1993-01-01

**HIV-1 Sequences in the Establishment of Chronic Virus Producers: a Thesis**

Farah Mustafa

*University of Massachusetts Medical School*

Let us know how access to this document benefits you.

Follow this and additional works at: [https://escholarship.umassmed.edu/gsbs_diss](https://escholarship.umassmed.edu/gsbs_diss)

Part of the [Cells Commons](https://escholarship.umassmed.edu/gsbs_diss), [Genetic Phenomena Commons](https://escholarship.umassmed.edu/gsbs_diss), and the [Viruses Commons](https://escholarship.umassmed.edu/gsbs_diss)

**Repository Citation**


This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in GSBS Dissertations and Theses by an authorized administrator of eScholarship@UMMS. For more information, please contact Lisa.Palmer@umassmed.edu.
HIV-1 SEQUENCES IN THE ESTABLISHMENT OF CHRONIC VIRUS PRODUCERS

A THESIS PRESENTED
BY
FARAH MUSTAFA

Submitted to the Faculty of the University of Massachusetts Medical School, Worcester, MA in partial fulfillment of the requirement for the degree of

DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES

January, 1993
Molecular Genetics and Microbiology
HIV-1 SEQUENCES IN THE ESTABLISHMENT OF CHRONIC VIRUS PRODUCERS

A Thesis presented

By

Farah Mustafa

Approved as to style and content by:

-----------------------------------------------
Dr. Trudy Morrison, Chairperson of Committee

-----------------------------------------------
Dr. Carel Mulder, Member of Committee

-----------------------------------------------
Dr. Janet Stein, Member of Committee

-----------------------------------------------
Dr. Klaus Strebel, Member of Committee

-----------------------------------------------
Dr. Gregory Viglianti, Member of Committee

-----------------------------------------------
Dr. Harriet L. Robinson, Thesis Advisor

-----------------------------------------------
Dr. Thomas B. Miller Jr., Dean of Graduate School of Biomedical Sciences

Molecular Genetics and Microbiology
January, 1993
This thesis is dedicated to my dear parents,

Tariq and Akhtar Mustafa,

and to my beloved land lady,

Anna E. Cannon
ACKNOWLEDGEMENTS

All praise be to Allah, the Beneficent, the Merciful.

Glory to God Most High, full of Grace and Mercy;
He created All, including Man.
To Man He gave a special place in His Creation.
He honoured man to be His Agent,
And to this end, endued him with understanding,
Purified his affections, and gave him spiritual insight;
So that man should understand Nature,
Understand himself,
And know God through His wondrous Signs,
And glorify Him in Truth, reverence, and unity.

(Yusuf Ali)

My heartfelt gratitude goes to my mentor and advisor, Dr. Harriet L. Robinson, for all her patience, hardwork, guidance, help, and understanding throughout the duration of my graduate school experience.

I would also like to thank all those who shaped and challenged me, inspired and encouraged me, and made graduate school as much of a pleasant experience as could have been possible—my professors, especially Dr. Carel Mulder, and my colleagues and classmates, especially Cindy, Cherie, Dave, Ellen, Fran, Kathy, Kishore, Lalitha, Mohan, Nancy, Nandini, Pracha, Rob, Roz, Shan, and Sharon. I would like to acknowledge Donna Zinkus for part of the work presented in Figure 9 of this thesis.

The past 5 1/2 years of my life in Worcester would not have been the same without the special love and friendship of my dearest friend Dr. Naseema, who was always there when I needed her, and who gave me the strength and fortitude to keep on going, and my beloved Ann (may God rest her soul in peace), who somehow always believed in my ability to accomplish almost anything in life, and who taught me the meaning of faith, courage and hope.
Of course, no thanks can be complete without remembering my loving family—Mama, Abbu, Baji, Hunu, Umar, Makoli, Nini, and especially Golo—each one of whom encouraged me to pursue my dreams in their own ways, and who patiently withstood the long separation in time and space during the accomplishment of those dreams. And this accomplishment would not have been so sweet, complete, and meaningful without my fiancé Ahmad, who kept me going in many strange ways.

I would also like to take a moment to thank so many others whose professionalism, guidance, help, and friendship made it such a pleasant experience to be a student at UMMC: the staff and friends at the Department of Pathology—especially Beryl, Rick and Rosemary—and the administration of UMMC—especially Linda Dexter at the Graduate School Office, Mary Phelan at Government Relations, Maria Durham at the Interpreter Services, Dr. Debora Hines at Student Affairs, and Eileen Consolmagno at the Registrar’s office.

And last but not least, though words cannot convey the depth of my feelings, my very, very special thanks goes to my roommate, Sajida, without whose tender loving care I would not have made it.
HIV-1 SEQUENCES IN THE ESTABLISHMENT OF
CHRONIC VIRUS PRODUCERS

January 1993

Farah Mustafa

Thesis Advisor: Harriet L. Robinson, Ph.D.

ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) infections have different patterns of expression in different T-cell lines. HIV-1 encodes regulatory as well as structural genes. The role of HIV-1 regulatory gene expression in determining different patterns of infection was explored in four T-cell lines: C8166, H9, A3.01, and Jurkat. The hypothesis being tested was that differences in the expression of regulatory genes would determine differences in the kinetics of infection.

To study patterns of regulatory and structural gene expression, RNA was isolated from cultures infected with HIV-1-NL4-3 (NL4-3). During the early and acute phases of infection, the absolute amounts of viral RNA differed in the four T-cell lines. However, the relative proportions of messages for regulatory and structural genes were similar. Thus, differences in the kinetics of infection in C8166, H9, A3.01 and Jurkat cells were not determined by differences in the relative levels of expression of regulatory and structural genes.
Analyses of RNA samples from the chronic phase of infection revealed the consistent appearance of novel RNase sensitive sites in H9 and Jurkat cultures. These marked the emergence of viral variants with high ability to establish chronic virus producers. These variants were specifically selected in the chronic phase since they did not undergo selection during serial passage of the virus through the lytic phase of infection. Sequence analysis of the region with the novel RNase sensitive sites revealed the co-mapping of nucleotide changes with each of the novel sites. Most of these differences represented a sense mutation in tat and the abrogation of the initiator methionine of vpu. However, the selected mutations in tat and vpu were not sufficient, by themselves, to affect the ability of NL4-3 to establish chronic virus producers (Chapters I and II).

Further studies on the roles of viral sequences in the chronic phase of infection were undertaken using constructed viruses. Two molecularly cloned viruses, NL4-3 and HIV-1-HXB-2 (HXB-2), were used as parents. NL4-3 has a low ability to establish chronic virus producers. In contrast, HXB-2 has a high ability to establish chronic virus producers. NL4-3 encodes all known HIV-1 genes, whereas HXB-2 is defective for three auxiliary genes: vpr, vpu, and nef. In addition, both viruses differ at other positions throughout the genome. The first series of constructed viruses tested whether differences in auxiliary gene expression determined differences in the ability of NL4-3 and HXB-2 to establish chronic virus producers. NL4-3 mutants containing all possible combinations of the three defective genes in HXB-2 were constructed. Analysis of the ability of
these mutants to establish chronic virus producers revealed that vpr and nef limit
the ability of NL4-3 to establish chronic virus producers. This was shown by
viruses with defects in both of these genes having high ability to establish chronic
virus producers (Chapter III).

The second series of constructs tested for the roles of non-auxiliary as well
as auxiliary gene sequences on chronic virus production by creating recombinants
between NL4-3 and HXB-2. Tests of these recombinants revealed that a gag, pol,
vif, and vpr fragment could affect the ability of fragments containing defective
auxiliary genes to establish chronic virus producers. Taken together, these results
indicate that vpr, nef, and 5’ internal sequences play important roles in
determining the ability to establish chronic virus producers (Chapter IV).
TABLE OF CONTENTS

ABSTRACT vi

TABLE OF CONTENTS ix

LIST OF FIGURES xv

LIST OF TABLES xvii

INTRODUCTION 1

HIV-1 Virion Structure and Genome Organization 2

Expression of Viral Genes 4

Structural Genes: gag, pol, and env 6

Regulatory and Accessory Genes 7

The CD4 Molecule is the Receptor for HIV-1 13

CD4-Independent Infection of Cells 14

HIV-1 Life Cycle 14

Control of HIV-1 Life Cycle 16

Clinical Stages of HIV-1 Infection 20

Immune Dysfunctions in the Infected Individuals 24

Macrophages as Reservoirs of HIV-1 in the Infected Individual 24

Quiescent T-cells as the Reservoir of Inducible Virus 25

Dynamics of HIV-1 Persistence with Disease Progression 29

Evolution of the Viral Genome 29
Degree of Defectiveness of HIV-1 Genes with Disease Progression

Increase Virulence of HIV-1 Isolates with Disease Progression

Genetic Determinants of Cytopathicity

Mechanism of HIV-1-Induced Cytopathicity

Goals of the Thesis

MATERIALS AND METHODS

Cells
DNA Constructs
Antibodies
Viral Stocks
Stock Titrations
Transfections and Infections
Indirect Immunofluorescence Assays
Fluorescence-Activated Cell Sorting (FACS) Analyses
RNA Extractions
Riboprobes
Northern Blot Analyses
RNase Protection Assays
Amplification, Cloning and Sequencing
Protein Extractions
$^{35}$S Metabolic Labeling of Cells
CHAPTER I

Introduction

Results

Comparative Analysis of HIV-1 Infections in T-cell Lines

Patterns of RNA Expression in the Four T-cell Lines

Mapping of the Novel RNase Sensitive Sites in Chronic Virus Producers

Analysis for Size Classes of RNAs

The Novel RNase Sensitive Sites are not Selected on Serial Passage of NL4-3 Through the Lytic Phase of Infection

The Novel RNase Sensitive Sites Represent the Emergence of Variant Viruses

Discussion

Temporal Expression of Structural and Regulatory Genes

Relative Usage of Splice Sites by HIV-1

Entry is the Major Determinant of Different Kinetics of Expression in T-cells.

Differences in the Ability of Cytopathic HIV-1 Isolates to Establish Chronic Virus Producers

Differences in the Ability of T-cell Lines to Establish Chronic Virus Producers
Differences in Selective Pressures in the Lytic and the Chronic Phases of Infection

CHAPTER II

Introduction

Results

Characterization of Variant Viruses

Origin of the Variant Viruses

The 600 bp Test Region of the Variants is not Sufficient to Change the Ability of NL4-3 to Establish Chronic Virus Producers

Discussion

Selection of Certain HXB-2-like Mutations in Chronic Virus Producers

Selection for Specific Mutations in Immunodeficiency Virus in Tissue Culture

Frequent Transitions of G to A in HIV-1 Genomes Undergoing Passage in Tissue Culture

Origin of HXB-2-like Sequences

The Region Encoding the HXB-2-like tat and vpu Mutations is not Sufficient for the Efficient Establishment of Chronic Virus Producers

CHAPTER III

Introduction

Results

NL4-3 and HXB-2 Have Distinct Abilities to Generate Chronic Virus Producers
Tests for the Effects of Vpr, Vpu, and Nef on the Ability of NL4-3 to Establish Chronic Virus Producers

Discussion

Roles of vpr and nef in the Establishment of Chronic Virus Producers

Low Cytopathic Potential does not Ensure the Efficient Establishment of Chronic Virus Producers

Vpu is not Important in the Establishment of Chronic Virus Producers

CHAPTER IV

Introduction

Results

Use of NL4-3-HXB-2 Recombinants to Map Sequences that Affect the Establishment of Chronic Virus Producers

Discussion

Involvement of HIV-1 Sequences in the Establishment of Chronic Virus Producers

Context-Dependent Requirement for 5’ Internal Sequences

Low Cytopathic Potential is not a Direct Correlate of the Ability to Establish Chronic Virus Producers

Env Sequences do not Determine the Ability to Establish Chronic Virus Producers
SUMMARY AND FUTURE DIRECTIONS

Roles of vpr, nef, and the 5' Internal Sequences in the Establishment of Chronic Virus Producers

Future Directions

Relevance of the Phenomenon of Chronic Virus Production to Infected Hosts

BIBLIOGRAPHY
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>HIV-1 virion structure</td>
<td>3</td>
</tr>
<tr>
<td>2.</td>
<td>Genomic organization of HIV-1 and overview of the known function of each gene product</td>
<td>4</td>
</tr>
<tr>
<td>3.</td>
<td>HIV-1 splicing patterns</td>
<td>5</td>
</tr>
<tr>
<td>4.</td>
<td>Model for the bimodal Rev function</td>
<td>9</td>
</tr>
<tr>
<td>5.</td>
<td>HIV-1 life cycle</td>
<td>15</td>
</tr>
<tr>
<td>6.</td>
<td>Schematic representation of the HIV-1-LTR and the locations of binding sites for nuclear transcription factors</td>
<td>18</td>
</tr>
<tr>
<td>7.</td>
<td>Natural course of HIV-1 disease in humans</td>
<td>22</td>
</tr>
<tr>
<td>8.</td>
<td>Cloning of the 600 bp PCR amplified fragments from the variant viruses into pNL4-3</td>
<td>47</td>
</tr>
<tr>
<td>9.</td>
<td>Temporal analysis of IIIB and NL4-3 infected C8166, H9, A3.01, and Jurkat cells</td>
<td>56</td>
</tr>
<tr>
<td>10.</td>
<td>Schematic for RNase protection assays</td>
<td>59</td>
</tr>
<tr>
<td>11.</td>
<td>RNase protection analysis of RNAs from timed infections of NL4-3</td>
<td>61</td>
</tr>
<tr>
<td>12.</td>
<td>Mapping of expected and novel RNase protected fragments using the S-S and R-K probes</td>
<td>63</td>
</tr>
<tr>
<td>13.</td>
<td>Northern blot analysis of RNAs from culture with the acute and chronic patterns of RNase protected fragments</td>
<td>65</td>
</tr>
<tr>
<td>14.</td>
<td>Schematic representing the protocol for selection of NL4-3-DNA-1 through the lytic phase of infection</td>
<td>67</td>
</tr>
<tr>
<td>15.</td>
<td>Selection of NL4-3-DNA-1 through the lytic phase of infection in H9 and A3.01 cells</td>
<td>69</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>16. RNase protection analyses of cellular and viral RNAs following different selections of pNL4-3 DNA</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>17. Temporal analyses of NL4-3-, NL4-3-C1-, and NL4-3-C4- infected H9 cells</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>18. Photomicrographs of the cultures in Figure 17 at 19 days post infection</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>19. Protein expression by the variant viruses</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>20. PCR recovery of fragment containing the novel RNase sensitive site from the variant virus</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>21. Schematic demonstrating the co-mapping of sequence changes with novel RNase sensitive sites</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>22. Immunoprecipitation of Vpu from cultures infected with NL4-3, NL4-3-C1, NL4-3-C4, and HXB-2</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>23. Analysis of pNL4-3-DNA-2- and pHXB-2-transfected H9 cultures for the generation of novel pattern of protected fragments</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>24. Temporal analysis of pNL4-3-, pHXB-2-, pC1h-, pC2r-, and pC4t-transfected H9 cultures</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>25. Temporal analysis of pNL4-3- and pHXB-2-transfected H9 cultures</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>26. Temporal analysis of H9 cultures transfected with pNL4-3, pHXB-2, and NL4-3 mutants</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>27. Temporal analysis of H9 cells transfected with pNL4-3, pHXB-2, and NL4-3-HXB-2 recombinants</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>28. Summary of data on the NL4-3-HXB-2 recombinants and schematic of their construction</td>
<td>121</td>
<td></td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Occurrence of variant viruses in NL4-3-DNA-1-transfected H9 and Jurkat cells</td>
<td>62</td>
</tr>
<tr>
<td>2. Amino acid changes encoded by the mutations in the variant viruses</td>
<td>87</td>
</tr>
<tr>
<td>3. Amino acid differences between NL4-3 and HXB-2</td>
<td>103</td>
</tr>
<tr>
<td>4. Comparison of vpr, vpu, and nef genes of NL4-3, NL4-3 mutants, and HXB-2</td>
<td>105</td>
</tr>
<tr>
<td>5. Summary of data on the NL4-3 mutants</td>
<td>108</td>
</tr>
</tbody>
</table>
INTRODUCTION
INTRODUCTION

Human Immunodeficiency Virus (HIV-1) is the etiological agent for the Acquired Immunodeficiency Syndrome (AIDS). CD4(+) T-cells are the prime target of the viral infection in this syndrome. Most infections of CD4(+) T-cells are cytopathic. However, in some instances, the virus can establish itself in T-cells and produce progeny without causing cytopathicity and death. Studies presented in this thesis were directed towards understanding the role of HIV-1 genes in the ability of the virus to establish cells that are chronic virus producers. The following introduction provides a broad overview of the virus itself and the disease that it causes. It ends with a perspective on why we chose to study viral determinants that affect the ability to establish chronic virus producers.

HIV-1 Virion Structure and Genome Organization

HIV-1 is a member of the Lentivirinae subfamily of the family Retroviridae (Barre-Sinoussi et al., 1983; Gallo et al., 1984). It is an enveloped RNA virus which is approximately 100 nm in diameter (Figure I). The HIV-1 virion contains two strands of genomic RNA which are assembled as a nucleoprotein within a toroidal capsid. In addition to the genomic RNA, the capsid contains three viral enzymes that are necessary for early and late steps in the viral life cycle. These include, the reverse transcriptase for DNA synthesis, the integrase for the integration of the provirus into the cellular genome, and the protease that cleaves precursor viral proteins to their mature forms. In addition, the capsid contains
the primer tRNA$_{198}$ needed for reverse transcription. The capsid is surrounded by a lipid membrane derived from the cytoplasmic membrane of the host cell. The lipid membrane contains the viral envelope glycoproteins (for recent reviews see Chatterjee, Basak, and Khan, 1992 and Kessler et al., 1992).

Figure 1. Structural characteristics of HIV-1 (Figure adapted from Kessler et al., 1992).

The HIV-1 genome organization is similar to that of other retroviruses. The 9.2 kb single stranded RNA genome resembles cellular mRNAs in that it has a 5' cap (Gppp) and a 3' poly(A) tail. At each end of the genomic RNA are short repeats (R) and unique 5' (U5) and 3' (U3) sequences. These contain important regulatory elements involved in reverse transcription, integration, transcription, and poly-adenylation. Sequences immediately adjacent to U5 encode the primer binding site (PBS) for (-) strand DNA synthesis whereas sequences immediately
adjacent to U3 encode the PBS for (+) strand DNA synthesis. Sequences thought to be important for packaging of the viral RNA into virions are also located near U5. The integrated virus is a double stranded DNA species containing long terminal repeat sequences (LTRs) at both ends. Each LTR consists of a copy of the U3, R, and U5 sequences. In between the LTRs are sequences encoding the structural and regulatory genes of the virus (Figure 2).

