Role of Mitochondria in HIV Infection

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ROLE OF MITOCHONDRIA IN HIV INFECTION

By

Gaofei Lu

A DISSERTATION

Submitted to the Faculty
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A dissertation submitted in partial fulfillment of
the requirements for the degree of
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ROLE OF MITOCHONDRIA IN HIV INFECTION

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HIV-1 hijacks our cellular machinery to complete its life cycle. A better understanding of the interactions between cellular proteins and viral components will certainly lead to the discovery of new ways to inhibit viral replication. My primary research goals are directed toward understanding the basis of molecular interactions between viral and host factors (proteins or organelles) during infection of human cells.

In my thesis, the role of mitochondria in the early stage of HIV infection has been evaluated by using a VSV-G pseudotyped HIV-based lentiviral vector. I have observed that cells lacking mitochondrial DNA (ρ0 cells), isolated from an established human osteosarcoma cell line, are defective in the ability to support virus infection when compared to their parental cells. This infection deficiency in ρ0 cells can be repaired by reintroducing mitochondria from ρ+ human cells (293T cells) using transmitochondrial technology. Inhibition of oxidative phosphorylation by mitochondrial inhibitors did not inhibit HIV infection in HOS cells (ρ+ cells) indicating that the reduced infection efficiency in ρ0 cells is not simply the result of reduced oxidative phosphorylation. Further analysis indicates that virus infection in ρ0 cells is blocked at steps after reverse transcription and before nuclear import. Confocal fluorescence microscope analysis
shows the subcellular location of the majority of virus nucleoprotein particles (identified by the presence of the viral capsid protein) to be near or in contact with mitochondria. Co-fractionation of viral complexes with mitochondria from infected cells also supports the hypothesis of association of viral complexes with mitochondria.

In conclusion, in my study I have shown that $\rho^0$ cells are defective in the ability to support HIV infection and viral infection is inhibited at steps after reverse transcription and before nuclear import. Mitochondria may play an important role in the intracellular transport by directly association with viral complexes. A role for mitochondrial association of viral intracellular complexes has not been previously explored. The elucidation of this role will open new avenues for investigation of the early steps in HIV infection.
This dissertation is dedicated to my mentor
Dr. Walter A. Scott
who passed away on January 28, 2013.
He became my mentor in 2006 and will always be a beacon of light in my career and life.
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# TABLE OF CONTENTS

## LIST OF FIGURES

- HIV-1 and disease ................................................................. vii
- Early stage of HIV-1 infection ............................................ 1
- Interaction of virus and cellular factors during the early stage of HIV-1 infection ........................................ 10
- Approaches to study the early stage of HIV-1 infection ........ 16
- Mitochondria and virus infection .......................................... 19
- Goals of this dissertation ...................................................... 23

## Chapter 1: INTRODUCTION

1. HIV-1 and disease ................................................................. 1
2. Early stage of HIV-1 infection ............................................ 1
3. Interaction of virus and cellular factors during the early stage of HIV-1 infection ........................................ 10
4. Approaches to study the early stage of HIV-1 infection ........ 16
5. Mitochondria and virus infection .......................................... 19
6. Goals of this dissertation ...................................................... 23

## Chapter 2: MATERIALS AND METHODS

1. Oligonucleotide primer sequences ........................................ 25
2. Cells and materials ............................................................ 26
3. VSV-G pseudotyped virus production .................................... 26
4. Analysis of virus infection by flow cytometry ....................... 27
5. Virus infection in $\rho^0$ and HOS cells ................................ 28
6. Analysis of the effect of mitochondrial inhibitors on virus infection ........................................ 28
7. Construction of cybrid cells by fusion of $\rho^0$ and enucleated HEK293T cells ........................................ 29
8. Viral cDNA isolation from the infect cells (HOS, $\rho^0$ and cybrid cells) ........................................ 30
9. Real-time PCR quantification .................................................. 31
- Mitochondrial DNA detection ................................................ 31
- Viral cDNA detection .......................................................... 32
  - Viral early and late reverse transcription product ............... 32
  - Integrated viral DNA ....................................................... 32
  - 2LTR circle ........................................................................ 33
- Cell number reference assay ............................................... 34
10. Immunochemistry and confocal fluorescence microscope .... 34
12. Detection of viral cDNA synthesis in infected cells by Click-it method ........................................ 36
13. Mitochondria morphology of HOS, $\rho^0$ and cybrid cells .......... 38
14. Cell extracts and virus DNA identification ......................... 38
15. Antibody-mediated binding of mitochondria and viral cDNA ........................................ 40
- Bind of anti-Tom22 antibody to the protein A beads ............... 40
- Isolation of mitochondria from infected cell ......................... 40
- Antibody-mediated binding ................................................ 41
Chapter 3: RESULTS

1. HIV Infection in $\rho^0$ cells is defective in comparison with HOS cells
   1.1. HIV infection in HOS and $\rho^0$ cells
   1.2. Effects of mitochondrial inhibitors to HIV infection in HOS cells
   1.3. Introduction of mitochondrial DNA into $\rho^0$ cells
   1.4. Mitochondria morphology in HOS, $\rho^0$ and cybrid cells

2. Quantification of different forms of viral DNA in the infected cells
   2.1. Reverse transcription (RT) product detection
   2.2. Early reverse transcription product (strong stop DNA)
   2.3. Late reverse transcription product (full length viral cDNA)
   2.4. Integrated viral DNA detection
   2.5. 2-LTR circle DNA

3. Interaction between virus and mitochondria
   3.1. Confocal fluorescence microscopy analysis
      3.1.1. Co-localization of viral complexes with mitochondria in the infected cells
      3.1.2. Detection of HIV cDNA in infected cells by EdC incorporation and the Click-it method
   3.2. HIV-1 intracellular complexes and mitochondria interaction
      3.2.1. Distribution of early and late reverse transcripts in cell extract
      3.2.2. Distribution of viral complexes and mitochondria
      3.2.3. Antibody-mediated binding of mitochondria and viral complexes

Chapter 4: DISCUSSION AND CONCLUSION

1. Infection defect in $\rho^0$ cells
2. Virus infection was blocked at steps after reverse transcription and before nuclear import
3. Mitochondria and viral complexes interaction
4. Co-fractionation of virus complexes with mitochondria
5. Conclusion

SIGNIFICANCE OF THIS STUDY

REFERENCES
LIST OF FIGURES

Figure 1. HIV-1 structure........................................................................................................3
Figure 2. HIV-1 genome...........................................................................................................4
Figure 3. $\rho^0$ cells are deficient in the support of HIV infection compared with HOS cells.................................................................43
Figure 4. Effect of antimycin A (AMA) to HIV infection in HOS cells.........................46
Figure 5. Effect of CCCP and oligomycin to HIV infection in HOS cells..................47
Figure 6. Mitochondrial DNA level in cybrid and HOS cells............................................49
Figure 7. Virus infection in HOS, cybrid, and $\rho^0$ cells..................................................50
Figure 8. Mitochondria morphology in HOS, cybrid, and Rho0 cell............................51
Figure 9. Steps during early stage of HIV replication.........................................................53
Figure 10. FACS analysis of infection in HOS, cybrid, and $\rho^0$ cell...............................53
Figure 11. Detection of early and late reverse transcripts................................................55
Figure 12. Reverse transcription activity is similar in HOS, cybrid and $\rho^0$ cell............57
Figure 13. Semi-nested PCR detection of integrated viral DNA..................................60
Figure 14. Standard curve generated for quantifying integrated viral DNA.................61
Figure 15. 2LTR circles and integrated DNA in $\rho^0$ cells are significantly lower than in HOS, and cybrid.............................................................63
Figure 16. Co-localization of HIV intracellular complexes with mitochondria in HOS cells.......................................................................................66
Figure 17. Viral nucleoprotein complexes and mitochondria in 293T cell....................67
Figure 18. Nanoscope images of viral complexes with mitochondria in 293T cell........................................................................................................68
Figure 19. Newly synthesized mtDNA detection by Click-it method............................70
Figure 20. Newly synthesized viral DNA detection............................................................71
Figure 21. Cell fractionation and viral cDNA detection…………………………..75
Figure 22. Co-fractionation of virus with mitochondria…………………………...…76
Figure 23. Co-immunoprecipitation of viral complexes with mitochondria............76
Figure 24. The role of mitochondria in the early stage of HIV infection…………….85
Chapter 1: Introduction

1. HIV-1 and disease

Since the discovery of HIV-1 as the cause of acquired immunodeficiency disease in 1983 (Evans, 1989; Weiss, 1993), HIV-1 has been one of the most extensively studied viruses. Many studies have been done to characterize HIV-1 and its lifecycle, and based on those knowledge, different new therapeutic methods have been developed to control HIV-1 infection (Alfano & Poli, 2004; Cann & Karn, 1989). The majority of antiviral drugs target virus enzymes and proteins including reverse transcriptase, integrase and protease. Even though currently there are many antiviral drugs and therapeutic treatments available, but the key to eliminate HIV-1 has remained elusive. Because of the highly mutagenic nature of HIV-1 replication, virus can mutate and evolve very quickly during the drug treatment (Ribeiro & Bonhoeffer, 2000). As a result, drug resistant strains of HIV-1 can emerge very rapidly. In order to overcome the emerging of drug resistance, several different antiviral drugs have to be used during the treatment of HIV-1 infection. This cocktail treatment called highly active antiretroviral therapy (HAART) has been proven to have a pronounced affect on HIV-1 replication in the infected patients. Another thing is that long time treatment with those drugs can lead to many side effects. New antiviral drugs and treatment strategies need to be developed. During the co-evolution
with host cells, HIV-1 has successfully adapted to utilize cellular factors to facilitate its replication. As a result, the interactions between virus and cellular compartments play a key role in the virus life cycle. Generally, HIV-1 life cycle can be divided into two stages: early stage infection and late stage infection (Bukrinskaya, 2004; Murray et al., 2011; Nisole & Saïb, 2004). The early stage infection includes entry into the host cell, reverse transcription, uncoating, intracellular transport, nuclear import and integration. The late stage infection includes transcription of viral gene, translation, viral particle assembly and budding. The late stage of HIV-1 infection has been studied in great detail (Bukrinskaya, 2004; Cullen, 1991; Greene, 1990), but the early stage of HIV-1 infection has not been fully understood. By better understanding the steps of early stage of HIV-1 infection, and through the identification of cellular factors interacting with viral intracellular complexes during the infection, better drugs and therapeutic methods can be developed.

1.1. HIV-1 virion structure

HIV-1 virion is about 120nm in diameter and roughly spherical. Like the classic lentiviruses, HIV-1 contains an envelope derived from the host cell membrane when the virus buds from the infected cell. Viral conical capsid core is constructed by capsid monomers (p24). This capsid core is surrounded by viral matrix protein (p17) which provides intermediary between the capsid core and envelope. Two copies of the
single-stranded RNA are enclosed in the viral capsid core. A specific tRNAlys from the host cell is hybridized with the viral RNA genome at primer binding site (PBS) and this tRNAlys serves as a primer for reverse transcription. The viral RNA genome is coated by nucleocapsid protein (p7 or NC). Several viral enzymes and regulatory proteins are included in the capsid core. Those proteins include reverse transcriptase (p66/p51 or RT), protease (p11 or PR), negative regulatory factor (Nef), virion infectivity factor (Vif) and viral protein R (Vpr) (Turner & Summers, 1999) (Figure 1).

Figure 1. HIV-1 structure (US National Institute of Health, Carl Henderson)
1.2. HIV-1 genome organization

HIV-1 RNA genome is approximately 9.8 kb in length and encodes 15 proteins (Figure 2). Like eukaryotic cellular mRNA, HIV-1 RNA has post-transcriptional modification such as 5’ cap and 3’ poly A tail (Coffin et al., 1997). HIV-1 contains three major genes called gag (coding for structural proteins), pol (coding for viral enzymes) and env (coding for envelope glycoproteins). HIV-1 genome also codes 2 regulatory proteins: transcriptional transactivator (Tat), regulator of virus protein expression (Rev) and several accessory proteins (Nef, Vif, Vpr and Vpu) (Frankel & Young, 1998). The regulatory proteins control HIV-1 expression in the host cells, and accessory proteins play essential roles in virus persistence, spread and the interactions with cellular proteins (Emerman & Malim, 1998; Strebel, 2003). Long terminal repeats (LTR) sequences regions are composed by short directed repeat sequence (R), unique 5’ (U5) and unique 3’ (U3) sequences (U5-R-U3). LTR sequence contains trans-activation response (TAR) element, HIV-1 promoter and enhancer which are important in virus gene expression (Gendelman et al., 1986; Velpandi et al., 1992).

Figure 2. HIV-1 genome.
2. Early stage of HIV-1 infection

HIV-1 life cycle, that usually lasts one day in normal condition, can be divided into an early and a late stage of infection. The early stage of infection begins with the association of virion with the cell membrane, and finishes with the integration of the proviral DNA into the host genome. The late stage of infection includes viral gene expression, viral particles assembling and release and maturation of the progeny virions (Turner & Summers, 1999). My thesis mainly focuses on the early stage of HIV-1 infection.

