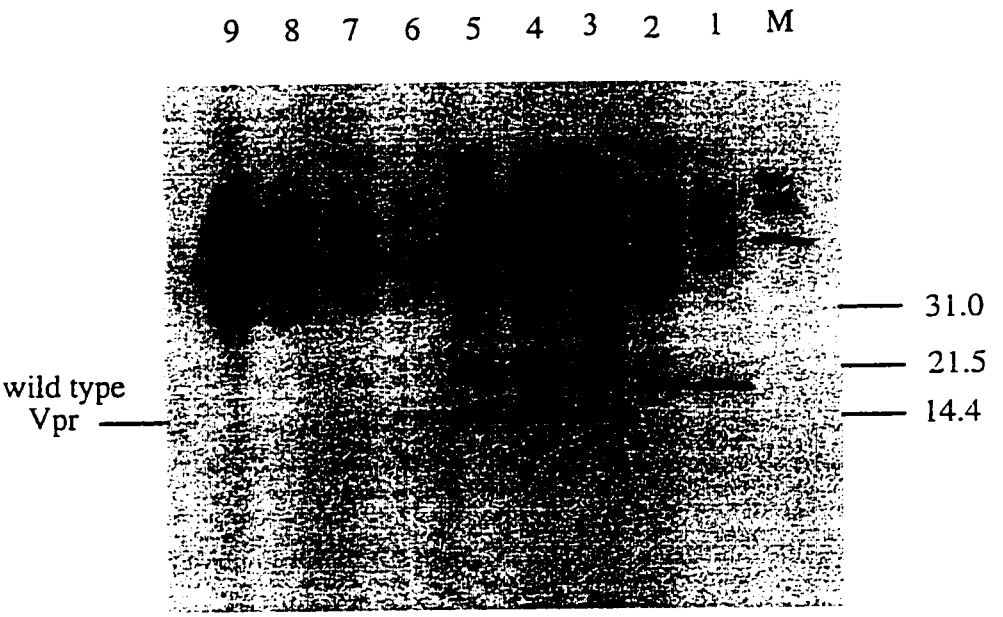


Figure 4.6 Western blot analysis of Vpr expression in mutant yeast cells

Mutant cells were grown in MM without thiamine and protein extracts were prepared as described in the Materials and Methods in Chapter III.

Lane M: Protein marker

Lane 1 to Lane 9: " Vpr-resistant " colonies from EMS mutagenesis

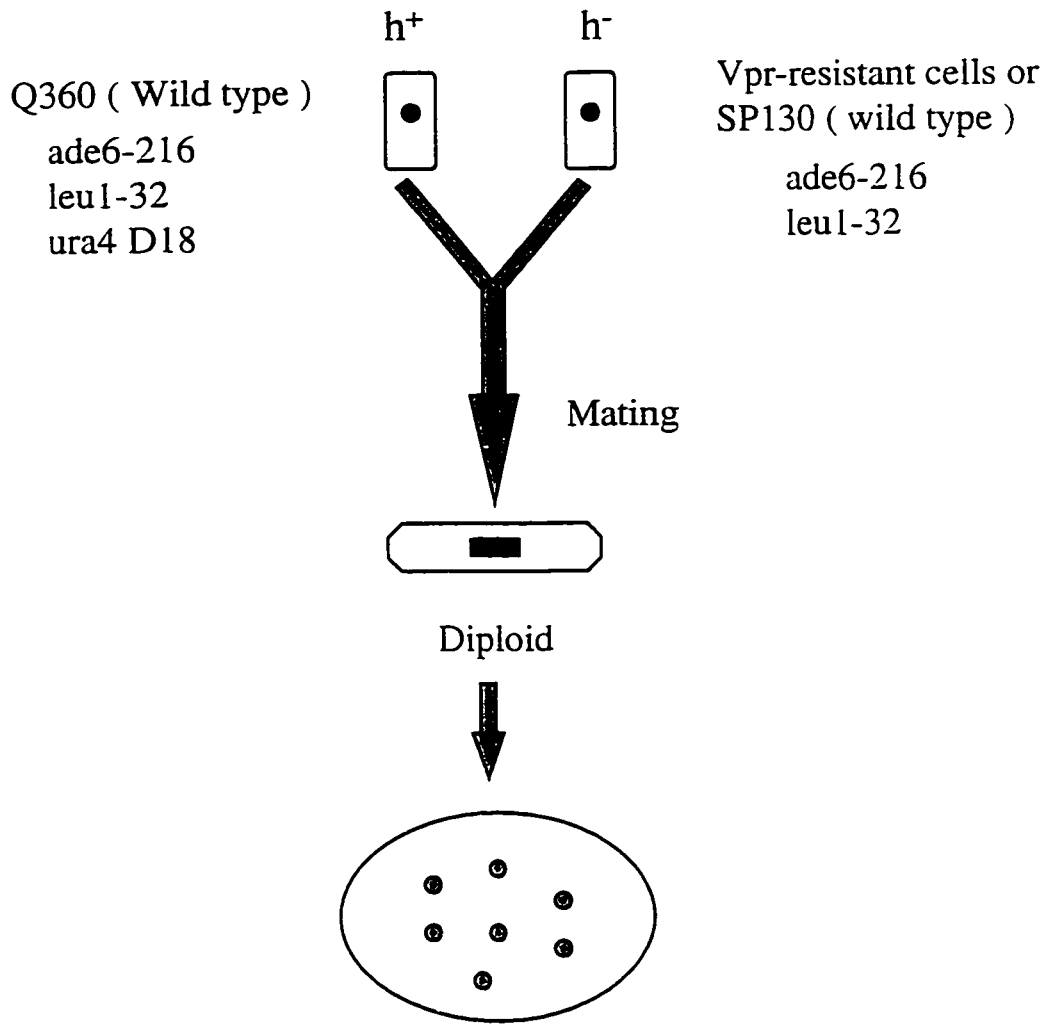


Figure 4.7. Construction of stable diploid cells

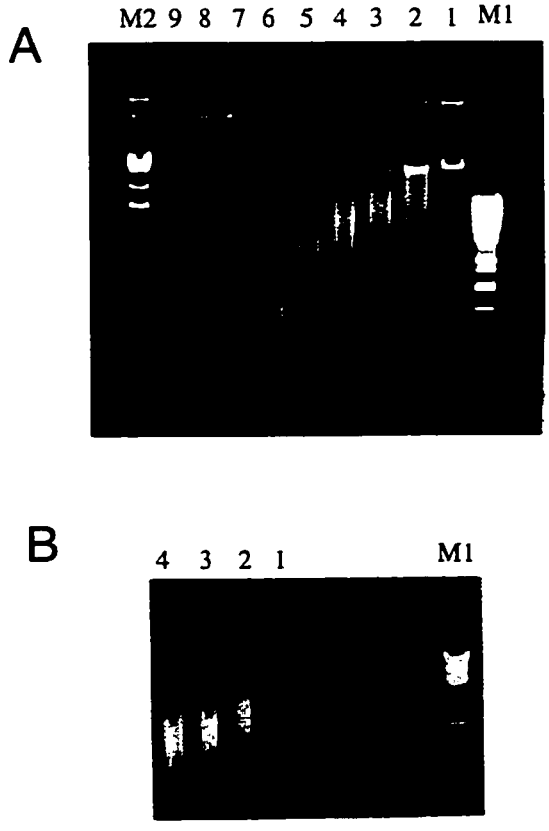


Figure 4.8. Partial digestion of genomic DNA with Asa1

Genomic DNA was partially digested with Asa1 as described under the materials and Methods.

A : Kinetics of partial digestion of the genomic DNA with Asa1.
M1: DNA marker (Lamda DNA-BstEII Digest, NEB).
M2: DNA marker (Lamda DNA-Hind III Digest, BRL).
Lane 1 to Lane 9 : Digested DNA samples at various time points (0, 1, 2, 5, 10, 15, 20, 25, 30 min, respectively).

B : Purification of the 2-3 Kb fragments from the partially digested DNA.
M1: DNA marker (Lamda DNA-BstEII Digest, NEB).
Lane 1 to Lane 4: Purified DNA fragments from the partially digested DNA (Figure 4.8A).

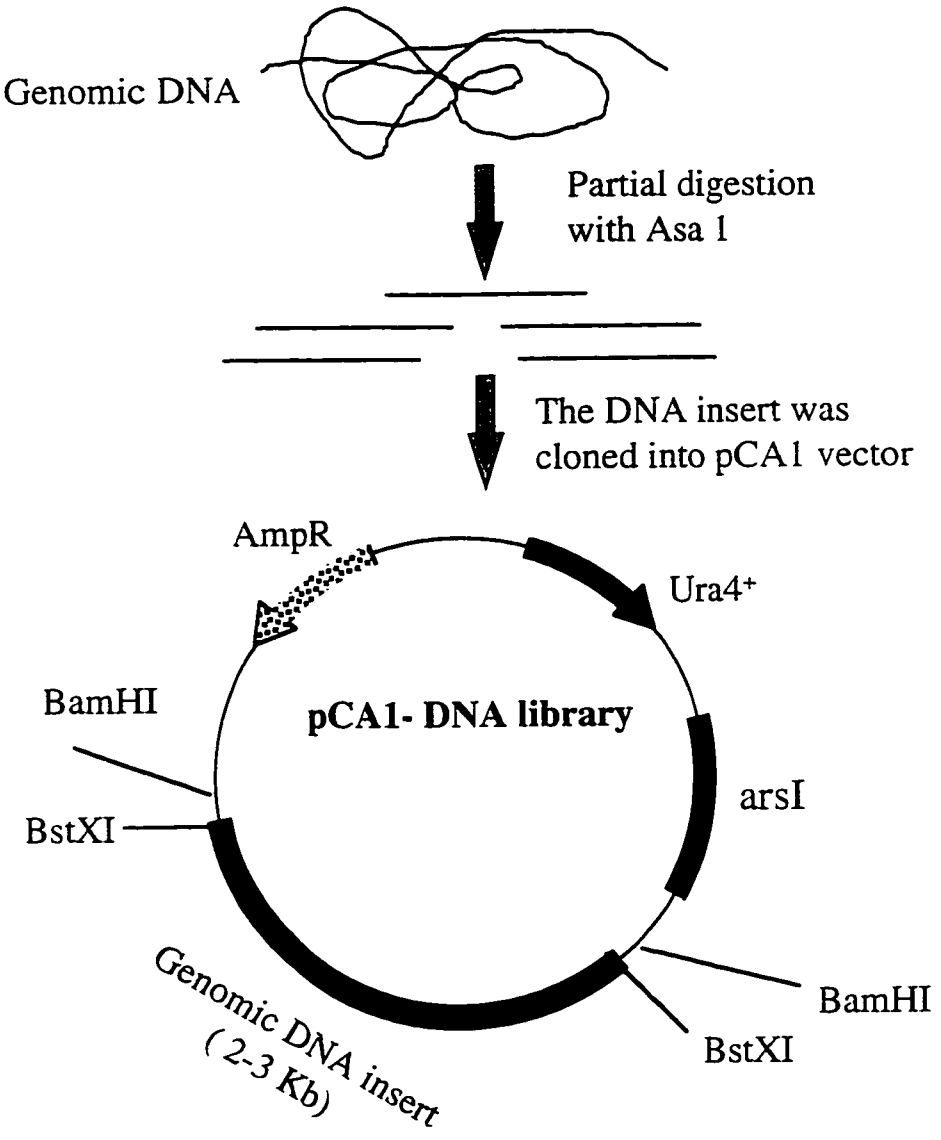


Figure 4.9. Schematic diagram of construction of genomic DNA library

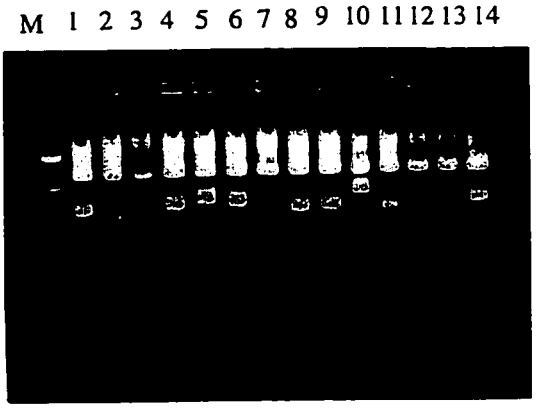


Figure 4.10. Checking the size of the DNA insert by enzymatic digestion.

To check the size of the DNA insert, plasmid DNA was prepared and digested as described in the Materials and Methods.

M : DNA molecular weight marker - Lamda DNA-BstE II Digest

Lane 1 to Lane 14 : Plasmid DNA isolated from fourteen clones of the pCA-1-genomic DNA ligation was digested with BamHI, respectively.

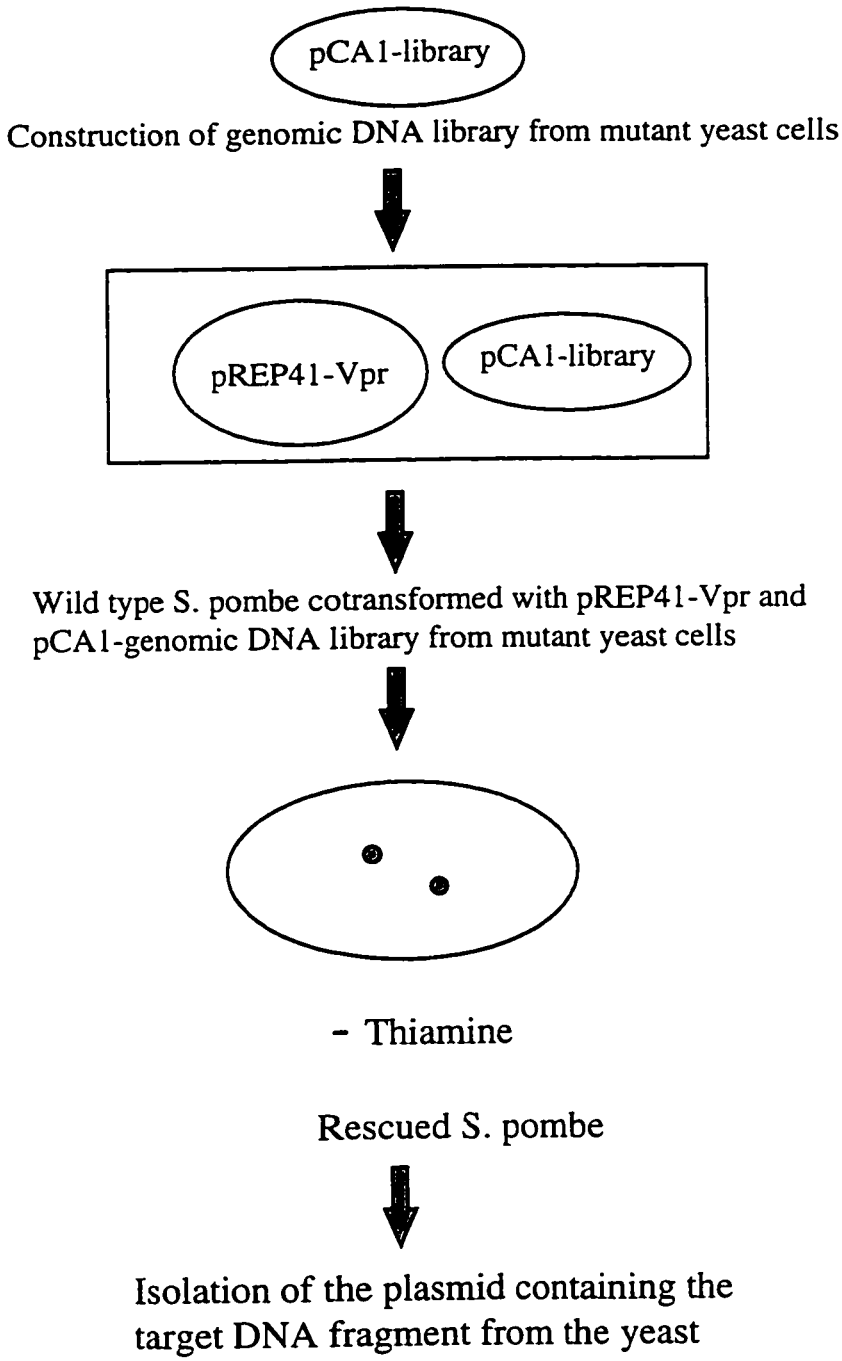


Figure 4.11. Schematic diagram of screening of genomic DNA library made from the Vpr-resistant mutant cells.

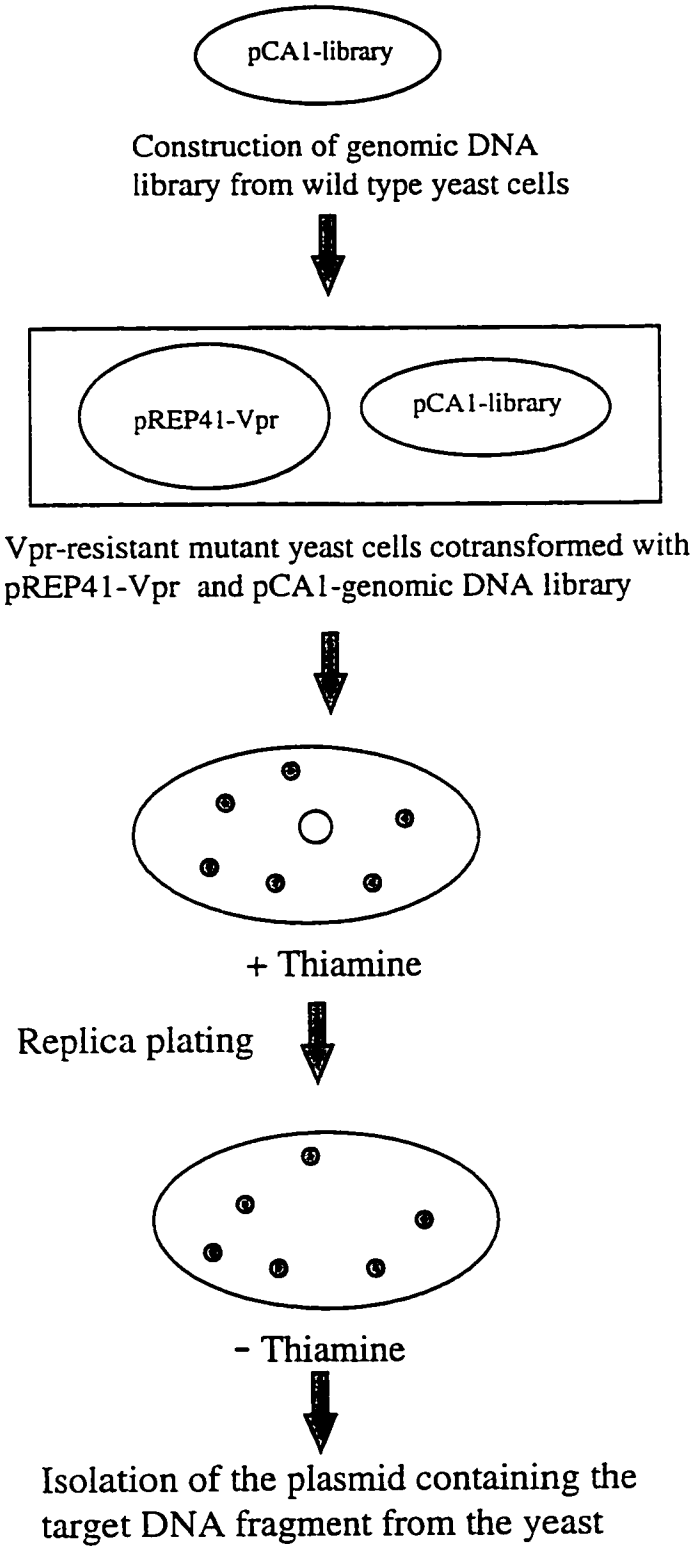


Figure 4. 12. Schematic diagram of screening of genomic DNA library made from wild type yeast cells.