**Figure 2.** Genomic organization of HIV-1 and overview of the known function of each gene product. LTR, long terminal repeat; TAR, trans-acting responsive element (Figure adapted from Feinberg and Greene, 1992).

**Expression of Viral Genes**

HIV-1 is a prototypic complex retrovirus (Cullen, 1991b). Complex retroviruses express a number of gene products other than gag, pol, and env. HIV-1 uses differential splicing, frameshifting and ribosome scanning to express at least nine gene products from a single RNA transcript. These include two
structural polyproteins (Gag and Env), a polymerase polyprotein (Pol) and at least six maturational or regulatory proteins (Figures 2 and 3). The Gag and Pol polyproteins are encoded by an unspliced 9.2 kb RNA. Five to 10% of the peptides initiated at the Gag AUG undergo frameshifting to produce Pol-specific products (Jacks et al., 1988; Wilson et al., 1988). A 4-5 kb class of mRNAs encodes the Env polyprotein and the maturational proteins Vif, Vpr and Vpu (Muesing et al., 1985; Rabson et al., 1985; Arrigo et al., 1990; Schwartz et al., 1990a and b; Furtado et al., 1991).

**HIV-1 mRNAs**

![Diagram](image)  

**Figure 3.** HIV-1 splicing patterns. The HIV-1 genome is shown at the top with the coding exons shown as boxes. Bold lines depict the mature mRNAs with their coding potential(s) shown along the sides (Figure adapted from Schwartz et al., 1990a).
Approximately 5 and \( \sim 4.5 \) kb species encode Vif and Vpr respectively (Arrigo et al., 1990; Schwartz et al., 1990a and b; Furtado et al., 1991). Vpu and Env are encoded by a bicistronic \( \sim 4 \) kb RNA (Arrigo et al., 1990; Schwartz et al., 1990b; Furtado et al., 1991). A 2 kb class of RNAs expresses the regulatory proteins, Tat, Rev, and Nef (Benko et al., 1990; Guatelli et al., 1990; Robert-Guroff et al., 1990; Salfeld et al., 1990; Schwartz et al., 1990a; Furtado et al., 1991). The 5' non-coding sequences for the 2 kb RNAs include different combinations of at least 3 small non-coding exons. Splicing events distinguish the translation of these 2 kb mRNAs with the AUG immediate to the 5' splice site determining the sites for protein synthesis (Schwartz et al., 1990a; Furtado et al., 1991).

**Structural Genes: gag, pol, and env**

The Gag and Pol proteins of the virus are produced as precursor molecules that are cleaved into the mature virion forms during virus maturation (Figures 1 and 2) (for reviews, see Chatterjee, Basak, and Khan, 1992 and Kessler et al., 1992). The gag region encodes the Gag precursor polyprotein, Pr55, that is endoproteolytically cleaved to form the structural proteins of the virus. These include the myristoylated matrix protein (p17), the capsid protein (p24), and the nucleocapsid proteins (p7 and p9). The pol region encodes enzymes necessary for virus replication. The Pol precursor polyprotein, Pr160, is cleaved to generate the viral protease (p10) (required for virus maturation), the reverse transcriptase (p66/51) (required for viral DNA synthesis), and the endonuclease/integrase (p31) (required for provirus integration). The env gene encodes a polyprotein
gp160 that is cleaved in the golgi to form the extracellular glycoprotein (gp120) and the transmembrane glycoprotein (gp41). The gp120 subunit of the Env protein contains the binding site for the virus receptor, the CD4 molecule. The gp41 trans-membrane protein is involved in the fusion of viral and cellular membranes.

**Regulatory and Accessory Genes**

The regulatory and accessory genes of HIV-1 can be grouped into two categories based on their formation by single (vif, vpr, and vpu) or multiple (tat, rev, and nef) splicing events (Figures 2 and 3). Of these, only tat and rev are essential for virus replication. The roles of the other gene products are not clearly understood and frequently disputed.

**tat**

Tat, an 86 amino acid, 14 kd nuclear protein, is a potent trans-activator for the transcription of proviral DNA (Arya et al., 1985; Sodroski et al., 1985a and b; Dayton et al., 1986) (Figure 2). Tat functions by binding to TAR, a cis-acting RNA stem-loop structure at the 5' end of all HIV-1 mRNAs (Rosen, Sodroski, and Haseltine, 1985; Muesing, Smith, and Capon; 1987; Feng and Holland, 1988; Berkhout, Silverman, and Jeang, 1989; Dingwall et al., 1990; Calnan et al., 1991; Weeks and Crothers, 1991). TAR functions only in the correct position and orientation (Selby et al., 1989). Tat has been proposed to modulate transcription by increasing the rate of initiation as well as the efficiency of the elongation of viral transcripts. Tat acts by binding to a U-rich bulge in the TAR element.
According to the RNA enhancer model, this binding facilitates the initiation of subsequent rounds of transcription by Tat. Tat’s effect on the rate of initiation is affected by the basal rate of LTR-directed transcription and is apparent only when the basal rate is low (Lasplia, Rice, and Mathews, 1990; Kessler and Mathews, 1991). Increasing evidence indicates that Tat also acts to stabilize the elongation of viral transcripts by RNA polymerase II (Selby et al., 1989; Lasplia, Rice, and Mathews, 1989; Marciniak and Sharp, 1991; Kessler and Mathews, 1992). In addition to functioning through an RNA binding site, Tat can function when bound to upstream promoter DNA (Southgate and Green, 1991). Tat requires cellular factors for acting through both RNA and DNA elements (Marciniak et al., 1990; Gatignol et al., 1991; Southgate and Green, 1991). In the absence of Tat, the HIV-1-LTR has very low activity (Dayton et al., 1986; Fisher et al., 1986).

**rev**

The 19 kd Rev protein is the second essential regulatory gene of HIV-1 (Figure 3) (Feinberg et al., 1986; Sodroski et al., 1986b). Primarily localized to the nucleus and the nucleolus, Rev controls the synthesis of viral structural proteins (Figures 2 and 4). In the nucleus, Rev inhibits the splicing and/or allows the export of unspliced and singly-spliced HIV-1 mRNAs (Malim et al., 1988; Arrigo et al., 1989; Emerman et al., 1989; Felber et al., 1989; Hadzopoulo et al., 1989; Hammarskjold et al., 1989; Malim et al., 1989a and b). In the cytoplasm, it increases the translation of HIV-1 mRNAs (Arrigo and Chen, 1991; Cochrane et al., 1991). Like Tat, Rev requires a cis-acting sequence, the Rev Responsive
Element (RRE), for both of its activities (Malim et al., 1988; Rosen et al., 1988; Arrigo et al., 1989; Emerman et al., 1989; Hammarskjold et al., 1989; Malim et al., 1989b) (see below). Formation of multimeric Rev structures are required for Rev binding to the RRE (Olsen et al., 1990; Malim and Cullen, 1991; Zap et al., 1991).

The RRE is a complex secondary RNA structure present in the env coding region of the viral genome (Figure 2). It is present in the unspliced, 9.2 kb, and the singly spliced, 4-5 kb, classes of mRNAs, but is removed by splicing from the multiply spliced 2 kb class of mRNAs (Figure 4) (Arrigo et al., 1989; Felber et al.,

Figure 4. Model for the bimodal Rev function. Rev is translated from an RNA that does not depend on Rev for expression. Rev localizes in the nucleus where it binds to RRE-containing RNAs, promoting their export from the nucleus. Rev remains bound to the RNA during transport. Once in the cytoplasm, Rev would allow the association of the RNA with the translational machinery of the cell (Figure adapted from Arrigo, Heaphy, and Haines, 1992).
1989; Hadzopoulou-Cladaras et al., 1989). Rev controls the transport of the 9.2 and 4–5 kb classes of mRNAs by binding to the RRE (Daly et al., 1989; Zap and Green, 1989; Felber et al., 1990; for review, see Feinberg and Greene, 1992). When levels of Rev are low (such as occurs early in infection or in cell lines in which an infection is latent), 2 kb mRNAs predominate (Guatelli et al., 1990; Kim et al., 1989; Pomerantz et al., 1990). When levels of Rev are high, the 9.2 kb mRNA can become the predominant species (Kim et al., 1989; Pomerantz et al., 1990). Since Rev requires multimerization for binding to the RRE, levels of Rev that are below the threshold for multimer formation (such as occur early in infection) can prevent the synthesis of viral structural proteins (for review see Pomerantz, Bagasra, and Baltimore, 1992) (see below).

\textit{nef}

The \textit{nef} gene of HIV-1 is the most 3' coding sequence of the viral genome. Its coding sequences partially overlap the 3' LTR (Figure 2) (Allan et al., 1985; Ratner et al., 1985b). Like Tat and Rev, Nef is an early gene product (Guatelli et al., 1990; Schwartz et al., 1990a; Hewlett et al., 1991; Klotman et al., 1991). Of the three multiply spliced mRNAs, Nef message is the most abundant in T-cells as well as monocyte/macrophages (Guatelli et al., 1990, Robert-Guroff et al., 1990; Klotman et al., 1991). The role of Nef is highly disputed and not clear (reviewed in Hovanessian, 1992). However, the fact that a Nef open reading frame is found in all primate lentiviruses suggests that this gene has an important function in the lentivirus life cycle \textit{in vivo} (Cullen, 1991a).
The biological significance of Nef *in vivo* has been demonstrated in the SIV/rhesus monkey model (Kestler et al, 1990 and 1991). In this model, Nef is essential for the maintenance of high viral loads and the induction of immunodeficiency. A premature stop codon is rapidly selected for a sense codon in infected animals with the resulting Nef-expressing virus causing immunodeficiency. By contrast, virus with a *nef* deletion (a mutation that cannot revert) is unable to cause immunodeficiency.

The most pronounced effects of Nef in cell culture are on the cellular factors involved in the activation of T-cells and monocyte/macrophages and not on virus replication. Nef down regulates the viral receptor, CD4, from cell surfaces (Garcia and Miller, 1991), limits the ability of mitogens to activate the transcription of the lymphokine, interleukin-2 (IL-2) (Luria, Chambers, and Berg, 1991), and inhibits the induction of nuclear factor kB (NFkB) by T-cell mitogens (Niederman et al., 1992). This has lead to the suggestion that Nef's targets may be the infected cell and not the virus itself (Feinberg and Greene, 1992).

*vif, vpr, and vpu*

Roles of Vif, Vpr, and Vpu, in the virus life cycle are not clear (Figure 2). Two of these proteins, Vif and Vpu, may be important for virus maturation. The viral infectivity factor, Vif, is a 23 kd protein (Kan et al., 1986; Sodroski et al., 1986c). An open reading frame corresponding to vif has been observed in all primate, feline, and bovine immunodeficiency viruses as well as such lentiviruses as visna-maedi (for references, see Fan and Peden, 1992). Vif is thought to act by
increasing viral infectivity during virus production (Fisher et al., 1987; Fan and Peden, 1992; Gabuzda et al., 1992).

Vpr is a 15 kd, 96 amino acid protein (Wong-Staal, 1987) (Figure 2). It is the only non-essential HIV-1 protein that is associated with the virion (Cohen et al., 1990a; Yuan et al., 1990). Multiple Vpr proteins are found in each virus particle and the protein is thought to be a dimer (Cohen et al., 1990a; Gras-Masse et al., 1990). Non-pathogenic strains of simian immunodeficiency viruses, such as SIV<sub>cpz</sub> and SIV<sub>md</sub>, do not contain the vpr open reading frame (Fukasawa et al., 1988). Vpr can weakly trans-activate the HIV-1-LTR as well as heterologous viral promoters (Cohen et al., 1990b). It does not seem to act through a cis-acting element and is thought to function by interacting with cellular proteins for trans-activation. Vpr can increase the rate of viral replication and the level of viral protein expression as well as the cytopathicity of infection for T-cells (Cohen et al., 1990b). The C-terminus of Vpr is essential for activity and is rich in arginine residues which could be involved in binding to RNA (Yuan et al., 1990). No specific function for Vpr has been associated in T-cells. However, Vpr appears to increase monocyte/macrophage tropism (Hattori et al., 1990; Westervelt et al., 1992). Preliminary data indicate that Vpr may have important functions for the maintenance of high viral loads and induction of immunodeficiency in infected animals (Lang and Fleckenstein, 1992).

Vpu is a 16 kd integral membrane phosphoprotein important for the release of virus particles from infected cells (Figure 2) (Cohen et al., 1988;
Strebel, Klimkat, and Martin, 1988; Strebel et al., 1989; Terwilliger et al., 1989; Klimkait et al., 1990). Vpu shares sequence homology with the membrane-associated M2 protein of influenza virus (Strebel, Klimkait, and Martin, 1988). M2 has been shown to be an ion channel (Pinto, Hoslinger, and Lamb, 1992). Vpu and Env are translated from a bicistronic mRNA suggesting a coordinant interaction of the two proteins (Arrigo et al., 1990; Schwartz et al., 1990b). This is consistent with Vpu increasing the processing of the Env precursor gp160 by disrupting complex formation of the Env precursor with the viral receptor molecule, CD4 (Willey et al., 1992a). Vpu dissociates gp160 from CD4 by inducing the degradation of CD4 in the endoplasmic reticulum (Willey et al., 1992b). Whether the ability of Vpu to enhance virus particle release is functionally related to its ability to disrupt gp160-CD4 complex formation remains to be determined.

The CD4 molecule is the Receptor for HIV-1

Several lines of evidence indicate that CD4 is the major receptor for HIV-1. Antibodies to CD4 molecules can block infection of otherwise permissive cells (Dalgleish et al., 1984; Klatzman et al., 1984). The viral envelope protein, gp120, can specifically bind to the CD4 molecule (McDougal et al., 1986). Transfection of human CD4(-) cells with the CD4 molecule results in the infection of these cells by HIV-1 (Maddon et al., 1986). Finally, soluble CD4 molecules as well as synthetic peptides of CD4 can block viral infection (Fisher et al., 1988; Lifson et al., 1988).
CD4-Independent Infection of Cells

CD4 may not be the only receptor used by the virus for the entry process. HIV-1 can infect cells that do not express CD4 molecules such as cells of the central nervous system, cells in the bowel, human foreskin fibroblasts, and muscle cells (Levy, 1989; Tateno et al., 1989; Li et al., 1990; Zack, Arrigo, and Chen, 1990). Galactosylceramid on glial cell surfaces, the complement receptor CR2, and the Fc receptor gamma III on monocyte/macrophages have been implicated as being alternative receptors for HIV-1 (Takeda et al., 1988; Tschachler et al., 1988; Clapham et al., 1989; Robinson, Montefiori, and Mitchell, 1990; Harouse et al. 1991).

Other cellular proteins may also be required for HIV-1 infection since HIV-1 cannot infect all CD4(+) human cells such as certain brain and skin cells (Cheseboro et al., 1990), nor can it replicate in murine cell lines transfected with the CD4 molecule (Maddon et al., 1986). One line of evidence indicates that a cellular protease, present in some but not all CD4(+) cells, may be required for infection (reviewed in Putney, 1992).

HIV-1 Life Cycle

The overall HIV-1 life cycle is similar to that of other retroviruses. Infection of permissive cells is initiated by the attachment of the virus to its receptor, the CD4 molecule (Figure 5) (reviewed in Chatterjee, Basak, and Khan, 1992). Sequences in the viral envelope gp120 mediate binding of the virus to CD4. Attachment is followed by the fusion of viral and cellular membranes. The
viral gp41 protein is involved in the fusion process. Once the viral capsid is inside the cell, the virion RNA undergoes reverse transcription by the virion-associated reverse transcriptase to produce a double stranded DNA copy of the viral RNA. Once inside the nucleus, the DNA integrates into the cellular genome using the virion encoded integrase. The double stranded linear DNA molecule is the precursor for integration. After integration, the viral genome becomes a cellular gene that is replicated and expressed as other cellular genes. Viral mRNAs

Figure 5. HIV-1 viral life cycle. See text for details.
transcribed from the integrated DNA are transported to the cytoplasm for translation. The Gag precursor molecules become anchored at the inner surface of the plasma membrane by the myristoyl moiety at their N-termini. The Gag precursors also bind genomic RNA by the zinc finger domains found in the nucleocapsid proteins. Final assembly and maturation takes place during budding. Essential to maturation is proteolytic cleavage of the Gag and Gag-Pol precursor polyproteins into their mature forms.

Control of HIV-1 Life Cycle

The HIV-1 viral life cycle is controlled by both viral and cellular factors. The most important viral genes in this control are the trans-acting, regulatory factors tat and rev. Other viral factors like Vif and Vpr may also be involved in the regulation of the HIV-1 life cycle, but since the functions of the genes are not clear, their roles in this regulation are also not clear.

Tat control of HIV-1 gene expression

Tat is a positive regulatory factor of LTR-directed gene expression (reviewed in Cullen, 1991a; Feinberg and Greene, 1992). The HIV-1-LTR is transcriptionally inactive in most cells. But once cells are stimulated (by mitogens, for example), the LTR produces small amounts of multiply spliced RNAs which include messages for Tat. Newly synthesized Tat upregulates its own production as well as that of all the other HIV-1 proteins. Tat acting on the TAR element is thought to be instrumental in activating the virus from its latent state in quiescent cells (see below).
Rev control of HIV-1 gene expression

The Tat/TAR activation loop of the virus interacts with another regulatory loop involving the Rev protein (Figure 4) (Arrigo, Heaphy, and Haines, 1992). Rev controls the transition from early (regulatory genes) to late (regulatory and structural genes) gene expression by controlling the extent of splicing (reviewed in Zack, Arrigo, and Chen, 1990; Cullen, 1991a; Feinberg and Greene, 1992). As Rev synthesis increases, Rev acts to allow the synthesis of viral structural proteins. This diversion of RNA from regulatory to structural genes limits the synthesis of regulatory genes (Figure 4). Early in infection, when the levels of Rev are low, predominantly the multiply spliced 2 kb species of mRNAs are made. With the progression of infection, higher levels of Tat increase the level of expression of all genes including Rev. Increased levels of Rev result in increased synthesis of the 4-5 kb and the 9.2 kb structural mRNAs (containing the RRE) concomitant with a decrease in the 2 kb regulatory mRNAs (lacking the RRE). This pushes the infection into the late phase resulting in the production of progeny virions.

The concerted action of Tat and Rev gives HIV-1 the flexibility to establish either a latent or a productive infection depending upon the activation or differentiation state of the infected cell (see below).