2.1. Entry

HIV-1 host cells are mainly helper T cells, lymphocytes, monocytes, dendritic cells and macrophages (Coleman & Wu, 2009; Herbein & Varin, 2010; Wu & KewalRamani, 2006). HIV-1 enters the target cell through a pH-independent membrane fusion process (Stein et al., 1987). During the infection, HIV-1 envelope glycoprotein interacts with cellular receptor CD4 and co-receptor CCR5 or CXCR4 (Alkhatib et al., 1996; Ashorn et al., 1990; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996). The envelope glycoprotein is encoded by the virus envelope gene. The mature form of this glycoprotein can be divided into two regions: the surface region gp120 and the trans-membrane region.
gp41. The interaction between gp120 and primary cell CD4 receptor results the exposure of co-receptor binding site through the conformation change of gp120 domain (Wyatt & Sodroski, 1998). Further interaction between co-receptor binding site and co-receptor (CCR5 or CXCR4) leads to the additional conformation change of HIV-1 glycoprotein and inserts a hydrophobic gp41 derived peptide into the host cell membrane, which leads to the membrane fusion between HIV-1 envelope and target cell membrane (Doms & Moore, 2000). As a result of membrane fusion, virus core particle is released into the cytoplasm of the target cells.

2.2. Post-entry and Reverse transcription

Uncoating

The HIV-1 capsid core is made up of 1500 capsid monomers which assemble into a conical core structure with 100-120nm in long and 20nm in diameter (Gitti et al., 1996; S. Li et al., 2000; Momany et al., 1996; Worthylake et al., 1999). The capsid shell makes up the basic structure of viral core and protects RNA genome. After entry into the target cell, capsid core structure has to be disrupted in order to transport the viral genome into nucleus through nuclear pore complex, a process called uncoating. The time when the uncoating occurs is still a matter of debate. The early studies showed that viral capsid is disassembled immediately after virus entry into the cell (Auewarakul et al., 2005;
Lehmann-Che & Saïb, 2004). Those studies showed that after successfully uncoating, reverse transcription complex is formed and converts the viral RNA genome into cDNA. More recent studies suggested that the capsid core remains intact until the complexes reach the nuclear membrane (Arhel, 2010). The intact capsid core might be important for maintaining HIV-1 proteins necessary for reverse transcription and integration, and protecting the viral genome from cellular degradation.

**Reverse transcription**

Upon injection of the viral core into the cytoplasm of the target cell, HIV-1 core undergoes a partial and progressive reorganization process and the resulting complex is called the reverse transcription complex (RTC) (Lehmann-Che & Saïb, 2004; Warrilow et al., 2009). The RTCs contain different viral proteins including capsid, matrix protein, reverse transcriptase, integrase, and several cellular host proteins which play different roles during virus infection (Fassati & Goff, 2001; Karageorgos et al., 1993; McDonald et al., 2002; Nermut & Fassati, 2003; Warrilow & Harrich, 2007). Initiation of reverse transcription is triggered by the exposure of the RTCs to a high concentration of deoxyribonucleotides in the cytoplasm (Lehmann-Che & Saïb, 2004). Once reverse transcription is initiated, the DNA synthesis proceeds through a series of steps to generate the double-stranded viral cDNA. This process is catalyzed by the viral reverse transcriptase (p51/p66). The p66 subunit has both polymerase and RNase H activity which is essential for the reverse transcription reaction and p51 is mainly the structure
protein (Kohlstaedt et al., 1992; Patel et al., 1995; Sarafianos et al., 2009). Reverse transcriptase lacks proofreading activity which leads to 10- to 100-fold increase in errors during DNA synthesis (Harpstead, 2006). Error prone synthesis of viral cDNA is the most important contributor to the high rate of HIV-1 mutation which is thought to play a key role in the emergence of antidrug resistant mutations. After reverse transcription, newly synthesized viral cDNA and protein complex is called the pre-integration complex (PIC) which consists of double stranded viral DNA and a number of both viral and host proteins (Miller et al., 1997; Warrilow & Harrich, 2007).

2.3. Intracellular transport

After entering into the target cell, HIV-1 has to deliver its newly synthesized viral DNA into nucleus. Considering the viscous nature of the cell cytoplasm, active transport mechanism has to be employed by the virus. In the cell, cytoskeleton network is used by the cell for trafficking cellular cargoes within the dense cell cytoplasm (Rogers & Gelfand, 2000). It is believed that virus complexes in the cells are transported through the cellular cytoskeleton network. Fluorescent microscope studies showed that some virus intracellular complexes move along microtubule network inside the cell (McDonald et al., 2002).
2.4. Nuclear import

In order to infect non-dividing cells, the HIV pre-integration complex (PIC) containing viral cDNA genome has to be transported into nucleus through the nuclear pore complex. PIC has a diameter of 50nm that is larger than the central channel of the nuclear pore complex (NPC) (25nm) (Fouchier & Malim, 1999; McDonald et al., 2002; Miller et al., 1997). So it is unlikely for the PIC to enter the nucleus by passive diffusion in non-dividing cells. Some viral proteins (MA, IN, CA, Tat and Vpr) have been identified to contain nuclear localization signal (Bukrinsky et al., 1993; Efthymiadis et al., 1998) or can interact with cellular proteins that have a nuclear localization signal (Maertens et al., 2004), which might allow the direct transport of PICs through nuclear pore complex. The central DNA flap in viral cDNA, generated during the reverse transcription has also been proposed to act as a cis-acting element for HIV-1 genome nuclear import (Zennou et al., 2000).

2.5. Integration

Following nuclear import, integration of viral cDNA into the host chromosome is catalyzed by viral protein integrase (IN). Briefly, the 3’ end of the viral cDNA is processed by IN, and after cleaving of cellular genome DNA, strand transfer reaction is
catalyzed, which leads to the insertion of viral DNA into the cell chromosome (Alan Engelman et al., 1991; Van Maele & Debyser, 2005). Gaps in the DNA strands are filled by host cellular DNA repair enzymes (L. Li et al., 2000; Sakurai et al., 2009; K. E. Yoder & Bushman, 2000). The enzymatic assay showed that IN has the ability to perform integration reaction without the help of other viral and cellular proteins in vitro (Esposito & Craigie, 1999), but several cellular proteins have been identified to be involved in the integration process in vivo (Van Maele & Debyser, 2005). Those factors play some functional roles in the integration reaction, such as integration site selection, DNA gap repair and cellular chromatin remodeling. It has been suggested that integration reaction preferentially happened in active transcribed units (F. Bushman, 2002; F. D. Bushman, 2003; Ciuffi & Bushman, 2006; Ciuffi et al., 2005; Schröder et al., 2002).

3. Interaction of virus and cellular factors during the early stage of HIV-1 infection

During the infection, some cellular proteins are recruited into viral intracellular complexes by HIV-1 to facilitate its replication. Those cellular factors act as co-factors in virus infection. Conversely, mammalian cells also have a number of proteins to suppress virus replication, which is called restriction factors. Interaction between the virus and the host cell factors is dynamic and has been observed at every step in HIV-1 life cycle (F. D. Bushman et al., 2009; Freed, 2004; Goff, 2007; Komano et al., 2005; Sorin & Kalpana,
A better understanding of the interaction between HIV-1 and its host cell will provide us new knowledge about the HIV-1 life cycle, which can lead to the development of new antiviral therapeutic methods.

### 3.1. Restriction factors

Currently, two restriction factors shown to be potent inhibitors of early stage of the HIV-1 infection have been widely discussed: APOBEC3G and TRIM5alpha (R. S. Harris & Liddament, 2004; Newman & Johnson, 2007). APOBEC3G is a member of the family of vertebrate proteins with polynucleotide cytidine deaminase activity. APOBEC3G can be packaged into the assembling HIV-1 virions through the combined action of its RNA binding activity and interaction between APOBEC3G and certain region in Gag protein (Alce & Popik, 2004; Cen et al., 2004; Schäfer et al., 2004). After entering the target cells, APOBEC3G in the viral complexes deaminates cytidine residues in newly synthesized viral cDNA, which leads to the lethal G-to-A hypermutations in the HIV-1 cDNA genome (Mangeat et al., 2003; Mariani et al., 2003; Zhang et al., 2003). Vif, a viral protein, counteracts APOBEC3G by directly binding to it and recruit it to a cellular ubiquitin ligase complex for degradation (Mehle et al., 2004; Sheehy et al., 2002; Stopak et al., 2003). In the presence of Vif, APOBC3G cannot be packaged into virions. TRIM5alpha is a cytoplasmic protein that acts after virus entering into the target cells.
TRIM5alpha binds directly to HIV-1 capsid and causes conformational change of the capsid. This process accelerates virus uncoating and leads to the disruption of viral reverse transcription complex architecture, which abolishes the reverse transcription of virus RNA genome (Black & Aiken, 2010; Sebastian & Luban, 2005; Tang et al., 2009; Zhao et al., 2011).

3.2. Cellular proteins that facilitate HIV-1 infection

HIV-1 genome only encodes 16 proteins. As a parasite in the host cells, HIV-1 has evolved to make use of host cellular machinery to replicate itself. A lot of cellular proteins have been identified to be used by the virus during the infection (F. D. Bushman et al., 2009). Those cellular proteins are described below in detail.

Virus entry

The well known cell surface receptors for HIV-1 cell entry are CD4 and CCR5 or CXCR4 proteins (Dragic et al., 1996; He et al., 1997; Lama & Planelles, 2007). They are chemokine receptors which are involved in intracellular signal transduction. Interaction between HIV-1 and CXCR4 causes the activation of its downstream signals leading to depolymerization of F-actin which is necessary for virus entry process (A. Yoder et al., 2008). Molecules inhibiting downstream signaling from CXCR4 can also inhibit HIV-1
infection significantly. There are some other cell surface molecules that also participate in HIV-1 entry, such as integrin alpha4beta7 which can interact with gp120 and are involved in viral synapsee formation and cell to cell transmission (Arthos et al., 2008; Gorry & Ancuta, 2011).

**Reverse transcription**

HIV-1 reverse transcriptase has both DNA polymerase and RNase H activity. In vitro, reverse transcriptase can synthesize DNA from RNA without the help of other factors. Endogenous reverse transcription (ERT) of HIV-1 has been reported and plays an important role in viral infection of non-dividing cells (Zhang et al., 1996). In vivo, reverse transcriptase (RT) and nucleocapsid (NC) protein are two viral proteins that perform the conversion of genomic RNA into DNA. The polymerization activity of RT is greatly facilitated by NC protein which has nucleic acid binding activity and serves as a chaperon by refolding of nucleic acids into the most thermal stable conformation and eliminating secondary structures formed in viral RNA (Johnson et al., 2000; Levin et al., 2005). A number of reports have shown that some host factors in the cell are required for the synthesis of HIV-1 proviral DNA. Recent studies suggest the cellular protein HuR, AKAP149 and DNA topoisomerase I are involved in reverse transcription by direct interaction with RT (Warren et al., 2009). AKAP149, a kinase anchoring protein, has been identified to have direct interaction with reverse transcriptase in both the yeast two-hybrid system and human cells. AKAP149 silencing by RNAi and mutation in
AKAP149 binding region of RT inhibit HIV infection at the reverse transcription step (Lemay et al., 2008).

**Transport of viral complex to the nucleus**

The intracellular trafficking of viral complexes is not well understood. It is suggested that the cellular transport system is utilized by virus to transport viral cDNA into the nucleus (Anderson & Hope, 2005; Dvorin & Malim, 2003; McDonald et al., 2002). Several genome wide siRNA screens have identified some proteins that may play a role in the cellular trafficking of viral complexes. Those cellular factors include AKAP13, some guanine exchange factors, and factors regulating actin filament and microtubule network (Brass et al., 2008; König et al., 2008; Liu et al., 2011). Translocation of viral complex across the intact nuclear membrane also needs the involvement of cellular factors. A number of HIV-1 proteins have been shown to bind to members of importin α family which can interact with importin β at the nuclear pore complex (Ao et al., 2010; Bukrinsky & Haffar, 1999; Goff, 2007; Suzuki & Craigie, 2007). Several cellular factors have been shown to have direct interaction with the viral pre-integration complex (PIC). Those factors include barrier to autointegration factor, Lap 2alpha, HMG I(Y) protein et al. (Chen & Engelman, 1998; Farnet & Bushman, 1997; Lin & Engelman, 2003; Van Maele et al., 2006). Some nuclear pore complex proteins (Nup98, Nup124p, Nup358 and Nup153) have also been identified to be involved in the process of nuclear import of HIV DNA genome (Ebina et al., 2004; Goff, 2008; König et al., 2008; Lee et al., 2010; Lever & Jeang, 2011; Woodward et al., 2009).
**Integration into host cell genome DNA**

Viral integrase is the main protein responsible for integrating of viral DNA into host cell DNA genome. However, studies suggest that some cellular proteins and factors are involved in this process (Farnet & Bushman, 1997; Van Maele et al., 2006). Barrier-to autointegration factor (BAF) has been identified to associate with PIC in the cell (Chen & Engelman, 1998; D. Harris & Engelman, 2000). BAF can prevent suicidal autointegration and also can interact with proteins having nuclear structure organization function. High mobility group chromosomal protein A1 (HMGA1), a non-histone DNA binding protein, has been shown to have the ability to restore in vitro PIC integration activity after salt-stripping treatment (Engelman, 2003; Van Maele et al., 2006). Integrase interactor 1 (INI1), a component of the chromatin remodeling SWI/SNF, has been identified to interact with HIV-1 integrase using the yeast-two-hybrid system and activates its DNA-joining activity (Kalpana et al., 1994; Morozov et al., 1998). LEDGF/p75 (lens-epithelium-derived growth factor) has also been identified to interact with HIV-1 integrase. Knock down of LEDGF/p75 by siRNA reduces HIV-1 replication significantly. It is suggested that the function of LEDGF/p75 is to tether HIV-1 integrase to the chromosome, which promote the interaction of viral DNA into cellular DNA (Busschots et al., 2007; Ciuffi et al., 2005; Alan Engelman & Cherepanov, 2008; Llano et al., 2006; Vanegas et al., 2005).
4. Approaches to study the early stage of HIV-1 infection

