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Structural and Functional Characterization of the Human Immunodeficiency Virus Type-1 Vpr

par

Ramu A. Subbramanian

Programme de Virologie

Faculté de Médecine

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Structural and Functional Characterization of the Human Immunodeficiency Virus Type-1 Vpr

présenté par:

Ramu A. Subbramanian

a été évaluée par un jury composé des personnes suivantes

Dr. José Menezes:	Président-rapporteur
Dr. Eric A. Cohen:	Directeur de recherche
Dr. Pierre Belhumeur:	Membre du jury
Dr. John Hiscott:	Examinateur externe

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To my parents

SOMMAIRE

Vpr est une petite protéine de régulation de 96 acides aminés codée par le virus de l'immunodéficience humaine (VIH), l'agent causatif du SIDA. Cette protéine est synthétisée de façon tardive dans le cycle viral et est incorporée efficacement dans les virions. De cette façon, Vpr entre dans la cellule nouvellement infectée; Vpr aurait alors la fonction de cibler au noyau l'ADN proviral à l'intérieur des cellules quiescentes, comme les macrophages. Cependant, il a aussi été suggéré que la protéine Vpr possède un rôle d'activateur transcriptionel tardif dans l'infection virale. La protéine Vpr du VIH-1 a deux activités biologiques qui peuvent contribuer à la fois à ses deux fonctions précoce et tardive: premièrement, elle est transportée au noyau cellulaire; deuxièmement, elle arrête activement la division de la cellule à la phase G2 du cycle cellulaire. Il demeure toutefois que les régions moléculaires impliquées qui contrôlent ces deux activités ne sont pas entièrement définies. De plus, comment ces deux activités s'apparentent aux fonctions précoce et tardive associées à la protéine Vpr demeure encore à être caractérisé.

Dans cette étude, nous avons d'abord caractérisé la structure de Vpr et avons démontré l'existence de deux régions hélicoïdales pertinentes pour les fonctions de la protéine. Notre analyse de la structure de Vpr avait comme but de déterminer les régions importantes de la protéine pour 1) son incorporation au virion, 2) sa localisation nucléaire ainsi que 3) sa capacité d'arrêter le cycle cellulaire. Du point de vue de la pathogénèse virale, nous démontrons que Vpr est essentiel pour la réplication virale optimale dans les macrophages, cellules quiescentes ciblées par le VIH-1 *in vivo*. Nos résultats indiquent que dans ces cellules, la protéine agit tôt lors de l'infection virale en facilitant le transport nucléaire de l'ADN proviral, ainsi que tard dans le cycle de réplication du VIH comme régulateur positif de transcription. Enfin, les observations présentées dans ce travail suggèrent fortement que les deux fonctions précoce et tardive de Vpr sont liées, respectivement, à la localisation nucléaire de la protéine et à son activité d'arrêt en G2 du cycle cellulaire.

SUMMARY

Vpr is a small, 96 amino acids long, regulatory protein that is encoded by the human immunodeficiency virus (HIV), the causative agent of AIDS. Vpr is synthesized late in the viral lifecycle and is efficiently packaged in the progeny virions. In this context, Vpr enters the cell with the incoming virion and appears to function in the nuclear targeting of proviral DNA in non dividing cells such as macrophages. However, the protein has also been suggested to function as a transcriptional activator late in the infection. HIV-1 Vpr has two biological activities which may contribute to these potential early and late functional roles: 1) it localizes to the cell-nucleus and 2) it growth-arrests actively dividing cells in the G2-phase of the cell-cycle. However, the molecular determinants that control these two activities are not fully defined. Also, how these two activities relate to the late and early functions associated with Vpr has not been characterized.

In this study, we first characterize HIV-1 Vpr structurally and show the existence of two functionally relevant helical motifs within the protein. Our structural analysis of Vpr was aimed at understanding the molecular determinants within Vpr that control 1) its ability to incorporate into the budding virions 2) its ability to localize to the nucleus and 3) its ability to growth-arrest dividing cells. In terms of functional relevance during viral pathogenesis, we demonstrate that Vpr is essential for optimal viral replication in human macrophages, a population of non dividing cells targeted by HIV-1 *in vivo*. Our results indicate that in these cells, Vpr functions both early in the infection by facilitating the nuclear import of proviral DNA and late in the infection by upregulating viral transcription. The data presented in this work strongly suggests that these early and late functions are linked, respectively, to the nuclear localization and G2-arrest activities of this regulatory protein.

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Scientific research is usually a team effort and a number of people have contributed to this work. I particularly wish to thank my colleagues and friends including Drs. Xiao-Jian Yao and Dominique Bergeron; Robert Lodge, Janique Forget, Marq Vaillantcourt, Gary Pignac-Korbinger, Florent Checroune, Simon Garcon, and Serge Dandache. I also wish to acknowledge the technical support provided by Nicole Rougeau, Hugo Dilhuydy, and Johanne Mercier. I particularly wish to thank Mr. Jean-Pierre Baril, Francoise Boivert, and the numerous blood donors without whose help much of this work would not have been possible.

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PREFACE

This Ph.D thesis was written according to the Guidelines Concerning Thesis Preparation from the Faculté des études supérieures at Université de Montréal. The thesis includes, as separate chapters or sections: (1) a table of contents, (2) a general summary in English and French, (3) an introduction which clearly states the rational and objectives of the study, (4) a comprehensive general review of the background literature pertinent to the objectives of the subject of the study, and (5) a final discussion including overall conclusions (6) significance of this research and original contributions to the lab.

I have included, as chapters in this thesis, one previously published article and two additional articles which have been submitted for publication. The three articles included in this thesis are as follows:

1. Yao, X.-J., R.A. Subbramanian, N. Rougeau, F. Boisvert, D. Bergeron, and E.A. Cohen. 1995. Mutagenic analysis of HIV-1 Vpr: role of a predicted N-terminal alpha helical structure on Vpr nuclear localization and virion incorporation. *J. Virol.* **69**: 7032-7044

2. Subbramanian R.A., X.-J. Yao, H. Dilhuydy, N. Rougeau, Y. Robitaille, D. Bergeron, and E.A. Cohen. 1997. Human immunodeficiency virus type 1 Vpr localization: nuclear transport of a viral protein modulated by a putative amphipathic helical structure and its relevance to biological activity. Submitted for publication. *J. Mol. Biol.*

3. Subbramanian R.A., A. Kessous-Elbaz, R. Lodge, J. Forget, X-J. Yao, D. Bergeron, and E.A. Cohen. 1997. Human immunodeficiency virus type 1 Vpr is a positive regulator of viral transcription and infectivity in primary human macrophages. Submitted for publication. *J. Exp. Med.*

ADDITIONAL PUBLICATIONS

In addition to articles included in this work (listed in the preface section), I have also contributed to the following studies which are not part of this academic thesis. These works have either been published or are submitted for publication.

Subbramanian R.A and E.A. Cohen. Molecular biology of the human immunodeficiency virus accessory proteins. *J. Virol.* **68**:6831-6835.

Cohen E.A., R.A. Subbramanian, and H.G. Gottlinger. Role of auxiliary proteins in retroviral morphogenesis. *Curr. Top. Microbiol. Immunol.* **241**:219-236.

Lisa F.K., C. Woffendin, M.O. Hottinger, R.A. Subbramanian, E.A. Cohen, and G.J. Nabel. Activation of HIV transcription by the accessory protein, Vpr, is mediated by the p300 co-activator. Submitted for publication. *Proc. Natl. Acad. Sci. USA.*

Lodge R, R.A. Subbramanian, J. Forget, G. Lemay, and E.A. Cohen. MuLV-based vectors pseudotyped with truncated HIV glycoproteins mediate specific gene transfer in CD4+ peripheral blood lymphocytes. Submitted for publication. *Gene. Ther.*

Nie Z., D. Bergeron, R.A. Subbramanian, X-J. Yao, F. Checroune, N. Rougeau, and E.A. Cohen. The putative alpha helix 2 of human immunodeficiency virus type 1 Vpr contains a determinant which is responsible for the nuclear translocation of the proviral DNA in growth- arrested cells. Submitted for publication. *J. Virol.*

Yao X-J, A. Mouland, R.A. Subbramanian, J. Forget, N. Rougeau, D. Bergeron, E.A. Cohen. Vpr stimulates viral production and induces cell-killing during human immunodeficiency virus type 1 infection in the dividing T-cells. Submitted for publication. *J. Virol.*

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LIST OF ABBREVIATIONS

AIDS	: Acquired immunodeficiency syndrome
CA	: Capsid protein (p24)
CAR	: *cis*-acting anti-repression sequences
CD4	: Cell surface marker for helper T-cells/ HIV receptor
CD8	: Cell surface marker for cytotoxic T-cells
CDC	: Centers for disease control
CK-II	: Casein kinase II
CRS	: *cis*-acting repression sequences
CTD	: C-terminal domain of RNA polymerase
CCR5	: HIV coreceptor for macrophage-tropic strains
CXCR4	: HIV coreceptor for lab adapted/T-tropic trains.
ER	: Endoplasmic reticulum
FCS	: Fetal calf serum
FITC	: Fluorescein isothiocyanate
gp	: Glycoprotein
GRII	: Glucocorticoid receptor type II
HIV	: Human immunodeficiency virus
HIV-1	: Human immunodeficiency virus type 1
HIV-2	: Human immunodeficiency virus type 2

HTLV : Human T-cell leukemia virus

IkB : Negative regulator of NFkB

IL-2 : Interleukin 2/ a T-cell stimulatory cytokine

IN : Integrase

Kb : Kilobases

LBP-1 : Leading binding protein

LTR : Long terminal repeat

M2 : Influenza ion-channel protein

MA : Matrix protein

MHC : Major histocompatibility complex

MMLV : Moloney murine leukemia virus

NCP7 : Nucleocapsid protein

Nef : Negative Factor (accessory protein)

NFAT-1 : Nuclear factor of activated T cells

NFkB : Nuclear factor-Kappa B

NRE : Negative regulatory elements

nt : Nucleotides

ORF : Open reading frame

PBMC : Peripheral blood mononuclear cell

PCR : Polymerase chain reaction

PHA : Phytohemagglutinin

RRE : Rev responsive element

RSV : Rous sarcoma virus

RT : Reverse transcriptase

SIV : Simian immunodeficiency virus

SP1 : Transcriptional factor binding to SP1 site

STLV : Simian T-cell leukemia virus

TAK : Tat associated kinase

TAR : Trans-activation response element

Tat : Transactivating protein

TCR : T-cell receptor

UBP-1 : Untranslated binding protein

USF : Upstream binding factor

Vif : Viral infectivity factor

Vpr : Viral protein R

Vpu : Viral protein U

Vpx : Viral protein X

CHAPTER 1

LITERATURE REVIEW

Epidemiology

The human immunodeficiency virus (HIV) is the causative agent of the lethal infectious disease Acquired Immune Deficiency Syndrome, AIDS (Barre-Sinoussi *et al.*, 1983; Gallo *et al.*, 1983; Gallo *et al.*, 1984; Levy *et al.*, 1984). Since the first reports of this disease caught the medical community's and the general public's attention back in 1981 (Gottlieb *et al.*, 1981; Masur *et al.*, 1981), the epidemiological trend for AIDS has been on a constant rise. In 1981, a total of 189 cases were reported to the Center for disease control (CDC), mainly from the states of New York and California. Seventy nine percent of this group was either homosexual or bisexual men and 6 cases (3.2%) occurred among women, and none occurred among children.

Ten years later, as of June 1991, both the numbers and the risk groups involved were changing. The CDC reported that the U.S carried a total of 179,694 cases, of whom 18201 (10.1%) were women and 3140 (1.7%) were children. Homosexual/bisexual contact (59%) and IV drug use(22%) or both (7%) still constituted the main mode of transmission in the United States. Heterosexual transmission, however, was on a constant rise, then accounting for 10011 (6%) of the cases in the U.S. Unlike in the United States, heterosexual contact was the main mode of transmission in the African countries. Both in Africa and the U.S (especially the urban centers) there was an emerging tread of high seroprevalence among women of child bearing age.

The numbers have drastically increased since the beginning of this decade and the world health organization (WHO) surveillance data indicates that nearly 12 million people have died of AIDS as of 1997. This year alone (1997), 2.3 million people died of AIDS of which almost half a million were children. Surveillance data also suggests that close to 30 million people live with AIDS today. Should currently unbroken trends in transmission continue, it is estimated that more than 40million people will be living with HIV in the year 2000. These numbers are a notable detraction from the relatively optimistic estimates that existed a decade ago when 12.2 to 18.3 million were expected to be infected by the year 2000 (Braun *et al.*, 1990). In 1988, AIDS was rated the fifteenth leading cause of death in the United States. In less than five years, AIDS was the leading cause of death among adults < 45 years of age, and children of the age group 1-5.

Retroviruses

HIV, the etiological agent of AIDS, belongs to the virus family Retroviridae. Retroviruses are enveloped RNA viruses and encode for a unique enzyme, reverse transcriptase, that enables them to replicate their RNA genome through a DNA intermediate. Traditionally, the retroviridae family has been subdivided into three major subfamilies: the Spuma- or foamy viruses, the Oncoretro- or RNA tumor viruses, and the HIV-family, the Lenti- or slow viruses (Coffin, 1992). Spumaviruses have been isolated from several primates including man even though they are not conclusively associated with any human disease at present (Saib *et al.*, 1995). However, at least in tissue cultures, they induce multinucleated giant cells with extensive vacuolation giving them their name the "foamy viruses." The second retroviral subfamily consists of the tumorigenic oncoretroviruses, transmitted either exogenously, or endogenously as a part of the germ line. These viruses have been shown in several animals, and in man to be transforming in nature with relatively little cytopathicity. Rous Sarcoma Virus (RSV) and the Human T-lymphotropic Viruses (HTLV) are typical examples of the oncoretroviruses. The third retroviral subfamily consists of exogenously transmitted immunosuppressive and neurotropic lentiviruses, which unlike the oncoretroviruses are highly cytopathic in some permissive cells (Sodroski *et al.*, 1986). HIV belongs to this latter group. Persistence and latency seems to be characteristic of all three groups. As discussed later, one of the key differences between the lentiviruses and oncoretroviruses is that lentiviruses appear to infect non dividing cells such as macrophages, while the oncoretroviruses only establish infection in dividing cells successfully (Lewis and Emerman, 1994). Oncoretroviruses rely on the nuclear membrane disintegration during mitosis to gain entry to the cellular genome while lentiviruses are considered to encode for specific gene products that facilitate the transport the proviral DNA into the nuclear compartment in a mitosis independent manner (Emerman, 1996) and this will be further elaborated in subsequent sections. The HTLVs and HIVs have been shown to target cells *in vivo* and *in vitro* that express the CD4 accessory molecule (Hattori *et al.*, 1981; Lifson *et al.*, 1986; McDougal *et al.*, 1986).

Morphologically, the three groups have both similarities and differences (Coffin, 1992). All three groups acquire their envelopes from the host's cell membrane during the budding process and display envelope-knobs readily visible in negatively stained electron micrographs. These knobs are presumed to be oligomers of the env-glycoproteins (Gelderblom, 1991). While the capsids of the oncoretroviruses (with the exception of HTLVs) and spumaviruses are fully assembled before budding

commences, the capsids of lentiviruses are assembled in parallel to the budding process. Also, the virion cores differ between the groups (Figure 1). Most oncoretro- and spumaviruses at maturity possess an isometric, ovoid capsid, while the lentiviruses and the D-type oncoretroviruses possess a distinct cone shaped core (Gonda *et al.*, 1985). In addition, the lentivirus group seems to possess a unique morphological structure, the lateral bodies, which is absent in all other retroviral groups (Gelderblom *et al.*, 1989; Gelderblom, 1991).

Members of the oncoretroviruses (HTLV-I and HTLV-II) and lentiviruses (HIV-I and HIV-II) have been identified as human pathogens with the HTLVs causing leukemias and the HIVs causing AIDS (Fauci *et al.*, 1996; Uchiyama, 1997). Though the ability of these viruses to affect cells of the immune system is a common feature, it should be noted that cells of the nervous system are also routinely affected leading to clinical disease. HTLVs cause a chronic inflammatory myelopathy, and HIVs are associated with sub-cortical dementia in adults and progressive encephalopathy in children . Also associated with HIV infection and AIDS are a subacute myelopathy and sensory neuropathy (Tyor *et al.*, 1995; Dalakas and Cupler, 1996; Uchiyama, 1997).

As etiological agents of diseases causing high morbidity and mortality these viruses have evoked enormous interest among retrovirologists. Though there is very little sequence homology between the oncoretroviruses and lentiviruses, members within each subfamily show relatively high homology. In this respect, it is worth noting that the oncoretrovirus group shows more homology within the group than does the lentivirus group. The oncoretroviruses, HTLV-I and STLV, share 95% homology (Homma *et al.*, 1984) as compared to the lentiviruses HIV and SIV sharing only 75% homology even among the more closely related members (Chakrabarti *et al.*, 1987; Franchini *et al.*, 1987). In fact, the two human lentiviruses, HIV-1 and HIV-2, share only 40% overall homology (Guyader *et al.*, 1987). Also HIV has a high rate of replication when compared to the HTLVs, and this may account for the high rate of mutation observed in the former. HIV isolates, even from the same patient, may differ extensively in the env protein regions as compared to the HTLVs which are highly homogenous even among different individuals (Reitz *et al.*, 1983; Hahn *et al.*, 1986).

Though both the oncoretroviruses and the lentiviruses belong to the *retroviridae* family, the lentiviral subfamily that includes HIV-1, is comparatively complex both from the point of view of the number of viral proteins encoded by these viruses and in the regulation of their expression. In addition to the *gag, pol,* and *env* open reading frames

(ORFs) present in all retroviruses, lentiviral genomes also contain novel ORFs generally not found in prototypic retroviruses (Figure 2). These ORFs encode for a variety of auxiliary proteins which account for the tight and often intricate regulation of gene expression observed in lentiviruses (Subbramanian and Cohen, 1994). Auxiliary protein involvement in viral replication has been well characterized in the extensively studied member of the lentiviral subfamily, the Human Immunodeficiency Virus (HIV), the causative agent of the Acquired Immune Deficiency Syndrome (AIDS).

HIV-1 encodes for at least six auxiliary proteins namely, Vif, Vpr, Tat, Rev, Vpu, and Nef. These proteins can be classified into two groups based on the temporal regulation of their expression. Tat, Rev, and Nef, are Rev-independent proteins expressed early in the viral life cycle, while Vif, Vpr, and Vpu are Rev-dependent late proteins that are expressed during active viral assembly. HIV-2 and the Simian Immunodeficiency Viruses (SIVs) generally do not encode for the Vpu protein, but for another late protein, Vpx, not found in HIV-1. The functional role of the two early auxiliary proteins, Tat and Rev, has been extensively reviewed elsewhere (Cullen, 1992) and are dealt with in this work only briefly. The remaining regulatory products are dealt with in the accessory protein section in greater detail.

Accumulating evidence suggests that the expression of the accessory proteins may ensure efficient virion assembly and release as well as optimal infectivity of virions generated in their presence. Most auxiliary proteins are not packaged in the progeny virions and have to be synthesized *de novo* in infected cells. Notable exceptions are the auxiliary proteins Vpr and its homologue Vpx, both of which are efficiently packaged in the virion (Cohen *et al.*, 1990a; Yu *et al.*, 1990; Yuan *et al.*, 1990; Yu *et al.*, 1993). As discussed in subsequent sections, other regulatory products such as Vif and Nef have also been identified in the virion, though at lower levels. Virion incorporation of viral products may allow effective regulation of functions that immediately follow infection, before *de novo* protein synthesis can be initiated. In fact, virion incorporated proteins may confer some advantageous qualities to lentiviruses not at the disposal of their oncoviral relatives. HIV-1 Vpr, the focus of this thesis, has been demonstrated to be one of the nucleophilic viral determinants that may aid proviral DNA transport to the nucleus in non dividing target cells such as macrophages (Heinzinger *et al.*, 1994). In this regard it is interesting to note that oncoretroviruses which generally lack a Vpr like protein do not efficiently infect non dividing cells (Lewis *et al.*, 1992; Lewis and Emerman, 1994). In addition to the virally encoded Vpr and Vpx proteins, some members of the lentiviral family appear to recruit and incorporate other cellular factors such as cyclophilins into

the progeny virions to benefit their own life cycle. In fact, it is likely that all HIV auxiliary proteins function, at least in part, by targeting specific cellular factors and thus manipulating existing cellular processes to optimize viral propagation. Coding for a panoply of such auxiliary viral products, which may exploit different cellular processes to advance viral life cycle, may actually distinguish the lentiviruses form other prototypic retroviruses (Cohen *et al.*, 1996).

HIV Structure

The plus strand RNA genome of HIV, similar to all retroviruses, can direct translation of the three archetypal genes: the *gag, pol* and *env.* The translation products of these three genes constitute the structural and enzymatic repertoire of the HIV-1 virion. All three genes direct translation of large polyproteins which are subsequently cleaved into modular proteins either by the action of the viral (Gag, Pol) or cellular proteases (Env). In addition, HIV unlike the oncogenic retroviruses, also codes for several regulatory proteins some of which are also packaged in the virion (to be discussed in a later section).

HIV, a lentivirus, like all retroviruses has in the most simplified terms an exterior envelope, a protein capsid in the middle, and a duplicate RNA genome in the interior. Details of the structure and the proteins involved are depicted in Figure 3. In HIV, 72 knobs of 9-10 nm long project from the envelope in a highly symmetrical manner. Current consensus is that the knobs are oligomers of the gp120, one of the envelope proteins mentioned above. Based on the knob's dimensions and triangular outline form sections, Gelderblom and coworkers suggested a gp120 trimer. Subsequent groups have suggested dimeric, trimeric, and tetrameric oligomers (Gelderblom, 1991). Evidence also suggests that host proteins such as the MHC antigens are also displayed along with the envelope proteins (Arthur *et al.*, 1992).

Located interior to the viral membrane is the Gag cleavage product, p17, which directly associates with the lipid bilayer through myristoylation (Gelderblom, 1991), This protein, usually referred to as the Gag matrix p17, has been suggested to play a vital role during virion morphogenesis (discussed in the assembly section). The two other Gag cleavage products, capsid p24 (CA) and nucleo-core protein p7 (NCP7) are associated more closely with the RNA interior, forming the cone shaped viral core (Gelderblom *et al.*, 1987). The *gag* gene product NCP7 binds the viral RNA through a zinc finger structural motif. A morphological structure linking the viral core with the

membrane associated p17 has been described as the core envelope link or CEL (Gelderblom, 1991). Though conformation is pending, it has been speculated to contain viral Gag protein, p6. The Pol cleavage products, namely, the protease, the reverse transcriptase, and the integrase, are also carried in the virion core. A unique feature of HIV and most lentiviral virions is the lateral bodies inside the envelope. It appears on either side of the viral core running along its long axis with a clear electrolucent space separating it from the core. The lateral bodies have been speculated to contain excess Gag proteins.

The viral RNA genome located in the very interior within the virion core consists of two identical copies of RNA. The genomic RNA contains, immediately preceding the Gag initiation codon, an untranslated leader sequence which is critical for specific encapsidation of viral RNA into budding virions (discussed later in the assembly section). Surrounding the leader sequence and the coding regions that lie downstream to it, there are unique sequences both on the 5' and the 3' end of the genome called, respectively, U5 and U3. Immediately following the U5 lies the primer binding site (PBS). Similarly, immediately preceding the U3 lies a polypurine stretch. An identical redundant sequence termed R appears immediately upstream to the U5 on the 5' end and down stream to the U3 on the 3'end. Hence it forms the terminus of both ends of the RNA. These redundant regions will play crucial role during the reverse transcription step. By the end of reverse transcription, along with the unique sequences, it will also create the proviral long terminal repeat (LTR) structures. Also a cellular tRNA lysine species is found associated with the genomic RNA with a complementary sequence for the primer binding site. As this RNA genome is transcribed by the cellular RNA polymerase II, it also shows two cellular RNA modifications: 5'cap structure and a 3' poly-A tail.

HIV Lifecycle and Gene Function

HIV replication in human cells can be viewed as a step-by-step process that starts with an incoming virion binding to specific cell types (tropism), entering and establishing infection, producing progeny viruses, that in turn repeat the process, thus generating a lifecycle (Figure 4). Though extensive coverage of each step would be beyond the scope of this work, the following sections try to provide a concise description of each of the replication steps.

Cell Tropism and Cytopathicity:

The first step in the lifecycle of HIV is the binding of the virus to specific populations of host cells and the subsequent entry of the virion into such cells. The CD4 molecule has been shown to be the primary cellular receptor for the virus. The CD4 antigen is an accessory molecule that is involved in T-cell activation (McDougal *et al.*, 1985). This antigen is expressed mainly by the T-helper cells (from here on referred to as CD4+ cells) and the monocyte/macrophage cell lineage, including microglia in the CNS and the Langerhans' cells in the skin, and are hence the primary targets for HIV. These cell populations play a central role in antigen presentation (macrophages, dendritic cells, and Langerhans' cells) and immune response amplification (CD4+ cells) in normal subjects. HIV infection is highly cytopathic to the CD4+ T-cells *in vitro* and leads to the drastic reduction in the absolute number of circulating CD4+ T-cells *in vivo* (Klatzmann *et al.*, 1984; McDougal *et al.*, 1985; Ho *et al.*, 1995). The exact mechanism(s) involved in the CD4+ T cell depletion process *in vivo*, however, are not well understood.

In addition to T-helper cells, other cells including glial cells have been shown to express varying levels of CD4, and hence, are capable of being infected by HIV (Maddon *et al.*, 1986; Adachi *et al.*, 1987; Funke *et al.*, 1987). The microglia which are of the monocyte-macrophage lineage in the CNS have been shown to be the most infected population in the brain (Johnson, 1995) and are currently considered to be the route of entry into the CNS for HIV (Gartner *et al.*, 1986).

The Macrophage and HIV

It is currently well established that the CD4+ T-cells are not the only population of immune cells that harbor HIV *in vivo* (Chun *et al.*, 1997). The monocyte/macrophage lineage of cells have been shown to be actively infected and function as viral reservoirs *in vivo* (Clarke *et al.*, 1990; Bagasra and Pomerantz, 1993; Crowe and Kornbluth, 1994; Blauvelt *et al.*, 1995; Crowe, 1995; Bornemann *et al.*, 1997; Cunningham *et al.*, 1997). In the early stages of the disease the virus can be isolated from the macrophages but not from peripheral blood lymphocytes (Popovic and Gartner, 1987). In the CNS, lungs, and lymph nodes the macrophages appear to constitute a major infected population (Meltzer and Gendelman, 1992; Meltzer *et al.*, 1993). Unlike in the CD4+ cells, however, HIV infection in the macrophages is more restricted and is not cytocidal. Hence these cells constitute a surviving population of infected cells carrying the virus to organs such as the brain and the lungs (Chayt *et al.*, 1986; Vazeux *et al.*, 1987; Eilbott *et al.*, 1989; Meltzer and Gendelman, 1992; Epstein and Gendelman, 1993). Also, in the macrophages, the virus may be produced in intercellular vacuoles protected from the

immune surveillance (Orenstein *et al.*, 1988). Macrophages have also been implicated to secrete neurotoxins, an indirect HIV effect, that may damage neural tissue (Giulian *et al.*, 1990). Viral isolates from non symptomatic patients show macrophage tropism and are less productive, while isolates from full blown AIDS patients show high productivity and lesser tropism toward the monocyte lines. In this context, the macrophage-tropic viruses are generally considered to be involved in the initial establishment of the infection as well as in carrying the virus into vital organs such as the brain in a Trojan horse-like manner (Balter, 1996).

Infection of CD4-negative cells

Though HIV has been shown to specifically recognize CD4 receptor expressing cells such as T-helper cells and macrophages in conjunction with coreceptors (discussed later), whether or not CD4 is the only viral receptor is currently controversial. Though it is currently well established that CD4 positive cells are a major target for the virus, other cell types including neurons and epithelia which do not express CD4 have also been shown to be infected, if poorly, by HIV (Clapham *et al.*, 1987; Dewhurst *et al.*, 1987; Harouse *et al.*, 1989). Generally, alternative antigens have been proposed as cell surface receptors for HIV in these cells that do not express CD4 to any appreciable level. It is interesting in this regard that HIV entry in neural cell lines via galactosyl ceramide has been reported (Bhat *et al.*, 1991; Harouse *et al.*, 1991; Bhat *et al.*, 1992). Some cells of the epithelial lineage, such as the colon derived cells, which do not express CD4 also appear to be infected via galactosyl ceramide (Yahi *et al.*, 1992). Interestingly, just as with macrophage tropism, the envelope determinant linked with galactosyl ceramide binding is the V3 loop region (Cook *et al.*, 1994). If anything, the infection of cells that are CD4 negative underscored the need to consider additional if not alternative receptors and co-receptors for HIV-1.

Coreceptors

The existence of coreceptors for HIV-1 has long been suspected as experimental evidence suggested, as discussed above, that the absence of CD4 in itself was not enough to preclude infection in some cell systems. Alternatively, in other systems, the presence of CD4 by itself was not enough to ensure infection. Murine cell lines that express CD4, bind gp120 but do not proceed with the internalization steps (Maddon *et al.*, 1986). Interestingly, direct transfection of viral DNA into the cells rescues the infection, showing that some other factor in addition to CD4, at the membrane level, is

required for infection. An interesting development in recent years has been the finding that chemokine receptors can function as co-receptors for HIV (Wells *et al.*, 1996). The chemokine receptor, CXCR4, previously termed Fusin, was shown to be involved in the infection of the T-cell tropic viruses (Feng *et al.*, 1996). Fusin, shows high sequence homology with IL-8 receptor and is a seven transmembrane G-protein linked glycoprotein. Some evidence exists that CD4-negative cells that express fusin can also be infected by strains of HIV-1 and may partly explain the infection of CD4- cells discussed earlier (Endres *et al.*, 1996; Hesselgesser *et al.*, 1997; Potempa *et al.*, 1997; Reeves *et al.*, 1997). In this regard, infection of epithelial cells via galactosyl ceramide likely occurs in the context of CXCR4 (Delezay *et al.*, 1997). Neurons have also been reported to be infected by HIV by CXCR4 usage in a CD4 independent manner (Hesselgesser *et al.*, 1997). Microglia were demonstrated to be infected by CCR3 and CCR5 coreceptor usage (He *et al.*, 1997). Similarly, the macrophage-tropic viruses require the coreceptor, CCR5, also a chemokine family receptor (Deng *et al.*, 1996; Dragic *et al.*, 1996). While these coreceptors belong to the G-protein coupled chemokine family of receptors, G-protein signaling does not appear to be a contributing factor during HIV-1 infection (Alkhatib *et al.*, 1997). Emerging evidence also suggests that viruses capable of using a variety of chemokine receptors exist, and emergence of different co-receptor usage may be one way of ensuring active cell-killing and disease progression (Unutmaz and Littman, 1997).

Cytotoxicity mechanisms

Several theories have been put forward to explain the cytopathicity observed in the CD4+ cell population. In vitro HIV infection of CD4+ cells leads to cell fusions leading to giant multinucleated cells which die subsequently (Lifson *et al.*, 1986; Sodroski *et al.*, 1986). These fusions are mediated by gp120 expressed on infected cells reacting with uninfected cells expressing CD4. However, this mechanism seems unlikely in the light of several opposing reports including cytopathicity in the absence of syncytia formation, and conversely, little cytopathicity even in the presence of syncytia (Somasundaran and Robinson, 1987).

Several suggestions in addition to the syncytial cell death have also been proposed. Some authors have suggested HIV may infect CD4+ cells precursors and other cells that may secrete immunostimulatory factors (Fauci, 1988; Rosenberg and Fauci, 1989; Zinkernagel and Hengartner, 1992; Fauci *et al.*, 1996). It has also been suggested that the budding process may weaken the membrane and cause ionic

imbalance leading to cell death (Leonard *et al.*, 1988; Lynn *et al.*, 1988). Apoptosis or programmed cell death has also been suggested as a cytopathic mechanism operative in AIDS leading to CD4+ T-cell depletion (Gougeon and Montagnier, 1993; Cotton *et al.*, 1996; Frost and Michie, 1996). In this regard it is interesting to note that many HIV-1 gene products have been associated with apoptosis including Tat (Li *et al.*, 1995; New *et al.*, 1997), the viral protease (Strack *et al.*, 1996), gp120 (Banda *et al.*, 1992; Tuosto *et al.*, 1995), and as discussed in a later section, HIV-1 Vpr (Poon *et al.*, 1997; Stewart *et al.*, 1997).

An autoimmune mechanism is suggested by experiments using ADCC assays. In these experiments, CD4+ cells clearance by anti-HIV-sera was possible using soluble gp120 or inactivated virus coating of the CD4+ cells. Autoantibodies and cytotoxic antibodies against HIV infected CD4+ cells have also been documented. Spontaneous gp120 shedding may undermine humoral neutralization and may label uninfected CD4 positive cell for immune clearance (Rosenberg and Fauci, 1989; Gelderblom, 1991). Also, the MHC class I and II molecules displayed on the viral surface, acquired during budding, and gp120's homology with class II molecules may lead to several autoimmune pathologic mechanisms (Ziegler and Stites, 1986; Golding *et al.*, 1988). The immunopathogenic mechanisms that may be operative during HIV-1 infection have been dealt with in detail in several reviews (Fauci, 1988; Rosenberg and Fauci, 1989; Pantaleo and Fauci, 1996).

In vivo, one or more of these mechanisms may be responsible for the observed CD4+ cells depletion as there is little evidence at present to presuppose the importance of one mechanism over another. However, it is increasingly being realized that direct cell killing alone cannot explain the drastic reduction of CD4+ cells population as only a fraction of cells are infected at any one given time. In addition to the CD4+ cells depletion, functional impairments of the CD4+ cells have been well documented. These include, reduced cloning ability, inability to induce B-cell functions, failure to respond to allo-and soluble antigens, and impaired IL-2 production. Defective B-cell response including failure to mount adequate immunoglobulin M to antigenic challenge may lead to fatal outcomes in infants who have not been exposed to pathogenic bacteria and must depend on the humoral response heavily.

Viral Envelope Protein Gp120 and Gp41

HIV, like all viruses can only replicate by utilizing the cellular metabolic machinery and hence access to the interior of the cell is a necessity to start replication. However, most viruses do show special preference or tropism toward particular cells in vivo. One can assume that viruses have evolved so they can infect cells most suited to sustain and advance their unique lifecycle and pathogenesis. Viruses that replicate in specific cells generally code for proteins that recognize receptors present on these target cells.

The recognition of the CD4 expressing cells is accomplished by gp120, one of the Env-proteins present on the outer viral membrane. This exterior membrane layer is derived from the host cell membrane during the budding process with the viral *env* gene products inserted in or displayed on the surface of the lipid bilayer. As the membrane is host derived, other host-specific MHC proteins have also been shown to be present on the envelope.

The virus expresses two envelope glycoproteins gp120 and gp41 the numbers denoting their molecular weights in kDa. Both are coded for by the *env* gene and are spliced form a larger precursor, gp160, by cellular proteases. These two proteins fulfill specific and coordinated functions in the early viral lifecycle. Gp120 resides on the exterior of the envelope and is used by the virus to recognize its primary targets, the CD4 positive cells, with very high affinity. Gp120-CD4 association constant has been determined to be around 10^9 M using both recombinant and purified gp120 from infected cells (Lasky *et al.*, 1987; Smith *et al.*, 1987). Gp120 is associated closely through a non covalent linkage to gp41, the second envelope protein. While gp120 is considered to be the CD4 specific anchor that binds to target cells, gp41, the transmembrane protein is considered to be involved in membrane fusion and internalization of the virion (Freed *et al.*, 1990; Helseth *et al.*, 1990). Receptor mediated endocytosis followed by pH change-mediated release of the core does not appear to be the primary mode of entry during HIV-1 replication. Rather, following recognition and attachment of gp120 to CD4, the transmembrane gp41 seems to accomplish the next step in the viral life cycle, namely, membrane penetration by direct fusion.

Current research into chemokine coreceptors has clearly added to the intricacy of envelope binding and fusion events during HIV-1 infection. It is now clear that CD4-binding by itself does not render the viral envelope capable of activating membrane fusion (Wells *et al.*, 1996). This is clear from the early observations that document successful gp120 binding to CD4 but subsequent failure to progress through infection in

non human cells (Maddon *et al.*, 1986). The need for additional interactions with a suitable coreceptor subsequent to, or in conjunction with, actual CD4 binding appears to be essential for fusion in these cells (Lapham *et al.*, 1996). Such interactions appear to transform the viral envelope to a "fusion-active" form. In terms of mechanism, evidence suggests that CD4 binding may reveal a high affinity binding site in the envelope protein for the co-receptor (Trkola *et al.*, 1996; Wu *et al.*, 1996). Such coordinated interactions, perhaps in a trimolecular form involving gp120, CD4, and the coreceptor, may induce allosteric changes in gp41 transmembrane subunit triggering fusion events. Though the exact nature of these molecular interactions and potential allosteric changes are not currently understood, the resulting viral and cellular membrane fusion appears sufficient to release the viral capsid into the cytoplasm.

The gp120 sequence among different HIV-1 isolates is highly variable (Myers and Gelfand, 1991). However, there are 18 highly conserved Cysteine residues in the molecule, all involved in specific disulfide bonds which produce intricate loop structures in the primary sequence (Leonard *et al.*, 1990). Also, there are 24 Asparagine N-linked glycosylation sites, accounting for approximately half the molecular weight of the glycoprotein. Also, O-linked immunogenic glycans have also been reported on gp120 (Myers and Gelfand, 1991). Though the total number of 24 glycosylation sites is retained among isolates, only 13 sites are regularly conserved. It has been shown that deglycosylation seems to reduce, but not abolish the CD4 binding ability of gp120 (Fenouillet *et al.*, 1990; Fenouillet *et al.*, 1994). However, while glycosylation doesn't seem to greatly affect gp120-CD4 binding function, it has been shown to be important in the intracellular gp160 processing, and release of infectious virions (Kozarsky *et al.*, 1989; Pal *et al.*, 1989).

Based on sequence data from several isolates, gp120 has been shown to contain both highly conserved as well as hypervariable regions (Myers and Gelfand, 1991). This has been suggested to be a highly advantageous evasive technique used by the virus against the host's immune responses. Sequence, and site directed mutation studies, have shown five conserved regions (labeled C1 - C5) in the primary sequence of various isolates some of which have been shown to be critical for recognition of CD4. The third, fourth and fifth conserved domains seem to be particularly important for CD4 binding (Kowalski *et al.*, 1987). These might constitute the three aspects of the CD4 binding site brought into geographical proximity by a disulfide bond in the region also conserved among strains that recognize CD4. Single substitution in the fourth

conserved region has been shown to either abolish binding to CD4 or alter cellular tropism (Cordonnier and Montagnier, 1989).

Also, most of the variability in gp120 that accounts for the strain differences appear in the five hypervariable regions which have been labeled V1 - V5. These highly immunogenic domains have been suggested to constitute decoys displayed by the virus to sidetrack the immune system from the essential conserved domains. The V3 domain, spanned by a disulfide bridge, is highly immunogenic and has been shown not critical for CD4 binding. However, the loop is required for membrane fusion (Freed *et al.*, 1991). Travis and coworkers, have shown that the V3 disulfide loop is required for cleavage of gp160, and the sequence essential for gp120 mediated syncytia formation (Travis *et al.*, 1992). The V3 loop has been implicated in an important additional function: determining differential cell tropism as in lymphocyte vs. macrophages and in regulating the efficiency of replication (Westervelt *et al.*, 1991; Cann *et al.*, 1992; De Jong *et al.*, 1992).

It has been shown that gp120 is shed continuously and spontaneously as the virion matures (Schneider *et al.*, 1986). Shedding of gp120 may also lead to several pathogenic mechanisms that might undermine the immune response against the virus. Free gp120 may compete for the neutralizing antibodies against the virus, may label uninfected bystander CD4+ cells making them targets for immune clearance, and may lead to immune complex formation. There have been recent reports showing that gp120 may also be toxic to neural cells, perhaps by induction of inflammatory cytokines such as TNFα (Giulian *et al.*, 1990; Giulian *et al.*, 1993; Yeung *et al.*, 1995).

Early Post Entry Events

In the simplest terms, following gp120 and gp41 mediated binding, membrane fusion, and entry into the cytoplasm, in order to advance its lifecycle, the virus needs to (a) have its RNA genome reverse transcribed to DNA (b) have this reverse transcript transported into the nucleus and (c) have it successfully integrated into the host genome. The following is a concise account of the present understanding of each of the above steps.

Reverse transcription

Kinetic studies show that the processes of reverse transcription and integration in infected cultures occur rapidly after penetration. PCR techniques were used to probe for

viral specific DNA species in acutely infected T-cell lines and these early events appear to be detectable as early as 4 hours post entry and reach a peak by 12 hours post infection (Kim *et al.*, 1989). Similar kinetics were noticed for primary cells though macrophages may have slightly slower kinetics (Collin and Gordon, 1994). Very little is currently known regarding virion-uncoating and how the RNA is released form its nucleoprotein core soon after fusion. Some evidence exists showing that total release from protein capsid may not happen.

Retroviral reverse transcriptases are complex proteins which show DNA polymerase activity. However, this enzyme has the unique ability to use both RNA or DNA as a template, an essential character that comes into play during the reverse transcription process. HIV reverse transcriptase enzyme is a cleavage product from the Gag-Pol protein precursor as mentioned earlier. Two species of this protein have been shown to exist in the virion, one of 51 kDa and the other of 66kDa, both sharing the same amino-terminal sequences. The p66 species, however, contains an RNase H domain. The two proteins co-purify during affinity chromatography and are considered to exist as a heterodimer as p66/p51 complex shows higher activity than any one of them (Katz and Skalka, 1994).

RNase H activity, or the ability to degrade RNA specifically in a RNA/DNA hybrid, plays a vital role in retroviral reverse transcription and cellular RNase H cannot substitute for this virion specific activity (Tisdale *et al.*, 1991). HIV RNase H activity was initially shown to co-purify with the reverse transcriptase. Subsequent studies have also shown a reverse transcriptase free 15 kDa protein with similar activity to be present in the virion . RNase H domain has been mapped at the carboxyl terminal of p66 and may result from proteolytic cleavage of this larger reverse transcriptase protein. HIV RNase H has been shown to exhibit both endonuclease and exonuclease activity (Hansen *et al.*, 1987; Krug and Berger, 1989; Nakamura *et al.*, 1991; Gopalakrishnan *et al.*, 1992; Lacey *et al.*, 1992). A brief and highly simplified account of reverse transcription as understood from several retroviral systems is given below. The structure of the genomic RNA essential to understand this process has been previously described in the virion structure section above.

Reverse transcription starts at the 5' end of the genomic RNA which forms the template for the proviral DNA synthesis (Figure 5). Lysine t-RNA molecules, which are efficiently packaged in the virion, bind to specific primer binding (PB) sites at the 5'end of the genomic RNA and start the DNA syntheis process. First, a short DNA segment

complementary to the U5 and the 5' redundant sequence is generated at the 5' end, following which the corresponding RNA template is degraded by the RNase H activity of the reverse transcriptase. This exposes the nascent DNA's 3' terminal containing the redundant sequence which is complementary to the template RNA's 3' redundant sequence on the other end of the same template or another RNA molecule (Peliska and Benkovic, 1992). This brings the RNA 3' unique sequence (U3) from the other end of the template collinear to the previously transcribed U5 and R regions of the DNA transcript. The DNA is further "read –through" this collinear U3 region to produce full length (-) strand DNA. From this (-) strand DNA, the reverse transcriptase using the RNase H created primer site, further generates the (+) strand DNA, yielding a double stranded DNA provirus (Varmus, 1987).

To summarize, the reverse transcription not only generates a double stranded DNA copy from a single stranded RNA genome, but it also results in a new arrangement of the unique sequences initially found in the RNA template. In the RNA template, the overall arrangement is "U5-R-----RNA genome--- -R-U3". However, in the DNA reverse transcript, the arrangement becomes "U3-R-U5-----DNA genome----- U3-R-U5". This new array flanking the genome is called the long terminal repeat or LTR and plays a major role in the transcriptional regulation of the provirus.

Preintegration complex and its nuclear transport

The end product of the reverse transcription process, a double stranded DNA viral genome, now called the provirus, exists in close association with a large molecular complex, consisting of both viral and cellular proteins (Miller *et al.*, 1997). This large conglomeration of proteins that associates with the provirus is generally referred to as the preintegration complex. To further advance its lifecycle, the virus needs to have the preintegration complex transported to the nucleus where the proviral DNA can be integrated in a stable fashion into the host's chromosome, which requires getting past the nuclear envelope of the cell. One obvious solution is to have the integration process occur when such a barrier doesn't exist as during cell-division when the nuclear membrane architecture disintegrates. Oncoretroviruses apparently rely on this mode of nuclear access and hence only replicate in dividing cells. However, in lentiviruses such as HIV, integration and consequently the preceding nuclear transport seems to occur in nondividing cells such as macrophages (Lewis *et al.*, 1992; Lewis and Emerman, 1994). The transport of the preintegration complex appears to be an active energy-dependent process that involves ATP hydrolysis (Bukrinsky *et al.*, 1992). It is clear that

the preintegration complex is a large high molecular weight conglomerate of both viral and cellular proteins which may be necessary for its stability and functional activity (Miller *et al.*, 1997). In fact, the presence of other virion-associated regulatory proteins such as Vif (Simon and Malim, 1996) appears to correlate with the stability of the preintegration complexes though it is not known if this is due to direct interaction of Vif with the preintegration complex. It is conceivable that this Vif-effect is due to morphological defects in virion cores generated in the protein's absence (see Vif section that follows). However, some virion-associated proteins have been demonstrated to physically associate with the preintegration complexes including the Gag matrix p17, integrase, reverse transcriptase, and the accessory product Vpr (Bukrinsky *et al.*, 1993; Heinzinger *et al.*, 1994). It is interesting in this regard that other core associated products such as capsid p24 are excluded (Bukrinsky *et al.*, 1993). Hence some amount of reorganization of the capsid and core structures appears to occur following viral entry in infected cells. However, the exact composition and the topologic organization of these viral and cellular proteins is currently not well understood.