Chapter V

Development of hu-PBL-SCID/beige mouse model and its utilization in the studies of HIV-1 infection *in vivo*

(Some of the data presented in this Chapter have been published in an article entitled " Protective immunity to HIV-1 in SCID/beige mice reconstituted with peripheral blood lymphocytes of exposed but uninfected individuals " by Chengsheng Zhang, Yan Cui. Stan Houston, and Lung-Ji Chang in Proc. Natl. Acad. Sci. USA. 93: 14720-14725. 1996).

A. Introduction and rationale

The ongoing studies in our laboratory, including the studies of HIV-1 infection, gene therapy, and cancer immunotherapy, have prompted us to develop a suitable animal model for *in vivo* assays. In particular, such an animal model is crucial for the studies of HIV-1 pathogenesis, vaccination, and chemotherapy (Chang et al., 1996).

The C.B-17-SCID/SCID mouse (SCID mouse) was first reported to lack serum immunoglobulins (Ig) and functional T and B lymphocytes in 1983 (Bosma et al., 1983a). Early studies have shown that SCID mice, due to their severe immuno-deficiency, were good recipients for the growth of human, rat, and mouse transformed cell lines (Ware et al., 1985; Reddy et al., 1987). Subsequently, SCID mice have been successfully reconstituted with the human immune system by engraftment of human peripheral blood lymphocytes (hu-PBL-SCID mice) or fetal lymphoid tissues (SCID-hu mice) (Mosier et al., 1988; McCune et al., 1988). The initial report of hu-PBL-SCID

mice showed that the human PBL injected into the SCID mice increased in number and survived for at least six months and that human Ig was secreted spontaneously in the hu-PBL-SCID mice (Mosier et al., 1988). This study also demonstrated that human lymphocytes could be found in the lymphoid tissues and peripheral blood of SCID recipients and that a specific human humoral immune response was induced following immunization with tetanus toxoid. This initial study suggested that a functional human immune system could be reconstituted in SCID mice by injection of human adult PBL (Mosier et al., 1988). Subsequently, a number of studies have demonstrated that the hu-PBL-SCID mice could be infected by HIV-1 *in vivo* (Mosier et al., 1991; Koup et al., 1994; Torbett et al., 1991). In addition, hu-PBL-SCID mice have also been successfully used in the studies of the efficacy of immunoprophylaxis against HIV-1 infection by passive immunization with anti-HIV-1 monoclonal antibodies (Cauduin et al., 1995; Parren et al., 1995; Safrit et al., 1993). However, it is still uncertain whether the cell-mediated immunity is intact or could be induced in the hu-PBL-SCID mice. It has been suggested that cellular immunity may play a crucial role in the protective immunity against HIV-1 infection *in vivo* (Shearer and Clerici, 1996). Thus it is very important to determine whether the hu-PBL-SCID mouse is a suitable model for the evaluation of anti-HIV-1 cellular immunity *in vivo*.

There remains some controversy regarding the reconstitution efficiency of human PBL and human immune responses in the hu-PBL-SCID model (Tary-Lehmann and Saxon, 1992; Tary-Lehmann et al., 1995). It has been suggested that the variation of reconstitution outcome in hu-PBL-SCID mice can be attributed to a variety of factors including the background of the SCID mice (i.e., the endogenous microbial flora, age, genetic background, and the extent of leakiness) and the human donors of PBL (Bosma and Carroll, 1991). In particular, attention has focused on the leaky phenotype of the mice. Some SCID mice (approximately 15-25%) have a leaky phenotype based

on the serum Ig level (> 1.0 μg/ml is considered to be leaky; normal non-SCID mice have Ig level > 800 μg/ml) (Bosma et al., 1991; Hesselton et al., 1993). The molecular basis for the leaky phenomenon is not clear yet. It is noteworthy that SCID mice have normal innate immunity, including functional macrophages, normal to elevated NK cell function, and elevated hemolytic complement activity (Dorshkind et al., 1985; Shultz et al., 1994). The leaky phenotype and the innate immunity have been suggested to be able to reduce the success rate of xenogenic engraftment into the SCID mice (Bosma et al., 1991). Indeed, when SCID mice were treated with anti-asialo-GM1(an anti-serum that could deplete mouse NK cells *in vivo*), or/and with irradiation, an improvement of the reconstitution efficiency of human PBL has been achieved (Sandhu et al., 1994; Shpitz., 1994).

In the current study, in an attempt to improve the reconstitution rate of human graftment, we have used another strain of immunodeficient mouse, designated as SCID/beige. In addition to lacking functional T and B lymphocytes, SCID/beige mice have much reduced NK cell activity compared to SCID mice (Dorshkind et al., 1985; MacDougall et al., 1990). Therefore we reasoned that SCID/beige mice may serve as a good recipients of human cells. Indeed, our preliminary experiments show an almost 100% success rate for tumor growth when the SCID/beige mice are injected subcutaneously with a variety of human tumor cell lines, whereas only 50-60% success rate is observed when the same tumor cells are injected into SCID mice (Taylor et al., 1992; Zhang and Chang, unpublished data). We have also find that SCID/beige mice can support a high reconstitution efficiency of human PBL and the hu-PBL-SCID/beige mouse chimera can be infected by HIV-1 with a near 100% success rate.

There are reports of individuals who have been frequently exposed to HIV-1, but remain seronegative for the virus, and it has been hypothesized that these individuals

are resistant to HIV-1 infection (Shearer and Clerici, 1996). However, little is known about the mechanism of immune protection against HIV-1 infection in these high-risk individuals because it is difficult to directly demonstrate protective immunity *in vivo*. To test this hypothesis directly, and to exploit the potential utilization of hu-PBL-SCID/beige mice model in the studies of anti-HIV-1 protective immunity *in vivo*, we have examined the HIV-1 infectivity of SCID/beige mice reconstituted with PBL isolated from the multiply exposed, HIV-1 seronegative individuals. Here we provided direct evidence suggesting that *in vivo* protective immunity may exist in some of these multiply exposed, HIV-1 seronegative individuals (Zhang et al., 1996). In addition, our study indicates that the hu-PBL-SCID/beige mouse model may be useful for the evaluating the anti-HIV-1 protective immunity *in vivo*.

B. Materials and Methods

Animals: SCID/beige mice, 4-6 weeks old, were purchased from Taconic or Charles River, USA. Except for HIV-1 infected mice, all the mice were bred and maintained under SPF (Special Pathogen Free) conditions at the Health Science Laboratory Service (HSLAS), University of Alberta. For the studies of HIV-1 infection, the experiments were performed in the biosafety level 3 facility at the Health Science Laboratory Animal Service (HSLAS), University of Alberta. All the protocols used in the animal study have been reviewed and approved by the Health Science Laboratory Service Committee, and are also in agreement with the federal guidelines for the care and use of experimental animals by the Canadian Council on Animal Care.

Preparation of human peripheral blood lymphocytes (PBL): Blood samples were generously provided by the Canadian Red Cross Blood Donation Center in Edmonton in the form of buffy coats and by standard venipuncture from normal volunteers in our research group. The blood samples from the HIV-1 infected individuals, or the high-risk, multiply exposed, HIV-1 seronegative individuals were collected by Dr. Stan Huston at the Infectious Diseases Clinic, University of Alberta Hospital in Edmonton. PBL were isolated from these blood samples by density gradient centrifugation with Histopaque (Sigma) as described in Chapter II. The isolated PBL were washed with PBS (four times) and resuspended in Hanks' balanced salt solution (Gibco, BRL) for injection. Usually the PBL were prepared and injected into the mice within 2-3 hr, but were also cryopreserved for later use.

Construction of hu-PBL-SCID/beige mice: SCID/beige mice were reconstituted with human PBL as described previously (Mosier et al., 1988). Briefly, each mouse was injected intraperitoneally (i.p) with 2 $x10^7$ PBL resuspended in 0.5 ml of Hanks'

balanced salt solution. Two weeks after the PBL injection, the mice were bled from the tail and the human Ig level measured by enzyme-linked immunosorbent assay (ELISA) as described below. Only the mice that tested positive for human Ig were considered successfully reconstituted and used for further studies.

Detection of human and mouse immunoglubolin (Ig) level by ELISA: Human and murine Ig levels were measured by standard ELISA protocols. Briefly, a 96-well ELISA plate was coated with affinity purified rabbit anti-human IgG/IgM antibody (Jackson labs, or anti-mouse IgG/IgM for murine Ig assay) and incubated at 37^{0}C for 30 min, followed by incubation at 4^{0}C overnight. The wells were washed with the washing buffer (0.05% Tween 20 in PBS), and then were incubated with the blocking solution containing 0.02% Tween 20 and 5% non-fat milk in PBS at room temperature for 2 hr. Serial dilution of standard human IgG (Sigma, or standard mouse IgG for murine Ig assay) and mouse serum samples were prepared and added to the wells in triplicates. The wells were incubated at room temperature for 2 hr. After washing the wells, HRP-goat-anti-human IgG (Sigma, or HRP-goat-anti-mouse IgG for murine Ig assay) was added to the wells and incubated at room temperature for 30 min. The wells were washed, and OPD (o-Phenylenediamine Dihydrochloride) solution (Sigma) was added to the wells to develop the reaction at room temperature for 30 min. The plate was read by an ELISA reader at wavelength 450-570 nm (UV max Kinetic Microplate Reader, Molecular Devices)

Flow cytometry analysis: For flow cytometry analysis, single cell suspensions were collected and prepared from the peritoneal lavage, spleen, and peripheral blood of the mice. The cell samples containing red blood cells were treated with ammonium chloride solution (containing 90 ml of 0.16 M NH_4Cl and 10 ml of 0.17 M Tris-pH 7.65). The cells were stained with PE-anti-mouse-H-2K^d (PharMingen) and FITC-

anti-human-CD45 (Becton Dickinson), or with PE-anti-human-CD4 and FITC-anti-human CD8 (Becton Dickinson) for 30 min on ice, followed by washing three times with 0.1% FBS, 0.02% NaN_3 in PBS. Isotype-matched mouse-Ig-PE and mouse Ig-FITC were used for negative control staining. The samples were analyzed using the LYSIS II program on a FACScan machine (Becton Dickinson).

Virus preparation: HIV-1 strains used in this study included laboratory-adopted HIV_{NL4-3} (T cell-tropic) and HIV_{NLAD8} (Macrophage-tropic). The virus stocks for infection were prepared from the infected primary PBL or macrophages as described previously (Chang and Zhang, 1995). The same virus stocks were used for both *in vitro* and *in vivo* experiments throughout the whole study. The multiplicity of infection (MOI) or $TCID_{50}$ were determined by the infection of HIV-1 negative, PHA-stimulated PBLs using serial dilution of the virus stock as described previously (Chang and Zhang, 1995; Ho et al., 1989; Chapter II in this thesis)

***In vitro* HIV-1 infection:** Human PBL were isolated and prepared as described above, and infected with HIV-1 as described in Chapter II. Briefly, PBLs were treated with phytohemaglutinin (PHA, 5μg/ml, Sigma) and infected with T-cell or macrophage tropic HIV-1 at various MOI. The culture was split at a 1: 3 ratio every 3-4 days and the supernatant was harvested for reverse transcriptase (RT) assay as described in Chapter II. HIV-1 Infections were also examined by immunohistochemical staining as described in Chaper II.

Immunohistochemical staining of HIV-infected cells in the hu-PBL-SCID/beige mice: To determine the frequency of HIV-1 infected cells, cell suspensions from peritoneal lavage, spleen, or peripheral blood of the mice were

prepared, and immunohistochemically stained for HIV antigens as described in Materials and Methods in Chapter II.

Depletion of human CD8$^+$ T cells in the hu-PBL-SCID/beige mice: CD8$^+$ T cells were depleted as previously described (Safrit et al., 1993) with the following modifications. Anti-human CD8 antibody was purified from the ascites of OKT8 hybridoma cells (ATCC) using protein A affinity column (ImmunoPure Plus, Pierce). The mice were injected intraperitoneally with 20 μg of the purified antibody five times in total: two days prior to PBL reconstitution; at the time of PBL injection; and at one week, two weeks and three weeks after PBL injection, respectively. To confirm the depletion of CD8 T cells, hu-PBL-SCID/beige mice were sacrificed and the lymphocytes were analyzed by FACS analysis as described above.

Challenge of hu-PBL-SCID/beige mice with HIV-1: To challenge the hu-PBL-SCID/beige mice, 100 $TCID_{50}$ of cell free HIV_{NL4-3} (T cell-tropic) or HIV_{NLAD8} (Macrophage-tropic) were injected intraperitoneally into the mice under metofane-induced anesthesia. At two to three weeks after the HIV-1 injection, the mice were sacrificed and single cell suspensions were prepared from the peritoneal lavage, spleen and peripheral blood, respectively. The cell samples were processed for the detection of HIV-1 infection by immunohistochemical staining or/and polymerase chain reaction (PCR), and for the determination of the human cell phenotype by flow cytometry analysis (see above). All the experiments were performed in a biosafety level 3 facility with protocols approved by the University of Alberta Biosafety Committee, Research Ethics Board, and Health Sciences Animal Welfare Committee.

ELISPOT analysis of human IFN-γ production and *in vitro* lymphocyte depletion (carried out by Dr. Yan Cui in our laboratory)**:** ELISPOT was performed

as described previously (Miyahira et al., 1995; Cui and Chang, 1997). To quantify the IFN-γ producing cells, a 96-well nitrocellulose-bottomed plate (MultiScreen-HA, Millipore) was coated with 75 µl per well mouse anti-human IFN-γ (10 µg/ml, PharMingen) at room temperature overnight. HIV-1 infected autologous PBLs were treated with mitomycin C (5 µg/ml) for 2.5 hr, washed and resuspended in RPMI-1640 containing 10% FBS and 20 units/ml IL-2 (Boehringer Mannheim), and seeded in the anti-IFN-γ coated 96-well plate at 1 x 10^5 per well as target cells (T). Frozen unstimulated autologous PBLs, which were thawed out and used as effector cells (E), were added to the wells at E/T ratio of 0.4:1, 2:1, and 10:1 in triplicates, and incubated in RPMI-1640 containing 10% FBS, 20 units/ml IL-2 at 37°C, 5% CO_2 for 24 h.

Thawed PBLs were depleted of CD4, CD8 and CD56 cells by incubation with mouse anti-human CD4-, CD8-, CD56-, or mouse IgG_1-labeled BioMeg magnetic beads (PerSeptive) at 50 beads per cell on ice for 30 min. The depletion was carried out by sorting the cells on a magnet for 5 min twice using mouse IgG_1 sorting as a control. After depletion, cells were washed with culture medium and seeded in triplicates with the HIV-1 infected autologous PBLs as target cells in an anti-IFN-γ coated 96-well plate for 24 h as described above. The E/T ratio was based on the starting PBL number before depletion. After overnight incubation, the wells were washed four times using PBS-Tween 20 (PBS-T, 0.05%), blocked with 20% FBS in PBS-T at room temperature for 15 min, and incubated with 100 µl of biotinylated-mouse-anti-human IFN-γ (PharMingen, 2.5 µg/ml in PBS-T) at 4^0C overnight. Each well was then washed four times using PBS-T and incubated with peroxidase-labeled streptoavidin (Caltag , CA) in PBS/T at room temperature for 1 h. IFN-γ producing cells were detected as purple brown spots after DAB (Sigma) and 0.3% $NiCl_2$ staining. The wells were washed four times with ddH_2O, air-dried and the number of IFN-γ producing cells was counted using a video-imaging and computer analysis system (Cui and

Chang, 1997). HIV-1-specific ELISPOT was shown as the mean ± standard error in triplicate wells after subtracting the background from controls. The control samples were prepared using the same effector cells plus autologous PBLs that were not infected with HIV-1.

C. Results

Reconstitution of SCID/beige mice with human PBL

Two weeks after intraperitoneal injection of 2×10^7 human PBL, the status of human PBL reconstitution in the mice was determined by the measurement of human immunoglobulin (Ig) level in the serum of the mice or/and the detection of human lymphocytes in the peritoneal cavity, spleen, or other tissues from the sacrificed mice (Figure 5.1).

Secretion of human immunoglobulin in the serum of hu-PBL-SCID/beige mice

Since Ig levels can be detected from the tail blood without sacrifice of the mice, the detection of the human Ig levels is a major parameter for determining the status of human lymphocyte reconstitution in the hu-PBL-SCID mice (Hesselton et al., 1993; Gauduin et al., 1995; Parren et al., 1995). To measure human Ig levels in the reconstituted hu-PBL-SCID/beige mice, tail blood samples were collected at various time points after PBL injection and human Ig measured by ELISA as described in the Materials and Methods. We found that there were very low levels of human Ig (usually < 20 μg/ml) in the mice within the first week after reconstitution (Figure 5.2). However, the human Ig level increased and peaked at 3-4 weeks after the reconstitution with an average concentration of 1155.6 ± 394.8 μg/ml (mean ± SD, Figure 5.2). Subsequently the concentration of human Ig decreased but remained at relatively high levels (367.3 ± 110.8 μg/ml) in the hu-PBL-SCID/beige mice up to eight weeks after the reconstitution (Figure 5.2).

Distribution of human lymphocytes in the hu-PBL-SCID/beige mice

To study the kinetics and phenotypes of the human lymphocytes distributed in the hu-PBL-SCID/beige mice, cells samples prepared from the peritoneal lavage, spleen, and other tissues of the mice at various time points after the PBL injection were examined by FACS analysis as described in Materials and Methods. While a high percentage of human $CD45^+$ cells could be detected in the peritoneal cavity, there were few human cells, if any, detected in the spleen and peripheral blood of the reconstituted mice within the first week post-reconstitution (Figure 5.3). However, human lymphocytes were detected in the spleen and peripheral blood two weeks after the reconstitution (Figure 5.3). The highest percentage of human cells could often be detected around 4 weeks after the reconstitution. The percentage of human $CD45^+$ cells in the peripheral blood, spleen, and peritoneal cavity of the hu-PBL-SCID/Beige mice were around 1-10%, 5-30%, and 10-60%, respectively, 4 weeks after reconstitution (Figure 5.4). In addition, human CD4 and CD8 lymphocytes were also detected in the peritoneal cavity, spleen and peripheral blood (Figure 5.5B & C). Interestingly, a normal CD4/CD8 ratio (> 1.00) was often observed in the hu-PBL-SCID/beige mice (Figure 5.5C). Generally speaking, the highest percentage of human lymphocytes was detected in the peritoneal lavage, followed by spleen and peripheral blood. However, there were few human cells detected in the thymus and bone marrow of the reconstituted mice.