4.1. Virus complexes isolation and characterization

Attempts to define the composition of RTCs or PICs isolated from infected cells have not yielded a clear answer, since the nature of the viral and cellular components found to be associated with the viral genome in the cell cytoplasm depends on the technique used for purifying the viral intracellular complexes, which are very sensitive to detergents and isolation conditions. In most experiments, RTCs or PICs were purified by velocity sedimentation and equilibrium density fractionation methods. The size and density are profoundly altered by different treatments even with mild detergents and moderate salt concentrations. Regardless of those problems, RTCs have been shown to have a sedimentation velocity of approximately 350 S and a density of 1.34 g/ml in equilibrium gradients (Fassati & Goff, 2001; Nermut & Fassati, 2003). Most studies show that HIV PICs contain protease (PR), reverse-transcriptase (RT), integrase (IN) and Vpr, and the presence of the structural proteins such as capsid, nucleocapsid and matrix is more controversial (Khiytani & Dimmock, 2002). The capsid (CA) proteins are thought to be released soon after infection and only trace amounts are found in PICs. Whereas nucleocapsid (NC) and matrix (MA) were initially thought to be associated with PICs, more recent studies revealed that the majority of these proteins are lost during the uncoating process. As some viral structural components are released, certain cellular
proteins associate with the PICs during their journey to the nucleus, such as the high
mobility group protein HMG I (Y), host lens epithelium-derived growth factor (LEDGF)
which have been proposed to be important for integration. But there is still no clear
conclusion about the molecular nature of RTCs/ PICs and cellular factors needed for
reverse transcription, intracellular trafficking, nuclear import and attachment to
chromosome. New technical approaches will have to be developed in order to unravel
this question.

4.2. Fluorescence microscope

Fluorescence microscope has become a widespread tool to investigate biological
processes including protein-protein interactions, protein localization, and organelles
movement in the cell. Studies about the intracellular trafficking of HIV-1 in the infected
cells have been benefited from this technology. Several fluorescence-based methods have
been developed to detect and characterize individual viral intracellular complexes in the
infected cells (McDonald et al., 2002). To make fluorescently labeled HIV-1 particles,
Vpr-GFP is commonly used to incorporate GFP into viral particles. Vpr is an accessory
protein and can be packaged into viral particles through the interaction with gag p6
region during virus assembly. Labeled virus are generated by cotransfection of HIV-1
proviral DNA with a plasmid that express GFP fused to the N-terminus of HIV-1 Vpr
Using this method, GFP labeled virus complexes can be observed in live cells. Intracellular HIV-1 complexes are shown to be associated with microtubules and use cytoplasmic dynein to move around in the cell (McDonald et al., 2002; Muthumani et al., 2000). The majority of virus particles entering into the target cells do not lead to a productive infection. The ratio of virus particles to infectious units is usually around 1000:1 (Welker et al., 2000). Study of those virus particles in the infected cell may generate misleading result because the majority of the particles in the infected cell may not be on the way to successful infection.

4.3. Genome-wide screen to identify cellular factors that is important for HIV-1 infection

RNA-interference (RNAi) is a powerful technique to study the loss-of-function phenotypes by specifically knocking down certain cellular gene. The RNAi screen studies have become a new way to explore the relation between virus and the host cells during the infection. Genome-wide screens using RNA-interference technologies have revealed a wealth of host factors that play important role in HIV-1 infection (Brass et al., 2008; F. D. Bushman et al., 2009; König et al., 2008; Yeung et al., 2009; Zhou et al., 2008). But the majority of those host factors identified by those RNAi screens have not been carefully analyzed and mechanisms of those factors in HIV infection are largely unknown.
5. Mitochondria and virus infection

Mitochondria are multifunctional organelles with diverse functions including ATP production, cell proliferation, apoptosis, synthesis of amino acids, nucleotides, innate immune response, diseases and aging. Recent studies have revealed that mitochondria play a key role in host antiviral responses (Belgnaoui et al., 2011; Seth et al., 2006; West et al., 2011). In order to evade host response started by mitochondria, viruses have developed various strategies to modulate the function of mitochondria during the infection. Many viral proteins target to mitochondria and interact with mitochondrial proteins.

5.1. Innate immune response and virus

Innate immune response to virus infection

After sensing the virus, host cell innate immune response is triggered in order to eliminate the invading virus. Host cell defense system detects pathogen-associated molecular patterns (PAMPs), which include exogenous nucleic acids, virus structure proteins, and transcriptional products (Nürnberger & Kemmerling, 2009). Pattern recognition receptors (PRRs) detect PAMPs generated during virus infection. There are three types of PRRs including toll-like receptor (TLRs), retinoic acid-inducible gene-1
like receptor (RLR), and nucleotide oligomerization domain like receptors (NLRs) (Kawai & Akira, 2006, 2007). Recognition of PAMPs initiates a variety of signal transduction pathways leading to the production of interferons (INFs), inflammatory cytokines, and chemokines. Mitochondria have been shown to be a key location for the RLR signaling pathway. Retinoic acid-inducible gene I (RIG-1) and melanoma differentiation-associated gene 5 (MDA-5) can recognize viral RNAs. Mitochondrial antiviral signaling protein (MAVS), a RIG-I binding protein, has been identified as signal transducer downstream of RIG-I and MDA5. It has been demonstrated that MAVS is localized on the outer membrane of mitochondria (Seth et al., 2005). Moreover, two other mitochondria associated proteins, stimulator of IFN gene and mitochondrial tethering protein mitofusin 2, are also involved in the RLR signaling pathway as signal transducers (Koshiba et al., 2011; Ohta & Nishiyama, 2011). On the other hand, the negative regulators of RLR-induced signals have recently been identified such as NLRX1 which is located on the out membrane of mitochondria and acts as a negative regulator of RLR-MAVS-mediated antiviral signaling. Those findings suggest that mitochondria are key organelles for the innate immunity and serve as an IFN-signaling platform (Arnoult et al., 2009; Arnoult et al., 2011; Koshiba, 2012; West et al., 2011).

Viral strategies against host immune response

Different strategies have been developed by viruses in order to escape from host immune responses like RLR-mediated IFN production. A lot of viral encoded proteins
have been identified to have ability to inhibit RLR signaling pathway. Those viral proteins associate with viral RNA and interfere with transcriptional factors downstream of RLRs, or regulate other mitochondria-associated signal transducers (Ohta & Nishiyama, 2011). Hepatitis C virus encodes NS3 which specifically inhibits RIG-I mediated signaling by cleaving MAVS and preventing MAVS anchoring into mitochondria surface. A serine protease, encoded by Picornaviridae hepatitis A virus, can cleaves MAVS at Gln428 and thus block antiviral immune signaling triggered by MAVS (Ohta & Nishiyama, 2011).

5.2. Apoptosis and virus infection

Apoptosis

Apoptosis is a programmed cell death characterized by membrane blebbing, condensation of the nucleus and cytoplasm and endonucleosomal DNA cleavage. Apoptotic cell death is an early host cell response to virus infection. Apoptosis can be triggered by two major pathways: extrinsic pathways and intrinsic pathways (Sanfilippo & Blaho, 2003). The extrinsic pathway is activated by binding of the extracellular ligands to the cell death receptors including TNF receptor-1, CD95/Fas and TNF-related apoptosis inducing ligand receptors-1. Ligand binding initiates apoptosis process and causes the assembly of death inducing signaling complex with the recruitment of other
proteins like caspases-8, caspase-9, caspase-3 and caspase-7. In the intrinsic pathway, following mitochondrial membrane permeabilization, intermembrane space proteins are released including cytochrome c, Smac/DIABLO, Omi/HtrA2 and caspase independent death effectors including apoptosis-inducing factor (AIF) and endonuclease G. These mitochondrial intermembrane space proteins serve as caspase activators and activate caspase-9 and caspase-3. Finally, cytochrome c, caspase-9, and apoptosis protease activating factor 1 form apoptosome, which activates effectors caspases and leads to cell death (Green, 1998, 2005; Ohta & Nishiyama, 2011).

**HIV-1 induced apoptosis**

HIV-1 infection leads to the progressive depletion of key immune cells including CD4-positive T lymphocytes, macrophages, monocytes and microglial cells. It has been demonstrated that apoptosis may be the major contributor to depletion of those immune cells during AIDS (Février et al., 2011). It has been showed that HIV-1 encoded proteins including Env, Nef, Tat, protease and Vpr are major players in HIV-1 induced apoptosis (Ohta & Nishiyama, 2011). Those viral encoded proteins can change mitochondrial membrane potential directly or indirectly, which is the common mechanism to induce apoptosis by viral proteins. Apoptosis in HIV-1 infected patients is predominantly associated with the expression of low levels of Bcl-2, an inhibitor of mitochondrial membrane permeability (Ohta & Nishiyama, 2011; Selliah & Finkel, 2001).
6. Goals of this dissertation

Study the important role of mitochondria in the virus infection

During the early stages after HIV-1 infection, the viral genome is reverse transcribed into a double stranded DNA that is subsequently modified by viral integrase and translocated into the nucleus where it is integrated into a host chromosome. Various cytoplasmic structures in the target cell have been implicated in this process and a large number of host cell factors have been shown to be essential for HIV-1 infection. Although mitochondria play a key role in innate immune response and apoptosis during virus infection, studies of HIV-1 biology have usually not focused on a role for mitochondria in early stage of HIV-1 infection. The studies in this thesis mainly focus on exploring the role of mitochondria in the early stage of HIV-1 infection.

ρ₀ cell as a model system to study the function of mitochondria

The contribution of mitochondria to the HIV-1 infection was investigated through infection of ρ₀ cell which is a cell line that was completely depleted of mtDNA. Cell lines lacking mtDNA (ρ₀ cells) have been isolated from various eukaryotic species by long-term exposure to ethidium bromide (King & Attardi, 1989, 1996). Depletion of mtDNA in ρ₀ cells produced functional and morphological changes in mitochondria and only had minor effects to the nuclear genome and encoded protein (Chevallet et al., 2006). Loss of mtDNA in ρ₀ cells was associated with significant distortion of mitochondrial
structure, decreased electron density of the matrix and disorganized inner and outer membranes, resulting in the appearance of ‘ghost-like’ mitochondria (Holmuhamedov et al., 2003). However, the number of mitochondria-like structures was not significantly different between mtDNA-deficient and parental cells. Generation of mtDNA depleted mutants provides a unique tool to directly study the role of mitochondrial DNA and encoded proteins.
Chapter 2. Materials and Methods

1. Oligonucleotide primer sequences

Synthetic oligodeoxynucleotides were purchased from Sigma (St. Louis, MO). Probes for real-time PCR were purchased from Operon (Huntsville, AL). The sequences for the oligonucleotides used are as follows:

MH533:  5'-ACCCACTCCCTCTTAGCCAA TA TT-3'
MH534:  5'-GTAGGGCTAGGCCCACCG-3'
Mito-probe:  5'-(TET)-CTAGTCTTTGC CGCCTGCGAAGCA-(TAMRA)-3'
MH535:  5’-AACTAGGAACCCACTGCTTAAG-3’
Early reverse:  5’-CTGCTAGAGATTTTTCCACAC-3’
MH603: 5’-(FAM)-ACACTACTTTGAAGCA CTCAAGGCAAGCTTT-(TAMRA)-3’
MH531:  5’-TGTGTGCCCGCTCTGGTGTG-3’
MH532:  5’-GAGTCCTGCGTCGAGAGGC-3’
LRT-P:  5’-(FAM)-CAGTGGCGCCCGAAGGAACAGGA-(TAMRA)-3’
Alu2:  GCCTCCCCAAAGTGCTGGGATTA CAG-3’
GST-LTR5: AGACAGATAGGGCG TTAAACTAGGGAACCCACTGCTTAAG-3’
GST:  AGACAGATAGGGCGTTAAAC-3’
dNK-11-26-5:  GCCTCCGCAAAGAAATAAAG-3’
dNK-11-26-3: CCAATCCGGCAACGCACTG-3’
2. Cells and materials

HEK293T cells and HOS cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM glutamine, 10% fetal calf serum and 50µg/ml gentamicin (complete medium) at 37°C in an atmosphere containing 5% CO₂. Human osteosarcoma (HOS) cells (HOS-CD4-CCR5), anti-p24 monoclonal antibody (mAb AG3.0) were received from NIH AIDS Reagent Program. ρ⁰ cells (143B206) were a derivative of the 143 B TK- human osteosarcoma (HOS) cell line. ρ⁰ cells were grown in DMEM supplemented with 2 mM glutamine, 10% fetal calf serum, 50µg/ml gentamicin, 10mM Sodium pyruvate and 50µg/ml Uridine. Alexa-488 conjugated anti-IgG antibody and MitoTracker Red were purchased from Invitrogen. DNeasy blood tissue kit was purchased from Qiagen. Oligonucleotides used in this study were synthesized by Sigma-Aldrich.