Recent developments in our understanding of the preintegration complex suggests viral proteins found in the preintegration complex are partly responsible for this ability to target proviral DNA into an intact cell nucleus (Gallay *et al.*, 1995; Gallay *et al.*, 1997). The Gag matrix p17, the viral integrase, and Vpr as mentioned earlier are all shown to be part of the preintegration complexes and have been shown to facilitate the nuclear targeting of the proviral DNA (this will be further elaborated in subsequent chapters). It is interesting to note that only a small portion of the viral matrix p17 associates with the preintegration complex, specifically, the phosphorylated form (Gallay *et al.*, 1995). As matrix proteins associate with the membrane structures due to myristoylation, phosphorylation of the matrix product has been suggested to conceivably relieve this effect, allowing the membrane bound core structures to migrate to the nucleus. It should be noted that some authors have questioned the role of matrix p17 in post-entry nuclear targeting. However, *in vitro* experiments suggest that the nuclear localization signals (NLS) present in the matrix protein may allow the preintegration complex to specifically interact with karyopherin-α family of proteins at the nuclear periphery (Gallay *et al.*, 1996). The viral integrase also appears to interact with the karyopherin-α, using a bipartite NLS (Gallay *et al.*, 1997). The Vpr product, however, appears to facilitate the proviral nuclear import through a currently unknown pathway that does not depend on karyopherin binding (Gallay *et al.*, 1996). Further molecular details and dynamics of how these proteins actually mediate nuclear

translocation of the proviral preintegration complex is not clear and is the focus of research in many laboratories.

Integration

Following nuclear transport of the preintegration complexes, the actual process of integration is accomplished by the retroviral integrase (Buchow *et al.*, 1989). HIV integrase is a 31 kDa protein which like the reverse transcriptase is a Gag-Pol cleavage product, and is found in the virion. It has been shown to contain zinc finger domain (Cai *et al.*, 1997) that allows it to bind to nucleic acids. It also contains a bipartite nuclear localization signal which targets the protein (and as discussed earlier, conceivably the preintegration complex it associates with) into the cell-nucleus, where viral integration occurs. While the linear form of the proviral DNA is currently considered to be the substrate for the integrase protein, circular forms are also found. These proviral circles are only present in the nucleus and are absent in the cytoplasm and may contain either one or two contiguous LTRs. Their exact role in the viral lifecycle is still not clear. As noted above, the integrase protein, as a part of the preintegration complex, may also function as one of the multiple nucleophilic factors that target the proviral DNA to the nuclear compartment.

Upon entry of the provirus into the nucleus, studies have established that a virally encoded integrase is necessary to accomplish the proviral integration step (Figure 6). Briefly, two nucleotides are removed from the 3' end of the viral DNA by the integrase following which host DNA is nicked in a staggered fashion and the viral DNA joined into this nick. The nicking and joining are probably simultaneous or can proceed through a DNA-protein intermediate though the former is favored in the literature. Overall, the integration process results in the loss of two bases form the termini of the provirus and the duplication (direct repeat) of the host sequences on either side integration site, which in case of HIV is 5 base pairs on either side (Goff, 1992). The preintegration complex may contain other cellular products which may be necessary for the optimal function of the viral integrase. In this respect, the HMG 1(Y) protein, a protein involved in chromosomal architecture has been shown to be necessary for integration (Farnet and Bushman, 1997). Though HIV does not seem to favor specific sites with a particular sequence for integration, some evidence suggests that integration sites may not be entirely random (Kitamura *et al.*, 1992; Bor *et al.*, 1996).

Synthesis of mRNA and its nuclear export

Following integration, the stage is set for the production of progeny virion. This requires the production of the viral Gag, Pol, and envelope proteins. These proteins are produced in HIV, similar to other retroviral systems, by rather intricate controls at the transcriptional, translational and post-transcriptional levels, some of which are unique to the retroviral lifecycle. At the transcriptional level, this control is accomplished through cellular and viral factors which act upon the viral LTR to attenuate or increase the basal transcription. Cellular activation associated factors such as NFκB and viral transactivator Tat can dramatically increase the transcription. On the other hand negative or repressive factors may reduce transcription through LTR interaction. The details of this level of control is further expanded below.

Retroviral transcription as such results in full length 9.7 Kb viral genome and is accomplished by the cellular RNA polymerase II. The transcription starts form the redundant sequences, and hence the RNA transcript is identical to the genomic RNA and only has the unique sequence arrangement found in the initial genomic RNA and not the proviral LTR (Figure 7A). The fate of the full length transcript (~9.7 kb) depends on the presence or absence of the regulatory protein Rev (Malim *et al.*, 1990). The mechanism of Rev action appears to involve secondary mRNA structures and is discussed in a subsequent section. In the presence of Rev, the mRNA is effectively transported to the cytoplasm unspliced as a full length product. Full length mRNA functions both as the genomic mRNA as well as a template for Gag and Gag-Pol precursors. In addition, Rev also aids the effective transport of singly spliced mRNA species (~4 kb) which code for the envelope precursor as well as the late regulatory proteins that modulate virion assembly and infectivity as discussed in a later section (Arrigo and Chen, 1991). Hence, Rev is essential for active virion production as it controls the availability of both genomic mRNA as well as the structural and regulatory proteins needed for effective virion production. In the absence of Rev both the full length and singly spliced mRNA species are retained in the nucleus where multiple splicing events render them into smaller mRNA species (~2 kb) that subsequently exit the nucleus in a Rev-independent manner. These multiply spliced mRNAs, favored in the absence of Rev, direct the translation of regulatory proteins Tat, Nef and Rev itself. The synthesis of Rev from multiply spliced mRNA species, the presence of which is itself negatively regulated by Rev, leads to a negative feed back loop that controls the levels of Rev in the cell (Felber *et al.*, 1990).

This alternative expression of the structural and regulatory proteins controlled by availability of Rev, which negatively regulates itself, has profound ramifications for the

establishment of latency. Maintaining a low level of Rev leads to lack of cytoplasmic exit of full length mRNA, reduced late protein synthesis, and hence, virion production. Release from latency appears to occur when activation signals increase overall proviral transcriptional activity in infected cells (Pavlakis and Felber, 1990; Haseltine, 1991; Rosen, 1991). Such increased mRNA generation in the absence of Rev could lead to their being multiply spliced. As multiply spliced mRNAs are themselves the templates used to synthesize regulatory proteins such as Rev, cellular activation of latent cells could lead to increased Rev accumulation following activation. Increased Rev levels in turn increase cytoplasmic export of full-length mRNA leading to augmented late protein synthesis and virion production. Hence one can conceptualize latency as a highly balanced low-level transcription and virion-production system controlled by Rev thresholds (Pomerantz *et al.*, 1992). Cellular activation can be conceptualized to tilt this deadlock toward a higher transcriptional activation and virion-production system (Butera *et al.*, 1994). The control and latency and productive infection by Rev has been discussed elsewhere in greater detail (Cullen, 1992).

Generation of virion proteins and viral assembly.

Following entry of the mRNA into the cytoplasm, in HIV, similar to other retroviral systems, two processes seem to further control the production of individual proteins: frameshifting and proteolytic processing (Haseltine, 1991). Gag and Pol proteins are synthesized as polymeric precursors from which individual proteins are cleaved. The full length RNA is either translated to produce the Gag precursor in which case the polyprotein synthesis is stopped soon after the p6 translation by a termination codon. Alternatively, just before p6 translation, the ribosome can make a -1 frameshift to another reading frame, producing a longer Gag-Pol fusion precursor. Hence Gag proteins can either be produced from the Gag precursor or form the Gag-Pol precursor protein. However, as the frameshift occurs in the p6 region, the p6 protein can only be cleaved from the Gag precursor and not from the Gag-Pol precursor. Similarly, the Pol proteins are made only in the context of Gag-Pol precursors. Following viral protein synthesis, assembly of infectious virions requires the coordinated targeting of not only the Gag, Gag-Pol, and Env precursors, but also the recruitment of accessory proteins, and importantly, the genomic RNA.

In a vast majority of cases, recruitment of virion factors is accomplished by specific interaction with the Gag precursor p55 which constitutes the minimal requirement for capsid formation and viral budding (Craven and Parent, 1996).

However, the envelope proteins and the Pol products which are cleaved from the Gag-Pol precursor are both essential for conferring infectivity to the viral particles (reviewed in Krausslich and Welker, 1996). In fact, optimal infectivity may require the participation of additional accessory proteins during virion assembly (Cohen *et al.*, 1996). Both the Gag and Gag-Pol precursor molecules contain the N-terminal Gag matrix p17 product which is known to be myristoylated. Such post translational modification in conjunction with additional electrostatic interactions provided by basic amino acids in the N-terminal region are currently considered to provide efficient targeting of the Gag precursor molecules to the lipid bilayer (Zhou *et al.*, 1994; McLaughlin and Aderam, 1995; Craven and Parent, 1996). The exact nature of how the precursors traffic to the membrane budding sites is currently not understood though cytoskeletal involvement has been suggested by some groups (Pearce-Pratt *et al.*, 1994; Rey *et al.*, 1996).

The precursors of Gag and Pol proteins assemble as immature viral particles at the plasma membrane and are cleaved into individual proteins by the viral protease late during the budding process (Figure 8). It should be noted that the p6 protein, present only in the context of the Gag, but not the Gag-Pol precursor, has been suggested to play a role in the release of the budding virion particles (Gottlinger *et al.*, 1991; Huang *et al.*, 1995; Yu *et al.*, 1995). As discussed later, p6 also plays a role in the specific incorporation of the accessory protein Vpr. Other viral proteins including Nef and Vif may also be packaged at this stage into the budding particles. The incorporation of Vif, unlike Vpr, does not appear to depend upon the presence of the p6 region (Bouyac *et al.*, 1997a). The mechanism by which Nef is targeted to the virion is not clearly understood though membrane association via myristoylation appears to play a role (Pandori *et al.*, 1996). Interestingly, cellular molecules such as Cyclophilin A have also been shown to be recruited into the budding virions by interacting with specific Gag structures (Luban *et al.*, 1993). The Gag capsid p24 domain has been shown to be the primary molecular determinant that confers the Cyclophilin-incorporation capacity to the Gag precursors (Luban *et al.*, 1993). Specifically, a Proline-rich region within the capsid protein appears to be essential for Cyclophilin interaction (Franke and Luban, 1995; Franke and Luban, 1996). Hence, specific interactions of both cellular and viral proteins to the lipid bilayer and viral Gag structures at the site of assembly appears to be a common theme in HIV-1 virion morphogenesis (reviewed in Cohen *et al.*, 1996).

The leader sequence present in the full length RNA preceding the Gag initiation codon is considered to be the major determinant responsible for efficient packaging of

genomic RNA into budding virions (Aldovini and Young, 1990; Clavel and Grenstein, 1990; Hayashi *et al.*, 1992). This region has been proposed to form stable secondary RNA hairpin structures (Harrison and Lever, 1992; Clever *et al.*, 1995). These hairpin structures appear to be functionally relevant as their disruption generally impairs RNA encapsidation (McBride and Panganiban, 1996; McBride and Panganiban, 1997). Current evidence clearly implicates the RNA binding nucleocapsid protein, NCp7, in the context of the Gag precursor, plays an important role in RNA encapsidation (Dorfman *et al.*, 1993; Poon *et al.*, 1996). The NCp7 protein comprises two zinc finger like regions, frequently referred to as Cystine-Histidine boxes (Bess *et al.*, 1992). *In vitro* binding experiments strongly suggest specific binding of Gag precursor, and specifically NCp7, to RNA occurs possibly at sequences that include RNA secondary structure elements in the leader sequence (Luban and Goff, 1991; Berkowitz *et al.*, 1993; Berkowitz and Goff, 1994). Affecting the integrity of the Cystine-Histidine box region of NCp7 impairs RNA encapsidation (Aldovini and Young, 1990; Gorelick *et al.*, 1993). Thus the viral genomic RNA is also recruited into the assembly machinery through specific interactions with Gag domains.

Unlike the Gag and Gag-Pol precursor trafficking, the envelope protein trafficking to the site of viral budding is well understood. The envelope proteins, gp120 and gp41 are translated from a bisistronic mRNA species that has the ability to code for both the envelope proteins and the accessory protein, Vpu. Such coordinated expression of an accessory protein with that of a viral structural protein is interesting in light of the fact that the Vpu protein augments viral budding as discussed later. The envelope precursor, gp160, like most retroviral glycoproteins, is transported through the ER-Golgi network where specific enzymes mediate its glycosylation and disulfide bridge formations the details of which are beyond the scope of this work (reviewed in Einfeld, 1996). A variety of cellular proteases (Hallenberger *et al.*, 1992; Kido *et al.*, 1993; Decroly *et al.*, 1994) have been proposed to cleave envelope polyproteins presumably during transit through the golgi network (Willey *et al.*, 1988; Bedgood and Stallcup, 1992). Envelope proteins, following cleavage and post translational modifications are packaged into budding virions, and some evidence suggests that specific interaction sites for gp41 cytoplasmic tail exist in the N-terminal matrix region of Gag and Gag-Pol precursors (Yu *et al.*, 1992; Dorfman *et al.*, 1994; Freed and Martin, 1995). In fact, direct interactions between the HIV-1 envelope and matrix p17 has been documented convincingly in a recent report (Cosson, 1996). Interestingly, the very same gp41 C-terminal tail region that has been shown to potentially interact with the Gag matrix

protein has also been shown to target the virion assembly machinery to the basolateral aspect of polarized cells (Lodge *et al.*, 1997). Such basolateral targeting may provide ready access to the blood stream and may play potentially a pivotal role in propagating the virus from mucosal entry sites during sexual transmission. In this regard it is interesting that Gag and Env precursor protein interactions lead not only to specific recruitment of structural proteins necessary for virion integrity and infectivity, but also to controlled targeting of the progeny virions to specific aspects of the cells where subsequent propagation can be maximized.

The LTR and the Viral Regulatory Protein Repertoire

The switch from the latent, low-transcriptional activity phase toward productive phase of high transcription rates and virion production seems to depend both on the viral and cellular factors as discussed above. To understand the viral genomic regulation, it is necessary to understand the structure and function of the proviral long terminal repeats (LTRs) and the viral and cellular regulatory proteins that interact with it (Figure 9). Though both LTRs on either side of the genome are identical the 5' LTR sequences seem to be preferentially utilized for the promoter and enhancer orientation, the reason for this preferential use of the 5' LTR are not clear. Following is a brief account of the salient features of the LTR and the regulatory proteins which contributes to our understanding of their role in cellular pathogenesis. Thus no attempt is made to give a comprehensive review of the cellular and viral factors, and readers are referred to works that deal with specific areas in further detail (Doppler *et al.*, 1992; Gaynor, 1992; Moses *et al.*, 1994; Chang and Zhang, 1995).

Regulatory Regions

The generation of the LTR has been described in detail in a previous section. It is a structure that exists only during the DNA stage of the virus and is generated by the action of the reverse transcriptase. The HIV LTR, indispensable for the regulatory functions extends approximately 600 base pairs on either side of the provirus. In the 5' to 3' direction it contains the U3, R, and U5 regions, with the U3 region lying directly upstream from the transcription start site and the R and U5 regions lying within. The U3 region going upstream form the transcription initiation site contains, the core promoter elements and the modulatory elements (Figure 9).

Directly upstream from the transcription site lies the basic promoter shown to contain a TATA region where TFIID binds, and three tandem repeats of SP-1 binding

sites. Further upstream in the U3, are two 10 base pair conserved NFκB binding sites. Several other cellular factors are shown to bind to the modulatory region possibly in conjunction or in competition with each other. These factors include TCF-1α, USF, NF-AT, COUP, and AP-1 (reviewed in Gaynor, 1992). A segment of the modulatory region, upstream of the NFκB sites has been referred to as the negative regulatory element or NRE as deletions in this region, such as the USF binding site, appear to augment viral replication (Rosen *et al.*, 1985; Lu *et al.*, 1990).

The trans-acting responsive (TAR) region is located in the U3/R border, starting at the transcription site and extending into the transcript (Gaynor, 1995). Hence the TAR sequences are present in all RNA transcripts. Around the same region as Tat, initiation activator LBP-1 and the untranslated binding protein UBP-1 have been shown to bind. Down stream to Tat binds the CTF/NF-1, a factor that is known to bind and activate HSV promoters. Polyadenylation signals are located in the U5 region. The details of LTR organization and its control by cellular factors has been discussed in greater detail elsewhere (Gaynor, 1992).

While the intricate interplay between the panoply of cellular factors that bind to HIV-1 LTR are not completely understood, consensus suggests that the NFκB-binding enhancer sequences play a pivotal role in modulating the HIV-1 promoter activity. NFκB is an inducible transcription factor which frequently plays an important role in the early immune and inflammatory responses by orchestrating gene expression. NFκB activation is immediate in that it is independent of protein synthesis. NFκB is maintained as an inactive complex with its inhibitor IκB in the cytoplasm. Various intra- and extracellular stimuli including viral replication or cytokine activation lead to the dissociation of IκB and the release of active NFκB. Subsequent to its release from IκB, NFκB is translocated to the nucleus, and binds to specific sites in the enhancer elements of various gene promoters thus activating gene expression. The details of NFκB regulation are beyond the scope of this review, though a brief mention of its involvement during HIV replication is appropriate. Earlier studies established that induction of NFκB upon monocytic differentiation (Griffin *et al.*, 1989) and T-cell activation resulted in the upregulation of HIV-1 LTR activity (Harrich *et al.*, 1990). Subsequent studies performed in the chronic monocytic infection system brought to light the fact that HIV-1 infection in itself may activate NFκB activity constitutively in these cells (Roulston *et al.*, 1993; Roulston *et al.*, 1995). The reasons for activation of NFκB upon HIV infection appear to be intricate. A possible mechanism appears to be the upregulation of promoter activity of one of the NFκB subunit precursors, p105, which

in turn leads to higher levels of NFκB that could potentially result in a self perpetuating loop (Paya *et al.*, 1992). It is also becoming apparent that the increased turnover of IκBα may contribute to the constitutive upregulation of NFκB DNA binding activity (McElhinny *et al.*, 1995; Hiscott *et al.*, 1997). While it is clear that HIV uses complex regulatory mechanisms to upregulate NFκB enhancer activity, the exact nature of these mechanisms and their interrelationships are only now being unraveled (reviewed in Roulston *et al.*, 1995).

Auxiliary Genes

Simple retroviruses contain three long, contiguous reading frames coding for the *gag, pol*, and *env* genes, which constitute their structural and enzymatic repertoire, all packaged in the progeny virion. However, HIV belongs to the lentivirus subfamily, members of which are characterized by several additional open reading frames (ORF) not found in simple retroviruses. These ORFs all appear following the Gag-Pol sequences, either immediately preceding the Env sequences or overlapping it, and at least in one case (Nef) extending well into the 3' LTR. These ORFs code for regulatory viral proteins that are readily detectable in infected cells. Some are also packaged in the progeny virions. Much evidence has accumulated in the past decade indicating that these gene products, collectively referred to as auxiliary proteins, are capable of modulating viral replication and infectivity.

HIV-1 possesses at least six such auxiliary proteins, namely, Vif, Vpr, Tat, Rev, Vpu, and Nef. The closely related HIV-2 does not code for Vpu, but codes for another unrelated protein, Vpx, not found in HIV-1 (Figure 2). Mutations affecting either Tat or Rev severely impair viral replication indicating that these two auxiliary proteins are essential for viral replication. However, at least i*n-vitro*, mutations affecting other auxiliary proteins result in minimal effect on the viral replication kinetics. Hence, these proteins have been dubbed dispensable or nonessential for *in-vitro* replication, and are usually referred to as accessory gene products.

Regulatory Genes

Tat and Rev: proteins that bind to mRNA secondary structures

Both Tat and Rev are nuclear proteins with Rev accumulating in nucleolar regions. A common theme unites both Tat and Rev functions in that their function is intricately tied to their ability to bind secondary structures present within the mRNA

transcripts (Karn *et al.*, 1991). Control mechanisms that regulate Tat and Rev functions utilize this specific protein-RNA binding event (Rosen, 1991). Though complete coverage of these two proteins and their functional mechanisms is beyond the scope of this work, in this section we provide a concise summary.

The multiply spliced *tat* gene codes for the 86 amino acids long protein (Tat) which functions as the primary viral transactivator (Arya *et al.*, 1985; Sodroski *et al.*, 1985; Goh *et al.*, 1986). This functional role is indispensable during viral replication as mutants involving defective Tat protein fail to replicate to any appreciable level *in vitro* (Dayton *et al.*, 1986; Fisher *et al.*, 1986). Tat's transactivation function is intricately linked with its ability to bind an RNA structure, the trans-acting responsive region, TAR (Cullen, 1992). The TAR RNA region, by virtue of its position downstream to the initiation site, is present in all mRNA transcripts and has been shown to form a stable stem-loop structure that interacts with purified Tat *in vitro* (Berkhout *et al.*, 1989; Dingwall *et al.*, 1989; Selby *et al.*, 1989).

Though earlier studies could not dissect Tat's activity to either the transcriptional initiation or the transcriptional elongation step, accumulating evidence suggests the primary mechanism of transcription mediated by Tat is at the level of elongation (Cullen, 1993; Garcia-Martinez *et al.*, 1995; Keen *et al.*, 1996; Parada and Roeder, 1996). It is becoming apparent that shortly after initiation of RNA synthesis, the RNA polymerase II stalls and accumulates immediately downstream of the initiation site (Bentley, 1995). This promoter-proximal pausing is ameliorated by nucleic acid binding activators such as Tat which typically recruit elongation factors to the complex (Zhou and Sharp, 1995; Keen *et al.*, 1996; Zhou and Sharp, 1996). Transcriptional elongation correlates with the phosphorylation of the C-terminal domain (CTD) of RNA polymerase II and elongation inhibition occurs in the absence of CTD phosphorylation (Kumar et al., 1997). Mechanistically, Tat mediated transactivation appears to correlate with its ability to interact with a kinase termed the Tat associated kinase, or TAK (Herrmann and Rice, 1995; Yang *et al.*, 1996). Current consensus suggests that catalytic subunits in TAK, and perhaps other kinases that associate with Tat, result in CTD phosphorylation leading to increased processivity of the RNA polymerase II (reviewed in Jones, 1997).

While Tat functions at the transcriptional level, increasing the processivity of the RNA polymerase II, Rev acts at the post transcriptional level on the full length RNA efficiently generated in the presence of Tat. The key to Rev action are the cis acting repression elements (CRS). The presence of CRS elements hinder the nuclear exit of

mRNA that contain them. These repressive sequences are present in the full length mRNA (~9.7 kb) strategically placed in the mRNA sequences of polyprotein precursors of late structural proteins (Cochrane *et al.*, 1991; Schwartz *et al.*, 1992a; Schwartz *et al.*, 1992b). Following nuclear retention, subsequent splicing events in the nucleus remove the CRS regions resulting in spliced mRNAs that efficiently exit the nucleus (Malim and Cullen, 1993). As the CRS are present in structural protein coding regions and not in the regulatory gene sequences, nuclear splicing events effectively preclude structural protein expression (Malim *et al.*, 1990) and only allow for the exit of small multiply spliced mRNA species that code for the early regulatory proteins Tat, Rev, and Nef (Robert-Guroff *et al.*, 1990).

One of these early regulatory genes, Rev, itself a product of multiply spliced mRNA species, upon reaching a certain threshold, appears to radically alter the splicing pattern in such a way that late protein synthesis could occur (Pomerantz *et al.*, 1992). The primary function of Rev is to enable the full-length or minimally spliced mRNAs to exit the nucleus which would otherwise be spliced into smaller mRNA species (Malim and Cullen, 1993). This function is brought about by another cis acting sequence, the rev-responsive element (RRE) also known as cis-acting anti-repression sequences (CAR) located in the *env*-sequences. The RRE sequence is located in the envelope coding region and is hence present both in the unspliced (~9.7 kb) and singly spliced (~4 kb) mRNA species (Dayton *et al.*, 1988; Rosen *et al.*, 1988). The RRE sequence, which forms a complex RNA stem-loop structure, upon binding Rev, promotes cytoplasmic entry of full length and singly spliced mRNA transcripts (Daefler *et al.*, 1990; Dillon *et al.*, 1990; Olsen *et al.*, 1990). Hence, it appears that the nuclear retention of unspliced and singly spliced transcripts mediated by one mRNA structure, the CRS, is overcome by Rev binding to another mRNA structure, the RRE. Hence, the Rev protein upregulates viral structural proteins while down regulating the regulatory genes including itself in an intricate feedback loop (Felber *et al.*, 1990) as discussed in an earlier section on the control of viral structural gene production. Thus, Rev is involved in the transition from the latent to the productive mode of HIV infection and bears great relevance to the disease progression.

Accessory Genes

Increasingly, it has become evident that the remaining auxiliary gene products, collectively called the "accessory" proteins, while not being absolutely essential for replication, are nonetheless capable of affecting specific replication events. In fact,

recent evidence from primary cell infections suggests that optimal viral replication even *in vitro* may only occur in the presence of these proteins. More importantly, accumulating evidence from animal models suggests that these proteins may play pivotal roles during viral pathogenesis *in vivo* (Subbramanian and Cohen, 1994). This section aims to bring the reader up-to-date on what is known regarding the lesser known auxiliary proteins: Vif, Vpr, Vpx, Vpu, and Nef. The Nef protein more closely resembles the regulatory proteins Tat and Rev, and is discussed here along with the accessory proteins solely to indicate its dispensability *in vitro*. Like Tat and Rev, Nef is an early protein coded from ~2Kb Rev independent multiply spliced mRNA species. The other accessory proteins (Vif, Vpr, Vpx, and Vpu) are unlike Nef in that they are all late proteins coded from ~4Kb Rev dependent singly spliced mRNAs.

Nef

The Nef protein is 25 to 27 kDa viral product coded by HIV-1 early in the infection. The protein has been shown to be both phosphorylated and myristoylated (Wolber *et al.*, 1992; Bandres *et al.*, 1994; Cullen, 1994; Harris and Neil, 1994). As an early protein, the expression of Nef is Rev independent and constitutes a large portion of the viral protein synthesized early in the viral lifecycle(Robert-Guroff *et al.*, 1990). Initial studies which were instrumental in designating the protein Nef (or negative factor) characterized it as a down regulator of viral replication, presumably acting through LTR directed transcriptional inhibition (Ahmad and Venkatesan, 1985; Niederman *et al.*, 1989). However, a slew of subsequent studies have clearly established the protein as a positive regulator of viral replication. Several reports by independent groups clearly demonstrated that viral kinetics of Nef mutants were impaired when compared to their wild-type counterparts and this positive effect on the viral replication was evident in various transformed and primary mononuclear cells (de Ronde *et al.*, 1992; Zazopoulos and Haseltine, 1993; Miller *et al.*, 1994; Spina *et al.*, 1994; Sinclair *et al.*, 1997). Though a consensus has evolved in recent years as to the positive nature of Nef function during viral replication, the mechanisms that underlie such positive regulation are only now being understood. One of the primary functional roles identified for Nef that could contribute to augmented viral replication is its effect on viral infectivity. The increased infectivity mediated by Nef manifests itself early in the infection in the form of augmented proviral DNA synthesis (Aiken and Trono, 1995; Schwartz *et al.*, 1995). This functional role is, interestingly, reminiscent of Vif function discussed in the next section.

From a mechanistic point of view, it has been suggested Nef could bring about this early infectivity function by interacting with cellular kinases capable of modulating the activation status of the infected cells. Specifically, Nef has been shown to interact with the Src family of kinases via a conserved PXXP motif, the target for the SH3 domain of Src family of kinases (Saksela *et al.*, 1995; Collette *et al.*, 1996). Studies also suggest other kinases, including PAK-related serine/theronine kinases, could interact with Nef (Sawai *et al.*, 1994; Sawai *et al.*, 1995; Nunn and Marsh, 1996; Sawai *et al.*, 1997). It is worth noting that some evidence exists that suggests Nef's ability to interact with kinases correlates with its ability to increase viral infectivity (Luo and Garcia, 1996; Wiskerchen and Cheng-Mayer, 1996). However, how the kinase interactions and modulation of cellular activation status by Nef lead subsequently to the positive regulation of viral replication, is currently an open-ended question. Some evidence suggests that Nef could potentially mediate this early infectivity function by either modifying viral components and/or by mediating incorporation of cellular kinases (Stevenson *et al.*, 1995; Wiskerchen and Cheng-Mayer, 1996; Swingler *et al.*, 1997). Recruitment of kinases is an interesting notion as Nef itself appears to be incorporated in the virion (Pandori *et al.*, 1996; Schorr *et al.*, 1996; Welker *et al.*, 1996). Regardless of the recruitment of functionally relevant kinases into the virion, consensus exists as to the ability of Nef to interact with cellular kinases in infected cells and its ability to alter cellular activation in general. However, there is very little consensus as to whether such interactions lead to negative or positive regulation of the cellular activation status. It is conceivable some of the differences may have to do both with the experimental systems used by different groups as well as the specific kinases involved (Du *et al.*, 1995; Greenway *et al.*, 1995; Greenway *et al.*, 1996; Luo and Garcia, 1996; Iafrate *et al.*, 1997). Hence, identification of Nef's ability to associate with various cellular kinases has been a mixed blessing in that while it has provided new insights into the muddled world of Nef function, it has also stirred up new controversies in the field. Ongoing experiments in various labs will undoubtedly settle some of the current uncertainties that surround Nef's functional mechanisms (Luo and Garcia, 1997).

In addition to the relatively recent recognition of Nef's ability to affect viral replication, it has long been known that Nef mediates effective down regulation of the cell surface CD4 expression in infected cells (Garcia and Miller, 1991; Garcia *et al.*, 1993). It appears that CD4 down modulation is not directly linked to the virion infectivity function of Nef (Goldsmith *et al.*, 1995). In fact, while Nef's ability to associate with cellular kinases appears to correlate with the viral infectivity phenotype, it

doesn't appear to correlate with the CD4 down regulation (Luo and Garcia, 1996; Wiskerchen and Cheng-Mayer, 1996). Hence, these two functions of the protein seem to utilize apparently separate mechanistic pathways. Surface down regulation of CD4 by Nef appears to be accomplished by endocytosis and lysosomal degradation of the protein (Aiken *et al.*, 1994). Nonmyristoylated Nef mutants were less efficient in down regulating CD4 suggesting an important role for membrane association of Nef (Aiken *et al.*, 1994). From a mechanistic point of view, recent evidence suggests that CD4 and Nef interact physically (Grzesiek *et al.*, 1996; Rossi *et al.*, 1996) and that such interactions may involve Nef acting as a linker between CD4 and the endocytic sorting pathways (Mangasarian *et al.*, 1997). It has been suggested that CD4 down modulation may be functionally relevant either due to protection from superinfection, and/or by releasing associated kinases which in turn contribute to positive regulation of viral lifecycle (Cullen, 1994).

Regardless of the exact mechanisms that underlie Nef function, the protein appears to be essential for optimal viral pathogenesis *in vivo*. It is noteworthy that in spite of extensive sequence variability in Nef, the ORF itself is frequently retained in HIV-strains isolated thus far (Ratner *et al.*, 1985). Importantly, Kestler and coworkers found that SIV Nef-mutants quickly reverted to functional ORFs in infected monkeys suggesting a selective drive to foster intact Nef ORFs *in-vivo* (Kestler *et al.*, 1991). Monkeys that showed no reversion did not progress to clinical disease. Studies performed with SCID-hu mice model of HIV infection also suggests Nef is essential for optimal viral replication (Aldrovandi and Zack, 1996). More recent studies provide additional support to the notion that selective pressures exist *in vivo* to reestablish the expression of a functional Nef protein (Wooley *et al.*, 1997).

Vif

The viral infectivity factor, Vif, is a 23 kDa regulatory protein (Kan *et al.*, 1992) coded late in the viral lifecycle to coincide with active virion production (Arrigo and Chen, 1991; Garrett *et al.*, 1991). From a functional standpoint, the Vif-deficient mutants were severely impaired in establishing cell free infections mediated by virions alone, while they retained the ability to spread efficiently by cell-to-cell transmission suggestive of a defect in the progeny virions generated in the absence of Vif (Fan and Peden, 1992). Cells infected with Vif-deficient HIV-1 mutants produced virions with comparable efficiency as the wild type strain, but the mutant virions were as much as a 1000 times less infectious than wild type virions (Sodroski *et al.*, 1986; Fisher *et al.*,

1987; Strebel *et al.*, 1987). Emerging consensus suggests that the lack of infectivity of virions generated in a Vif-negative context is due to a striking impairment of synthesis of proviral DNA and/or its subsequent integrity (Sova and Volsky, 1993; vonSchwedler *et al.*, 1993; Simon and Malim, 1996).

It has become clear that the requirement for Vif differs dramatically in different cells. While some established cell lines are non-permissive for Vif-deficient strains, most cell lines appear to be either permissive or semipermissive (Fan and Peden, 1992; Gabuzda *et al.*, 1992; Sakai *et al.*, 1993; Sova and Volsky, 1993). However, peripheral blood T lymphocytes and monocyte/macrophages are primarily non-permissive to Vif-deficient viruses. These primary cells show highly restricted permissivity to Vif deficient viruses suggestive of a requirement for Vif in these cells *in vivo* (Gabuzda *et al.*, 1992; Gabuzda *et al.*, 1994; Park *et al.*, 1994). Virions produced by Vif-deficient mutants in semipermissive cells are poorly infectious even for permissive cells (Gabuzda *et al.*, 1992; vonSchwedler *et al.*, 1993). However, after one round of replication in permissive cells, *vif*-deficient mutants continue to spread with wild type kinetics (vonSchwedler *et al.*, 1993). Conversely, Vif-deficient viruses produced in permissive cells can efficiently infect semipermissive as well as non-permissive cells and proceed through one replication cycle with close to wild type kinetics (Gabuzda *et al.*, 1992; vonSchwedler *et al.*, 1993). However, further transmission of the virus, which requires additional replication cycles, is impaired (vonSchwedler *et al.*, 1993). Evidently, the consequences of a defect in the *vif* gene depend on the cell type in which the virus is produced rather than on the target cells. This conclusion is consistent with the observation that Vif-deficient HIV-1 mutants can be complemented in producer but not in target cells by providing Vif *in trans* (Gabuzda *et al.*, 1992; Gabuzda *et al.*, 1994; Park *et al.*, 1994).

The fact that Vif is required during virion production prompted studies that explored if Vif affected the either incorporation and/or processing of viral components. Current consensus suggests that the amount and processing of viral proteins in Vif-deficient virions are not significantly affected (Fouchier *et al.*, 1996; Bouyac *et al.*, 1997b). Also, no difference in the viral RNA content of virions produced in the presence or absence of Vif was detectable (Gabuzda *et al.*, 1992; vonSchwedler *et al.*, 1993). Interestingly, however, the morphology of Vif-deficient virus particles itself was affected (Hoglund *et al.*, 1994). The wild type virions upon maturation contain a characteristic cone-shaped core of electron-dense material homogeneously distributed throughout the core structure. The electron-dense material is thought to represent the

viral genomic RNA complexed with viral proteins. By contrast, virions produced in semipermissive cells in the absence of Vif frequently have a core structure that exhibits nonhomogeneous packing of the electron-dense material (Hoglund *et al.*, 1994). In these mutant viruses, the viral ribonucleoprotein complex is concentrated at the broad end of the cone-shaped core, while the narrow end of the core appears transparent. These morphological changes are likely to be functionally relevant, since they are only apparent in cells in which Vif is required (Hoglund *et al.*, 1994). More recent studies have provided additional support to the notion of morphologically abnormal virions being generated in a Vif-negative context (Borman *et al.*, 1995; Bouyac *et al.*, 1997b).

The fact that Vif's effect on the virions, as mentioned earlier, are primarily determined on the basis of the cell from which the virions are produced and the fact that virions generated in the absence of Vif are morphologically aberrant has led to the notion that the events leading to the augmented infectivity effect are set in motion by Vif late in the preceding viral lifecycle at the level of virion morphogenesis. It has become clear that Vif exists both in a soluble cytosolic as well as a membrane-associated form in infected cells (Michaels *et al.*, 1993; Goncalves *et al.*, 1994; Goncalves *et al.*, 1995). It is conceivable that the membrane-bound Vif affects viral assembly either by coordinating yet unknown events at the budding site, or by interacting with virion components at the plasma membrane. Consistent with this hypothesis, the membrane-association of Vif appears to be important for the infectivity function (Goncalves *et al.*, 1995). In fact, colocalization of Vif and Gag proteins have been documented in infected cells (Simon *et al.*, 1997). Interestingly, earlier studies have documented that Vif itself is incorporated into progeny virions, if only at low copy numbers (Liu *et al.*, 1995; Camaur and Trono, 1996; Fouchier *et al.*, 1996). Subsequent studies have also shown direct binding of Vif to the p55 Gag precursor (Bouyac *et al.*, 1997a). Vif appears to interact specifically with two major Gag regions, NCp7 and the region located between the matrix p17 and the capsid p24 (Bouyac *et al.*, 1997a). Given the virion incorporation of this protein, it is not clear whether it is the activity of Vif late during assembly or its physical presence in the virion that is actually responsible for the aberrant viral morphology and the subsequent reduction in the synthesis and stability of proviral DNA following entry (Simon and Malim, 1996; Bouyac *et al.*, 1997b).

Vpu

Vpu is 16 kDa phosphoprotein unique to the HIV-1 group of primate immunodeficiency viruses with the exception of SIVcpz (Cohen *et al.*, 1988; Strebel *et*

al., 1988; Huet *et al.*, 1990; Schubert *et al.*, 1994). The expression of Vpu is Rev-dependent; hence Vpu, like Vif and Vpr, is a late protein (Arrigo and Chen, 1991). However, unlike Vpr and Vif, Vpu does not appear to be incorporated into progeny virions. Biochemical studies show that Vpu is a type 1 integral membrane protein with an hydrophobic amino-terminal which constitutes the membrane-anchor and a hydrophilic carboxyl-terminal which constitutes the cytoplasmic domain of this protein. It was also shown that Vpu is capable of homo-oligomerization (Maldarelli *et al.*, 1993).

One of the primary phenotypes associated with Vpu is its ability to increase virion release from infected cells (Strebel *et al.*, 1989; Terwilliger *et al.*, 1989). Vpu does not augment viral protein synthesis or the assembly of structural proteins into viral particles. Rather, Vpu facilitates the release of assembled virions from the cell surface (Strebel *et al.*, 1989; Terwilliger *et al.*, 1989). This view is supported by electron microscopic studies which revealed an accumulation of budding virions at the cell surface in the absence of Vpu. In addition, Vpu may be involved in the proper assembly and maturation of virions, as aberrant budding structures are frequently seen in the absence of Vpu (Klimkait *et al.*, 1990; Yao *et al.*, 1993). Furthermore, lack of Vpu leads to an increased frequency of budding at intracellular membranes, rather than at the plasma membrane (Klimkait *et al.*, 1990). It is likely that this results from the endocytosis of unreleased viral particles tethered at the membrane back into the cell surface in the absence of Vpu. It is clear that Vpu need not be expressed from the viral genome, but can function equally well when supplied *in trans* (Terwilliger *et al.*, 1989; Yao *et al.*, 1992). The effect of Vpu on virion release is not dependent on virion maturation since Vpu facilitates release both in the presence and absence of a viral protease (Gottlinger *et al.*, 1993). Also, Vpu can significantly enhance particle production by Gag proteins from retroviruses distantly related to HIV-1, suggesting that the effect of Vpu is unlikely to require highly specific interactions with Gag proteins (Gottlinger *et al.*, 1993). It is tempting to speculate that Vpu enhances retroviral budding indirectly through modification of the cellular environment.

A second function associated with Vpu expression is its ability to selectively mediate CD4 degradation in the Endoplasmic Reticulum (ER). In cells infected with HIV-1, the viral envelope precursor gp160 and CD4 can form complexes which are retained in the ER thus affecting the maturation and transport of this viral protein (Crise *et al.*, 1990; Jabbar and Nayak, 1990; Bour *et al.*, 1991). Vpu can release gp160 from these intracellular complexes, thus allowing the maturation of gp160 to gp120 and gp41 during transport through the secretory pathway (Willey *et al.*, 1992b). The release of

gp160 from intracellular complexes appears to be a consequence of Vpu-induced degradation of CD4 retained in the ER (Willey *et al.*, 1992a). Evidence suggests that for the effective degradation of CD4, both Vpu and CD4 need to be associated with the same membrane compartment (Chen *et al.*, 1993; Schubert and Strebel, 1994; Paul and Jabbar, 1997). In fact it is now clear that Vpu and CD4 interact physically (Bour *et al.*, 1995). NMR studies predict that in addition to its N-terminal transmembrane helix, Vpu might contain two alpha helical motifs in its carboxyl terminal on the cytoplasmic aspect (Wray *et al.*, 1995; Federau *et al.*, 1996; Willbold *et al.*, 1997). CD4-Vpu interaction likely occurs between the first cytoplasmic helix of Vpu and the membrane proximal helical motif of CD4 cytoplasmic tail (Tiganos *et al.*, 1997).

The facilitation of virion release does not appear to be directly linked to the Vpu mediated degradation of CD4 since augmented capsid release was evident even in the absence of the envelope glycoproteins and CD4 (Yao *et al.*, 1992; Geraghty and Panganiban, 1993). It was recently shown that phosphorylation of Vpu affects both known functions of Vpu, though clearly to varying extents. While the virion release function was only partially affected by Vpu phosphorylation mutants, CD4 degradation was adversely affected by the same mutants (Schubert and Strebel, 1994; Friborg *et al.*, 1995; Paul and Jabbar, 1997; Tiganos *et al.*, 1997). While facilitation of virion release mediated by Vpu occurs at the plasma membrane, degradation of CD4 mediated by Vpu occurs in the ER (Schubert and Strebel, 1994). Furthermore, unlike for the CD4 degradation function, which is dependent on the cytoplasmic region of Vpu, the virion release function is dependent on the transmembrane anchor motif of Vpu. Hence, it appears that the two Vpu-functions are mediated by two distinct molecular determinants of the protein and occur in two different cellular compartments (Schubert and Strebel, 1994; Schubert *et al.*, 1996a).

Based on Vpu's weak structural similarity to the influenza ion channel protein M2, it has been speculated that Vpu may wield similar ion channel activities (Strebel *et al.*, 1988). Ion channels are known to regulate cytoskeletal elements which may participate in the terminal events during viral budding. Vpu may also affect local conditions in the ER which may induce degradation of the retained CD4. Though some evidence supporting a ion channel activity for Vpu exists (Ewart *et al.*, 1996; Schubert *et al.*, 1996b; Grice *et al.*, 1997), the functional relevance of such reports still remains unclear (Lamb and Pinto, 1997).

Vpr

Vpr gene and the putative structure of its gene product

All primate immunodeficiency viruses possess an open reading frame that codes for a Viral protein R (Vpr) product. The *vpr* gene sequences frequently overlap Vif sequences on the 5' end and Tat sequences on the 3' end. Expression of this gene product in infected individuals was formally shown in 1987 by the existence of antibodies in infected individuals that recognized recombinant protein of the predicted sequence (Wong-Staal *et al.*, 1987). Subsequent studies have shown that antibody response to Vpr occurs frequently in HIV-1 infected individuals (Gras-Masse *et al.*, 1990; Reiss *et al.*, 1990) and may have pathogenic significance as discussed later (Levy *et al.*, 1994). The Vpr protein is expressed primarily from a singly spliced Rev-dependent mRNA (Arrigo *et al.*, 1990; Arrigo and Chen, 1991; Garrett *et al.*, 1991; Schwartz *et al.*, 1991). The Rev-dependency of Vpr-expression ensures that the protein is generated late in the infection along with other virion proteins. Consistent with this coordinated expression, Vpr is efficiently packaged in the progeny virions in very high copy numbers, at molar equivalents to Gag (Cohen *et al.*, 1990a; Yu *et al.*, 1990; Yuan *et al.*, 1990).

HIV-1 Vpr, the focus of this thesis, is a 14 kDa, 96 amino acids long protein. Though no crystallographic evidence currently exists, mutational studies and computer assisted structural analyses including the data provided in this work identify at least two putative helical motifs within the protein: one in the N-terminal (Chapter 2, Figure 7) and another in the central region of the protein (Chapter 3, Figure 1C). These two structures are referred to here as the N-terminal helix and the central hydrophobic helix, respectively. There exists a short stretch of amino acid residues preceding the first helix (amino acids 1 to 15) which is not presumed to form a highly ordered structure. The putative N-terminal helix that follows this structure discussed in chapter 2 of this work extends approximately from amino acids 16 to 30 (Mahalingam *et al.*, 1995c; Mahalingam *et al.*, 1995d; Yao *et al.*, 1995). This helix is followed by a region (residues 30 to 54) whose secondary structure is currently unclear. The putative central hydrophobic helix discussed in chapter 3 of this work extends approximately from amino acid 53 to 74 and is predicted to form an amphipathic structure with a preponderance of hydrophobic residues (Zhao *et al.*, 1994a; Mahalingam *et al.*, 1995a; Mahalingam *et al.*, 1997). The secondary structure of Vpr at the C-terminus is currently not known, though it is not predicted to form a highly ordered structure such as a helix (Yao *et al.*, 1995). Though the functional relevance of these regions are discussed in subsequent sections, it should be noted here that one of the functions attributed to the C-

terminal region of the protein is its ability to affect the overall stability and/or the conformation of the protein (Mahalingam *et al.*, 1995b; Mahalingam *et al.*, 1995e; Yao *et al.*, 1995). It is also known that a highly conserved Glycine-Cystine motif in Vpr C-terminal may be of particular importance to Vpr stability (Mahalingam *et al.*, 1995b). Formal proof for stabilizing disulfide bridges through the Cystine residues, however, is currently lacking.

Limited information derived from *in vitro* experiments suggests that Vpr is capable of oligomerization perhaps existing as a hexamer (Zhao *et al.*, 1994b). Though the N-terminal portion of the protein comprising of the first helical domain is presumed to be sufficient for this effect, the oligomerization may well extend into the central hydrophobic region generating a compact structure for the protein (Zhao *et al.*, 1994b). Though the exact nature of the biochemical interactions operative in Vpr oligomerization is not known, the involvement of the N-terminal amphipathic helix suggests a conceivable role for hydrophobic interactions between adjacent molecules through this interface. Though oligomerization is likely to play a role in some of the Vpr-associated phenotypes, a suggestion put forward by some authors as a possibility, no studies currently exist that formally demonstrate such functional relevance.