Donor effects on the reconstitution rate

It is controversial whether the donors's background affects the rate of PBL reconstitution in SCID mice. While some studies suggested that the variation of reconstitution rate was related to the donor's background (e.g., status of EBV infection. Mosier et al, 1988; Hesselton et al., 1993), others indicated that the differences in human cell engraftment was not associated with the human donors (Hesselton et al., 1995). To further address this issue, we have examined several

groups of hu-PBL-SCID/beige mice varying only the donor PBL. We found that there were variations in terms of the PBL reconstitution efficiency in the SCID/beige mice among different donors. For instance, reconstitution with one of the donors' PBL (donor J) resulted in very high levels of human cells in the mice with approximately 60%, 40%, and 20% of human CD45 cells in the peritoneal lavage, spleen and peripheral blood, respectively (Figure 5.4. donor J). In contrast, the percentage of human CD45 cells in mice reconstituted with the PBL from donor C was much lower than that of donor J (Figure 5.4 ; $P< 0.01$ by t-test). However, the factors which caused these variations remain unknown.

The effect of mouse Ig on human cells reconstitution in SCID/beige mice

It has been proposed that the leaky SCID mice may affect the reconstitution efficiency of human PBL in SCID mice (Bosma and Carrol, 1991). Leaky SCID mice are screened based on the murine Ig levels, and the mice will be considered to be leaky if the murine Ig level is higher than 1.00 μg/ml in the serum (Hesselton et al., 1993; Keyk et al., 1994). There is no previous report about the leaky phenotype in SCID/beige mice. Since SCID/beige mice were generated from SCID mice, we reasoned that SCID/beige mice may also have leaky phenotype. To test this hypothesis, we have reconstituted both leaky and non-leaky SCID/beige mice with the same donor's PBLs, and compared the levels of human Ig and CD45 cells in the reconstituted mice. However, in our study, most of the leaky SCID/beige mice had a similar reconstitution efficiency to the non-leaky mice, as determined by human Ig and $CD45^+$ lymphocytes levels (Table 5.1).

Infection of hu-PBL-SCID/beige mice with HIV-1 *in vivo*

Having demonstrated that SCID/beige mice could support high levels of human PBL engraftment, we next examined the HIV-1 infectivity in hu-PBL-SCID/beige mice.

Previous studies by Dr. Mosier's group have demonstrated that almost 100% of the hu-PBL-SCID mice could be infected with cell free HIV-1 at various $TCID_{50}$ including 50, 100, and 1000 (Mosier et al., 1991; Mosier et al., 1993a and 1993b). We have also challenged the hu-PBL-SCID/beige mice reconstituted with the healthy donor's PBL with 50 and 100 $TCID_{50}$ of cell free HIV_{NL4-3} or HIV_{NLAD8} at two weeks after the PBL reconstitution by intraperitoneal injection. The HIV-1 infected mice were then sacrificed at various time points post-infection and the single cell suspensions were prepared from the peritoneal lavage, spleen, and peripheral blood. To detect the HIV-1 infection, the collected cell samples were immunohistochemically stained for HIV-1 antigens as described in Materials and Methods. HIV-1 infected cells could be detected in the peritoneal lavage, spleen, and peripheral blood cells one to two weeks after challenge (Figure 5.6). The percentage of HIV-1 infected cells in hu-PBL- SCID/beige mice often peaked at 2-3 weeks post-infection. We also observed that HIV-1 infection often results in the reversal of human CD4/CD8 ratio in the HIV-1 infected hu-PBL-SCID/beige mice (Figure 5.7B and 5.7C). HIV-1 infected cells could be detected 2-3 months post infection. It is noteworthy that an almost 100% success rate of HIV-1 infection was attained with hu-PBL-SCID/beige mice challenged with either 50, or 100 $TCID_{50}$ in this study. Our data indicate that the hu-PBL-SCID/beige mice are a potentially excellent model for HIV and AIDS research.

Susceptibility of HIV-1-exposed, high-risk individual PBLs to HIV-1 infection *in vitro*

Having demonstrated that hu-PBL-SCID/beige mice could be easily infected by HIV-1, we next asked whether the model could be used for the investigation of anti-HIV-1 protective immunity *in vivo*. To address this issue, we identified seven individuals who have been engaged in regular unprotected heterosexual or homosexual relations with HIV-positive partners over a period of up to 10 years, but have remained HIV-negative

as determined by periodical serological assays, polymerase chain reaction (PCR), and peripheral blood lymphocyte (PBL) cocultivation. Two of these individuals were chosen for these studies. Donor 1 is the heterosexual partner of a bisexual individual who had a CD4 count of 400-500 at the time of positive HIV diagnosis. The frequency of unprotected sex between donor 1 and his partner is estimated at approximately 144 times prior to the diagnosis. Donor 2 is the homosexual partner of an individual known to be HIV-1 positive since 1986 and whose CD4 count has remained in the 300-400 range; unprotected anal sex is estimated at approximately 225 times prior to this study and unprotected sexual contact is still ongoing. To determine if these individuals were susceptible to HIV-1 infection, their PBLs were activated with PHA in culture and then incubated with T cell- or macrophage-tropic HIV-1. Infection was scored on the basis of HIV-1 reverse transcriptase (RT) activity and immunohistochemical staining with AIDS patients' sera. Both the RT assay and the immunostaining results demonstrated that PBLs from these high-risk (HR), HIV-1-seronegative individuals were as susceptible as PBLs from normal low-risk (LR) donors to HIV-1 infection in tissue culture (Figure 5.8, and Figure 5.9A). To further determine whether the high-risk individuals' PBLs were more resistant to HIV-1 infection, the tissue culture infection was performed using a series of diluted virus preparations (from MOI of 10^{-1} to 10^{-4}). The result showed that even with MOI of 10^{-4}, the high-risk donor's PBLs were still infected (Figure 5.9B). Similar results have been reported for high-risk, HIV-1 seronegative hemophiliacs (Lederman et al., 1995). However, little is known about the possible nature of the protective immunity in these individuals, mainly because it is impossible to carry out direct studies *in vivo* .

PBLs isolated from high-risk, HIV-1 seronegative individuals were resistant to HIV-1 infection in hu-PBL-SCID/beige mice

To overcome the problem of the lack of an *in vivo* HIV-1 infection model, we used the human PBL-SCID/beige mice model to investigate the nature of the protective immunity in the HIV-exposed but uninfected individuals. In the current study, we used this *in vivo* model to test the susceptibility of the two high-risk, HIV-negative individuals to HIV-1 infection. PBLs were prepared by standard methods (see Materials and Methods) and cells from each donor were used to reconstitute 6-7 SCID/beige mice (2 x 10^7 PBLs per mouse). Two weeks after PBL reconstitution, the reconstituted hu-PBL-SCID/beige mice were challenged with T cell- or macrophage-tropic HIV-1 using a 100 $TCID_{50}$ which was equivalent to a MOI of 0.001. The mice were sacrificed two weeks after HIV-1 challenge, and peritoneal lavage, splenocytes and peripheral blood mononuclear cells were collected and analyzed by flow cytometry using anti-mouse MHC class I antibody (H-2kD) and anti-human CD45, CD3, CD4 and CD8 antibodies. At the same time, the extent of HIV-1 infection was examined by a sensitive single-cell immunohistochemical staining method using either serum from HIV-positive patients or a monoclonal anti-p24 antibody as described previously (Chang and Zhang, 1995). The results of immunostaining demonstrated that hu-PBL-SCID/beige mice reconstituted with PBLs from control individuals were infected by either T cell- or macrophage-tropic HIV-1, at frequencies of about 1-4 % of cells from the peritoneal lavage (Figure 5.10A), 0.8-5% of splenocytes (Figure 5.10B). In contrast, the mice reconstituted with PBLs from the two high-risk, HIV-uninfected individuals were all negative for the HIV immunostaining (Figure 5.10C & D, peritoneal lavage and spleen, respectively). However, one of the negative mice showed positive for HIV-1 DNA sequences by PCR analysis, suggesting that infection was established at the time of challenge. These results are summarized in Figure 5.13. The difference observed between the *in vivo* and the *in vitro* studies indicates that anti-HIV-1 protective

immunity cannot be properly assessed by tissue culture infection study alone. Here we have not examined the viral dose-response in the mice. The main reason for failing to do that was the limitation to get blood samples from the donors, especially the high-risk donors. However, it will be very informative if higher viral dose could be used to challenge these mice in this study.

Cell-mediated immune responses in the high-risk, HIV-1 seronegative individuals

To investigate if the *in vivo* protection is due at least in part to HIV-1-specific, cell-mediated immune responses in the high-risk, seronegative individuals, we adapted a convenient and very sensitive ELISPOT assay to determine the frequency of IFN-γ producing cells after HIV antigen presentation. Unstimulated PBLs from the control and high-risk donors were incubated with mitomycin C-treated, HIV-infected autologous PBLs in a 96-well nitrocellulose-bottomed plate which had been coated with an anti-IFN-γ antibody. Production of IFN-γ was detected 24 h later using a secondary HRP-conjugated anti-IFN-γ antibody. As shown in Figure 5.12, no HIV-specific IFN-γ producing responders were observed in PBLs of four low-risk donors except for donor 3 who showed a frequency of IFN-γ producing cells at one in 1 x 10^6 PBLs at the highest E/T ratio. In contrast, the existence of HIV-specific responders in the PBLs of high-risk seronegative donors were easily detected; at the E/T ratio of 10:1, the frequencies of HIV-specific IFN-γ producing cells were 14, 21 and 28 per 10^6 cells for the three high-risk donors, which was significantly different ($P< 0.05$, t-test) from that of the low-risk donors. Even at an E/T ratio of 0.4:1, HIV-specific IFN-γ producing cells were still detectable in all three high-risk donors. The contribution of the individual subset of immune effector cells in this HIV-specific reaction was further studied by depleting CD4, CD8 or CD56 cells from PBLs of two of these high-risk donors prior to the *in vitro* ELISPOT assay. Results of this quantitative analysis

indicated that CD8 T cells contributed significantly to the production of IFN-γ (48% - 71%).

In vivo resistance of HR's PBLs to HIV-1 infection is CD8 T cell-dependent

The above information was used to further delineate the mechanism of *in vivo* protection from HIV-1 in the reconstituted SCID/beige mice. We used a high-risk donor (HR-1) PBLs and depleted CD8 T cells from the reconstituted mice using a monoclonal antibody (OKT8) intraperitoneally injected prior to, during and after PBL reconstitution (CD4 cells are the targets of HIV-1 infection and thus cannot be depleted in this study). Reconstitution and CD8 depletion were confirmed by FACS analysis four weeks later. In mice receiving no antibody, we observed 14 % human CD45 cells (Figure 5.13A), 7% human CD4 cells and 8% human CD8 cells (Figure 5.13B) in spleens four weeks after reconstitution. In mice treated with anti-CD8 antibody, there were similar numbers of CD45 cells in the spleen (12%, Figure 5.13C), but the CD8 cells were virtually absent (0.1%, Figure 5.13D). These mice were inoculated with the macrophage-tropic HIV-1_{NLAD8} on day 14 after reconstitution and sacrificed 14 days later for analysis as before. Results of this study are summarized in Figure 5.14. Mice reconstituted with PBLs from high-risk donor 1 continued to be resistant to HIV-1 infection as long as CD8 cells were present. However, after CD8 cell depletion, similarly reconstituted mice became susceptible to HIV-1 infection (Figure 5.14 hu-PBL-SCID/beige mouse #14, 15, 16). These results strongly suggest that *in vivo* protection from HIV-1 infection in the reconstituted mice is mediated by human CD8 lymphocytes.

In terms of the overall numbers of human CD45 cells, a decreased human CD45 cell population in the CD8-depleted mice was expected. However, we did not observe

changes in the percentage of human CD45 cells after the depletion of human CD8 T cells. It is not clear why we did not see this difference in this study. One of the possible explanations is T cell homeostasis. Previous studies have shown that the overall numbers of T cells and B cells remain at constant levels despite the depletion of T cell by antibody treatment (Rocha et al., 1989). These observations also suggest that there is a sensor mechanism in the host immune system which can detect the overall number of T cells (or a subset) and maintain the T cell homeostasis (Butcher et al., 1996). In addition, clonal expansion of human cells have been reported in the hu-PBL-SCID mice, and cell proliferation and death could occur simultaneously during the clonal expansion (Tary-Lehmann et al., 1995). Some of the human cells in the reconstituted mice could be killed by the CD8 mediated pathways (Tary-Lehmann et al., 1995). Therefore, the depletion of CD8 cells in the anti-CD8 treated mice will also lead to the loss of CD8 mediated cell killing effect on the clonally expanded cells. Taken together, the T cell homeostasis and other unknown mechanisms may play a role in the regulation of overall numbers of CD45 cells in the anti-CD8 treated mice.

D. Summary of results

1. Hu-PBL-SCID/beige mice model have been developed by the intraperitoneal injection of 2 X 10^7 human PBL into SCID/beige mice.

2. High levels of human immunoglobulin could be detected in the peripheral blood of all the hu-PBL-SCID/beige mice.

3. Various percentages of human lymphocytes including $CD4^+$, $CD8^+$ subsets could be detected in the peritoneal lavage, spleen, and peripheral blood of the reconstituted hu-PBL-SCID/beige mice by FACS analysis.

4. A normal CD4/CD8 ratio (> 1.00) is observed in the hu-PBL-SCID/beige mice.

5. Donor effects on the reconstitution efficiency of human PBL in SCID/beige mice were observed. However, the murine Ig levels seem not to be correlated with the reconstitution efficiency.

6. The hu-PBL-SCID/beige mice could be easily infected by cell-free HIV-1 viruses including T cell tropic and macrophage tropic HIV-1. In addition, the reversal of CD4/CD8 ratio can occur in the HIV-1 infected mice.

7. Using this hu-PBL-SCID/beige mice model, we have show that some multiply exposed, HIV-1 seronegative individuals have *in vivo*, but not *in vitro*, protective immunity to HIV-1 infection.

8. These data suggest that this hu-PBL-SCID/beige mouse model will be very useful for the studies of HIV-1 infection, particularly for the assessment of the immune response to HIV-1 vaccines.

E. Discussion

In the current study, we have attempted to improve the reconstitution efficiency of human cells in immunodeficient mice by using SCID/beige mice rather than SCID mice. Compared with the previous studies conducted in SCID mice (Mosier et al., 1988; Hesselton et al., 1993; Greiner et al., 1995), we found that SCID/beige mice can also support a high level of human PBL engraftment and have high levels of human Ig circulating in peripheral blood. These data suggest that SCID/beige mice are good recipients for human PBL engraftment. Interestingly, a recent study has compared another immunodeficient mouse (NOD/LtSz-SCID) with SCID mouse, and found that much higher levels of human PBL reconstitution were attained in this animal than in SCID mice (Hesselton et al., 1995). Since NOD/LtSz/SCID mice have been demonstrated to be deficient in macrophage development and function, and NK cell activity, as well as T and B cell functions (Shultz et al., 1995), this mouse strain may also be potentially used to improve the reconstitution efficiency of human engraftment in the mice.

Since the immunodeficiencies of the recipient mice could affect the reconstitution efficiency of the human cells in the mice, it is important to determine the immune status of the mice before doing experiments. One of the documented approaches is to screen the "leaky mice " based on the murine Ig levels. We have also examined the murine Ig levels in the SCID/beige mice to establish any correlation between the murine Ig levels and human PBL reconstitution. However, our data are not in agreement with the previous notion suggesting that the " leaky mice " had lower success rate in the human PBL reconstitution than the non-leaky mice (Hesselton et al., 1993). Therefore, we argue the reliability and significance of the screening of "leaky mice " based on the murine Ig levels. Further studies including the test of NK cell activity may be required to examine the relevant immune status of the mice.

In addition to the variations generated from the genetic background of the mice, the donor's effects have also been considered to be one of the factors which may influence the success rate of the human engraftment. One of the well-documented donor factors is the status of EBV-infection. It has been shown that the SCID mice injected with EBV^+ PBL have had active B cell clonal expansion and subsequently develop B cell lymphoma in the mice (Rochford and Mosier et al., 1995; Torbett et al., 1991). We also observed the donor-dependent engraftment in this study. However, we do not know the exact factors which caused these donor-dependent variations. One possibility is that the recipients' responses to the foreign engraftment will influence the outcome.

We have further demonstrated that the hu-PBL-SCID/beige mice could easily be infected by HIV-1 (both T cell tropic and macrophage tropic HIV) *in vivo*. HIV-1 infected cells were quantified by a very sensitive immunhistochemical staining at a single cell level. We found that HIV-1 infection induced $CD4^+$ T cell depletion and caused the reversal of CD4/CD8 ratio in the hu-PBL-SCID/beige mice, both of which are consistent with other studies conducted in hu-PBL-SCID mice (Mosier et al., 1991; Mosier et al., 1993). Our study suggest that hu-PBL-SCID/beige mice are an excellent model for the studies of HIV-1 infection *in vivo*.

We have successfully used the hu-PBL-SCID/beige model to study the anti-HIV-1 protective immunity *in vivo*. We find that some multiply exposed, HIV-1-seronegative individuals have *in vivo* protective immunity against HIV-1 even though their PBLs are susceptible to HIV-1 infection in tissue culture. To obtain statistically valid results, a large number of reconstituted SCID/beige mice for each study subject was necessary. Due to the difficulty in acquiring large volumes of blood from high-risk, HIV-negative donors and the laborious nature of handling and analyzing a large number of reconstituted SCID/beige mice, we chose two representative high-risk, HIV-1-negative

subjects for the *in vivo* infection study in depth. The reconstitution efficiency and the percentage of infection of human lymphocytes in three different organ compartments were analyzed for each reconstituted mouse. The results of the *in vivo* HIV-1 challenge suggest that the cell-mediated, protective immunity developed in these high-risk subjects reacts to multiple strains of HIV-1: the challenge strains used in this study, T cell- and macrophage-tropic HIV-1, are unrelated to the endogenous HIV-1 strains in the corresponding partners of these high-risk individuals under investigation.