LTR Control of HIV-1 Life Cycle

The HIV-1 viral life cycle is influenced not only by the virus-encoded regulatory factors, but also by the presence of host transcription factors. A combination of viral and host factors ultimately determine the transcriptional state
of the viral genome which can extend from being completely inactive to being fully active. HIV-1 has achieved this level of control by the evolution of an LTR that contains binding sites for a number of host cell transcription factors. Some of these are constitutively expressed, while others are inducible (Figure 6) (for a recent review, see Reddy and Dasgupta, 1992).

The initial rate of HIV-1 proviral DNA transcription is determined by interaction of cellular factors with binding sites present near the transcription start site in the LTR (Figure 6). The LTR has been divided into three functionally

![Diagram of HIV-1 LTR and binding sites](image)

**Figure 6.** Schematic representation of the HIV-1-LTR and the locations of binding sites for nuclear transcription factors. The boundaries of U3, R, and U5 regions as well as the negative regulatory element (NRE), enhancer (ENH), and trans-activating response (TAR) regions are indicated. Locations of the binding sites for various transcription factors are indicated below the LTR structure (Figure adapted from Reddy and Dasgupta, 1992).
distinct regions relative to the transcriptional start site: an upstream negative regulatory element (NRE), an enhancer region (ENH), and a downstream trans-activating region (TAR) (Figure 6). The NRE has been shown to contain sites for nuclear factors of activated T-cells, NFAT-1 (Shaw et al., 1988), upstream stimulatory factor, USF (Garcia et al., 1987), and AP-1 (Franza et al., 1988) binding factors. The enhancer region includes a set of three tandem sites for transcription factor Sp1 (Jones et al., 1986; Garcia et al., 1987; Harrich et al., 1989), TATA box factor, TFIID (Jokobovits et al., 1988), two tandem binding sites for nuclear factor, NFkB (Nabel and Baltimore, 1987), and the leader-binding protein, LBP (Jones, Luciw, and Duchange, 1988). The TAR region includes a site for the CCAAT binding protein, CTF/NF-1 (Jones, Luciw, and Duchange, 1988) and the TAR binding proteins, UBP-1 and EBP-1 (Wu et al., 1988). In addition to these sites, the LTR contains three sites for the proto-oncogene product Myb, one of which is contained within the NRE, one within the enhancer region, and one upstream of the trans-activation region (Dasgupta et al., 1990).

Three of these transcription factors have been shown to have major effects on LTR-dependent transcription. Two of these are constitutively expressed, while one has tissue specific and inducible expression. Sp1 and TFIID are the constitutively expressed cellular transcription factors important in mediating promoter function of many cellular and viral genes (for a review see Lewin, 1990). Both are critical to the functioning of the HIV-1-LTR in vitro and deletion of their binding sites results in a defective HIV-1 provirus in cells without NFkB.
NFkB is important for tissue specific and inducible gene expression, especially during the activation of resting T-cells (Nabel and Baltimore, 1987; Tong-Starksen, Luciw, and Peterlin, 1987; Bohnlein et al., 1988). Thus, T-cell activation allows transcriptional activation of the HIV-1-LTR. This has led to the hypothesis that intracellular levels of activated NFkB may determine whether the integrated HIV-1 provirus will undergo an active lytic life cycle or a latent one (Nabel and Baltimore, 1987; Tong-Starksen, Luciw, and Peterlin, 1987). NFkB is also important in cells of the monocyte/macrophage lineage since mature macrophages constitutively produce NFkB (Griffin et al, 1989). Phorbol esters and mitogenic lectins as well as tumor necrosis factor alpha (TNF-alpha) and interleukin-1 (IL-1) activate HIV-1 production in T-cells by acting through NFkB (see Zack, Arrigo, and Chen, 1990 for references). However, NFkB binding sites are not essential for viral replication since HIV-1 can replicate in cells that do not produce significant levels of NFkB (Maddon et al., 1986). In addition, deletion of both NFkB sites in the HIV-1-LTR has little effect on the replication of the virus in activated peripheral blood lymphocytes (PBLs) (Leonard et al., 1989). Thus, NFkB probably acts to enhance the rate of viral transcription in cells responding to mitogenic stimuli (see below).

Clinical Stages of HIV-1 Infection

Acquired Immunodeficiency Syndrome (AIDS), the pathological consequence of HIV-1 infection, initiates as a self-limited, acute viral syndrome
that develops into a long, clinically asymptomatic stage, ending with the
development of severe immunodeficiency in the infected individual and eventual
death (for recent reviews, see Feinberg and Greene, 1992; Kessler et al., 1992).
Detailed analysis of each period is described below.

**The Early Acute Period**

AIDS is characterized by an early acute phase of infection which can have high levels of virus replication (Figure 7). During this early phase (lasting for a few weeks), the infected individual may experience flu-like symptoms including fever, headaches, lymphadenopathy, and rash. Plasma viremia of up to $10^3$-$10^4$ tissue culture infectious doses (TCID)/ml may be observed with as high as 1% of circulating CD4(+) cells becoming infected (Clark et al., 1991; Daar et al., 1991). During the acute phase, a sharp decline in the numbers of CD4(+) cells may occur (Figure 7) (Clark et al., 1991).

**The Long Asymptomatic Period**

The high plasma viremia observed in the early acute phase of the disease falls with the appearance of cytotoxic T-cells and antibody responses to the infection (Figure 7). The length of the asymptomatic period, from seroconversion to developing AIDS, varies with a median of $\sim$12 years. Some individuals (such as transfusion-related cases in children) develop the disease within a year, while others take more than a decade. Throughout the asymptomatic period, virus continues to replicate, but at a very low level in the peripheral blood (Coombs et al., 1989; Ho, Moudgil, and Alam, 1989; Bagnarelli et al., 1992; Michael et al.,
Viral replication is thought to be kept in check by the activation of the humoral and cellular arms of the immune system. Neutralizing antibodies to the virus appear concomitant with the appearance of cytotoxic T-cells, natural killer cells, and antigen-presenting monocyte-macrophages. With the progression of the disease, a gradual decline in the levels of CD4(+) T-cells is observed (50-100 cells/mm²/year) (Fahey et al., 1990).

Figure 7. Natural course of HIV-1 infection in humans. The graphs present relative levels of viral replication and CD4(+) lymphocyte counts with disease progression (Figure adapted from Kessler et al., 1992).

The rapid seroconversion noted in infected individuals is primarily a response to the structural proteins of the virus. Early antibodies to Env include isolate-specific neutralizing activity (Albert et al., 1990). However, with disease progression, viral variants emerge which are resistant to the neutralizing effects of the early antibodies (Robert-Guroff et al., 1986). As viral variants emerge with new epitopes, a fresh antibody response is generated to these new viral epitopes.
These viral variants are no longer neutralized by the earlier antibodies.

Patients in the long asymptomatic period have very low levels of infected cells in the peripheral blood with as few as 1 in 1000 to 1 in 10,000 circulating T-cells having viral DNA (Brinchmann et al., 1991; Saag et al., 1991; Hsia and Spector, 1991). Of the DNA containing cells, only one in 10 typically displays viral antigens on its cell surface (Brinchmann et al., 1991). With the development of better techniques like in situ PCR, higher percentages of infected cells have been reported during this phase with up to 1% of the peripheral blood mononuclear cells now thought to potentially harbor HIV-1 provirus (Bagasra et al., 1992).

Peripheral blood contains only about 2% of the total body lymphocyte pool (Feinberg and Greene, 1992), with the major reservoir of CD4(+) lymphocytes being found in the lymphoid organs (tonsils, adenoids, and lymph nodes). Studies of virus load in the lymphoid organs have revealed that these tissues consistently contain ten times higher proportions of infected lymphocytes than peripheral blood. This is true for individuals from all stages of disease (Fox et al., 1991; Pantaleo et al., 1991). Infection of the lymphoid tissues is thought to be important in the development of immune dysfunction.

The Final Symptomatic Period

The symptomatic stage of disease appears as the immune system fails to control opportunistic infections. High levels of virus replication reappear with the virus load increasing ten times in the peripheral blood as well as the lymphoid
organisms (Psallidopoulos et al., 1989; Schnittman et al., 1989, 1990a; Simmonds et al., 1990). The levels of plasma viremia rise and can exceed $10^4$ TCID/ml (Figure 7) (Ho, Moudgil, and Alam, 1989; Schnittman et al., 1990a; Saag et al., 1991).

With the rise of viral antigens in plasma, there is a concomitant increase in the rate of CD4(+) T-cell decline. The host immune response is no longer able to suppress virus replication, most likely due to the increasing severity of defects in the immune system. The result is pathologies related to full-blown AIDS.

**Immune Dysfunctions in the Infected Individuals**

Disturbances in many facets of the immune system occur in infected individuals. These include alterations in the numbers and functions of memory T-cells, macrophages and B-cells (for references, see Kessler et al., 1992; Feinberg and Greene, 1992). The CD4(+) class of memory T-cells undergoes depletion. Those that remain are compromised in their ability to produce IL-2 and in their responsiveness to antigen stimulation. These latter defects are often detected before a decline in the numbers of CD4(+) cells occurs. Impairment of macrophage functions include defective intracellular killing, diminished ability to present processed antigens due to reduced expression of Class II HLA-DR molecules, and increased production of tumor necrosis factor alpha. Functional defects for B-cells are observed as early as three months post infection. These include loss of mitogen-induced Ig synthesis and chronic B-cell activation.

**Macrophages as Reservoirs of HIV-1 in the Infected Individual**

CD4(+) T-cells and macrophages are the major targets of HIV-1 in the
infected individual. Of these, macrophages are the major reservoir of HIV-1 in solid tissues (Ho, Pomerantz, and Kaplan, 1987; for a recent review, see Pomerantz, Bagasra, and Baltimore, 1992). In situ DNA hybridization has revealed chronic infection of macrophages from the bone marrow, lungs, lymph nodes, skin, and the brain (Levy et al., 1989; Kessler et al., 1992). Unlike T-cells where viral maturation occurs at the plasma membrane, viral maturation in the macrophage can occur at the endoplasmic reticulum as well as the cell membrane. Thus, intracellular stores of virus in macrophages may act as reservoirs of virus throughout the body.

**Quiescent T-cells as the Reservoir of Inducible Virus**

In addition to macrophages, resting T-cells may also act as reservoirs of HIV-1 in the peripheral blood. Unlike the macrophages that can contain complete progeny virions, resting T-cells are an inducible source of the virus. Productive infection of T-cells is dependent upon the proliferative state of the cells (Zagury et al., 1986; Stevenson et al., 1990c; Zack, Arrigo, and Chen, 1990). The virus can productively infect stimulated but not unstimulated T-cells (McDougal et al., 1985). In unstimulated cells, virus can enter and undergo reverse transcription, but it cannot make progeny virions.

**The Pre-Integration State of Latency**

Two states of viral latency have been reported in quiescent T-cells. In the pre-integration state of latency, extrachromosomal HIV-1 DNA can be detected in quiescent lymphocytes with PCR (Stevenson et al., 1990c; Zack et al., 1990;
Pomerantz, Bagasra, and Baltimore, 1992). The state of this DNA is controversial. According to Zack et al., 1990, it is the result of inefficient, incomplete reverse transcription. These incomplete reverse transcripts are labile and lose the ability to produce progeny virions on mitogenic stimulation by 15 hours post infection (Zack et al., 1990 and 1992).

In contrast, Stevenson et al., 1990 have shown extrachromosomal DNA in resting T-cells to be complete reverse transcripts (Stevenson et al., 1990c; Bukrinsky et al., 1991). This completely reverse transcribed DNA is transcriptionally active and can synthesize p24 Gag, Env, and Tat proteins (Stevenson et al., 1990b and c). In addition, it is stable and capable of integration for up to two weeks (Stevenson et al., 1990b and c). The reason for the different results obtained by the two groups is not clear.

Complete reverse transcripts also appear to occur in quiescent T-cells in infected hosts. In the asymptomatic stage of infection, resting T-cells have been shown to contain unintegrated, complete reverse transcripts (Bukrinsky et al., 1991). This DNA retains the ability to integrate upon T-cell activation. By contrast, in patients with full blown AIDS, an increasing proportion of DNA is found in the integrated state.

The block giving rise to the pre-integrative state of latency in quiescent T-cells may be due to a lack of necessary cellular factors in these cells (Zack et al., 1990; Stevenson et al., 1990c). These may include NFkB and protein kinase C (PKC)-- two factors important in T-cell activation as well as the activation of the
viral LTR and enhancement of Tat function. Tat's potency may be enhanced by PKC in infected cells (Jakoborits, Rosenthal, and Capon, 1990). Quiescent T-cells contain low levels of both NFkB and PKC. Thus, low levels of these factors in non-proliferating cells may be a possible mechanism of keeping HIV-1 infection dormant until levels of these factors increase such as after T-cell activation (see Feinberg and Greene, 1992). Whatever the cause of the block, the ability of resting T-cells to become latently infected with HIV-1 is of significance as such may be the major source of inducible virus in the peripheral blood.

**The Post-Integration State of Latency**

In addition to the pre-integration form of latency, a post-integration form of latency has also been observed in T-cells both in tissue culture and in infected individuals (Pomerantz et al., 1990; Pomerantz, Bagarsa, and Baltimore, 1992). In contrast to the pre-integration form of latency, low levels of virus replication can occur in post-integration latency.

Post-integration latency has been characterized in the monocytic cell line U1 and the T lymphocytic cell line ACH-2 (Pomerantz et al., 1990). In the unstimulated state, these cells are found to be arrested in an aberrant state of RNA expression typical of cells in the early stage of infection. This state is characterized by low levels of predominantly multiply spliced 2 kb and some 4 kb mRNAs with very low expression of the unspliced 9.2 kb mRNAs. The same pattern of expression is observed in cells infected with Rev mutants (Feinberg et al., 1986). Stimulation of these cells with mitogens relieves the block leading to a
productive viral life cycle. The block observed in these cells may be due to sub-threshold levels of Rev. Mitogenic stimulation probably activates NFκB which in turn activates the viral LTR. Small increases in the levels of all proteins, especially Tat, then trigger the LTR-directed increase in the transcription of all viral genes including Rev which removes the block and allows a productive viral life cycle to ensue (Pomerantz et al., 1990; Pomerantz, Bagasra, and Baltimore, 1992).

Preliminary work on HIV-1 specific RNA expression in the peripheral blood mononuclear cells (PBMC) at various stages of the disease has provided some evidence for the existence of regulatory gene-encoded latency in the infected individual (Seshamma et al., 1992). The ratios of the multiply spliced to unspliced RNA were greatly increased in the asymptomatic individuals compared to individuals with AIDS. Over 70% of the PBMC in the asymptomatic patients seem to express multiply spliced but not unspliced RNA. This is in contrast to the AIDS patients where over 80% of PBMC express unspliced RNAs. Cells expressing only multiply spliced RNAs may be spared by the immune system due to the lack of expression of viral structural proteins, thus providing another source of an inducible reservoir of virus. More importantly, this reservoir should not be sensitive to treatment with reverse transcriptase inhibitors since the virus has already undergone reverse transcription (Pomerantz, Bagasra, and Baltimore, 1992).
Dynamics of HIV-1 Persistence with Disease Progression

Thus, whether the viral latency is pre-integrative, post-integrative, or both, HIV-1 is able to persist in the infected individual for extended periods of time and survive. Clinical latency, the asymptomatic stage of HIV-1 infection, as opposed to viral latency, is by no means a state of absence of virus expression and replication. Instead, it is characterized by low levels of virus production which may be the result of pockets of "smoldering" infections in the infected individual. These pockets may be exemplified by the lymphoid organs such as the lymph nodes in which certain populations of cells are producing virus, while others are latently infected with no virus expression or production. Circulating cells in the blood and lymphatic vessels passing through these centers of active virus replication, may occasionally get productively infected and die releasing a constant amount of virus. Thus, a certain virus load could be maintained during the long asymptomatic stage without completely depleting the main target cells and at the same time causing immune dysfunction (Fox and Cottler-Fox, 1992).

Evolution of the Viral Genome

One of the hallmarks of retroviral infection is the evolution of the viral genomes with time (Coffin, 1986; Goodenow et al., 1989). Many mutations are the result of the high error rate of reverse transcription. The viral reverse transcriptase introduces ~1 misincorporation per genome per replication cycle (Dougherty and Temin, 1988; Leider et al., 1988; Goodenow et al., 1989). The error rate of the HIV-1 reverse transcriptase may exceed that of other retroviruses
(Vartanian et al., 1992). The populations of related HIV-1 genomes in a patient (or a stock) over time are referred to as a "quasispecies" (Steinhauer and Holland., 1986; Goodenow et al., 1989).

Sequence data of viral isolates from patients reveals heterogeneity of viral genomes both within and among infected individuals. Within an individual at a given time period, the quasispecies may vary between 0-2% of nucleotides (nts) in env (Hahn et al., 1986; Saag et al., 1988; McNearny et al., 1990). Viral genomes vary between 0-9% (of nts in env) among close contacts (Burger et al., 1991; Balfe et al., 1990; McNearny et al., 1990), and between 6-22% (of nts in env) among isolates from unrelated individuals (Ratner et al., 1985a; Alizon et al., 1986; Myers et., 1990). Within one patient in a three-week period, the maximum amino acid variation observed in a series of genes was: 14.9% for env, 1.0% for vif and vpr, 1.9% for nef, 7.9% for tat, 8.6% for vpu, and 10.2% for rev (Groenink et al., 1992).

Degree of Defectiveness of HIV-1 Genes with Disease Progression

Analyses of the degree of defectiveness of viral genomes from uncultured patient isolates have revealed that there are limited signs of defectiveness for regulatory and accessory genes within the viral genomes present in vivo (Balfe et al., 1990; Delassus et al., 1991; Martin et al., 1991; Li et al., 1991 and 1992; Blumberg et al, 1992). Biological tests of cloned proviruses in animal model systems have revealed that there is selective pressure to preserve open reading frames for at least two viral accessory genes, vpr and nef. The functions of both
of these genes appear to be important for the maintenance of high viral loads and the induction of immunodeficiency (Kestler et al., 1991; Lang and Fleckenstein, 1992).

**Increased Virulence of HIV-1 Isolates with Disease Progression**

Biological analyses of viruses isolated from patients over a period of time, as well as patients from different disease stages, have revealed increases in the virulence of isolates with disease progression (Wong-Staal et al., 1985; Cheng-Mayer et al., 1988; Fenyo et al., 1988; Meyerhans et al., 1989; Tersmette et al., 1988; Schwartz et al., 1989). Viruses isolated from the asymptomatic stage of the disease are generally slowly replicating, non-syncytium inducing viruses that grow to only low titers in primary cells and not at all in established cell lines. These are the slow/low viruses. With the progression of the disease, viruses isolated from ARC (AIDS related complex) or AIDS patients can replicate rapidly and to high titers in both primary cells as well as established cell lines and can cause syncytia (Asjo et al., 1986; Cheng-Mayer et al., 1988; Fenyo et al., 1988; Tersmette et al., 1988). These latter isolates display not only higher cytopathicity, but also a broadened host range.