3. VSV-G pseudotyped virus production

VSV-G pseudotyped HIV-1 vector stocks (HIV-GFP) were generated by co-transfecting HEK293T cells with plasmids (pCMV-dR8.91, pMD2.G and pWPI).

HEK293T cells (1X 10⁷) were seeded in 75 cm² flasks and transfected the following day by polyfect transfection reagent (Qiagen) with 4 µg of plasmid pWPI (containing the
green fluorescent protein cDNA), 4µg of plasmid pCMV-dR8.91 (containing HIV-1 genes pol, gag, tat, and rev, but contains deletions in the accessory genes and env), and 4 µg of plasmid pMD2.G (containing the vesicular stomatitis virus envelope gene) per flask. The medium was replaced with fresh completed medium after 24 hours incubation in the incubator. The virus-containing supernatant was collected between 48 hours and 96 hours after transfection and filtered through a 0.22-µm-pore-size filter (Millipore, Billerica, MA). The virus (HIV-GFP) was concentrated using centrifugal filter units (Ultracel-100K) (Millipore, Billerica, MA), and aliquots were frozen and stored at −140°C. The capsid contents of the virus stocks were quantified by p24 antigen enzyme-linked immunosorbent assay following the manufacture’s instruction (Perkin Elmer, Massachusetts).

4. Analysis of virus infection by flow cytometry

HOS cells (0.25X10⁵/well) were seeded in 24-well plate and incubated overnight at 37°C in an atmosphere containing 5% CO₂. Cells were inoculated with various concentrations of virus (HIV-GFP) in a total volume of 500µl. Two days after infection, the cells were detached using 0.025% trypsin and washed one time using 1ml PBS. The cells were subsequently fixed by the addition of 0.4ml phosphate-buffered saline (PBS) containing 1% paraformaldehyde (PFA). Green fluorescent protein (GFP) expression was
quantified by fluorescence-activated cell sorting (FACS) by using a FACSCalibur instrument (Becton Dickinson) and the percentage of GFP-expression cells was quantified with Flow Jo 7.6.1. A minimum of 10,000 cells were analyzed for each sample. Data was analyzed and plot was generated in Excel.

5. Virus infection in ρ₀ and HOS cells

HOS and ρ₀ cells were seeded in 24-well plate at the concentration of 0.25X10⁴/well. Cells were grown in DMEM supplemented with 50 µg/ml uridine and 1mM sodium pyruvate. The next day, different amounts of HIV-GFP (2, 5, 10, 20ng of p24) were added to the cells in a total volume of 0.5ml/well. Two days after infection, virus infection efficiency was analyzed by FACS as described in method 4.

6. Analysis of the effect of mitochondrial inhibitors on virus infection

HOS cells (0.5 X 10⁵/well) were cultured in 12-well plate overnight. The next day, different amounts of antimycin A, oligomycin and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were added to the cultured HOS cells with or without
the supplementation of 1mM sodium pyruvate and 50 µg/ml uridine. Six hours later, 10µl of virus (10ng p24) was added in the presence of 10µg/ml polybrene. Two hours after infection, medium was replaced with fresh complete medium containing appropriate inhibitors. Two days after infection, cells were harvested and fixed with 1% paraformaldehyde. Virus infection efficiency was measured by FACS as described in method 4.

7. Construction of cybrid cells by fusion of ρ⁰ with enucleated HEK293T cells

HEK293T cells were seeded in 6-well plate and grown until they were 70~80% confluent. To enucleate the cells, 0.5~5µg/ml actinomycin D were added to the cells and incubated for 20 hours. Cells were washed twice with PBS and 1X10⁶ ρ⁰ cells were added on top of actinomycin D treated HEK293T cells and incubated at 37 °C for 5 hours allowing ρ⁰ cells to attach and make contact with HEK293T cells. Cells were washed three times with plain DMEM. Freshly prepared 1ml of fusion medium (1g/ml PEG 1450, 20% DMSO in DMEM) were added to the cells and incubated for 2 minutes at room temperature. Cells were washed with DMEM three times and incubated overnight in complete medium (10% FBS). The next day, cells were detached and reseeded at very low concentration (1:20 dilution) in 6-well plate in DMEM supplement with 10%
dialyzed FBS and 100µg/ml BrdU. Medium was changed at 3 days interval. The colonies were visible after 3 weeks incubation at 37 °C. Single clones were isolated using cloning rings and trypsin treatment. Isolated cybrid cell clones were grown in complete DMEM in the absence of pyruvate and uridine. Total cellular DNA was isolated by using DNeasy blood tissue kit and the presence of mtDNA in cybrid was measured by real-time PCR as described below. Infection in HOS, ρ⁰ and cybrid cells were carried out as described in method 4.

8. Viral cDNA isolation from infected cells (HOS, ρ⁰ and cybrid cells)

HOS, ρ⁰ or cybrid cells (2X10⁵) were seeded in 6-well plates and incubated at 37 °C overnight. The next day, HIV-GFP stocks were treated with 200U/ml DNase I at 37°C for 2 hours to remove contaminating plasmid DNA. Inoculation were normalized by p24 content to 200ng of p24 per well in a total of 1.5ml medium in the presence of 10µg/ml polybrene. Medium was changed at 2 hours after inoculation. At different times (2, 6, 10, 24, 48 and 72 hours) after inoculation, cells were washed 5 times with 1ml PBS and then detached with 0.0025% trypsin. The trypsin was deactivated by the addition of 1ml complete medium and then cells were pelleted and washed three times with 1ml PBS. The cell pellets were resuspended in 200µl PBS and total DNA was isolated using
the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer’s instructions. Infection efficiency was measured by FACS using the cells collected at 72 hours after inoculation.

9. Real-time PCR quantification

Unless otherwise specified, quantitative PCR was performed in triplicates, in TaqMan Universal PCR master mix using 200nM of each primer and 250nM TaqMan probe. A 4.6µl sample was used in each PCR measurement in a total volume of 10 µl. Thermal-cycling conditions were 3 minutes at 95 °C, 15 second at 95 °C and 1 minute at 60 °C for 40 cycles.

9.1. Mitochondrial DNA detection

The primer sets used to detect mitochondrial DNA (encompassing nucleotide position 10620-10710) were forward primer (MH533), reverse primer (MH534) and probe (mito-probe). A mitochondrial DNA fragment (nucleotide position 10620-10710) was cloned into a plasmid containing pGEX-3X (Addgene) backbone. The recombinant plasmid (pGST-MT) was used as a standard to generate the standard curve in this experiment. Thermal-cycling condition was as described above.
9.2. Viral cDNA detection

9.2.1. Viral early and late reverse transcription product

Viral late reverse transcription products were detected using primers that amplify the region flanking U5 and primer binding site (PBS) site. Viral early reverse transcription products were detected using primers that amplify the region in U5. The primer sets used to detect virus early reverse transcription product were forward primer (MH535), reverse primer (Early reverse) and probe (MH603). The primer sets used to detect virus late reverse transcription product were forward primer (MH531), reverse primer (MH532) and probe (LRT-P). Linearized plasmid WPI (digested by restriction enzyme XbaI) was used as the standard in real-time PCR to generate the standard curve. Thermal-cycling condition was as described above.

9.2.2. Integrated viral DNA

Alu-LTR based real-time nested-PCR

Detection of integrated proviruses was accomplished by Alu-LTR based real-time nested-PCR. In the first round Alu-LTR PCR, forward primer (GST-LTR5) and reverse primer (Alu2) were used. In the second-round nested real-time PCR, forward primer (GST), reverse primer (early reverse) and probe (MH603) were used. The first round of PCR was performed by using 4.6µl of each sample (total DNA from infected cells) in a
20µl reaction mixture containing 1X TaqMan PCR master mix, 100nM Alu2 primer, and 600nM GST-LTR5 primer. The first-round PCR cycle conditions were as follows: a denaturation step of 2 min at 94°C and then 20 cycles of amplification (94°C for 30 s, 50°C for 30 s, and 72°C for 2 min). Linear, one-way amplification was also monitored by performing the pre-amplification PCR with either the GST-LTR5 alone or the Alu 2 primer alone.

Sample from the first-round PCR product (1µl) was used as template for second round quantitative real-time nested PCR in a total volume of 10µl PCR reaction mix containing 200nM GST primer, 200nM MH535 primer and 200nM probe (MH603). The nested PCR conditions were as follows: a denaturation step of 2 minutes at 94°C and then 40 cycles of amplification (94°C for 30 s, and 60°C for 30 s). Total cellular DNA isolated from infected cells (17% GFP positive, 40 days after infection) was used as a standard. Integrated viral DNA in the standard was quantified using the late reverse transcription primer set (MH531, MH532 and Late RT-probe). A standard curve was generated by two-stage PCR amplification of a serial dilutions of the standard. The copy number of integrated HIV DNA in the samples was determined in reference to the standard curve.

9.2.3 2LTR circle

The 2-LTR circle primers amplify the linkage region between 5’ and 3’ LTR ends. The primer sets used to detect virus 2-LTR circles were forward primer (MH535), reverse
primer (2LTR-3) and probe (MH603). A DNA fragment containing 2LTR junction sequence (U5U3) was cloned into a plasmid containing pGEX-3X (Addgene) backbone. The recombinant plasmid (p2LTR) was used as a standard in the measurement of 2LTR circles. Thermal-cycling conditions were 9 min at 95 °C, 15 s at 95 °C and 15 s at 60 °C for 40 cycles.

9.3. Cell number reference assay

Cell number variation was measured using real-time PCR for RNase P gene DNA by using Taqman Copy Number Reference Assay, RNase P (Invitrogen). Primers are premixed in the master mix from manufacture. Cellular DNA isolated from a certain number of HOS cells was used as the standard to generate standard curve in the PCR analysis. Cell number in the measured samples was calculated based on the standard curve. Thermal-cycling conditions were as described above.

10. Immunochemistry and confocal fluorescence microscope

HEK293T cells (1X10^5) or HOS cells were seeded on the glass coverslip in 6-well plates. Cells were incubated at 37 °C overnight and then infected with 20µl virus in the presence of 10 µg/ml polybrene (MOI ~10). Six hours after infection, 300nM
MitoTracker was added to the cells and incubated for another 30 minutes. Cells were washed 3 times using DMEM and one time with PBS. Samples were fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature and subsequently washed 5 times with PBS. Fixed cells were permeabilized with 0.0025% digitonin in PBS for 20 minutes at room temperature and subsequently blocked with blocking buffer (3% BSA, 0.0025% digitonin in PBS). Samples were incubated with monoclonal anti-p24 antibody (diluted 1:100 in wash buffer) overnight at 4 °C in a humidified atmosphere followed by incubation with Alexa 488-conjugated anti-mouse IgG secondary antibody (1:300 in wash buffer) for 2 hours at room temperature. Samples were mounted onto glass slides with Prolong Gold Anti-fade reagent (Invitrogen). Imaging was performed by using Leica SP5 spectral confocal inverted microscope (SP5 inverted) or a laser-scanning confocal microscope (LSM510, Carl Zeiss). Cell images were acquired by using the 63X oil objective, with zoom factor of 2~3. Optical slices were taken every 200nm interval along the z axis covering the whole depth of the cells. Appropriate laser lines for each fluorophore were used: 488nm line for Alexa Fluor 488; 543-nm line for MitoTracker Red; 633-nm line for Alexa Fluor 647. Nanoscope image was taken by Dr. George McNamara in Seattle. Images were analyzed using ImageJ, Zeiss LSM Image Browser and softWoRx Explore.
11. Detection of newly synthesized mitochondrial DNA

HOS cells (1X10^5) were seeded on the coverslip in 6-well plates. Cells were incubated at 37 °C overnight. The next day, aphidicolin (APH, Sigma, St. Louis, MO) was added into the cells at a concentration of 10µM. Two hours later, 100µM EdC was added into the cell. After 6 hours incubation, 300nM MitoTracker was added to the cells and incubated for another 30 minutes. And then cells were washed and fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. Coverslip was incubated with 300µl Image-it signal enhancer solution (Invitrogen) for 1 hour at room temperature to block no specific binding of the fluorescent dye to the samples. Click-it assay was performed following the manufacturer’s instructions. To label newly synthesized DNA, samples were incubated with 200µl Click-it labeling mix containing Alexa 488-azide for 30 min at room temperature. Coverslips were washed and mounted onto glass slides with a drop of Prolong Gold Anti-fade reagent. Imaging was performed as described before.