Molecular determinants of Vpr virion targeting

Our understanding of how Vpr is targeted to the virion has greatly improved in recent years. It is now clear that neither the viral envelope glycoproteins nor the genomic viral RNA play a role in Vpr incorporation (Lavallée et al, 1994; Paxton et al, 1994, Lu et al, 1993). As Vpr is not synthesized as part of a HIV-1 polyprotein precursors, it must independently associate with the assembling capsid structure. Several recent studies have clearly shown that Vpr uses a virion-association motif in the C-terminal p6 domain of the viral Gag polyprotein. Removal or truncation of the p6 domain abolished the incorporation of Vpr into HIV-1 virions (Checroune et al, 1995; Paxton et al, 1994). More importantly, when the HIV-1 p6 domain was fused to the C-terminus of the Gag polyprotein of Moloney murine leukemia virus, a distantly related retrovirus that lacks a p6 domain, efficient incorporation of Vpr into the heterologous capsids was achieved (Kondo et al, 1995). Further mutational analysis showed that the Vpr virion-association motif is located within residues 1 to 46 of the HIV-1 p6 domain (Kondo et al, 1995). A Leucine repeat region, LXXL, present in the carboxyl terminal of the protein has been demonstrated to play an essential role in this interaction (Lu *et al.*, 1995; Kondo and Gottlinger, 1996). Consistent with its role in Vpr incorporation, a p6 domain is present

in the Gag polyproteins of all primate immunodeficiency viruses, but is not found in other retroviruses.

While it is possible that p6 directly interacts with Vpr as an independent domain, anchoring it to the budding virion structures, this has not yet been formally shown. Though genetic studies suggest adding the p6 molecular determinant also results in active Vpr incorporation, they do not prove that p6 interacts with Vpr directly as an independent entity. Also in light of the fact that the NCP7 ribonucleocapsid protein has also been shown to be involved in Vpr incorporation (Li *et al.,* 1996), does p6 function in the context of other Gag structures, or is p6 a separate entity, that co-evolved with Vpr, to mediate its incorporation? These questions may be clarified if future *in vitro* experiments show direct binding of p6, or perhaps p15 (NCP7 and p6 encoding Gag cleavage product) can bind Vpr in isolation of other Gag structures. However, it is currently clear that the p6 "domain" provides the genetic requirements for Vpr virion incorporation.

Recent years have also seen rapid progress in the dissection of molecular determinants within the Vpr protein itself that mediate its virion incorporation. In contrast to the Gag p6 domain, which is highly variable among different HIV-1 genotypes, the Vpr protein is well conserved and an earlier section briefly dealt with the protein's putative structure. The C-terminal basic domain of the protein, and the central hydrophobic helix appear to play a lesser role in the virion-association of Vpr (Mahalingam et al, 1995; Yao *et al.,* 1995). In fact, current consensus suggests that this N-terminal helix is necessary and sufficient to mediate the virion incorporation of Vpr. Mutations which are predicted to disrupt the helix or which affect either the hydrophobic or hydrophilic interface residues within the helix impair Vpr incorporation, suggesting that this amphipathic helix constitutes an interface involved in the incorporation process (Mahalingam et al, 1995; Yao *et al.,* 1995). It is known, however, that particular residues within the C-terminus can affect virion incorporation indirectly as they affect the overall stability of the protein. Though Vpr is targeted to the virion in the context of the Gag precursors, following virion maturation Vpr appears to be localized between the viral envelope and the viral core (Wang et al 1995, Liska et al, 1994, Yu et al, 1993). Recent studies also suggest that Vpx, an HIV-2/SIV homologue of HIV-Vpr, in the context of the mature virions, may interact with the Gag matrix product p17 (Sato *et al.,* 1996). Such interactions may have functional significance as both these gene products have been implicated in the nuclear transport of proviral DNA (discussed in a later sections).

Vpr's ability to growth-arrest proliferating cells

It has been known for sometime that one or more accessory proteins, frequently including Vpr, was responsible for the cytotoxic mechanisms that prevented establishment of HIV-1 chronic infection (Nishino *et al.*, 1991; Nishino *et al.*, 1994). In other words, target cells failed to divide and propagate following an acute infection with viruses that had an intact open reading frame for accessory proteins such as Vpr (Planelles *et al.*, 1995). This concept is supported by the interesting finding that Vpr, in the absence of any other viral proteins, induces differentiation and growth arrest in a rhabdomyosarcoma cell line, demonstrating that Vpr can modify basic cellular pathways (Levy *et al.*, 1993). It was not until Rogel and coworkers formally proved that this was the result of Vpr's ability to growth-arrest proliferating cells in the G2-phase of the cell-cycle a mechanistic understanding of the lack of chronic infection began to emerge (Rogel *et al.*, 1995). Currently, it is clear that Vpr is sufficient to induce G2-arrest in not only T-cells, but also other cells types including Human fibroblasts (HeLa), kidney derived 293 cells, and the muscle cell lineage (Levy *et al.*, 1993; Di Marzio *et al.*, 1995; He *et al.*, 1995; Re *et al.*, 1995; Ayyavoo *et al.*, 1997). In fact, recent evidence suggests that Vpr may perturb the growth of eukaryotic yeasts and prokaryotic bacteria as well (Zhao *et al.*, 1996; Bodeus *et al.*, 1997; Zhang *et al.*, 1997). Hence it is highly likely that the HIV-1 Vpr mediated growth-arrest is brought about by manipulating biochemical pathways which are evolutionarily conserved.

In spite of the evolutionary conservation of the growth-arrest phenotype itself, some species specificity exists among the Vpr alleles coded by different lentiviral groups. Earlier experiments performed using a laboratory adapted strain suggested that HIV-2 Vpr, due to lower protein stability, may not mediate growth-arrest as efficiently as the highly stable HIV-1 Vpr (Kewalramani *et al.*, 1996). However, subsequent experiments suggest that this is not a general property of naturally occurring viruses as Vpr derived from primary HIV-2 isolates were capable of inducing G2-arrest regardless of their reduced stability (Stivahtis *et al.*, 1997). In fact, in spite of clearly distinct steady state levels in infected cells, both human immunodeficiency viruses (HIV-1 and HIV-2) appear to retain the ability to mediate efficient growth-arrest in human cells (Stivahtis *et al.*, 1997). Similar species specificity was also evident among the simian immune deficiency viruses in that all SIV isolates regardless of the origins arrested simian cells but failed to growth-arrest human cells. However, SIV derived from sooty mangabey, SIVsm, was capable of partial G2-arrest activity in human cells perhaps due to its close resemblance to HIV-2 Vpr. Interestingly, more divergent simian isolates from African

green monkey (SIVagm) and sykes' monkey (SIVsyk) were totally non functional for the G2-arrest phenotype in human cells.

Mechanistically, studies suggest that the cdc2 kinase, a key player in the G2-phase to mitosis transition, is inactivated by hyperphosphorylation in the presence of Vpr (He *et al.*, 1995; Re *et al.*, 1995). As Vpr does not appear to directly bind cdc2, it is likely that the cdc2 hyperphosphorylation is the result of upstream events (Re *et al.*, 1995). Upstream events may involve modifying key cellular phosphatase activities that control cdc2 phosphorylation status. In fact, some evidence suggests that Vpr directly activates the protein phosphatase 2A (PP2A) in the conjunction with the Gag NCp7. As activated PP2A is a negative regulator of kinases that activate cdc2, this could explain the downstream inactivation of cdc2 (Tung *et al.*, 1997). The exact nature of Vpr mediated PP2A activation are currently not clear. Earlier studies suggested that Vpr mediated G2-arrest may be distinct form pathways operative during DNA damage checkpoints (Bartz *et al.*, 1996). However, this observation is not in agreement with recent evidence that suggests that Vpr's ability to induce growth-arrest resembles pathways that are operative during DNA damage (Poon *et al.*, 1997; Stewart *et al.*, 1997). DNA damage pathways could be relevant to Vpr function as the protein is known to interact *in vitro* with cellular proteins involved in excision repair machinery, namely the Uracil DNA glycosylase (UDG) and HHR23A (Banapour *et al.*, 1991; Withers-Ward *et al.*, 1997). Regardless of the exact mechanism involved, it is clear that Vpr mediated G2-arrest leads to cell death by apoptosis (Poon *et al.*, 1997; Stewart *et al.*, 1997). This has lead to the suggestion that one of the key functions of Vpr may be to prevent the expansion of CD4+ T cells specific for viral antigens. Vpr may thus contribute to the functional anergy of helper cells documented in AIDS patients. Though this is a highly likely possibility, formal data that supports this hypothesis is currently lacking. Also unclear from published literature is the functional relevance of this G2-arrest activity in non dividing target cells such as macrophages.

Molecular determinants that control Vpr nuclear targeting

One of the biological activities associated with Vpr is its ability to localize to the nuclear compartment. How the protein is targeted to the nucleus is an active area of research in many labs that research the protein, including ours. The trafficking of Vpr within the infected cell can readily be traced by immunofluorescence. Vpr expressed in the absence of Gag proteins localizes to the nucleus. However, Vpr becomes associated

with membrane compartments late in the replication cycle, when virion assembly and budding occurs (Lu et al, 1993).

As will be discussed at greater lengths in subsequent chapters, both the integrity of the N-terminal and the central hydrophobic helical motifs appear to be essential for optimal nuclear localization of Vpr (Zhao *et al.*, 1994a; Mahalingam *et al.*, 1995a; Mahalingam *et al.*, 1995c; Mahalingam *et al.*, 1995d; Yao *et al.*, 1995; Mahalingam *et al.*, 1997). The C-terminal region does not appear to greatly affect this phenotype, though gross deletions appears to indirectly affect the phenotype by affecting stability and/or, conceivably, the confirmation of the protein (Yao *et al.*, 1995). However, studies described in this thesis suggests that the nuclear localization of Vpr may proceed by a multi step process whereby the protein is first targeted to the nuclear periphery and is subsequently imported into the nuclear interior. The central hydrophobic helix appears to be at least one of the molecular determinants that controls the nuclear targeting and import phenotype of the protein as discussed later. Interestingly this hydrophobic region has been suggested to constitute a membrane spanning domain of Vpr by studies performed in lipid bilayers (Piller *et al.*, 1996). While, whether or not such membrane insertion occurs in intact cells is currently unclear, studies have shown a portion of Vpr expressed in cells does associate with cellular membrane structures (Sato *et al.*, 1990; Lu *et al.*, 1993). Another possibility, which may not be mutually exclusive with physical membrane insertion, could be Vpr's ability to bind integral membrane associated structures involving strong protein-protein interactions. In this regard, some studies have suggested that the central hydrophobic region may constitute a Leucine zipper like domain that interacts with cellular partners (Zhao *et al.*, 1994a; Wang *et al.*, 1996b). Several cellular proteins that could potentially target Vpr to the cell-nucleus have been identified to date. Vpr has been suggested to interact with nuclear transcription factors TFIIB and SP1 (Wang *et al.*, 1995; Bouhamdan *et al.*, 1996). An unknown 200kDa protein termed RIP (for Vpr interacting protein) has been suggested to bind Vpr through this hydrophobic domain and transport it to the nucleus in a "piggy-back" fashion (Zhao *et al.*, 1994a). In fact, Vpr has also been suggested to bind the glucocorticoid receptor type II complex which is known to shuttle between the cytoplasm and the nucleus (Refaeli *et al.*, 1995). Vpr is also known to directly interact with HHR23A another nuclear protein involved in DNA excision repair (Withers-Ward *et al.*, 1997). As the relevance of most of these interactions in the context of viral replication is not known, the functional contributions of these interactions to Vpr's nuclear localization, and to viral replication in general, remain to be formally proved. In

fact, whether or not nuclear localization of Vpr is linked to various functions attributed to the protein (discussed in the next few sections) is not clearly understood and this issue is further addressed in chapter 4 of this work.

The functional roles of Vpr

Several studies indicate that Vpr confers rapid growth kinetics to viruses expressing the protein *in vitro*. Vpr-positive strains *in vitro* grow faster and release moderately higher levels of virus than their Vpr-negative counterparts, an effect that is particularly pronounced in primary macrophages (Hattori *et al.*, 1990; Westervelt *et al.*, 1992; Connor *et al.*, 1995). The functional mechanisms that underlie this augmented replication phenotype has been an active area of research in the past few years. Current consensus associates two major biological activities with Vpr expression. Vpr, in dividing cells, is considered to mediate G2-arrest. On the other hand, in non dividing cells such as macrophages, Vpr is associated with efficient nuclear transport of the proviral DNA. In addition to these two primary activities, extracellular Vpr also appears to trigger cellular responses relevant to viral replication. The relevance of these activities to Vpr function are elaborated in the following sections.

The early functional role

As discussed earlier in detail, one of the key characteristic of Vpr is its ability to incorporate into progeny virions specifically and at high copy numbers. As a protein packaged in the virions, Vpr is available early in the next round of infectious cycle. In fact, early studies that characterized Vpr's virion incorporation put forward the possibility that the protein may function at a very early step immediately following virion entry (Cohen *et al.*, 1990a). This possibility was formally demonstrated when Vpr was shown to facilitate the nuclear localization of the viral DNA in non dividing cells (Heinzinger *et al.*, 1994). In contrast to onco-retroviruses, HIV-1 does not require mitosis for entry of the viral genome into the nucleus, which is a necessary step of the retroviral life cycle (Lewis *et al.*, 1992; Lewis and Emerman, 1994). In this context, Vpr is reported to function as one of the multiple nucleophilic determinants that target the retroviral preintegration complex to the nuclear compartment where it can be integrated in the cellular genome in a stable fashion (Stevenson *et al.*, 1995; Emerman, 1996). This appears to be an intricately controlled event in the viral life cycle with the participation of at least two other virion-associated viral proteins, the integrase and the Gag matrix p17. It appears that Vpr mediated proviral nuclear import may occur by pathways distinct from the viral integrase and matrix p17, both of which appear to bring about this effect

by interacting with karyopherin-α family of proteins at the nuclear periphery (Gallay *et al.*, 1996; Gallay *et al.*, 1997). The need for such multiple nucleophilic determinants utilizing apparently distinct pathways has not been explored in detail by current studies and a later chapter in this work will address this question formally. It remains to be seen whether this function of Vpr early in the infection is in some way connected to its effects on cellular proliferation and/or nuclear localization property of the protein. Such a correlation may help unify the pleiotropic effects associated with this auxiliary protein.

Vpr late functional role

One of the very first studies that addressed the functional relevance of Vpr suggested that it could function as a moderate transactivator of gene expression driven not only by the HIV-1 LTR, but also by heterologous promoters (Cohen *et al.*, 1990b). One possibility is that this transactivating ability of Vpr could be advantageous early in the infectious cycle, before the strong viral transactivator Tat becomes available (Subbramanian and Cohen, 1994). It is conceivable that Vpr has a role in augmenting the basal promoter activity of the long terminal repeat during a period immediately following integration, in which Tat is not yet available. Such activation of the long terminal repeat may occur via changes in the cellular environment induced by the incoming Vpr or Vpr synthesized following integration. Some evidence exists suggesting that the augmented replication evident in macrophages may primarily result from Vpr synthesized *in situ* and not by the virion associated Vpr (Connor *et al.*, 1995). However, the authors could not preclude the contribution of the early function as the ability of Vpr to target the proviral DNA to the nucleus was not monitored in this study. It is likely that Vpr in the macrophage lineage of cells may act upon transcriptional regulation of other cellular genes, and indirectly galvanize a cellular milieu that will support optimal viral replication. Recent evidence also suggests that Vpr can alter NFκB activity by modulating IκB levels (Ayyavoo *et al.*, 1997). It is interesting to note that, Vpr has been biochemically shown to interact with other transcriptional factors, including SP1 and glucocorticoid receptor type II (GRII) which are known to shuttle from the cytoplasm to the nucleus (Refaeli *et al.*, 1995; Wang *et al.*, 1995). Vpr has also been shown to interact with the TFIIB basal transcriptional factor (Agostini *et al.*, 1996).

Extracellular Vpr

Recent studies show that extracellular Vpr is a powerful activator of HIV-1 expression. Exogenously added Vpr reactivated virus expression in latently infected cell

lines and induced virus replication in newly infected resting peripheral blood mononuclear cells (Levy *et al.*, 1994; Levy *et al.*, 1995). This observation of transcriptional reactivation also provides support to the late function of Vpr. Purified recombinant Vpr in minute concentrations was also capable of inducing a long lasting state of increased cellular permissiveness for HIV-1 replication when added prior to infection. It is conceivable that extracellular Vpr provides the same nucleophilic functions, or induces the same changes in the cellular milieu, as does the Vpr which is carried in the incoming virion. Such pervasive alteration of the cellular environment most likely involves specific associations with both viral and cellular proteins as discussed earlier. However, the functional relevance of these associations is not known.

A role for Vpr *in vivo*

The *in vivo* relevance of the accessory proteins, including Vpr, has been controversial. However, emerging consensus suggests these proteins may play a vital role that may be overlooked in *in vitro* experiments. It appears that Vpr plays an important role in pathogenesis *in vivo*, as rhesus monkeys infected with a Vpr-defective SIV_{mac} either reverted to a Vpr-positive phenotype or were associated with a low viral burden with subsequent inability to progress to clinical disease (Lang *et al.*, 1993). Vpr expressing molecular clones maintained high viral loads *in vivo* in SIV infected rhesus monkeys while Vpr mutants were associated with lower viral burden in the same system (Lang *et al.*, 1993). A subsequent study by Gibbs and coworkers also suggests that affecting either Vpr or its homologue Vpx in the SIV genome resulted in attenuated viruses that showed lower viral burdens (Gibbs *et al.*, 1995). Similarly, Hoch and coworkers also find that SIV-Vpr-mutant viruses show transient antigenemia and viral attenuation, allowing some animals to resist disease progression (Hoch *et al.*, 1995). Perhaps the most interesting observation that indicates the need for Vpr *in vivo* has been the experiments performed by Wooley and coworkers where recombination events *in vivo* reassemble a functional open reading frame for Vpr (Wooley *et al.*, 1997). It is also clear that frequent mutations in the C-terminus of the protein occur *in vivo,* which has been correlated with low viral burden and long term non progression to disease (Wang *et al.*, 1996a). It is likely that the higher viral burdens discussed above, documented by various groups *in vivo,* are mechanistically linked to the higher levels of viruses produced *in vitro* in the presence of Vpr both in T-cells (Ogawa *et al.*, 1989; Cohen *et al.*, 1990b) and in macrophages (Westervelt *et al.*, 1992; Connor *et al.*, 1995).

Vpx

The 12 to 16 kDa Vpx protein is unique to the HIV-2/SIV_{mac} group of primate immunodeficiency viruses and is not present in HIV-1 (Henderson *et al.*, 1988). Vpx shows considerable sequence conservation among various isolates of HIV-2 (Tristem *et al.*, 1992). The members of the HIV-2/SIV_{mac} group, in addition to the Vpx protein, also code for an additional Vpr protein which shares notable sequence homology with Vpx. Based on such sequence similarity and the overall genome organization, it has been suggested that the *vpx* gene could have arisen from the duplication of the *vpr* gene in a common ancestor for the HIV-2/SIV_{mac} group of primate immunodeficiency viruses (Tristem *et al.*, 1990; Tristem *et al.*, 1992). However, an alternative hypothesis, specifically, gene acquisition as opposed to gene duplication, has also been put forward recently (Sharp *et al.*, 1996). Regardless of their origins, as discussed later, emerging consensus suggests that these two gene products mediate apparently separable functions during HIV-2/SIV replication (Fletcher *et al.*, 1996).

Like its homologue Vpr, Vpx is packaged into virions at high copy numbers (Yu *et al.*, 1993). Also, like Vpr, Vpx incorporation is mediated via its interaction with the carboxyl terminal p6 domain of the viral Gag polyprotein (Wu *et al.*, 1994). While the same Gag domain mediates the virion-association of both Vpx and Vpr, the precise requirements for the incorporation of these proteins differ. Carboxyl-terminal sequences within the p6 domain, which are required for the incorporation of Vpr, are dispensable for the incorporation of Vpx. Also, in spite of its relative homology, Vpx is not efficiently incorporated into HIV-1 virions, indicating that the HIV-1 p6 domain lacks an efficient virion-association motif for Vpx (Kappes *et al.*, 1993; Wu *et al.*, 1994). Vpx, like Vpr, appears to form higher order oligomers and its close association with virion core structures has been documented, presumably as a homodimer (Kewalramani and Emerman, 1996).

Phenotypically, Vpx appears to be essential for optimal viral replication in primary cells. Vpx deficient mutants lag behind their wild-type counterparts in viral kinetics (Kappes *et al.*, 1991; Park and Sodroski, 1995), and this effect is particularly pronounced in macrophages (Yu *et al.*, 1991). In fact, current consensus suggests that this phenotype results from the efficient nuclear import of proviral DNA in the presence of Vpx in non dividing cells such as macrophages. This function, as discussed earlier, is mediated in the HIV-1 system by its homologue Vpr. Interestingly, however, HIV-2/SIV Vpx does not mediate the second function of HIV-1 Vpr, that is, the ability to growth-arrest proliferating cells in the G2-phase of the cell-cycle (Di Marzio *et al.*, 1995;

Re *et al.*, 1995; Fletcher *et al.*, 1996). The G2-arrest function is mediated in HIV-2/SIV systems rather by a second HIV-1 Vpr like protein named Vpr-2. Interestingly, Vpr-2 of HIV-2/SIV fails to mediate the proviral nuclear import function associated with HIV-1 Vpr (Fletcher *et al.*, 1996). It has been suggested that Vpr-2 and Vpx arose either through a gene duplication or through a gene acquisition event phylogenetically in the HIV-2/SIV lineage (Tristem *et al.*, 1990; Sharp *et al.*, 1996). Hence, it appears, that two functions mediated by one gene in HIV-1, namely Vpr, are mediated in HIV-2/SIV by two related but separate genes, namely, Vpr-2 and Vpx.

Vpx has been reported to localize to the nucleus and this is in accordance with its proposed proviral nuclear import function. Localization studies in the mature virion also place the protein in a functionally relevant position, in close association with the core, though the exact location has been somewhat controversial. Earlier studies localize Vpx, like its functional homologue HIV-1 Vpr, between the nucleocapsid core and the outer protein shell formed by the matrix protein (Yu *et al.*, 1993; Liska *et al.*, 1994). However, a subsequent report suggests that Vpx may associate with the core structure itself, perhaps in a homodimer form (Kewalramani and Emerman, 1996). Also, some evidence suggests that Vpx may associate with the core capsid protein p27 domain (Horton *et al.*, 1994). Early work by Henderson and coworkers indicated that Vpx could interact with single stranded RNA (Henderson *et al.*, 1988). Taken together, these observations suggest that Vpx could remain associated closely with the viral core and perhaps also with the viral nucleic acids. In fact, a recent study demonstrated that Vpx associates with the preintegration complex in infected cells (Fletcher *et al.*, 1996; Hansen and Bushman, 1997). The exact mechanisms by which Vpx targets the preintegration complex to the nucleus, however, is currently not known.

OBJECTIVES OF THE PRESENT STUDY

While the previous section on HIV-1 Vpr highlighted some of the biological activities and functional roles associated with the protein, our understanding of the mechanisms that underlie these phenotypes is still far from complete. The two major biological activities associated with HIV-1 Vpr are its ability to localize to the nucleus and its ability to growth arrest proliferating T-cells in the G2-phase of the cell cycle. At the functional level, Vpr has been suggested to play an early role by targeting the provirus to the cell nucleus. It is also considered to be functionally active late in the life cycle, perhaps as a transactivator. However, how the two major biological activities associated with the protein contribute to the protein function is unclear. Also, the molecular determinants within the protein that mediate these biological phenotypes has not been well characterized.

The objectives of my study are:

1) Characterize the structural elements within HIV-1 Vpr that control the various biological activities of the protein (dealt with in Chapters 2 and 3).

2) Characterize the functional relevance of the two primary biological activities associated with Vpr, namely, its nuclear localization and its ability to growth arrest T-cells (Chapter 4)

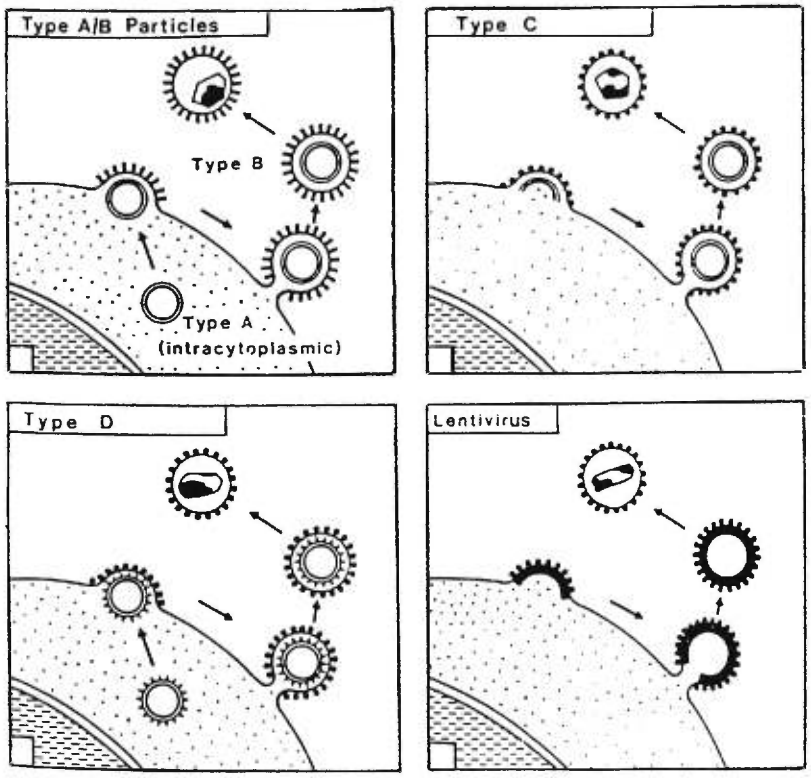

Figure 1: Morphological grouping of retroviruses. A-type particles are strictly intracytoplasmic and may include intermediary forms of type B- or D-type viruses. Capsid structures of both B- and D-type viruses assembled first in the cytoplasm and subsequently bud at the plasma membrane. Type-D differs from type-B in that they do not have prominent surface envelope projections characteristic of mature type-B virions. On the other hand, in type-C viruses, both capsid assembly and budding occurs at the plasma membrane hand in hand. Lentiviruses can be conceptualized as a variation on the type-C theme. Like type-C viruses, lentiviruses also assemble and bud at the plasma membrane though their cores mature into cone/bar shaped bodies (original illustration derived from *Fields Virology,* second edition/Lippincott-Raven publishers).

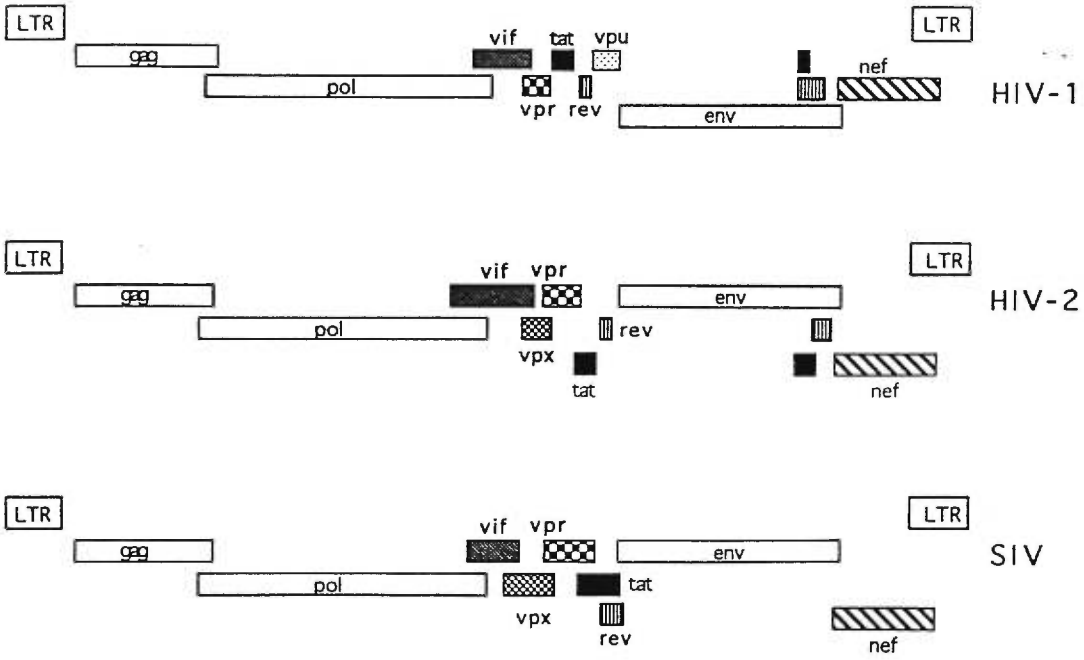

Figure 2: Lentiviral genome organization. A common feature is are the ORFs that code for the structural proteins Gag, Pol, and Env. Additional regulatory proteins are also present though not all are present among the different lentiviral members. As discussed in the regulatory protein section, Vpu is unique to HIV-1 and Vpx is unique to HIV-2 and SIV(discussed later in further detail in the regulatory protein section). Original illustration derived from *Principles of Molecular Virology*, Second Edition, Alan J. Cann (1997) Academic Press.

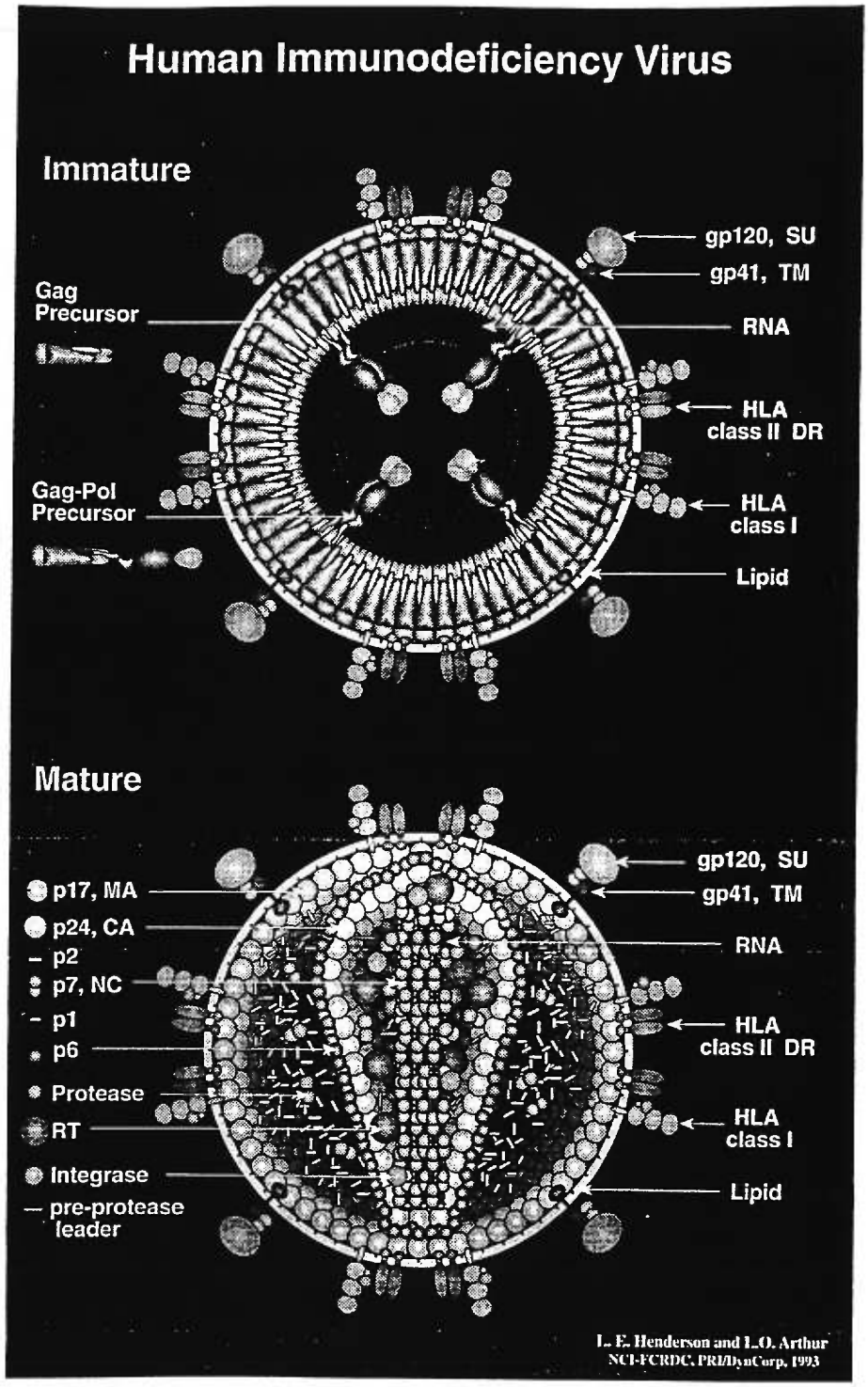

Figure 3: Structure of HIV-1 virions both in the mature and immature form. Location of the virion proteins are depicted in each case. Note that the precursors proteins which predominate in immature virion are cleaved into separate protein products in the process of maturation. The details of the structure and the assembly processes are discussed in appropriate sections in the text. Original illustration derived from *Viral Pathogenesis*, Neal Nathanson, 1997, Lippincott-Raven Publishers.

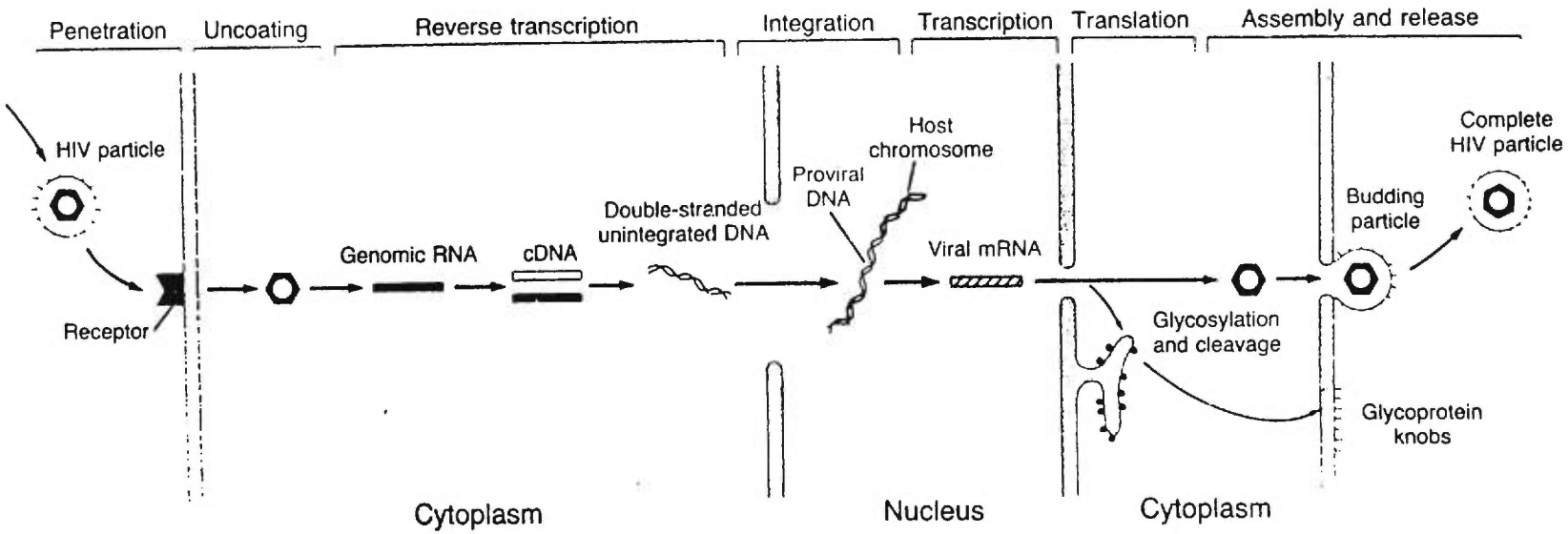

Figure 4: Schematic of the HIV lifecycle. The picture depicts 1) viral entry through fusion/penetration, 2) uncoating of genomic RNA, 3) reverse transcription of RNA to a DNA provirus and its nuclear transport, 3) integration 4) transcription of viral mRNA from integrated provirus and its export to the cytoplasm 5) translation of RNA to generate viral proteins 6) assembly of viral proteins and virion release. The details are discussed in the text. Original illustration derived from *Fields Virology,* second edition/Lippincott-Raven publishers..

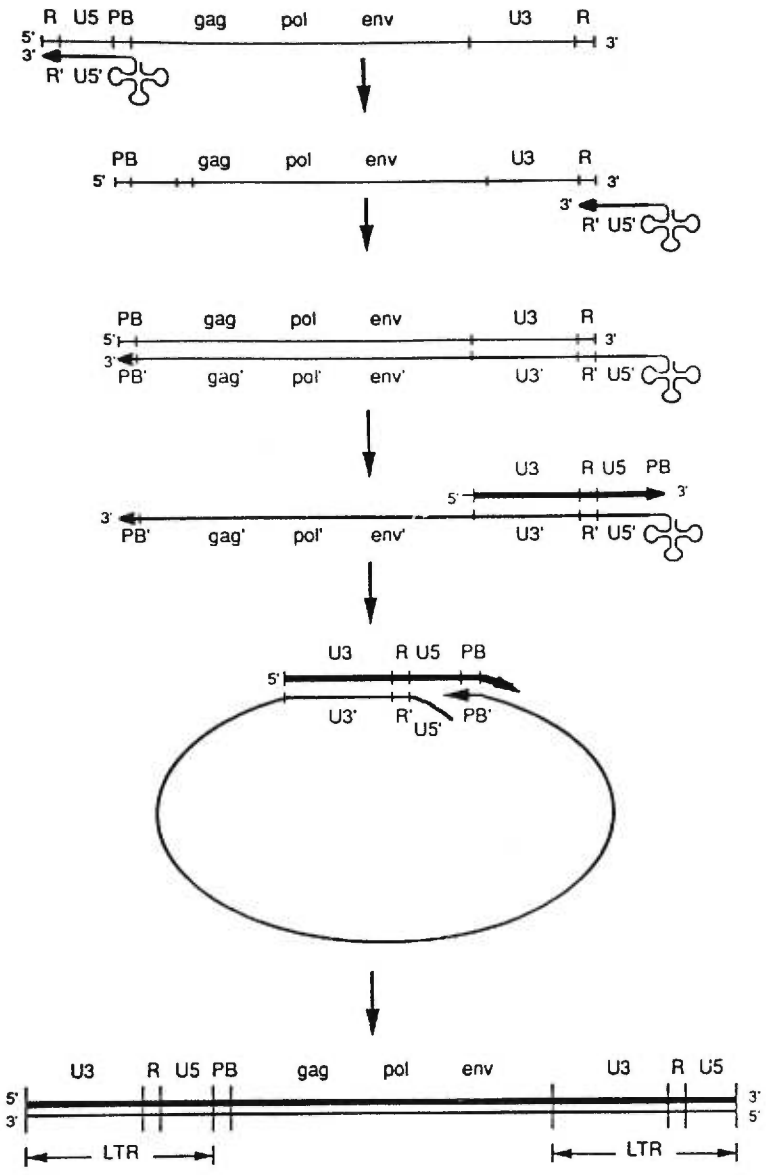

Figure 5: Schematic of retroviral reverse transcription. Thin lines represent original viral RNA. Thick lines represent newly synthesized DNA provirus. The stem loop structure represents the t-RNA lysine bound at the primer binding site. The original unique sequences (U3 and U5), the terminally redundant sequences (R), and the resulting LTR organization (U3-R-U5) is indicated. Original illustration derived from *Fields Virology,* second edition/Lippincott-Raven publishers.

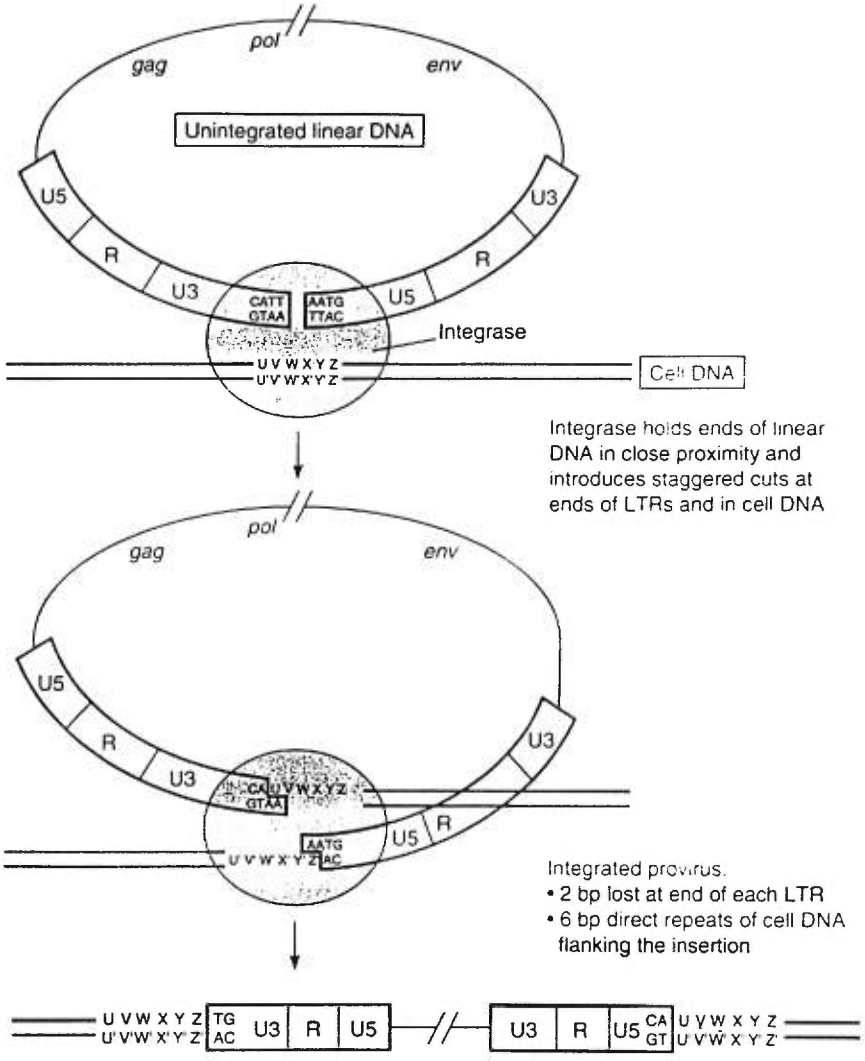

Figure 6: Schematic of retroviral integration. The figure depicts the nicking and joining reactions that ensure stable integration into the cellular genome. Original illustration derived from *Principles of Molecular Virology*, Second Edition, Alan J. Cann (1997) Academic Press.

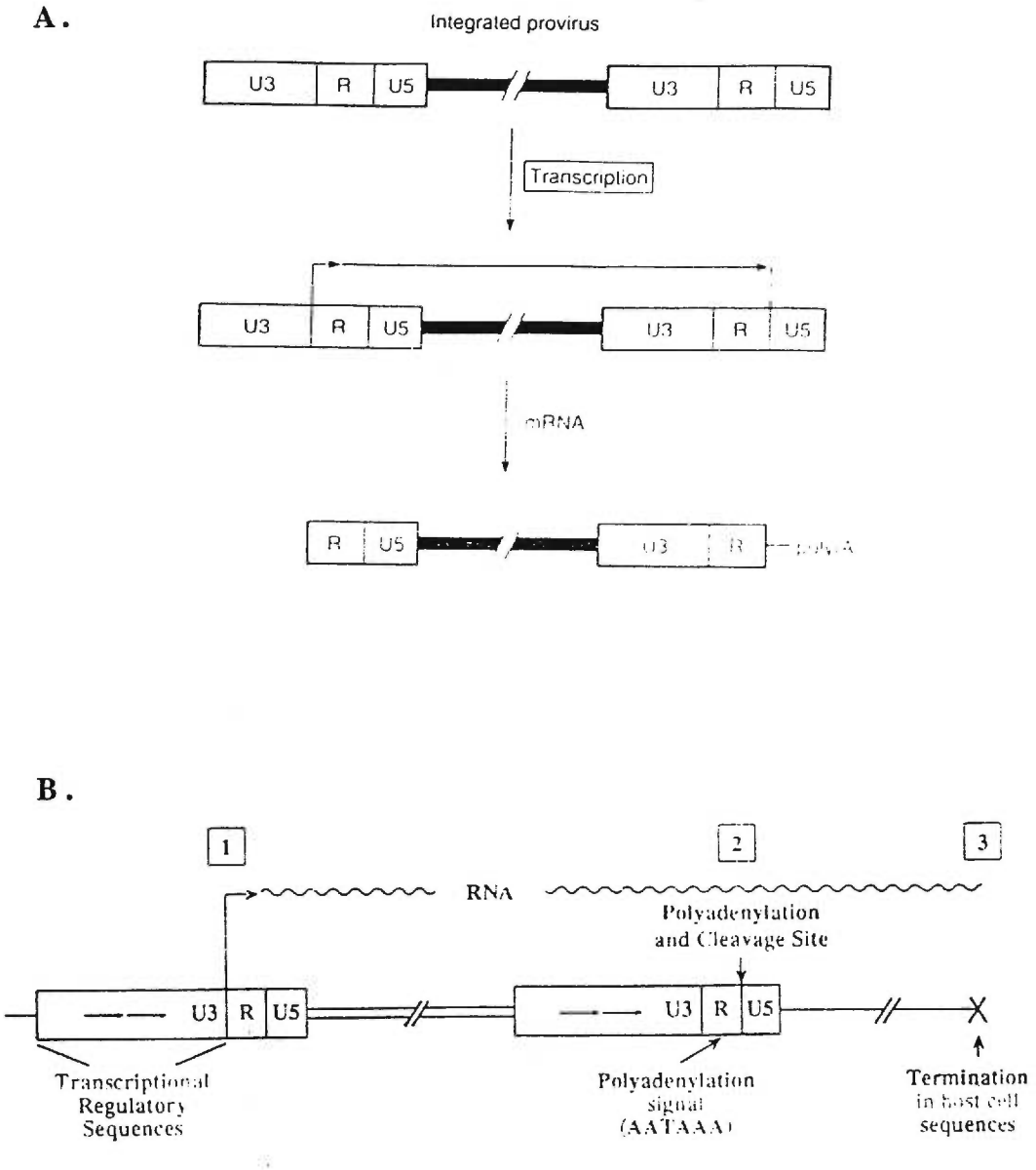

Figure 7. Generation of viral mRNA. Panel A depicts schematically how the process of transcription driven off the viral LTR regenerates the unique and redundant sequences found in the original genomic RNA. Panel B depicts the start site (1) transcription start site at the U3/R border, (2) the polyadenylation signals at the R/U5 region and (3) termination signals further down stream in the cellular genome. Original illustration derived from *Principles of Molecular Virology*, Second Edition, Alan J. Cann (1997) Academic Press.