Other studies have demonstrated the development of cell-mediated immune responses in HIV-1-exposed individuals (Clerici et al., 1992; Clerici et al., 1994; Pinto et al., 1995; Rowland-Jones et al., 1995; Taylor, 1994). In addition, Dr. Mosier's group has used the hu-PBL-SCID mouse model to evaluate the anti-HIV-1 protective immunity in the vaccinated individuals with vaccinia gp160 and recombinant gp160 (Mosier et al., 1993). Interestingly, they have demonstrated that some of the SCID mice reconstituted with the vaccinated donor's PBLs were resistant to HIV-1 infection, and the resistance seems to be mainly related to cell-mediated immunity (Mosier et al., 1993). Furthermore, studies of the simian models also suggest that cell-mediated immune responses play an important role in protection against simian immunodeficiency virus infection (Salvato et al., 1994). It is possible that some genetic factors including HLA haplotype may also contribute to the development of resistance to HIV-1 infection (Detels et al., 1996; Haynes et al., 1996; Kaslow et al., 1996). However, the two high-risk subjects who participated in the hu-PBL-SCID mouse challenge study do not share common HLA haplotypes. Any possible genetic association with the development of protective immunity requires further investigation.

In vivo protective immunity against HIV-1 has been hypothesized to involve the functions of CD8 T cells that secret anti-HIV chemokines (Cocchi et al., 1995). In

addition, CD4 T cells from 2 of 25 frequently exposed but uninfected individuals have recently been shown to have relative resistance to HIV-1 infection (Paxton et al., 1996). Subsequent studies have indicated that these two individuals have homozygous defects in the CCR5 loci that encode the co-receptor for the Macrophage tropic HIV-1 (Liu et al., 1996). In addition, it has been shown that the SCID mice reconstituted with PBLs from the CCR5 Δ32/Δ32 homozyous individuals were resistant to macrophage-tropic HIV-1 infection and showed delayed replication of dual-tropic HIV-1 (Picchio et al., 1997). We have followed up this study by examining the genotypes of six multiply exposed, HIV-1 seronegative individuals including the two HR subjects in the present *in vivo* study. Our results of the PCR analysis have shown that one is heterozygous and five are wild type of CCR5 loci. Both of the two HR subjects have wild type CCR5 loci. This is consistent with the result that PBLs of those two HR subjects were susceptible to HIV-1 infection *in vitro*.

In vitro ELISPOT analysis indicate that, when exposed to autologous HIV-1 infected cells, Th1-associated, HIV-1 antigen specific IFN-γ production was higher from CD8 cells than from CD4 or natural killers cells in the HR donors. However, the possible contribution of CD4 cells to resistance due to the loss of double positive cells ($CD4^+$ $CD8^+$) in the depletion assay can not be excluded. Thus, it is possible that these two HR, HIV-1 seronegative individuals participating the *in vivo* SCID/beige mice challenge study may have also protective immunity developed in their CD4 T cell population. In addition, human CD8 mediated non-specific anti-HIV effects, such as chemokines, may also play a role in the protection of these high-risk individuals against HIV-1 infection *in vivo*. Further studies are necessary to characterize the possible mechanisms of CD8, CD4, or NK cell mediated resistance to HIV-1 infection.

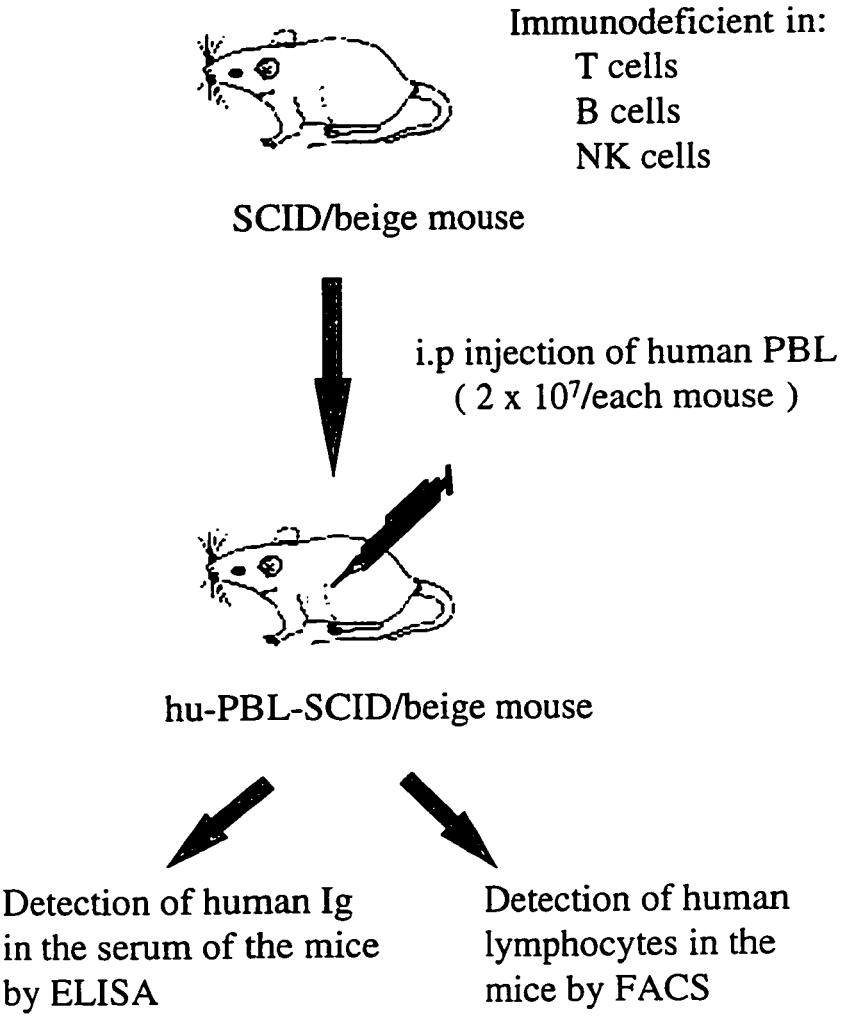


Figure 5.1 Schematic diagram of reconstitution of SCID/beige mice with human peripheral blood lymphocytes (PBL).

To develop hu-PBL-SCID/beige mice, 2 x 10^7 human PBLs were injected into SCID/beige mice intraperitoneally. At two weeks after PBL injection,the status of the reconstitution was determined by the detection of human Ig and lymphocytes in the mice.

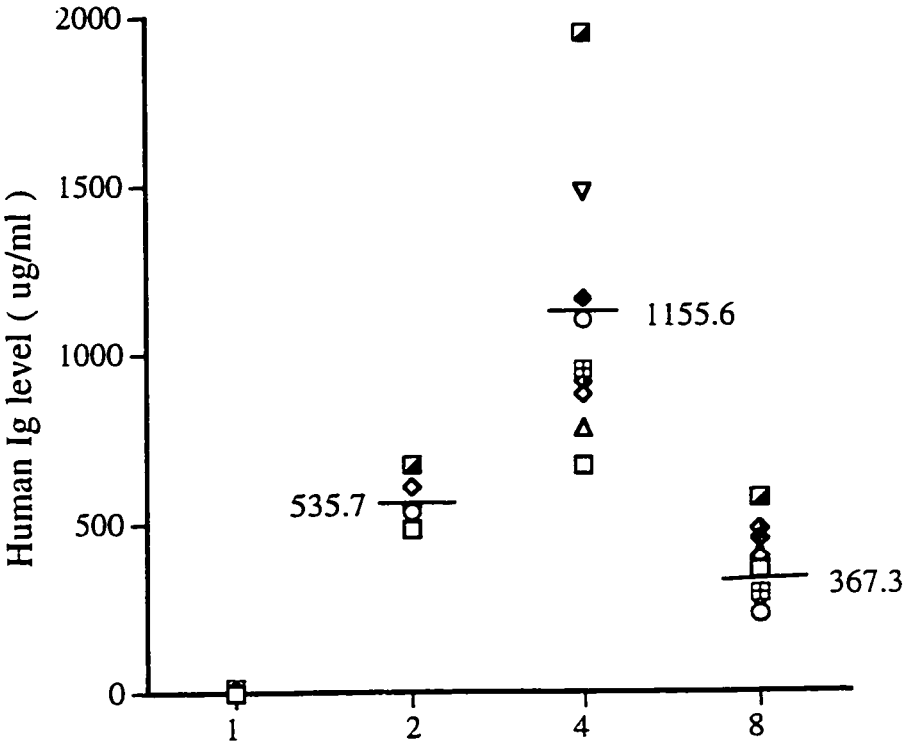


Figure 5.2 Human Ig in the peripheral blood of hu-PBL-SCID/beige mice

To measure the human Ig levels, tail blood samples were collected from the hu-PBL-SCID/beige mice at one, two, four, and eight weeks after PBL injection, and human Ig was detected by ELISA. The data represent the human Ig levels in ten different mice (one symbol permouse). Consistent results were obtained in two independent experiments in this study. The mean value of each group was shown.

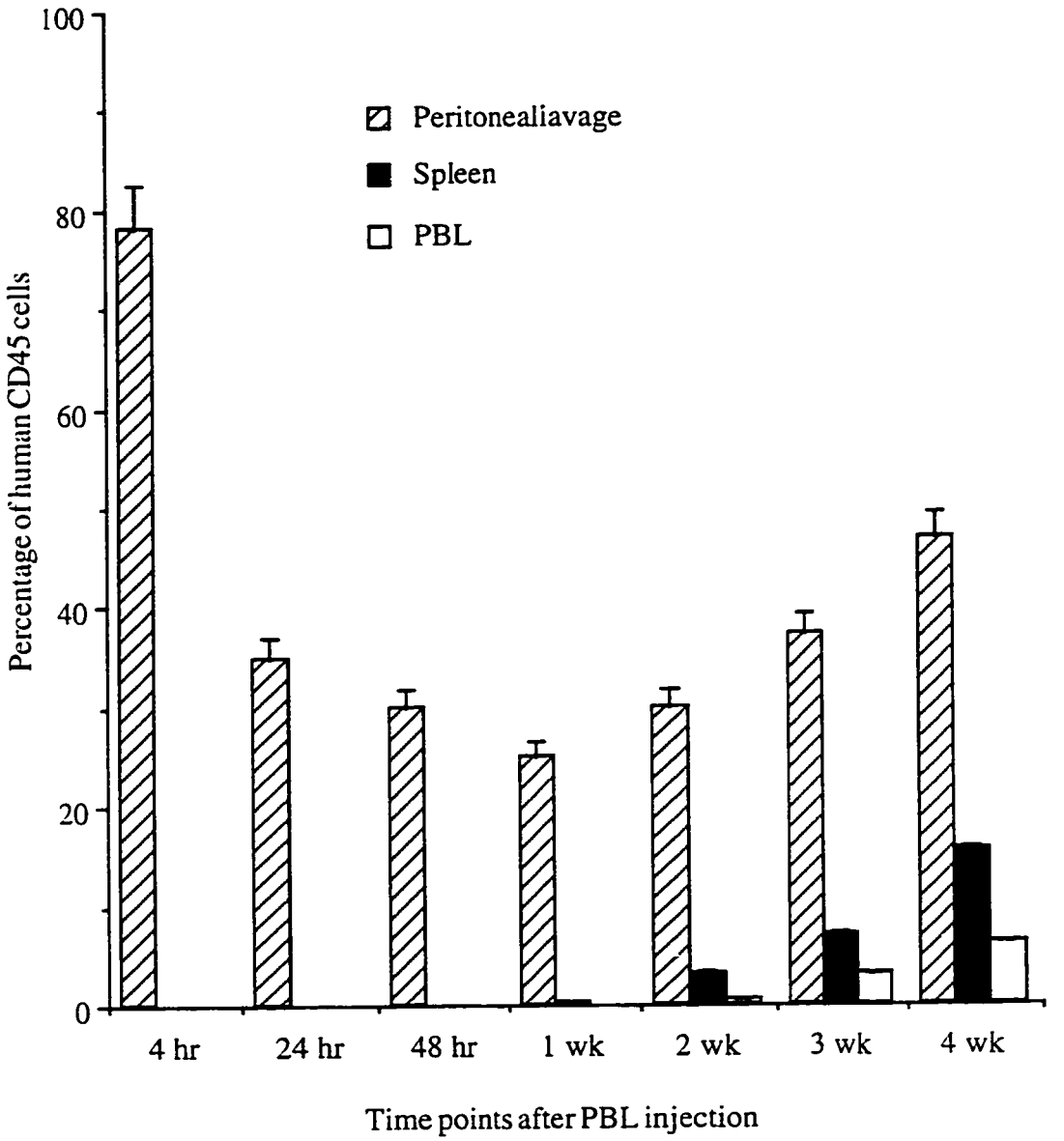


Figure 5.3. Kinetics of the distribution of human CD45 cells in the hu-PBL-SCID/beige mice

SCID/beige mice were reconstituted with human PBL and sacrificed at various time points as described in the text. Single cell suspensions consisting of human and mouse cells were prepared from peritoneal lavage, spleen, and peripheral blood of the mice and stained with human CD45 and mouse H2kD antibodies. Three mice were sacrificed at each time point and the data were shown as mean ± SD.

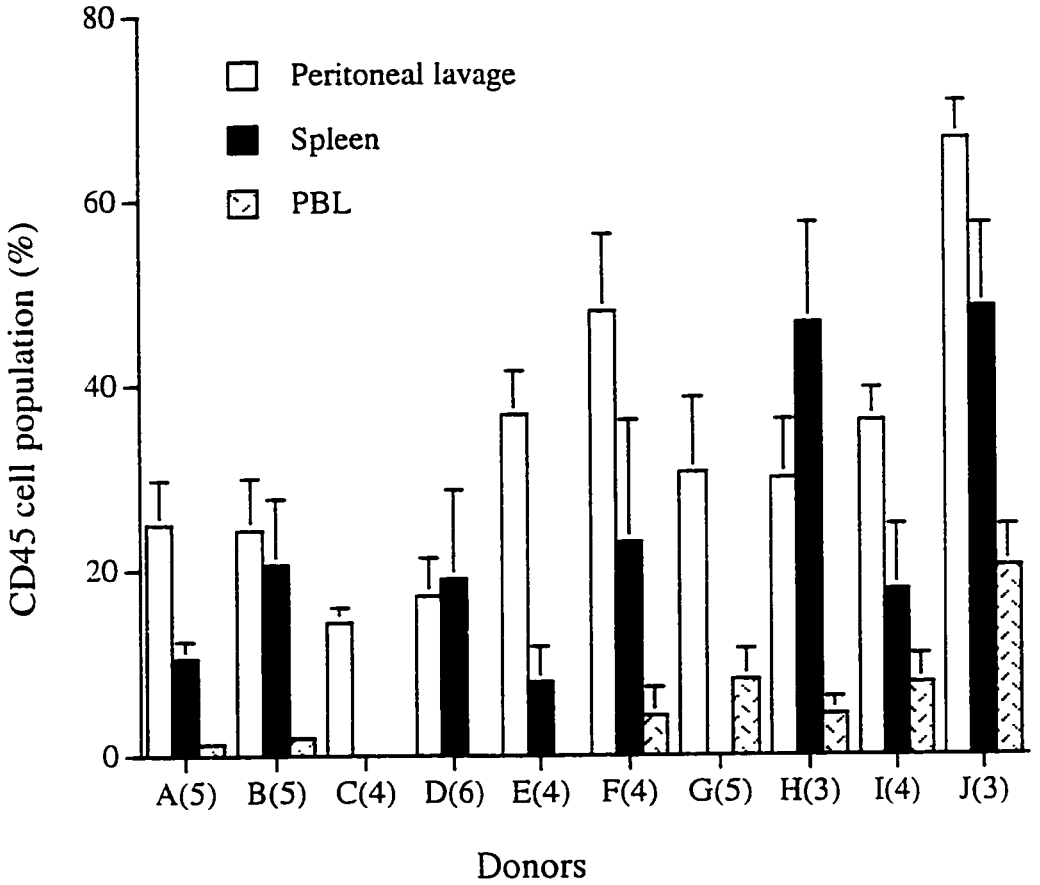


Figure 5.4 Distribution of human CD45 cells in the hu-PBL-SCID/beige mice four weeks after reconstitution.

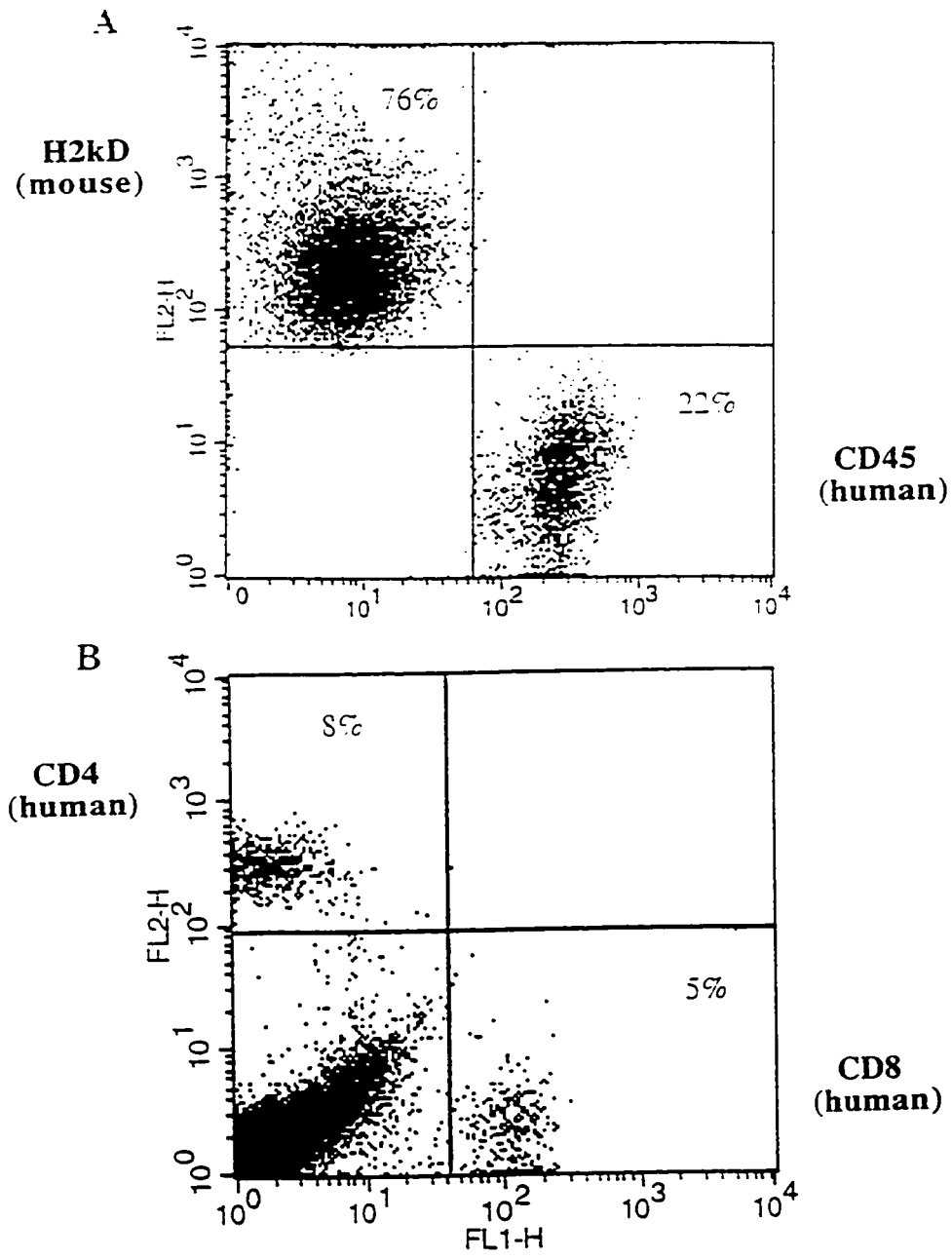
To detect the distribution of human CD45 cells in the hu-PBL-SCID/beige mice, single cell samples were prepared from the peritoneal lavage, spleen, and peripheral blood of the mice at four weeks after PBL injection, and analysed by flow cytometry. The numbers in the parenthesis on the x-axis (Donors) indicate the number of mice used in a particular study. The percentage of CD45 cells was represented by the mean value and its standard deviation (SD) in each group.