12. Detection of viral cDNA synthesis in infected cells by Click-it method

HOS cells (1X10^5) were seeded on the coverslip in a 6-well plates. Cells were incubated at 37 °C overnight. The next day, 100µM EdC was added into the cells and incubated for 1 hour. Cells were subsequently infected with 100µl virus (MOI~5) in the
presence of 10µg/ml polybrene. Six hours after infection, 300nM MitoTracker was added to the cells and incubated for another 30 min. Cells were washed 3 times using DMEM and one time with PBS. Samples were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and washed 5 times with PBS. Fixed cells were permeabilized with 0.0025% digitonin in PBS for 20 min at room temperature and subsequently subjected to the Click-it assay as described in method 11. Samples were washed and blocked with blocking buffer (3% BSA, 0.0025% digitonin in PBS) for 30 min at room temperature. Samples were then incubated with monoclonal anti-p24 antibody (diluted 1:100 in wash buffer) overnight at 4 °C in a humidified atmosphere and followed by incubation with Alexa 647-conjugated anti-mouse IgG secondary antibody (1:300 in wash buffer) for 2 hours at room temperature. Samples were mounted onto glass slides with a drop of Prolong Gold Anti-fade reagent. Imaging was performed by using Leica SP5 spectral confocal inverted microscope (SP5 inverted). Cell image were acquired by using the 63X oil objective, with zoom factor of 2~3. Appropriate laser lines for each fluorophore were used: 488nm line for Alexa Fluor 488; 543-nm line for MitoTracker Red; 633-nm line for Alexa Fluor 647. Images were analyzed using LAS AF Lite software.
13. Mitochondria morphology of HOS, ρ₀ and cybrid cells

HOS, ρ₀ or cybrid cells (1X10⁵) were seeded on the coverslip in 6-well plates. Cells were incubated at 37 °C overnight. The next day, 300nM MitoTracker Red was added to the cells and incubated for another 30 min. Cells were washed twice with PBS. Samples were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and washed 5 times with PBS. Fixed cells were permeabilized with 0.0025% digitonin in PBS at room temperature for 20 min. Samples were mounted onto glass slides with Prolong Gold Anti-fade reagent. Imaging was performed by using Leica SP5 spectral confocal inverted microscope (SP5 inverted). Cell image were acquired by using the 63X oil objective lens. 543-nm laser line was used to detect MitoTracker Red signal.

14. Cell extracts and virus DNA identification

Approximately 1X10⁷ HEK293T cells or 1X10⁶ HOS cells were infected in 75-cm² flasks with 300 µl of virus stock in the presence of 5µg/ml polybrene (Sigma). Cells were then incubated at 37°C for 6 hours, washed 5 times with DMEM, trypsinized and washed again with PBS. Cells were incubated with 50µM nocodozal (Sigma) and 10µM taxol (Sigma) at 37 °C for 30 min and then pelleted again. The pellet was washed with ice-cold PBS twice. The pellet containing the infected cells was re-suspended in 1ml hypotonic
buffer (10mM HEPES, pH 7.6, 1.5mM MgCl₂, 10mM KCl) and centrifuged for 4 min at 3000 rpm (500g) in an Eppendorf centrifuge. The cell pellet was re-suspended in 1ml hypotonic buffer and incubated for 10 min on ice. Cells were lysed by pipetting up and down 50 times using 1ml pipette to break the cell membrane. Nuclei and unbroken cells were removed by centrifugation at 3000 rpm (500g) for 4 minutes (Pellet 0). The cytoplasmic extract was centrifuged at 7000 rpm (2700g) for 4 minutes, and the pellet was called pellet 1 (P1). The resulting supernatant was centrifuged again at 14000 rpm (11000g) for 4 minutes, and the pellet was called pellet 2 (P2). The final supernatant was called Sup. The pellets (P1 and P2) in each of the fractions were resuspended in approximately 400μl of isotonic buffer (10mM HEPES, pH 7.6, 150mM KCl, 5mM MgCl₂). Some oligonucleotides were added into all the samples (P1, P2 and Sup) to serve as a carrier for DNA purification. dNK gene fragments (500 bps) (1ng) were added to each fraction to serve as internal control for the measurement of DNA isolation efficiency. All the fractionation were lysed with 0.5% SDS and digested with proteinase K for 1 hour at 56 °C. DNA was purified by phenol/chloroform extraction and ethanol precipitation method. Virus cDNA was identified by real time PCR using the specific primers and probes as described before. DNA isolation efficiency was evaluated by SYBR green real-time PCR using primers for dNK gene. The primers used to quantify dNK in the samples were dNK-11-26-5 and dNK-11-26-3. Thermal-cycling conditions were 3 min at 95 °C, 15 s at 95 °C and 1 min at 60 °C for 40 cycles.
15. Antibody-mediated binding of mitochondria and viral cDNA

15.1. Bind of anti-Tom22 antibody to the protein A beads

Protein A beads (20µl) were incubated with 5µl anti-Tom22 antibody (in 100µl PBS) for 1 hour at room temperature. Unbound anti-Tom22 antibody was then washed out with PBS. The beads were further incubated with 3% BSA at room temperature for 1 hour and then kept on ice.

15.2. Isolation of mitochondria from infected cells

The infection and cell lysis preparation were the same as described in method 14. PFA was added to cytoplasmic extract to a final concentration of 4% and the sample was incubated at room temperature for 20 min. The fixed cell extract was centrifuged at 14000 rpm (11000g) for 10 min. The mitochondria pellet was re-suspended in PBS and re-pelleted again. This process was repeated for 3 times. Finally, the mitochondria pellet was re-suspended in 1ml isotonic buffer.
15.3. Antibody-mediated binding

Fixed mitochondria from infected cells (200µl) were pre-incubated with BSA (3%) at room temperature for 30 min to block the non-specific binding. The suspension (20µl) was incubated with 5µl beads (protein A beads, protein A beads with anti-Tom22 antibody). After 2 hours incubation at room temperature, beads were collected with a magnet. The beads were re-suspended and treated with the magnet again. This wash step was repeated for 4 times. Before the last wash, the beads were transferred into a fresh tube to avoid the material carryover in the tube. After the wash steps, binding material was eluted by 0.5% SDS and incubated at 65 °C overnight to reverse the crosslink. Oligonucleotides (1µg) was added into each sample to serve as a carrier for DNA isolation. dNK gene fragments (1ng) were added to each sample to serve as internal control for the measurement of DNA isolation efficiency. DNA was purified by phenol/chloroform extraction and ethanol precipitation method. Purified DNA was dissolved in 100µl H₂O. Viral cDNA and mtDNA were quantified by quantitative real time PCR as described in method 9. DNA isolation efficiency was evaluated by SYBR green real-time PCR using primers for dNK gene as described before.
The $\rho^0$ cell line used in this study is a derivative of the 143 B.TK$^-$ osteosarcoma cell line which was generated by culturing 143B.TK$^-$ cells in the presence of low concentration of ethidium bromide. Depletion of mtDNA in $\rho^0$ cells produced functional and morphological changes in mitochondria and only had minor effect to the nuclear genome and encoded proteins. Loss of mtDNA in $\rho^0$ cells was associated with significant distortion of mitochondrial structure, decreased electron density of the matrix and disorganized inner and outer membranes, resulting in the appearance of ‘ghost-like’ mitochondria. Generation of mtDNA depleted mutants provides a unique tool to directly study the role of mitochondrial DNA and encoded proteins in the cell. The contribution of mitochondria to the HIV-1 infection was investigated through virus infection in $\rho^0$ cells.

1. **HIV infection in $\rho^0$ cells is defective in comparison with HOS cells**

1.1. **HIV infection in HOS and $\rho^0$ cells**

The infection efficiency of HIV in $\rho^0$ cells and HOS cells (parental cell line of the $\rho^0$ cell) was compared in this study. HIV-GFP viral genome contains a green fluorescent protein (GFP) gene and a successful infection will lead to the expression of GFP. Virus infection efficiency was evaluated by measuring the percentage of GFP positive cells.
The infection assay was performed as described in the method section. Briefly, cells were seeded in 24-well plates and incubated overnight. The next day, serial dilutions of virus were added to the cells. Two days after infection, cells were harvested and GFP positive cells were measured by FACS analysis (Figure 3. A). The result showed that the virus infection efficiency in \( \rho^0 \) cells was reduced by 10–20 fold when compared with the infection in HOS cells (Figure 3. B). Low infection efficiency in \( \rho^0 \) cells suggested that some steps during virus infection were inhibited in \( \rho^0 \) cells.

Figure 3. \( \rho^0 \) cells are deficient in the support of HIV infection compared with HOS cells.

(A) Infection HOS and \( \rho^0 \) cells. Cells (0.25X10^5/well) were seeded in 24-well plate and incubated overnight at 37°C. The next day, cells were inoculated with virus in a total volume of 500\( \mu \)l. Virus input was shown in the figure. Two days after infection, the cells were detached and fixed with 1% PFA. GFP expression was quantified by FACS. Data was analyzed with Flow Jo 7.6.1. (B) GFP fluorescence in HOS cells and \( \rho^0 \) cells. Histograms are from the cells infected with high concentration of virus (20ng of p24 in 500\( \mu \)l medium). The value labeled in the histogram indicates the percentage of total GFP positive cells. Grey bar indicates the gate for eGFP expression. Similar results were obtained in three independent experiments.
1.2. Effects of mitochondrial inhibitors to HIV infection in HOS cells

\( \rho^0 \) cells lack a functional respiratory chain and cannot perform oxidative phosphorylation (OXPHOS) which is important for some viruses infection such as rubella virus (Claus et al., 2011). To test the contribution of functional OXPHOS to HIV-1 infection, OXPHOS in HOS cells was inhibited and virus infection efficiency was measured in those cells.

In this study, three different mitochondrial OXPHOS inhibitors (antimycin A, oligomycin and CCCP) were used. HOS cells were treated with different mitochondrial inhibitors for 6 hours and subsequently infected with virus. Infection efficiency was measured by FACS analysis 2 days after infection. Figure 4.C shows cell images at different time after antimycin A treatment. Without the supplementation of pyruvate and uridine, cell proliferation was inhibited by antimycin A at low concentration (1.25µg/ml). The growth inhibition by antimycin A can be abolished by the supplementation of pyruvate and uridine. But the growth rate is slower than the control cells (without antimycin A treatment) (Figure 4. C). Oligomycin and CCCP did not inhibit cell growth significantly at all concentrations used in this experiment (data not shown). Virus infection efficiency in the cells treated with different mitochondrial inhibitors was evaluated by comparing the percentage of GFP positive cells. I observed that antimycin A did not decrease virus infection efficiency (Figure 4 A and B). Interestingly, the infection
efficiency was doubled in the cells treated with antimycin A (without the supplementation of pyruvate). After entering into the target cell, it takes around 24 hours for the virus to complete the infection steps including reverse transcription, transport of viral cDNA into nucleus and integration of its DNA into cellular DNA. The viral cDNA cannot be duplicated before the integration. If the cell division happened before the viral cDNA integrates, the infection efficiency in those cells will be decreased comparing to the cell having normal growth rate. My result showed that after antimycin A treatment, HOS cells cannot grow without the supplementation of pyruvate. So the infection efficiency is 2-fold higher than the untreated control HOS cells which will divide one time during 24 hours period. In this experiment, I also observed that the infection efficiency in the cells treated with oligomycin and CCCP was similar with the control cells (untreated cells) (Figure 5). This experiment showed that inhibition of oxidative phosphorylation by mitochondrial inhibitors did not lead to the decrease of virus infection in HOS cells, which indicated that oxidative phosphorylation defect was not the direct reason of virus infection defect in \( \rho^0 \) cells.
Figure 4. Effect of antimycin A (AMA) to HIV infection in HOS cells.

(A) Infection efficiency of the virus in the HOS cells under different treatment. HOS cells were treated with AMA and subsequently infected with virus with or without the supplementation of pyruvate. The infection efficiency was measured by FACS analysis 2 days after infection. (B) eGFP fluorescence in HOS cells treated with AMA (5 µg/ml). Histograms of the infected cells with different treatment are indicated in the figure. The value labeled in the histogram indicates the percentage of total eGFP positive cells. Grey bar indicates the gate for eGFP expression. (C) Cell images at different time after different treatments indicated on top of the images. AMA concentration was 1.25 µg/ml. Similar results were obtained in three independent experiments.
Figure 5. Effect of CCCP and oligomycin to HIV infection in HOS cells. 
(A, C) Infection efficiency in HOS cells treated by CCCP or oligomycin. HOS cells were treated with CCCP or Oligomycin and subsequently infected with virus with or without the supplementation of pyruvate. The infection efficiency was measured by FACS analysis 2 days after infection. (B, D) eGFP fluorescence in HOS cells treated with CCCP (10µM) or oligomycin (10µg/ml). Histograms of the infected cells with different treatment are indicated in the figure. The value labeled in the histogram indicates the percentage of total eGFP positive cells. Grey bar indicates the gate for eGFP expression. Similar results were obtained in three independent experiments.
1.3. Introduction of mitochondrial DNA into $\rho^0$ cells

Transmitochondrial cybrid has been proposed as a very useful tool for studying the biological function of mitochondria and its encoded proteins (Vithayathil et al., 2012). To further explore the relationship between virus infection and mitochondria, cybrid cells were generated by introducing mitochondria containing mt-DNA into $\rho^0$ cells. In this study, we want to test whether the introduction of mitochondria containing mtDNA can rescue the infection defect in $\rho^0$ cells. HEK293T cells were used as mitochondria donor cells. Actinomycin D can intercalate into DNA duplexes, thereby interfering with the action of enzymes engaged in replication and transcription. It is unclear why actinomycin D does not block mtDNA replication, or damage mtDNA as severely as it does the nuclear DNA.

In this experiment, 293T cells were enucleated by actinomycin D treatment and fused to $\rho^0$ cells by PEG mediated membrane fusion method (Bayona Bafaluy et al., 2003). Fusion cells were cultured in the medium supplemented with dialyzed FBS. The medium also contains BrdU for the selection against nonenucleated HEK293T cells which are thymidin kinase (TK) positive cells. The clones were visible after 3 weeks of incubation. The mtDNA from isolated cybrid cells was quantified and compared with mtDNA in HOS cells (Figure 6). Clone 1 and 8 were two selected clones of cybrid cells. I observed that there was no significant difference in mtDNA level between clone 8 and HOS cells.
The mtDNA level in clone 1 was higher than in HOS cells. Infection assay was performed as described before. Infection efficiency in cybrid cells was compared with HOS and $\rho^0$ cells (Figure 7). In this experiment, the infection efficiency in cybrid cells was between $\rho^0$ cells and HOS cells. This result suggested that introduction of mitochondria containing mt-DNA significantly rescued the HIV-1 infection defect in $\rho^0$ cells.