**Genetic Determinants of Cytopathicity**

Genetic analyses of the patient isolates have revealed that the env gene is the major determinant of cytopathicity (Sodroski et al., 1986a; Cheng-Mayer et al., 1990; Koga et al., 1990; Stevenson et al., 1990a; York-Higgins et al., 1990; Hoxie et al., 1991; Kowalski et al., 1991; de Jong et al., 1992). The major effect of Env
on cytopathicity is due to its effect on the ability of the virus to induce syncytia. Specifically, the V3 (the third variable domain) loop within the gp120 portion of the envelope protein has been implicated in the syncytium-inducing capability of the virus. Single amino acid substitutions altering positively charged residues within this loop can drastically affect the ability of virus to induce syncytia (de Jong et al., 1992).

However, other regions of the viral genome such as the 5' gag/pol sequences, have also been implicated in determining the cytopathic potential of the virus (Hirsch et al., 1992; de Mareuil et al., 1992). In addition, certain auxiliary genes of the virus have been shown to influence the cytopathicity of the virus. These include: tat (Cheng-Mayer, Shioda, and Levy, 1991), vif (Sakai et al., 1991), vpr (Cohen et al., 1990b), and vpu (Terwilliger et al., 1989). The fact that cytopathicity can be modulated by such a variety of genetic elements suggests that there may be several mechanisms for cell killing.

**Mechanism of HIV-1-Induced Cytopathicity**

As apparent from the fact that many regions of the virus have been implicated in the cytopathic potential of a virus, cytopathicity is not a well understood phenomena. Syncytium formation can cause cell death (Sodroski et al., 1986a). Cell death can also occur in the absence of syncytium formation (single-cell killing) (Somasundaran and Robinson, 1987; Leonard et al., 1988; Tersmette et al., 1989). Fusion of infected cells with uninfected cells leads to the formation of syncytia. The viral envelope glycoproteins expressed on the surfaces
of infected cells interact with the CD4 receptor on the surfaces of adjacent uninfected cells resulting in the formation of multi-nuclear giant cells (Lifson et al., 1986; Sodroski et al., 1986a; Stein et al., 1987; Rusche et al., 1988; Kowalski et al., 1991). This explains why Env protein has been found to be the major determinant of cytopathicity of viruses in cell culture.

Though syncytium formation is amply observed in cell culture with most cytopathic HIV-1 isolates, few syncytia have been observed in vivo in the lymph nodes of patients with AIDS and none has been observed in the peripheral blood (Racz et al., 1986). In addition, in vitro, cell death has been observed in the absence of syncytia (Somasundaran and Robinson, 1987; Cloyd and Moore, 1990) and syncytia have been observed without cell death (Stevenson et al., 1990a; Sakai et al., 1988). Thus, cell fusion may not be a very important determinant of cell death in vivo.

Death by single-cell killing could result from many factors including: i) death by disruption of cellular membranes due to massive viral budding (Leonard et al., 1988); ii) viral protein products causing disruption of cellular functions (Hoxie et al., 1986; Koga et al., 1990) or iii) direct toxicity due to the intracellular accumulation of viral protein products (Stevenson et al., 1988; Terwilliger et al., 1989). These observations, once again, point to the complex nature of "cytopathicity" and emphasize that no one mechanism or determinant can explain its basis.

In short, HIV-1 is a complex retrovirus capable of using an intricate web of
control mechanisms, both at the cellular and viral levels, to ensure its long term survival. Armed with Tat and Rev, it is capable of a latent or a productive infection depending upon the cell type it infects and the proliferative and/or differentiation state of the cell.

Goals of the Thesis

This thesis was undertaken to study the role of regulatory genes in determining different kinetics of replication of HIV-1 in T-cells. Preliminary work had shown that both the virus and the cell line affected the kinetics of HIV-1 expression in culture. At the time, very little was known about the nature of the regulatory genes and the hypothesis had been presented that regulatory genes controlled the tempo of virus replication. Based on this, our hypothesis was that differential expression of viral regulatory genes would determine the kinetics of virus replication in T-cell lines.

Actual analyses of the temporal expression of regulatory and structural genes revealed that regulatory genes were not determining differences in the tempos of infection in T-cell lines (Chapter I). During these studies, novel RNA fragments were observed in the chronic phase of infection. These fragments were characterized and shown to reflect the emergence of viral variants (Chapters I). Characterization of the variants revealed that they had enhanced ability to generate chronically infected, virus producing cells (chronic virus producers) (Chapter II). Studies in Chapter II also identified two molecularly cloned viruses with different abilities to generate chronic virus producers. In Chapters III and
IV, these two viruses were used to map HIV-1 sequences that affect the ability to establish chronic virus producers. Results of these analyses revealed that the ability to establish chronic virus producers is affected by vpr, nef, and a 5' fragment encoding gag, pol, vif, and vpr.
MATERIALS AND METHODS
MATERIALS AND METHODS

CELLS: C8166, H9 (American Type Culture Collection, ATCC TIB 161), Jurkat clone 8, and A3.01 cell lines were obtained from the NIAID AIDS Repository (Rockville, MD). Cells were maintained at densities of 0.5 to 2X10^6 cells/ml in RPMI 1640 supplemented with 10-20% fetal bovine serum, penicillin and streptomycin (growth media).

DNA CONSTRUCTS: Parental DNAs: Viruses HIV-1-NL4-3 and HIV-1-HXB-2 were obtained as plasmids from the NIAID AIDS repository. pNL4-3 is a laboratory construct that was created by ligating the 5' half (till the unique EcoRI site) of HIV-1-NY5 with the 3' half (from the EcoRI site onwards) of HIV-1-LAV-1 (Adachi et al., 1986). The plasmid pHXB-2 contains a non-permuted HIV-1 DNA that was molecularly cloned from HIV-1-IIIb infected cells (Fisher et al., 1985).

NL4-3 Mutants: The Vpu(-)NL4-3 (NLΔU) was obtained from K. Strebel. It was created by inserting an 8 bp XhoI linker at the SspI site in the vpu gene (Strebel, Klimkat, and Martin, 1988). The Nef(-)NL4-3 (NLΔN) was obtained from S. Venkatesan. It was created by filling-in the unique XhoI site in nef (Ahmed and Venkatessan, 1988). The Vpr(-)NL4-3 (NLΔR) was created by filling-in the EcoRI site in vpr using the Klenow fragment of E. coli DNA Polymerase I. The EcoRI to BamHI fragment of the NLΔU mutant was
exchanged with the homologous NLΔN virus to generate the NLΔUΔN mutant.

To create the NLΔRΔU, NLΔRΔN, and NLΔRΔUΔN mutants, the EcoRI sites in vpr of the NLΔU, NLΔN, and NLΔUΔN mutants were filled-in with the Klenow fragment of E. coli DNA Polymerase I. Restriction endonuclease sites that were created or abrogated in constructions were used to verify the presence of mutations.

**NI3-HXB-2 Recombinants:** Recombinants between NI3 and HXB-2 were constructed using conserved SpeI, ApaI, EcoRI, SalI, and XhoI sites to interchange homologous regions. Constructions were verified using EcoRI, NcoI, and SpeI restriction sites that distinguish pHX-2 from pNI3. Recombinant DNAs were grown in E. coli HB101.

**ANTIBODIES:** i) Leu 3a, a monoclonal antibody against the virus-binding region (CDR2) of CD4 was obtained from Becton Dickinson (Mountain View, CA); ii) OKT4A, a monoclonal antibody against CD4 outside the virus-binding site (next to CDR2) was obtained from Ortho Diagnostics (Raritan, NJ); iii) a patient sera from an HIV-1 sero-positive individual for use in indirect immunofluorescence assays (IFA) and western blot analyses was provided by Dr. J. Sullivan (Department of Pediatrics and Program in Molecular Medicine, University of Massachusetts Medical Center, Worcester, MA); iv) IgG-IG, a pooled sera from HIV-1 sero-positive individuals, for use in immunoprecipitations and western blot analyses was obtained from Abbot Laboratories (Chicago, IL); v) an anti-Vpu sera
raised in rabbits against an *E. coli* derived fusion protein was obtained from Dr. Klaus Strebel (Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, MD).

**VIRAL STOCKS:** HIV-1-IIIb (IIIb) was produced by serial passage of a stock originally obtained from Dr. R. C. Gallo (Gallo et al., 1984; Shaw et al., 1984). HIV-1-NL4-3 (NL4-3) was recovered 4 to 6 days post transfection of H9 cells with pNL4-3. HIV-1-HXB-2 (HXB-2) was recovered 14 to 16 days post transfection of H9 cells with pHXB-2. Transfections were carried out as described below. Stocks were titered for infectious units on C8166 cells as described below.

**STOCK TITRATIONS:** Titers (infectious units) of virus stocks were determined by infecting C8166 cells with serial dilutions of the test stock in the presence of 2 ug/ml Polybrene. Leu 3a (240 ng/ml) was added to cultures at 3 hours after infection to prevent syncytium formation (Sattentau et al., 1986). At 24 hours after infection, cells were assayed for the fraction expressing viral antigen by IFA (see below). Stock titers were calculated as (percentage of virus-expressing cells) X (concentration of C8166 cells at the time of infection) X (test dilution). Titers obtained by this method agreed well with those obtained by endpoint dilution on C8166 cells. The highest-titered stocks (~10^6 infectious units per ml) were obtained from cultures undergoing a spreading infection. Multiplicities of infection are given as C8166 infectious units per cell.
TRANSFECTIONS AND INFECTIONS: For transfections, 1 ug of plasmid DNA was used per 1X10^6 H9 cells. For each transfection, 10^7 exponentially growing cells were used. Cells were washed in RPMI/Tris (RPMI 1640 medium buffered with 0.1 M Tris-HCl, pH 7.5), trypsinized in 0.025% Trypsin (Gibco/BRL, Grand Island, NY) for five minutes at room temperature, and washed once in RPMI/Tris. Washed cells were resuspended in 2 ml of RPMI/Tris and incubated for 20 minutes at room temperature with 10 ug of DNA and 400 ug DEAE-Dextran (200 ug/ml) (Pharmacia, Sweden). Transfections were stopped by adding a five-fold excess of growth medium. Cells were washed once in RPMI/Tris before culture. The medium of transfected cultures was changed daily during the early and lytic phases of infection and on alternate days during the chronic phase. At the time of feeding, cultures were monitored for the numbers of live and dead cells using trypan blue exclusion and the percent of infected cells using IFA as described below.

Infections were carried out in the presence of 2-10 ug/ml polybrene. Cultures were washed at 24 hours after infection. As explained above, infected C8166 cultures were grown in the presence of 240 ng/ml of Leu3a. Both the transfected and infected cultures were fed daily during the early and lytic phases of infection and on alternate days during the chronic phase of infection. At the time of feeding, cultures were monitored for virus-expressing cells by IFA. Virus-induced cytopathic effects were followed by scoring numbers of live (trypan blue excluding) and dead (trypan blue staining) cells.
**INDIRECT IMMUNOFLUORESCENCE ASSAYS (IFA):** IFAs for the detection of viral antigen-positive cells were performed as follows: 2.5X10⁵ cells were washed with phosphate buffered saline (PBS), suspended in fresh 20 ul PBS, and allowed to settle on 10-well siliconized slides (Cell Line Associates, Newfield, NJ) by sequentially placing the 20 ul samples over a series of wells. Excess liquid was removed after approximately one minute and the wells were allowed to air dry. Slides were stored at room temperature till assayed for the presence of HIV-1 antigens. HIV-1 antigens were detected by fixing the air-dried cells for 5-10 minutes at room temperature in methanol. Slides were washed with PBS and fixed cells incubated for 30 minutes at 37°C with an appropriately diluted heat-inactivated (57°C, 30 minutes) serum from an HIV-1-seropositive individual. After the first incubation, the slides were again washed three times with PBS and incubated for 30 minutes at 37°C with an appropriately diluted fluorescein-conjugated goat anti-human serum (H and L chain specific; Organon Teknika-Cappel, Durham, NC). Finally, the slides were washed three times with PBS and mounted in 90% glycerin. Virus-positive and -negative cells were counted with a Zeiss fluorescence microscope using a counting grid. Stained slides could be stored at 4°C for several weeks.

**FLUORESCENCE-ACTIVATED CELL SORTING (FACS) ANALYSES:** FACS was carried out using the OKT4A monoclonal antibody as described above. 1-2X10⁶ cells were washed twice in cold PBS and resuspended in 25 ul of cold
RPMI 1640 medium supplemented with 5% FBS and 0.2% Azide. Cells were incubated with 5 ul of OKT4A (50 ug/ml) for 30 minutes on ice with intermittent mixing and washed twice with cold PBS. Washed cells were resuspended in 25 ul of 1:20 dilution of fluorescein-conjugated anti-mouse IgG (H and L chain specific, Ortho Diagnostics, Raritan, NJ) and incubated on ice for 30 minutes with intermittent mixing. After washing twice in cold PBS, cells were fixed in 200 ul of fresh 2% paraformaldehyde for 10 minutes at room temperature, washed once more in PBS at room temperature, and resuspended in the azide-containing medium at 1-2X10^6/ml.

**RNA EXTRCTIONS:** Cellular RNAs were extracted by solubilization of washed cells in a 4 M solution of guanidine isothiocyanate (GIT) followed by cesium chloride (CsCl) density fractionation (for reference see, Davis, Dibner, and Battey, 1986). Solubilized cells (5X10^7/ml) were layered over a 4 ml cushion of 5.7 M CsCl and centrifuged for >36 hours at 32,000 rpm in a SW41 rotor at 20°C. The RNA pellet was dissolved in 0.3 M sodium acetate (pH 6.0), phenol extracted, and precipitated by adding 2-3 volumes of 95% ethanol. The resulting precipitate was collected by centrifugation and dissolved in diethylpyro-carbonate-treated water. The optical density at 260 and 280 nm was determined and the RNA was aliquoted and stored at -80°C as a 70% ethanol slurry.

Virion RNAs were prepared by concentrating virion particles using high speed centrifugation. First, the viral stocks were cleared of cellular debris by
centrifugation at 6,000 rpm for 30 minutes in the table top Sorval RT 6000. Virions in the cleared supernatants were pelleted by ultracentrifugation in the SW 28 rotor at 20,000 rpm for 90 minutes at 4°C. The pelleted virus particles were solubilized in the 4 M GIT buffer and processed for RNA extraction as described for cellular RNAs.

**RIBOPROBES:** Riboprobes were prepared from fragments of pNL4-3 or pHXB-2 that had been subcloned into pBlueskript SK(+) (Stratagene, La Jolla, CA). Riboprobes were produced by the modification of the protocol described by Melton et al., 1984. Briefly, 1 ug of linearized template DNA was incubated with excess GTP, ATP, and UTP (1ul of 10 mM stocks), 12 uM of cold CTP (if used for RNase protection assays), [³²P]CTP (800Ci/mmole) (50 uCi for RNase protection assays and 80 uCi for northern blot analyses), appropriate buffers, RNase inhibitor (RNasin, Promega, Madison, WI), and bacteriophage T3 or T7 polymerase (Promega, Madison, WI). The transcription mixtures were incubated for one hour at 37°C for the polymerization of the probe and DNase treated (DNase RQ1; Promega, Madison, WI) for 15 minutes at 37°C to remove any DNA template. Radiolabeled RNAs were phenol extracted in the presence of yeast tRNA as carrier, separated from unincorporated nucleotides by passing through a Sephadex-G50 spin-column, analyzed for the amount of incorporated radioactivity by Cerenkov counts and stored in water at 4°C if destined for RNase protection assays, or in a 50% formamide buffer at -20°C if to be used for
northern blot analyses. The size and integrity of radiolabeled RNAs were verified by electrophoresis on 6% polyacrylamide gels prepared in the presence of 7 M urea (6% PAGE-7M urea).

**NORTHERN BLOT ANALYSES:** Three to 5 μg of cellular RNAs were mixed with 5 μg of ethidium bromide and separated on 1% agarose gels in 2.2 M formaldehyde and 1x MAE (0.2 M MOPS (pH, 7.0), 5 mM NaAc, and 1 mM EDTA) for 16 to 20 hours at 24 volts with vigorous recirculation. Gels were rinsed in water and photographed to visualize the positions and integrity of rRNA. Using capillary action, transfer to Duralon membranes (Stratagene, La Jolla, CA) was carried out for 16-18 hrs in 20X SSC (0.3M sodium citrate, 3M sodium chloride, pH 7). Transferred RNA was cross-linked to the filters using two exposures of 0.12 Joules of UV irradiation in a Stratalinker (Stratagene, La Jolla, CA). Prehybridizations were done for 3-4 hours at 55-60°C in 0.05 X dried skim milk (Blotto)/50% formamide/6X SSC (Johnson et al, 1984). \(^{32}P\)-labeled riboprobes were added to this solution at 1-2 \(\times\) 10^6 cpms/ml and hybridized overnight at 55-60°C. The filters were washed thrice for 20-30 minutes each in 0.1X SSC/0.1% sodium dodecyl sulfate (SDS) at 67°C and subjected to autoradiography for variable lengths of time using Kodak X-OMAT film. The 0.24-9.5 kb RNA ladder from Bethesda Research Laboratories (Gaithersberg, MD) was used for sizing.
RNase Protection Assays: RNase protection assays were carried out by a modification of the procedure of Zinn, De Maio, and Maniatis, 1984 as described by Beberich and Stoltzfus, 1991. Specifically, one to 20 ug of RNA was pelleted, dried, and dissolved in 35 ul of hybridization buffer (80% formamide, 40 mM PIPES (pH 6.7), 400 mM NaCl, 1 mM EDTA) containing 2-3 x 10^5 cpm of 32P-labeled riboprobe. The mixture was denatured at 95°C for 10 minutes and immediately incubated at 45°C for 10-12 hours. Three hundred ul of RNase digestion buffer (16 ug/ml RNase A and 2 ug/ml RNase T1 in 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 0.3 M NaCl) were added to each sample and the reactions were incubated at room temperature for one hour. Fifty ug of proteinase K, 17 ul of 10% SDS and 3.6 ug of the ribonuclease inhibitor polyvinyl sulfate were added and the samples incubated for 15 minutes at 37°C. Incubation mixtures were extracted once with 400 ul phenol, 2 times with 400 ul of chloroform and precipitated with 3 volumes of 95% ETOH at room temperature in the presence of 20 ug of carrier tRNA. After a 70% ethanol wash at 0-4°C, RNA pellets were dried and the RNA resuspended in 5 ul of loading buffer (80% deionized formamide, 5 mM EDTA, 0.1% each of bromophenol blue and xylene cyanol dyes), denatured at 95°C for 10 minutes, and fractionated on 6% PAGE-7 Maurea sequencing gels at 1000 V for 4 hours. The gels were dried for one hour at 80°C and autoradiographed using Kodak X-OMAT film for variable lengths of times. Denatured 32P end-labeled (using T4 polynucleotide kinase) pBR322-MspI and bacteriophage X174-HaeIII fragments were used as markers for sizing.
AMPLIFICATION, CLONING, AND SEQUENCING: Virion RNA was reverse transcribed using an antisense (as) oligonucleotide primer spanning the KpnI site at nt 6343 of pNL4-3 proviral DNA (Go et al., 1990). The reverse transcripts were PCR amplified using Taq polymerase and a sense (s) oligonucleotide spanning the EcoRI site at nt 5743 of pNL4-3 proviral DNA and the KpnI(as) oligonucleotide (Go et al., 1990). PCR products were then substituted into pNL4-3 for the homologous EcoRI to KpnI fragment (see Figure 8 below and Figure 20 in Chapter II). To accomplish this, the 2.73 and 12.1 kb EcoRI to BamHI fragments of pNL4-3 were isolated by separating the fragments on a 1% low melting point agarose gel. The 2.73 kb fragment was KpnI digested, and the 2.13 kb KpnI to BamHI fragment was isolated as described above. A three way ligation was carried out with this 2.13 kb fragment, the 0.6 kb EcoRI and KpnI digested PCR products, and the 12.1 kb pNL4-3 fragment. Recombinants were screened for intact proviral DNA using KpnI and EcoRI plus BamHI digests. The PCR-derived region of recombinants was sequenced using double strand DNA sequencing primed by anti-sense oligonucleotides (Sequenase version II protocol, United States Biochemical, Cleveland, OH). The oligonucleotides used for cloning and sequencing (and shown in Figure 20) were:

EcoRI (s) 5'-GCCATAATAAGAATTCTGCAACAACCTGC-3' (5733-5760);
EcoRI (as) 5'-GCAGTTGTTGCAGAATTCTTATTATGGC-3' (5760-5733);
KpnI (s) 5'-GTCTATTATGGGTACCTGTGTGGAAGG-3' (6332-6359);
KpnI (as) 5'-CCTTCCACACAGGTACCCCATATAAGAC-3' (6359-6332);
Figure 8. Cloning of the 600 bp PCR amplified fragments from the variant viruses into pNL4-3. The EcoRI to KpnI fragment of pNL4-3 was exchanged with the RT-PCR amplified 600 bp fragment from the variant viruses. R, EcoRI; K, KpnI; and B, BamHI restriction endonuclease sites. RT, reverse transcription; PCR, polymerase chain reaction.
Oligo a (as) 5'-CAATAGCAATTGGTACAAGC-3' (5909-5890);
Oligo b (as) 5'-TACTGCTTTTGTAGAGAAGC-3' (6047-6028);
Oligo c (as) 5'-ATGATTACTATGGACCACAC-3' (6140-6121).

PROTEIN EXTRCTIONS: Protein extracts were made by the method of Felber, Drysdale, and Pavlaks, 1990. In brief, cells were washed in PBS and lysed in 0.5X RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) at 10^7 cells/ml. To prevent oxidation and proteolysis, beta-mercaptoethanol was added at 50 ul/ml, leupeptin was added at 2 ug/ml and phenylmethylsulfonyl fluoride at 1 mM. Nuclei were spun down and supernatants (containing the protein extracts) were frozen at -80°C.

35S METABOLIC LABELING OF CELLS: Cells were metabolically labeled using the procedure of Felber, Drysdale and Pavlaks, 1990. 5x10^6 cells were washed and incubated in 1 ml of cysteine- and methionine-free modified Eagle's medium (buffered with 12 uM N-2-Hydroxyethylproperazine-N'2-Ethane sulfonic acid) for 30 minutes at 37°C. Cellular proteins were metabolically labeled by adding 200 uCi/ml of 35S-Translabel (15% 35S-L-Cysteine and 70% 35S-L-Methionine) and 200 uCi/ml of 35S-L-Cysteine (ICN, Radiochemicals, Irvine, CA) for 3-4 hours at 37°C. Protein samples were prepared for immunoprecipitation according to Felber, Drysdale, and Pavlaks, 1990 as described above.
**IMMUNOPRECIPITATIONS:** Immunoprecipitations were carried out by a modification of the procedure of Felber, Drysdale, and Pavlakis, 1990. Briefly, cell lysates were pre-cleared with pre-immune human sera for 1 hour at 4°C and protein-A Sepharose beads (CL-4B; Sigma chemicals, St. Louis, MO) (15-20 ul bed volume) in RIPA buffer for 30 minutes at 4°C. Cleared lysates were incubated overnight with the appropriate antibodies (1:4000 dilution of IgG-IG, and 1:100 dilution of anti-Vpu sera at 4°C. Immune complexes were precipitated with protein-A Sepharose beads (4 hours at 4°C), and separated on 12-14% SDS-polyacrylamide gels as described below.

**WESTERN BLOT ANALYSES:** Proteins were separated on 12-14% SDS-polyacrylamide gels using the denaturing-discontinuous buffer system of Laemmli, 1970. Samples were boiled in SDS-containing Laemmli buffer prior to loading and run overnight at 8mA (gel size, 11X14 cm). For sizing, the commercially available 14-200 kD Rainbow Markers from Amersham (Arlington Heights, IL) were used. Gels containing 35S-labeled samples were fixed in 10% acetic acid/30% methanol for 1 hour and treated with En3hance (New England Nuclear, Dupont, Boston, MA) for another hour. After rinsing in water and drying, treated gels were exposed to Kodak X-OMAT film.

Gels containing non-radiolabeled samples were prepared for wet electrophoretic transfer onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH) using the SDS-containing transfer buffer (50 mM Tris, 380 mM
glycine, 0.1% SDS (wt/vol), and 20% methanol) (Harlow and Lane, 1988). Transfer was carried out overnight at 300 mA at 4°C. Transferred proteins were cross-linked to membranes using two exposures of 0.12 Joules of UV irradiation in a Stratalinker (Stratagene, La Jolla, CA). Membranes were incubated in 5% dried skim milk (Blotto) (Johnson et al. 1984) and 0.2% Tween-20 in PBS (PBS-Tween) for 1 hour at room temperature. Blocked membranes were incubated with the primary antibody (1:4000 dilution of IgG IG or 1:100 dilution of patient sera) for 2 hours at room temperature in Blotto-PBS-Tween, washed 3 times (10 minutes each) in the PBS-Tween, and incubated with 1:1000 dilution of 125I-labeled protein-A (10 uCi/ul) (Amersham, Arlington Heights, IL) in Blotto-PBS-Tween for 2 hours at room temperature. Membranes were washed 3-5X in PBS-Tween for 10-15 minutes each, and exposed to Kodak X-OMAT film.
CHAPTER I
INTRODUCTION

HIV-1 infections have different tempos and patterns of expression in different T-cell lines (Evans et al., 1987; Somasundaran and Robinson, 1987; Fenyo et al., 1988; Cloyd and Moore, 1990; Kim et al., 1990; Ma et al., 1990). HIV-1 encodes regulatory as well as structural genes. The initial goal of this thesis was to test for the role of HIV-1 regulatory gene expression in determining different patterns of infection. The hypothesis under test was that differences in the temporal expression of HIV-1 regulatory and structural genes would determine differences in the kinetics of infection.

Four T-cell lines were selected for analyses. These differed in the tempo of infection, susceptibility to syncytium formation, and relative surface densities of CD4. C8166 cells were used because of their unusual susceptibility to infection and syncytium formation (Somasundaran and Robinson, 1988). C8166 cells are a line of Human T-cell leukemia virus type 1- (HTLV-1) immortalized, cord blood lymphocytes (Salahuddin et al., 1983). They do not express detectable levels of HTLV-1 capsid proteins, but do express the HTLV-1 tax gene (Giam et al., 1986). C8166 cells have an intermediate surface density of CD4 (Somasundaran and Robinson, 1987; Srivastava et al., 1991). H9 cells were studied because of their unusual ability to establish cells that are chronically producing virus (Popovic et al., 1984). H9 cells are intermediate in susceptibility to infection, are intermediate in ability to form syncytia, and have intermediate densities of surface
CD4 (Somasundaran and Robinson, 1987; Srivastava et al., 1991). H9 cells represent a clone of the Hut78 line which was derived from a human cutaneous T-cell lymphoma (Gazdar et al., 1980). A3.01 cells were studied because of their high surface density of CD4 and their relative resistance to syncytium-induction (Somasundaran and Robinson, 1987; Srivastava et al., 1991). A3.01 cells have an intermediate susceptibility to infection. They are a clone of the human lymphoblastoid cell line CEM (Foley et al., 1985; Folks et al., 1985). Jurkat clone 8 cells were analyzed because of their ability to undergo T-cell activation in culture. Jurkat cells have an intermediate susceptibility to infection, an intermediate ability to form syncytia, and low levels of surface CD4 (Somasundaran and Robinson, 1987; Srivastava et al., 1991). Jurkat cells were derived from a human leukemia cell line (Weiss, 1984).

Two laboratory strains of HIV-1 were used to analyze the role of regulatory genes in controlling virus expression in T-cells. One of these, HIV-1-IIIB (IIIB) is a stock that has been continuously passaged in tissue culture (Popovic et al., 1984). Most clones of IIIB are defective for one or more of the regulatory gene products (for a compilation of these sequences, see Myers et al., 1990). The second, HIV-1-NL4-3 (NL4-3), is a laboratory construct that encodes all known HIV-1 gene products (Adachi et al., 1986).

The temporal expression of HIV-1 structural and regulatory genes was examined using RNase protection assays. The Gag-Pol mRNA of HIV-1 is unspliced. The mRNAs for Vif, Vpr, and Vpu-Env are singly spliced and the
mRNAs for Tat, Rev, and Nef are multiply spliced. Each of the spliced mRNAs is created by the use of unique and specific splice sites (Sadaie et al., 1988; Arrigo et al., 1990; Schwartz et al., 1990a and b; Furtado et al., 1991; Garret, Tiley, and Cullen, 1991). These splicing patterns allowed the design of an RNase protection assay in which one probe could distinguish the mRNAs for the regulatory gene products Tat, Rev, and Nef, as well as the bicistronic Vpu-Env mRNA. This probe could also distinguish the above mRNAs from a single fragment representing Gag-Pol, Vif, and Vpr messages. Thus, a single probe could be used to provide a quick and convenient screen for the mRNAs of regulatory and structural genes.

As expected, the temporal analyses of HIV-1 mRNAs in the T-cell lines revealed that the absolute amounts of all mRNAs increased with the progression of infections. However, the relative ratios of mRNAs for the structural and regulatory genes remained similar in the early and the acute phases of infection. Furthermore, no major differences in these ratios were observed in the four T-cell lines during the early and acute phases of infection. Unexpectedly, a novel pattern of RNase protected fragments appeared in the chronic phase of H9 and Jurkat infections. Analysis of this pattern revealed that it was due to the selection of variants of NL4-3. Selection of these variants was specific to the chronic phase of infection as it did not occur when virus was selected by serial passage through the lytic phase of infection. Chapter II will present a detailed analysis of these variants and their origin.
RESULTS

Comparative analysis of HIV-1 infections in T-cell lines. *T-cell lines differ in the temporal appearance of virus-expressing cells and the ability to establish chronic virus producers.* Infections of C8166, H9, A3.01, and Jurkat cells were initiated with IIIB and NL4-3 at MOI of \( \sim 0.25 \) C8166 infectious units. Since C8166 cells are highly susceptible to HIV-1-induced syncytia, the monoclonal antibody leu3a was added to these cultures at 4 hours post infection to prevent syncytium formation. The other infected cultures were grown in the absence of leu3a.

Infections in the four T-cell lines differed in the temporal appearance of virus-expressing cells (Figure 9). C8166 cells were highly permissive with \( \sim 20\% \) of the cells expressing virus, as determined by the indirect immunofluorescence assay, within 18 hours of infection. By 3 days post infection, more than 90\% of the cells in C8166 cultures were expressing virus. H9, A3.01 and Jurkat cells were less permissive with low numbers of virus-expressing cells first appearing between one and three days post infection. By one to two weeks post infection, 40 to 80\% of cells in these cultures became virus-expressing. In each of the infected cultures, virus infection caused cell death. In general, a one to two day lag occurred between the appearance of virus-expressing cells and the appearance of dead cells.
Figure 9. Temporal analyses of IIIb and NL4-3 infected C8166, H9, A3.01, and Jurkat cells. Infections were followed for virus-expressing cells and dead cells. C8166 cells were grown in the presence of 240 ng of leu3a per ml to prevent syncytium formation. % IFA(+), o; % dead, •.
The four T-cell lines also differed in the ability to establish chronic virus producers (Figure 9). C8166 cells did not establish chronic virus producers as these cells did not survive the lytic phase. In contrast, the lytic phase of infection in H9, A3.01, and Jurkat cultures was followed by the appearance of surviving populations of cells. In H9 and Jurkat cultures, cells that survived the lytic phase contained both virus-expressing cells and non-virus-expressing cells. In A3.01 cultures, cells that survived the lytic phase did not express virus. Thus, only H9 and Jurkat cells were able to establish chronic virus producers.

**IIIB and NL4-3 differ in the ability to establish chronic virus producers.** The overall patterns for the acute phase of infection were similar for IIIB and NL4-3 in each of the four T-cell lines. Both viruses replicated equally well in C8166 cells. NL4-3 appeared to replicate slightly better than IIIB in H9, A3.01, and Jurkat cells. However, more pronounced differences were observed in the ability of IIIB and NL4-3 to establish chronic virus producers. Cultures that grew out from the lytic phase of IIIB infections contained higher proportions of virus-expressing cells than the survivors of NL4-3 infections. In IIIB infections, 90-100% of the H9 survivors, and ~20% of the Jurkat survivors were chronic virus producers. In contrast, in NL4-3-infections, only 5-20% of the H9 survivors, and ~5% of the Jurkat survivors were chronic virus producers (Figure 9).

**Patterns of RNA expression in the four T-cell lines.** RNase protection assays were undertaken to test whether differences in patterns of expression of regulatory and structural genes determined differences in the time courses for the
appearance of infected cells in the four T-cell lines. Assays using RNA from IIIB infections and probes generated from subclones of pHXB-2 (a molecular clone of IIIB) did not give interpretable patterns of protection. This may reflect the accumulation of mutations in the IIIB stock leading to mismatches between the probe and viral sequences. Therefore, further protection assays were done on infections initiated with NL4-3 produced by pNL4-3 transfected H9 cells.

RNase protection assays used a combination of single and overlapping probes, between nt 5661 and 6343 of NL4-3 proviral DNA. Single probes were used to screen for the relative levels of regulatory and structural mRNAs in infected cultures. Three overlapping probes were then used to verify the assignment of bands to specific mRNA species. These distinguished: (1) mRNAs that do not undergo splicing between nt 5661 to 6343 (mRNAs for Gag-Pol, Vif, or Vpr), (2) the Tat and Tev mRNAs that use nt 5776 as a splice acceptor and nt 6044 as a donor, (3) the bicistronic Vpu-Env mRNA that utilizes the splice acceptor at 5975, (4) the bicistronic Rev-Nef mRNAs that utilize splice acceptors at nt 5953 or 5959 and the donor at nt 6044, and (5) the Nef mRNA that utilizes the splice acceptor at 5975 and the donor at 6044 (Figure 10). The predicted sizes of fragments representing each of these mRNAs are summarized for each of the probes in Figure 10C.

RNA expression in the early and acute phases of infection. The temporal analysis of cellular RNAs revealed a steady increase in the absolute amount of both spliced and unspliced mRNAs as the infections progressed from the early to
Figure 10. Schematic for RNase protection assays. A) The region mapped for RNase protected fragments. Known splice acceptor and donor sites; protein start sites; possible locations of the ends of the novel RNase protected fragments. B) Overlapping probes used for protection assays. Numbers denote nucleotide positions in NL4-3 proviral DNA. The probes are designated by bounding restriction endonuclease sites; R-K, EcoRI-KpnI; S-S, SphI to SspI; R-S, EcoRI-SspI. C) Sizes and coding potentials of the mRNA(s) represented by expected fragments. *, an expected fragment that was not observed. D) Sizes and potential positions of novel protected fragments. Doublets or triplets of novel fragments within the different size classes are indicated by presenting schematics in duplicate or triplicate. ..., approximate boundaries of novel fragments.
the acute phase. However, the protection analyses did not reveal temporal changes in the relative proportions of regulatory and structural mRNAs (Figure 11). The fragment representing Gag-Pol, Vif, and Vpr as well as fragments representing Tat, Vpu-Env and Nef were present at the earliest time points analyzed in each of the cell lines.

A high background of non-specific bands was detected in each of the samples from the acute phase of infection. This may reflect the degradation of cellular RNAs during the lytic phase of HIV-1 infections (Agy, 1990).

**A novel pattern of RNase protected fragments in chronic virus producers.** A novel pattern of protected fragments came to dominate the pattern of protected fragments as chronic virus producers emerged in H9 and Jurkat cultures (Figure 11, pattern C). The novel fragments appeared earlier in H9 cultures (between 18 and 28 days) than in Jurkat cultures (between 36 and 64 days) (Table 1). Once the novel pattern appeared, it was stably present for more than two months (see 64 day point for H9 cells in Figure 11). The novel pattern appeared in each of 10 infections initiated by virus recovered from different transfections of one preparation of pNL4-3 DNA (pNL4-3-DNA-1) (Table 1).