Figure 5.5. Detection of human lymphocytes in the spleen of hu-PBL-SCID/beige mice by flow cytometric analysis.

Hu-PBL-SCID/beige mice were sacrificed 4 weeks after human PBL injection, single cell suspensions were prepared from the peritoneal lavage, spleen, or peripheral blood, and stained with anti-mouse H2Kd, anti-human CD45, anti-human CD4 or anti-human CD8 as described in the Materials and Methods.

A. Human CD45 lymphocytes in the spleen of the mice. CD45 cells were also detected in the peritoneal lavage and peripheral blood.
B. Human CD4 and CD8 T cell subsets in the spleen of the mice. CD4 and CD8 T cells were also detected in the peritoneal lavage and peripheral blood.

C. Percentage of human CD4 and CD8 cells in the spleen of hu-PBL-SCID/beige mice. Except mouse #6, all of the hu-PBL-SCID/beige mice in this study had a CD4/CD8 ratio > 1.0.


A
H2kD
(mouse)
76%
22%
CD45
(human)
FL2-H
B
CD4
(human)
8%
5%
CD8
(human)
FL1-H

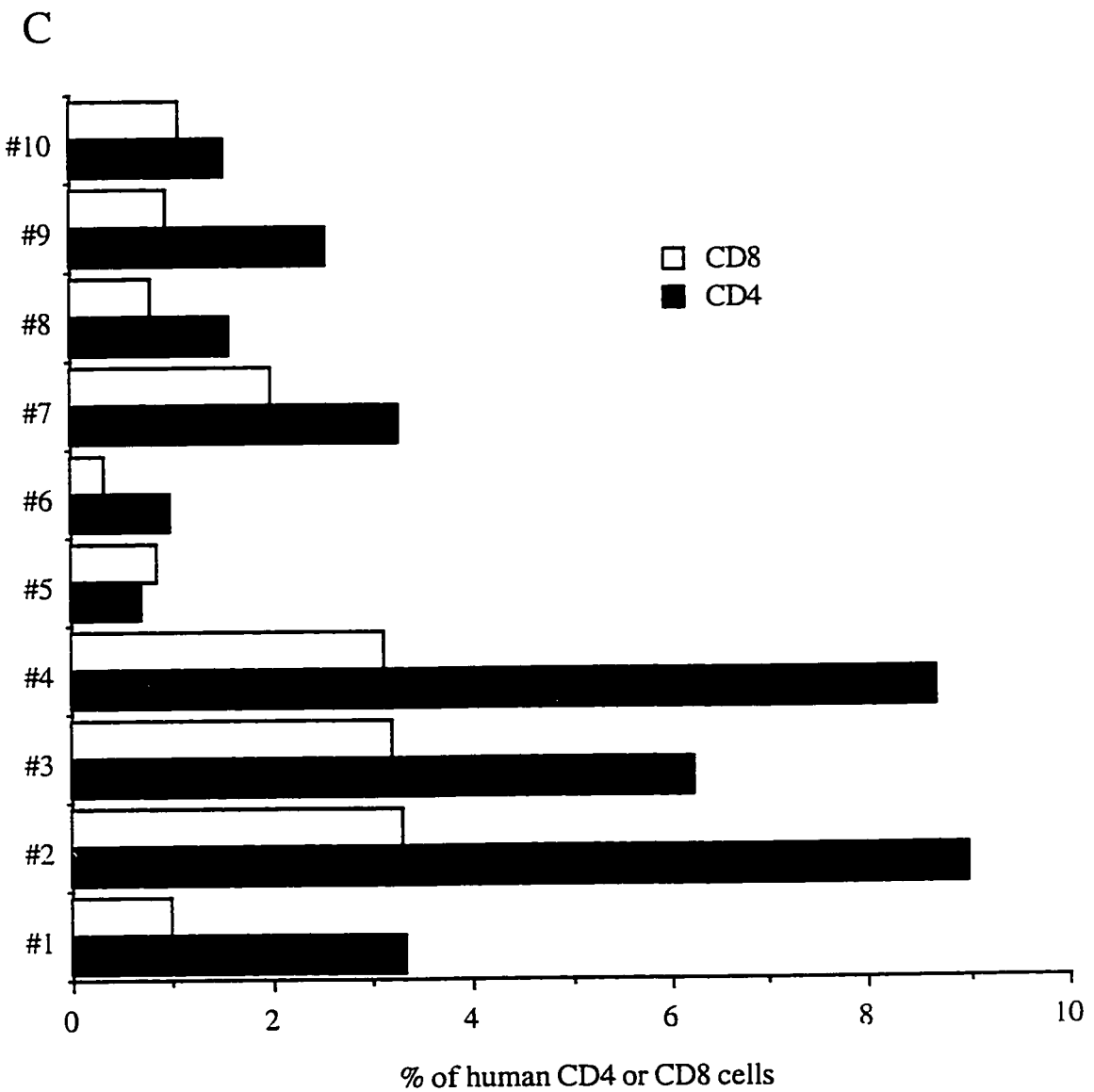


Figure 5.5C. Percentage of human CD4 and CD8 cells in the spleen of hu-PBL-SCID/beige mice four weeks after PBL reconstitution.

Figure 5.6. Immunostaining of cells from the control and HIV-1 infected hu-PBL-SCID/beige mice.

Hu-PBL-SCID/beige mice were infected with 100 $TCID_{50}$ of HIV_{NLAD8}. The mice were sacrificed two weeks post infection, and single cell suspensions were prepared from peritoneal lavage, spleen, and peripheral blood, and then fixed and immunostained with an AIDS patient's serum as described in the Materials and Methods.

A. Cells from peritoneal lavage of mock-infected mice (x400).

B. Cells from peritoneal lavage of the infected (x400).

C. Splenocytes from the infected mice (x400).

D. Peripheral blood leukocytes from the infected mice (x400).

Similar results were obtained when hu-PBL-SCID mice were infected with HIV_{NL4-3}.

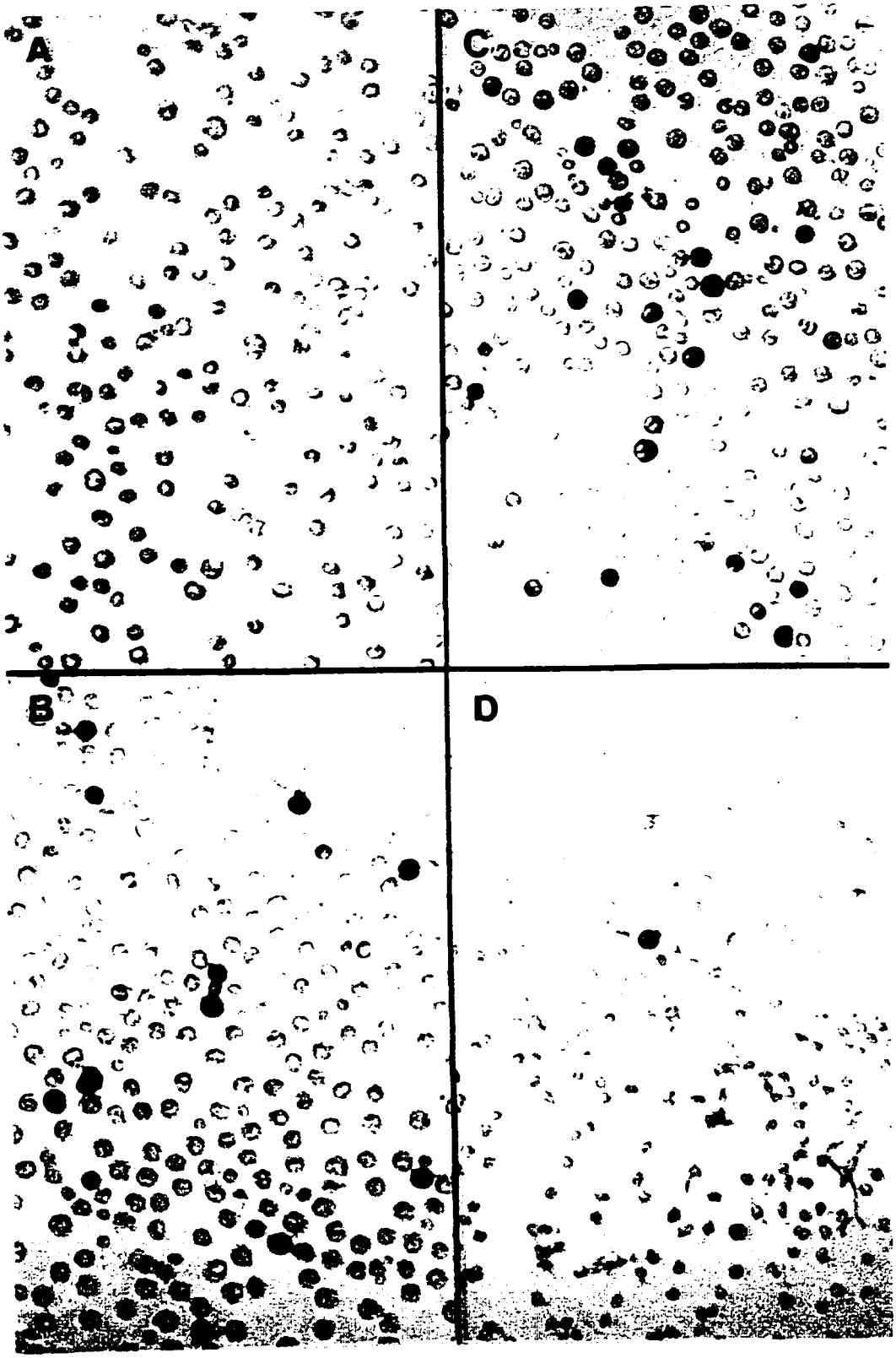

A
C
B
D

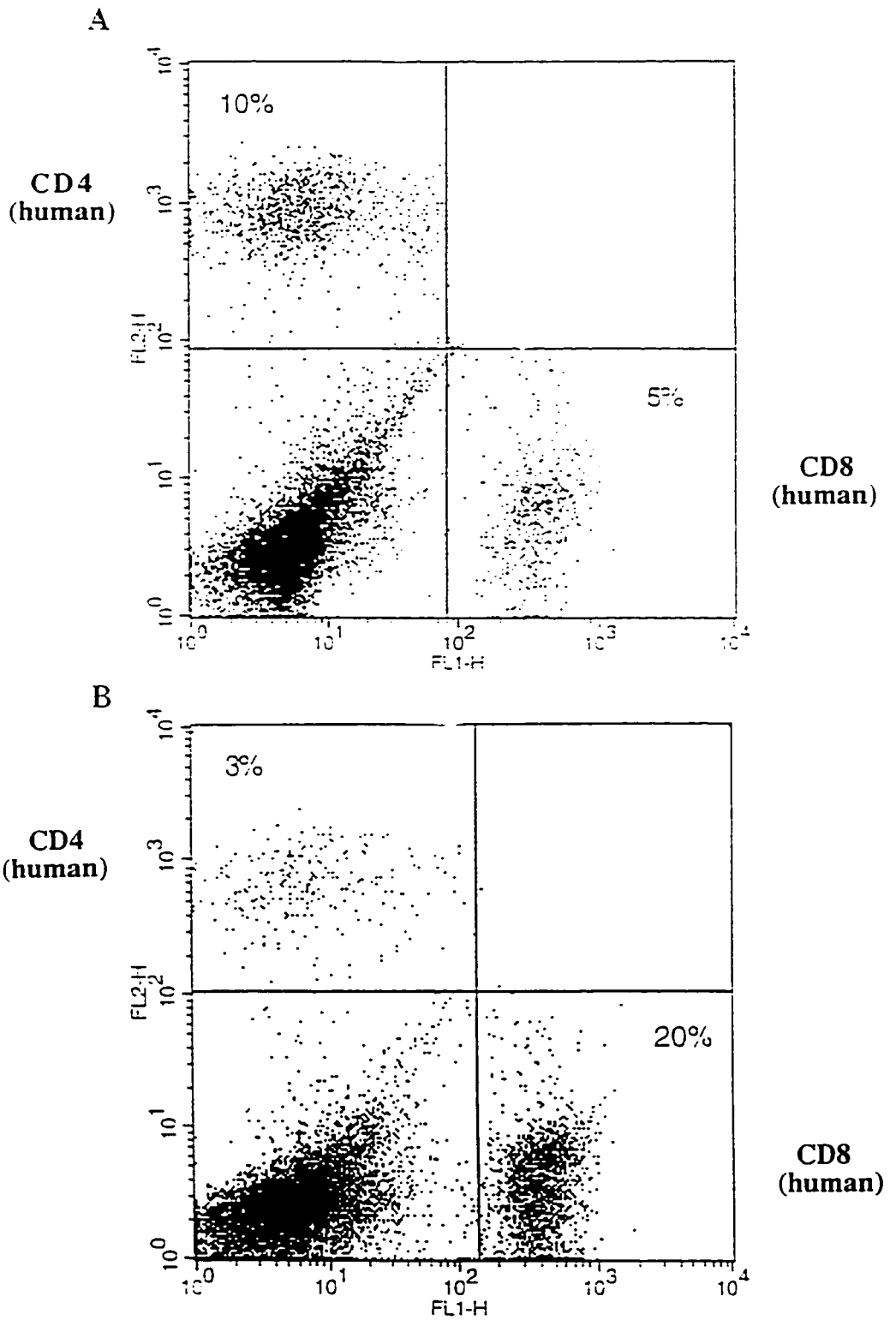
Figure 5.7. Reversal of CD4/CD8 ratio in the HIV-1 infected hu-PBL-SCID/beige mice by flow cytometric analysis.

The mock-, or HIV-1 infected hu-PBL-SCID/beige mice were sacrificed at 2 weeks after challenge. Single cell suspensions were prepared from the peritoneal lavage, spleen, or peripheral blood, and stained with anti-mouse H2Kd, anti-human CD45, anti-human CD4, and anti-human CD8 as described in the Materials and Methods.

A. Cells from the peritoneal lavage of mock-infected hu-PBL-SCID/beige mice. A normal CD4/CD8 ratio (> 1.0) was observed (also in Figure 5.5). A similar ratio was observed with spenocytes.

B. Cells from the peritoneal lavage of HIV-1 infected hu-PBL-SCID/beige mice. A reversed human CD4/CD8 ratio (< 1.0) was observed. A similar ratio was observed with spenocytes.

C. Reversal of CD4/CD8 ratio in the HIV-1 infected hu-PBL-SCID/beige mice.
Control mice: Mock-infected, 10 mice in total .
(one symbol per mouse).
Mean value and SD (2.3 ± 0.6) were shown.
HIV-infected mice: 10 mice in total.
(one symbol per mouse).
Mean value and SD (0.6 ± 0.3) were shown.


A
CD4
(human)
10%
5%
CD8
(human)
FL2-H
FL1-H
B
CD4
(human)
3%
20%
CD8
(human)
FL2-H
FL1-H

C

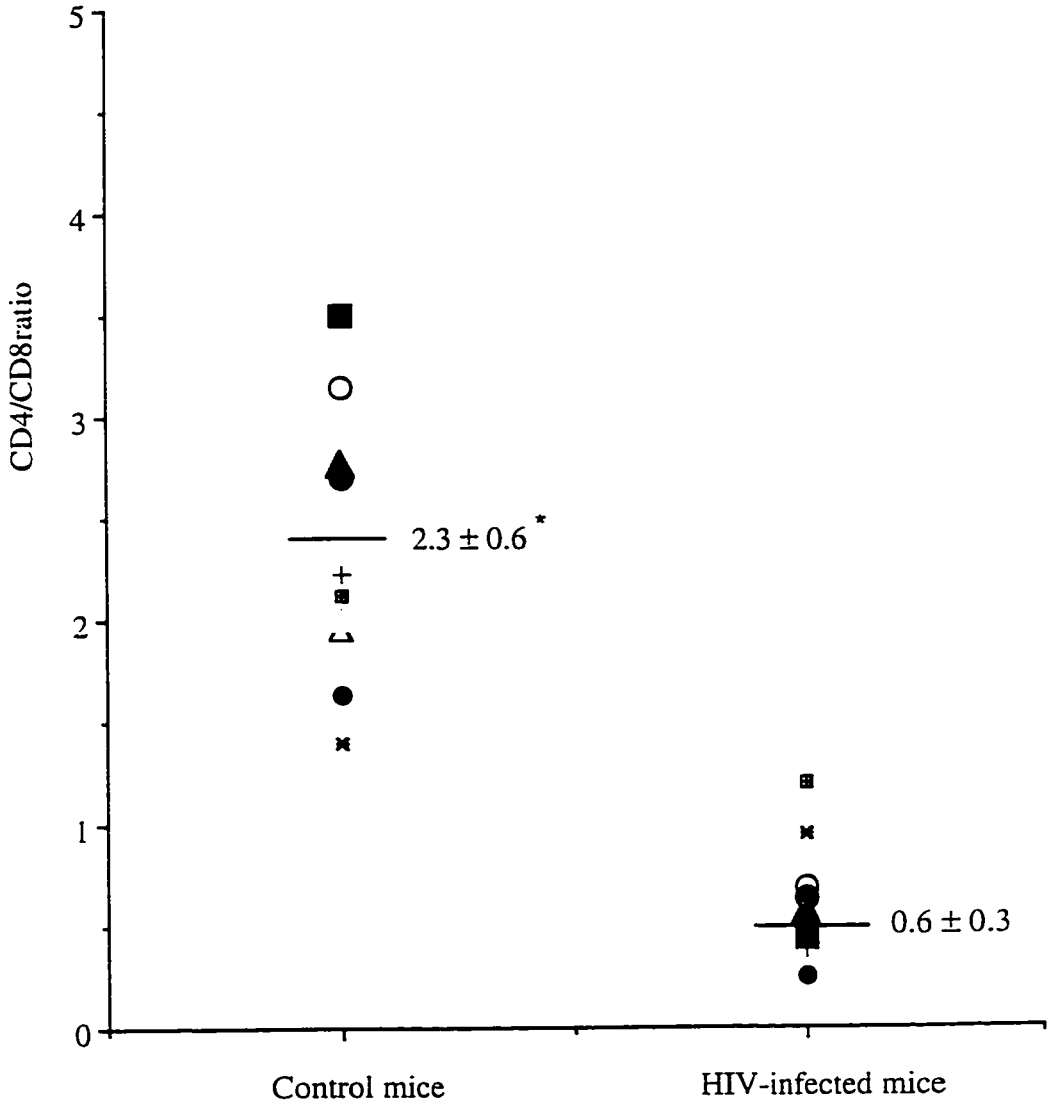


Figure 5.7C. Reversal of CD4/CD8 ratio in the HIV-1 infected hu-PBL-SCID/beige mice.