**Figure 6. Mitochondrial DNA level in cybrid and HOS cells.**
Total cellular DNA was isolate and mtDNA was quantified by real-time PCR. Cell number variation was measured using real-time PCR for RNase P DNA by using Taqman Copy Number Reference Assay, RNase P (Invitrogen). Clone 1 and Clone 8 are two different clones of cybrid cells. Error bars indicate standard deviations (n=3). $P$ values (student’s T test) between the results are shown in the figure.
Figure 7. Virus infection in HOS, cybrid, and $\rho^0$ cells.

(A) Infection HOS and $\rho^0$ cells. Cells (0.25X10^5/well) were seeded in 24-well plate and incubated overnight at 37°C. The next day, cells were inoculated with concentrated virus in a total volume of 500µl in the presence of 10µg/ml polybrene. Virus input was shown in the figure. 5 hours after infection, medium was replaced with fresh complete medium. Two days after infection, the cells were detached and fixed with 1% PFA. eGFP expression was quantified by FACS. Data was analyzed with FlowJo 7.6.1. (B) eGFP fluorescence in HOS cells, Cybrid, and $\rho^0$ cells. Histograms are from the cells infected with high concentration of virus (5ng of p24 in 500µl medium). Grey bar indicates the gate for eGFP expression. Similar results were obtained in three independent experiments.
1.4. Mitochondria morphology in HOS, $\rho^0$ and cybrid cells

Mitochondria morphology in HOS, $\rho^0$ and cybrid cells was examined in this study. Mitochondria were visualized by MitoTracker Red (Figure 8). I observed that mitochondria morphology was different in HOS, $\rho^0$ cells and cybrid cells. In HOS cells, most mitochondria were short straight tubules and small globules which were undergoing normal fission and fusion processes. MitoTracker Red signal in $\rho^0$ cells were not as bright as in HOS cells due to the abnormal mitochondria. In $\rho^0$ cells, donut-shaped mitochondria were observed more often and swollen globules and twisted tubules forms of mitochondria were also dominant in some cells. Interestingly, in cybrid cells, most mitochondria formed long tubules structure.

Figure 8. Mitochondria morphology in HOS, cybrid and $\rho^0$ cell
HOS, Cybrid and $\rho^0$ cells were seeded on coverslips and incubated overnight. Mitochondria were labeled with MitoTracker Red. Cells were fixed with 3.75% PFA and mounted onto glass slides with Prolong Gold Antifade reagent. Imaging was performed by using Leica SP5 spectral confocal inverted microscope (SP5 inverted). Cell image were acquired by using the 63X oil objective lens. 543-nm laser line was used to detect MitoTracker Red signal.
2. Quantification of different forms of viral DNA in the infected cells

Our data showed that the virus infection in $\rho^0$ cells was significantly lower than in HOS cells, which suggested that some steps during the virus infection were blocked in $\rho^0$ cells. At different steps during HIV infection, viral cDNA exists in different forms (Figure 9). By analyzing the viral cDNA of different forms in the infected cells, different steps during virus infection can be monitored.

In this experiment, HOS, $\rho^0$ and Cybrid cells were infected with HIV and infected cells were collected at 2, 6, 10, 24, 48 and 72 hours post infection. Total cellular DNA from infected cells was purified using DNasey tissue and blood kit. Different forms of viral cDNA in the samples were quantified by real-time PCR using the well established methods (Brussel & Sonigo, 2003; Butler et al., 2001). Cell number variation in each sample was quantified by real-time PCR and was used for normalization of viral cDNA in the infected cell. Virus infection efficiency in different cells (HOS, $\rho^0$ and Cybrid cells) was measured by FACS at 72 hours post infection (Figure 10).
Figure 9. Steps during early stage of HIV replication.
After entry, HIV RNA genome is reverse transcribed into DNA. The first step of reverse transcription is the strong stop DNA synthesis (step 1) which is the early reverse transcripts. In step 2, a full-length double stranded DNA is generated, which is the late reverse transcripts. 1-LTR circles and 2-LTR circles are generated in nucleus following the nuclear import of the full-length viral DNA. The last step is the integration of viral cDNA into chromosomal DNA. In the figure, Alu represents the Alu repetitive elements in human genome.

Figure 10. FACS analysis of infection in HOS, cybrid, and $\rho^0$ cells.
Histograms of the infected cells (HOS cells, Cybrid, and $\rho^0$ cells) were shown in the figure. The percentage of eGFP positive is as follows: HOS (15.7%), $\rho^0$ (2.47%) and Cybrid (7.36%).
2.1. Reverse transcription (RT) product detection

Virus reverse transcripts were quantified by real-time PCR using different primer sets (Figure 11). The early primers (MH535 and Early reverse) used in this experiment can detect all forms of the viral cDNA including strong stop DNA and full length viral cDNA. The late primers (MH531 and MH532) can only measure full length viral cDNA (Butler et al., 2001). Viral cDNA copy number in the samples was calculated based on the standard curve generated by serial dilution of linearized plasmid pWPI. In Figure 12, we observed that the viral cDNA reached the maximal level between 6 hours and 10 hours after infection and then decreased rapidly. The pattern of viral cDNA in HOS, cybrid, and \( \rho^0 \) cells at different time post infection was similar.

2.2. Early reverse transcription product (Strong stop DNA)

Synthesis of the strong stop DNA is the first step of reverse transcription and most viral complexes are capable of performing this reaction. The strong stop DNA copy number per cell was calculated using the formula described as follows: \( \text{Strong stop DNA} = \{\text{PCR}(A) – \text{PCR}(B)\} \times 4 \) (Figure 11). We observed that the level of viral strong stop DNA was rapidly decreased (Figure 12C) after 2 hours. The strong stop DNA in cybrid cells at 2 hours was lower than in HOS and \( \rho^0 \) cells (Figure 12C). This is probably
because that virus entry in cybrid cells was not as efficient as in HOS and \( \rho^0 \) cells in this experiment, so there were fewer viruses entering into the cybrid cells comparing to HOS and \( \rho^0 \) cells. This result showed that strong stop DNA synthesis in \( \rho^0 \) cells is as same as in HOS cells.

**Figure 11. Detection of early and late reverse transcripts.**

Arrows indicate the approximate locations of primers and probes used in quantitative real-time PCR. Linearized transfer vector pWPI plasmid serve as the standard for real-time PCR. The levels of strong stop DNA and late reverse transcripts are calculated using the following formula: Strong stop DNA copy number = \{PCR(A) – PCR(B)\} \times 4; Late reverse transcripts = PCR(B). PCR (A) and PCR (B) represent the PCR results of corresponding real-time PCR reactions. PCR (B) using the late primers (MH531 and MH532) measures full length viral cDNA. In PCR (A), the early primers (MH535 and Early reverse) can detect all forms of the viral cDNA including strong stop DNA and full length viral cDNA. And there are two amplicons (U3RU5 fragment) in the full length DNA that can be recognized by early primers set. In the standard, there are also two amplicons (U3RU5 fragment) that can be detected by early primers. And also the strong stop DNA is a single stranded DNA. So the strong stop DNA copy number should be calculated using the formula described above.
2.3. Late reverse transcription product (full length viral cDNA)

The second step of reverse transcription is the first strand transfer which is a rate limit step. After that, a serial steps of reverse transcription lead to the synthesis of full length viral cDNA (late RT product). The copy number of late RT product in HOS, cybrid and $\rho^0$ cells was measured by using late primer set (Figure 11). Late RT products (maximal level) in HOS, cybrid and $\rho^0$ cells at 6 or 10 hours after infection were compared (Figure 12 A). The result showed that there was no significant difference between HOS, cybrid, and $\rho^0$ cells in the level of late RT product (maximal level). Reverse transcription ability in the cells can be evaluated by the ability of converting strong stop DNA to full length RT product. My result showed that the level of strong stop DNA in HOS and $\rho^0$ cells was similar (Figure 12). In this experiment it was observed that in HOS and $\rho^0$ cells around 25% of strong stop DNA was converted into full length viral cDNA. As for cybrid cells, around 30% strong stop DNA was converted into full length viral cDNA. This suggested that there was no significant differences in the virus reverse transcription ability between HOS and $\rho^0$ cells (Figure 12D), which suggested that the reverse transcription in $\rho^0$ cells was normal and the defective infection was caused by the inhibition of some steps after reverse transcription in $\rho^0$ cells.
Figure 12. Reverse transcription activity is similar in HOS, cybrid and $\rho^0$ cells. Cells were infected with DNase I treated HIV-GFP virus (20ng p24/well, 6-well plate) for 2 h and then incubated in virus free medium. Total cellular DNA was isolated from infected cells at 2, 6, 10, 24, 48, 72 h postinfection and HIV late (A) and total RT (B) product, and cell RNase P were quantified by real-time PCR. Viral cDNA values are shown per cell (RNase P). Linearized pWPI plasmid was used as the standard for late and total RT products measurement. Primer pair for total RT products is specific for the R/U5 region of the LTR and this primer pair detects all HIV cDNA synthesized including strong stop DNA (early RT products), late RT products and other partial reverse transcripts. In this analysis, only (-) single-stranded strong stop DNA and late RT products were considered. One molecule of linearized pWPI (standard) contains two LTR sequences which equal to 4 molecules of single-stranded strong stop DNA in PCR measurement. So the strong stop DNA (C) was calculated using the following formula: Strong stop DNA = (Total RT-Late RT) X 4. The % of late RT (2, 6, 10 h postinfection) per strong stop DNA (2 hours post-infection) is shown (D) to demonstrate the progress and completion of the reverse transcription. The Error bars indicate standard deviation in triplicate values. Data are representative of three independent experiments. *, $P<0.01$. 
2.4. Integrated viral DNA detection

Detection of integrated proviruses DNA was accomplished by amplification with primers complementary to the HIV LTR and chromosomal *Alu* repeats. In this study, Alu-LTR based real-time nested-PCR method was used to measure integrated virus DNA ([Figure 13](#)) (Brussel & Sonigo, 2003). In the first round of PCR, integrated HIV-1 sequences were amplified with *Alu* primer (Alu2) and HIV LTR specific primer (GST-LTR5). *Alu* primer can anneal within conserved regions of the *Alu* repeat element in chromosome DNA. LTR primer contains a GST sequence (sequence from Glutathione S-transferase plasmid) at the 5’end of the oligonucleotide ([Figure 13](#)). Distance between LTR and *Alu* element is variable. So the products generated from the first round of PCR contain fragments of various lengths. In the second round of quantitative real-time PCR using the GST primer and the LTR primer (MH535), only products containing viral LTR from the first-round PCR could be amplified. The products from the second round PCR contain discrete DNA fragments.

In this experiment, total cellular DNA isolated from infected HOS cells (40 days after infection) was used as a standard. Infected cells that have been cultured for 40 days should not contain extra-chromosomal forms of viral DNA (Butler et al., 2001). The concentration of integrated viral DNA in the standard was quantified by real-time PCR using the primers for late reverse transcripts. The standard curve was generated by
two-round PCR amplification of serial dilutions of the standard. In this experiment, the linear regression obtained from amplification of serial dilutions of the standard sample was linear over several log\textsubscript{10}-units range (Figure 14. A). Linear, one-way amplification was monitored by performing the pre-amplification PCR with GST-LTR5 primer alone. The products generated by one way amplification of viral cDNA do not necessarily represent the integrated viral DNA, because any form of viral cDNA (integrated and un-integrated DNA) containing LTR sequences could be amplified in LTR one-way amplification process. In this experiment, \(C_T\) value resulting from LTR one-way amplification was 8-units higher than that from LTR-Alu PCR (Figure 14.B), which meant that signal generated from LTR one-way amplification (300-folds lower than LTR-Alu PCR) will not have influence over the measurement of integrated viral DNA.