**Mapping of the novel RNase sensitive sites in chronic virus producers.**

The ends of the novel protected fragments were mapped to three approximate positions within the NL4-3 genome: ~nt 5945, ~nt 6070, and ~nt 6233 (Figure 10D). An example of this mapping using the S-S and R-K probes is presented in Figure 12. The novel ~410 nt fragment detected with the S-S probe correlates
Figure 11. RNase protection analyses of RNAs from timed infections of NL4-3. Mapping was done with the S-S probe. Designations at the tops of autoradiographs indicate the culture and the time after infection of the RNA being analyzed. Designations at the bottom of the lanes denote whether the fragment sizes are characteristic of the early and lytic (A) or chronic (C) phase of infection. The sizes and coding potentials of the fragments are indicated along the sides. (?) denote novel species.
Table 1: Occurrence of Novel Protected Fragments in Chronic Virus Producers

<table>
<thead>
<tr>
<th>NL4-3 Stock</th>
<th>Cultures with Novel Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfection 1 (DNA-1)</td>
<td>DAY 20 (C2)*</td>
</tr>
<tr>
<td>Transfection 2 (DNA-1)</td>
<td>DAY 18 (C6)</td>
</tr>
<tr>
<td></td>
<td>DAY 23 (C3)</td>
</tr>
<tr>
<td></td>
<td>DAY 24 (C10)</td>
</tr>
<tr>
<td></td>
<td>DAY 27 (C7)</td>
</tr>
<tr>
<td></td>
<td>DAY 28 (C1)</td>
</tr>
<tr>
<td></td>
<td>DAY 31 (C8)</td>
</tr>
</tbody>
</table>

* Designation of variant virus in culture medium

Day refers to the time point (post infection) the novel pattern was first observed
Figure 12. Mapping of expected and novel RNase protected fragments using the S-S and R-K probes. RNase protection data for RNA from one acute (A) and two independently established chronic (C1 and C2) cultures of NL4-3 infected H9 cells are presented. The probes used for the analyses are indicated below the lanes. The sizes and the coding potentials of the protected species are indicated along the sides.
with an ~80 nt shorter fragment (~330 nt) detected with the R-K probe. As the S-S probe would protect ~82 nt of RNA 5’ to the R-K probe, the 5’ end of this fragment is positioned at the 5’ end of the S-S probe. This places the 3’ end of the ~410 nt fragment at ~nt 6070 of proviral DNA. Similarly the ~84/80 nt fragments detected with the S-S probe correlate with the ~274/270 fragments protected by the R-K probe. As the ~84/80 and ~274/270 fragments differ by ~190 nt and the R-K probe extends 190 nt 3’ to the S-S probe, the 3’ end of these fragments is positioned at the 3’ end of the R-K probe and the 5’ end at ~nt 6070 of proviral DNA. The ~123/124/127 cluster of fragments which lie within each of the tested probes was positioned between ~nt 5945 and ~6070, because the length of these fragments was the same as the distance between the novel sensitive sites at positions ~5945 and ~6070. The novel ~110 nt fragment was detected only with the R-K probe and was therefore positioned at the unique end of the R-K probe.

**Analysis for size classes of RNAs.** HIV-1 expresses three major size classes of mRNAs: 9.2 kb unspliced, 4-5 kb singly spliced, and ~2 kb multiply spliced. To determine if the appearance of the novel protected fragments was associated with the appearance of a new size class of HIV-I messages, cellular RNAs from the acute and chronic phases of infection were analyzed by northern blotting (Figure 13). Membranes were hybridized with a 675 nt antisense RNA for sequences common to all HIV-1 mRNAs (extending from the XhoI site in nef to a SacI site in the 3’ LTR). No new size class or shift in the proportions of mRNAs
Figure 13. Northern blot analysis of RNAs from cultures with the acute (A) and chronic (C) pattern of RNase protected fragments. Lanes: 1, uninfected H9 cells; 2-4, RNAs from three independently established acute infections; 5-7, RNAs from the same cultures as in lanes 2-4 after their conversion to the novel pattern of protected fragments. The sizes of RNA species and the locations of rRNAs are presented on the sides. The % of viral RNA present as 2 kb mRNA (determined using a Betascope, Waltham, MA) is indicated at the bottom of lanes. See text for probe.
in the three size classes of mRNAs were observed.

The novel RNase sensitive sites are not selected on serial passage of NL4-3 through the lytic phase of infection. To test whether the novel pattern of protected fragments was specific to selective pressures in chronic virus producers, a stock harvested from pNL4-3-DNA-1 was selected by serial passage through the lytic phase of infection in H9 cells. Five serial passages were initiated with 1:10 dilutions of serial virus stocks harvested from the peak or just past the peak of the lytic phase of infection (Figures 9 and 14). The first three passages were continued into the chronic phase of infection. Cells were harvested from the acute (A) and chronic (C) phases of each serial passage (Figure 14). Cellular RNAs from these phases were analyzed for the RNase sensitive sites characteristic of chronic virus producers.

Analysis of RNAs from the acute phase of the serial passages revealed that the novel protection pattern did not emerge on selection through the lytic phase of infection (Figure 15, panel A). These RNA samples, from the acute phases of the serial passages, displayed the expected pattern of protected fragments (see lanes AI, AII, AIII, and AIV in Figure 15A). However, when each passage was followed into the chronic phase of infection, the novel pattern of protected fragments appeared (see lanes CI and CIII in Figure 15A). These results demonstrate that selective pressures present in the chronic phase of NL4-3 infection are different from the pressures present in the lytic phase of infection.

Passage of pNL4-3-DNA-1 through the lytic phase of infection in the A3.01
Figure 14. Schematic representing the protocol for selection of NL4-3-DNA-1 through the lytic phase of infection. AI-AV represent the five serial passages through the acute phase of infection of DNA-1. CI-CIII represent the passage of three of the serial infections through the chronic phase of infection.
cells also did not select for the novel RNase protected fragments (see lanes AI, AII, AIII, AIV, and AV in Figure 15B). Because A3.01 cells do not support the establishment of chronic virus producers, serial passages of these infections could not be followed into the chronic phase of virus production.

The novel RNase sensitive sites represent the emergence of variant viruses.

Selection of the novel pattern of protected fragments in chronic virus producers could have resulted either from the use of novel splice sites or from the selection of specific mutations in the genomic RNA. To distinguish between these two possibilities, viral RNA (which is unspliced) was tested for the presence of novel sites. For these analyses, cellular and viral RNAs were harvested from H9 cultures infected with (i) unselected NL4-3 (NL4-3 recovered from pNL4-3-DNA-1-transfected H9 cells), (ii) NL4-3 that had been selected in H9 cells by serial passages through the lytic phase of infection (lytic selection), and (iii) NL4-3 that had been selected through the chronic phase of infection (chronic selection).

RNase protection analysis of the viral RNAs harvested from infections that had been selected for chronic virus production revealed the same novel fragments in virion and cellular RNAs (Figure 16, panel (iii)). The presence of these fragments in virion RNA suggested that the novel RNase sensitive sites represented the selection of mutations in genomic RNA rather than the use of novel splice sites.

By contrast, NL4-3 that had undergone lytic selection had the same pattern of protected fragments as unselected NL4-3 (Figure 16, panels (i) and (ii)).
**Figure 15.** Selection of NL4-3-DNA-1 through the lytic phase of infection in A) H9 and B) A3.01 cells. AI-AV represents RNase protection analyses of RNAs harvested from cells undergoing serial passages of pNL4-3-DNA-1 through the lytic phase of infection. CI-CIII represent RNase protection analyses of RNAs harvested from cells of the chronic phases of infections of the first three serial passages (see Figure 14). Designations on sides indicate the sizes and coding potentials of protected fragments.
Figure 16. Analyses of cellular and viral RNAs following different selections of NL4-3 in culture. RNase protection analyses of infections initiated with NL4-3 stocks recovered from pNL4-3 DNA: i) NL4-3 harvested from transfected H9 cells, ii) NL4-3 selected by serial passage through the lytic phase of infection, iii) NL4-3 selected in chronic producers. RNase protection assays were with the S-S probe. Designations at the tops of the lanes indicate whether RNA was prepared from cells (C) or virus (V). Designations on sides indicate the sizes and coding potentials of protected fragments.
Protection assays of the cellular RNAs from cultures infected with both the lytically selected virus and the unselected virus revealed the expected pattern of protected fragments. These represented full length Gag-Pol, singly spliced Vpu-Env, and multiply spliced Tat and Nef mRNAs. Protection assays of the virion RNAs produced by these cultures revealed the single, unspliced fragment characteristic of full length genomic RNA with no mismatches between the probe and the genome.

**DISCUSSION**

The studies presented in this chapter on the temporal expression of HIV-1 regulatory and structural genes in infected cells revealed several findings. First, they suggested that temporal differences in NL4-3 regulatory and structural gene expression do not determine differences in the kinetics of HIV-1 expression in T-cells. Second, they showed that cytopathic HIV-1 strains differ in their ability to establish chronic virus producers. Third, they again demonstrated that T-cell lines differ in their ability to establish chronic virus producers. And fourth, they revealed that selection pressures in the chronic phase of HIV-1 infection differ from selection pressures in the lytic phase of infection.

**Temporal expression of structural and regulatory genes.** Differences in the temporal expression of regulatory and structural genes did not determine the 2-3 day differences in the time course of appearance of virus expressing cells. The
usage of signature splice sites for regulatory and structural genes was similar during the different stages of infection and in each of the infected T-cell lines (Figure 11). Kim et al., 1989 reported temporal differences in the expression of the size classes of mRNAs representing the 9.2 kb, the 4-5 kb, and the ~2 kb mRNAs. This temporal difference occurred between 12-16 hours post infection of H9 cells. The fact that our assays did not detect this temporal difference may have been due to our harvesting the first sample at >18 hours post infection. By this time, irrespective of the tempo of infection, bands representing structural and regulatory genes were present at the same relative ratios as in fully infected cultures. We did not look earlier because we were interested in the role of gene expression in determining differences in infection that were most clearly displayed between 1 and 3 days post infection (Figure 9).

**Relative usage of splice sites by HIV-1.** The splice site usage that we observed was like that previously reported for IIIb, HIV-1-BA-L, and HIV-1-BRU. The splice acceptors for Nef and the bicistronic Vpu-Env mRNAs were more efficient than the acceptor for Tat and Tev or the acceptors for Rev (Figures 11 and 13) (Guatelli et al., 1990; Robert-Guroff et al., 1990; Klotman et al., 1991).

Our assay was not sensitive enough to discriminate between the relative efficiencies of the use of the Tat/Tev and Rev acceptors as we did not detect fragments representing Rev mRNAs (Figures 10A, 11 and 12). Our failure to detect Rev mRNA most likely represents these relatively low abundance messages
being represented by two fragments (91 and 84 nt), each of which would be expected to contain a 3 fold lower level of radioactivity than a single Tat/Tev fragment (268 nt). The high intensity of the fragment representing unspliced Gag-Pol as well as singly spliced Vif and Vpr mRNAs as opposed to multiply spliced mRNAs in the protection assays reflects (1) the presence of fragments representing multiple mRNAs in one band and (2) the intensity of fragments being directly related to their length.

**Entry is the major determinant of different kinetics of expression in T-cells.** Our results reveal that differences in the expression of the regulatory genes of HIV-1 were not the major determinant of the different kinetics of infection observed in the four T-cell lines. Subsequent work in our and other laboratories has shown that the major determinant of permissiveness of cell lines occur at the level of virus entry (Kim et al., 1990; Srivastava et al., 1991). These studies showed no major differences in the timings of reverse transcription, use of the long terminal repeat, expression of viral RNA or proteins, and production of virus particles once entry had taken place. Single cycle infections of C8166, H9, A3.01, and Jurkat cells with NL4-3 revealed that the time needed for viral entry into the cells was 30 minutes for the most permissive C8166 cells, 4 hours for the less permissive H9 and A3.01 cells, and 5 hours for the least permissive Jurkat cells. All four cell lines required about 3.5 hours for reverse transcription. The time required for the expression of viral antigens was somewhat variable with C8166 requiring an average of 17 hours, H9 cells requiring 22 hours, and A3.01 and
Jurkat cells requiring 25 hours (Srivastava et al., 1991). Thus, the biggest difference (eight folds) in the times required for steps in the virus life cycle occurred at the entry step.

Differences in the ability of cytopathic HIV-1 isolates to establish chronic virus producers. Our data demonstrate that NL4-3 and IIIB have different cytopathic effects and substantially different abilities to establish chronic virus producers (Figure 9). The low ability of NL4-3 to establish chronic virus producers is similar to what we have observed for two PBL-passaged patient isolates, SF94 and SF216 (Cheng-Mayer et al., 1988; York Higgins et al., 1990). These viruses undergo lytic as well as chronic phases of infection on H9 cells. Only 10 to 20% of the survivors become chronic virus producers (Robinson and Zinkus., 1991). Thus, the high ability of IIIB and a clone of IIIB, HXB-2 (see Chapter II), to establish chronic virus producers may be relatively unique to IIIB, a laboratory strain that was heavily selected for the ability to establish continuously producing cultures (Popovic et al., 1984).

Differences in the ability of T-cell lines to establish chronic virus producers. Only two of the four tested T-cell lines (H9 and Jurkat) generated easily detected levels of chronic virus producers (Figure 9). Of these two, H9 cells underwent both more rapid and efficient generation of chronic virus producers. The differentiated state of H9 and Jurkat cells is distinct from that of C8166 and A3.01 cells in displaying the T-cell receptor (TCR) and CD3 (Srivastava and Robinson, 1990). Both of these T-cell markers interact with CD4
during T-cell activation (for reviews see Bierer et al., 1989; Robey and Axel, 1990). Whether a differentiated state that is characterized by these markers or other host cell factors influence the efficiency of the establishment of chronic infections remains to be determined.

**Differences in selective pressures in the lytic and the chronic phases of infection.** Passage of virus recovered from NL4-3-DNA-1 through the chronic phase of infection resulted in the emergence of viral variants. By contrast, serial passage of virus recovered from NL4-3-DNA-1 through the lytic phase of infection did not select for the variants that emerged in the chronic phase of infection (Figures 15 and 16). This demonstrates that different selection pressures exist in the acute and chronic phases of HIV-1 infection. These two phases of infection differ from each other in several ways. Cultures from the acute phase of infection contain both CD4(+) and CD4(-) cells. During this phase, virus is undergoing spread and high titer production. Infected cells may die either by syncytium formation or as mononuclear cells.

In contrast, cells that become chronic virus producers produce lower levels of virus and remain healthy. These cells have down regulated CD4 from their cell surfaces. Preliminary data indicate that cells in the chronic phase may express increased levels of several cell surface markers that are involved in T-cell activation and cell-to-cell communication. These include the T-cell receptor, CD3, and CD45 (data not shown). In contrast, surface density of the CD3 antigen on acutely infected H9 cells has been reported to remain the same as on
uninfected cells (Meerlo et al., 1992). What the involvement may be of the T-cell markers, if any, in the establishment of chronic virus producers remains to be determined (see Summary and Future Directions).
CHAPTER II
INTRODUCTION

Work presented in Chapter I demonstrated the selection of NL4-3 variants in the chronic phase of infection of T-cells. These variants were not selected when virus was serially passaged through the lytic phase of infection. The goal of the studies presented in this chapter was to characterize these variants and to establish their origin.

Temporal analyses of H9 cultures infected with the variants revealed that the variants had high ability to establish chronic virus producers. Partial cloning and sequencing of the variants revealed that they contained HXB-2-like mutations in tat and vpu. These mutations were not sufficient, by themselves, to increase the ability of NL4-3 to establish chronic virus producers. Thus, despite the fact that mutations in tat and vpu were selected in chronic virus producers, other mutations within the HIV-1 genome are essential to the efficient establishment of chronic virus producers.

RESULTS

Characterization of variant viruses: *Temporal analysis of H9 cultures infected with the variants*. The variants that emerged in the chronic virus producers were present in 10-30% of the initially infected cells. The *de novo* ability of these variants to establish chronic virus producers was tested by infecting fresh H9 cells
with these variants. The infections were initiated at an equal MOI (0.1 infectious units per cell) with NL4-3 and two of the variants. These variants had been independently selected in chronic H9 (NL4-3-C1) or chronic Jurkat (NL4-3-C4) cultures (Table 1). Infections were followed with time for cell growth, virus-expressing cells, and cell death (Figure 17, panels A-C). Both of the variants were much more effective at establishing chronic virus producers than the unselected NL4-3 stock (Figures 17 and 18). In addition, cultures infected with the variants grew much better and had lower cell death during the acute phase of infection than NL4-3-infected culture (Figure 17, panels A and B). Thus, the variants that had evolved in the chronic phase of infection had a much higher ability to establish chronic virus producers and a lower cytopathic potential than the parental NL4-3 virus.

Proteins expression by the variants. To compare protein expression by the variants with that of NL4-3, protein extracts were prepared from NL4-3-, NL4-3-C1-, NL4-3-C2-, and NL4-3-C9-infected H9 cells (for origin of the variants, see Table 1). Proteins extracts were also prepared from NL4-3-C1 virions. Figure 19 presents the immunoprecipitation and western analysis of these proteins. A pooled patient sera (IgG-IG) was used for all immunoprecipitations. No apparent differences were observed in the expression of p55, gp41, p24, or p17 (Figure 19) and gp120 (data not shown) among the different viruses. However, this analysis did not preclude differences in proteins not recognized by the IgG-IG sera.

Cloning and sequencing of the variants. To evaluate the genetic basis for
Figure 17. Temporal analysis of NL4-3-, NL4-3-C1- (C1), and NL4-3-C4- (C4) infected H9 cultures. A) Growth of infected cultures; B) temporal appearance of virus-expressing cells; C) temporal appearance of dead cells. Infections were initiated with an MOI of 0.1 infectious units per cell. C1 and C4 represent independently selected variants in chronic H9 and Jurkat cells, respectively.
Figure 18. Photomicrographs of the cultures in Figure 17 at 19 days post infection. Immunofluorescent stain is for HIV-1 antigen. A) uninfected; B) NL4-3-infected; C) NL4-3-C1-infected; D) NL4-3-C4-infected H9 cells.
Figure 19. Protein expression by the variant viruses. Immunoprecipitation and western analysis of HIV-1-specific proteins expressed by 1) uninfected H9 cells and by H9 cells infected with 2) NL4-3; 3) NL4-3-C9; 4) NL4-3-C1, 5) NL4-3-C1 virions; and 6) NL4-3-C2. A pooled patient sera, IG-G, was used for the immunoprecipitations. Designations along the sides indicate the HIV-1-specific proteins observed and the sizes of the molecular weight markers.
the novel RNase sensitive sites that had appeared in the variants, the region containing the novel sites was cloned and sequenced. To accomplish this, sequences encompassing a 600 bp EcoRI to KpnI fragment were reverse transcribed, PCR amplified and subcloned (nt 5743 to nt 6343) (Figure 20). Subcloning was done into pNL4-3 so that the biological significance of the encoded mutations could be tested in a NL4-3 background (see Figure 8 in Materials and Methods for the cloning strategy). This analysis was done for three viruses that had been independently selected through the chronic phase of infection (NL4-3-C1, C2, and C4) (Table 1), and two viruses that had been independently selected by passage through the lytic phase of infection (NL4-3-L1, and L2). Two to three subclones of PCR-amplified products were sequenced for each of the selected viruses (Figure 21).