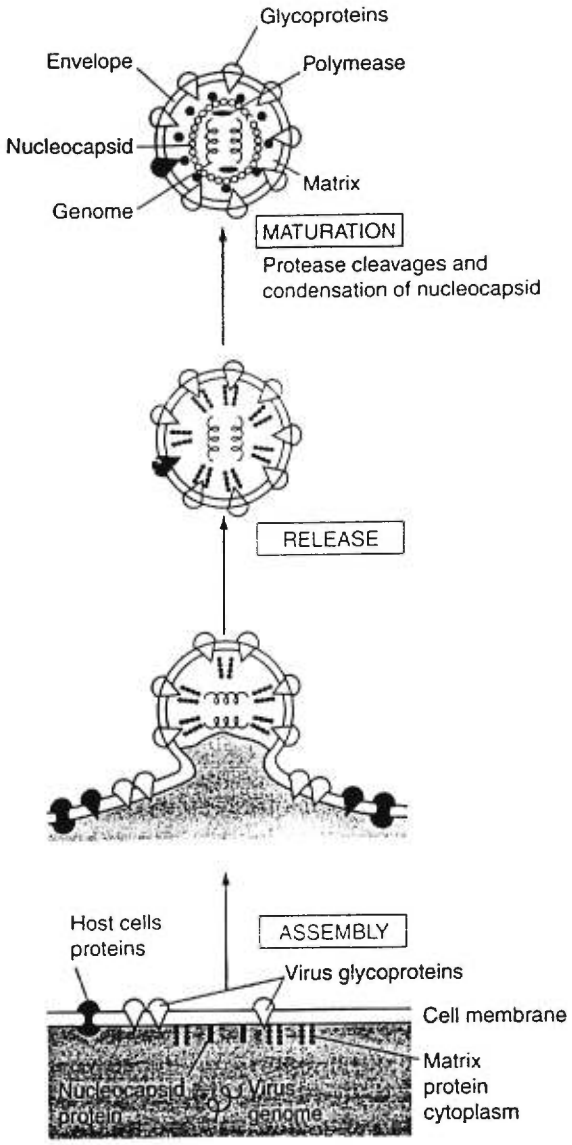

Figure 8: Virion assembly at the cell membrane. The assembly process brings together viral and cellular factors necessary to generate progeny virions. Viral genomic RNA, Gag/Gag-Pol, and Env precursor proteins are trafficked to the site of assembly (see text for details) where immature virions bud out of the infected cells. Viral protease acts during the late stages of viral budding presumably continuing after release to generate the mature, infectious virion. Original illustration derived from *Principles of Molecular Virology*, Second Edition, Alan J. Cann (1997) Academic Press.

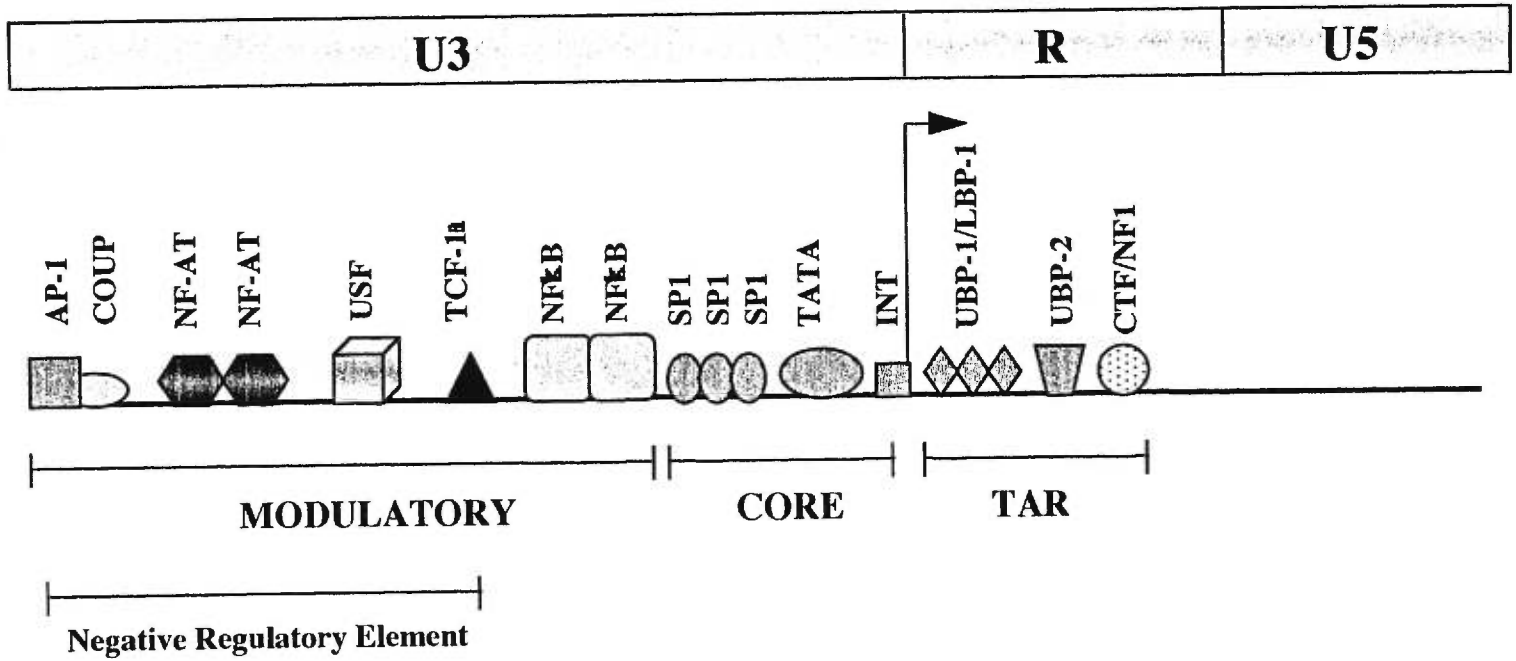

Figure 9: Schematic of the HIV-1 LTR organization. The modulatory, core, and TAR regions are indicated in the figure. The text discusses the features of this unique viral promoter region. Figure adapted from Gaynor *et al.* (1992).

Chapter 2

Mutagenic analysis of HIV-1 Vpr: role of a predicted N-terminal alpha helical structure on Vpr nuclear localization and virion incorporation

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Mutagenic Analysis of Human Immunodeficiency Virus Type 1 Vpr: Role of a Predicted N-Terminal Alpha-Helical Structure in Vpr Nuclear Localization and Virion Incorporation

XIAO-JIAN YAO, RAMU A. SUBBRAMANIAN, NICOLE ROUGEAU, FRANÇOISE BOISVERT, DOMINIQUE BERGERON, AND ERIC A. COHEN*

Laboratoire de Rétrovirologie Humaine, Département de Microbiologie et Immunologie, Faculté de Médecine, Université de Montréal, Montréal, Québec, Canada H3C 3J7

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The Vpr gene product of human immunodeficiency virus type 1 is a virion-associated protein that is important for efficient viral replication in nondividing cells such as macrophages. At the cellular level, Vpr is primarily localized in the nucleus when expressed in the absence of other viral proteins. Incorporation of Vpr into viral particles requires a determinant within the p6 domain of the Gag precursor polyprotein $Pr55^{gag}$. In the present study, we have used site-directed mutagenesis to identify a domain(s) of Vpr involved in virion incorporation and nuclear localization. Truncations of the carboxyl (C)-terminal domain, rich in basic residues, resulted in a less stable Vpr protein and in the impairment of both virion incorporation and nuclear localization. However, introduction of individual substitution mutations in this region did not impair Vpr nuclear localization and virion incorporation, suggesting that this region is necessary for the stability and/or optimal protein conformation relevant to these Vpr functions. In contrast, the substitution mutations within the amino (N)-terminal region of Vpr that is predicted to adopt an alpha-helical structure (extending from amino acids 16 to 34) impaired both virion incorporation and nuclear localization, suggesting that this structure may play a pivotal role in modulating both of these biological properties. These results are in agreement with a recent study showing that the introduction of proline residues in this predicted alpha-helical region abolished Vpr virion incorporation, presumably by disrupting this secondary structure (S. Mahalingam, S. A. Khan, R. Murali, M. A. Jabbar, C. E. Monken, R. G. Collman, and A. Srinivasan, Proc. Natl. Acad. Sci. USA 92:3794–3798, 1995). Interestingly, our results show that two Vpr mutants harboring single amino acid substitutions (L to F at position 23 [L23F] and A30F) on the hydrophobic face of the predicted helix coded for relatively stable proteins that retained their ability to translocate to the nucleus but exhibited dramatic reduction in Vpr incorporation, suggesting that this hydrophobic face might mediate protein-protein interactions required for Vpr virion incorporation but not nuclear localization. Furthermore, a single mutation (E25K) located on the hydrophilic face of this predicted alpha-helical structure affected not only virion incorporation but also nuclear localization of Vpr. The differential impairment of Vpr nuclear localization and virion incorporation by mutations in the predicted N-terminal alpha-helical region suggests that this region of Vpr plays a role in both of these biological functions of Vpr.

The human immunodeficiency virus type 1 (HIV-1), the causative agent of AIDS, is a retrovirus that belongs to the lentiviral subfamily. Unlike simple retroviruses which generally code only for the structural and enzymatic proteins, lentiviruses code for a number of auxiliary proteins (Vif, Vpr, Tat, Rev, Vpu, and Nef, in the case of HIV-1) not found in most retroviruses. These proteins play pivotal regulatory functions which account for the tightly regulated replication of these viruses (reviewed in references 9 and 37). Both HIV-1 and HIV-2 and most simian immunodeficiency viruses code for the highly conserved open reading frame R, which lies in the central region of the genome (3, 7, 10, 13, 17, 18, 32). Vpr is a 14-kDa, 96-amino-acid protein expressed primarily from a singly spliced Rev-dependent mRNA (1, 7, 12). A recent study has characterized the Vpr protein as an oligomer (47). HIV-2 and most simian immunodeficiency viruses in addition also code for a second protein, Vpx, which shares notable sequence homology with Vpr. It has been suggested that Vpx may have arisen from Vpr by gene duplication in these viruses (40). Both of these homologous proteins are packaged efficiently in the viral particles at high copy numbers and at molar amounts comparable to those for Gag (6, 16, 31, 44). Virion localization studies place both Vpr and Vpx outside the core structure (41, 45). As Vpr and Vpx are not part of the Gag structural polyprotein, their incorporation requires an anchor to associate with the assembling capsid structures. Though the molecular mechanism is currently unknown, the C-terminal region of the Gag precursor corresponding to the p6 protein appears to constitute such an anchor, essential for the incorporation of both of these accessory proteins (4, 21, 33, 43). Recent studies suggest that the virion incorporation of Vpr involves a predicted alpha-helical structure located near the protein N terminus (29, 30).

Functionally, Vpr was first identified as a moderate and nonspecific transactivator, capable of augmenting reporter gene expression from both the HIV-1 long terminal repeat and other heterologous promoters (7). The ability to replicate in the nondividing host cell distinguishes HIV from the oncoretroviruses (27, 35). Also, Vpr was subsequently shown to induce differentiation and growth arrest in several tumor cell lines, even in the absence of any other viral proteins, indicating that the presence of Vpr by itself is sufficient to alter cellular events independent of HIV replication (25, 36). Functional studies

* Corresponding author. Phone: (514) 343-5967. Fax: (514) 343-5995. Electronic mail address: cohenea@umontreal.ere.ca.

have shown that Vpr accelerates HIV-1 replication in some T-lymphoid cell lines, and this augmentation of virus production is more pronounced in primary macrophages in both HIV-1 and HIV-2 systems (2, 7, 8, 15, 32, 42). The virion association of Vpr suggests that the protein may play a role early in replication. A recent study has determined that in addition to $p17^{gag}$, Vpr may also mediate the nuclear transport of HIV-1 proviral DNA in nondividing target cells (16). Interestingly, results from another study suggest that Vpr function may also require the de novo synthesis of this protein, as the virion-associated protein alone was insufficient to augment replication in cell types such as macrophages (8). Recent studies have also characterized Vpr as having transcellular activity (26). Both Vpr purified from the plasma acquired from HIV-seropositive individuals and recombinant-purified Vpr were capable of inducing latent cells into high-level viral producers when added to the culture medium at low concentrations (26). This report also suggests that Vpr can continue to act following integration events, as latent cells presumably have already progressed through these early steps. Mechanistically, it is conceivable that this transcellular activity is mediated by the same mechanisms that are operative in modifying cellular growth and differentiation. At the cellular level, Vpr has been localized mainly in the nucleus when expressed in the absence of other HIV-1 proteins (28). Even though no classical nuclear localization signal in this protein has been clearly identified, it has been suggested that Vpr may specifically interact with other nuclear proteins and may thus gain access to the nucleus (46). In this regard, two groups have identified proteins of various molecular weights that interact with Vpr (34, 46). Interestingly, one of these Vpr targets, designated Vpr-interacting protein, or RIP-1, appears to translocate to the nucleus following its interaction with Vpr and glucocorticoid receptor (34).

In this study, we have used mutational analysis to explore the region(s) of the Vpr protein involved in its nuclear localization and virion incorporation properties. Our studies indicate that the C-terminal truncation of Vpr, but not substitution mutations in this region, result in the impairment of Vpr nuclear localization and virion incorporation. These impairments are likely due to reduced protein stability and/or altered structural conformation associated with these truncated Vpr proteins. Also, we demonstrate that a region extending from amino acids 16 to 34 near the N terminus of Vpr with a predicted alpha-helical structure is involved in both Vpr nuclear localization and virion incorporation. Moreover, we identify residues located on the hydrophobic face of this predicted N-terminal alpha-helical structure capable of affecting Vpr virion incorporation without affecting the protein's ability to localize to the nucleus.

MATERIALS AND METHODS

HIV molecular clones. HIV-1 provirus plasmid HxBRU used in this study is a hybrid between the two closely related proviruses, HxBc2 and BRU/LAI (24). The genotype of this molecular clone is 5′ long terminal repeat *gag*$^+$ *pol*$^-$ *vif*$^-$ *vpr*$^+$ *tat*$^+$ *rev*$^+$ *vpu env*$^+$ *nef* 3′ long terminal repeat. HxBRUR$^-$ was constructed by replacing the sequences from a *Stu*I site to a *Sal*I site (nucleotides [nt] 4989 to 5367; +1 = start of BRU initiation of transcription) of HxBRU with the corresponding sequences of ptrENVR$^-$, in which the initiation codon (ATG) of Vpr has been changed to GTG (24). HxBRUR$^-$ and HxBRU are isogenic except for the expression of Vpr. The HxBH10 provirus clone encodes a functional Vpu and a truncated Vpr (R72f) (39). This truncated R72f is due to a frameshift caused by insertion of a single T at nt 5315 (+1 = start of HxBc2 initiation of transcription) (23). HxBRUR77f was generated by digestion of HxBRU with *Sal*I located at nt 5367 and religation following blunt ending with the Klenow fragment of DNA polymerase I. These treatments created a frameshift within the Vpr open reading frame at the amino acid position 78, as described before (28).

HIV HxBRU proviral constructs harboring different Vpr substitution mutations were generated by using a two-step PCR-based method essentially as described previously (19). The 5′ primer (5′-AACACCATATGTATGTTT-3′) is located at a *Nde*I site in *vif* (nt 4705; +1 = start of BRU initiation of transcription), and the 3′ primer (5′-TGTATCATATGCTTTAGC-3′) is located at the *Nde*I site in *env* (nt 5946; +1 = start of HxBc2 initiation of transcription). Complementary oligonucleotide primers containing the desired mutations in Vpr were used to generate *Nde*I-*Nde*I PCR fragments containing the different Vpr mutations. These PCR fragments were digested with *Stu*I and *Sal*I or with *Sal*I and *Kpn*I restriction enzymes, depending on the Vpr region involved in mutagenesis. The resulting DNA fragments were subcloned into one of the intermediate vectors; one vector contained the *Apa*I-*Sal*I fragment derived from HxBRU, while the other contained the *Sal*I-*Bam*HI fragment from HxBc2. Either the *Apa*I-*Sal*I fragment or the *Sal*I-*Bam*HI fragment containing the various Vpr mutations was cloned back into the proviral construct HxBRU. The resulting HxBRU proviruses harboring the different Vpr mutations used in this study are shown in Fig. 1. Three additional Vpr mutants (EA29,30FK, IL63,64KR and LI68,70RK) were only cloned into Vpr expressor, as described later and shown in Fig. 6A. The nucleotide sequences of the sense mutagenic oligonucleotides are as follows: RE12,13PG, sense, 5′-AGGGCCACAGCCCGGGCCACACAAT-3′; L23F, sense, 5′-ACTAGAGCTCTTCGAGGAGCTT-3′; E25K, sense, 5′-GCTTTTAGAGAAGCTTAAGAA-3′; A30F, sense, 5′-TAAGAATGAATTCGTTAGACATT-3′; H33I, sense, 5′-AGCTGTTAGAATATTTCCTAGGA-3′; EA29,30FK, sense, 5′-GCTTAAGAATTTTAAAGTTAGACATT-3′; I63F, sense, 5′-ATAATAAGATTTCTGCAAC-3′; IL 63,64KR. sense, 5′-ATAATAAGAAAACGGCAACAACTG-3′; LI68,70RK, sense, 5′-CAACAACTGCGGTTTAAACATTTCAGA-3′; R73S, sense, 5′-TATCCATTTCAGCATTGGGTGTC-3′; G75N, sense, 5′-TTTCAGAATTAATTGTCGACATA-3′; SR79,80ID, sense, 5′-TGTCGACATATCGATATAGGCGTTA-3′.

Eukaryotic Vpr expression vectors. To construct Vpr expression vectors for the various mutations, we first amplified the Vpr sequences from the respective HxBRU proviruses by PCR, using a 5′ primer (5′-ACTTCTAGAGGATAGATGGAACAAGCC-3′) with an *Xba*I site engineered preceding the Vpr ATG and a 3′ primer (5′-CAGGAGCTCAGTCTAGGATCTACTGGC-3′) which harbors an engineered *Sac*I site following the stop codon of Vpr. These PCR fragments were digested with *Xba*I and *Sac*I restriction enzymes and then used to replace the corresponding Vpr fragment in a Vpr expression vector SVCMVER, which contains the cytomegalovirus immediate-early gene promoter (24). Four Vpr substitution mutants WEL, EAHF, HFRI, and RR90,95NN (as shown in Fig. 6A) based on the Vpr sequence derived from the HIV-1 ELI strain were also generated in the same expression vector by using an oligonucleotide-directed mutagenesis method, as described before (48). The sense mutagenic oligonucleotides used are as follows: WEL, 5′-ATACAATGAAGCAGCATTACTTCTGCAGGAGGAGCTTA-3′; EAHF, 5′-GCTTAAGAGTTTCAGAGTTAGACTGCAGCCTAGGATAT-3′; HFRI, 5′-ACTGTTTATTGGATCCGAACAGGGGTGTCAAC-3′; RR90,95NN, 5′-AGGAGAGCAAATAATGGAAGTAGTAATTCCTAGACT-3′. All expression vectors so generated were subsequently analyzed by DNA sequencing to confirm the mutations.

Cell lines and antisera. The transformed lymphoid cell line (MT4 cells) used in this study were maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS). This cell line has been shown to be highly sensitive to HIV-1 virus infection (14). COS-7, an African green monkey kidney cell line transformed by an origin-defective mutant of simian virus 40, was propagated in Dulbecco modified Eagle medium supplemented with 10% FCS. An HIV-1-positive human serum 162 and a rabbit anti-Vpr serum raised against an *Escherichia coli*-derived ELI Vpr protein were used in this study and have been previously described (24). Fluorescein-labeled goat anti-rabbit $F(ab')_2$ was purchased from Gibco BRL Co.

Transfection of cells. MT4 cells (5×10^6) were transfected with 10 μg of either Vpr$^-$ or mutated Vpr provirus plasmids by using the DEAE-dextran method as described before (39). The transfected cells were maintained in RPMI 1640 containing 10% FCS for 48 to 84 h. To test for Vpr expression, COS-7 cells (2.5×10^4) cultured in shell vials were transfected with 1 μg of either wild-type or mutated VPR expressors, using the calcium-phosphate method (24). The transfected cultures were maintained in Dulbecco modified Eagle medium containing 10% FCS for 48 h.

Immunofluorescence microscopy. At 48 h posttransfection, COS-7 cells were fixed in acetone for 30 min at 4°C. After fixation, the cells were first incubated with the rabbit anti-Vpr serum in phosphate-buffered saline (PBS) containing 2% Carnation instant skim milk powder (Nestlé) for 12 h at 37°C and then incubated with fluorescein-labeled goat anti-rabbit antibody for 2 h. After being washed with PBS, the cells were observed at a magnification of ×100 (oil emulsion) with a Zeiss fluorescence microscope.

Cell labeling and radioimmunoprecipitation. At 48 to 84 h posttransfection, MT4 cells were metabolically labeled with 100 μCi of [^{35}S]methionine per ml and 100 μCi of [^{3}H]leucine per ml for 12 h. After labeling, virions were pelleted from supernatants by ultracentrifugation at 30,000 rpm through a 20% sucrose cushion in a Beckman SW41 rotor and lysed in radioimmunoprecipitation assay buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.25% sodium deoxycholate, and 0.2% phenylmethylsulfonyl fluoride and immunoprecipitated with a mixture of

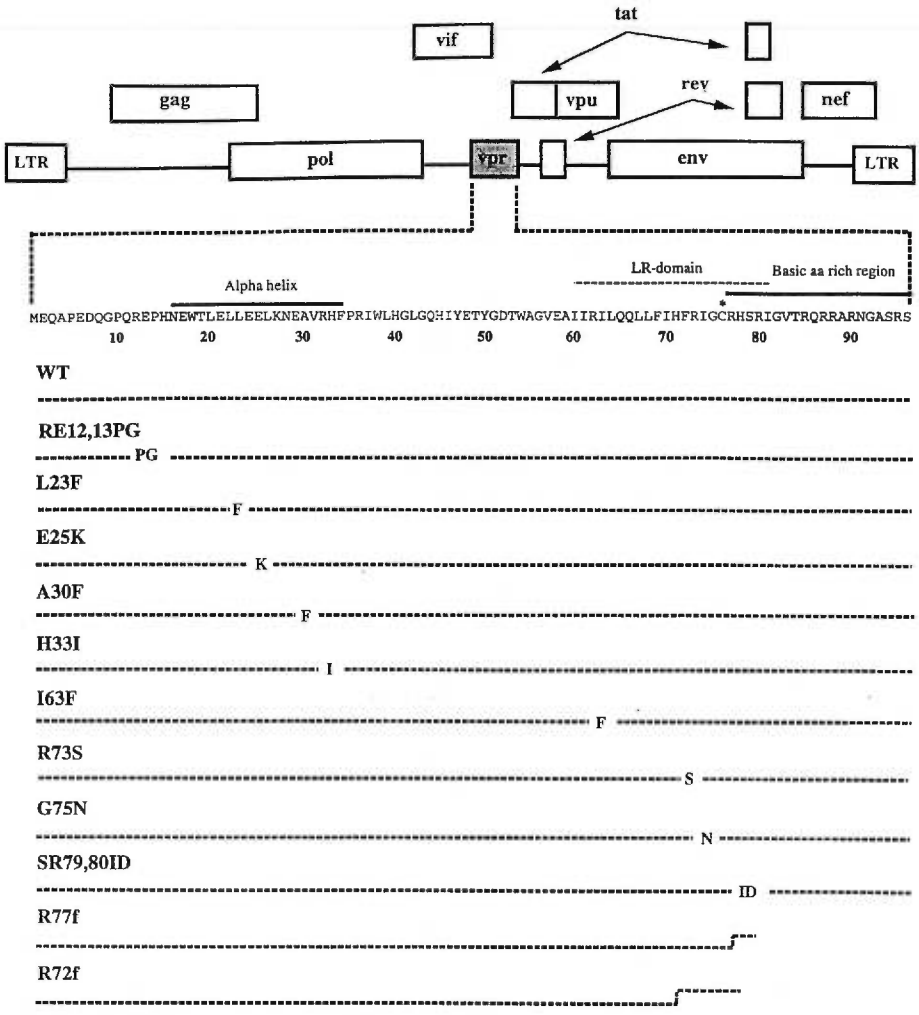


FIG. 1. Genetic organization of the HIV-1 genome and Vpr substitution and deletion mutations in proviral plasmid constructs. The schematic organization of the HIV-1 genome and the amino acid sequence of Vpr are shown at the top. The position of the predicted amphipathic alpha-helical structure extending from amino acids (aa) 16 to 34 near the N terminus of Vpr, the highly conserved cysteine residue at position 76, the previously described LR domain (46), and the basic amino-acid-rich region are indicated. The positions of the mutated amino acids of Vpr mutants are shown at the bottom. The positions of the C-terminal truncations (HxBRUR77f and HxBH10R72f) are also indicated. LTR, long terminal repeat.

HIV-1-positive human serum 162 and rabbit anti-Vpr serum, as previously described (24). Immunoprecipitates were run on an SDS–14% polyacrylamide gel and subsequently analyzed by autoradiography. In the experiment whose results are shown in Fig. 4C, the virions in the supernatants were pelleted by ultracentrifugation through a 20% sucrose cushion, lysed, directly run on SDS–14% polyacrylamide gels, and analyzed by autoradiography without additional immunoprecipitation steps.

For pulse-chase metabolic labeling, MT4 or COS-7 cells were starved 48 h posttransfection in methionine- and leucine-free RPMI 1640 medium for 15 min. The COS-7 cells were scraped off the petri dish during the starvation. Subsequently, the cells were metabolically labeled with 250 μCi of [^{35}S]methionine and 100 μCi of [^{3}H]leucine in 0.5 ml of medium for 30 min. The labeling medium was then removed, and the cells were washed once with complete RPMI 1640 containing 10% FCS. Equal aliquots of labeled cells were resuspended in 0.5 ml of complete RPMI 1640 containing 10% FCS and incubated at 37°C. At each indicated time point, the cells were lysed with radioimmunoprecipitation assay buffer and immunoprecipitated with a mixture of HIV-1-positive human serum 162 and rabbit anti-Vpr serum or with the rabbit anti-Vpr serum alone. The immunoprecipitates were analyzed as described above. Densitometric analysis of autoradiograms was performed with a Molecular Dynamics Personal densitometer, using ImageQuant software, version 3.22.

Computer-assisted protein secondary-structure analysis. The secondary structure of the Vpr protein was analyzed by using the MacVector sequence analysis software from International Biotechnologies Inc., New Haven, Conn. The prediction of the secondary structure of proteins by this software is based on the Robson-Garnier and Chou-Fasman methods (5, 11).

RESULTS

Construction of HIV-1 proviruses harboring different Vpr mutations. In order to identify the domain(s) of Vpr involved in its capacity to be incorporated into virions, we constructed a series of infectious HIV proviruses that coded for the mutated versions of Vpr (Fig. 1). In designing these mutants, we targeted three putative structural regions in the Vpr sequence: (i) the acidic N-terminal domain containing a putative amphipathic alpha helix extending from amino acids 16 to 34 as predicted by computer-assisted analysis, (ii) the previously identified leucine-isoleucine-rich region (LR domain) (46), and (iii) the arginine-rich C-terminal domain (Fig. 1).

Substitution mutations were designed to affect specific amino acid residues found to be highly conserved among Vpr sequences from different HIV-1 isolates (40). Generally, sub-

stitutions were designed to introduce either a charge or a steric change in the amino acid sequence as described in Fig. 1. One mutant carrying substitutions of amino acids Arg-12 and Glu-13 to Pro and Gly (Fig. 1, RE12,13PG) was generated to modify the proximal N terminus of Vpr. Also, four substitution mutations were introduced in the predicted alpha-helical structure located near the N terminus of Vpr. This predicted alpha helix contains a notable acidic hydrophilic face and an aliphatic hydrophobic face, as shown in Fig. 7B. To explore the importance of each face of this predicted helical structure, two amino acids, Leu-23 and Ala-30, located at the hydrophobic face were replaced by an aromatic amino acid Phe to generate mutants L23F (an L-to-F substitution at position 23) and A30F, respectively. In two other mutants, nonconservative substitutions were made on the same helical structure, where the charged residues Glu-25 and His-33 were replaced by Lys (mutant E25K) and Ile (mutant H33I), respectively (Fig. 1). As shown in Fig. 7B, one of these residues, Glu-25, is located on the hydrophilic face of the predicted alpha-helical structure. None of these four mutants (L23F, E25K, A30F, and H33I) disrupted this putative helical structure, as predicted by computer-assisted analysis (data not shown). In addition, two Vpr frameshift mutants, R77f and R72f, which prematurely terminate the wild-type Vpr and effectively remove the last 19 and 24 amino acids, respectively, were also used in this study. Four mutants carrying substitutions of Ile-63 for Phe (I63F), Arg-73 for Ser (R73S), Gly-75 for Asn (G75N), and Ser-79 and Arg-80 for Ile and Asp (Fig. 1, SR79,80ID) were constructed to introduce changes in the LR domain and/or arginine-rich region of Vpr. Moreover, none of the Vpr mutations introduced modified other open reading frames or known splicing sites in the HIV sequence, with the exception of the mutant RE12,13PG, which changed the residues Arg-184, Gly-185, and Ser-186 in the Vif sequence to Ser, Pro, and Gly, respectively. However, this particular mutant was not restricted for replication in T-lymphoid MT4 cells.

In addition to being tested for their virion incorporation capacity, all mutated Vpr proteins were evaluated for their stability during viral replication in MT4 cells as described in the following sections. In the case of mutants that showed a reduced steady-state protein level, we performed pulse-chase experiments to further explore their stability status. Most of the Vpr mutants were also subcloned into an expression vector, as described in Materials and Methods, in order to evaluate, in parallel, Vpr stability in the absence of other viral proteins following transfection of Vpr expressors in COS-7 cells.

Effect of C-terminal truncation on Vpr stability and virion incorporation capacity. To determine the effect of the C-terminal region on Vpr stability, we transfected HIV provirus constructions expressing either the wild-type (HxBRU) or the C-terminal-truncated Vpr proteins (HxBRUR77f and HxBH10R 72f) in MT4 cells. At 48 h posttransfection, expression of viral proteins was analyzed by metabolic labeling and immunoprecipitation. Briefly, equal numbers of transfected MT4 cells were pulse-labeled with [^{35}S]methionine and [^{3}H]leucine for 30 min and subsequently chased at different intervals of time. The viral proteins in the cell lysates were immunoprecipitated with HIV-1-positive human serum 162 mixed with rabbit anti-Vpr serum. Immunoprecipitated proteins were analyzed by autoradiography after SDS–14% polyacrylamide gel electrophoresis (SDS–14% PAGE). The viral structural proteins were abundantly detected in cell lysates from viruses expressing both the wild-type and the truncated Vpr proteins (Fig. 2A). A protein of 14 kDa corresponding to the Vpr product was specifically immunoprecipitated from cells transfected with the wild-type Vpr expressing HxBRU (Fig. 2A, lanes 1 to 4). Different faster-migrating proteins corresponding to the two truncated Vpr proteins were evident in lysates from cells transfected with HxBRUR77f or HxBH10R72f. The molecular mass of these two truncated Vpr proteins ranged from approximately 9 to 10 kDa (Fig. 2A, lanes 5 to 8 and lanes 9 to 12, respectively). Moreover, as shown by pulse-chase labeling, these truncated Vpr species (R77f and R72f) were less stable than the wild-type Vpr. While the half-life of wild-type Vpr was more than 7 h, the half-lives of the truncated Vpr proteins (R77f and R72f) were approximately 3 and 1 h, respectively (Fig. 2B). These data clearly indicate that the removal of the last 19 or 24 amino acids from the Vpr C terminus severely impairs Vpr stability in T-lymphoid cells (MT4).

In order to evaluate the ability of these truncated Vpr proteins (R77f and R72f) to incorporate into progeny virions, MT4 cells were transfected with the different HIV proviruses (HxBRU, HxBRUR77f, and HxBH10R72f). At 48 h posttransfection, the cells were labeled with [^{35}S]methionine and [^{3}H]leucine for 12 h. The cells were then lysed, immunoprecipitated, and analyzed by SDS–14% PAGE. The results indicate that the levels of truncated Vpr mutants R77f and R72f in cell lysates were notably reduced, compared with the wild-type Vpr level (data not shown). Also, the virions in the culture supernatants were pelleted and immunoprecipitated in parallel. As truncated forms of Vpr comigrated with an unknown nonspecific protein, we performed parallel immunoprecipitations with either a mix of HIV-1-positive human serum 162 and anti-Vpr-specific antibodies or a mix of HIV-1-positive human serum 162 and normal rabbit serum. As shown in Fig. 3, Vpr proteins can be specifically immunoprecipitated from virions collected from MT4 cultures transfected with proviruses containing wild-type or truncated Vpr proteins (Fig. 3, compare lanes 1, 3, and 5 with lanes 2, 4, and 6). These results indicate that the truncated R77f and R77f are incorporated into virions even though the detected levels of these two truncated Vpr proteins in virions are much lower than that of the wild type. Such low levels of truncated Vpr proteins in the virions can be partially attributed to the instability of these mutants. These data suggest that as the truncated Vpr proteins were capable of being packaged in the virions, albeit at lower levels, the C terminus of Vpr may not be absolutely necessary for virion incorporation but may modulate this Vpr property by affecting the stability and, conceivably, the optimal protein conformation necessary for virion targeting.

Substitution mutational analysis indicates that a predicted N-terminal alpha-helical structure is necessary for Vpr virion incorporation. We first determined the effect of different substitution mutations on Vpr stability. MT4 cells were transfected with the proviral constructs harboring the wild-type or substitution mutations in the Vpr sequence. At 48 h after transfection, the cells were labeled with [^{35}S]methionine and [^{3}H]leucine for 12 h during the peak of replication, as determined by the appearance of HIV-induced syncytia in the cell cultures. Radiolabeling was done at 48 h posttransfection with proviruses harboring wild-type and mutated Vpr, with the exception of HxBRUL23F and HxBRUA30F (72 to 84 h), which showed a delay of viral replication. After labeling, the cells were lysed, immunoprecipitated with HIV-1-positive human serum 162 mixed with rabbit anti-Vpr serum, and analyzed by SDS–14% PAGE followed by autoradiography (Fig. 4A, B, and C, left panels). From each HIV provirus transfection, viral structural proteins were abundantly detected in cell lysates. However, the 14-kDa Vpr protein was specifically immunoprecipitated only from cultures transfected with proviruses expressing the wild-type or mutated Vpr but not in cells transfected with Vpr^{-} provirus as indicated. The mobility of

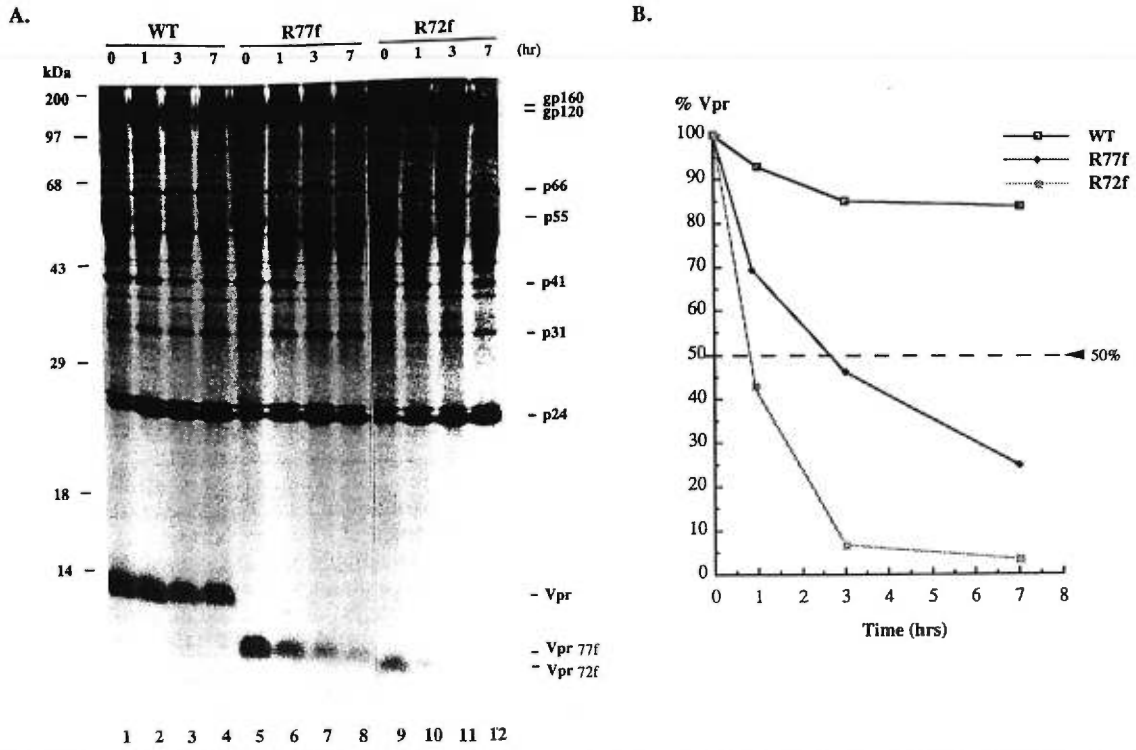


FIG. 2. Effect of C-terminal deletions on Vpr stability. (A) MT4 cells were transfected with 10 μg of HxBRUwt, HxBRUR77f, or HxBH10R72f provirus plasmid. At 48 h posttransfection, the cells were pulse-labeled with 250 μCi of [^{35}S]methionine and 100 μCi of [^{3}H]leucine for 30 min and chased at the indicated times in media containing excess unlabeled methionine and leucine. Subsequently, the cells were lysed with radioimmunoprecipitation assay buffer, and the cell lysates were immunoprecipitated with HIV-1-positive human serum 162 mixed with rabbit anti-Vpr serum. The positions of viral proteins and Vpr (wild-type [WT] and truncated forms) are indicated. The molecular mass markers are shown on the left. (B) The quantitative analysis of the wild-type (WT) and truncated Vpr bands was performed with a Molecular Dynamics Personal densitometer, using ImageQuant software, version 3.22. The percentage of Vpr proteins recovered relative to the amount present at the end of the pulse (0 min) is plotted as a function of time.

mutated Vpr (SR79,80ID) was shown to be a little faster than that of wild-type Vpr (Fig. 4B, compare lanes 4 and 2). The levels of expression and stability of all mutated Vpr proteins, with the exception of Vpr H33I, were similar to the wild-type Vpr level.

To further confirm Vpr stability results, we also performed pulse-chase immunoprecipitation analysis of some mutated Vpr proteins expressed either in a proviral context or from Vpr expression vectors in the absence of other viral proteins. Vpr expression vectors containing the substitution mutation L23F, E25K, A30F, or H33I were transfected in COS-7 cells. At 48 h posttransfection, the cells were pulse-labeled with [^{35}S]methionine and [^{3}H]leucine for 30 min and were subsequently chased at different intervals of time. The Vpr proteins in the cell lysates were immunoprecipitated with rabbit anti-Vpr serum and analyzed by autoradiography after SDS–14% PAGE. The levels of Vpr protein at different time intervals were evaluated by densitometric analysis and are presented in Fig. 5A. The half-life of the wild-type Vpr and that of the mutated Vpr (L23F, E25K, and A30F) in COS-7 cells were shown to be more than 7 h, and as expected from the preceding results, the mutated Vpr H33I was shown to be less stable, with a half-life of approximately 5.5 h. When MT4 cells were transfected with HIV proviruses expressing the same Vpr mutants, followed by pulse-chase labeling and immunoprecipitation, similar results were also obtained, indicating that the stability of these mutated Vpr proteins (L23F, E25K, and A30F) is not affected by the presence or the absence of other viral proteins (data not shown).

To determine the level of virion incorporation of the mutated Vpr proteins, virions were pelleted from the supernatants of the labeled MT4 cultures transfected with each proviral plasmid. The pelleted virions were then run on an SDS–14% polyacrylamide gel either directly after lysis (Fig. 4C, right panel) or following immunoprecipitation with the mix of the anti-Vpr and anti-HIV serum 162 (Fig. 4A and B, right panels). The results presented in Fig. 4 show that the wild-type Vpr protein was specifically immunoprecipitated from the virion lysates, indicating that wild-type Vpr can be efficiently incorporated into progeny viruses (Fig. 4A, B, and C, right panels, lanes 2). Mutated Vpr proteins were detected at levels comparable to that of the wild-type Vpr in the progeny virions obtained from proviruses harboring mutations in the proximal N-terminal region (RE12,13PG) or in the C-terminal half of the protein (I63F, R73S, G75N, or SR79,80ID) (Fig. 4A, right panel, lanes 5 and 6; B, right panel, lanes 3 and 4; C, right panel, lane 5). In contrast, low levels of Vpr products were detected from the virions obtained from proviruses harboring mutations in the region extending from amino acids 16 to 34, which is predicted to form an alpha-helical structure (L23F, E25K, A30F, or H33I) (Fig. 4A, right panel, lanes 3 and 4; Fig. 4C, right panel, lanes 3 and 4).

To determine the incorporation efficiency of the mutated Vpr proteins in the virions, the level of Vpr found in the virions

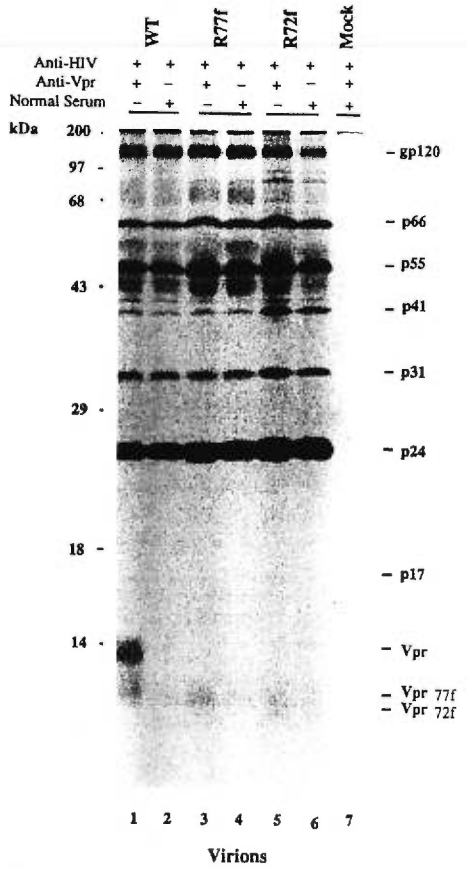


FIG. 3. Effect of C-terminal truncation on Vpr virion incorporation. MT4 cells were transfected with 10 μg of HxBRUwt, HxBRUR77f, or HxBH10R72f provirus plasmid. At 48 h posttransfection, the cells were labeled with [^{35}S]methionine and [^{3}H]leucine for 12 h. Labeled virions were collected by ultracentrifugation through a 20% sucrose cushion and immunoprecipitated with HIV-1-positive human serum 162 mixed with either rabbit anti-Vpr serum or normal rabbit serum. The positions of viral proteins and Vpr (wild-type [WT] and truncated forms) are indicated.

was evaluated by densitometric analysis of the autoradiograms presented in Fig. 4. The virion-associated Vpr level was evaluated as a ratio of virion-associated Vpr to that of the reverse transcriptase (RT) p66, which served as an internal control in each sample. The results indicate that the mutations at positions R-12 and E-13, I-63, R-73, G-75, and S-79 and R-80 allowed the efficient incorporation of mutated Vpr into virions, compared with the wild type (Fig. 5B). However, the mutated Vpr at positions L-23, E-25, A-30, and H-33 resulted in a remarkable decrease in incorporation level (Fig. 5B). In the case of H33I, the low level of Vpr incorporation could partially result from its instability (Fig. 4A, left panel, lane 4).

These results indicate that substitution mutations at residues R-12 and E-13 in the N terminus or residues I-63, R-73, G-75, and S-79 and R-80 in the C-terminal portion of Vpr affect neither its stability nor its virion incorporation. In contrast, the presence of mutations at position L-23, E-25, or A-30, all located in the putative N-terminal alpha helix, leads to the expression of stable Vpr proteins in the cells with low capacity to be incorporated in the progeny virions. From this mutational analysis, it is clear that residues L-23, E-25, and A-30 are involved in Vpr virion incorporation.

Effect of mutations on Vpr nuclear localization. Previous studies have shown that Vpr localizes in the nucleus when expressed in the absence of other viral proteins (28, 46). To identify the region(s) of Vpr involved in its nuclear localization, we generated a series of Vpr expressors (SVCMV-Vpr) which contain a number of substitution mutations in the different regions of Vpr as shown schematically in Fig. 6A. In addition to the BRU-derived mutants, we also analyzed substitution mutations spanning a Vpr sequence derived from another HIV isolate, ELI. The BRU mutants tested for nuclear localization by immunofluorescence analysis included RE12,13PG, located in the N terminus of Vpr, four mutants spanning the predicted alpha-helical region (L23F, E25K, A30F, and EA29,30FK), three mutants in the LR domain (I63F, IL63,64KR, and LI68,70RK), and two mutants in the C-terminal basic amino-acid-rich region (SR79,80ID and R77f) (Fig. 6A). The ELI mutants tested included the WEL and EAHF mutants, which were located in the proximal and distal regions of the N-terminal helix, respectively, while the HFRI and RR90,95NN mutants were located in the distal half of the protein (Fig. 6A).