The total DNA isolated from the infected cells at different time after infection was subjected to two-round PCR amplification along with a serial dilutions of the standard. The concentrations of integrated viral DNA in the unknown samples were determined in reference to the standard curve. The results showed that the viral DNA integration happened between 10 hours and 48 hours after infection, and the integrated viral DNA per cellular genome in \(\rho^0\) cells was significantly lower than in HOS and cybrid cells (Figure 15A, B). The integrated DNA level in \(\rho^0\) cells at 72 hours after infection was \(~10\) fold lower than in HOS cells and \(~6\) fold lower than in cybrid cells (Figure 15A, B).
Figure 13. Semi-nested PCR detection of integrated viral DNA.
Detection of integrated proviruses was accomplished by two steps PCR amplification. Primers Alu2 and GST-LTR5 were used in the first-round PCR for the amplification of fragments between viral LTR and Alu elements in cellular chromosome. The LRT containing fragments generated in the first-round PCR were quantified by second-round nested real-time PCR using GST and early reverse primers.
Figure 14. (A) Standard curve generated for quantifying integrated viral DNA. Total cellular DNA isolated from infected cells (17% GFP positive, more than 40 days after infection) was used as a standard. The number of integrated proviral DNA in the standard was quantified using the late reverse transcription primer set (MH531, MH532 and Late RT-probe). This standard curve was generated by two-round PCR amplification of a serial dilution of the standard. The x-axis shows the known number of proviruses in the cellular DNA sample (input in the first-round LTR-Alu PCR). The y-axis shows the PCR cycles at which the amplification signal entered the exponential range (cycle of threshold) during the second-round PCR. (B) Comparison of LTR-Alu PCR and LTR one-way amplification. Linear, one-way amplification was monitored by performing the preamplification PCR with GST-LTR primer alone and integrated viral DNA input was 20896 copies per reaction. Products from the first-round PCR (LRT-Alu PCR or LTR one-way amplification) were subjected to the second-round quantitative real-time PCR. The y-axis shows the $C_T$ values of second round real-time PCR. Error bars indicate standard deviations (n=3). $P$ value (student’s T test) between the results is shown in the figure.
2.5. 2-LTR circle DNA

HIV 2LTR circles are the products of non-homologous end joining DNA repair event and exclusively found in the nucleus, and usually serve as a marker of viral nuclear import in the studies of viral trafficking. The 2-LTR circle primers amplify the linkage region between 5’ and 3’ LTR ends. In cybrid and HOS cells, 2-LTR circles reached to the maximum level at 24 hours post infection and decreased after 72 hours (Figure 15 C). In contrast, 2-LTR circles in ρ^0 cells peaked at 48 hours post infection and then rapidly decreased (Figure 15 C). This delayed accumulation of 2-LTR circles suggested that virus intracellular transport in ρ^0 cells was slower than in HOS and cybrid cells. The ratio between 2-LTR circles and late RT products (maximum level) reflects the ability of transporting viral DNA from cytoplasm into nucleus which was significantly lower in ρ^0 cells compared with that in HOS and cybrid cells (Figure 15 D). This result showed that HIV infection in ρ^0 cells was blocked at the steps before nuclear import.
Figure 15. 2LTR circles and integrated DNA in $\rho^0$ cells are significantly lower than in HOS, and cybrid.

Integrated DNA and 2-LTR circles in the samples described in Figure 12 were detected by real-time PCR. HIV cDNA values are shown per cell (RNase P). Cellular DNA isolated from infected HOS cells at 40 days post infection was used as standard for Alu-LTR PCR to measure integrated viral DNA. A DNA fragment containing 2LTR junction sequence was cloned into a plasmid (pGEX backbone, Invitrogen) and served as standard for the measurement of 2LTR circles. Error bars indicate standard deviation in triplicate values. Data are representative of three independent experiments. *, $P<0.01$; **, $P<0.05$. 
3. Interaction between virus and mitochondria

The infection deficiency in \( \rho^0 \) cells suggested that mitochondria may play an important role during the early stage of HIV-1 infection. Further analysis showed that virus infection was blocked at steps after reverse transcription and before nuclear import. We proposed that mitochondria may play a role in the intracellular transport of viral complexes. In this study, the localization of viral complexes in the infected cells was investigated.

3.1. Confocal fluorescence microscopy analysis

3.1.1. Co-localization of viral complexes with mitochondria in the infected cells

Viral complexes and mitochondria in the infected cells were visualized by fluorescent immunohistochemistry method (McDonald et al., 2002). Mitochondria in the cells were stained with MitoTracker Red, a dye that can be transported into mitochondria specifically. Viral complexes were detected by an anti-p24 monoclonal antibody (mAb AG3.0) and visualized using fluorescently labeled second antibody (Figure 16). Figure 16 shows the images from a representative HOS cell. The majority of viral nucleoprotein complexes were found to be associated with or located in very close proximity to
mitochondria. A similar result was observed in another cell line (293T cells) (Figure 17). Optical slices of the infected 293T cells were taken every 200nm interval along the z-axis covering the whole depth of the cell. The representative images selected from all the optical slices were shown, which showed the co-localization of viral complexes (green) with mitochondria (red). The 3D reconstruction of the cells also showed that the viral complexes were associated with mitochondria (Figure 17.B).

The resolution of normal confocal fluorescence microscopy is limited. Generally, viral complex has a size of 100nm. It is hard to make a conclusion about the direct interaction between viral complexes and mitochondria. Do they stay very close to each other or do they actually interact with each other? To answer this question, Nanoscope technology was used to generate images with resolution below 100nm. Two representative Nanoscope images of viral complex with mitochondria were shown in Figure 18. The image showed that there was not a visible gap between viral complexes and mitochondria, which suggested that viral complexes may interact with mitochondrial surface proteins directly.
Figure 16. Co-localization of HIV intracellular complexes with mitochondria in HOS cells.
HOS cells seeded on the coverslips were infected virus (MOI~10). Six hours after infection, mitochondria were labeled with MitoTracker Red. Infected cells were fixed and viral complexes were detected with monoclonal anti-p24 antibody and visualized with Alexa 488-conjugated anti-mouse IgG secondary antibody. Imaging was performed by using Leica SP5 spectral confocal inverted microscope (SP5 inverted). Mitochondria were labeled in Red and viral nucleoprotein complexes were labeled in Green. Nucleus was marked by circle in (A). (B) Enlarged image of the area marked with square in (A). (1-10) Enlarged images of the area marked with white arrow in (B) show viral complexes and mitochondria. The scale bars shown in the images are as follows: (A) 50µm, (B) 10µm, (1-10) 500nm.
Figure 17. Viral nucleoprotein complexes and mitochondria in 293T cell.

(A) Optical sections of infected 293T cell. 293T cells seeded on the coverslips were infected with virus (MOI~5). Mitochondria were labeled with MitoTracker red (Red). Viral complexes were detected with monoclonal anti-p24 antibody and visualized with Alexa 488-conjugated anti-mouse IgG secondary antibody (Green). Imaging was performed by using a laser-scanning confocal microscope (LSM510, Carl Zeiss). Appropriate laser lines for each fluorophore were used: 543-nm line for MitoTracker Red; 488 nm line for Alexa Fluor 488. A single cell images were acquired by using the 63X oil objective lens, with zoom factor of 2. Optical slices were taken every 200nm interval along the z-axis covering the whole depth of the cell. Images were analyzed using ImageJ and Zeiss LSM Image Browser. Images (a-i) are representatives of a series of images captured at different focal depths along the z-axis. (B) 3D reconstruction from the images. 3D reconstruction was performed using Image J and 3D viewer plug-in software.
Figure 18. Nanoscope images of viral complexes with mitochondria in 293T cells. 293T cells were seeded on the coverslip and incubated overnight. The next day, cells were infected with virus (MOI~0.1). 6 hours after infection, mitochondria were labeled with MitoTracker Red. Cells were fixed with 4% PFA and viral complexes were detected with anti-p24 monoclonal antibody and visualized with Alexa-488-conjugated anti-mouse IgG secondary antibody. Nanoscope image was taken by Dr. George McNamara in Seattle.

3.1.2. Detection of HIV cDNA in infected cells by EdC incorporation and the Click-it method

The immunohistochemistry studies described above showed that a large fraction of viral complexes were co-localized with mitochondria, but still there were a lot of viral complexes that did not associate with mitochondria. It has been suggested that most viral complexes entering into the target cell do not lead to productive infection. Identification of the viral complexes that can lead to successful infection is necessary for our understanding the steps during virus infection. Because viral cDNA synthesis is the first
step of successful infection, it is necessary to directly visualize viral cDNA in the infected cells. A new method was developed in this study to detect viral cDNA and also preserve the cellular cytoplasm structure.

EdC is a dC analogue and can be converted into EdCTP in the cell and subsequently incorporated into cellular newly synthesized DNA. EdCTP incorporated in the DNA can easily be detected using Click-it labeling method (Cappella et al., 2008). To develop the method for the detection of the newly synthesized viral DNA, mitochondrial DNA (mtDNA) was used as a positive control (Lentz et al., 2010). Figure 19 shows the newly synthesized mtDNA detected by using Click-it method. Newly synthesized mtDNA was labeled in green. This result showed that newly synthesized mtDNA (around 16k bps) could be detected using this method, which suggested that newly synthesized viral cDNA (6k bps) may also be detected. To label viral cDNA, cells were infected with virus at high MOI (~10) and Click-it labeling method was performed as described above. Figure 20 showed one viral complex that was found to be associated with mitochondria and also contained newly synthesized DNA signal. This result showed that viral complex associating with mitochondria has the ability to perform reverse transcription.
Figure 19. Newly synthesized mtDNA detection by Click-it method.
HOS cells seeded on the coverslips were treated with aphidicolin to inhibit nuclear DNA synthesis. And EdC was added into the cell. After 6 hours incubation, mitochondria were labeled with MitoTracker Red and cells were fixed with PFA. EdC Click-it detection was performed following the instruction of the manufacture. Imaging was performed as described before. Appropriate laser lines for each fluorophore were used: 488nm line for Alexa Fluor 488; 543-nm line for MitoTracker Red. Images were analyzed using LAS AF Lite software. Mitochondria were labeled in Red and newly synthesized DNA was labeled in Green. Image in (A) shows one representative cell. Image B is the enlarged image of area marked with white square in (A).
Figure 20. Newly synthesized viral DNA detection.
HOS cells were infected with virus (MOI~10), and EdC incorporation and Click-it labeling methods were performed as described above. Mitochondria were labeled with MitoTracker Red. Viral complexes were detected with monoclonal anti-p24 antibody and visualized with Alexa 647-conjugated anti-mouse IgG secondary antibody. EdC in newly synthesized DNA was detected with Alexa-488 azide. In this image, mitochondria were shown in Blue; viral complexes were shown in Red; and newly synthesized DNA was shown in Green. (A) Image of an infected HOS cell. (B) Enlarged image of area marked by red square in (A) with different channels (separated and merged). (C) Overlay of different channels.
3.2. HIV-1 intracellular complexes and mitochondria interaction

3.2.1. Distribution of early and late reverse transcripts in cell extract

Cytoplasmic extract from infected HOS cells was fractionated by centrifugation method (Figure 21.A). Viral cDNA in each fraction was measured by quantitative real-time PCR assay. In this experiment, cell cytoplasmic extract was fractionated into supernatant and pelleted by centrifugation at 10000g for 5 minutes and DNA in each fraction was isolated. Viral DNA were identified by real-time PCR as described before. The distribution of strong stop DNA and full length viral cDNA in different fractions was compared (Figure 21.B). I observed that the majority of strong stop DNA was found to be in the supernatant, and interestingly, the majority of full length cDNA was found to be in the pellet fraction. Because of the small size, viral complexes alone should not be pelleted by centrifugation at 10000g for 5 minutes. The presence of viral full length cDNA in the pellet suggested that viral complexes containing full length cDNA may associate with cellular organelles, which can be pelleted at relative low speed during centrifugation (10000g, 5 minutes) and caused viral cDNA to be recovered in the pellet fraction. Strong stop DNA represents viral complex which is in the early stage of reverse transcription. Full length cDNA is the product of late stage reverse transcription. The
result in this experiment suggested that at different stages of reverse transcription, viral complexes associated with different cellular compartments which caused viral complexes to be recovered in different fractionations.

### 3.2.2. Distribution of viral complexes and mitochondria

Extract from infected cells was fractionated and the fractions containing viral complexes were identified by the presence of viral cDNA. It was reported that HIV-1 intracellular complexes are unstable and could be disrupted even under very mild condition. In my experiment, infected 293T cells were lysed by hypotonic breakage of cell membrane in order to preserve the natural interaction between viral complexes and cellular compartments. Some studies also suggested that HIV-1 intracellular complexes are associated with microtubules and transported through cellular cytoskeleton network in the host cells. In this study before lysing the cell, infected cells were incubated with nocodozal and taxol to disrupt microtubule network in the cells. Incubation on ice before the cell extraction procedure also helped to depolymerize microtubules. After removing the nucleus and unbroken cells, cytoplasmic extract containing reverse transcription complexes was divided into 3 fractions by centrifuging at different speeds (Figure 22. A). DNA was purified from each fraction and virus cDNA was identified by real-time PCR using specific primers detecting virus late reverse transcription products. We observed that the majority of viral cDNA (57%) was recovered in the pellet fraction (P1) (Figure
This result suggested that viral complexes may associate with some cellular organelles which were fractionated into the pellet by centrifugation at 2700g. The distribution of mitochondria was measured by detecting mtDNA using real-time PCR. The fractionation of mtDNA had similar pattern with viral cDNA: the majority of mtDNA was found in the first pellet (Figure 22 B). Those results suggested that viral complexes containing viral cDNA may associate with mitochondria in the infected cells.

3.2.3. Antibody-mediated binding of mitochondria and viral complexes

The cell fractionation results in this study showed that there is a possible interaction between HIV-1 virus complexes and mitochondria, which may be the reason that causes virus cDNA to be recovered in the pellet fraction. To explore whether viral complexes associated with mitochondria during the infection, an antibody-mediated binding method was used. Because of the instability nature of viral complexes, a PFA fixation treatment was used to stabilize interaction between viral complexes and cellular compartments. In this study, cell cytoplasmic extract containing viral complexes and mitochondria was fixed with 4% PFA before any further fractionation. A specific antibody to the translocase of the outer membrane of mitochondria 22 (TOM22) was used in the antibody-mediated binding assay to pull down mitochondria. After antibody-mediated binding, DNA was isolated and purified from eluted material. Viral cDNA and mtDNA were detected by real time PCR (Figure 23). We observed that about 29% mtDNA was pulled down by Protein
A beads having anti-Tom22. Similarly 24% viral cDNA was also found to be in the same fraction (Figure 23). The result showed that only 29% mtDNA was bound to the beads. Because of the heterogeneous nature of mitochondria, not all the mitochondria can be pulled down by the beads equally. Or some mitochondria were removed during the washes. This result suggested that there is a certain interaction between mitochondria and viral complexes containing viral cDNA.