* There is significant difference in the CD4/CD8 ratio between the control and HIV-infected mice ($P < 0.01$, t-test).

Figure 5.8. PBLs from LR and HR seronegative individuals were susceptible to infection by both T cell- and macrophage-tropic HIV-1 *in vitro*.

Heparinized PBLs were collected from HR and LR HIV-negative donors and infected with different cytotropic strains of $HIV\text{-}1_{NL4\text{-}3}$ as described in the Materials and Methods.

A. mock-infected PBLs (x400).

B. Infection of PBLs from HR-1 with HIV_{NLAD8} at 0.2 MOI (x400).

Similar results were obtained when the PBLs from HR-2, LR-1, and LR-2 were infected with HIV_{NLAD8} or $HIV_{NL4\text{-}3}$.

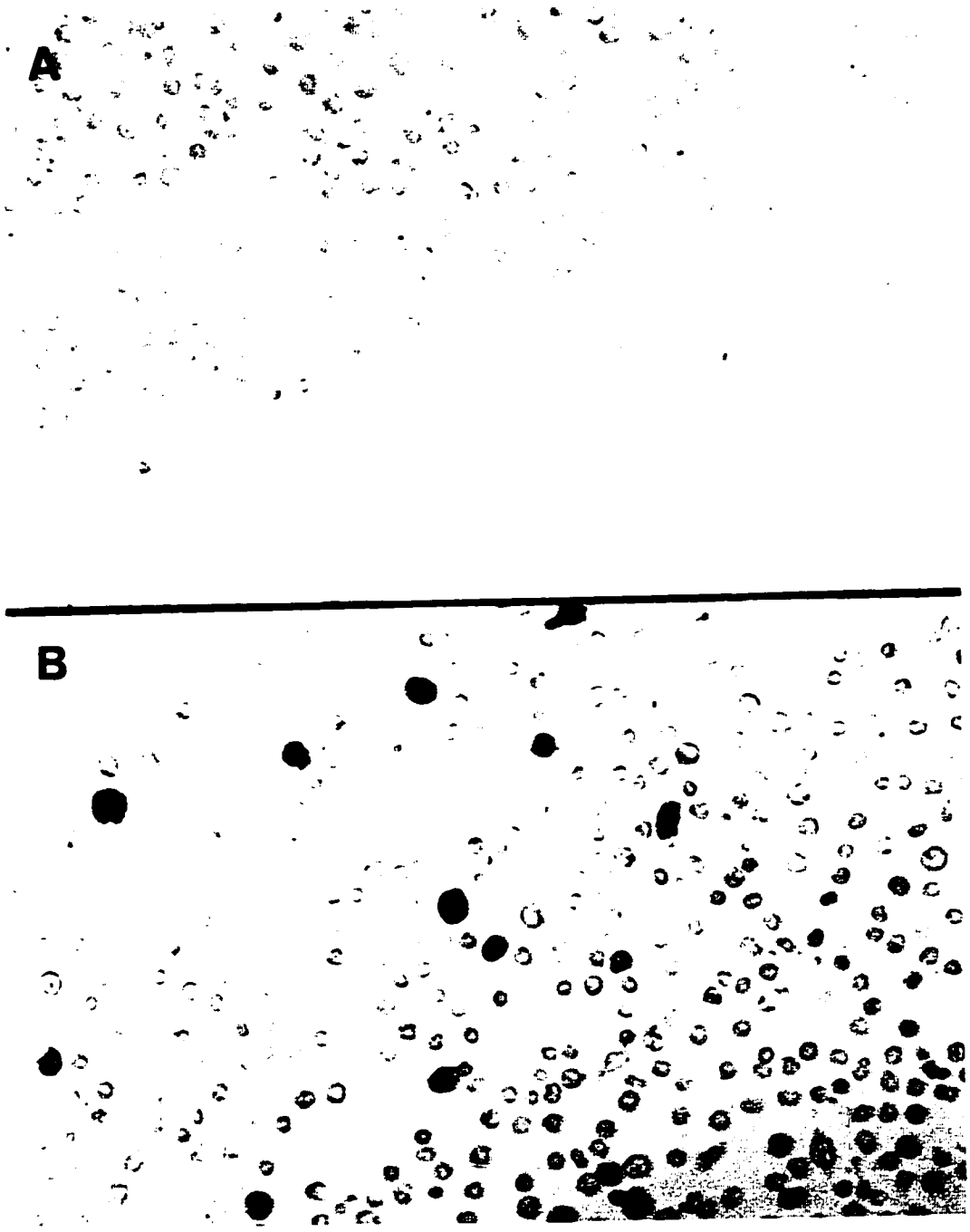

A
B

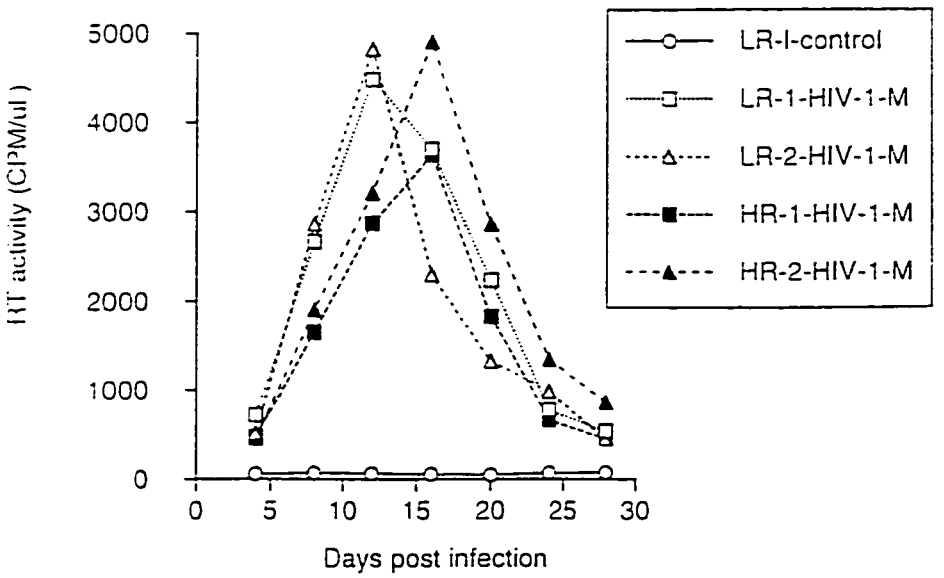
Figure 5.9. Infection of PBLs from low-risk (LR) and high-risk (HR), seronegative individuals with M-tropic HIV-1.

Heparinized PBLs were collected from HR and LR HIV-negative donors and infected with different cytotropic strains of HIV-1_{NL4-3} as described previously.

A. Infection of PBLs from two low-risk (LR-1 and LR-2) and two high-risk (HR-1 and HR-2) seronegative individuals with HIV_{NLAD8} (macrophage-tropic, M) at 0.2 MOI. Similar results was obtained with the T cell-tropic strain HIV-1_{NL4-3}.

B. Infection of PBLs from a low-risk (LR-1) and a high-risk (HR-1) seronegative individuals with HIV_{NL4-3} at MOI of 0.001 to 0.0001. The MOI 0.005 is equivalent to a $TCID_{50}$ of 100.

A


RT activity (CPM/ul)
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
30
Days post infection
LR-I-control
LR-1-HIV-1-M
LR-2-HIV-1-M
HR-1-HIV-1-M
HR-2-HIV-1-M

B

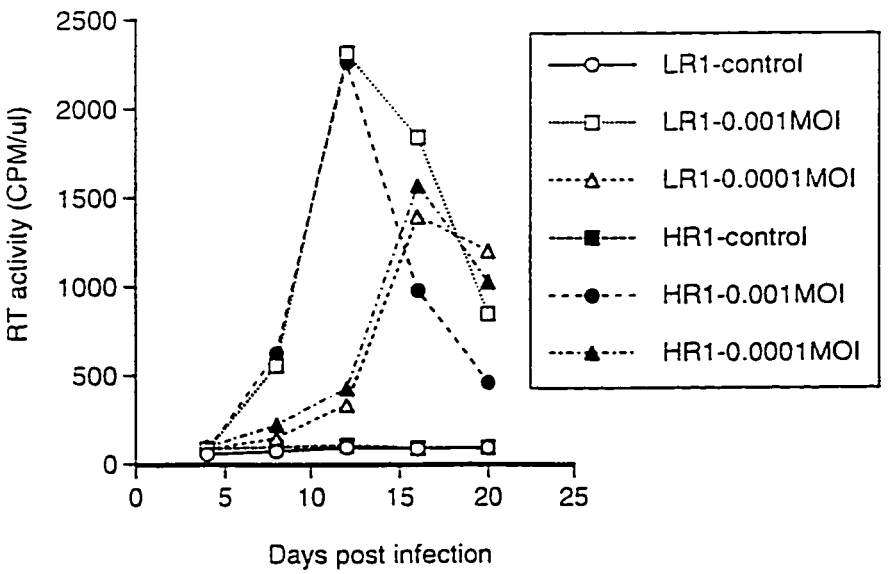
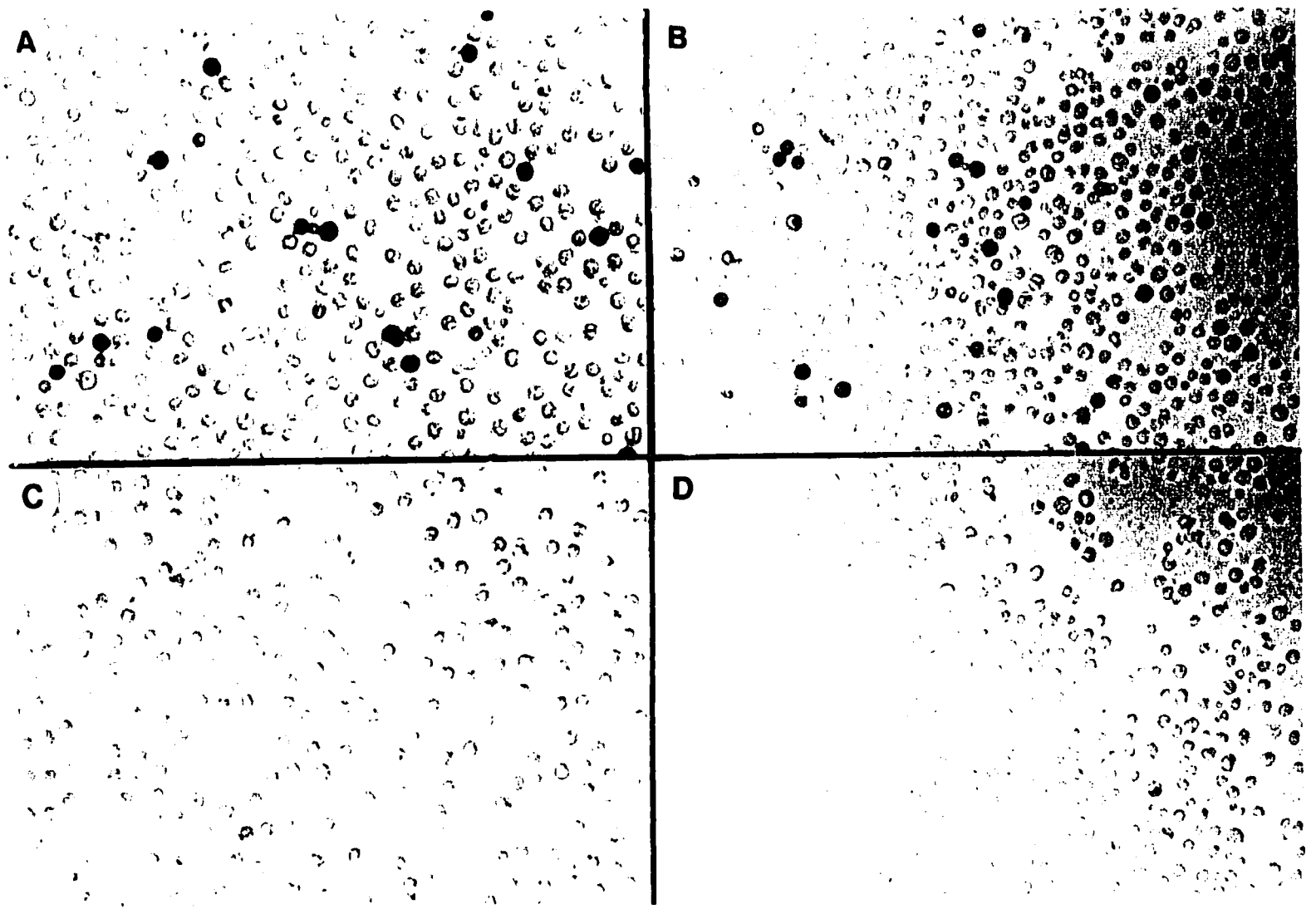

RT activity (CPM/ul)
2500
2000
1500
1000
500
0
0
5
10
15
20
25
Days post infection
LR1-control
LR1-0.001MOI
LR1-0.0001MOI
HR1-control
HR1-0.001MOI
HR1-0.0001MOI

Figure 5.10. *In vivo* infection of hu-PBL-SCID/beige mice.

Cell suspensions were prepared from the mice for immunohistochemical staining as described in the Materials and Methods.

A. & B. HIV-1-positive immunostaining of peritoneal lavage and splenocytes, respectively, of mice reconstituted from a low-risk individual (x400).

C. & D. HIV-1-negative immunostaining of peritoneal lavage and splenocytes, respectively, of mice reconstituted from a high-risk individual (x400).


A
B
C
D

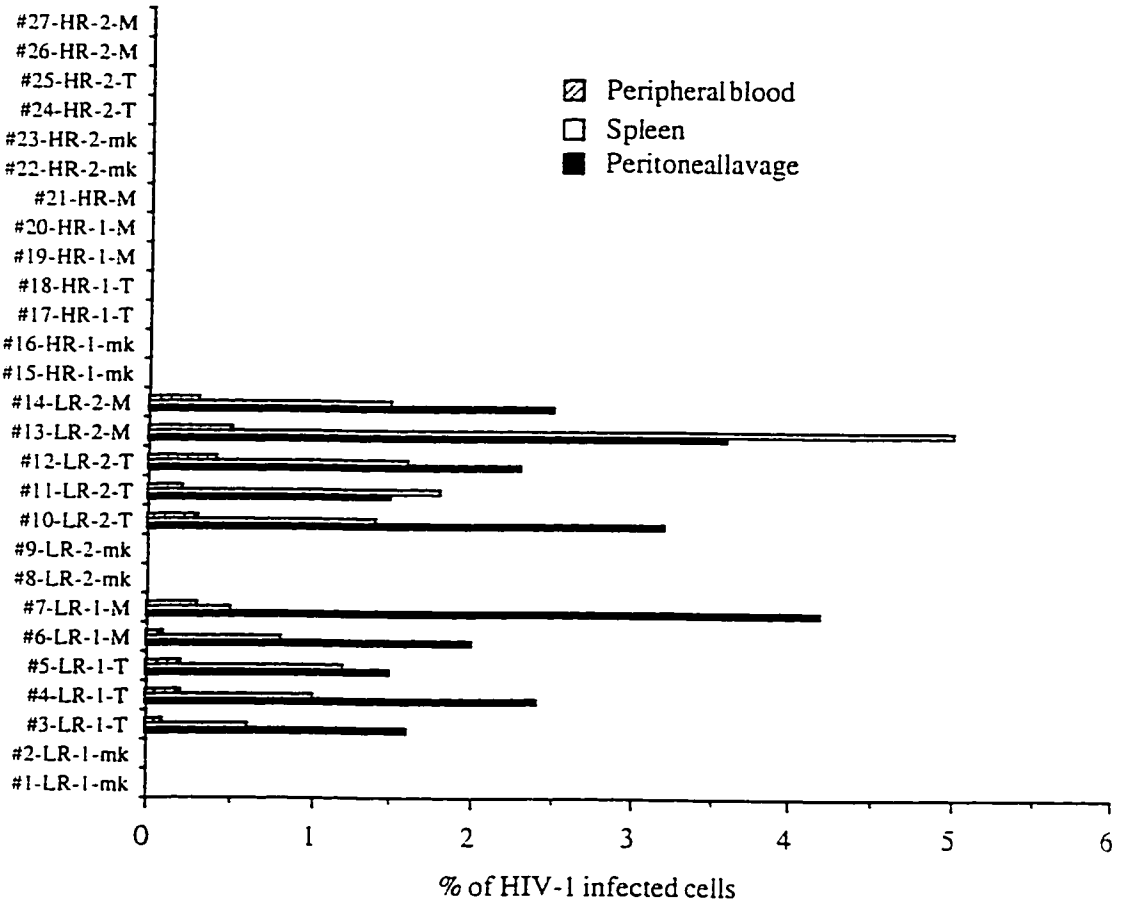


Figure 5.11. T cell and macrophage-tropic HIV-1 challenge of hu-PBL-SCID/beige mice reconstituted with low- and high-risk (LR and HR) donors' PBLs.

Reconstitution of mice with PBLs from LR-1 donor (#1-#7), the LR-2 donor (#8-#14), the HR-1 donor (#15-#21), and the HR-2 donor (#22-#27) were confirmed by ELISA for human Ig and FACS analysis for the human CD45 leukocytes marker. The reconstituted hu-PBL-SCID/beige mice were challenged with 100 TCID50 of T-cell- (T) or macrophage-(M) tropic HIV-1 by i.p. injection. All the mice were sacrificed four weeks after reconstitution, and single cell suspensions were prepared from the three different organ compartments for immunostaining. HIV-1-positive cells were counted under an inverted microscope and the percentage of infected cells was determined by taking the average of more than three representative counts of 1000-10,000 cells as previously described in the text.