To determine the nuclear localization of these Vpr mutants in the absence of other viral proteins, we transfected these Vpr expressors in COS-7 cells. At 48 h posttransfection, the cells were fixed, labeled with anti-Vpr serum, and analyzed by indirect immunofluorescence analysis. To evaluate the ability of various Vpr mutants to be targeted to the nucleus, the number of cells exhibiting a Vpr-specific signal from each experiment was determined in random fields. We also compared the pattern of immunofluorescence of the Vpr-positive cells to the wild-type pattern. The data presented in Fig. 6A represent the mean percentage of Vpr-positive cells with specific nuclear staining over total numbers of Vpr-positive cells from three independent experiments. As exemplified in Fig. 6B, specific nuclear staining for Vpr was clearly observed in COS-7 cells transfected with wild-type Vpr^+ expressor (Fig. 6B, panel b) but not in cells transfected with the Vpr^- expressor (Fig. 6B, panel a). In a majority of the cells (92%) that expressed the wild-type protein, specific Vpr staining was evident predominantly in the nucleus, with occasional perinuclear staining. In contrast, as shown in Fig. 6A and exemplified in Fig. 6B, panel o, the C-terminal Vpr truncation mutant (R77f) severely impaired nuclear localization, with only 38% of Vpr-positive cells showing Vpr staining in the nucleus. Even in cells that exhibited such nuclear staining, a notable portion of the protein was still retained in the cytoplasmic compartment. This result correlates with the data obtained in previous fractionation experiments that demonstrated a severe impairment of Vpr nuclear association when the last 19 amino acids were deleted (28). Interestingly, however, neither of the substitution mutants (the ELI mutant RR90,95NN and the BRU mutant SR79,80ID) in the same region were impaired in their ability to localize to the nucleus (Fig. 6A and B, panel n). Also, several substitution mutants located in the other Vpr regions, including the BRU (RE12,13PG, L23F, EA29, 30FK, A30F, I63F, IL63,64KR, and LI68,70RK) and the ELI (HFRI) mutants, were evaluated for their capacity to translocate to the nucleus. With these mutants, approximately 70 to 93% of the cells exhibiting Vpr-specific staining had some proportion of their signal in the nucleus, suggesting that the determinant to reach the nucleus was present in these mutants. However, analysis of the immunofluorescence pattern indicated that the distribution of the protein among the cytoplasmic and nuclear compartments varied. Some mutants (RE12,13PG, A30F, EA29,30FK, and LI68,70RK), like the wild type, had predominantly nuclear staining, while other mutants had notable amounts of the pro-

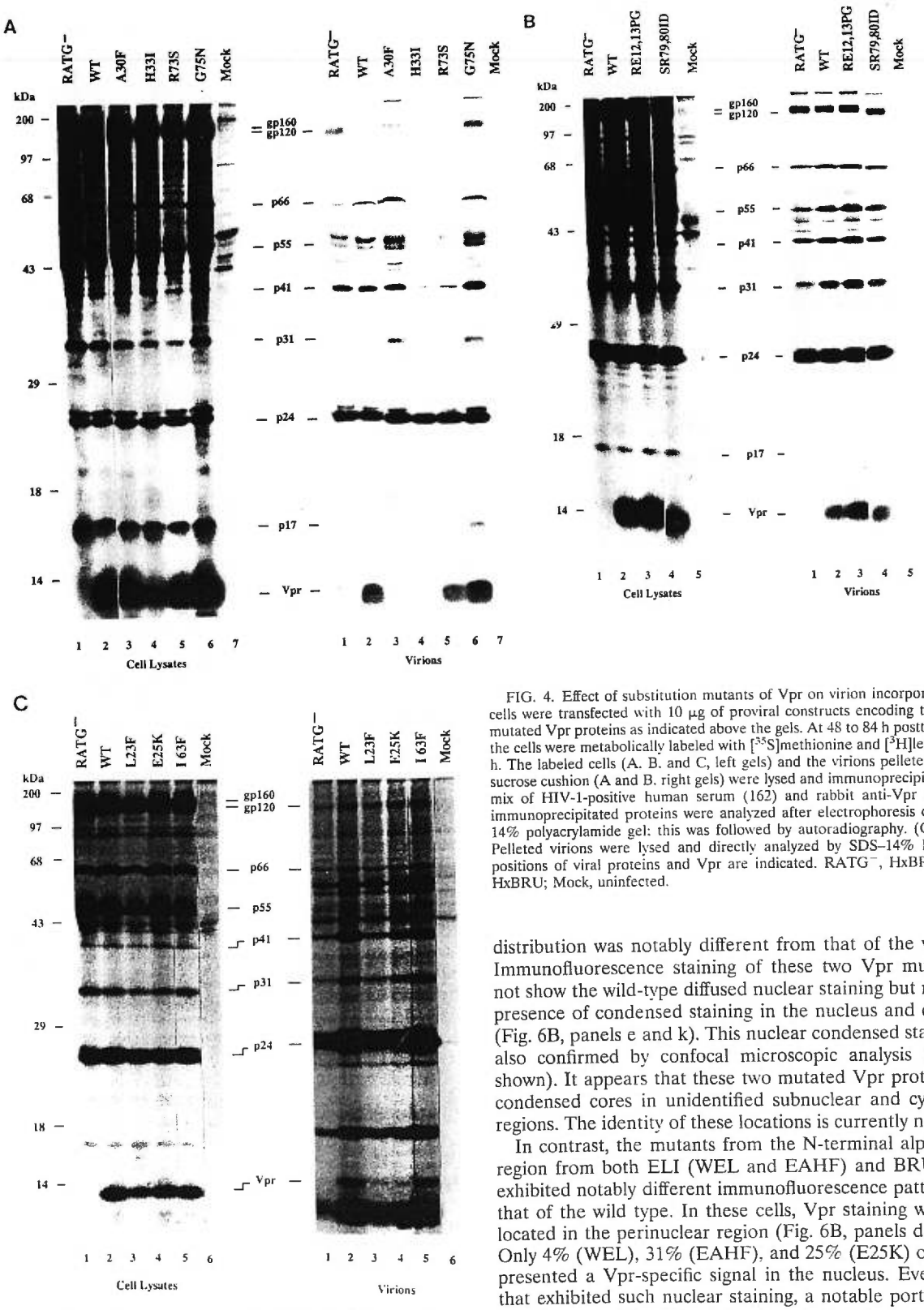


FIG. 4. Effect of substitution mutants of Vpr on virion incorporation. MT4 cells were transfected with 10 μg of proviral constructs encoding the different mutated Vpr proteins as indicated above the gels. At 48 to 84 h posttransfection, the cells were metabolically labeled with [^{35}S]methionine and [^{3}H]leucine for 12 h. The labeled cells (A. B. and C, left gels) and the virions pelleted through a sucrose cushion (A and B. right gels) were lysed and immunoprecipitated with a mix of HIV-1-positive human serum (162) and rabbit anti-Vpr serum. The immunoprecipitated proteins were analyzed after electrophoresis on an SDS–14% polyacrylamide gel: this was followed by autoradiography. (C, right gel) Pelleted virions were lysed and directly analyzed by SDS–14% PAGE. The positions of viral proteins and Vpr are indicated. RATG⁻, HxBRUR⁻; WT, HxBRU; Mock, uninfected.

tein in both the nucleus as well as the cytoplasm (Fig. 6A and B, panels c, e, g, h, and j to m).

Although two of the mutated Vpr proteins (L23F and IL63,64KR) were targeted to the nucleus, their intranuclear distribution was notably different from that of the wild type. Immunofluorescence staining of these two Vpr mutants did not show the wild-type diffused nuclear staining but rather the presence of condensed staining in the nucleus and cytoplasm (Fig. 6B, panels e and k). This nuclear condensed staining was also confirmed by confocal microscopic analysis (data not shown). It appears that these two mutated Vpr proteins form condensed cores in unidentified subnuclear and cytoplasmic regions. The identity of these locations is currently not known.

In contrast, the mutants from the N-terminal alpha-helical region from both ELI (WEL and EAHF) and BRU (E25K) exhibited notably different immunofluorescence patterns from that of the wild type. In these cells, Vpr staining was mainly located in the perinuclear region (Fig. 6B, panels d, f, and i). Only 4% (WEL), 31% (EAHF), and 25% (E25K) of the cells presented a Vpr-specific signal in the nucleus. Even in cells that exhibited such nuclear staining, a notable portion of the protein was still retained in the cytoplasmic compartment. These results are consistent with the notion that this putative N-terminal alpha-helical structure is essential for the preferential nuclear targeting of the Vpr protein.

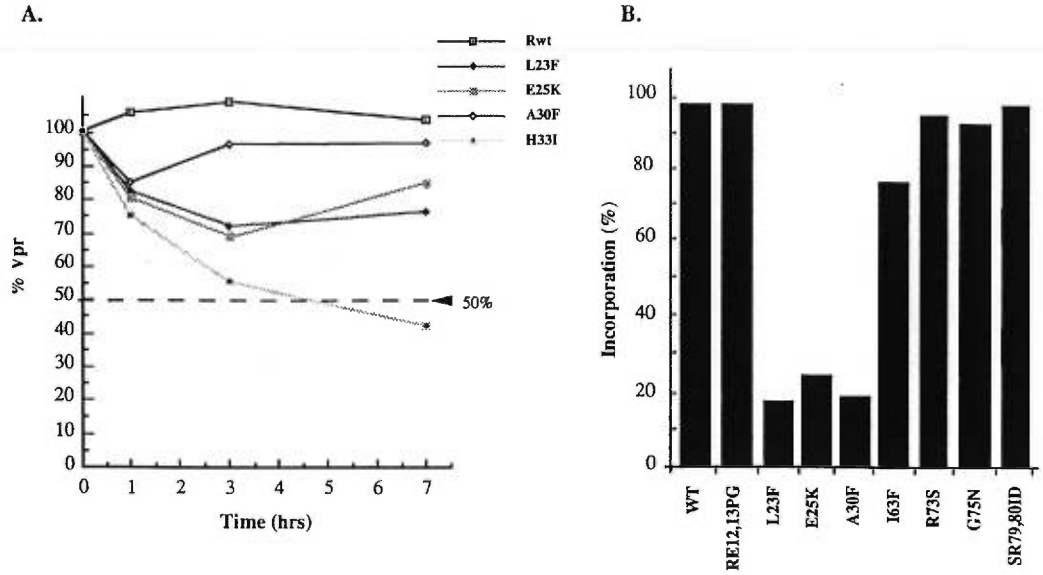


FIG. 5. Evaluation of stability and virion incorporation of different mutated Vpr proteins. (A) 10^6 COS cells were transfected with different Vpr expressors as indicated. After 48 h, the transfected cells were pulse-labeled with [^{35}S]methionine and [^{3}H]leucine for 30 min and were subsequently chased at different intervals of time. The Vpr proteins in the cell lysates were immunoprecipitated with rabbit anti-Vpr serum and analyzed by autoradiography after SDS–14% PAGE. Expression levels at different time intervals for each of the substitution mutants were evaluated by using a Molecular Dynamics Personal densitometer. The percentage of Vpr recovered relative to the amount present at the end of the pulse (0 min) is plotted as a function of time. Rwt, wild-type Vpr. (B) Autoradiograms were scanned to determine the densitometric values of Vpr and p66 RT proteins immunoprecipitated from pelleted virions. The level of p66 RT served as an internal control in each sample. The incorporation rate of Vpr in the virions was determined by the ratio of virus-associated Vpr to RT. The wild-type (WT) Vpr RT expression ratio was arbitrarily set at 100%.

DISCUSSION

Of the six HIV-1 auxiliary proteins (Vif, Vpr, Tat, Vpu, Rev, and Nef), Vpr is the only gene product that is incorporated efficiently into the progeny virions (6, 38). The presence of Vpr in the virions is a strong indication that this protein may have a functional role early in viral replication. When expressed in the absence of other viral proteins, Vpr was shown to localize in the nucleus (28). These two disparate Vpr cellular localizations are likely mediated through specific protein-protein interactions during viral replication. In order to identify regions of Vpr involved in its nuclear localization and virion incorporation, we generated a number of mutations in different putative regions of the Vpr sequence: a putative N-terminal alpha-helical structure predicted by computer-assisted sequence analysis, a previously characterized region termed the LR domain toward the distal half of the protein. and a carboxyl-terminal region rich in basic residues. Our results clearly show that several individual substitution mutations in this helical region, including L23F, E25K, and A30F, resulted in relatively stable proteins that showed markedly reduced virion incorporation. One of these mutants, E25K, also impaired Vpr nuclear localization, indicating that the putative N-terminal alpha-helical region extending from amino acids 16 to 34 plays a significant role in virion incorporation as well as in the nuclear targeting of Vpr. Furthermore, the results from this study indicate that the C-terminal truncation of Vpr, but not the substitution mutations in this region, resulted in a less stable Vpr protein and in the impairment of both virion incorporation and nuclear localization. These impairments may be due to altered structural conformation and/or stability of these truncated Vpr proteins.

Amphipathic helices are commonly observed in structural motifs that involve protein-protein and protein-lipid interactions (37). One of the important structural features of Vpr includes an N-terminal domain predicted to form an amphipathic alpha helix (from amino acids 16 to 34), as shown in Fig. 7A. This putative helical structure exhibits well-demarcated hydrophilic and hydrophobic faces, as shown in Fig. 7B. Interestingly, Vpx, another virion-associated protein encoded by HIV-2 and simian immunodeficiency virus, also contains a predicted amphipathic helical structure near its N terminus (20). In addition, the C-terminal halves of the alpha-helical structures of Vpr and Vpx share notable homology in terms of the amino acid sequence and, hence, amphipathicity (Fig. 7A). Recent data indicate that, like incorporation of Vpr, Vpx incorporation also depends on the presence of a region extending from amino acids 439 to 497 at the carboxyl-terminal end of the Pr55^{gag} polyprotein corresponding to the p6 region (43). These findings strongly tie the sequence homology found in these proteins with that of a common packaging mechanism for these related accessory products. Recent reports indicate that the presence of an alpha-helical structure near the N terminus of Vpr is important for its virion incorporation, since introduction of proline residues in this predicted alpha-helical region abolished Vpr virion incorporation (29, 30). Moreover, it was shown that substitution of alanine for the four leucine residues in the helical region resulted in a mutant which was not incorporated into virus-like particles, suggesting the importance of these hydrophobic amino acids in the helical region for Vpr virion incorporation (30). It is of interest to note in the present study that amino acids L-23 and A-30 are close to one another on the hydrophobic face of the predicted helix (Fig. 7B). Though substitution of L-23 or A-30 with phenylalanine conserves the hydrophobicity of this face, it notably impairs Vpr incorporation, suggesting that specific residues located on the hydrophobic face may be involved in protein-protein interactions relevant to virion targeting. Also, our

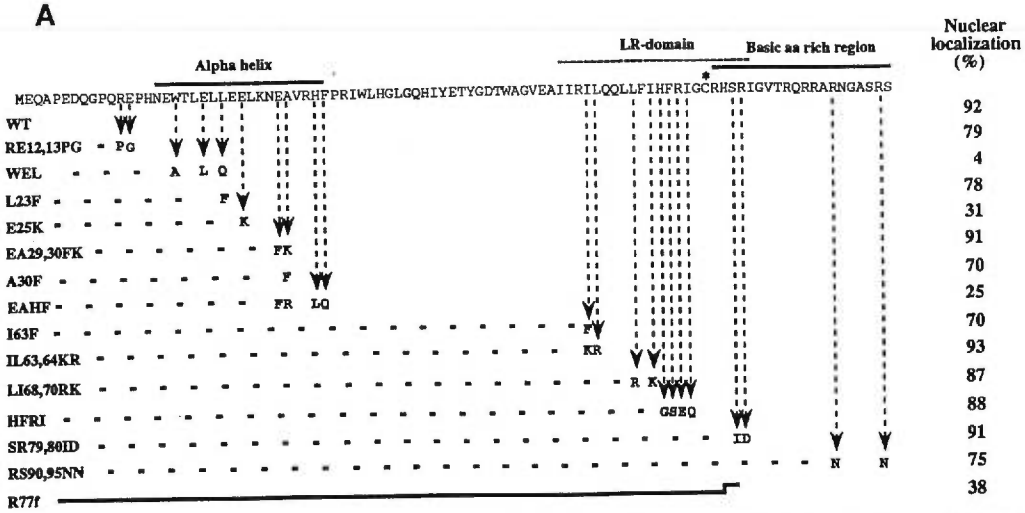


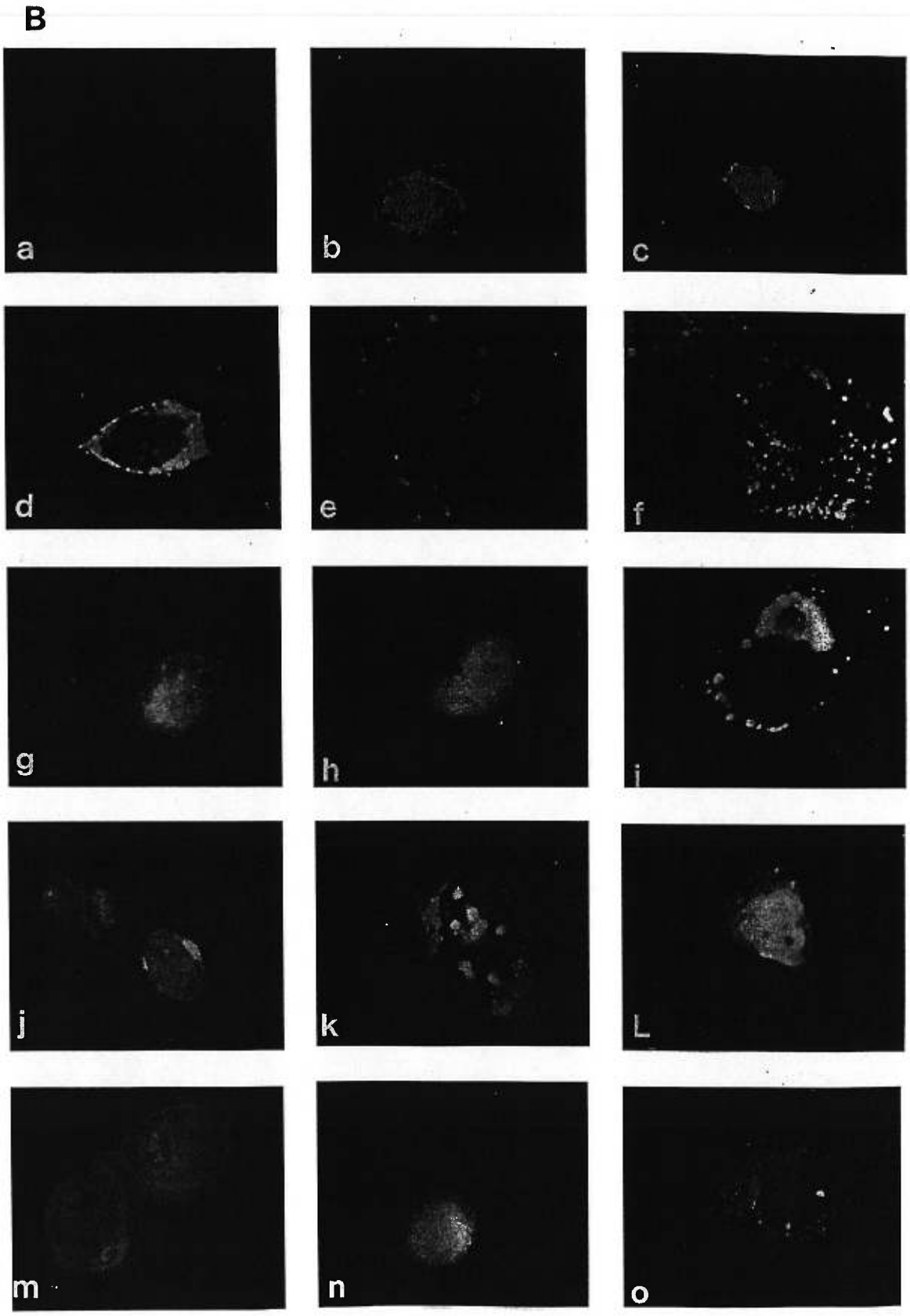
FIG. 6. Nuclear localization of different mutated Vpr proteins in COS cells. (A) The amino acid sequence of Vpr is shown at the top. The position of the predicted amphipathic alpha-helical structure, the highly conserved amino acid cysteine at position 76, the LR domain, and the basic amino-acid (aa)-rich region in the Vpr are also indicated. A series of mutated Vpr expressors (SVCMV-Vpr) were constructed (as described in Materials and Methods), and the position of each mutation on the Vpr sequence is shown by the arrow. For evaluating the nuclear localization of Vpr, COS cells cultured in shell vials were transfected with each of these Vpr expressors. After 48 h, the transfected cells were fixed, labeled with anti-Vpr antibodies, and analyzed by the indirect immunofluorescence technique (as described in Materials and Methods). The percentage of Vpr-positive cells having nuclear localization was evaluated by determining the numbers of Vpr-positive cells with specific nuclear staining over total numbers of Vpr-positive cells (shown on the right). WT, wild type. (B) Intracellular localization of wild-type and different mutated Vpr proteins in COS cells. (a) Vpr^-; (b) wild-type Vpr; (c) RE12,13PG; (d) WEL; (e) L23F; (f) E25K; (g) A30F; (h) EA29,30FK; (i) EAHF; (j) I63F; (k) IL63,64KR; (l) LI68,70RK; (m) HFRI; (n) SR79,80ID; (o) R77f. The localization of RR90,95NN is not shown.

results indicate that an individual substitution mutation, E25K, located at the hydrophilic side of this predicted helical region severely impairs the capacity of Vpr to be incorporated into virions. These results further extend the conclusion reached by a recent study (30), in which virion incorporation was not affected when multiple glutamic acid residues in the putative helix were replaced by a similarly charged aspartic acid residue. Interestingly, however, the authors noted a drop in incorporation when these acidic residues were replaced by an uncharged, but polar, glutamine residue. In our mutational analysis, one of the glutamic acids tested by the previous study was replaced by the oppositely charged lysine in order to disrupt the acidity associated with this hydrophilic face. Our results document a notable impairment of virion association of this mutant, which suggests the importance of the acidity rather than the identity of the amino acid residues associated with this hydrophilic face for effective virion incorporation of Vpr. Taken together, these results indicate that both sides of this secondary structure can affect Vpr incorporation.

Our results from immunofluorescence analysis clearly indicate that the predicted alpha-helical structure near the N terminus of Vpr plays an important role in Vpr nuclear localization in addition to Vpr incorporation. The importance of this predicted alpha-helical structure for nuclear targeting is also supported by the impairment of nuclear localization of a multiple substitution mutant (EAHF) which was shown to disrupt the conformation of the distal half of the helical structure, as predicted by our computer analysis (data not shown). It is of interest to note that the Vpr mutants WEL and E25K, which affect nuclear localization, carry amino acid substitutions on the hydrophilic face of the putative helix (Fig. 7B). In contrast, mutations that targeted the hydrophobic face of the Vpr N-terminal helix (L23F or A30F) resulted in mutant proteins that retained their ability to translocate to the nucleus but were impaired in their virion incorporation property.

The immunofluorescence patterns of the various Vpr mutants were also investigated. Two mutated Vpr proteins (L23F and IL63,64KR), even though clearly localized in the nucleus, still displayed different staining patterns, compared with the wild-type Vpr. Interestingly, these mutations are located either in the predicted alpha-helical region or in a previously described LR domain extending from amino acids 60 to 81 in Vpr (46) as depicted in Fig. 6A. This LR domain has been shown to be involved in the interaction with a cellular protein (RIP), and point mutations in this region (amino acids 60 to 81) abolished this interaction (46). RIP has been shown to be present in the cytoplasm and nucleus (46). So, it might be possible that the proper nuclear translocation of Vpr is related to its association with the RIP protein.

Previous studies have shown that the C-terminal portion of Vpr is involved in efficient Vpr incorporation into progeny virions (33, 41). We also tested the importance of the C-terminal portion of Vpr for its stability and biological properties. The results indicate that two premature truncation mutants that eliminate the C-terminal sequences were relatively unstable proteins, compared with the wild-type or the N-terminal mutants (Fig. 2). As the level of the virion-associated protein in these truncation mutants was lower than the level observed with the wild type, it is conceivable that the C-terminal region may also modulate the efficiency of virion association (Fig. 3). However, we cannot rule out the possibility that this is partially related to the unstable nature of the C-terminal truncation mutants. Nonetheless, the detection of these truncated Vpr proteins in the virions clearly indicates that the minimal determinant(s) required for virion incorporation is located not in the deleted C-terminal region (up to the last 24 amino acids) but in the proximal region of the protein. This is also supported by the fact that the homologous HIV-2–simian immunodeficiency virus proteins (Vpr-2 and Vpx) capable of effective virion association do not share C-terminal sequences with HIV-1 Vpr (40). Additional support suggesting the lack of specific motifs directing virion incorporation comes from our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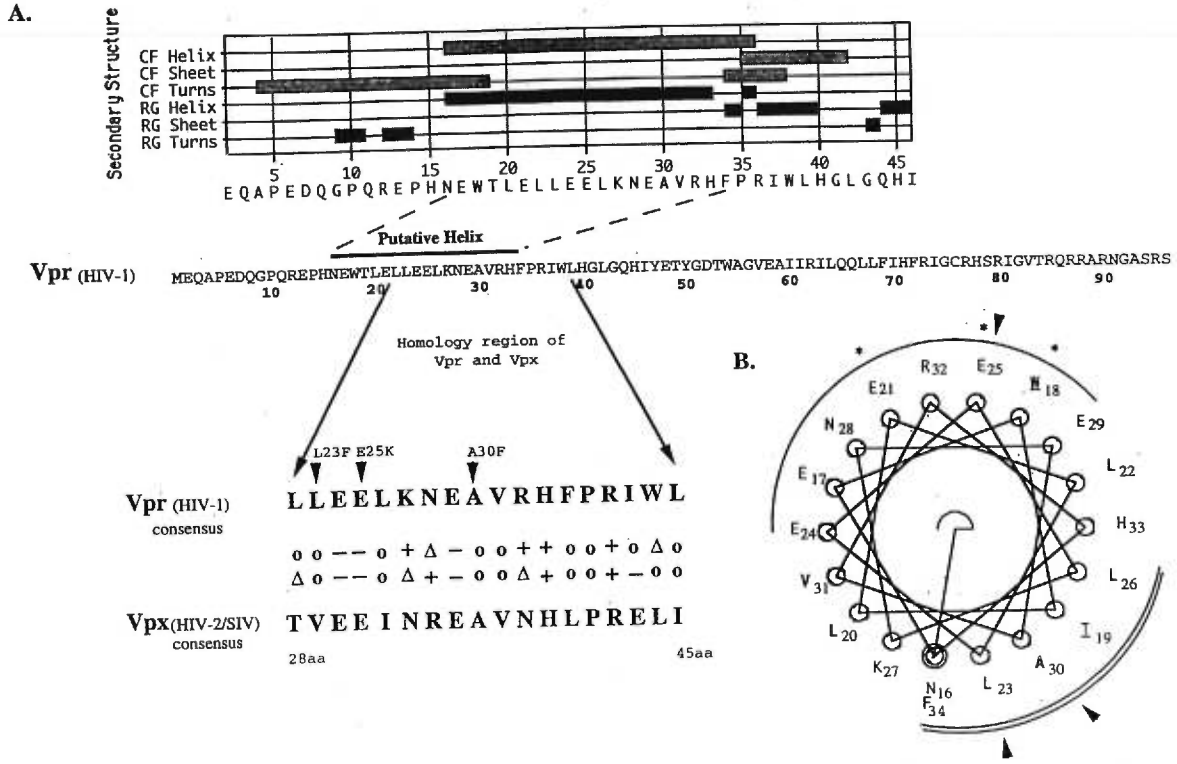

FIG. 7. The computer-predicted N-terminal alpha-helical structure in Vpr protein. (A) The amino acid sequence of Vpr and the positions of the alpha helix, β sheet, and β turn predicted by computer analysis are shown at the top. (RG, Robson-Garnier method; CF, Chou-Fasman method). A region with high sequence homology between Vpr and Vpx is shown at the bottom, and this region comprises the C-terminal half of the amphipathic helix region. The incorporation-related amino acids are indicated by arrows. O, hydrophobic amino acids (aa); Δ, polar aa; —, negatively charged aa; +, positively charged aa. (B) Helical-wheel diagram of the predicted alpha-helical structure extending from amino acids 16 to 34 in the HIV-1 Vpr sequence. The hydrophilic face is indicated by a single line, and the hydrophobic face is represented by a double line. The incorporation-related amino acids are indicated by arrows, and the amino acids involved in Vpr nuclear localization are indicated by asterisks.

substitution analysis of the C-terminal region where no single residue tested directly affected the efficiency of virion incorporation (Fig. 4 and 5). Given these independent lines of evidence which suggest the lack of a minimal required determinant for virion incorporation in this region, the highly reduced levels of virion-associated truncated Vpr proteins is likely mediated by simply modulating the stability of the protein (Fig. 2) and/or by impairing the optimal conformation of the protein necessary for virion incorporation. However, the fact that some protein is clearly present in the virion excludes the possibility of a major conformational change.

Our results from immunofluorescence analysis using the same R77f mutant indicate that the essential determinant(s) for the nuclear localization of Vpr is not located within the last 19 amino acids of the Vpr C terminus. However, as is the case with virion incorporation, eliminating the last 19 amino acids resulted in a dramatic reduction in the ability of Vpr to translocate to the nucleus (Fig. 6). These results correlate with fractionation experiments that show an impairment of nuclear localization, using a similar 19-amino-acid-truncated Vpr (28). A recent study shows that the deletion of the last 9 amino acids (amino acids 87 to 96) or an internal deletion from amino acids 77 to 87 in the arginine-rich C terminus of Vpr did not abolish Vpr nuclear localization (46). Consistent with this study, our analysis of a number of substitution mutations in the C-terminal region of Vpr did not identify any specific amino acid(s) involved in Vpr nuclear localization. Taken together, these results suggest the lack of a primary determinant necessary for nuclear localization in this region. Therefore, as with virion incorporation, the impairment of nuclear localization of a large C-terminal-truncated Vpr may also be due to altered structural conformation. Though a number of mutations were included in our study, we cannot formally rule out the possibility that other single residues in this region are capable of affecting these properties.

These results strongly suggest that a predicted N-terminal alpha-helical structure is involved in protein-protein interactions which mediate virion incorporation and nuclear localization of Vpr. Our results also distinguish between the hydrophilic and hydrophobic faces of this putative secondary structure in their ability to affect nuclear localization and virion incorporation of Vpr. It is clear that the efficiency of both nuclear and virion targeting of the mutant E25K is highly impaired. However, a small, but observable, quantity of the mutant still reaches the nucleus and the virions, ruling out the possibility of a global disruption of the Vpr protein. This may suggest that a common mechanism is operative both in transporting Vpr to the nucleus as well as in incorporating it in the virion. On the other hand, proteins that show relatively high levels of nuclear localization (A30 and L23) are still incapable of effective incorporation in the virion. This clearly indicates that determinants in the hydrophobic region, in addition to those in the hydrophilic region, influence Vpr virion targeting.

These results are interesting, in light of a recent study that

suggests that Vpr may associate with a 41-kDa protein (termed Rip-1) that specifically binds the type II glucocorticoid receptor which traffics between the cytoplasm and the nucleus (34). Hence, it is conceivable that specific mutations within the Vpr sequence that affect its ability to reach the nucleus may also affect its efficient association with such partners. As the hydrophobic mutants, in spite of active nuclear localization, still affect virion incorporation, it suggests that this side of the helix either forms a direct hydrophobic interface with virion structures or, alternatively, mediates homooligomerization of the protein. In this regard, it is worth noting that Vpr oligomers have been documented previously (33, 47). We are currently exploring Vpr-Vpr and Vpr-cellular protein interactions and their ability to influence virion incorporation and nuclear localization.

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Chapter 3

Human immunodeficiency virus type 1 Vpr localization: nuclear transport of a viral protein modulated by a putative amphipathic helical structure and its relevance to biological activity.

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Human Immunodeficiency Virus Type 1 Vpr localization: nuclear transport of a viral protein modulated by a putative amphipathic helical structure and its relevance to biological activity

Ramu A. Subbramanian[1] , Xiao-Jian Yao[1], Hugo Dilhuydy[2], Nicole Rougeau[1], Dominique Bergeron[1], Yves Robitaille[2], and Eric A. Cohen[1,3]

[1]Laboratory of Human Retrovirology

Department of Microbiology and Immunology

[2]Department of Pathology

Faculty of Medicine, University of Montreal, Quebec, Canada H3C 3J7

[3]Corresponding author

Tel: (514)-343-5967 Fax: (514)-343-5995

Electronic mail: cohenea@ere.umontreal.ca

Running Title: HIV-1 Vpr function and localization

ABSTRACT

Protein import into the nucleus is generally considered to involve specific nuclear localization signals (NLS) though it is becoming increasingly clear that efficient and well controlled import of proteins which lack a canonical NLS also occurs in cells. Human immunodeficiency virus type 1 (HIV-1) Vpr is one such protein which does not have an identifiable NLS and yet efficiently localizes to the nuclear compartment. In this study, we use confocal microscopy to demonstrate that mutations in the putative central hydrophobic helix of Vpr result in the retention of the protein in highly localized ring-like structures around the nuclear periphery with striking impairment in their ability to enter the nuclear interior. By characterizing other biological activities associated with this protein, such as its ability to incorporate into budding virions and its ability to arrest cells in G2, we show that this helical domain is specific for the nuclear translocation of the protein with very little effect on these other functions. Interestingly, however, perturbation of this helical motif also perturbs the protein's ability to augment viral replication in primary human macrophages indicating that the integrity of this secondary structure is essential for optimal infection in these non dividing cells.

Keywords: Cell cycle arrest/HIV-1 Vpr/Infection of non dividing cells/Nuclear localization/Virion-incorporation

Introduction

Unraveling the mechanisms operative in protein trafficking between the nuclear and cytoplasmic compartments is of key importance as the process ultimately controls access of macromolecules to the cellular genome both during normal homeostasis and during cellular pathology. Nuclear import of proteins in general appears to proceed through a targeting step where cytoplasmic proteins are docked onto the nuclear pores at the nuclear periphery, and a subsequent import step, where such docked proteins are translocated through the nuclear pore complex into the nuclear interior (Corbett and Silver, 1997; Nigg, 1997). A vast array of proteins destined for nuclear import contain a clearly defined nuclear localization signal (NLS) exemplified by the SV40 T-antigen NLS. The series of events that result in the nuclear import of proteins carrying an NLS has been well characterized (Schlenstedt, 1996). However, some proteins do not have a canonical NLS and are yet effectively imported into the nucleus following synthesis in the cytoplasm (Weighardt *et al.*, 1995). However, the exact mechanism operative in the nuclear import of these proteins is not clearly understood. This study focuses on one such protein, the human immunodeficiency virus type 1 (HIV-1) Vpr, a small viral accessory protein that effectively localizes to the nucleus though it lacks a canonical NLS (Lu *et al.*, 1993; Zhao *et al.*, 1994; Mahalingam *et al.*, 1995a; Yao *et al.*, 1995b). A previous study has identified a Leucine and Arginine rich region in Vpr (LR domain) which appears to play a role in its association with an uncharacterized cellular partner termed RIP-1 (Zhao *et al.*, 1994). Such an interaction is presumed to transport the Vpr protein in a "piggy-back" fashion into the cell nucleus.

From a functional point of view, Vpr is a late protein that is synthesized during active virion production. This protein is packaged efficiently in the progeny virions via its interaction with viral Gag proteins in high copy numbers (Cohen *et al.*, 1990; Paxton *et al.*, 1993; Lavallée *et al.*, 1994; Kondo *et al.*, 1995). As the Vpr protein is incorporated in the virion, it is also available during early replication events, immediately following entry of the new virion into a target cell, even before *de novo* viral protein synthesis could start. Based on such late expression during virus production and early availability during initial infection, two main functions have been proposed for this protein (Emerman, 1996). One of these functions involves an early event following virion entry: the efficient import of viral preintegration complexes into the nuclear compartment of cells which are not undergoing division (Heinzinger *et al.*, 1994). Unlike HIV-1, prototypic retroviruses, in general, do not replicate efficiently in

nondividing cells as they rely on the disintegration of the nuclear envelope during mitosis to gain access to the nuclear compartment. However, it is currently clear that HIV-1 codes for proteins such as Vpr that ensure effective transport of the viral preintegration complexes to the nucleus even in non dividing cells (Heinzinger *et al.*, 1994; Gallay *et al.*, 1996). This unique ability to side step mitosis manifests itself as the ability to infect nondividing target cells *in vivo*, such as macrophages, and can be assessed *in vitro* by the augmented replication of viruses encoding Vpr in such cells (Westervelt *et al.*, 1992; Balliet *et al.*, 1994; Connor *et al.*, 1995).

The second function that is associated with Vpr is the ability to arrest cells expressing the protein in the G2 phase of the cell-cycle (He *et al.*, 1995; Re *et al.*, 1995; Rogel *et al.*, 1995). The first indication that Vpr may perturb cell division came from observations that document the inability of the wild-type Vpr expressing cells to grow out of the acute infection stage to generate chronic virus-producing cell lines (Rogel *et al.*, 1995). Yet another report documented withdrawal of Vpr expressing cells from cell division into terminal differentiation (Levy *et al.*, 1993). Currently it is well established that this outcome is the result of effective cell-cycle block induced by Vpr in the G2 phase following DNA duplication (Di Marzio *et al.*, 1995; He *et al.*, 1995; Re *et al.*, 1995). This effect most likely involves a well conserved cellular pathway as it is evident not only in human cell lines but in non-human primate cells as well as in yeasts (Planelles *et al.*, 1996; Zhao *et al.*, 1996; Stivahtis *et al.*, 1997).

However, how these functions relate to the protein localization and its structure remains to be elucidated. To date, detailed analysis of the protein localization by the powerful confocal microscopic technique has not been employed to further characterize the localization of this nuclear protein which lacks a canonical NLS. Confocal laser microscopy allows for three dimensional imaging of protein localization in morphologically intact cells with high resolution by laser scanning. Here we use this methodology in conjunction with mutational analysis of a region involved in the protein's nuclear import in an effort to understand localization and its functional relevance in the context of protein structure. Results presented here suggest that the central hyrophobic helical region though not capable of affecting either Vpr's ability to incorporate into virions, or its ability to G2-arrest dividing cells, is capable of affecting the protein's nuclear localization as well as its ability to augment viral replication in non dividing cells such as primary human macrophages.

Results

Amino acids 53 to 74 of HIV-1 Vpr forms a putative alpha helical structure

The HIV-1 Vpr is a small, 96 amino acids long protein that is efficiently packaged in the virion. We have previously identified an N-terminal helix extending from residues 16 to 34 of Vpr (Yao *et al.*, 1995b). However there is limited information available on the secondary structure of the region distal to the N-terminal helix that is rich in hydrophobic residues. Though a putative helical motif has been suggested by different groups, little consensus exists as to the extent of the helical structure and functional domain mapping of this region (Zhao *et al.*, 1994; Mahalingam *et al.*, 1995a; Piller *et al.*, 1996). Here we use Rost and Sander's neural network-based prediction that uses algorithms that provide an average accuracy of more than 72% for the helical, sheet, and loop motifs (Rost and Sander, 1993; Rost, 1996). Such prediction suggests that the central hydrophobic region of Vpr that falls within the previously identified Leucine-Arginine rich "LR" domain could structurally form a helical motif which extends from residues 53 to 74, distal to the previously identified N-terminal helix (Figure 1A). The detailed predictions and its probability coefficients are indicated in Figure 1B. As can be seen in the scale of 1 to 10 for helical state, the central hydrophobic helix rates a 9 for a vast majority of residues. However, the probability of a loop or an extended beta structure, on the other hand, is limited ranging from zero to 1 in the coefficients, again correlating with the high probability of prediction for a helical motif. Taken together, this analysis indicates that the region has a high propensity to form a helical structural motif that falls within a previously identified "functional" LR domain.

In an effort to gauge hydrophobicity and solvent accessibility of this helical domain, we again used a neural network based protocol that is cross validated on a variety globular protein standards (Rost and Sander, 1994). Such analysis indicates that this region is highly hydrophobic though the small number of residues which are hydrophilic cluster on one aspect of the helix. This provides a clearly amphipathic helical structure with an extended hydrophobic interface and a well defined, albeit, small hydrophilic interface (refer to the helical wheel representation in Figure 1C). Multiple sequence alignment analysis of this region suggests that the sequence in this area is conserved across distinct HIV-1 isolates derived from different consensus groupings

(data not shown). Such conservation of this structure and its sequence is highly indicative of this region being important for Vpr function.

Choice of mutations and expression systems

In an effort to identify the functional importance of this putative central hydrophobic helix, we undertook a mutational analysis approach that targeted the different regions of the helical structure. In an effort to understand both steric and charge importance of residues in this region to protein function, we chose conserved residues and mutated them systematically. One mutant carrying substitution of amino acid Valine 57 to Leucine (V57L) was designed to assess the importance of steric interference that would result from the introduction of a branched chain amino acid. In order to directly assess the importance of charge and specific amino acid importance of the hydrophobic interface, the conserved uncharged Isoleucine residue at position 63 was changed either a) to a Lysine (I63K) that introduces a basic charge in this region or b) a Phenylalanine (I63F) residue that introduces an aromatic structure in place of the aliphatic Isoleucine. The latter mutation (I63F) has been previously described (Yao *et al.*, 1995b). The hydrophilic interface was targeted by mutating the uncharged Glutamine residue to a charged Glutamic Acid residue (Q65E). Further toward the carboxyl terminal, another previously described mutation (Yao *et al.*, 1995b) was included (LI68/70RK) which targeted simultaneously both aspects of the extended hydrophobic interface by changing hydrophobic residues, Leucine 68 and Isoleucine 70, to charged amino acids, Arginine and Lysine, respectively. None of the mutations described above were predicted to disrupt the putative helical structure by computer assisted analysis. This allowed us to address the relevance of the hydrophobic interface and specific residues as opposed to radically affecting the formation of the putative helix. However, Proline residues, when introduced in long linear helices, due to hydrogen bonding constraints, will ensure that an extended helical structures is broken or bent at the site of introduction. This has been used effectively to gauge the importance of helical structural conformation in a putative motif in earlier studies (Yao *et al.*, 1995a). In an effort to understand, what role, if any, the putative helix played in Vpr phenotypes, we substituted a Proline residue in the middle of the structure at position R62.

The mutations described above (systematically represented in Figure 1B) were cloned directly into a previously characterized expression vector driven by a

Cytomegalovirus (CMV) immediate early (IE) promoter (Yao *et al.*, 1995b). The vectors were transfected in COS-7 cells to monitor nuclear localization at 48 hours. All the mutants described in this study generated a very strong Vpr specific signal following radioimmunoprecipitations after a brief 30 min pulse with radioactive methionine indicating that both the wild-type and mutant proteins were being expressed efficiently with this vector system (data not shown). As this study also addressed the issue of virion incorporation, a selected panel of the mutations were also directly cloned in the context of the complete HXBRU based provirus (Yao *et al.*, 1995b). The envelope region of the well characterized macrophage tropic strain ADA (Westervelt *et al.*, 1992) was transferred into selected proviruses to render them macrophage tropic. Alternatively, to address the G2-arrest associated with these mutations, the proviral constructs were rendered envelope negative (see materials and methods) so they could be pseudotyped with the highly efficient vesicular stomatitis virus G envelope glycoprotein (VSV-G) for highly efficient single-cycle replication (Bartz *et al.*, 1996). This allowed us to express the Vpr protein coordinately with other viral structural proteins both during virion incorporation and G2-arrest.

HIV-1 wild-type Vpr is targeted to the nuclear periphery and accumulates in the nucleoplasm

HIV-1 Vpr has been localized primarily in the nuclear compartment by our group and others using conventional optical immunofluorescence (Lu *et al.*, 1993; Zhao *et al.*, 1994; Di Marzio *et al.*, 1995; Mahalingam *et al.*, 1995a; Yao *et al.*, 1995b; Mahalingam *et al.*, 1997). While such conventional analysis grouped anything closely associated with the nucleus as nuclear targeting, a clear understanding of the protein's localization in the cells is still lacking. In this study, we use powerful scanning confocal microscopy to delineate the localization of the protein in laser sections of intact cells.

Cos-7 cells were transfected with the wild-type and the ATG- negative control plasmids and were analyzed 48 hours post transfection for nuclear localization with a primary antisera raised against recombinant Vpr protein (Lavallée *et al.*, 1994) and an FITC conjugated secondary antibody against rabbit IgG. As we have previously described with this antisera (Yao *et al.*, 1995b), the cultures transfected with the wild-type vector showed a strong signal primarily in the nucleus (Figure 2, Panels A and B and Figure 3, Panel A), while cultures transfected with the ATG- mutant showed no

apparent signal (data not shown) indicating that the signal detected in the wild-type cultures was indeed specific to the Vpr protein. Two main patterns and a third infrequent pattern were seen among the cultures consistently: Pattern A) a pronounced intranuclear staining with no preferential accumulation at the nuclear periphery; Pattern B) staining both at the nuclear periphery and the nuclear interior; Pattern C) primarily perinuclear staining with little or no intranuclear staining. A random sample of cells derived from three independent experiments encompassing the three patterns were chosen for further analysis by laser sectioning confocal microscopy. Almost two out of three cells (65%) showed pattern A with primarily intranuclear accumulation with no preferential accumulation at the nuclear periphery (Figure 2, panel A). The second most obvious pattern (32%) of staining showed, again, primarily nuclear accumulation as in Pattern A above, but with clearly identifiable accumulation at the nuclear periphery as well. This pattern is illustrated in Figure 2B by serial sections through a representative positive cell (arrowhead points to the perinuclear accumulation). An extremely small number of cells (3%) showed primarily perinuclear staining with no apparent signal in the nuclear interior (data not shown).