Figure 21. Cell fractionation and viral cDNA detection
(A) Schematic representation of the procedure for fractionating infected cells.
(B) Strong stop DNA and full length viral cDNA in pellet and supernatant were identified by real-time PCR as described before.
Figure 22. Co-fractionation of virus with mitochondria.
(A) Schematic representation of the procedure for fractionating infected cells.
(B) Full length viral cDNA and mtDNA in Pellet 1 (P1), Pellet 2 (P2) and supernatant II (Sup) were identified by real-time PCR as described before.

Figure 23. Co-immunoprecipitation of viral complexes with mitochondria.
Suspension of fixed cytoplasmic extract was incubated with protein A beads (Control) or protein A beads with Tom22 antibody (anti-Tom) for 2 hours. Beads were washed and DNA eluted from the beads was purified. Full length viral cDNA and mtDNA in the samples were identified by real-time PCR as described before.
Chapter 4: Discussion and conclusion

The contribution of mitochondria to the HIV-1 infection was investigated through infection of \( \rho^0 \) cell. My results showed that \( \rho^0 \) cells are defective in the ability to support HIV based lentiviral vector infection when compared to their parental cells. The mechanism of the infection defect in \( \rho^0 \) cells was explored in this study. My study suggested that mitochondria play an important role in the early stage of HIV infection.

1. Infection defect in \( \rho^0 \) cells

Data in my experiment showed that HIV infection in \( \rho^0 \) cells was defective compared with infection in their parental cells (HOS cells) (Figure 3). This phenomenon suggested that mitochondria may play a role during the HIV infection. As we know, \( \rho^0 \) cells lack a functional respiratory chain and cannot perform oxidative phosphorylation (OXPHOS) which is important for the cells. Inhibition of OXPHOS leads to the change of metabolism in the cell and also influences the expression of some nuclear genes.

Functional OXPHOS are also important to some virus infection. It has been shown that inhibition of OXPHOS leads to the decrease of rubella virus infection. To test the contribution of functional OXPHOS to HIV infection, OXPHOS in HOS cells was inhibited by several mitochondrial inhibitors (Figure 4 and 5). Antimycin A inhibits the
oxidation of ubiquinol in the electron transport chain and disrupts the formation of the proton gradient across the inner membrane. Oligomycin inhibits mitochondrial H⁺-ATP synthase. CCCP is an uncoupling agent and destroys the membrane potential across the mitochondrial membrane. Treatment of cells with those inhibitors abolishes mitochondrial OXPHOS.

The results from my study showed that inhibition of OXPHOS did not lead to the inhibition of HIV infection in this study. ρ⁰ cell line used in this study was a gift from Dr. Barrientos’ laboratory. ρ⁰ cells were generated from 143B.TK- cells which may not have the same genetic background with the HOS cells (143B.TK-) used in this study. So transmitochondrial technology was used to introduce mitochondria containing mtDNA into ρ⁰ cell. The constructed Cybrid cells in this study have the same genetic background with ρ⁰ cell. Cybrid cells were successfully generated using 293T cells as mitochondria donor cells in this study. The infection assay showed that introduction of mtDNA into ρ⁰ cells rescued the infection defect in ρ⁰ cells. Those results suggested that mitochondria may be directly involved in the early stage of HIV infection. Another possibility is that introduction of mtDNA into ρ⁰ cells had some influences to the expression of some nuclear gene which is important to viral infection.
2. HIV infection was blocked at steps after reverse transcription and before nuclear import

The infection assay in \( \rho^0 \) cells suggested that some steps during the virus infection were blocked in \( \rho^0 \) cells. As we know, cellular factors play an important role during the virus infection. A better understanding of the steps of virus infection in \( \rho^0 \) cells may help us to identify cellular factors that are necessary for virus infection. At different steps during HIV infection viral cDNA exists in different forms which could serve as very useful marks for us to monitor different steps of virus infection (Figure 9). After entry, reverse transcription is initiated and strong stop DNA synthesis is the first step of reverse transcription. The synthesis of strong stop DNA is not a rate limit step, so strong stop DNA can be used to evaluate the virus entry in this study. The synthesis of full length viral cDNA indicates the completion of reverse transcription. Reverse transcription ability in the cells can be evaluated by comparing the level of full length viral cDNA with the strong stop DNA. 2LTR circles are the products of non-homologous end joining DNA repair event which is mediated in the nucleus as a protective host response to the presence of double stranded viral DNA. 2LTR circles are exclusively found in the nucleus, and may serve as a marker of viral nuclear import in the studies of viral trafficking. Integration of viral cDNA into the cellular chromosome DNA is the last step of the early stage of virus infection. The virus integration can be measured by the detection of integrated viral DNA. In this study, different forms of viral cDNA in the infected cells were quantified by real-time PCR. Virus entry in different cells was evaluated by the
level of strong stop DNA at early time post infection. The results showed that there is no
significant difference in the strong stop DNA level between HOS cells and ρ⁰ cells at 2
hours post infection (Figure 12). The strong stop DNA in Cybrid cells at 2 hours post
infection was lower than in HOS and ρ⁰ cells (Figure 12). This result showed that virus
entry in Cybrid cells was not as efficient as in HOS and ρ⁰ cells in this experiment, so
there were fewer viruses entering into the Cybrid cells comparing to HOS and ρ⁰ cells.
Those results indicated that the infection defect in ρ⁰ cells is not due to the difference of
virus entry.

The synthesis of full length viral cDNA indicates the completion of reverse
transcription. Through reverse transcription, strong stop DNA was converted into full
length viral cDNA. So the reverse transcription ability can be evaluated by comparing the
level of full length viral cDNA (product) with the strong stop DNA (substrate). In this
experiment it is observed that in HOS and ρ⁰ cells around 25% of strong stop DNA can
be converted into full length viral cDNA. As for Cybrid cells, around 30% strong stop
DNA can be converted into full length viral cDNA (Figure 12). This suggested that there
is no significant differences in the virus reverse transcription ability between HOS and ρ⁰
cells. So the reverse transcription in ρ⁰ cells is normal and the defective infection was
cau sed by the inhibition of some steps after reverse transcription in ρ⁰ cells.
Integration of viral cDNA into the cellular chromosome DNA is the last step of the early stage of infection. The integration efficiency was measured by the detection of integrated viral DNA. Under the same virus input, the integrated viral DNA per cellular genome in $\rho^0$ cells is significantly lower than in HOS and Cybrid cells (Figure 15). Those results suggested that HIV infection was inhibited at the steps before integration. Both 2-LTR and integrated DNA were reduced 6~9 fold in $\rho^0$ cells at 24 hours post infection (Figure 15) compared with Cybrid and HOS cells. Because nuclear import is the step prior to integration, the decrease of nuclear import will certainly lead to the decreased level of integrated DNA. The delayed accumulation of 2-LTR circles in $\rho^0$ cells suggests that the intracellular transport of viral complexes is impaired in $\rho^0$ cells. Data in this experiment indicates that HIV infection in $\rho^0$ cells is blocked at the steps after reverse transcription and before nuclear import.

3. Mitochondria and viral complexes interaction

The infection assays showed that mitochondria play an important role during the early stage of HIV-1 infection, and functional OXPHOS, the major function of mitochondria, is not important for virus infection. Further analysis showed that virus infection in $\rho^0$ cells was inhibited in the steps after reverse transcription and before nuclear import. We think that maybe mitochondria play a role in the process of viral complex intracellular transport. So virus complex intracellular localization was investigated in this study.
Immunofluorescent micrographic technology was used to study the localization of viral intracellular complexes. The confocal images showed that a large fraction of viral nucleocapsid complexes were co-localized with mitochondria (Figure 16, 17, 18). The association of HIV nucleocapsid with mitochondria has never been reported before, although several viral capsid proteins have been shown to interact with mitochondria. The studies done by Subash C. Das et al showed that vesicular stomatitis virus nucleocapsids may attach to mitochondria and move along with mitochondria in the infected cells. Rubella viral capsid also has been showed to interact with p32 (mitochondrial protein), which is important to Rubella virus infection. My results showed that HIV complexes containing p24 may interact with mitochondrial surface proteins and associate with mitochondria during the infection. In this way, mitochondria may facilitate the intracellular transport of viral nucleocapsid complexes.

Considering the fact that a lot of viral complexes enter the cells cannot perform reverse transcription, only a small fraction of viral complexes entering into the cell can lead to the productive infection. So it is necessary to identify the viral complexes that can lead to functional infection. HIV cDNA synthesis is the first step leading to successful infection, so labeling viral cDNA in the cell will provide useful information for our understanding of virus infection. Cytoplasmic viral cDNA has been detected using fluorescence in-situ hybridization (FISH) method. But proteinase K treatment and DNA denaturation steps used in the FISH assay could lead to the disruption of the cellular
cytoplasm structure, which will disrupt the localization of viral complex in the cytoplasm.

A new labeling method was developed in my study. Newly synthesized mitochondrial DNA can easily be detected using EdC incorporation and click-it labeling method (Figure 19). I have showed that viral complexes associating with mitochondria contained newly synthesized DNA signal. But the detection of viral cDNA is not as efficient as detection of mt-DNA (Figure 20). The reason is unclear. Maybe the EdC incorporation in viral complexes is very inefficient because of the difference in EdCTP pool accessibility between reverse transcriptase and mitochondrial DNA polymerase. Another reason is that only a small fraction of viral complexes can finish reverse transcription. So it is normal that the majority of viral complexes in the cell cannot perform reverse transcription and will not contain newly synthesized DNA signal. My data suggested that mitochondria association may be important to virus reverse transcription.

4. Co-fractionation of virus complexes with mitochondria

The interactions between virus and cellular factors play an important role during virus infection. It has been suggested that HIV-1 viral complexes interact with cellular transport machinery during the movement towards nucleus. In this study, cytoplasmic extract of infected cells was fractionated by centrifugation. The distribution of early reverse transcripts and late reverse transcripts was found to be different (Figure 21),
which suggested that the localization of viral complexes containing different reverse transcription products was different. Because of the small size, viral complexes alone cannot be pelleted by centrifugation at 10000g in the study. The majority of late reverse transcripts were found in the pellet, which indicated that late reverse transcripts may associate with cellular organelles which caused viral DNA to be recovered in the pellet fraction. The fact of most strong stop DNA in the supernatant suggested that early reverse transcripts may not associate with cellular organelles. At the different stages during the infection, viral complexes may interact with different cellular machinery and the association with different cellular factors may play an important role in the virus infection.

Cell fractionation experiment showed that viral complexes associated with cellular organelles during the infection. Confocal fluorescence microscope image showed that some viral complexes associated with mitochondria in the infected cells. The association of viral complexes with mitochondria could be the reason that caused viral cDNA recovered in the pellet fraction. The antibody mediated binding assay showed that viral complexes can be immunoprecipitated with specific antibody against mitochondrial surface protein (TOM22), which provided additional evidence supporting my hypothesis of the association between viral complex and mitochondria.
5. Conclusion

In this thesis work, the role of mitochondria in the early stage of HIV infection has been evaluated. I have observed that cells lacking mitochondrial DNA (ρ0 cells) are defective in the ability to support HIV infection when compared to their parental cells. My results showed that HIV infection in ρ0 cells is blocked at steps after reverse transcription and before nuclear import. This result indicated that viral intracellular transport in ρ0 cells is inhibited due to the absence of mtDNA. Results from confocal microscopy and co-fractionation experiments suggested that there is a certain interaction between viral intracellular complexes and mitochondria. My conclusion is that mitochondria play an important role during the early stage of HIV infection probably by associating with viral intracellular complexes and facilitating the intracellular transport of viral complexes (Figure 24)

Figure 24. The role of mitochondria in the early stage of HIV infection. After entry, HIV intracellular complexes associate with mitochondria by interact with mitochondrial surface proteins. Mitochondria play an important role during the early stage of HIV infection by facilitating the intracellular transport of viral complexes. Mitochondria may also play a role in reverse transcription.
Significance of this Study

HIV-1 hijacks our cellular machinery to complete its life cycle. A better understanding of the interactions between cellular proteins and viral components will certainly lead to the discovery of new ways to inhibit viral replication. Knowledge of viral-cellular protein interactions will also assist our understanding of important cellular pathways involved in HIV/AIDS-related disease. In my study, the contribution of mitochondria to the HIV-1 infection was investigated through infection of $\rho^0$ cells. I have shown that mitochondria play an important role during the early stage of HIV-1 infection probably by associating with viral intracellular complexes and facilitating the intracellular transport of viral complexes. The role for mitochondrial association of HIV-1 intracellular complexes has not been previously explored. The elucidation of this role will open new avenues for investigation of the early steps in HIV-1 infection. Therefore, I believe my thesis work will be of general interest to researchers in the virology field. In the long run, characterization of the interaction between HIV-1 intracellular complexes and mitochondria may lead to identification of new targets for antiviral drug development. In this study, I also showed that $\rho^0$ cells can serve as a very useful model system to study the early stage of HIV-1 infection due to the specific inhibition of HIV-1 infection in $\rho^0$ cells. Unravel the reason of HIV-1 infection defect in $\rho^0$ cells could lead to the new discovery of the factors involved in the interaction between HIV-1 and host cells.
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