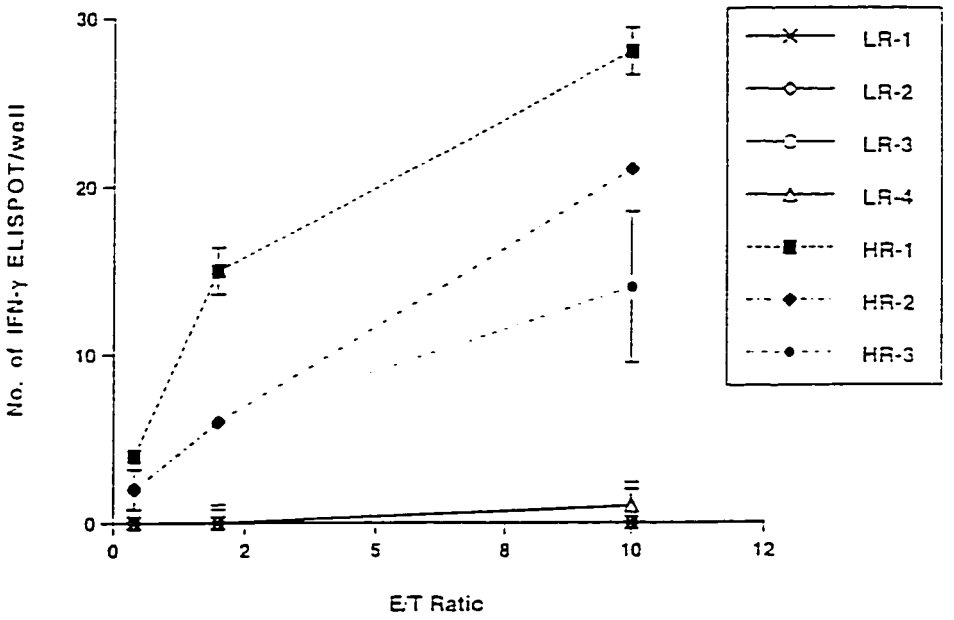


Figure 5.12. ELISPOT analysis of HIV-1-specific IFN-γ production in PBLs of HR and LR uninfected individuals.

ELISPOT assays were performed as described in the Materials and Methods. The number of IFN-γ producing cells was determined using an computerized video-imaging system as described previously (Cui and Chang, 1997). HIV-1-specific ELISPOT was shown as the mean ± standard error in triplicate wells after subtracting the background from controls which were prepared using the same effector cells with autologous HIV-1-negative PBLs and went through the same treatment as described. The frequencies of HIV-specific IFN-γ producing cells in the three high-risk donors (HR-1, HR-2, and HR-3) are significantly different ($P < 0.05$, t-test) from that of the four low-risk donors (LR-1, LR-2, LR-3, and LR-4).

Figure 5.13. Depletion of human CD8 T cells in the hu-PBL-SCID/beige mice.

Human CD8 T cells were depleted in the hu-PBL-SCID/beige mice as described in the Materials and Methods. To confirm the depletion of human CD8 T cell, cell suspensions were prepared from the spleen of the untreated or treated mice, and the samples were analyzed by FACS.

A and **B**, Untreated mice.

A: Huamn CD45 (13.8%).

B: Human CD4 (6.8%).

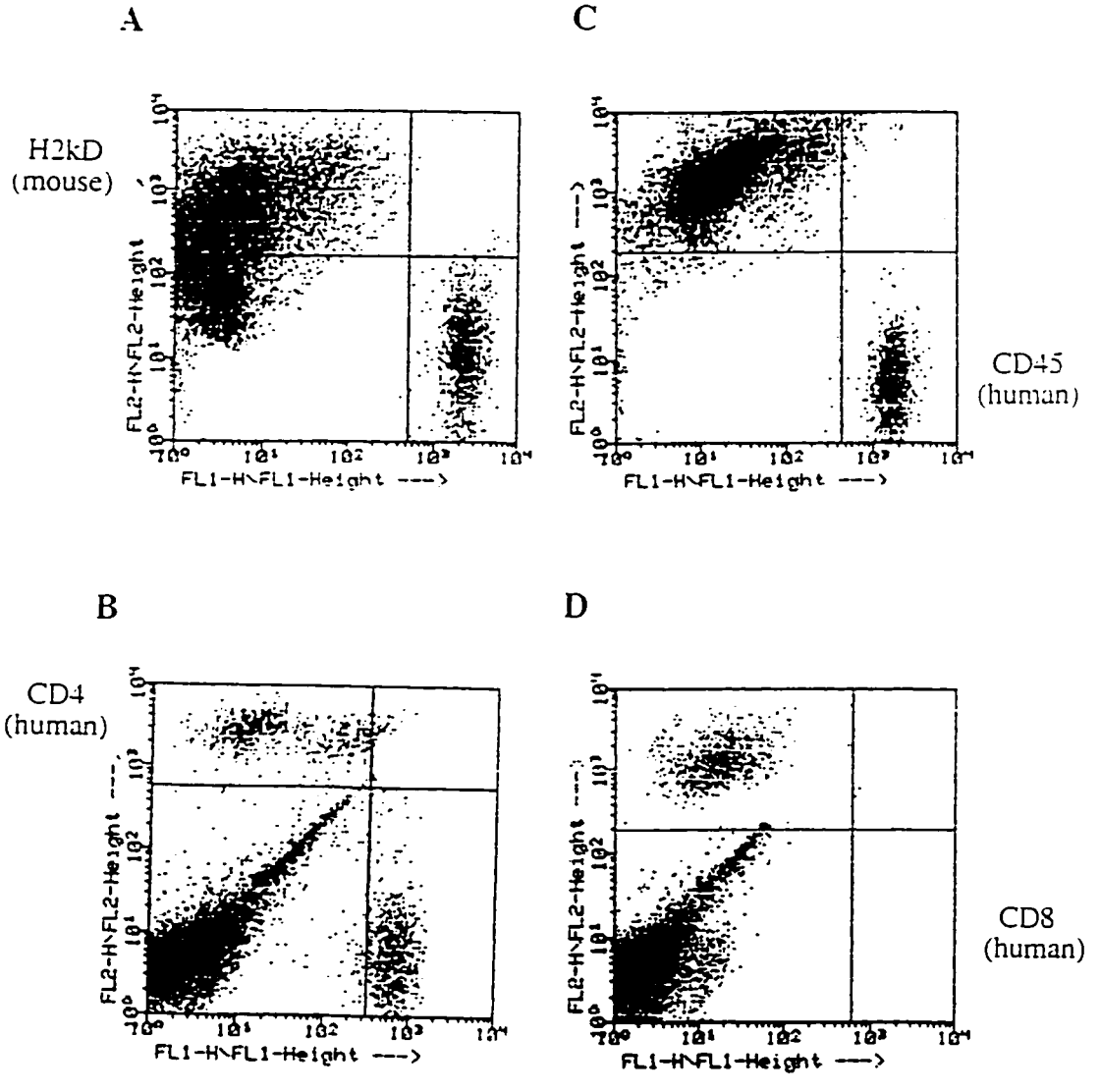
Human CD8 (8.2%).

C and **D**, Anti-CD8 antibody treated mice.

C: Human CD45 (11.65).

D: Human CD4 (8.3%).

Human CD8 (0.1%)


A
C
H2kD
(mouse)
CD45
(human)
B
D
CD4
(human)
CD8
(human)
FL2-H\FL2-Height --->
FL1-H\FL1-Height --->

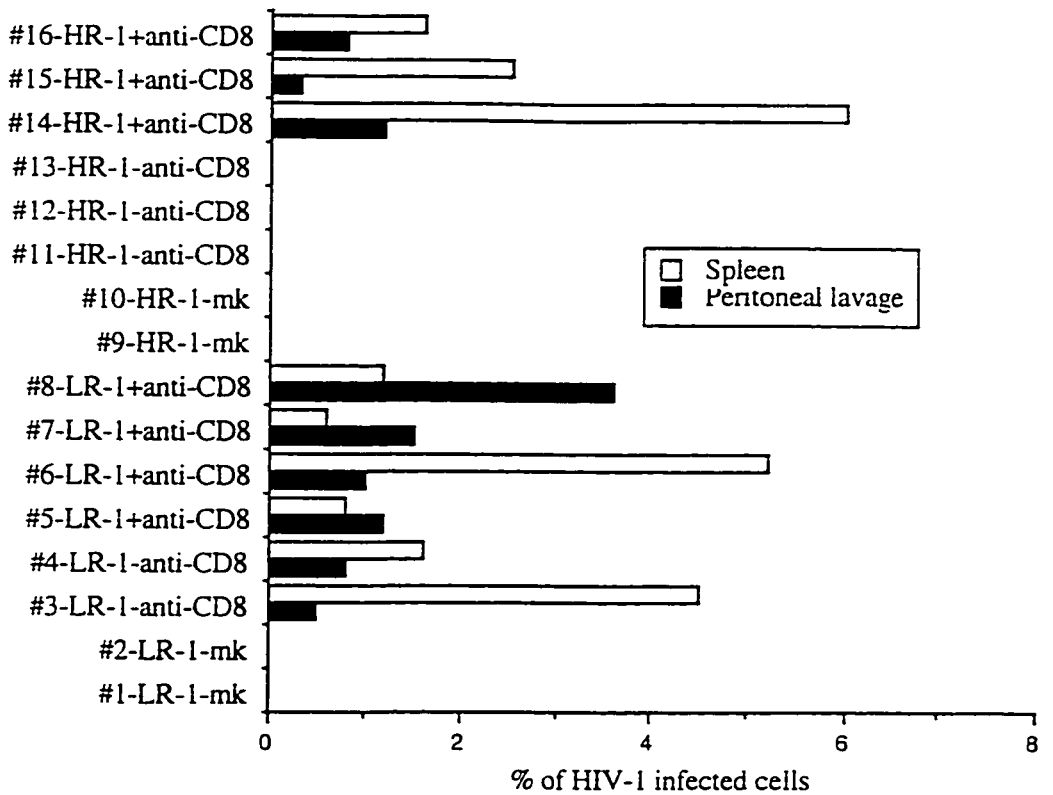


Figure 5.14. HIV-1 infection of human lymphocytes in hu-PBL-SCID/beige mice treated or untreated with anti-CD8 antibody.

Infection of SCID/beige mice reconstituted with HR-1 PBLs or LR-1 PBLs after CD8 T cell depletion. CD8 T cells were depleted by five sequential i.p. injections with 20 μg of purified OKT8 monoclonal antibody. The reconstituted mice, untreated (-anti-CD8) or anti-CD8 treated(+anti-CD8) as indicated, were infected with HIV-1_{NLAD8}, and the percentage of infected cells was determined in the spleen and in the peritoneal lavage two weeks after virus challenge as described in the Materials and Methods. The mock-infected (mk) mice were not treated with anti-CD8 antibody.

Table 5.1 Comparison of murine and human Ig levels, and percentage of human $CD45^+$ cells in peritoneal lavage of the hu-PBL-SCID/beige mice*

Mice #	Murine Ig (μg/ml)	Human Ig (μg/ml)	$CD45^+$ cell (%)
"Leaky mice"			
1	1.58	2250.22	56.00
2	5.38	1478.59	71.91
3	4.53	528.77	42.00
4	1.71	551.60	22.84
5	3.80	307.77	24.60
Mean ± SD	3.4 ± 1.7	1024 ± 820	43.6 ± 20.8
" Non-leaky mice"			
1	0.08	1161.78	72.00
2	0.00	714.47	59.24
3	0.00	534.19	31.01
4	0.23	1949.27	67.00
5	0.00	496.61	22.00
Mean ± SD	0.062 ± 0.10	971 ± 543	50.2± 22.4
t-test	$P < 0.01^a$	$P > 0.05^b$	$P > 0.05^c$

* Both " leaky" and "non-leaky" SCID/beige mice were reconstituted with 2×10^7 human PBL from the same donor. The mice were sacrificed 4 weeks after PBL injection, and human and mouse Ig as well as human CD45 cells were examined as described in the text.

a: indicates significant difference in mouse Ig levels between the two group of mice.

b: indicates no significant difference in human Ig levels between the two group of mice.

c: indicates no significant difference in the percentage of human CD45 cells between the two group of mice.

Chapter VI

General discussion and future direction

General discussion

While a tremendous volume of knowledge has been accumulated on the basic molecular biology of HIV-1 and its related lentiviruses since HIV-1 was identified in 1984, many critical issues regarding to the pathogenesis of HIV-1 infection *in vivo* remain unknown. The finding that SIV Nef plays a crucial role in the pathogenesis of SIV-induced simian AIDS has dramatically changed some previous thoughts on the potential roles of HIV or SIV accessory proteins in the pathogenesis of HIV or SIV infection (Daniel et al., 1992; Kestler et al., 1991). In fact, increasing evidence suggests that HIV-1 accessory proteins may play an important role in viral replication and pathogenesis (Trono, 1995). In addition to Nef, another accessory protein, referred to as Vpr, has also been suggested to play a vital role in HIV and SIV viral replications and possibly in pathogenesis as well (Emerman, 1996; Subbramanian and Cohen, 1994).

Vpr and HIV-1 infection : One of the key questions regarding the pathogenesis of HIV infection is how HIV-1 infects $CD4^+$ T cell and kills the infected cells *in vitro*, or induces $CD4^+$ T cells depletion *in vivo*. HIV-1 can infect a variety of human $CD4^+$ cell lines and primary PBL, and often results in acute infection and cell death. Several mechanisms including syncytium formation, accumulation of unintegrated HIV DNA, toxicity of viral proteins, interference of cellular signal transduction, and induction of apoptosis have been proposed to cause cell killing by HIV infection (Pang et al., 1990;

Tang et al., 1992; Stevenson et al., 1988; Sabatier et al., 1991; Hofmann et al., 1990; Somasundaran and Robinson, 1987; Bergeron and Sodroski, 1992; Cohen et al., 1992; Groux et al., 1992; Laurent-Crawford et al., 1991). On the other hand, persistent HIV-1 infections have been established in some lymphoid cell lines (Folks et al., 1987; Levy, 1993 ; Poli et al., 1990). Previous studies suggested that mutations in the HIV-1 accessory genes, such as vpr, vif, vpu, and nef, may contribute to the survival of $CD4^+$ cells from an acute HIV-1 infection (Nishino et al., 1991; Kishi et al., 1992; Nishino et al., 1994). Other studies indicate that HIV-1 vpr mutations may play an important role in the establishment of persistent infection (Mustafa, et al., 1993; Nakaya et al., 1994; Kishi et al., 1995). In this study, we have shown that Vpr expression was absent in all the chronically infected cell cultures (Figure 2.9) and that some of the vpr clones amplified from these cell lines have been found to have a novel stop codon at the N-terminal region of vpr. There is accumulating evidence indicating that Vpr expression may prevent cell proliferation during chronic infection (Planelles et al., 1995; Rogel et al., 1995). However, it is not completely understood how HIV-1 Vpr prevents the establishment of chronically infected cell lines, inhibits cell growth, and induces cell differentiation in some tumor cell lines (Levy et al., 1993).

Vpr and cell cycle arrest: The above-mentioned effects of Vpr expression on cell growth and proliferation may be related to HIV-1 Vpr-induced cell cycle arrest at G2/M in mammalian cells (Re et al., 1995). In this study (Chapter III), we have demonstrated that HIV-1 Vpr can also arrest cell cycle at G2/M in *S. pombe*. We also found that HIV-2/SIV Vpr and Vpx affect cell cycle progression in *S. pombe*, an observation contrary to other studies indicating that Vpx has no obvious effect on cell cycle progression (Fletcher, et al., 1996; Planelles, et al., 1996). It has been shown that the effects of Vpr or/and Vpx on cell proliferation are cell-type dependent. HIV-1 Vpr has been shown to induce cell cycle arrest in both human and simian cells. The G2

arrest induced by HIV2/SIVmac Vpr in human cells was attenuated compared with that of HIV-1 Vpr. In addition, SIVagm Vpr arrested simian cells but not human cells in G2 (Plannelles et al., 1996; Stivahtis et al., 1997). Vpr cloned from the primary isolates of HIV-2 could efficiently arrest human cells at G2 (Stivahtis et al., 1997). Vpr of SIVsm, which is most closely related to HIV-2, causes partial G2 arrest in human cells, whereas the SIVagm and SIVsyk Vpr are completely unable to arrest human cells. In contrast, all SIV Vpr have been shown to induce G2 arrest in African green monkey cells. In the HIV-2/SIVsm lineage, which have both Vpr and Vpx, only Vpr has been shown to arrest cell cycle at G2, while Vpx, but not Vpr, is required for efficient infection of macrophages (Marzio et al., 1995; Fletcher et al., 1996; Kewalramani et al., 1996). In this study, we have shown that HIV-1 Vpr can completely arrest cell cycle in yeast. HIV-2/SIVmac Vpr and Vpx have similar cell cycle arrest in yeast but have a attenuated phenotype compared with HIV-1 Vpr. It is not clear why Vpx is able to arrest cell cycle in yeast but not in mammalian cells. Taken together, all these data suggest that the Vpr or Vpx induced cell cycle arrest may be dependent on the availability of suitable cellular targets in the host cells. In other words, the species specificity of Vpr functions are more likely dictated by species-specific cellular substrates.

Vpr and its cytotoxic effects: While HIV-1 Vpr has been consistently demonstrated to be able to induce cell cycle arrest in yeast and mammalian cells, the fate of the arrested cells is still uncertain. In this study, we have clearly shown that HIV-1 Vpr could inhibit colony formation in yeast cells expressing Vpr, indicating Vpr is lethal to *S. pombe*. Our data is consistent with other studies showing that expression of HIV-1 Vpr could inhibit colony formation in *S . cerevisiae* and *S. pombe* (Macreddie et al., 1996; Zhao et al., 1996). Previous studies have also shown that a region of Vpr containing H (S/F) RIG is critical for Vpr-induced growth arrest and structural defects

in *S. cerevisiae* (Macreadie et al., 1995). Further studies have demonstrated that addition of Vpr peptides containing the H (S/F) RIG amino acid sequence motif can permeabilize cell membranes and kill a variety of yeast cells including *S. cerevisiae*, *S. pombe*, *Candida glabrata*, and *Candida albicans*. A similar toxicity induced by these peptides have also been observed in human cells (Macreadie et al., 1996). HIV-1 induced alternations of plasma membranes have been proposed to play an important role in AIDS pathology (Garry, 1989). Interestingly, it has been shown that HIV-1 Vpr can form cation-selective channels in planar lipid bilayers *in vitro*. This suggests that Vpr may form ion channel across cell membrane, change cell permeability, and thereby disrupt normal ion concentration gradients essential for normal cell function (Piller et al., 1996). It is noteworthy that the M2 protein of influenza virus is able to form a cation-selective channel that has a vital role in viral replication (Pinto et al., 1992). The finding that HIV-1 Vpr could form a cation-selective channel suggests that Vpr may also play such a role in HIV-1 replication. In addition, the peptides of two other HIV-1 proteins, Vpu and gp41, have also been shown to affect cell membrane permeability, a property considered to be important for viral replication (Carrasco, 1995). In addition to its potential effects on cell membranes, HIV-1 Vpr has recently been demonstrated to disrupt actin cytoskeleton in the budding yeast *S. cerevisiae* (Gu et al., 1997). Taken together, HIV-1 Vpr may disrupt normal cellular functions and induce cytotoxicity by a variety of mechanisms.