Mutations in the central hydrophobic region radically affect nuclear translocation of the protein: highly localized retention at the nuclear periphery

In an effort to understand what role, if any, the central hydrophobic helix played in the nuclear translocation, we investigated how mutations in this region (Figure 1B) affected the protein's ability to localize to the nucleus by utilizing the confocal scanning laser microscopy technique. COS-7 cells were analyzed 48 hours post transfection with plasmids expressing the above mutants for nuclear localization. We were able to detect clear Vpr specific signal for all the mutants tested though their ability to localize to the nucleus varied dramatically from the wild-type protein (compare Figure 3A to 3B, C, D,E and F).

Unlike the wild-type protein, the second helix mutants were clearly impaired in their ability to reach the nuclear interior (Figure 3, Panels B, C, D, E, and F). Though additive representations in Figure 3 show what appears to be general cytoplasmic retention of mutant proteins, interestingly, laser sections show that mutations which affect the amphipathicity of the helix actually result in the accumulation of the mutant proteins in strikingly localized ring-like regions around the nucleus (exemplified in Figure 4A by the I63K mutation). However, the Proline mutation (R62P) that disrupts

the helical structure itself is retained in the cytoplasm with no preferential accumulation at the nuclear periphery (Figure 4B). Taken together, our data suggests that the maintenance of the helical structure is necessary for the preferential targeting of the protein to the nuclear periphery and that the amphipathicity of the helical motif is important for the subsequent nuclear translocation of Vpr.

G2-Arrest mediated by Vpr is not dependent on the protein's ability to accumulate in the nucleus

One of the major functions attributed to Vpr includes its ability to growth-arrest cells in the G2 phase of the cell-cycle. We were interested in understanding if there is a correlation between the protein's ability to localize to the nucleus and its ability to G2-arrest as this will provide insights into the mechanism behind the Vpr mediated G2-arrest. To address this question, we chose to compare the G2-arrest associated with the wild-type protein to the mutant proteins R62P and V57L which showed clear impairment in their ability to localize to the nuclear interior. The system used here allowed for efficient single step infection of target cells with VSV-G pseudotyped HIV-1 virions expressing either the wild-type or the mutant Vpr proteins (see materials and methods). The control cells, as well as the cells infected with the Vpr-negative pseudotyped virus, showed a normal cell-cycle profile with a majority of cells in the G0/G1 phase of the cell-cycle. However, expression of the wild-type Vpr resulted in rapid accumulation in the G2 phase of the cell-cycle (Figure 5A) as previously reported (Di Marzio *et al.*, 1995; He *et al.*, 1995; Re *et al.*, 1995; Rogel *et al.*, 1995). FACS analysis for DNA content of the cells expressing the V57L mutant, which showed perinuclear accumulation, indicated that in spite of its inability to accumulate inside the nuclear compartment it was still capable of inducing G2-arrest at levels comparable to the wild-type as depicted by the G2/M ratio histogram in Figure 5B. Similarly, the R62P mutation that breaks the putative helical motif also showed a phenotype very similar to the wild-type indicating that this secondary structure is dispensable for the G2-arrest phenotype (Figure 5B).

Vpr expression results in DNA fragmenting into apoptotic bodies, an effect not dependent on the ability of Vpr to accumulate in the nucleus.

The localization of the Vpr protein in the wild-type cells clearly suggested the presence of the protein at the nuclear periphery and the interior of the nucleus. To further characterize the localization of the wild-type protein in the nucleus and its relationship to the DNA we performed double staining experiments that would help us identify the location of Vpr in the DNA context. Cells were first fixed with acetone and washed repeatedly on the coverslip. FITC staining for Vpr detection was performed on RNase treated cells which were subsequently stained with Propidium Iodide to localize DNA. The dual color analysis identified the DNA-specific Propidium Iodide in red and Vpr-specific FITC in green. Highly sensitive confocal laser sectioning and subsequent computer analysis indicated that the wild-type Vpr diffusely colocalized with the nuclear DNA. Not surprisingly, mutant cultures that were defective in their ability to translocate to the nucleus failed to show colocalization to any appreciable level. Notably, however, even in case of the wild-type Vpr, a strong and readily apparent colocalization of Vpr with the DNA was not supported by this study.

In terms of nuclear morphology, interestingly, control cells which were negative for Vpr-specific FITC signal (Figure 6A) showed normal nuclear morphology with even distribution of DNA staining (Figure 6B). However, wild-type Vpr expression resulted in clearly evident DNA fragmentation reminiscent of apoptosis (Figure 6E and 6F). The nucleus was frequently pignotic with DNA being concentrated in large dense fragments displaced toward the membrane. As expected, these cells also showed DNA laddering pattern typical of cells undergoing apoptosis in agarose gels (data not shown). Interestingly, the central hydrophobic domain mutations that radically affected the nuclear accumulation of Vpr also showed comparable levels of DNA fragmentation and apoptotic condensation. This was evident in all the mutations that showed nuclear exclusion and is exemplified in the illustration by mutant R62P (Figure 6H and 6I). Hence, accumulation of Vpr in the nucleus does not appear necessary to induce the apoptotic phenotype. It is also clear that minimal amounts of the protein can ensure apoptotic changes as cells expressing low levels of Vpr were also routinely apoptotic (Figure 6, Panel I, arrowhead).

Minimal virion-targeting domain does not lie within the central hydrophobic region of Vpr.

We have previously described the existence of a virion targeting region in the N-terminal helical region of the protein (extending from residues 16 to 34). This earlier study did not extensively analyze the central helical region that lies distal to the N-terminal helix targeted by the study (Yao *et al.*, 1995b). To analyze the central hydrophobic helix mutants, MT-4 cells were transfected with an infectious HIV-1 clone (HXBRU) expressing in *cis* the wild-type or mutant Vpr proteins which resulted in the coordinated expression of the Vpr and Gag products. Following transfection, the cells were radiolabeled and the ability of Vpr to be incorporated in the virion was monitored by immunoprecipitating both cell and virion associated viral proteins. We have previously described a ratio system to assess the amount of Vpr found in the virion as a proportion of the total amount found in the cell using other virion proteins as standards (Yao *et al.*, 1995b). This approach side-stepped the varying steady state levels of the different mutants and allowed for an objective look at virion targeting of the protein. It should be noted that such varying steady state levels observed in this proviral system where Vpr is expressed in *cis* in a intricately controlled fashion by the virus, was not apparent in the expressor system used to study nuclear localization where the protein expression was driven efficiently off a CMV promoter.

The data suggests that all mutants studied contained the minimal region necessary to target the protein to the virion. In fact, the virion incorporation levels of the mutants ranged from near wild-type levels to even higher than wild-type levels suggesting some experimental variation (Figure 7B). Even the mutant I63K, which showed clearly detectable, but lower steady state levels in this proviral system (Figure 7A) was targeted to the virion compartment at a ratio comparable to its cellular levels. It is clear then that the mutations did not affect the absolute determinants necessary for targeting the protein to the virion. This is underscored by the Proline mutation with high steady state levels that disrupts the secondary structure but not the virion-targeting indicating that this region is indeed dispensable for the virion incorporation of the protein. This is in accordance with our previous results (Yao *et al.*, 1995b) and work done by other groups where the virion-targeting domain was mapped to the N-terminus of the protein (Di Marzio *et al.*, 1995; Mahalingam *et al.*, 1995b).

Disruption of the central hydrophobic helical structure impairs viral replication in primary human macrophages.

Given the fact that the central hydrophobic helix did not affect either the protein's ability to G2-arrest dividing cells, or its ability to incorporate into virions, we sought to understand if disrupting this helical motif affected the second major biological activity attributed to Vpr, namely, its ability to augment viral replication in non dividing target cells such as macrophages. To address the issue we chose to monitor the replication of a macrophage tropic HIV-1 encoding a mutant Vpr protein in which the secondary structure of the putative central helix has been disrupted by introducing a helix-breaking Proline residue (R62P). Primary human macrophages, kept in culture for seven days were infected with equal amounts of macrophage tropic HIV-1 proviruses that either did not code for a Vpr protein (ATG-mutant), or alternatively, coded for the wild-type Vpr, or for the R62P mutant. Low viral inputs were chosen to assess this function as earlier reports have shown a stringent requirement for Vpr at lower input multiplicities than at higher viral inputs (Balliet *et al.*, 1994). These observations are not surprising as it is currently clear that HIV codes for multiple nucleophilic determinants which can effectively complement the nuclear transport function of Vpr (Heinzinger *et al.*, 1994; Gallay *et al.*, 1996). It can be seen from Figure 8, that while viruses coding for the wild-type Vpr replicated at high levels in primary macrophages even at low viral inputs tested, the Vpr-negative ATG- mutant failed to establish a productive infection in these cells. Interestingly, the R62P variant showed greatly impaired replication kinetics during macrophage infection. In fact, the R62P and ATG- mutations consistently failed to establish a productive infection in three experiments performed with macrophages derived from different donors infected with independent viral stocks. These observations strongly suggest that the structural integrity of the central hydrophobic region plays an indispensable role in the protein's ability to augment viral replication in non dividing target cells.

Discussion

A Leucine-Isoleucine rich region capable of affecting nuclear localization has been previously characterized by Zhao and coworkers in the central region of Vpr termed the LR-domain (Zhao *et al.*, 1994). Here we identify a helical motif that falls within this region which when mutated generally results in the retention of the protein in highly localized ring-like structures around the nucleus. We also provide concurrent functional evidence that suggests that this region is primarily involved in the nuclear translocation of the protein and does not affect either the protein's ability to affect the

cell-cycle or its ability to be incorporated in the virion. Virion incorporation occurs late in the infection when *de novo* synthesized Vpr is re-routed to the plasma membrane for packaging into budding virions through its interaction with viral Gag precursor proteins (Lu *et al.*, 1993; Paxton *et al.*, 1993; Lavallée *et al.*, 1994; Wang *et al.*, 1994; Kondo *et al.*, 1995).Hence, the nuclear localization of Vpr is likely to be relevant early in the infection when Vpr found in the virion helps translocate the viral preintegration complex to the nuclear compartment (Heinzinger *et al.*, 1994; Gallay *et al.*, 1996).

In fact, this ability of the virion-associated Vpr to act as a nuclear translocation signal for the viral preintegration complexes, and its ability to growth arrest infected cells in the G2-phase of the cell cycle are the two major functions that current consensus attributes to this protein. However, very little is known about how exactly these functions relate to the protein's ability to localize to the nucleus. In terms of the nuclear localization, it is not clear where exactly the wild-type Vpr is present *in situ.* This is mainly due to the limitations imposed by conventional optical microscopy used to address the issue to date. Also, previous studies failed to discern specific patterns of staining observed in the wild-type protein which frequently included both focal and diffuse nuclear signals (Zhao *et al.*, 1994; Mahalingam *et al.*, 1995a; Yao *et al.*, 1995b; Mahalingam *et al.*, 1997). Here we use confocal analysis to first dissect the localization of the wild-type protein and the central hydrophobic helix mutations *in situ.* Such analysis demonstrates that while the wild-type protein is efficiently translocated to the nuclear interior, the second helix mutations are either retained at the nuclear periphery in highly localized ring-like structures (Figure 4, Panel A) or in the cytoplasm with no preferential accumulation at the nuclear periphery (Figure 4, Panel B).

As understanding the cellular localization of Vpr during G2 may provide insights into the mechanism of Vpr, we sought to characterize the G2-arrest associated with the wild-type protein that showed typical nuclear localization and mutants that were retained at the nuclear periphery. We chose to look at the R62P and V57L mutations as they showed clear cytoplasmic retention and nuclear membrane accumulation, patterns identifiably different from the wild-type protein. FACS analysis of the DNA content indicated that both the V57L and R62P mutations, in spite of their inability to accumulate in the nucleus, still efficiently induce G2-arrest. Hence our results clearly suggest that even mutations showing severe impairment in their ability to accumulate in the nucleus, and hence retained at the level of the cytoplasm or nuclear periphery, are still capable of inducing G2-arrest at levels comparable to the wild-type. Given the nuclear accumulation of wild-type protein, we attempted to understand the status of the cellular

DNA during Vpr expression. Dual color immunofluorescence analysis suggests that the DNA staining pattern associated with wild-type Vpr expression did not show the normal pattern associated with control cells that did not express the protein (Figure 6). The nucleus in control cells showed even distribution of DNA with intact nucleoli. However, expression of wild-type Vpr resulted in abnormal condensation of DNA into clearly identifiable apoptotic bodies in agreement with a recent study (Stewart *et al.*, 1997). Interestingly, the mutations that were clearly impaired in their ability to accumulate in the nucleus also showed the very same aberrant apoptotic DNA condensation seen in the wild-type. This is exemplified in (Figure 6G, H, and I) with the R62P mutation. Hence, it appears that the aberrant DNA fragmentation, similar to the G2-arrest, does not depend on the ability of the protein to localize in the nucleus. This leads to the question what further advantage such nuclear accumulation seen in the wild-type protein may confer to the virus. This may be related to the already well characterized second function associated with the Vpr protein: its ability to facilitate the transport of the proviral DNA to the nuclear compartment in non dividing cells (Heinzinger *et al.*, 1994; Fletcher *et al.*, 1996; Gallay *et al.*, 1996). This function at least in part is responsible for the well documented ability of the protein to increase viral replication in non dividing macrophage cultures (Westervelt *et al.*, 1992; Balliet *et al.*, 1994; Connor *et al.*, 1995).

In this regard, we demonstrate that affecting the secondary structure of the central hydrophobic helix, in addition to affecting the nuclear localization of Vpr, also affects the ability of the protein to augment viral replication in macrophages. This is in keeping with current consensus that suggests that Vpr is one of the nucleophilic determinants coded by HIV-1 (Heinzinger *et al.*, 1994). Hence, the same secondary structure involved in the nuclear import of the protein also appears to be essential for facilitating early events that lead up to nuclear import of the proviral DNA and augmentation of viral replication in macrophages. While proteins of karyopherin-α family at the nuclear pore complex have been identified as targets for at least one of the viral nucleophilic determinants, the Gag matrix protein, it is currently established that Vpr governs nuclear import through a distinct pathway (Gallay *et al.*, 1996). It is conceivable that following interaction with targets at the nuclear periphery, the helical region identified in this study activates events that result in the effective nuclear import of the proviral DNA.

In this regard, we demonstrate that the central helical motif characterized in this study plays a key role in the nuclear translocation of Vpr protein itself. It is currently clear that the nuclear import of cytoplasmic proteins, in general, is a multi-step process

where proteins are first targeted to pore-complexes at the nuclear periphery, translocated through the complex, and subsequently released at the nuclear interior (Corbett and Silver, 1997; Nigg, 1997). This appears to be the case for the wild-type Vpr which is targeted to the nuclear periphery and subsequently translocated into the nuclear interior at an efficient pace as documented by its two predominant localization patterns in this study (Figures 2A and B). The results presented here also suggest that the integrity of the central helical motif itself is essential for the efficient targeting of the protein to the nuclear periphery as disrupting this secondary structure by a Proline substitution (R62P) results in the effective loss of nuclear targeting (Figure 4B). It is unlikely that this is a result of global misfolding of this small protein due to the Proline substitution as this mutant still retains its ability to efficiently mediate other biological functions such as G2-arrest and virion incorporation. However, the mutations that do not perturb the secondary structure, but affect the amphipathicity of hydrophobic helix, are efficiently targeted to the nuclear periphery but are radically affected in the subsequent translocation step into the nuclear interior. This best explains the highly localized ring-like accumulation of these mutant proteins around the nuclear periphery (Figure 4A). At the molecular level, it is conceivable that following efficient targeting of the protein to the nuclear periphery, this amphipathic interface interacts either directly or indirectly with components at the nuclear periphery, such as the nuclear pore complexes, triggering events that lead to efficient nuclear translocation. Further studies are clearly needed to understand what molecular determinants interact with the central hydrophobic helix of Vpr at the nuclear periphery and how perturbing this structure, as we identify in this study, results in the loss of the protein's biological activity in macrophages.

Materials and methods

Computer prediction and analysis

HIV-1 sequences were acquired through the National Institutes of Health, Bethesda, U.S.A (NIH) bioserver. Secondary structure predictions of the central hydrophobic domain was performed by the predict protein protocols at the European Molecular Biology Laboratory (EMBL) bioserver services using "predict protein" analysis. Hydrophobicity and hydrophilicity associated with the helical motif was analyzed using solvent accessibility analysis also provided by the EMBL Bioserver services.

Eukaryotic expression vectors and proviral constructs

The eukaryotic Vpr expression vectors were based on the previously described CMV promoter containing plasmids (Yao *et al.*, 1995b). The construction of the wild-type, ATG-, I63K, and LI68,70RK mutations was previously described (Yao *et al.*, 1995b). The additional mutations in the central hydrophobic domain for this study were constructed in the same plasmid backbone. The HxBRU wild-type provirus containing the intact Vpr gene was used as a template, and specific mutation containing oligonucleotides were used to amplify the Vpr sequences to be cloned into the expressor system using a two step PCR methodology. The sense mutagenic oligos used in the first step are as follows: V57L, 5'-TTG GGC AGG ACT CGA GGC CAT AAT AA-3'; R62P, 5'- AGC CAT AAT ACC CAT TCT GCA AC-3'; I63K 5'-GCC ATA ATA AGA AAG CTT CAA CAA CTG C-3'; Q65E 5'-ATA AGA ATT CTC GAG CAA CTG CTC T-3'. In the first step, short PCR fragments were generated with mutagenic oligos in the sense direction and the antisense oligo spanning the termination codon of the gene (5'-CAG GAG CTC AGT CTA GGA TCT ACT GGC-3') with an engineered Sac-1 site. These fragments were separated on an agarose gel, purified, and used as primers in the second PCR step where they were used as antisense primers in conjunction with a sense oligo spanning the methionine of Vpr (5'-ACT TCT AGA GGA TAG ATG GAA CAA GCC-3') and containing an Xba-1 site engineered prior to the ATG codon of the protein. Following generation of the mutations, the PCR fragments containing the mutations were digested with Xba-1 and Sac-1 site and cloned into the previously described SVCMV Vpr wt expressor which is driven by a CMV-IE promoter (Yao *et al.*, 1995b). DNA sequencing was used to confirm that the resulting positive clones did contain the designed mutation. Using an Apa-1 Sal-1 intermediate cassette, the mutations were transferred into an infectious proviral chimeric clone as previously described (Yao *et al.*, 1995b). Subsequently, the envelope expression of these proviruses was prevented for one-cycle replication by transferring Sal-1/BamH-1 fragments containing a mutated envelope initiation codon with an added frameshift at a Kpn-1 site as previously described in detail (Yao *et al.*, 1992). To analyze G2-arrest (see section on cell-cycle analysis below) during high-titer single-cycle infection, we pseudotyped these env-negative proviral clones above with the highly efficient expressor of VSV-G which has been previously described (Lodge *et al.*, 1997). For macrophage infections, the Sal-1 BamH-1 fragment (nucleotides 5785 to 8474) containing the macrophage tropic ADA envelope from NLX-ADA-SM was transferred into the HX-

BRU based Wild-type, ATG-, and R62P mutants described above. The construction of the well characterized NLX-ADA-SM proviral clone has been described earlier in detail (Westervelt et al., 1992).

Cell lines and antisera

The COS-7 cell line used in this study is an African green monkey kidney cell line transformed by an origin-defective mutant of Simian virus 40 and was maintained in Dulbecco modified Eagle Medium supplemented with 10% FCS. The Jurkat and the MT4 cells are of T-lymphoid origin and were propagated in RPMI-1640 medium containing 10% FCS. The 293T cells are of human embryonic kidney origin and were maintained in Dulbecco modified Eagle medium supplemented with 10%FCS. The antibiotics Penicillin-streptomycin were added to all cell culture media to a final concentration of 1%. The Vpr specific antisera used in this study for both immunofluorescent and immunoprecipitation detection of Vpr was raised in rabbits against recombinant Vpr protein and has been described in previous studies (Lavallée *et al.*, 1994). Other HIV-1 viral proteins were detected by a human serum (#162) derived from a HIV-1 seropositive individual. The secondary Fluorescein-labeled antibody raised in goat against the rabbit F(ab')2 was purchased from Gibco/BRL.

Radioimmunoprecipitation and virion incorporation assay

Virion incorporation assay was performed as previously described with slight modifications (Yao *et al.*, 1995b). Briefly, exponentially growing MT4 cells (5 X 10^6) were transfected using the DEAE-dextran method with either 10μg of wild-type provirus constructs or proviruses harboring mutations in the central hydrophobic domain. At 48 hours post transfection cells were metabolically labeled with 100μCi of [^{35}S]methionine per ml for 12 hours. Post labeling, virions were pelleted through a 20% sucrose gradient, lysed in buffer containing 10mM Tris-HCl (pH 7.4), 1mM EDTA, 100mM NaCl, 1% Triton, 0.2% phenylmethylsulfonyl fluoride and immunoprecipitated with a mixture of rabbit anti-Vpr and HIV-1 seropositive human serum, loaded on a 14% SDS gel and analyzed by autoradiography. A ratio system previously used to assess levels of virion association of mutant proteins relative to the

levels found in the cell using other virion proteins as standards was used (Yao *et al.*, 1995b).

Immunofluorescence and image analysis

Cos-7 cells grown on coverslips were transfected with the wild-type and mutant Vpr expression vectors. 48 hours post transfection, cells were washed in phosphate buffered saline (PBS) and fixed in acetone for 30 min at 4°C. Following fixation, the cells were processed for immunolabeling as previously described with slight modifications (Yao *et al.*, 1995b). For single labeling protocols, the cells were labeled with the first antibody diluted in PBS containing 2% Carnation instant skim milk powder (Nestlé) for 6 hours, washed repeatedly, and incubated with FITC-labeled goat anti-rabbit antibody for an hour. For concurrent DNA labeling with Propidium Iodide, cells were repeatedly washed after the acetone preparation and treated with DNase-free RNase (10μg/ml in PBS) for 30 minutes following which the cells were processed as above for indirect immunofluorescence methodology for Vpr detection. Following treatment with the second fluorescent antibody, the cells were washed and treated with 50 μg/ml Propidium Iodide in PBS for ten minutes, washed and mounted in nonquenching mounting medium. A minimum of 30 randomly chosen cells derived from three independent experiments were analyzed to objectively gauge the nuclear patterns associated with the mutations.

Confocal laser microscopy was performed on a Zeiss LSM 410 (Carl Zeiss, Germany) equipped with a Plan-APOCHROMAT 63X oil immersion objective and a Ar/Kr laser. The FITC images were obtained by scanning the cells with the 488 nm laser and filtering the emission with a 515-540 nm band-pass. For the Propidium Iodide images, the 568 nm laser was used in combination with a 590-610 nm band-pass filter. For each cell studied an image of the additive signal through its whole thickness was first digitized. Then the confocal serial sections were scanned.

Cell-cycle analysis

In order to monitor the ability of wild-type and mutant Vpr proteins to induce cell-cycle arrest we used a previously characterized technique that involves achieving

high titer infection of cells with isogenic viruses expressing either the wild-type or the mutant protein (Bartz *et al.*, 1996). Controls included uninfected mock cells or cells infected with a virus that did not express Vpr (ATG-mutant). Pseudotyped viruses expressing the VSV envelope glycoprotein were generated by cotransfection of the env-negative proviral constructs lacking HIV-1 envelope with a VSV envelope glycoprotein expressor construct in 293T cells (see constructs section above). Virus containing supernatant was collected at 48 to 72 hours post transfection, clarified, and concentrated by ultracentrifugation. Exponentially growing Jurkat T-cells were infected with high multiplicities to achieve 100% infection and the cells were harvested at 48hrs post infection, washed in PBS and fixed with 80% ethanol. Cells were treated with RNase A for 30 minutes and subsequently stained with 50µg/ml Propidium Iodide and analyzed by FACS.

Primary human macrophage culture and infection

Primary monocyte derived macrophages were purified from normal HIV-1 seronegative volunteers by double adherence method as previously described (Westervelt *et al.*, 1992). 2.5 X 10^6 macrophages were cultured in six well plates and maintained in RPMI-1640 media containing 10% FCS supplemented with 100 µg/ml of penicillin streptomycin and glutamine. Macrophage tropic virus stocks were prepared by transfecting COS-7 cells with 20 µg of ADA based proviruses (see expression vectors section above). Supernatants from transfected cultures were collected 72 hours post-transfection, clarified, and concentrated by centrifugation, and stored at -80^{o}C. Macrophages kept in culture for seven days were infected at low m.o.i (1.8 ng p24 as determined by ELISA) for eight hours and subsequently maintained in complete RPMI-1640 media. Viral replication was monitored by measuring p24 levels in the culture supernatants by ELISA every three to four days.

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Figure legends

Figure 1

Structure prediction and consensus of HIV-1 Vpr. Panel A: linear sequence of Vpr from HIV-1 isolate HXBRU. The 96 amino acids of the protein are marked in 10 residue intervals. The schematic immediately below the sequence depicts the previously identified N-terminal helix from residues 16 to 34 as well as the central helical domain identified in this study from residues 53 to 74. Panel B: Secondary structure prediction. The residues that were selected for mutagenesis are indicated by an arrow head. The abbreviations as provided by the EMBL server are as follows: AA: amino acid sequence. H=helix, E=extended (sheet), blank=other (loop). PHD: Profile network prediction HeiDelberg. Rel: Reliability index of prediction (0-9). prH: probability for assigning helix. prE: probability for assigning strand. prL: probability for assigning loop (the probabilities are scaled to the interval 0-9). SUB: a subset of the prediction, for all residues with an expected average accuracy > 82%. Solvent accessibility: 3st: relative solvent accessibility (acc) in 3 states: b = 0-9%, i = 9-36%, e = 36-100%. P_3: predicted relative accessibility in 3 states. 10st:relative accessibility in 10 states: = n corresponds to a relative acc. of n*n %. PHD acc: Profile network prediction HeiDelberg. Rel acc: Reliability index of prediction (0-9). SUB: a subset of the prediction, for all residues with an expected average correlation > 0.69. Note: for this subset "." means that no prediction is made for this residue, as the reliability is < 4. Panel C: Helical wheel representation of the predicted structure denoting the alpha-helical organization of the motif. The two headed arrow points to the extensive hydrophobic interface. Charged amino acids, are indicated in bold and cluster on one side of the helix forming a well delineated, albeit small, hydrophilic interface.

Figure 2

Laser sectioning analysis of the wild-type Vpr protein: Panel A: Serial laser sections through a representative cell that shows pattern A localization (primarily nucleoplasmic signal with no apparent peripheral ring like concentration in any of the sections through the cell). The cytoplasm becomes more obvious as the laser reaches the center of the cell in sections 6 and 7. Panel B: Serial laser sections through a representative cell expressing wild-type Vpr with a pattern B localization (again nuclear but with annular ring-like accumulation at least in some sections). The arrowheads point to the localized accumulation at nuclear periphery.

Figure 3

Localization of the wild-type and mutant Vpr proteins: This panel provides additive signals of the wild-type and mutant Vpr proteins tested in this study. Additive pictures capture and represent the Vpr specific FITC-signal emanating from the whole thickness of the cell. (A) wild-type (B) mutant V57L (C) mutant R62P (D) mutant I63K (E) mutant Q65E (F) mutant IL68/70RK. Note that the additive signal that shows some apparent nuclear signal in I63K panel (D) is due to signal emanating from the top of the nucleus which becomes clear in serial sections through the cell (refer to Figure 4A).

Figure 4

Laser sectioning analysis of second helix mutations: Panel A. Serial sections through a representative cell (depicted also in the Figure 3, panel D, as an additive image) expressing the Vpr mutant I63K. Sections through this flattened cell represented here are a subset of the actual sections made through the whole thickness of the cell. They are numbered from 1 at the base where the cell is adsorbed to the slide through 9 at the nuclear periphery. Due to strikingly high concentration of the signal as annular nuclear rings, planes that fall above section 9 showed no apparent signal at all and are not included in this illustration panel. Note that the signal in the cytoplasmic region is already barely visible by sections 7, 8, and 9, where only the nuclear "dome" is labeled with decreasing radius as one would expect for a curved surface. Panel B: Serial sections through a representative cell expressing the R62P Vpr mutant.

Figure 5

G2-arrest analysis of the wild-type and central hyrophobic mutants R62P and V57L: Panel A. Histogram representations of cell-cycle profile of Jurkat cells infected with pseudotyped HIV-1 virions packaged with the highly efficient VSV envelope glycoprotein at high multiplicity of infection. The mock infected control cells depict a normal cell-cycle histogram with a majority of cells in G0/G1. Note that cells infected with Vpr negative (R-) proviruses show a similar phenotype. However, cells infected with wild-type Vpr expressing viruses accumulate efficiently in the G2 phase of the cell-cycle. Panel B: ratios of G2M/G1 derived from histograms for the mock, Vpr-negative, wild-type, and two selected mutants. The G2-arrest associated with the mutant V57L that altered the steric interface of the central hyrophobic helix and the mutant R62P that disrupts the alpha-helical conformation of this motif are shown.

Figure 6

Aberrant DNA fragmentation in wild-type and R62P expressing cells. The left column, labeled as panels A, D, and G are representations of the Vpr-specific green (FITC) additive signals in the control, wild-type and R62P mutation expressing cells respectively. As expected, control cells did not show any Vpr specific labeling. Note the nucleoplasmic labeling in panel D for the wild-type protein and the exclusion from the nucleus in the R62P mutant (panel G). Panels B, E, and H are depictions of DNA-specific (red) Propidium Iodide signals for the control, wild-type and R62P mutations, respectively. Note the diffuse labeling of DNA in the control cell and the aberrant condensation reminiscent of apoptosis in the wild-type and R62P cultures. The images on the far right column labeled as panels C, F, and I depict superimposed images of the Vpr-specific (green) and DNA specific (red) signals in the same cells. Note that large quantities of Vpr are not needed to induce apoptotic changes as demonstrated by a positive cell expressing low levels of Vpr (arrowhead).

Figure 7

Virion incorporation of the wild-type and mutant Vpr proteins. Panel A: autoradiogram of a radioimmunoprecipitation used to analyze the ability of the wild-type and mutant Vpr proteins to associate with the virion. Lanes 1 to 7 show the cell-associated proteins, while lanes 8 to 14 show the protein profile from virions. The major viral proteins and Vpr are labeled and the molecular markers are denoted with a bold arrowhead. Panel B: Relative virion incorporation levels of the wild-type and Vpr mutants based on autoradiographic scanning is depicted in this graph (see materials and methods).

Figure 8

Replication kinetics of macrophage tropic viruses expressing either the wild-type, ATG-, or the R62P Vpr-mutants in primary human macrophage cultures. Macrophages were kept in culture for seven days following purification, infected at low input multiplicity (1.85ng of p24). The depicted graph is representative of three independent experiments where wild-type viruses resulted in high levels of virus production while the R62P and ATG- mutations showed drastically reduced supernatant p24 levels.

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Figure 1

Structure prediction and consensus of HIV-1 Vpr.

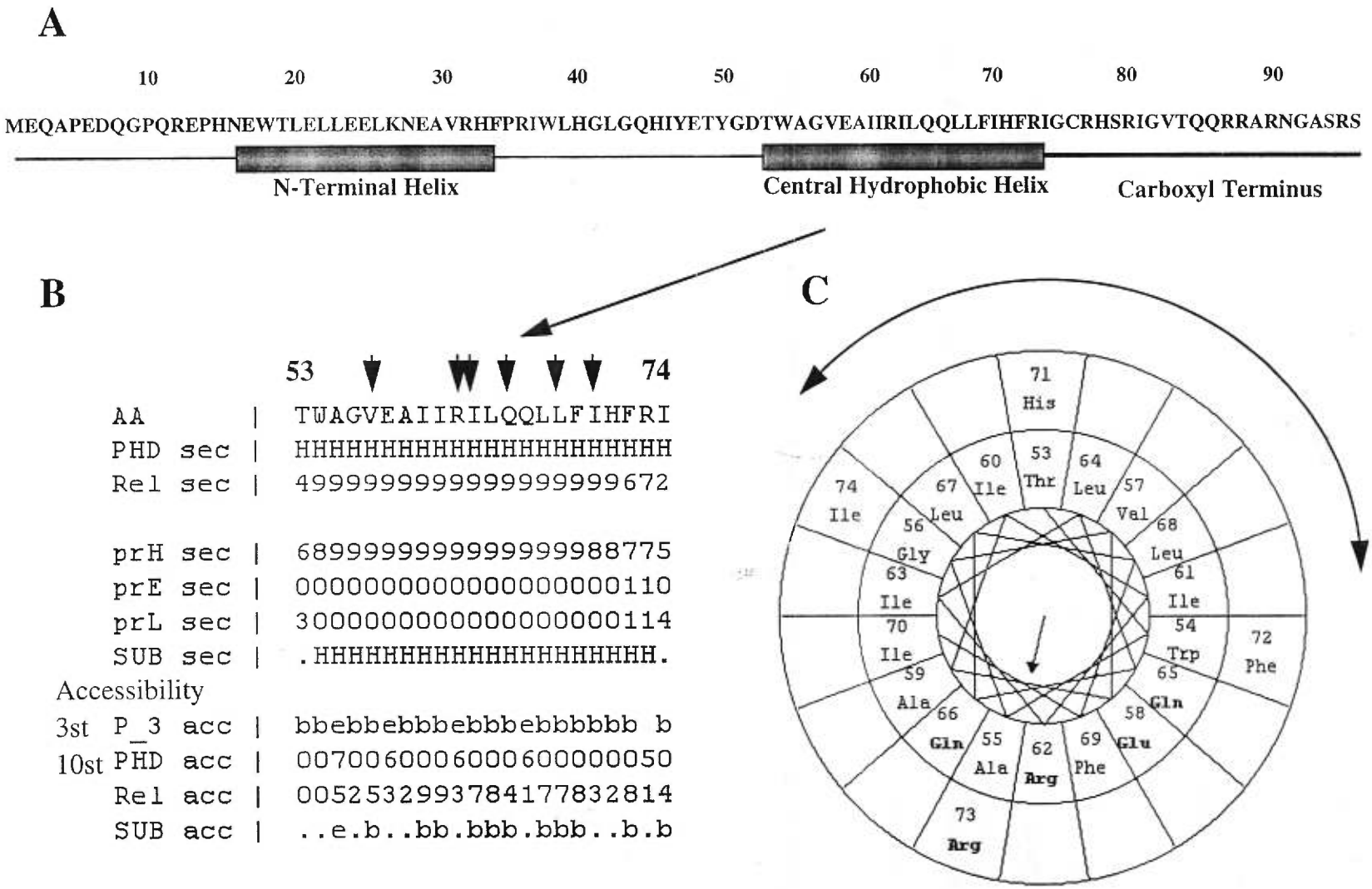
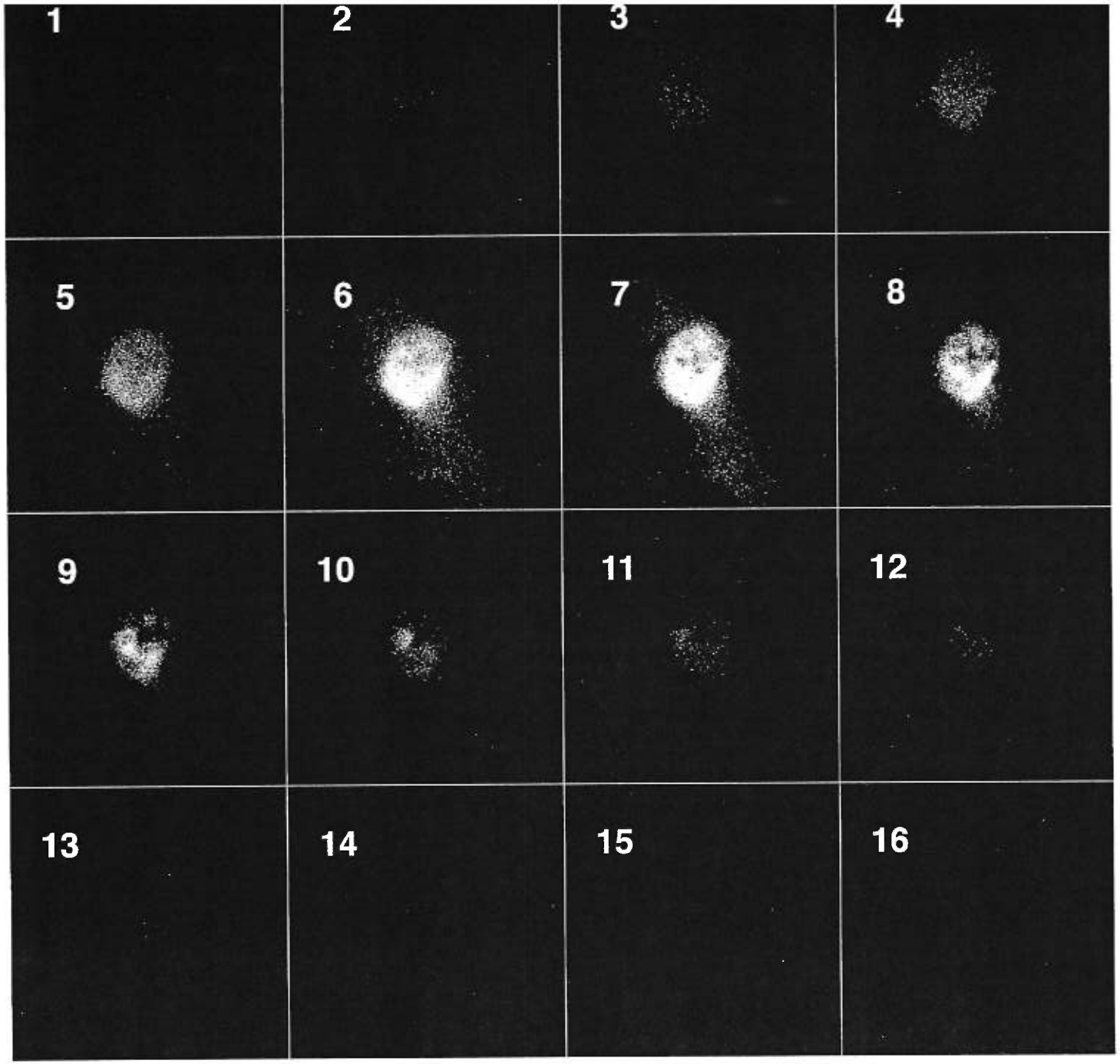

A
10
20
30
40
50
60
70
80
90
MEQAPEDQGPQREPHNEWTLELLEELKNEAVRHFPRIWLHGLGQHIYETYGDTWAGVEAIIRILQQLLFIHFRIGCRHSRIGVTQQRRARNGASRS
N-Terminal Helix
Central Hydrophobic Helix
Carboxyl Terminus
B
53
74
AA | TWAGVEAIIRILQQLLFIHFRI
PHD sec | HHHHHHHHHHHHHHHHHHHHH
Rel sec | 49999999999999999672
prH sec | 68999999999999988775
prE sec | 00000000000000000110
prL sec | 30000000000000000114
SUB sec | .HHHHHHHHHHHHHHHHHHH.
Accessibility
3st P_3 acc | bbebbebbbebbbebbbbbb b
10st PHD acc | 00700600060006000000050
Rel acc | 00525329937841778328l4
SUB acc | ..e.b..bb.bbb.bbb..b.b
C
71 His
53 Thr
64 Leu
57 Val
68 Leu
61 Ile
54 Trp
72 Phe
65 Gln
58 Glu
69 Phe
62 Arg
55 Ala
66 Gln
59 Ala
70 Ile
63 Ile
56 Gly
67 Leu
60 Ile
74 Ile
73 Arg

Figure 2

Laser sectioning analysis of the wild-type Vpr protein


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

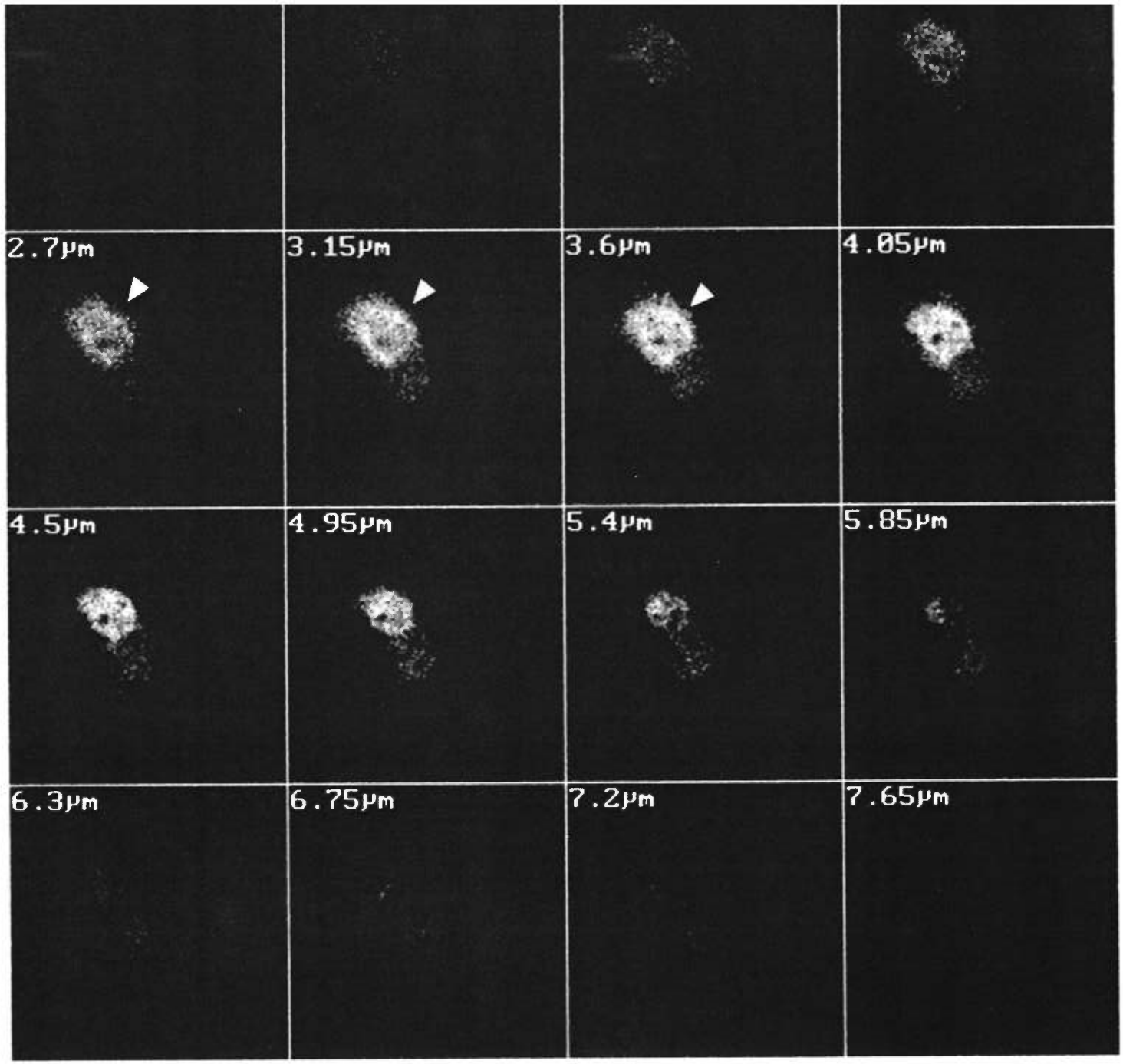

2.7µm
3.15µm
3.6µm
4.05µm
4.5µm
4.95µm
5.4µm
5.85µm
6.3µm
6.75µm
7.2µm
7.65µm

Figure 3

Localization of the wild-type and mutant Vpr proteins

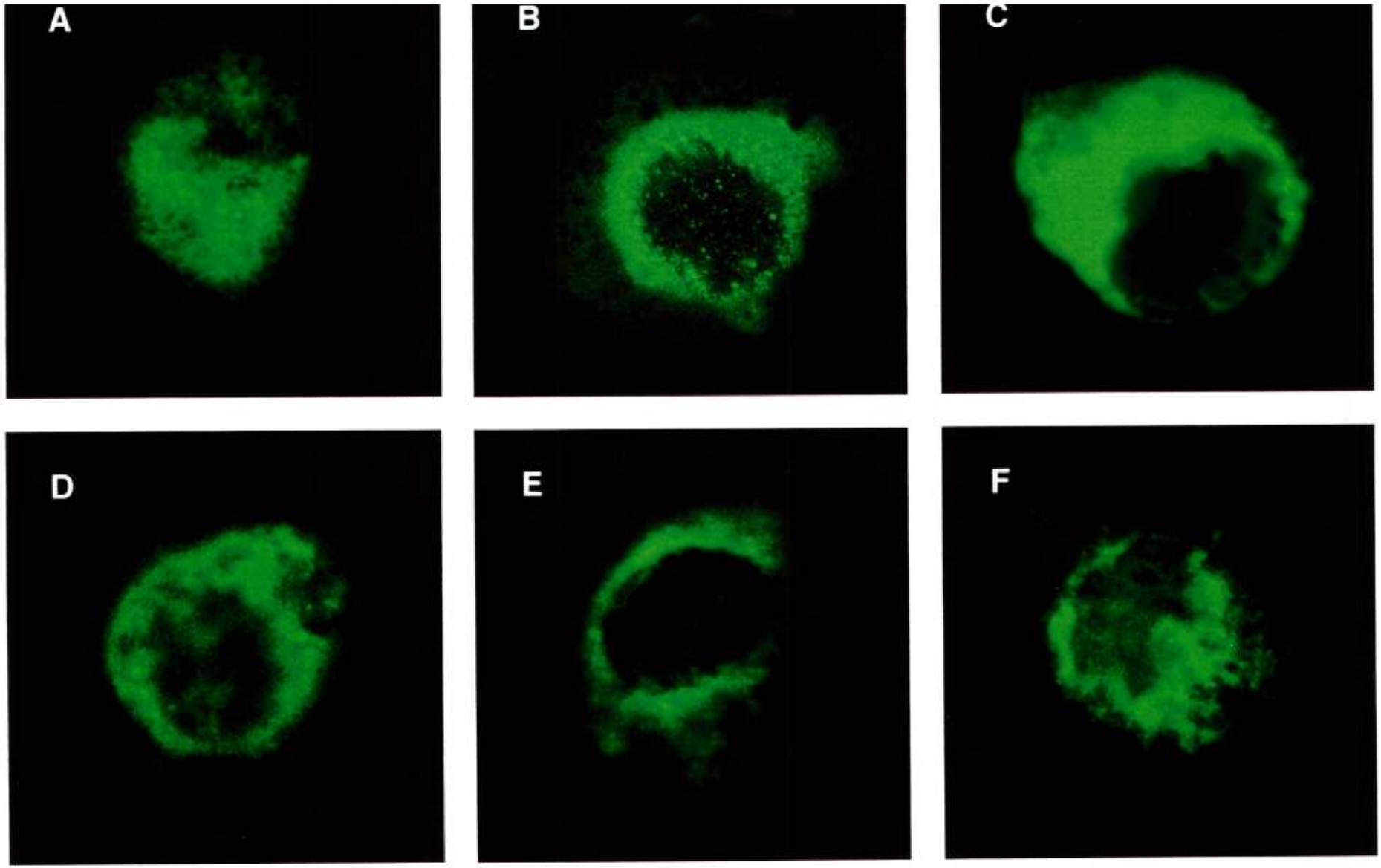
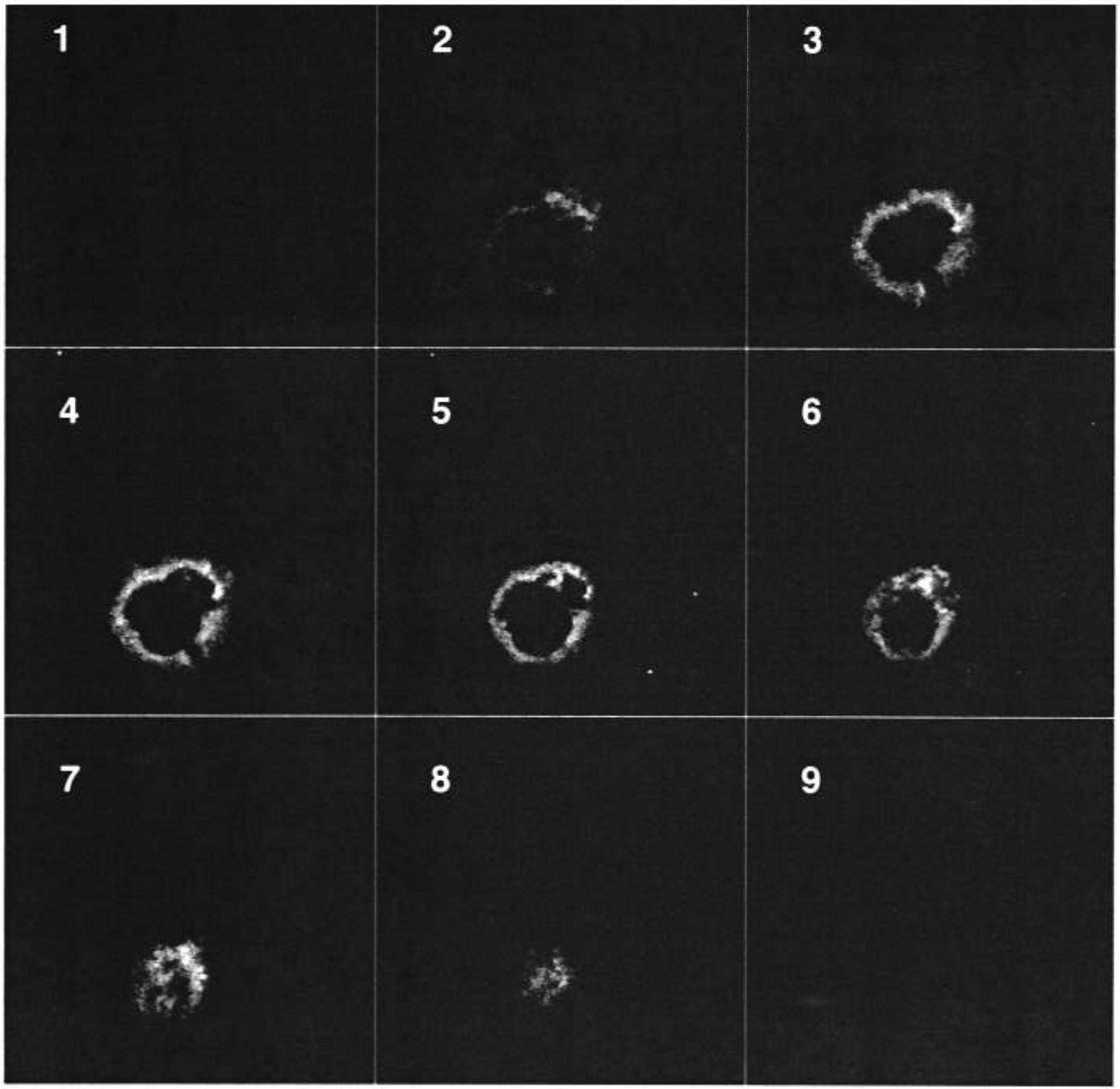