Specific nucleotide changes co-map with the RNase sensitive sites in the variants. Sequence analyses of the amplified regions revealed the presence of specific nucleotide changes at each of the novel RNase sensitive sites. Some of the fragments from each of the viruses that had undergone selection in the chronic phase contained mutations that co-mapped with the novel RNase sensitive sites (Figures 10 and 21). By contrast, none of the fragments from the virus that had undergone lytic selection contained mutations. Six out of the 9 clones from the viruses that had undergone chronic selection contained base changes that co-mapped with the RNase sensitive sites tentatively mapped to positions 5945 (site 1) and 6070 (site 2) (Figures 10 and 21). Three out of the 6 contained mutations
Sequences of the primers and oligos are as follows:

RI (+) 5' GCCATAATAAGAATTCTGCAACAACTGC 3' (5733-5760)
RI (-) 5' GCAGTTGTTGCAGAATTCTTATTATGGC 3' (5760-5733)
Kpn (+) 5' GTCTATTATGGGGTACCTGTGTGGAAGG 3' (6332-6359)
Kpn (-) 5' CCTTCCACACAGGTACCCCATAATAGAC 3' (6359-6332)

a (-) 5' CAATAGCAATTGGTACAAGC 3' (5909-5890)
b (-) 5' TACTGCTTTGTAGAGAAGC 3' (6047-6028)
c (-) 5' ATGATTACTATGGACCACAC 3' (6140-6121)

Figure 20. PCR amplification strategy. The 600 bp of the variant viruses analyzed in the protection assays was PCR amplified using the EcoRI and KpnI primers. After digestion of the amplified products with the appropriate restriction enzymes, the products were subcloned into pNL4-3 (see Figure 8). The subclones were sequenced using the a, b, and c as well as the EcoRI and KpnI antisense oligonucleotides.
**Figure 21.** Schematic demonstrating the co-mapping of sequence changes with novel RNase sensitive sites. Positions in pNL4-3 DNA are given at the top. NL4-3 sequences are presented in capital letters. HXB-2 sequences are presented in lower case letters with differences between NL4-3 and HXB-2 being presented in bold type. Sequence data for independently selected chronic (C) and lytic (L) stocks are presented. Subclones of each stock are grouped and indicated in lower case letters (e.g. C1b, C1c, and C1h for NL4-3-C1). Sequences that differ from NL4-3 in the selected viruses are indicated in lower case letters with those that are HXB-2-like being shown in bold. -, nt identical to that in NL4-3; ^, an insertion, ..., sequence present in HXB-2 but not in NL4-3.
co-mapping with the third RNase sensitive site (position 6233, Figures 10 and 21).

The mutations observed at each site were analyzed for the type and frequency of change. All of the 6 mutations at site 1 represented the same change (a transition from G to A). Mutations at site 2 included identical multiple changes in a 10 bp region (a transition from T to C and insertion of a CCA codon). In contrast to sites 1 and 2, site 3 contained different mutations that were less heavily selected. Two different mutations were present in the three clones with site 3 mutations (either a transition from A to G at nt 6236 or a transition from G to A at nt 6238). Mutations that did not co-map with RNase sensitive sites did not appear to undergo uniform selection. These were unique to clones from independent variants. In agreement with RNase protection assays, none of the 4 clones from virus that had undergone lytic selection contained base changes. These data suggest that mutations at the RNase sensitive sites appeared to represent the selection of specific mutations in NL4-3 in chronic virus producers.

The variants are deficient in the expression of Vpu. A site by site analysis of the mutations in the PCR amplified regions of the variant viruses revealed that most either changed or did not affect codons in the tat, rev, and env genes (Table 2). However, mutations in vpu had a more pronounced effect on protein expression with the changes in vpu abrogating Vpu expression by the replacement of the initiator methionine with a threonine.

The absence of Vpu expression in the variants was verified using immunoprecipitation of 35S-labeled cells. H9 cells infected with NL4-3, NL4-3-C1,
Table 2: Amino Acid Changes Encoded by Mutations in the Variant Viruses

<table>
<thead>
<tr>
<th>Position (Site)</th>
<th>Amino Acid Change</th>
<th>Protein</th>
<th>Mutation Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>5946 (Site 1)</td>
<td>M → I</td>
<td>Tat</td>
<td>Sense</td>
</tr>
<tr>
<td>5960</td>
<td>G → D</td>
<td>Tat</td>
<td>Sense</td>
</tr>
<tr>
<td>5976</td>
<td>R → R</td>
<td>Tat</td>
<td>Wobble</td>
</tr>
<tr>
<td></td>
<td>G → E</td>
<td>Rev</td>
<td>Sense</td>
</tr>
<tr>
<td>6026</td>
<td>Q → R</td>
<td>Tat</td>
<td>Sense</td>
</tr>
<tr>
<td></td>
<td>K → E</td>
<td>Rev</td>
<td>Sense</td>
</tr>
<tr>
<td>6062 (Site 2)</td>
<td>^M → T</td>
<td>Vpu</td>
<td>iAUG knockout*</td>
</tr>
<tr>
<td>6072 (Site 2)</td>
<td>^P**</td>
<td>Vpu</td>
<td>Insertion</td>
</tr>
<tr>
<td>6239 (Site 3)</td>
<td>K → E</td>
<td>Env</td>
<td>Sense</td>
</tr>
<tr>
<td>6241 (Site 3)</td>
<td>K → K</td>
<td>Env</td>
<td>Wobble</td>
</tr>
<tr>
<td>6270</td>
<td>K → R</td>
<td>Env</td>
<td>Sense</td>
</tr>
<tr>
<td>6311</td>
<td>A → T</td>
<td>Env</td>
<td>Sense</td>
</tr>
</tbody>
</table>

* Initiator AUG

** Insertion of a codon
Figure 22. Immunoprecipitations of $^{35}$S-labeled protein extracts to test for the presence of Vpu in cultures infected with variant viruses. Lanes: 1) uninfected; 2) NL4-3; 3) NL4-3-C1; 4) NL4-3-C4; and 5) HXB-2-infected cells. For experimental details, see text. Designations along the sides indicate the sizes of the molecular weight markers used.
NL4-3-C4, and HXB-2 (a virus known to be defective for the expression of vpu) were metabolically labeled with $^{35}$S-methionine and $^{35}$S-cysteine for 4 hours. Protein extracts were prepared in the RIPA buffer and analyzed for the expression of vpu by immunoprecipitation with an anti-Vpu sera. As seen in Figure 22, only NL4-3-infected cultures contained the Vpu protein. The other viruses were deficient in the expression of vpu, as predicted by the sequence analysis (Figure 21).

**Nucleotide changes reflect the selection of HXB-2-like sequences.** The changes in the mutant sequences were compared with mutations in other HIV-1 sequences to learn more about their relationship to other laboratory strains and naturally occurring isolates (Myers et al., 1990). This comparison revealed that most of the mutations represented differences in the sequences of NL4-3 and HXB-2 (Figure 21). In particular, the CCA insertion at site 2 is characteristic of HXB-2. HXB-2 is a molecular clone from the IIIB/LAV family of viruses with a high ability to generate chronic virus producers. This suggested that the mutations might have arisen from recombination of pNL4-3-DNA-1 with a low level of contaminating pHXB-2 DNA.

**Origin of the variant viruses.** To test whether the variant viruses emerged as a result of de novo generation and selection of mutations or to contamination of pNL4-3-DNA-1 with pHXB-2 DNA, an independent preparation of pNL4-3 DNA was tested for the generation of the variant viruses. In addition, RNA isolated from the acute phase of infection of pHXB-2-transfected cells was
analyzed for the novel pattern of protected fragments.

A single colony of pNL4-3-transformed bacteria was used to produce pNL4-3-DNA-2 (DNA-2). Virus produced from DNA-2 was passaged through the chronic phase of infection (till day 41) in H9 cells and analyzed for the generation of the novel RNase sensitive sites. The novel sites did not emerge in chronic producers of virus recovered from this DNA (Figure 23A). This is in contrast with a parallel infection initiated with a stock from DNA-1. The novel RNase sensitive sites were apparent in this culture by day 24 (Figure 23A).

The RNase protection analysis of RNA harvested from pHXB-2-transfected cells revealed the same novel pattern of protected fragments as observed in the chronic virus producers of pNL4-3-DNA-1-transfected cells (Figure 23B). These results suggest that the variants observed in infections initiated with pNL4-3-DNA-1 might have reflected the selection of specific regions of a contaminating HXB-2 genome.

The 600 bp test region of the variants is not sufficient to change the ability of NL4-3 to establish chronic virus producers. Sequence analysis of the 600 bp test region of the variant viruses had revealed that only certain sequences from the HXB-2 genome had been selected in the chronic phase of infection. Therefore, it was of interest to test if the 600 bp region of the variants, sub-cloned into pNL4-3, could alter the ability of NL4-3 to establish chronic virus producers. To test for this possibility, recombinant NL4-3 clones containing the 600 bp region were transfected into H9 cells along with pNL4-3 and pHXB-2 DNAs. Two of
Figure 23. Analysis of pNL4-3-DNA-2- and pHXB-2-transfected H9 cultures for the generation of the novel pattern of protected fragments. A) RNase protection analysis of RNAs harvested from pNL4-3-DNA-2- and pNL4-3-DNA-1-transfected cultures at day 41 post transfection. The S-S probe was used for this analysis; B) RNase protection analysis of RNAs harvested from several time points of pHXB-2-transfected H9 cultures. The R-K probe was used for this analysis. The panel on the right shows the typical RNase protection pattern obtained with the R-K probe. The sizes and coding potentials of the protected species are indicated along the sides.
the recombinant clones, pCh1 and pC2r, represented subclones of variants that had arisen in independently transfected H9 cells, while pC4t represented a subclone from the variants originating in the Jurkat cells. The cultures were followed for virus-expressing cells, cell growth, and cell death (Figure 24).

Results of the temporal analysis of cultures transfected with these DNAs showed that the 600 bp region containing the HXB-2-like sequences did not increase the ability of NL4-3 to generate chronic virus producers (Figure 24A). C2r had a low ability to generate chronic virus producers. It was NL4-3-like in its ability to establish chronic virus producers. C1h was somewhat better than NL4-3 in its ability to generate chronic virus producers. No expression was observed for the pC4t clone. Cultures transfected with C1h and C2r grew slightly better than NL4-3-transfected cultures (Figure 24B) and had intermediate levels of cell death (Figure 24C). Thus, the 600 bp region had some effects on the growth of these cultures, but was not sufficient, by itself, to make NL4-3 an efficient chronic virus producer.

DISCUSSION

Characterization of the variant viruses that emerged in the chronic phase of infection of H9 cells and Jurkat cells revealed that the variants contained HXB-2-like mutations in the tat and vpu genes. However, only certain HXB-2 sequences were present in the 600 bp region that was analyzed for mutations with
Figure 24. Temporal analysis of NL4-3, HXB-2, pC1h-, pC2r-, and pC4t-transfected H9 cultures. 
A) Temporal appearance of virus-expressing cells B) Growth of infected cultures C) Temporal appearance of dead cells. pC4t did not give any expression. H9C, uninfected H9 cells.
the remainder being NL4-3-like. Biological test of NL4-3 recombinants with the selected HXB-2-like mutations demonstrated that these mutations were not sufficient for the establishment of chronic virus producers. Thus, the ability to establish chronic virus producers requires sequences other than (or in addition to) the 600 bp region that had been analyzed for mutations.

Selection of certain HXB-2-like mutations in chronic virus producers.

Sequence analysis of three independently derived variants in the region containing the novel RNase sensitive sites revealed that the variants contained sequences common to both NL4-3 and HXB-2. This common region (from position 5780 to position 5945 of proviral DNA) was followed by sequences characteristic of HXB-2. In two of the variants, HXB-2-like sequences were found between position 5946 and 6073. In the third, they were present from 5946 to 6270. Thereafter, sequences were again characteristic of NL4-3. Other changes (found in neither NL4-3 and HXB-2) were scattered and not common to independently generated variants. Presumably these reflected polymerase errors that had occurred in the infected cell or during reverse transcription and PCR amplification. Taq polymerase has no 3'-5' exonuclease activity and has a strong bias for T--->C transitions (Tindall and Kunkel, 1988), while the HIV-1 reverse transcriptase is prone to GpA dinucleotide transitions (see below) (Vartanian et al., 1991).

HXB-2-like mutations that were selected at RNase sensitive sites 1 and 2 introduced coding changes into the viral genome. The selected mutation at RNase sensitive site 1 changed the 39th amino acid of Tat from a methionine to
an isoleucine. This position in Tat varies in different isolates of HIV-1 with isoleucine or threonine being present in ~80% of the isolates in the Los Alamos data base (Myers et al., 1990). Amino acid position 39 is contained within Region II of Tat, one of the two trans-activation domains of the protein (Garcia et al., 1988; Green et al., 1988). This mutation in tat does not seem to affect the ability of NL4-3 to establish chronic virus producers (see pCh1 and pCr2 in Figure 24A).

The mutations at RNase sensitive site 2 changed the initiator methionine for Vpu to a threonine. Analysis of the proteins expressed by the variants revealed that the variants were deficient for Vpu (Figure 22). Biological analysis of the recombinant NL4-3 viruses encoding the vpu mutation showed that the absence of Vpu did not favor the establishment of chronic virus producers (Figure 24A). The inability of vpu mutants to generate higher levels of chronic virus producers is not surprising since vpu mutants of NL4-3 are, at least as cytopathic as the NL4-3 parent (Strebel, Klimkat, Martin, 1988; Cohen et al., 1988). Our recombinant NL4-3 viruses containing the HXB-2-like vpu mutation were somewhat less cytopathic than the parental NL4-3 (Figure 24C). This may reflect the presence of the additional HXB-2-like tat mutation at amino acid 39 (Table 2).

Mutations at RNase sensitive site 3 underwent less strong selection than those at sites 1 and 2. This had been noted in the RNase protection assays where the intensity of novel bands representing site 3 varied among independently selected variants (data not shown). The nucleotide change that marks this site in
HXB-2 is silent. Thus, this site, which is within 177 bp to the more highly selected mutations at site 2, may be incidental to the recombination events that generated the variants.

Selection for specific mutations in immunodeficiency virus in tissue culture. Precedence for the efficient selection of specific variants of an immunodeficiency virus is found in work on simian immunodeficiency virus macaque (SIV mac) where truncated forms of the transmembrane protein, TMP, were strongly selected for or against depending on whether SIVmac251 was cultured in non-permissive (human) or permissive (monkey) PBLSs (Kodama et al., 1989). This selection took place in the first 20-30 days of culture with selected mutations being stable during subsequent culture.

Frequent transitions of G to A in HIV-1 genomes undergoing passage in tissue culture. Of the eight positions within the 600 bp region showing nucleotide changes (excluding the CCA insertion) seven of the changes involved a transition between the G and A bases (Figure 21). Of these five involved G to A transitions. Four of the eight positions containing transitions were non-HXB-2-like. Such frequent transitions between G to A have been observed in tissue culture-passaged HIV-1 isolates. Varnatian et al., 1991 have shown that multiple passages of HIV-1-B40 on peripheral blood mononuclear cells results in extensive G to A mutations. These have been called "hypermutations" due to their frequent occurrence. Such mutations are apparently the result of dislocation of the primer with respect to the template during reverse transcription of the viral genome by
the virion-associated reverse transcriptase.

**Origin of HXB-2-like sequences.** Results presented in this chapter suggest that the variant viruses that had emerged in the chronic virus producers could have originated as a result of recombination of NL4-3 with low levels of contaminating HXB-2 sequences (Figure 23). Recombination between retroviral genomes is a well known phenomenon (Coffin, 1979). Clavel et al., 1989 have studied HIV-1 recombination in cells harboring two defective HIV-1 genomes. Their results suggest that phenotypically complete, heterozygous virions are the intermediate in the recombination process with the actual recombination taking place in the next round of replication.

Based on these observations, we could hypothesize on the likely scenario responsible for the generation of the variants. Possible co-transfection or infection of the same cell with two viral genomes (primarily NL4-3 and only occasionally HXB-2) could have resulted in the co-packaging of heterozygous RNA templates. Once these heterozygotic virions had infected fresh cells, recombination could occur during reverse transcription of the co-packaged genomes. The apparent selection of very short HXB-2 regions in the 600 bp fragment suggests that multiple recombination events took place between NL4-3-HXB-2 recombinants and NL4-3.

The region encoding the HXB-2-like *tat* and *vpu* mutations is not sufficient for the efficient establishment of chronic virus producers. The factors that allow a virus to generate chronic virus producers are not understood. Selection of
specific HXB-2-like mutations in the chronic and not the lytic phase of infection suggested that these mutations might be important in the generation of chronic virus producers (Figure 15). However, biological tests of NL4-3 recombinants with these mutations revealed that, by themselves, the mutations were not sufficient to convert NL4-3 from an inefficient to an efficient chronic virus producer (Figure 24). Thus, other mutations are important to the establishment of chronic virus producers. Analyses presented in the next two chapters use NL4-3 and HXB-2 to map the auxiliary as well as the non-auxiliary gene sequences important in the establishment of chronic virus producers.
CHAPTER III
INTRODUCTION

The ability of HIV-1 to establish chronic virus producers is a poorly understood phenomenon. Work presented in Chapters I and II identified two molecularly cloned viruses with distinct abilities to establish chronic virus producers. One of these, NL4-3, had a low ability to generate chronic virus producers, whereas the second, HXB-2 had a high ability to generate chronic virus producers. NL4-3 is a laboratory construct that encodes all known HIV-1 gene products (Adachi et al., 1986). HXB-2 is a molecular clone from a stock of HIV-1-IIIB that was selected for the ability to establish chronic virus producers (Popovic et al., 1984; Fisher et al., 1985). HXB-2 is defective for three non-essential auxiliary genes: vpr, vpu, and nef. The goal of studies presented in this chapter was to determine whether defective vpr, vpu, or nef genes could convert NL4-3 from a virus with low ability to establish chronic virus producers to a virus with high ability to establish chronic virus producers.

To elucidate the potential role of vpr, vpu, and nef genes in the establishment of chronic virus producers, a series of NL4-3 mutants encoding all possible combinations of defective vpr, vpu, and nef genes were constructed. Tests of these NL4-3 mutants revealed that vpr and nef limit the ability of NL4-3 to establish chronic virus producers.
RESULTS

NL4-3 and HXB-2 have distinct abilities to generate chronic virus producers. To test the relative abilities of NL4-3 and HXB-2 to establish chronic virus producers, exponentially growing H9 cells were transfected with equal amounts of either pNL4-3 or pHXB-2 DNA. As expected, the parental viruses exhibited distinctive profiles for the establishment of chronic virus producers (Figure 25). NL4-3 established low levels of chronic virus producers (15-20%) whereas HXB-2 established high levels (>80%) (Figure 25A). The parental viruses also differed in their overall effects on cell death (Figure 25C) and cell growth (Figure 25B). HXB-2-transfected cultures had relatively low levels of cell death with the surviving cells having normal growth characteristics, similar to those of uninfected H9 cells. In contrast, NL4-3-transfected cultures exhibited more cell death and overall poorer cell growth. NL4-3 and HXB-2 transfections also differed in that HXB-2-transfected cells exhibited an ~10 day lag over NL4-3 transfections for the appearance of infected cells (Figure 25A). This lag was not observed if virus was used to initiate infections (data not shown). The reason for this lag is not clear.