Vpr and apoptosis: Apoptosis has been suggested to play an important role in the $CD4^+$ T cell depletion in AIDS patients (Terai et al., 1991). However, the mechanisms of HIV-induced apoptosis are not fully understood. HIV-1 Tat protein induces apoptosis in uninfected lymphocytes (Li et al., 1995). More recent studies show that HIV-1 Vpr also induces apoptosis in a variety of human cells including lymphocytes (Ayyavoo et al., 1997; Stewart et al., 1997; and L-J Chang et al., unpublished data).

Interestingly, HIV-1 Vpr can mimic glucocorticoids (GCs)-induced apoptosis in T cells (Ayyavoo et al., 1997; Ucker et al., 1987; Helmberg et al., 1995). Like GCs, Vpr induces apoptosis in T cells in the absence of T cell activation, but blocks TCR-triggered T cell death. It is noteworthy that activation-induced cell death in T cells has been shown to be dependent on the activation of cyclin B-p34/cdc2 kinase activity (Fotedar et al., 1995). Therefore, it makes sense that Vpr could block the activation-induced apoptosis in T cells since Vpr has shown to inactivate the p34/cdc2 kinase activity in human and yeast cells (Re et al., 1995; Zhang et al., 1997).

Correlation between cell cycle arrest and apoptosis: A recent study has also suggested that the Vpr-induced G2 arrest was required for Vpr-induced apoptosis to occur, and the extent of Vpr-induced G2 arrest correlated with the levels of apoptosis in human T cells (Stewart et al, 1997). Interestingly, in this study, we have demonstrated that the Vpr mutants which lost their effects on cell cycle arrest did not have cytotoxicity in *S. pombe* (Chapter III). On the other hand, HIV-1 Vpr has been shown to cause cell cycle arrest in both human (HeLa) and simian (CV-1 and Vero) cells, but only induces apoptosis in human cells, indicating that the Vpr-arrested cells may have different fates which may be dependent on the apoptotic machinery existing in the host cells. This may explain why Vpr-induced cell cycle arrest is not directly linked to viral pathogenicity. Vpr-induced G2 arrest seems not to affect viral pathogenicity. For instance, SIVagm Vpr could induce efficient G2 arrest in African green monkey cells, but it does not cause clinical disease in its natural host, suggesting that G2 arrest is not sufficient for viral pathogenicity (Stivahtis et al., 1997).

The potential role of Vpr in HIV-1 pathogenesis *in vivo*: Increasing evidence has indicated that Vpr may play a role in HIV-1 pathogenesis *in vivo*. The first evidence suggesting that Vpr may play a role in HIV-1 pathogenesis was from the

SIV-infected Rhesus macaque model. It has been shown that infection of macaques with Vpr-depleted SIV virus resulted in the attenuated viral pathogenicity (Lang et al, 1993). Further study has demonstrated that macaques infected with both vpr- and vpx-depleted mutant SIV exhibited much lower virus burdens and a lack of disease progression (Gibbs et al., 1995). Since HIV-1 Vpr has been proposed to combine the functions of the SIV Vpr and Vpx (Fletcher, III et al., 1996), it is possible that HIV-1 Vpr may also be crucial for viral replication in humans. Indeed, one recent study on an HIV-1 infected Long-Term Non-Progressor (LTNP) mother and child pair has shown that all the vpr clones made from the PBMC and plasma of the LTNP individuals have mutations clustered at the C-terminus of the vpr gene, whereas the vpr clones derived from the AIDS patients have shown no gene mutations in the C-terminus of Vpr (Wang et al., 1996). Interestingly, another study on an HIV-1 infected LTNP has also shown that all vpr clones derived from the LTNP individual have mutations between the residues 83-90 at the C-terminus of Vpr (Saksena et al., 1996).

Extracellular Vpr protein has been found in the serum of HIV-1-positive patients (Levy, D.N. et al., 1994a). In addition, this extracellular Vpr protein has been shown to increase the cellular permissiveness to HIV-1 replication, and to reactivate HIV-1 viral replication from latent infection (Levy, D.N. et al., 1994b; Levy, D.N. et al., 1995). Furthermore, HIV-1 Vpr has been shown to suppress the TCR-mediated T cell activation and reduce the production of various cytokines including IL-2, IL-10, and IL-12 through the regulation of nuclear factor kB (NFκB) (Ayyavoo et al, 1997), indicating that Vpr may play a role in the immunopathogenesis of HIV-1 infection.

Other Possible consequences of Vpr-induced cell cycle arrest: Vpr-induced cell cycle arrest may enhance viral replication. It has been suggested that both HIV-1 virus production and the expression of viral RNA are higher in G2 phase than that of

in G1 phase (Emerman, et al., 1996). In addition, it may be beneficial to the virus to arrest the cell cycle and maximize the viral gene expression since HIV-1 infected cells have an average life span of only 2.2 days (Perelson et al., 1996).

In addition, it has been shown that cytotoxic T lymphocytes (CTL)-induced apoptosis is dependent on the activation of cyclin B-p34/cdc2 kinase (Greenberg and Litchfield, 1995; Chen et al., 1995). In addition, the activation-induced T cell death has also been shown to be dependent on the activation of cyclin B-p34/cdc2 kinase (Fotedar et al., 1995). Interestingly, one recent study has demonstrated that HIV-1 Vpr could suppress TCR-triggered apoptotic cell death (Ayyavoo et al., 1997). These data suggest that Vpr may play a role in the establishment of persistent HIV-1 infection *in vivo* by suppressing CTL killing of infected cells.

The molecular mechanism of Vpr-induced cell cycle arrest: To understand the molecular mechanisms of HIV-1 Vpr-induced cell growth inhibition and cell cycle arrest, a number of studies have been conducted to identify the possible Vpr-interacting proteins. A 180-kDa cellular protein designated as Vpr-interacting protein (or RIP) has been found in HeLa cells by co-immunoprecipitation assay (Zhao et al., 1994). Interestingly, one of the Vpr mutants with the mutations in LR-domain at the C-terminal of Vpr abolished Vpr-interaction with RIP. We have examined the effect of this mutant on cell proliferation and found that this mutant also had lost its effect on cell cycle arrest in *S. pombe* (Chapter III). These observations suggest that RIP may mediate Vpr-induced cell cycle arrest. Another 41-kDa cellular protein, designated as RIP-1 (Vpr-interacting protein 1), has been detected in U937 and human embryonal rhabdomyosarcoma cells by Vpr ligand-immunoblot assay (Refaeli et al., 1994). This RIP-1 protein appears to be translocated to the nucleus after its interaction with Vpr or after triggering by glucocorticoid receptor (GR) II ligands, suggesting that the

glucocorticoid steroid pathway may be involved in the Vpr mediated cellular functions. HIV-1 Vpr has also been shown to interact with the cellular transcription factor Sp1, suggesting that Vpr may affect the cellular gene expression and cellular functions (Wang et al., 1995). More recently, HIV-1 Vpr has been shown to be able to bind to the Uracil DNA glycosylase (UNG), a cellular DNA repair enzyme (Bouhamdan et al, 1996). The significance of the interaction between Vpr and UNG is not clear yet. However, further studies have demonstrated that the binding between Vpr and UNG did not correlate with the cell cycle arrest (Selig et al., 1997).

Since p34/cdc2 kinase activity was apparently suppressed in mammalian cells expressing Vpr, it was hypothesized that Vpr may induce cell cycle arrest via the p34/cdc2 pathway (Re et al., 1995). In this study, we have also demonstrate that expression of Vpr or Vpx could dramatically decrease p34/cdc2 kinase activity in *S. pombe*. We have further examined the potential roles of Cdc25, Wee1, and Mik1 in the Vpr-induced cell cycle arrest. However, our data indicated that Vpr may not directly act on Cdc25, Wee1, or Mik1. In addition, protein phosphatase 2A (PP2A) has also been suggested to play a role in Vpr-induced cell cycle arrest (Re et al., 1995). However, it is still inconclusive whether PP2A is involved in this process.

In attempt to identify Vpr-suppressers in *S. pombe*, we have employed a classical yeast genetic analysis, i.e., chemical mutagenesis by EMS, to generate Vpr-resistant mutant strains (Chapter IV). We have successfully generated two Vpr-resistant mutant yeast strains which carried recessive mutations. In addition, we have subsequently constructed and screened the genomic DNA libraries made from either the mutant cells or wild type yeast cells. However, we were unable to identify any Vpr-interacting proteins by this approach. It is not clear why we failed to identify some possible Vpr-suppressers in our study. A similar approach has been used by a recent study in which

a premade cDNA library of *S. cerevisiae* was screened and, a small heat shock protein (sHSP) was identified (Gu et al., 1997). This sHSP could suppress HIV-1 Vpr induced cytoskeletal defects in budding yeast.

hu-PBL-SCID mice: The finding that SCID mice could be successfully reconstituted with a human immune system by engraftment of human peripheral blood lymphocytes (hu-PBL-SCID mice) has created a valuable model for the studies of HIV-1 infection (Mosier et al., 1988). Some early studies have suggested that a functional human immune system could be established in the hu-PBL-SCID mice (Aaberge et al., 1992; Mosier, et al., 1988; Markham et al., 1992). In addition, hu-PBL-SCID mice have been shown to be very useful in some aspects of HIV-1 studies, including the studies of the efficacy of immunoprophylaxis against HIV-1 infection by passive immunization with anti-HIV-1 monoclonal antibodies (Cauduin et al., 1995; Parren et al., 1995; Safrit et al., 1993) and the pathogenesis of HIV-1 *in vivo* (Mosier et al., 1991; Koup et al., 1994; Torbett et al., 1991). However, subsequent studies have also provided some controversial data regarding the reconstitution efficiency of human PBL and the potency of human immune responses in the hu-PBL-SCID mice (Tary-Lehmann and Saxon, 1992; Tary-Lehmann et al., 1995). In addition, it is still unclear whether cell-mediated immunity is intact or could be induced in the hu-PBL-SCID mice. Cellular immunity has been suggested to play a crucial role in the protective immunity against HIV-1 infection *in vivo* (Shearer and Clerici, 1996). Thus it will be very important to determine whether the hu-PBL-SCID mouse is a suitable model for the evaluation of anti-HIV-1 cellular immunity *in vivo*. Furthermore, the possible variations in the background of the SCID mice (i.e., the endogenous microbial flora, age, genetic background, and the extent of leakiness) and the human donors of PBL (Bosma and Carroll, 1991) are also considered to be related to the reconstitution efficiency of human immune system in the mice (Bosma et al., 1991; Hesselton et al.,

1993). In this study, we have used SCID/beige mice as the recipients of human PBL. Importantly, we have demonstrated that SCID/beige mice could support a high reconstitution efficiency of human PBLs. We have also found that the hu-PBL-SCID/beige mouse chimera could be infected with HIV-1 at a near 100% success rate. Furthermore, we have examined the HIV-1 infectivity of SCID/beige mice reconstituted with PBLs isolated from the multiply exposed, HIV-1 seronegative individuals and provided direct evidence suggesting that *in vivo* protective immunity may exist in some of these multiply exposed, HIV-1 seronegative individuals (Zhang et al., 1996). These studies indicate that the hu-PBL-SCID/beige mouse model may be useful for evaluating anti-HIV-1 protective immunity *in vivo*.

In summary, hu-PBL-SCID mice have been used in different aspects of HIV-1 infection, including study of the pathogenesis of acute HIV-1 infection *in vivo*, examination of anti-HIV therapeutic compounds, and evaluation of HIV-1 vaccine candidates. However, it is still controversial whether a primary antibody immune response can be induced in hu-PBL-SCID mice. It is likely that the induction of cellular immune responses in this mouse system will be difficult since human T cell activation and memory require cytokines and effectors cells to interact in the milieu of human lymphoid organs which do not exist in the hu-PBL-SCID mice. It will be necessary to overcome this obstacle before the live HIV vaccine can be extensively studied using the hu-PBL-SCID mouse model.

Future directions

It is still a mystery why and how HIV-1 Vpr inhibits cell growth and induces cell cycle arrest. Further studies are required to understand the precise molecular mechanisms of Vpr-induced cell growth inhibition and cell cycle arrest. We and others have demonstrated that HIV-1 Vpr can cause profound effects on the cellular functions of *S. pombe*, including cell growth inhibition, cell cycle block at G2/M, and cytotoxicity. These results argue that the *S. pombe* model will continue to be a valuable model for the studies of Vpr functions and the mechanisms involved. In addition, as discussed previously, since HIV-1 Vpr may likely act on a cellular target which is highly conserved in yeast and mammalian cells, it will be much simpler and more convenient to identify this target in yeast than in mammalian cells. In addition, further studies are also required to understand the signal pathways through which Vpr induce cell cycle arrest or/and apoptosis. This study will not only help us to further understand the role of Vpr in HIV pathogenesis, but also may advance our knowledge on the basic cell cycle regulation.

Interestingly, like HIV-1, SIV_{agm} also only has Vpr. Recent studies have shown that SIV_{agm} Vpr has all of the functions of HIV-1 Vpr protein and combines the properties of both Vpr and Vpx of SIV_{mac} (Campbell and Hirsch, 1997). In addition, it has been shown that SIVagm is pathogenic in pigtailed macaques. Therefore, the SIVagm-infected pigtailed macaques may provide an excellent model to determine the potential roles of Vpr in viral pathogenesis *in vivo*.

To further address the potential roles of Vpr in HIV-1 pathogenesis *in vivo*, more cohort studies are required to examine the relationship between Vpr mutations and disease progression. In particular, it will be very important to further elucidate the functional status of Vpr in individuals Long-Term Non-Progressors (LTNP).

Regarding the hu-PBL-SCID/beige model, although we have provided some data demonstrating its potential in the studies of HIV-1 infection and evaluation of anti-HIV-1 protective immunity, we have not examined the status of human immune responses in detail in this model. Further studies are required to characterize some basic aspects of human immune responses in hu-PBL-SCID/beige mice, e.g., the distribution of human naive T cells and generation of primary immune responses, especially cell-mediated immunity in the mice. These studies are not only important for the further understanding of this model, but also critical for applying the hu-PBL-SCID/beige model in other research areas including HIV vaccine studies.

Chapter VII

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IMAGE EVALUATION
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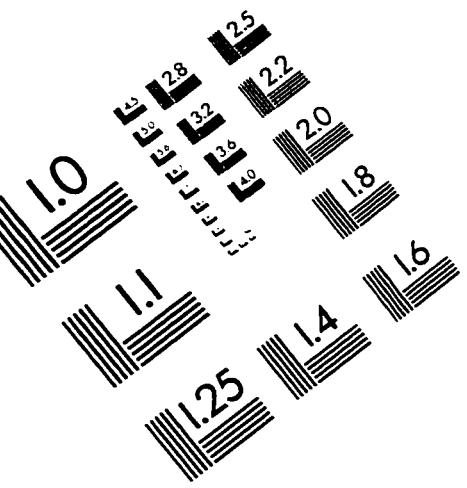

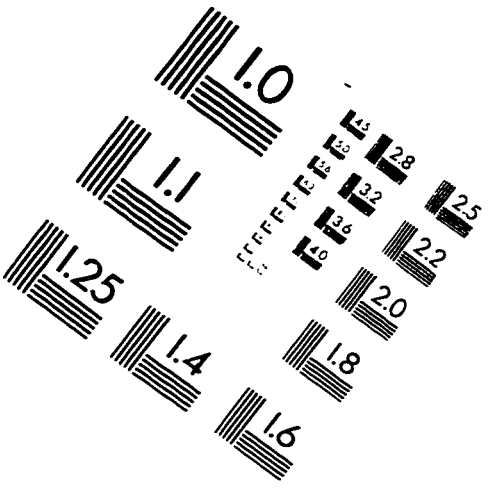

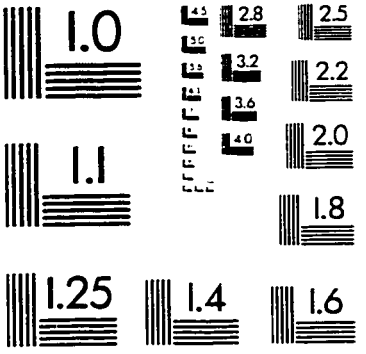

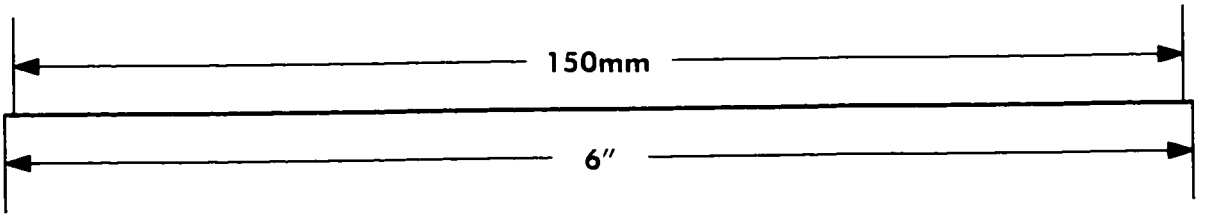

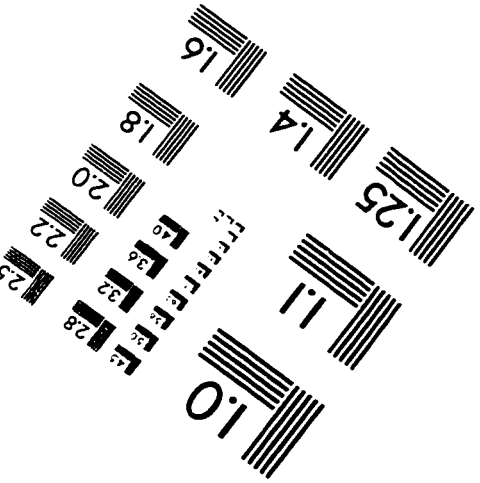

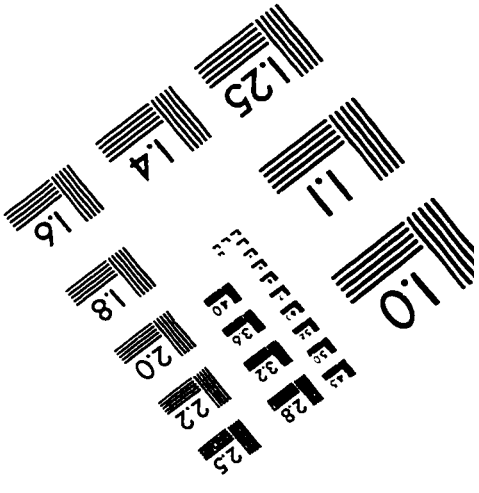

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