A
B
C
D
E
F

Figure 4

Laser sectioning analysis of second helix mutations


1
2
3
4
5
6
7
8
9

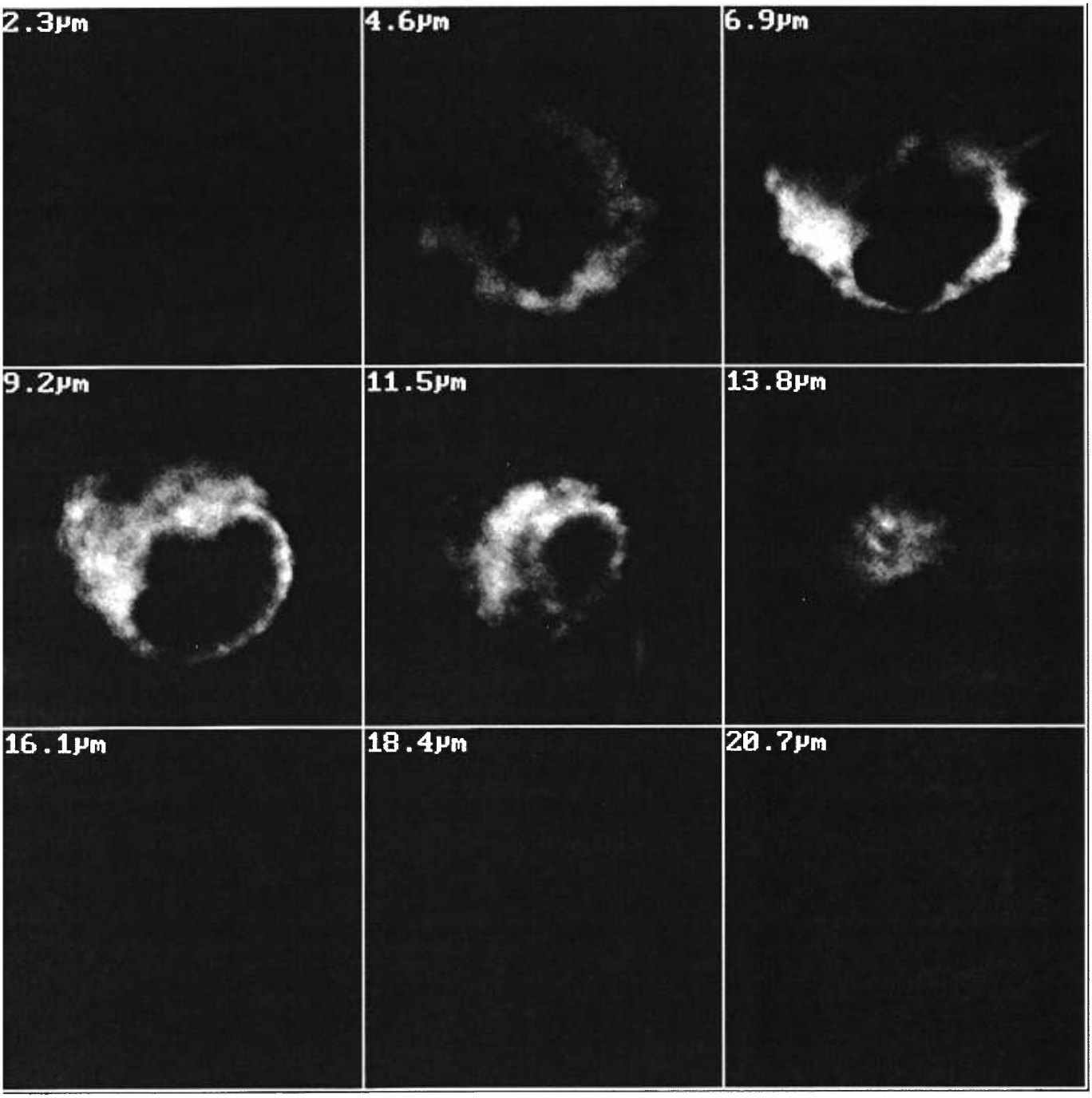
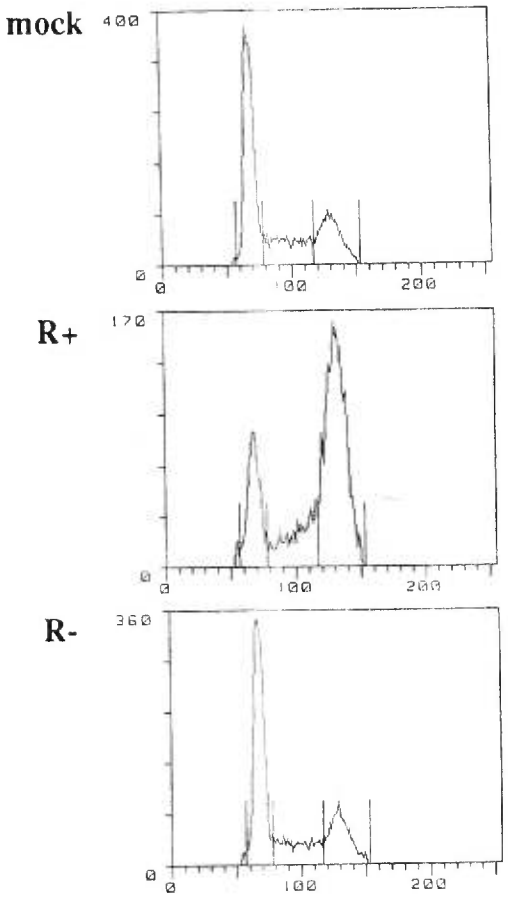

2.3μm
4.6μm
6.9μm
9.2μm
11.5μm
13.8μm
16.1μm
18.4μm
20.7μm

Figure 5

G2-arrest analysis of the wild-type and central hyrophobic mutants R62P and V57L

A.


mock
400
0
0
100
200
R+
170
0
0
100
200
R-
360
0
0
100
200

B.

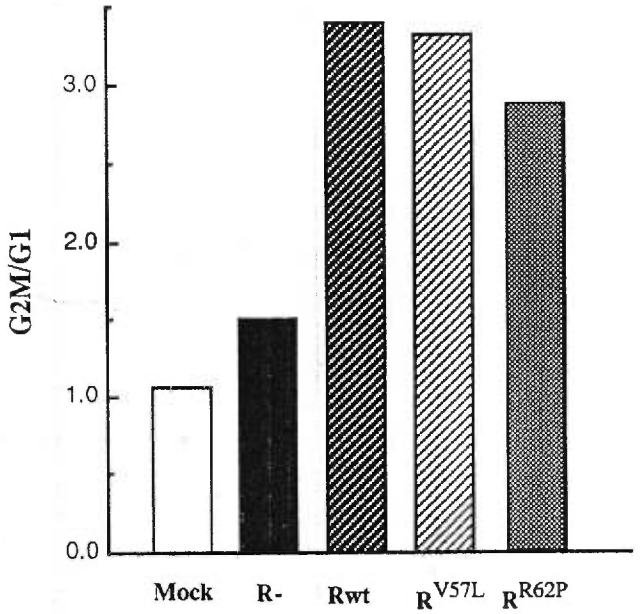

G2M/G1
3.0
2.0
1.0
0.0
Mock
R-
Rwt
R^V57L
R^R62P

Figure 6

Aberrant DNA fragmentation in wild-type and R62P expressing cells

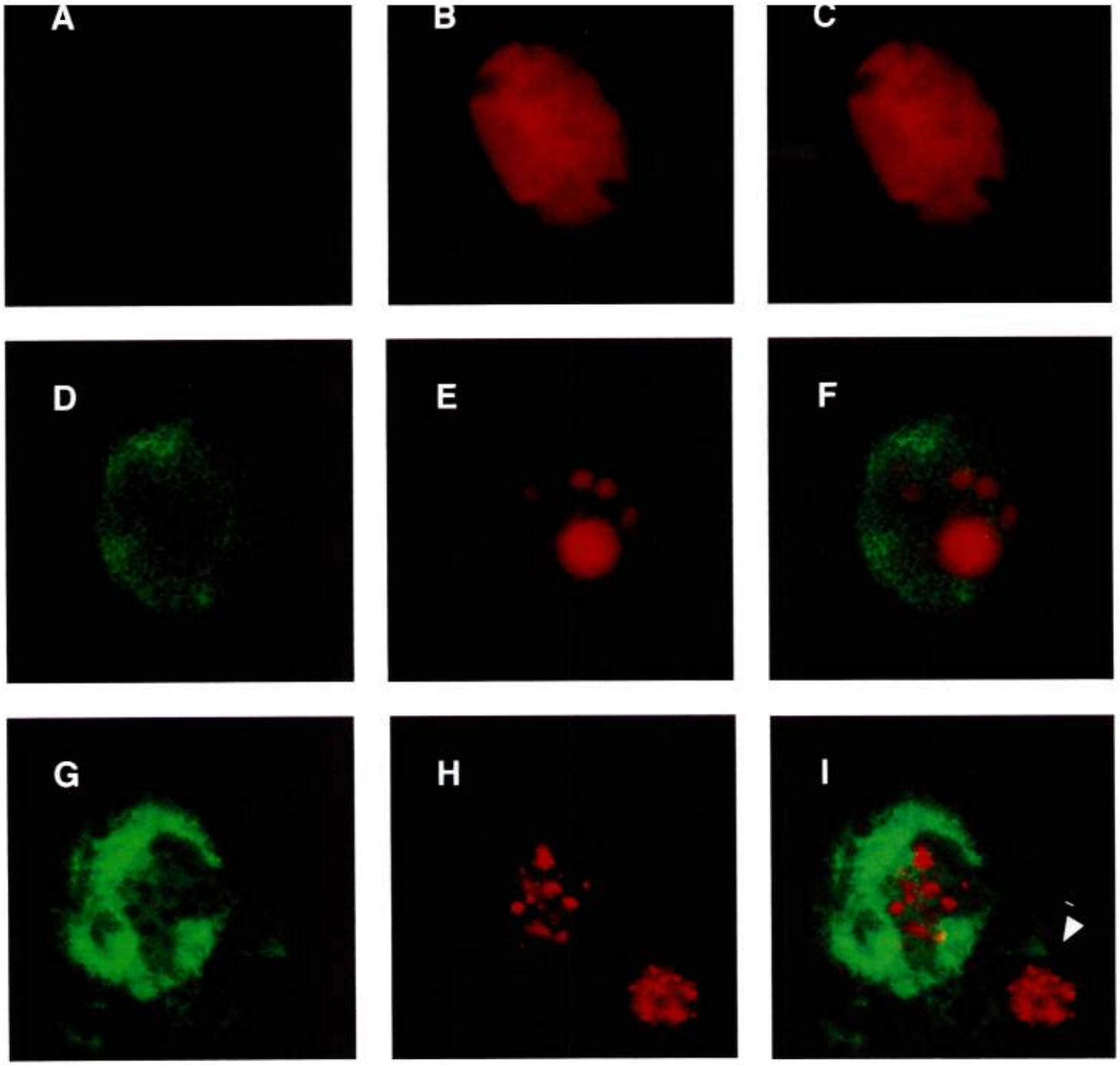
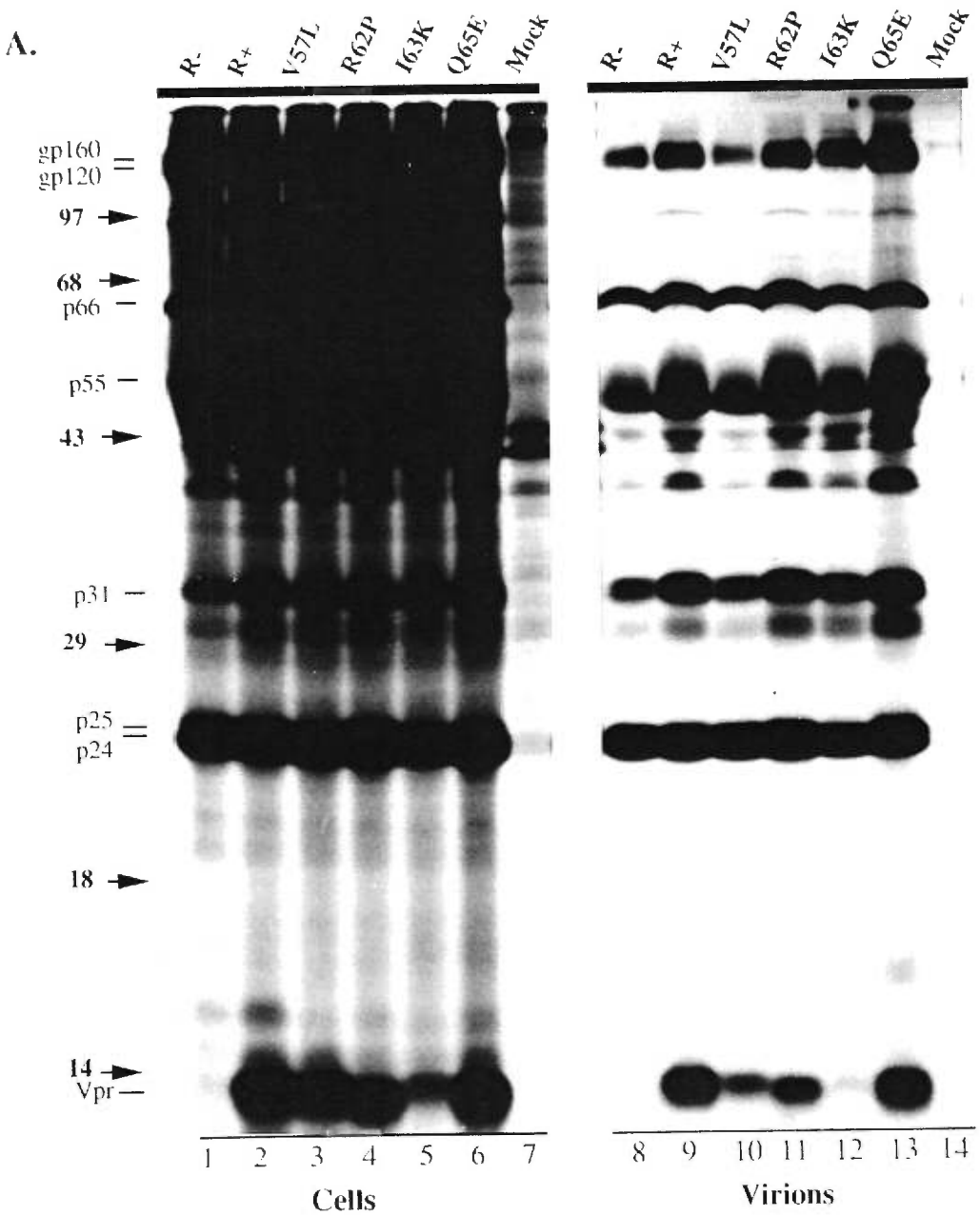

A
B
C
D
E
F
G
H
I

Figure 7

Virion incorporation of the wild-type and mutant Vpr proteins


A.
R-
R+
V57L
R62P
I63K
Q65E
Mock
gp160
gp120
97
68
p66
p55
43
p31
29
p25
p24
18
14
Vpr
1 2 3 4 5 6 7
Cells
8 9 10 11 12 13 14
Virions

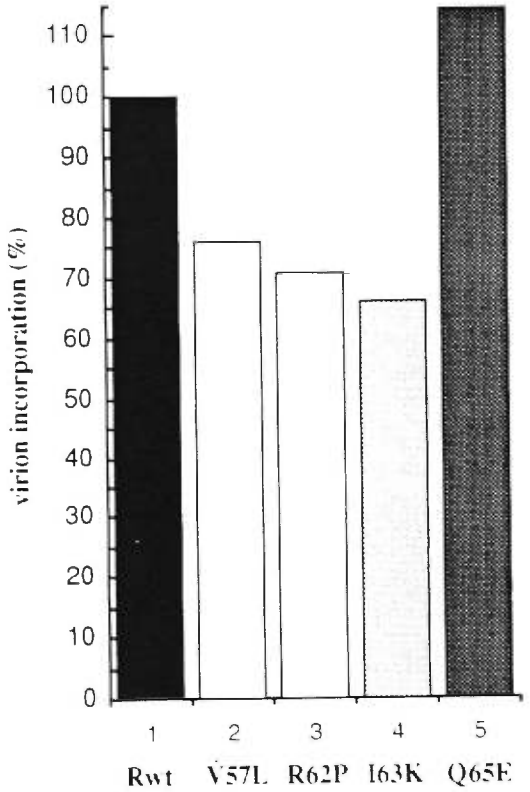
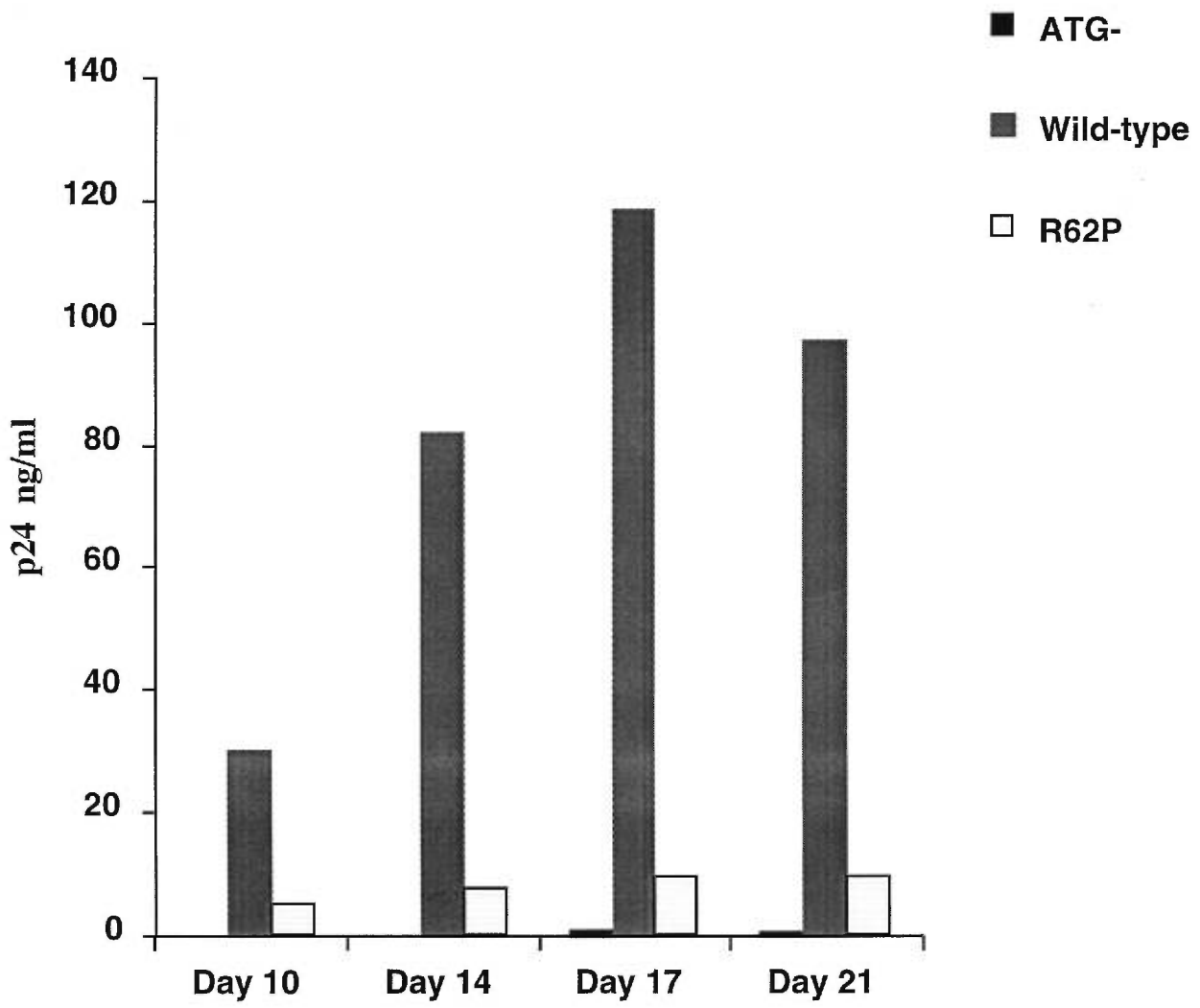

B.
virion incorporation (%)
110
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5
Rwt V57L R62P I63K Q65E

Figure 8

Replication kinetics of macrophage tropic viruses expressing either the wild-type, ATG-, or the R62P


ATG-
Wild-type
R62P
140
120
100
80
60
40
20
0
p24 ng/ml
Day 10
Day 14
Day 17
Day 21

Chapter 4

Human immunodeficiency virus type 1 Vpr is a positive regulator of viral transcription and infectivity in primary human macrophages.

Subbramanian R.A., A. Kessous-Elbaz, R. Lodge, J. Forget, X-J. Yao, D. Bergeron and E.A. Cohen. 1997. Submitted for publication. *J. Exp. Med.*

Human Immunodeficiency Virus Type 1 Vpr is a Positive Regulator of Viral Transcription and Infectivity in Primary Human Macrophages

Ramu A. Subbramanian[*], Allegria Kessous-Elbaz[‡], Robert Lodge[*], Janique Forget[*], Xiao-Jian Yao[*], Dominique Bergeron[*], and Eric A. Cohen[*]

§[*]Laboratory of Human Retrovirology

Department of Microbiology and Immunology

[‡]Department of Pathology

Faculty of Medicine

University of Montreal

C.P. 6128, Succursale Centre Ville, Montreal, Quebec, Canada H3C3J7

§Corresponding Author

Tel: (514)-343-5967

Fax: (514)-343-5995

Email: cohenea@ere.umontreal.ca

Abstract

It is currently well established that HIV-1 Vpr augments viral replication in primary human macrophages. In its virion-associated form, Vpr has been suggested to aid efficient translocation of the proviral DNA into the cell-nucleus. Though, Vpr growth arrests dividing T-cells, the relevance of this biological activity in non dividing macrophages is unclear. Here we use Vpr-mutants to demonstrate that the molecular determinants involved in G2-arresting T-cells are also involved in increasing viral transcription in macrophages, even though these cells are refractive to the diploid DNA status typical of G2-phase. Our results suggest that the two phenotypes, namely the nuclear localization and the G2-arrest activity of the protein, segregate functionally among the late and the early functions of Vpr. The nuclear localization property of Vpr correlates with its ability to effectively target the proviral DNA to the cell-nucleus early in the infection, while the G2-arrest phenotype correlates with its ability to activate viral transcription following establishment of an infection. These two functions may render Vpr's role essential and not accessory under infection conditions that closely mimic the *in vivo* situation, that is, primary cells being infected at low viral inputs.

Key Words: Vpr / Macrophage / G2-arrest / Nuclear localization / accessory proteins.

Introduction

The Vpr protein is one of the regulatory gene products encoded by the human immunodeficiency virus type 1 (HIV-1), the etiological agent of the Acquired Immune Deficiency Syndrome (AIDS) and is expressed late in the infection cycle (1, 2). All primate lentiviruses, including HIV-1 code for a Vpr-like product. However, some lentiviruses such as Human immunodeficiency virus type 2 (HIV-2) and most simian immunodeficiency viruses (SIV) encode not only for Vpr but also for a second protein named Vpx, which shares considerable sequence homology with Vpr (3, 4). Phenotypically, several studies demonstrate that viruses that encode for a Vpr protein replicate to much higher levels in macrophages than their Vpr-negative counterparts (5-9). However, exactly how the protein contributes to this effect is not clearly understood.

The fact that Vpr is packaged in the progeny virions at high copy numbers strongly suggests that the protein could play a role early in the infection (10). Experimental support for an early functional role comes from reports where Vpr contributed to the nuclear import of proviral DNA in non dividing cells such as macrophages (11). In addition, a late function is also suggested by experiments in which excluding the late *de novo* expression of Vpr effectively abolished the augmented replication even though the protein was made available in the virion early in the infection (7). However, the ability of Vpr to target the proviral DNA to the nuclear compartment early in the infection was not monitored in this study, and hence, the authors could not formally preclude an additional role for the protein early in the infection (7).

Hence, several issues remain unclear about our understanding of Vpr's ability to increase viral replication in macrophages. Does Vpr play a role late in the viral life cycle that can be segregated from its function early in the infection, and if so, at what replication step does this late function manifest itself? Given the fact that other virion-associated proteins such as Gag matrix p17 (11, 12) and viral integrase (13) also appear to facilitate proviral nuclear import, is Vpr's nuclear targeting function essential? If not, what is the relevance of evolutionary conservation of multiple nucleophilic determinants? If so, why has it not been detected in earlier studies? Finally, is Vpr's ability to growth-arrest proliferating (14-19) cells, an effect confined to cycling T-cells or does it have functional correlates in non dividing macrophages which are refractive to cell-cycle changes?

In this study we attempt to address these questions by systematically monitoring the relative contribution by HIV-1 Vpr to the early and late viral replication events in primary human macrophage cultures. We monitor the levels of proviral DNA targeted to the cell-nucleus in the presence and absence of Vpr, as well as the ensuing virus production and the pattern of RNA expression at the single cell level using *in situ* analysis. We also monitor the replication of Vpr-mutants that lack either the protein's ability to localize to the nucleus, or its ability to growth-arrest T-cells in an effort to gauge the relevance of these phenotypes during macrophage infection.

Materials and Methods

Isolation and Culture of Peripheral Blood Derived Macrophages.

Blood Units from HIV seronegative volunteers were obtained from the Hôpital Maisonneuve Rosemont, Montreal. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque centrifugation essentially as recommended by the manufacturer (Pharmacia), washed thoroughly to remove contaminating platelets, and cryopreserved. An enriched population of monocyte derived macrophages (MDM) were first isolated by adherence to plastic as previously described (5). Further purification was achieved by negative selection where the enriched macrophage population was incubated with a cocktail of monoclonal antibodies against T- and B-cells followed by subsequent depletion using a secondary antibody conjugated to magnetic beads (Dynal) essentially as described earlier (20). Purified macrophages were plated in six well plates at a concentration of 2.5 X 10^6 cells per well and maintained in endotoxin-free RPMI 1640 media (Gibco/BRL) containing 10% FCS, L-glutamine, penicillin (100 U/µl) streptomycin (100 U/µl), and Gentamycin (10 µg/ml). The resulting adherent macrophages were >93 % positive by nonspecific esterase test (Sigma, St.-Louis Missouri).

Proviral Constructs, Infections, and Replication Kinetics.

The previously well characterized isogenic HxBRU based clones (21) were rendered macrophage-tropic by transferring the Sal-1/BamH-1 fragment from another well characterized macrophage-tropic construct ADA (NLHXADA-SM) kindly provided by Dr. Lee Ratner, University of Washington, School of Medicine (9). The construction of the HxBRU parental clones that code for the wild-type Vpr or the Vpr-negative ATG-mutant (where the Vpr ATG-initiation codon has been mutated to prevent protein translation) have been previously described (21). The R62P and R80A were subcloned in this background using a two step polymerase chain reaction based method essentially as described earlier (21).

Viral stocks were generated in Cos-7 cells plated at 8 X 10^5 cells per 100 mm plates, cultured overnight, and transfected with proviral constructs by calcium phosphate method. Supernatants were collected 72 hours post transfection, concentrated by ultracentrifugation at 35,000 RPM for 2.5 hours, filtered through 0.45 µm filters, treated with DNase (25 U/ml) to remove contaminating plasmid DNA, and stored at

-80°C. In vitro differentiated macrophages that have been in culture for seven days were adsorbed with virus for eight hours at 37°C and were subsequently washed thoroughly to remove free virus and maintained in RPMI-1640 complete medium containing 100 nM Palinavir (BioMega-Boehringer Ingelheim), a previously well characterized protease inhibitor (22). Half the medium was changed every three to four days with fresh media supplemented with 100 nM of Palinavir. Virus production in cultures was determined by measuring supernatant p24 levels by standard ELISA techniques. Cell cycle analysis of control and infected cultures was performed using Propidium Iodide staining essentially as described earlier (15).

In Situ Hybridization

For *in situ* hybridization purposes, purified macrophages were maintained following purification for seven days in Lab-Tech slides (Nunc™) and subsequently infected at varying viral inputs under conditions similar to the ones used for one-step replication studies in the presence of 100nM Palinavir. Hybridization procedures were performed using essentially standard protocols (23, 24) utilizing an antisense probe described earlier in detail (25). This probe contains a 92 bp sequence complementary to the Sty-1 (nucleotide 7591) to Hind-III (nucleotide 7683) fragment overlapping the HxBc2 envelope sequences found in the ADA chimeric clones (9). Thus the hybridization procedure identified both full-length and singly spliced mRNAs both of which contain the target envelope sequences. Based on earlier optimization procedures, RNA pattern was determined at 14 days post infection as all donors tested clearly showed the Vpr-mediated phenotype of augmented replication by this time.

PCR Analysis and Southern Blotting.

Total DNA lysates were prepared to monitor the efficiency of reverse transcription at different time points early in the infection as described earlier (26). Alternatively, proviral levels found in the nuclear compartment were monitored also as previously described (26). PCR analysis to detect HIV-1 proviral products was performed using the following primers in the Gag sequence: sense oligonucleotide 5' ATA-ATC-CAC-CTA-TCC-CAG-TAG-GAG-AAA-T 3', and antisense oligonucleotide 5' TTT-GGT-CCT-TGT-CTT-ATG-TCC-AGA-ATG-C 3'. These primers amplified a fragment of 114 bp corresponding to the sequence 1090 to 1204 in the HxBRU strain. Alternatively, the cellular β2-adrenergic receptor (β2-AR) gene sequences were detected using the following primers: Sense 5' TAG-GCC-TTC-AAA-GAA-GAC-CTC-C 3',

and antisense 5' CGT-CTA-CTC-CAG-GGT-CTT-TCA-G 3'. PCR products from this amplification generated a 399 bp fragment that corresponded to the sequence 1465 to 1821 of the human β2-AR gene. Amplification was performed using *Taq* DNA polymerase (Perkin Elmer) for 30 cycles of 1 minute at 94°C, 2 minutes at 50°C and 3 minutes at 72°C. [α^{32}P]-dATP labeled probes capable of recognizing the PCR products were generated as follows: HIV-1 Gag probe was generated by nick translating with [α^{32}P]-dATP (3000 Ci/mmol, ICN Radiochemicals) the 591 base pair Apa-1 to Pst-1 digestion fragment corresponding to positions 964 to 1555 in the HxBc2 provirus sequence. β2-AR probe was generated by similarly nick translating a fragment corresponding to the sequence 1465 to 1821 from a well characterized β2-AR eukaryotic expressor construct (27).

Results

Correlation of the G2-Arrest and Nuclear Localization Phenotypes of Vpr with its Ability to Establish a Productive Infection in Macrophages.

To explore what role, if any, Vpr-mediated G2-arrest and nuclear localization played during viral replication in non dividing target cells such as macrophages, we compared the replication of a virus that coded for a wild-type Vpr, or no Vpr at all (ATG-mutant), to that of viruses coding for mutations in the gene that selectively affected either the G2-arrest or the nuclear localization activity of the protein. For this purpose, the macrophage-tropic envelope region from the well characterized NLHX-ADA-SM construct (9) was transferred into HIV-1 constructs that coded either for the wild-type Vpr or for a mutated version of the protein (21). The two mutations used in this study to gauge how the G2-arrest and nuclear localization phenotypes of Vpr contribute to HIV-1 replication in macrophages were chosen based on previously published reports which clearly separate these functions at the structural level (28, 29).The R62P and R80A proviruses expressed, respectively, a Vpr-mutant which substituted the Arginine residue at position 62 with a Proline or the Arginine residue at position 80 with an Alanine. The Proline substitution mutant, R62P, retained its ability to G2-arrest cells but lost its ability to localize to the nucleus as it disrupted a helical domain shown by earlier studies to affect the nuclear localization phenotype specifically (29-32). The R80A mutant was capable of localizing to the nucleus but was impaired in its ability to growth-arrest T-cells, also as previously shown (28). These observations are in agreement with a number of earlier functional studies that have characterized central helical and C-terminal domains of Vpr (21, 28, 29) and are summarized in Table 1.

Whether or not Vpr is dispensable for viral replication in macrophages has been controversial as there is limited consensus on the extent to which the virus is impaired in the absence of the protein. Reports from independent groups have documented anywhere from two fold to greater than 100 fold impairment (5, 7, 9). As some investigators have suggested that the viral input may contribute to the *in vitro* replication kinetics of viruses mutated in another nucleophilic determinant, matrix p17 (33), as well as viruses mutated in the accessory protein, Nef (34, 35), we tested if the input multiplicity can compensate for the Vpr mediated augmentation of viral replication. To address this issue, we infected purified macrophages kept in culture for seven days with the wild-type or Vpr-mutant viruses at either a high input titer (50 ng) or one third serial

dilutions thereof (16.7 ng, 5.6 ng, 1.85 ng, 619 pg). One step replication was ensured by maintaining the infected cultures in the presence of the potent protease inhibitor Palinavir (Boehringer Ingelheim), which effectively prevented multiple rounds of infection from newly generated viruses in this system (data not shown). We have previously shown that treatment with this inhibitor results in the release of noninfectious particles into the supernatant due to a block at the virion-maturation step (22). This culture system allowed us to look at a one step viral replication without the complication of progression of viral infection through the culture.

Our data suggests that at low viral inputs, both the ability of the protein to localize to the nucleus and its ability to G2-arrest dividing cells appeared to be essential for increased viral replication in macrophages. The wild-type protein capable of both activities established a readily apparent infection even at the lowest multiplicity tested (Figure 1). However, The R62P mutant, which lacks the nuclear localization activity and the R80A mutant which lacks the G2-arrest phenotype (Table 1) are both impaired at low viral inputs (Figure 1). The ATG-mutant, which lacks both these biological activities, is clearly impaired at the low multiplicities tested (Figure 1).

However, at high viral inputs, the requirement for the nuclear localization is no longer essential for augmented replication, while the G2-arrest ability is still required. Note that the R80A mutant which lacks the G2-arrest activity shows impairment of replication at the high viral inputs even though it is capable of localizing to the nucleus while the R62P mutant that possesses the G2-arrest activity is rescued even though it fails to localize to the nucleus (Figure 1).

Ability of the Wild-Type and Mutant Vpr Proteins to Target Proviral DNA to the Nuclear Compartment.

We sought to understand if either the nuclear localization or the G2-arrest activity associated with Vpr affected the protein's ability to target the proviral DNA to the nuclear compartment. To formally address this issue, we used a semi quantitative PCR approach to monitor the total levels of HIV-1 proviral generation and the levels of provirus found in the nuclear compartment at various time points following addition of the initial viral inoculum essentially as described earlier (26). Single step viral replication conditions identical to the ones used to monitor productive infection by p24 were employed for this purpose. The total proviral synthesis was approximately the same both in the presence or absence of Vpr indicating that neither viral entry nor reverse

transcription was radically affected by the protein (data not shown) an observation which is in agreement with previous reports (11, 12). However, absence of Vpr drastically reduced its ability to target the synthesized proviral DNA subsequently to the nuclear compartment and this effect, interestingly, only became apparent at low viral inputs (Figure 2, Panel A and Panel B 1.85 and 0.619 ng lanes). It is also clear that the drastic impairment in nuclear targeting is not due to a temporal delay in transport as nuclear proviral levels remain constant after 72 hours (Figure 2, Panel A). However, at high viral inputs the PCR data clearly demonstrates (Figure 2, Panel B) that the proviral import function proceeds efficiently in spite of the absence of Vpr (ATG-), or its inability to localize to the nucleus (R62P). This is likely due to complementation of the nuclear import function at high viral inputs by the viral integrase protein (13) and the Gag matrix p17 product (11, 12, 36). Note however, that at lower viral inputs Vpr was essential for efficient nuclear targeting of the provirus regardless of the presence of these complementary viral nucleophilic factors (Figure 2B, compare 1.85 ng lanes). Also, note that at lower viral inputs, inability of Vpr to localize to the nucleus impairs its ability to target the proviral DNA to the nuclear compartment (compare 1.85 ng lanes of R62P to that of wild-type).

However, an additional contribution to the augmented replication is provided by the protein's G2-arrest ability, as similar nuclear levels of proviral DNA present at higher viral inputs (Figure 2 compare 50ng lanes), still lead to a non productive infection in the R80A and ATG- cultures (Figure 1). This clearly suggests that access to the nucleus by itself is not sufficient to ensure productive infection, and that G2-arrest ability is essential to induce productive infection after achieving efficient proviral nuclear targeting. This conclusion is underscored by the productive infection of R62P mutation once efficient targeting of its proviral DNA is achieved by the use of high viral titers (Figure 1).

RNA Expression Pattern Among Cultures Infected with Wild-Type and Vpr-Mutant Viruses.

Though analysis of replication kinetics by monitoring supernatant p24 levels allowed us to gauge productive infection, this did not clarify if the highly reduced viral production apparent in some cultures (Figure 1, ATG- and R80A) is due to a reduction in the number of cells which are infected or, alternatively, due to the same number of infected cells producing lower amounts of viruses per cell. For example, it is conceivable that a sub-population of macrophages are susceptible to HIV-1 infection in

the absence of Vpr, while others aren't. This is a possibility that needs to be considered as it is clear that blood derived macrophages are a heterogeneous population of cells. Also, it is conceivable that infection itself is established in a small fraction of proliferating cells as previously suggested (37). Hence, in theory, the saturation seen in the p24 assay (Figure 1) may result from saturation of this sub-population, after which, increasing the viral input results in no further infection as there are no further susceptible cells.

To explore the issue we performed *in situ* hybridization in lab-tech slides under conditions identical to the ones used to monitor viral replication by p24 and proviral DNA targeting to the nucleus. Hybridization procedures were performed using essentially standard protocols (23, 24) utilizing an antisense probe described earlier in detail (25). This probe identified both full length and singly spliced mRNAs in HIV-1 infected primary cells (20). We chose to look at two input titers in this experiment, one at the higher saturating level (50 ng) and another at the lower end (1.85 ng).

Note that the *in situ* evidence clearly suggests that Vpr upregulates transcription in these non dividing cells, an effect linked to its ability to induce G2-arrest: mutants that lack the Vpr-G2-arrest capability (ATG- and R80A) clearly show drastic reduction in productively infected cells, even though the total number of infected cells were similar (in the range of 90%) at high viral inputs (Table 2). Interestingly, as exemplified by the R62P mutation at low viral inputs, the lack of nuclear targeting takes precedence over the ability to increase transcription as lack of proviral nuclear targeting impedes the establishment of infection (Figure 2B). However, the importance of this late transcriptional role is underscored by the lack of productive infection in the R80A mutant where close to wild type levels of nuclear import occurs. Also, the Vpr-negative ATG-mutant still fails to show productive infection (Figure 1 and Table 2) even after effective targeting of its proviral DNA to the nuclear compartment at high viral inputs (50 ng lane Figure 2B).

Also, both the wild-type and R62P cultures (both of which growth-arrest proliferating cells in the G2-phase), as expected, clearly failed to change the DNA profile of these terminally differentiated macrophages. Both the uninfected and infected cultures showed less than 1% of the cells in the G2-phase of the cell-cycle (Figure 4) even though in the range of 90% of the cells were shown to be infected (Table 2). Hence, the observed correlation between G2-arrest activity and the increased replication

in macrophages is likely due to biochemical events rather than actual DNA duplication that characterize the G2-arrest status in proliferating T-cells.

Discussion

Current consensus suggests HIV-1 Vpr mediates two biological activities in target cells. One activity has been described for non dividing and quiescent cells, such as macrophages, where Vpr targets the HIV-1 proviral DNA to the cell's nucleus even in the absence of mitosis (38). The second function has been described in T-cells, where Vpr induced growth arrest in the G2-phase of the cell cycle. Most prototypic retroviruses fail to infect non dividing cells, as mitosis, and the nuclear membrane disintegration that occurs during cell-division, is a prerequisite to their gaining access to the cell-nucleus. However, Vpr, and at least two other HIV-1 virion proteins, matrix p17 (11, 12, 36) and integrase (13), have been known to ensure effective targeting of proviral DNA to the nuclear compartment. This leaves open the question why HIV-1 codes for apparently redundant nuclear targeting determinants.

The data presented here suggests that Vpr's contribution to proviral nuclear targeting is an essential rather than an accessory function under conditions that closely mimic *in vivo* situations, that is, primary cells being infected at low viral inputs. At low viral inputs, lack of Vpr results in the lack of infection, a function not mitigated by the presence of other nucleophilic determinants such as Gag matrix p17 or viral integrase (11-13, 36, 39). This also provides support to the evolutionary conservation of multiple, and apparently redundant, nucleophilic determinants in HIV. It is conceivable that optimal nuclear targeting occurs due to interdependent nature of these multiple determinants. This may be due to the multivalent nature of the interaction of the preintegration complex with cellular targets. We assume lack of even one such valancy, could reduce the chance of effective targeting, especially under restrictive conditions such as low viral inputs. These results underscore the importance of using low multiplicity *in vitro* studies and provides support to previous suggestions that higher m.o.i may mask certain biological activities *in vitro* (33).

Mechanistically, data presented here directly links the ability of Vpr to localize to the nucleus with that of its ability to target the provirus to the cellular genome at low multiplicities. This is illustrated by the notable reduction in proviral targeting at low viral inputs associated with mutants which either fail to code for a Vpr product (ATG-), or alternatively, code for a Vpr protein (R62P) incapable of reaching the nucleus (Figure 2B). The R80A mutant that is capable of reaching the nucleus, performs the proviral targeting function at levels comparable to its nuclear localization abilities (Table 1).

In this study we also provide direct evidence that suggests HIV-1 Vpr upregulates transcription in infected macrophages. This provides formal proof for a late function for this protein independent of its nuclear targeting function. Note that at high viral inputs, where a vast majority of cells are infected (>90%) in both the wild-type and Vpr-negative (ATG-) cutures, the wild-type Vpr expressing viruses generate higher levels of HIV-1 mRNA than the ATG-mutant. We also demonstrate that the G2-arrest activity of Vpr, first identified in proliferating T-cells, correlates with this increased transcriptional activity in macrophages. As macrophages are refractive to actual proliferation, it is conceivable that Vpr induces biochemical changes in macrophages, that in the context of the dividing cells leads to cell cycle arrest. Clearly, further studies are needed to understand the changes brought about by Vpr in the macrophage's cellular milieu.

Our results demonstrating a transcriptional role for Vpr in a relevant target cell provides biological relevance to a number of biochemical observations made earlier. We have previously shown that Vpr can increase reporter gene activity from variety of viral promoters (10). In fact, extracellular Vpr has been shown to induce latently infected cells into productive expression, again, suggesting a role for the protein following integration (40, 41). It has also been shown that preventing the late expression of Vpr by using antisense approaches drastically reduces viral replication (6). It is interesting in this regard that a recent report suggests that Vpr can modulate the NFkB pathway by upregulating IκB transcription (42).

Hence, there appears to be a clearly identifiable functional contribution to both early and late viral replication events by HIV-1 Vpr. However, in *in vitro* experiments, it may not be possible to demonstrate early and the late functions in exclusion of each other except by using specific mutants, or physiologically relevant low viral inputs. It is conceivable, that similar mutants favoring one or the other function may be favored in some stages of the disease *in vivo*. For instance, *in vitro* studies show that mutating the C-terminal region primarily impairs the G2-arrest function of the protein (28, 29) and in this study we correlate this activity with increased viral replication. In this regard, it is interesting to note that such C-terminal mutations are associated with low viral loads and lack of disease progression *in vivo* (43). Furthermore, the late and the early functions of this protein are also separated in the HIV-2 and SIV systems where the two functions segregate among two HIV-1 Vpr homologues, namely Vpx and Vpr-2 (44).

Some of the regulatory products coded for by HIV-1 have been deemed dispensable or "accessory" based on the fact that viral replication *in vitro*, albeit attenuated, still occurs even in their absence (45). However, several lines of evidence suggest that accessory genes are not likely to be dispensable *in vivo*. The ability to code for these genes is maintained in distant lentiviral relatives and specifically within the HIV-1 family of viruses. The open reading frames are invariably retained in all HIV-1 clades and notable conservation of the gene sequences is a common feature. It is particularly difficult to conciliate such conservation of non essential genes in a highly adaptable system such as HIV-1. In this context, it is interesting to note that some experiments have documented repair or recombinational mechanisms that reestablish the expression of Vpr protein *in vivo*, suggesting a functional need for this protein during natural infection (46, 47). The potential *in vivo* relevance of accessory proteins such as Vpr is also suggested by some *in vitro* experiments in which the inability to code for these proteins appears to affect HIV-1 replication acutely when primary rather than transformed cells are used as targets (reviewed in reference 45). This observation again suggests experimental conditions more representative of *in vivo* milieu may be necessary to truly assess the functional relevance of these genes. Our results suggest that HIV-1 Vpr, one such accessory protein, plays an essential and not a dispensable role in the infection of non dividing cells under conditions that closely mimic *in vivo* situations, that is, primary cells being infected at low viral inputs.

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Table 1

Nuclear Localization and G2-Arrest Phenotypes of Various Vpr Genes.

Vpr gene	Wild-Type	ATG-	R62P	R80A
G2-Arrest Ability	Positive	Negative	Positive	Negative
Subcellular localization	Nuclear	N/A*	Cytoplasmic	Nuclear

Legend: Summary of G2 arrest and nuclear localization phenotypes associated with the wild-type and mutant Vpr expressing viruses. * The ATG- mutant involved elimination of the Vpr initiation codon and hence did not translate a Vpr product as described earlier (21).

Table 2

Pattern of RNA Expression Among HIV-1 Infected Primary Human Macrophage Cultures

Vpr gene	ATG-		WILD-TYPE		R62P		R80A	
Viral inoculum *	High	Low	High	Low	High	Low	High	Low
Total number of Positive cells‡	91.0%	6.5%	89.5%	67.5%	94.5%	8.0%	93.5%	42.5%
Productively infected cells§	2.50%	0.0%	43.5	29.0%	39.0%	3.0%	4.5%	1.5%

Legend: RNA expression pattern as assessed by *in situ* hybridization. * high signifies 50 ng of viral input and low signifies 1.85 ng of viral input as assessed by p24 ELISA. ‡ positivity is defined as cells expressing more than 20 grains over background levels. § productive infection is defined as the proportion of cells expressing 100 grains or more over background levels. Consistent results were also obtained from macrophages derived from a second donor with independent viral stocks. A minimum of 200 cells were quantified using methods we have previously described (20).

Figure Legends

Figure 1: Single cycle replication kinetics of viruses expressing the Wild-Type and mutant Vpr proteins in primary human macrophages. Peripheral blood monocyte derived macrophages cultured for seven days were infected with increasing viral inputs ranging from 619 pg to 50 ng (represented in the horizontal axis). Viral production following infection was monitored at regular intervals by measuring p24 levels in the culture supernatants (represented in the vertical axis as p24 ng/ml). The lateral axis represents days post infection spanning the peak of viral replication. Independent experiments performed with different donors and viral stocks consistently showed similar kinetics.

Figure 2: Levels of HIV-1 DNA found in the nuclear compartment among viruses expressing the wild-type and mutant Vpr proteins. Panel A: Kinetics of proviral DNA detection in the nuclear compartment for wild-type and Vpr negative mutants at a non saturating viral input (1.85 ng). HIV Gag sequences found targeted to the nucleus reached a plateau by day 3 and remained constant indicating that multiple rounds of infection in the presence of the protease inhibitor does not occur in this system. Panel B: HIV-1 proviral detection in the nuclear compartment at day 14 for the wild-type, ATG-, R62P, and R80A mutants. Similar levels of cellular DNA was analyzed as indicated by the β2-AR detection. Standards for HIV-Gag detection were measured by diluting uninfected macrophages with a known number of chronically infected ACH-2 cells (48).

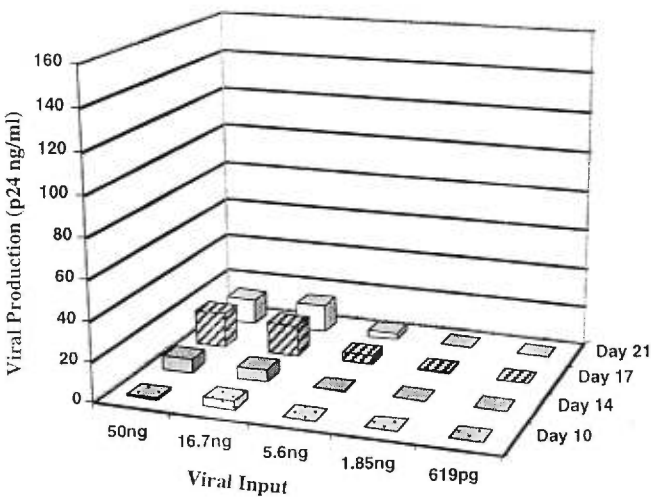
Figure 3: Productive and silent infection patterns associated with wild-type and Vpr-mutant cultures as assessed by in situ hybridization. Panels depict *in situ* patterns found in macrophage cultures infected on day seven following isolation with high viral titers (50 ng) and monitored for RNA expression at two weeks post infection where a clear difference in virion production was evident as assessed by supernatant p24 levels (Figure 1). Uninfected culture (panel a), wild-type (panel b), ATG- (panel c), R62P (panel d), and R80A (panel e).

Figure 4: G2-arrest levels found in macrophage cultures: ATG- (panel a), wild-type (panel b), R62P (panel c), R80A (panel d), and mock infected (panel e). Cells were monitored for G2-arrest following infection at 50 ng at day 14 post infection by Propidium Iodide staining and FACS analysis.

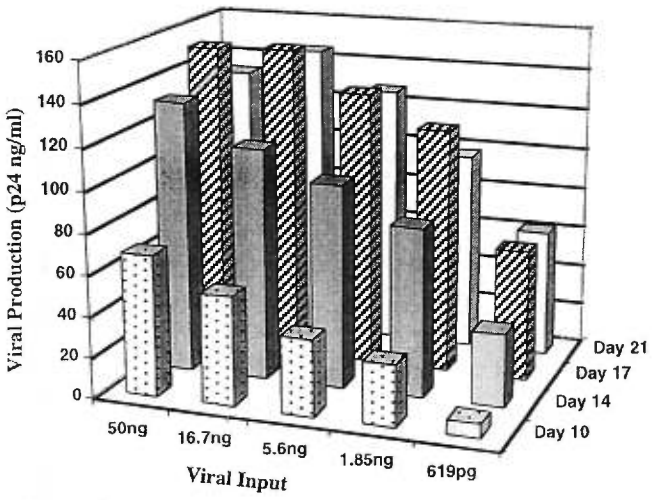
Figure 1

Single cycle replication kinetics of viruses expressing the Wild-Type and mutant Vpr proteins in primary human macrophages.

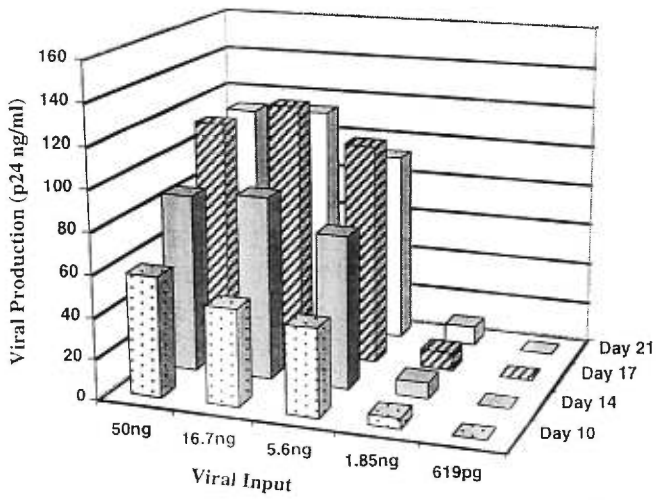
ATG-



Wild-Type



R62P



R80A

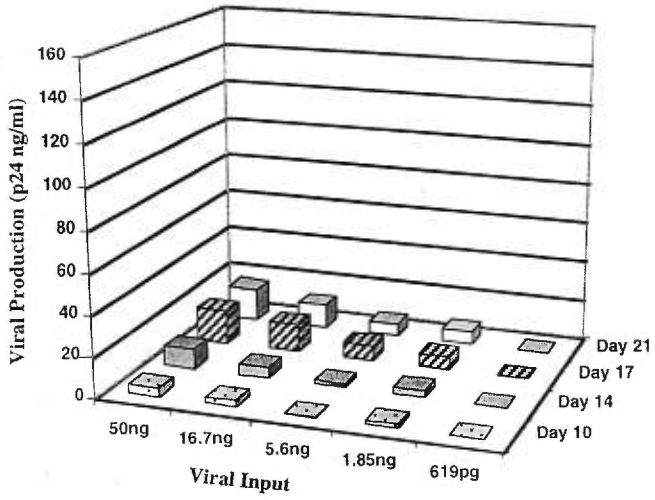
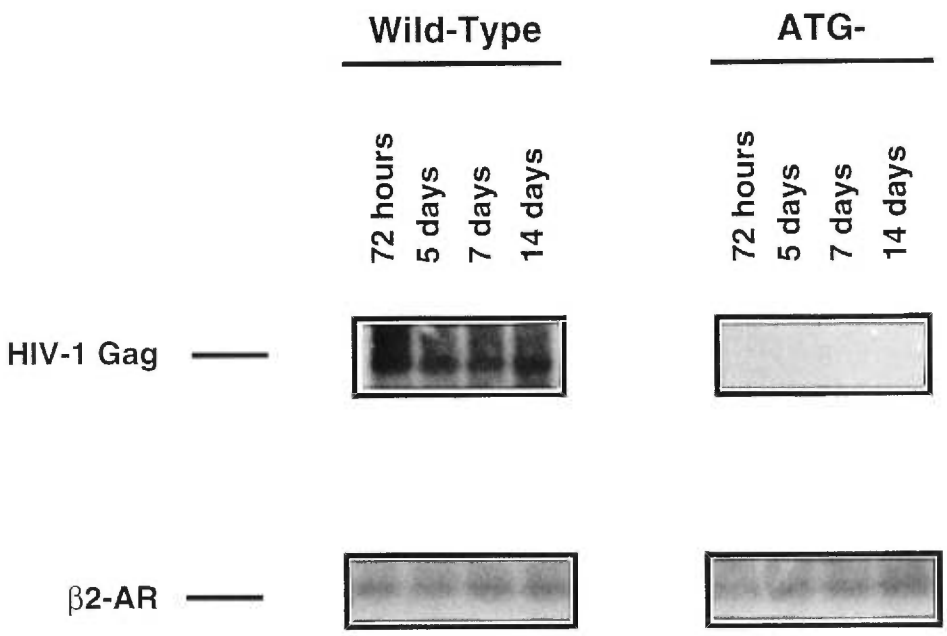


Figure 2

Levels of HIV-1 DNA found in the nuclear compartment among viruses expressing the wild-type and mutant Vpr proteins. Panel A


Wild-Type
ATG-
72 hours
5 days
7 days
14 days
72 hours
5 days
7 days
14 days
HIV-1 Gag
β2-AR

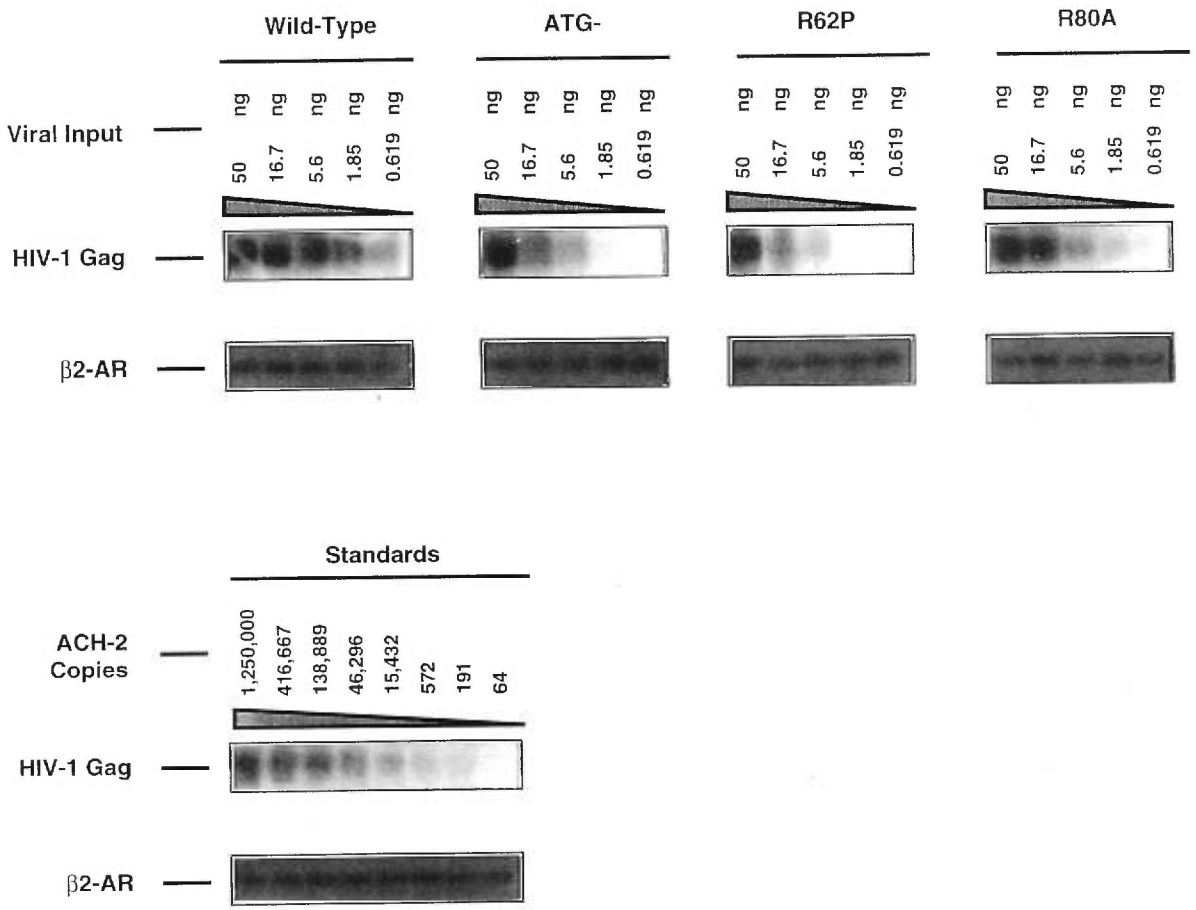
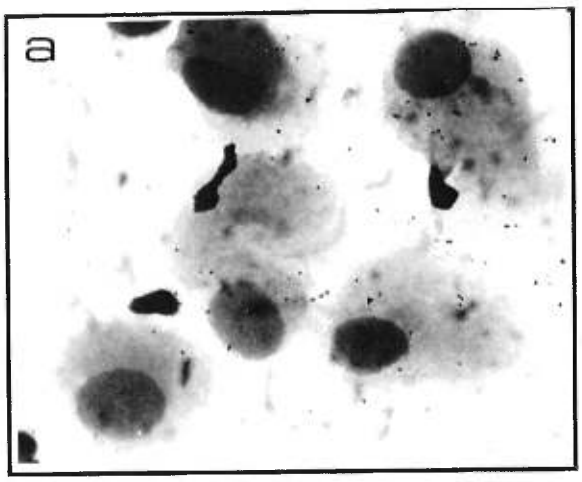
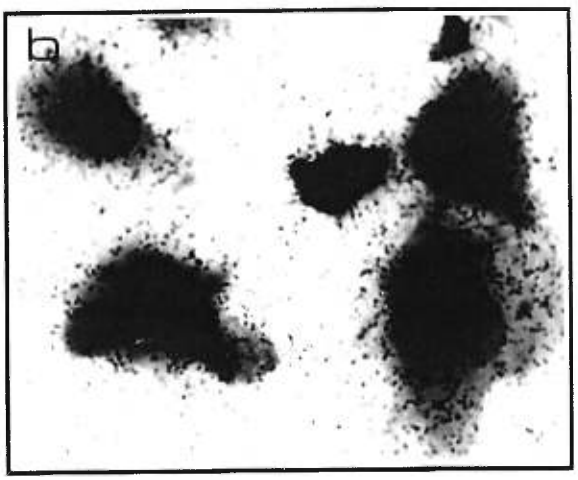
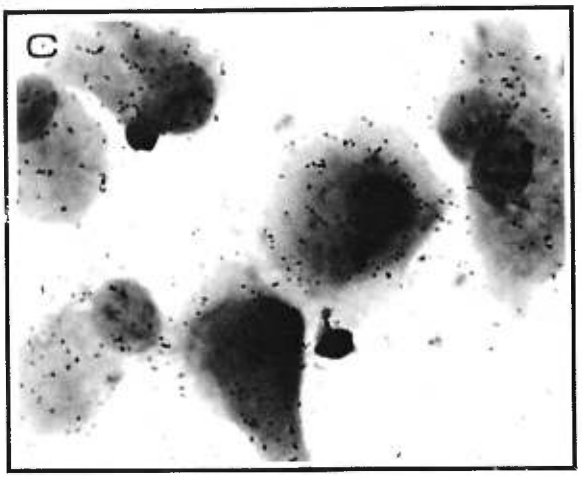

Wild-Type
ATG-
R62P
R80A
Viral Input
50 ng
16.7 ng
5.6 ng
1.85 ng
0.619 ng
HIV-1 Gag
β2-AR
Standards
ACH-2 Copies
1,250,000
416,667
138,889
46,296
15,432
572
191
64
HIV-1 Gag
β2-AR

Figure 3

Productive and silent infection patterns associated with wild-type and Vpr-mutant cultures as assessed by in situ hybridization


a


b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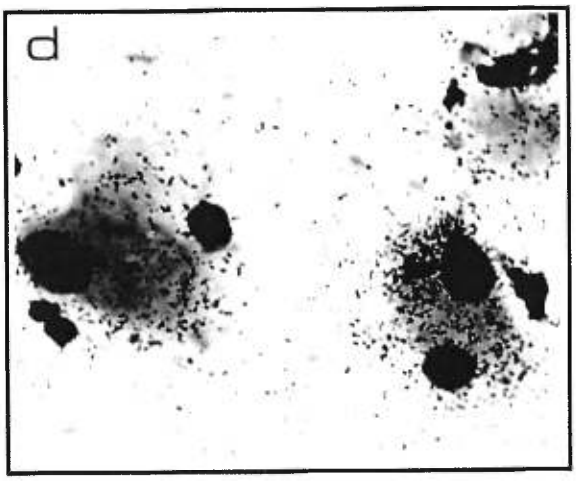
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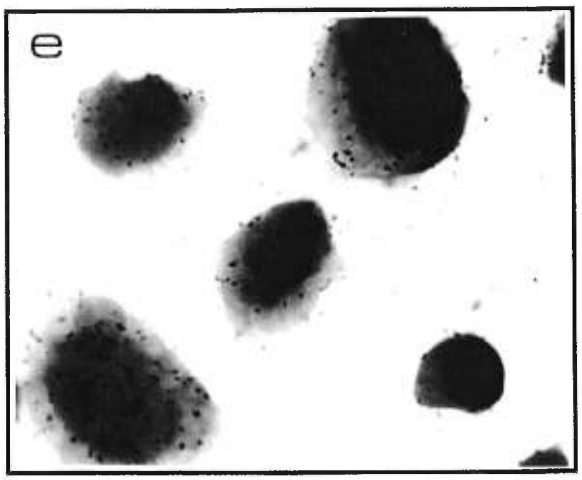
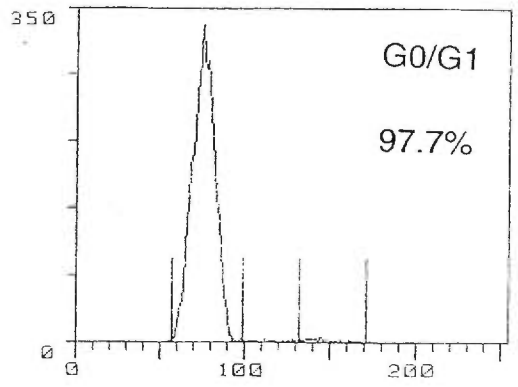

e

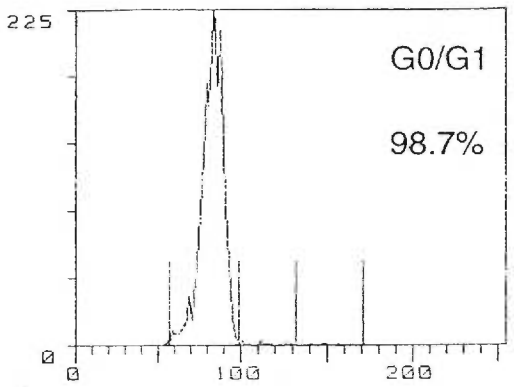
Figure 4

Cell cycle analysis of primary human macrophage cultures

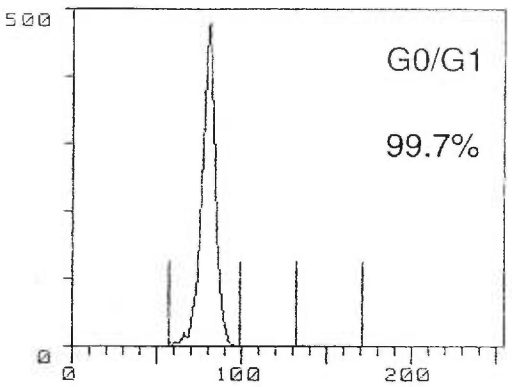
a


350
0
0
100
200
G0/G1
97.7%

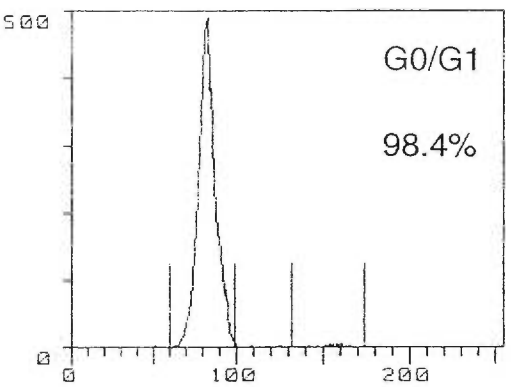
b


225
0
0
100
200
G0/G1
98.7%

c


500
0
0
100
200
G0/G1
99.7%

d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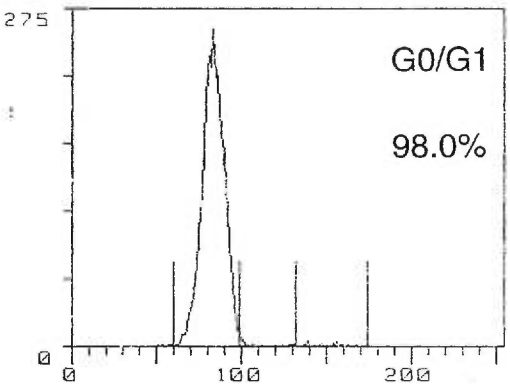
0
0
100
200
G0/G1
98.4%

e


275
0
0
100
200
G0/G1
98.0%

Chapter 5

Discussion

The pathogenesis of HIV, the causative agent of AIDS (Goedert and Gallo, 1985; Blattner *et al.*, 1988; Gallo, 1990; Lusso and Gallo, 1992), is characterized by progressive immune deficiency that in a majority of cases leads to death. The genes coded for by this virus have been extensively studied in an effort to unearth molecular mechanisms that could be exploited for therapeutic purposes. Both HIV-1 and the closely related HIV-2 are lentiviruses that belong to the greater family of retroviridae (Coffin, 1992). Lentiviruses, typified by the HIV group of viruses, differ from prototypic retroviruses in that they are associated with long progressive diseases usually associated with immunodeficiency (Gonda *et al.*, 1985; Griffin, 1986). Genomically, lentiviruses, like all retroviruses, code for the *gag*, *pol*, and *env* genes which constitute their basic structural and enzymatic repertoire. However, lentiviruses also code for a number of additional regulatory proteins which are unique to this subfamily and not found in the prototypic retroviruses referred to as auxiliary proteins (Subbramanian and Cohen, 1994; Trono, 1995). In HIV-1, these auxiliary proteins are temporally expressed as early (Tat, Rev, and Nef) and late (Vif, Vpr, and Vpu) products controlled primarily by the Rev protein (Cullen, 1995). While the early proteins, Tat, Rev, and Nef have been studied extensively, very little is known about the late auxiliary gene products such as Vpr. All late products are currently thought to work during virion morphogenesis or during early infection steps though these proteins also show pleiotropic phenotypes not well understood currently (Cohen *et al.*, 1996). The 96 amino acids long HIV-1 Vpr protein is one of these less characterized auxiliary products and the focus of this study.

One of the functions clearly associated with Vpr is the ability of the viruses coding this protein to replicate effectively in non dividing cells such as macrophages (Heinzinger *et al.*, 1994; Connor *et al.*, 1995). Oncoretroviruses, unlike their lentiviral cousins, do not replicate in nondividing cells as they rely on the disintegration of the nuclear envelope during mitosis to gain access to the nuclear compartment. However, it is currently clear that lentiviruses code for proteins that ensure effective transport of the preintegration complexes to the nucleus even in nondividing target cells (Stevenson *et al.*, 1995; Emerman, 1996). Vpr, a virion-associated protein that is available throughout the early steps of viral infection, has been shown to be one of the nucleophilic determinants that ensures effective transport of the preintegration complexes into the nuclear compartment of the nondividing cells (Heinzinger *et al.*, 1994).

An additional function associated with Vpr was first identified in proliferating T-cells: its ability to arrest cells in G2 phase of the cell cycle (Di Marzio *et al.*, 1995; He *et al.*, 1995; Jowett *et al.*, 1995; Re *et al.*, 1995). The first indication that Vpr may perturb cell division came from observations that document the inability of the wild type Vpr expressing cells to grow out of the acute infection stage to generate chronic virus producing cell lines (Planelles *et al.*, 1995; Rogel *et al.*, 1995). This corroborated with previous results suggesting that chronic virus producing cell lines were frequently mutated in this gene (Nishino *et al.*, 1991; Nishino *et al.*, 1994). Yet another report documented withdrawal of Vpr expressing cells from cell division into terminal differentiation (Levy *et al.*, 1993). Currently it is well established that this outcome is the result of effective cell-cycle block induced by Vpr in the G2 phase that follows DNA duplication. Biochemical events which normally characterize the G2 phase of the cell cycle such as hyperphosphorylation of the cdc2 and the cdc2-cyclin B associated kinase activity has been reported (He *et al.*, 1995; Re *et al.*, 1995). However, it appears cdc2 itself is not a direct target for Vpr as no binding was evident with either cdc2 or cyclin B (Re *et al.*, 1995). Thus, very little is known about the mechanism behind the G2-arrest associated with the Vpr protein. Regardless of the mechanism involved, it has been suggested that the G2-arrest activity may contribute significantly to the pathogenesis of AIDS which is primarily associated with T-cell depletion (Fauci *et al.*, 1996). It has been argued convincingly that the G2-arrest leads to lack of proliferation of antigen specific T-cell clones that could potentially fight off the virus potentially leading to clonal anergy (Jowett *et al.*, 1995; Planelles *et al.*, 1995; Rogel *et al.*, 1995). In fact the recent finding that apoptosis and cell death follows G2-arrest (Poon *et al.*, 1997; Stewart *et al.*, 1997) adds more weight to the notion of T-cell function perturbation by Vpr as killing of viral specific cells could lead to clonal deletion, which in essence could be an effective weapon wielded by the virus to subvert the immune response mounted against it. Thus, while a functional role for Vpr mediated G2-arrest in proliferating T-cells is slowly gaining support, whether this G2-arrest function has functional correlates in non dividing macrophages has not been formally addressed.

To understand the relevance of nuclear localization and G2-arrest contribution to protein function, it was essential to have a good understanding of the structural determinants that control these phenotypes in the wild type protein. Toward this end we analyzed the protein structure using computer assisted prediction algorithms. The two studies included in this work identify two helical motifs: one at the N-terminus and another spanning the central hydrophobic region of the protein. Both these helical motifs

are amphipathic with clearly identifiable hydrophobic and hydrophilic interfaces. The second helical motif in the central region of the protein, however, has a preponderance of hydrophobic residues in its amphipathic primary sequence. Highly ordered structures were not noted for the C-terminal region of the protein in our analysis.

These putative helical domains, both in the N-terminal and the central hydrophobic helical regions are conserved across HIV-1 isolates. Such evolutionary conservation indicates that this hydrophobic helical domain plays a significant functional role. In this work we first characterize an N-terminal helix extending from residues 16 to 34 (Yao *et al.*, 1995). We provide evidence that virion targeting is primarily mediated by this N-terminal structure and that the distal regions play a minimal role in the ability of the protein to associate with the virions. This earlier study, however, only analyzed one mutation in the central hydrophobic region described in the subsequent study (Isoleucine 63 to Phenylalanine). In the subsequent study we have included a panel of mutations in the central helical region and show that as previously suggested, this region does not comprise the minimal region required for the virion targeting of the protein. Relative to the levels found in the infected cells, all central helix Vpr mutants were also found in the virion with ratios hovering around the wild-type ratios. This suggests that the minimal domains necessary for virion targeting was not affected by the mutations in this helical region. This is different from the previously characterized N-terminal mutations which radically affected the virion associated levels of the protein.

Further functional characterization of these two putative helical structures and the C-terminal region suggests that Vpr structure is most likely organized in a modular fashion (Figure 1 in this chapter) and can be summarized as follows: the C-terminus primarily mediates the G2-arrest activity. Neither the N-terminal helix nor the central hydrophobic helix contribute significantly to this phenotype. This is in agreement with studies published by other groups (Di Marzio *et al.*, 1995; Mahalingam *et al.*, 1997). Similarly the ability of the protein to incorporate into budding virions is mediated primarily by the N-terminal helix while the central hydrophobic helix and the C-terminus of the protein did not greatly contribute to this phenotype (Mahalingam *et al.*, 1995c; Mahalingam *et al.*, 1995e; Yao *et al.*, 1995). However the nuclear localization of the protein was controlled by both the N-terminal and the central hydrophobic helical structures (Zhao *et al.*, 1994a; Mahalingam *et al.*, 1995a; Yao *et al.*, 1995). The mutations in the C-terminal does not appear to affect this function in any significant way. Specific mutations within the C-terminus, however, may indirectly affect nuclear localization and virion incorporation as it is clearly necessary for the stability and

perhaps proper confirmation of the protein (Mahalingam *et al.*, 1995b; Mahalingam *et al.*, 1995e; Yao *et al.*, 1995). Hence, the domains that code for the G2-arrest and nuclear localization of the protein are clearly separable (Di Marzio *et al.*, 1995; Mahalingam *et al.*, 1997). The virion incorporation and the nuclear localization functions, however, are not totally separable as they can both be affected by affecting the N-terminal helical motif.

A logical extension of this structure-function characterization is that the G2-arrest phenotype is mediated by Vpr even when the protein is impaired in its ability to reach the nucleoplasm. This suggests that the G2-arrest mediated by Vpr is controlled from the cytoplasm and/or the nuclear membrane. Hence, the accumulation of Vpr within the nucleus is dispensable for this activity. The G2-arrest phenotype may be of functional relevance to the viral pathogenesis on two accounts. One possibility that has been put forward suggests that the G2-arrest could lead to higher virus production as transcription levels are augmented at this phase in the cell cycle (Bartz *et al.*, 1996), and this is formally demonstrated in this work by experiments detailed in chapter 4. Another possibility is that the G2-arrest will lead to prevention of clonal expansion of the infected T-cells as discussed earlier. Recent studies indicate that the G2-arrest mediated by Vpr is also followed by death by apoptosis (Poon *et al.*, 1997; Stewart *et al.*, 1997), an observation supported in our present study by direct demonstration of apoptotic bodies in cells expressing Vpr but not in the control cells. Hence the G2-arrest function will be operative in dividing T-cells where the goal is not only to delay growth by blocking cells in G2 but subsequently eliminating them by inducing apoptosis. Hence, Vpr expression could lead to clonal deletion of antigen specific T-cells thus compromising the immune clearance function, which in turn could contribute to high viral loads associated with Vpr expression documented in previous in-vivo studies (Lang *et al.*, 1993; Gibbs *et al.*, 1995).

If the G2-arrest function can be effectively mediated from the cytoplasm, what then is the relevance of the nuclear localization of the protein? Results presented in this work suggest that this activity may be related to the second function associated with the Vpr protein: its ability to facilitate the transport of the preintegration complex to the nuclear compartment in non dividing cells. While the wild-type protein demonstrating the intra-nuclear accumulation was capable of targeting the proviral DNA to the nucleus effectively, the R62P-mutant which was retained in the cytoplasm was not capable of this function.

The relevance of these two biological activities in non dividing macrophages, one of the major targets for HIV-1 *in vivo* was addressed in Chapter 4 of this work. As the structural determinants of these activities segregate to different domains on the protein (Di Marzio *et al.*, 1995; Mahalingam *et al.*, 1995a) it was possible to design mutations that primarily affected one but not the other activity. Our functional analysis clearly suggests that the G2-arrest activity is responsible for the transcriptional upregulation seen in macrophages in the presence of Vpr. Interestingly, what leads to transcriptional activation in non dividing cells also leads to growth arrest in proliferating cells. As we formally prove that DNA replication does not occur in our macrophage cultures, it appears, then, the biochemical events rather than the DNA duplication status of the cell is a common factor that underlies this phenotype. We also link the protein's nuclear localization activity with that of its ability to target the proviral DNA to the nuclear compartment. It is interesting that this proviral nuclear targeting function is only apparent *in vitro* at low viral inputs. Higher inputs clearly mask this functional attribute conceivably due to complementation by other nucleophilic proteins in this system. However, it is highly unlikely that this function is dispensable *in vivo* where high viral titers frequently used *in vitro* are rarely achievable. Our observations also provide insight into the conservation of apparently redundant nucleophilic factors in the lentiviral systems. It is conceivable that the targeting of the preintegration complex to the nucleus is mediated by multivalent interactions with cellular proteins at the nuclear periphery, or cytoplasmic partners that function as adapter proteins which shuttle the complex to the nuclear pore structure. In this regard both matrix p17 and integrase have been suggested to interact with karyopherin-α at the nuclear periphery (Gallay *et al.*, 1996; Gallay *et al.*, 1997). Vpr is not known to utilize this karyopherin interaction-based mechanism to mediate proviral nuclear import. However, Vpr itself has been shown to bind Gag structures, specifically p17 directly (Gallay *et al.*, 1996). Hence it is likely that Vpr could lead to recruitment of cellular factors that modify or activate other preintegration components. Alternatively, Vpr may also recognize specific cellular factors that help shuttle it in the context of the preintegration complex to the nuclear compartment. There is currently no compelling evidence to chose one or the other possibility and they may not be mutually exclusive. In fact, as discussed earlier, Vpr has been shown to interact with a number of cellular factors shown to shuttle between the cytoplasm and the nucleus, including GRII and the novel nuclear protein HHR23A involved in DNA excision repair. These cellular proteins may not only shuttle the protein to the nucleus but may be intricately involved in subsequent Vpr-associated phenotypes such as induction of DNA damage and apoptosis (Refaeli *et al.*, 1995; Withers-Ward *et al.*,

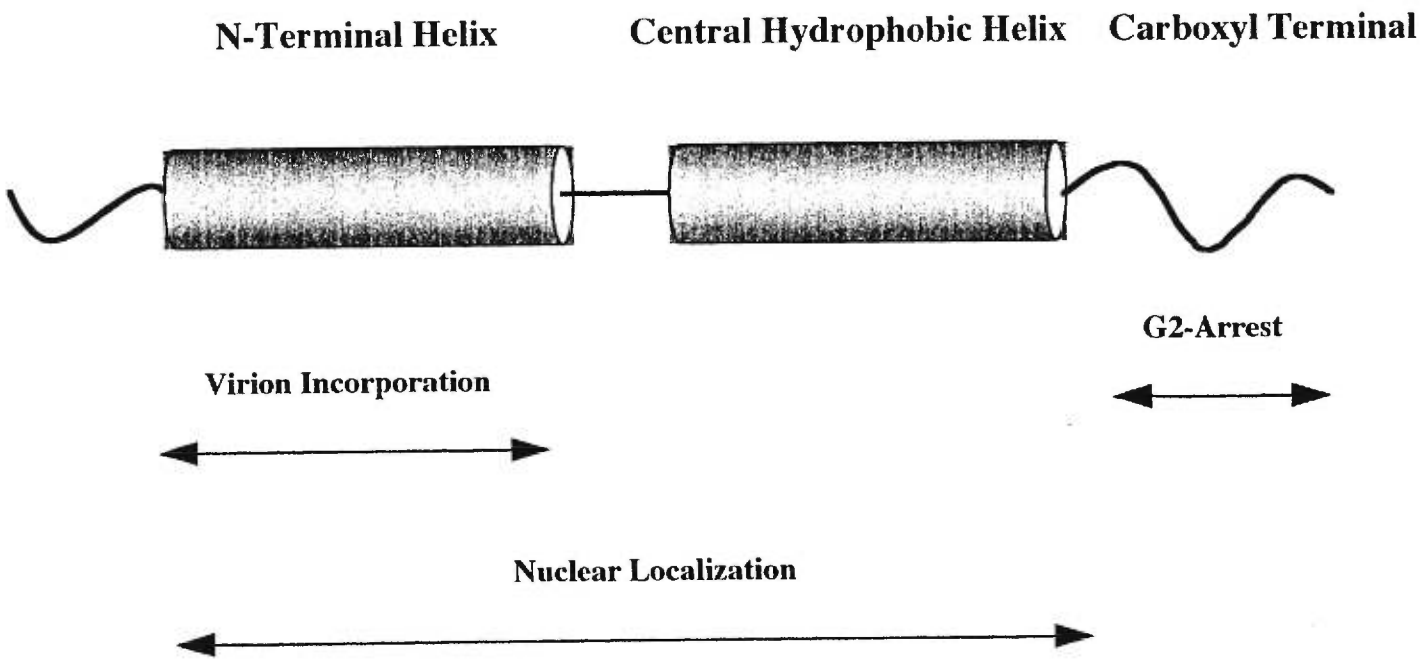
1997). Direct evidence that could help unravel the exact mechanism(s) involved in the nuclear transport mediated by Vpr is lacking, and this aspect of the protein function is being explored both by our group and other laboratories.

In vitro results, in biological sciences has been indiscriminately used to derive *in vivo* conclusions and this has especially been the case in categorizing some of the regulatory products of HIV-1 as being functionally "accessory". Though *in vitro* results have been enormously rewarding by themselves in elucidating the molecular biology of the virus, out of experimental constraints, numerous *in vivo* extrapolations have been made over the years. This can be primarily attributed to the lack of an appropriate, affordable, and unendangered animal model for *in vivo* experiments. SIV has proved to be an useful model virus in macaques, however, recently the SCID mice system has also emerged as a promising model (Mosier *et al.*, 1992; Luciw *et al.*, 1995; Aldrovandi and Zack, 1996; Mosier, 1996).

Several lines of evidence suggest that the accessory proteins coded by HIV play an important role in the viral life cycle and disease pathogenesis: accessory protein reading frames are highly conserved among HIV and distant lentiviral relatives which is indicative of a selective pressure to foster these ORFs. While it is clear that unlike the regulatory proteins Tat and Rev other auxiliary proteins are not essential for productive viral replication *in vitro,* several independent studies have shown that optimal viral replication, even *in vitro*, requires one or more of these accessory proteins. Viruses carrying mutations in one or more of these genes are severely impaired in cell types such as macrophages that may play a pivotal role in viral pathogenesis (Subbramanian and Cohen, 1994). *In vitro* evidence from both primary and transformed cell systems already indicate that a majority of these proteins aid, rather than inhibit HIV replication, either generally, or in a cell specific manner. In this work, we dissect one such accessory protein at the structural level and show that the two biological functions associated with the protein, the nuclear localization and G2-arrest, contribute respectively to early and late functions in macrophages. Our results also suggest that these two activities render the protein's functional contribution essential and not accessory under conditions that closely mimic *in vivo* conditions, that is, primary cells being infected at low viral inputs.

Figure 1: Schematic of HIV-1 Vpr structure. The a simplified structure function relationship diagram relevant to this discussion. The specifics of the N-terminal and the central hydrophobic helices have already been dealt with in earlier chapters. Please refer to figure 7, Chapter 2 and Figure 1 from Chapter 3 for additional information on these helical motives.

Schematic of Functionally Relevant Putative Structures within HIV-1 Vpr



ORIGINAL CONTRIBUTION

In terms of cloning, a necessary process in Virology, I have been involved either in the choice and oligonucleotide design of a majority of Vpr mutations in the lab, or alternatively, in the actual physical cloning itself. First, I have cloned several versions of macrophage-tropic ADA and Bal-based viruses in the lab including Vpr-mutations. Also, the PCEP vector system, introduced by me in the lab, though not suitable for gauging transient G2-arrest, was a good system to look at the Vpr effect in long-term cultures. In fact, it has been used by colleagues in the lab to gauge the growth-effects associated with Vpr-fusion proteins in long term cultures. Importantly, the envelope-negative versions of HIV-1 which were instrumental for all the G2-arrest work done in the lab using pseudotyped VSV envelopes were both designed and cloned physically by me. Demonstration of apoptosis and DNA fragmentation in Vpr-expressing cells including DNA ladder patterns in the lab were first identified by me and subsequently utilized by others in their works. Needless to say, scientific research is a group effort to which I have not only contributed to, but have also benefited from. In this regard, the efforts of Yao Xiao-Jian particularly need to be recognized, as we have contributed to each other's works and have collaborated on a wide variety of projects.

I have established protocols in the lab that pertain to primary cell cultures of lymphocytes and macrophages. Purification protocols developed include ones that provide partial, but rapid, enrichment to ones that use multi-step process providing highly purified cell-subsets. FACS, immunofluorescence, and cell-specific enzymatic assays pertinent to primary cells were also needed to routinely test the purity of these cultures. I have also optimized infection in lymphocytes and macrophages which generally vary significantly from transformed cells both in terms of tropism as well as in the protracted nature of their viral kinetics. While two-circle LTR detection has been previously used in our lab to gauge nuclear transport of proviral DNA, I have optimized a cell fractionation process to detect DNA in the nuclear compartment by semi-quantitative PCR. Though the actual semi-quantitative PCR reactions on cell fractions and gel separation were performed by me, I wish to acknowledge Robert Lodge who performed the radioactive [^{32}P] Southern blotting on them. In turn, I have collaborated with Robert Lodge on his project where purification of specific primary lymphocyte subsets was required. I consider this collaboration as another positive experience where I have had the opportunity to both contributed to, and benefit from a mutual collaboration within the lab.

In the larger scheme of things, these protocols were of course established to understand the correlates of HIV-1 replication in primary human macrophages as it pertains to the accessory protein Vpr. The results presented in this work show that these correlates are likely to be complex with viral expression being controlled both before and after integration. In addition to primary cell purification, infection, PCR, and *in situ* protocols, the articles presented in this work have also required computer assisted structural analysis, nuclear localization of Vpr mutations with subsequent optimization for confocal microscopy. I have also had the good fortune of co-authoring two reviews with my mentor, Dr. Eric A. Cohen. It is my hope that the data I have presented here and in other works performed in collaboration will provide a small, but hopefully, a significant boost to the field of HIV-1 accessory protein research.

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