Tests for the effects of Vpr, Vpu, and Nef on the ability of NL4-3 to establish chronic virus producers. Table 3 lists the amino acid differences between NL4-3 and HXB-2. As apparent, the most prominent differences between the two viruses occurred at the coding sequences for vpr, vpu, and nef.
Figure 25. Temporal analysis of pNL4-3- and pHXB-2-transfected H9 cultures. A) Temporal appearance of virus expressing cells; B) growth of cultures; C) temporal presence of dead cells. H9C, uninfected H9 cells.
Table 3: Amino Acid Differences Between NL4-3 and HXB-2

<table>
<thead>
<tr>
<th>Protein</th>
<th>No. of Changes</th>
<th>Changes in HXB-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gag</td>
<td>25</td>
<td>Sense mutations</td>
</tr>
<tr>
<td>Pol</td>
<td>33</td>
<td>Sense mutations</td>
</tr>
<tr>
<td>Vif</td>
<td>20</td>
<td>Sense mutations</td>
</tr>
<tr>
<td>Vpr</td>
<td>9+</td>
<td>Sense + 18aa truncation</td>
</tr>
<tr>
<td>Tat</td>
<td>2</td>
<td>Sense mutation</td>
</tr>
<tr>
<td>Tev</td>
<td>3</td>
<td>Sense mutations</td>
</tr>
<tr>
<td>Rev</td>
<td>1</td>
<td>Sense mutation</td>
</tr>
<tr>
<td>Vpu</td>
<td>1+</td>
<td>Abrogation of iAUG*</td>
</tr>
<tr>
<td>Env</td>
<td>28</td>
<td>Sense mutations</td>
</tr>
<tr>
<td>Nef</td>
<td>4+</td>
<td>Sense + 83aa truncation</td>
</tr>
</tbody>
</table>

* Initiator methionine
genes. Therefore, to test for the effects of auxiliary gene differences in the establishment of chronic virus producers, mutants of NL4-3 containing all possible combinations of defective \textit{vpr}, \textit{vpu}, and \textit{nef} genes were constructed. These mutants were tested for their ability to establish chronic virus producers in H9 cells. Table 4 summarizes the construction of these NL4-3 mutants and compares the defects in their \textit{vpr}, \textit{vpu}, and \textit{nef} genes with those in HXB-2. Tests for the ability of the mutant genomes to establish chronic virus producers were initiated by transfecting H9 cultures with plasmid DNAs. Transfections with parental DNAs were carried out in parallel to allow direct comparison of the ability of mutant and parental genomes to establish chronic virus producers. Transfected cultures were monitored over time for virus-expressing cells, cell growth and cell death.

The NL4-3 auxiliary gene mutants varied in their abilities to establish chronic virus producers. Overall three patterns were observed: inefficient establishment of chronic virus producers (Figure 26A), intermediate establishment of chronic virus producers (Figure 26B) and efficient establishment of chronic virus producers (Figure 26C). Two mutants, NL\Delta U and NL\Delta U\Delta N, had low abilities to generate chronic virus producers (Figure 26A). The efficiency with which these mutants generated chronic virus producers was similar to that of the NL4-3 parent. Three mutants, NL\Delta R, NL\Delta N, and NL\Delta R\Delta U, showed intermediate abilities to establish chronic virus producers (Figure 26B). Cultures infected with these viruses exhibited transient high levels of virus-expressing cells.
Table 4: Comparison of *vpr*, *vpu*, and *nef* genes of NL4-3, NL4-3-mutants, and HXB-2.

<table>
<thead>
<tr>
<th></th>
<th>NL4-3</th>
<th>NL4-3 MUTANTS</th>
<th>HXB-2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vpr</strong></td>
<td>96 amino acid protein.</td>
<td>Fill-in of EcoRI site (nt 5743) creating a frameshift at amino acid 63 and a truncation 16 amino acids later.</td>
<td>Mutation creating a frameshift at amino acid 71 and a truncation 7 amino acids later.</td>
</tr>
<tr>
<td><strong>Vpu</strong></td>
<td>81 amino acid protein.</td>
<td>Addition of an <em>XhoI</em> linker at <em>SspI</em> site (nt 6153) creating a frameshift at amino acid 32 and a truncation 3 amino acids later.</td>
<td>Loss of initiator Methionine.</td>
</tr>
<tr>
<td><strong>Nef</strong></td>
<td>206 amino acid protein.</td>
<td>Fill-in of the <em>XhoI</em> site (nt 8887) creating a frameshift at amino acid 35 and a truncation 11 amino acids later.</td>
<td>Mutation generating a stop codon after amino acid 123.</td>
</tr>
</tbody>
</table>
Figure 26. Temporal analysis of H9 cultures transfected with pNL4-3, pHXB-2, and NL4-3 mutants. A) Mutants that were inefficient at the establishment of chronic virus producers; B) mutants with intermediate abilities to establish chronic virus producers; and C) mutants that were efficient at establishing chronic virus producers. H9C, uninfected H9 cells. The open symbols present data for the mutants. The closed symbols present data for NL4-3 and HXB-2.
days 5-15 of culture). Two viruses, the double mutant NLΔRΔN and the triple mutant, NLΔRΔUΔN, had high abilities to generate chronic virus producers (Figure 26C). There was no difference in the relative ability of these two mutants to generate chronic virus producers. This suggests that defects in both vpr and nef are sufficient for NL4-3 to establish high levels of chronic virus producers with mutations in vpu not further enhancing this ability.

In general, the ability to establish chronic virus producers correlated inversely with the amount of cell death in cultures. This can be seen most clearly in Table 5 where mutants are listed from decreasing to increasing ability to establish chronic virus producers. The two NL4-3 mutants that had a low ability to establish chronic virus producers (NLΔU and NLΔUΔN) exhibited high levels of cell death during the acute phase of infection. By contrast, the two mutants that were efficient at generating chronic virus producers, NLΔRΔN and NLΔRΔUΔN, caused only low levels of cell death. In keeping with this trend, cultures infected with two of the three viruses with intermediate ability to generate chronic virus producers (NLΔR and NLΔN) had intermediate levels of cell death. However, the culture infected with the third virus with an intermediate ability to establish chronic virus producers (NLΔRΔU) had low levels of dead cells. This exception indicates that low cytopathicity does not ensure the efficient outgrowth of chronic virus producers.

The efficient establishment of chronic virus producers did not necessarily correlate with cultures having a normal growth potential. The growth potential of
Table 5: Summary of data on the NL4-3 mutants

<table>
<thead>
<tr>
<th>Test Virus</th>
<th>Auxiliary Genes</th>
<th>Chronic Producers</th>
<th>Growth of Culture</th>
<th>Cell Death</th>
<th>Post Transfection</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL4-3</td>
<td>+ + +</td>
<td>+</td>
<td>+</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>NLAR</td>
<td>- + +</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>NLARU</td>
<td>+ + -</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>NLARUAN</td>
<td>- + -</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>NLARAN</td>
<td>- - +</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>NLARARUAN</td>
<td>- - -</td>
<td>++++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>HXB-2</td>
<td>- - -</td>
<td>++++</td>
<td>++++</td>
<td>+</td>
<td>++++</td>
</tr>
</tbody>
</table>

(+), presence of; (-), absence of auxiliary genes

The last four columns present the extent of the various tested phenotypes using a scale of (-) for least effect to (+++++) for maximum effect.
cultures was determined both by the amount of cell death during the acute phase of infection and by the doubling times of the surviving cells. Following the acute phases of infection, the doubling times for NLΔRΔN- and NLΔRΔUΔN-expressing cultures were slower than for uninfected or HXB-2-expressing cultures. These cultures had only intermediate growth characteristics and did not exhibit the normal growth characteristic of uninfected or HXB-2-expressing H9 cells (Figure 26C).

The efficient establishment of chronic virus producers did not require the long post-transfection lag observed in the HXB-2-transfected cultures. In NLΔRΔN- and NLΔRΔUΔN-transfected cultures, infected cells were detected within 1 to 3 days of their appearance in NL4-3-transfected cultures (Figure 26C).

The infection and growth patterns summarized in Table 5 were highly reproducible with similar results being obtained in independent growth tests (compare growth tests on NL4-3- and HXB-2-transfected cultures in the three independent tests shown in Figures 25, 26, and 27).

**DISCUSSION**

We have demonstrated that two auxiliary genes, vpr and nef, have the potential to limit the ability of HIV-1 to establish chronic virus producers. For NL4-3 infections, defective vpr and nef genes were sufficient to change the
efficiency of the establishment of chronic virus producers from low to high (Figure 26 and Table 5). The potential function of these two auxiliary genes in limiting the ability of NL4-3 to establish chronic virus producers is discussed below.

**Roles of vpr and nef in the establishment of chronic virus producers:** The *gene products.* Both vpr and nef are non-essential HIV-1 genes. Vpr is a 96 amino acid, 15 kd, virion-associated protein (Wong-Staal, Chanda, and Ghrayeb, 1987; Cohen et al., 1990a and b; Yuan et al., 1990). It is not essential for virus assembly. Western blots of sub-cellular fractions suggest that Vpr may be membrane associated (Sato et al., 1990). Multiple Vpr molecules (probably dimers) are found in each viral particle (Cohen et al., 1990a; Gras-Masse et al., 1990). The entire Vpr protein is important for its function since even a three amino acid truncation in its arginine rich carboxy terminus can abrogate function (Cohen et al., 1990b; Yuan et al., 1990). Arginine rich regions are important in nuclear localization as has been demonstrated for Tat and Rev (Hauber et al., 1989; Cochrane et al., 1990; Subramanian et al., 1990). They are also found in RNA binding proteins like lambda phage N protein (Lazinski et al., 1989). Thus, Vpr may exert its effect by binding to genomic RNA and/or nascent viral or cellular transcripts.

Nef is a non-virion associated, non-structural protein (reviewed in Hovanessian, 1992). It has two protein products: p25 and p27. Most forms of p25 are N-terminal truncated forms of Nef that are produced by the use of an internal
methionine, 19 codons downstream from the initiator codon (Kaminchik et al., 1990 and 1991). Such forms of Nef are cytoplasmic proteins (Kaminchik et al., 1991). A second p25 form of Nef can result from an anomalous mobility of certain alleles of Nef on denaturing gels. This is seen for Nef proteins that contain alanine instead of an aspartate at position 54 (Obaru, De Clue, and Haseltine, 1992). NL4-3 expresses the p27 form of Nef though it contains an alanine at position 54 (Luria, Chambers, and Berg, 1991).

The full-length p27 Nef is a myristoylated protein that is associated with the plasma membrane (Allan et al., 1985; Franchini et al., 1986; Kaminchik et al., 1991). p27 Nef can down regulate the CD4 receptor for HIV-1 from cell surfaces of T-cells, B-cells, monocyte/macrophages, and the non-lymphoid HeLa-CD4 cells (Guy et al., 1987; Garcia and Miller, 1991). It can also limit the ability of mitogenic stimuli to induce the transcription factor NFkB (Niederman et al., 1992) and the lymphokine IL-2 (Luria, Chambers, and Berg, 1991). N-terminal myristoylation and plasma membrane association are critical for p27 Nef function (Allan et al., 1985; Franchini et al., 1986; Guy et al., 1990; Kaminchik et al., 1990 and 1991; Zazopoulous and Haseltine, 1992).

p27 Nef protein can also be phosphorylated by protein kinase C (PKC) at Thr15 (Guy et al., 1987 and 1990b; Nebreda et al., 1991). The significance of this phosphorylation is not known. However, there seems to be selective pressure present both in vitro and in vivo to substitute Thr15 with an Ala. A long term evolutionary study of Nef in an infected individual has revealed high rates of
substitution of Thr15 with an Ala with disease progression (Delassus et al., 1991). Nef-induced down-regulation of surface CD4, inhibition of IL-1 mRNA, and inhibition of NFkB induction seem to be independent of phosphorylation by PKC at this site. NL4-3 Nef has an Ala at position 15.

Some evidence suggest that Nef may play a role as a signal transducer. Structurally, Nef has similarities to leucine zipper-like sequences found in transcriptional factors (Samuel et al., 1991) and can interact with nuclear factors that bind the Negative Regulatory Element of the HIV-1-LTR (Guy et al., 1990a). Thus, it may have the potential to influence the transcriptional activity of HIV-1-LTR (Guy et al., 1990b). Nef has also been reported to have homology to the thyrotropin receptor (Burch et al., 1991) as well as scorpion peptides that interact with potassium channels in the brain (Werner et al., 1991).

One study suggests that certain forms of Nef may be selected in chronic virus producers (Zweig et al., 1990). Stocks of HIV-1-IIIB contain sub-populations of viruses which express either a p25 or a p27 form of Nef. Both of these forms are myristoylated and react with antibodies to the N-terminal peptide of Nef. Thus, the two forms most likely reflect the presence of a mutation(s) that affects the electrophoretic mobility of the full length form of Nef. At low passage (<8), IIIB-infected cultures express both forms of Nef in about the same ratios. By contrast, at high passage (>100) IIIB-infected cultures are enriched for virus expressing the p25 form of Nef.
**Role of vpr and nef in cell culture.** In cell culture, both vpr and nef are dispensable for virus replication. The presence of Vpr has been reported to accelerate cytopathic effects (Cohen et al., 1990b). It has also been shown to increase virus replication in both T-cells and monocyte/macrophages (Ogawa et al., 1989; Cohen et al., 1990b; Hattori et al., 1990; Westervelt et al., 1992). Vpr can somewhat increase the activity of the HIV-1-LTR as well as the transcriptional control elements of a number of other viruses. Effects of Vpr on LTR activity do not involve Vpr binding to the LTR (Cohen et al., 1990b).

The expression of Nef has a variety of effects on cells. These effects have depended upon the use of specific alleles of nef, the multiplicity of infection, and the target cell line (reviewed in Hovanessian, 1992). Nef can have both a positive and a negative effect on viral transcription and replication as well as no effect at all. The inhibitory effects of Nef in vitro have implicated it in the establishment of viral latency (Haseltine, 1991). However, in vivo, Nef seems to have a more positive effect, as discussed below.

**Roles of vpr and nef in infected animals:** Most primary HIV-1 isolates contain intact vpr and nef genes suggesting that functional forms of these genes confer a selective advantage to the virus in the infected host (Balfe et al., 1990; Delassus, Cheynier, and Wain-Hobson, 1991; Li et al., 1991; Blumberg et al., 1992; Li et al., 1992). In the SIVmac/rhesus monkey model, the presence of a functional nef has been shown to increase virulence. In studies with the infectious molecular clone SIVmac239, a premature stop codon at the 93rd amino acid in nef
reverts to a sense codon within 2 weeks of inoculation into monkeys. Monkeys inoculated with either the virus containing the full length nef gene, or the stop codon mutant, maintain high viral loads and develop immunodeficiency within months of inoculation. However, monkeys inoculated with viruses containing deletions in nef (that cannot revert by simple point mutation) have low viral loads and remain healthy (Kestler et al., 1991). Recent results suggest that the same may also be true for vpr (Lang and Fleckenstein, 1992). Monkeys inoculated with vpr stop codon mutants that do not revert have remained healthy and without viremia in contrast to monkeys in which the stop codons reverted.

Low cytopathic potential does not ensure the efficient establishment of chronic virus producers. Temporal analyses of infections with the NL4-3 mutants revealed that low cytopathicity does not necessarily correlate with the efficiency of the establishment of chronic virus producers. Low cytopathicity appears to be a prerequisite for the efficient establishment of chronic virus producers. This can be seen by the fact that none of the mutants with high cytopathic potential could generate high levels of chronic virus producers (see NLΔU and NLΔUAΔN in Figure 26A). However, low cytopathicity was not sufficient for the efficient establishment of chronic virus producers. This is best shown by the mutant NLΔRΔU (Figure 26A and Table 5). NLΔRΔU had low cytopathic potential, yet it had only an intermediate efficiency of establishing chronic virus producers.

Analyses of the cultures transfected with the NL4-3 mutants also revealed that the NL4-3 backbone was more toxic for cells than HXB-2. Analysis of the
growth potential of cells transfected with the parental and the mutant viruses showed that none of the NL4-3-based viruses had the growth potential exhibited by the uninfected or HXB-2-transfected cultures (Figure 26). Even cultures transfected with mutants having a high ability to establish chronic virus producers, NLΔRΔN and NLΔRΔUΔN, had only intermediate doubling times.

**Vpu is not important in the establishment of chronic virus producers.** Tests of the NL4-3 mutants again revealed that *vpu* was not important in the establishment of chronic virus producers. The presence of the *vpu* mutation in the triple mutant, NLΔRΔUΔN, did not increase the ability of this mutant to establish chronic virus producers over that of the double mutant, NLΔRΔN (Table 5). This is puzzling since one would have predicted some involvement of a defective *vpu* gene due to the highly selected nature of this defect in the variant viruses (Chapters I and II). Therefore, it is possible that the *vpu* defect was not tested with the right combination of other genes to score for effects on chronic virus production. Also, selection of the *vpu* defect in the variants may have reflected the selection of variants in fully infected cultures. Assays on NL4-3 mutants were initiated in spreading infections and did not select for the ability of a virus to emerge in a fully infected culture.
CHAPTER IV
INTRODUCTION

The studies presented in this chapter were undertaken to address the possible roles of non-auxiliary gene sequences in the establishment of chronic virus producers. HXB-2 and NL4-3 contain many sequence differences in addition to those that encode the defective vpr, vpu, and nef genes of HXB-2. These differences could have influenced the relative ability of NL4-3 and HXB-2 to establish chronic virus producers (Table 5).

To map the role of non-auxiliary gene sequences, as well as auxiliary gene sequences, in the generation of chronic virus producers, six reciprocal recombinants between NL4-3 and HXB-2 were constructed using conserved restriction sites. The recombinants were designed to ensure that no region of HXB-2 was left out in the exchange process. Tests of the NL4-3-HXB-2 recombinants revealed that 5' internal sequences (3' gag, pol, vif, and 5' vpr), as well as fragments encoding defective auxiliary genes, were important for the establishment of chronic virus producers.

RESULTS

Use of NL4-3-HXB-2 recombinants to map sequences that affect the establishment of chronic virus producers. To test for effects of non-auxiliary as well as auxiliary gene sequences on the generation of chronic virus producers,
reciprocal recombinants between NL4-3 and HXB-2 were constructed and tested for their ability to establish chronic virus producers. The genomes and the expression of vpr, vpu and nef by these recombinants are illustrated in Figure 28. As with the NL4-3 mutants, results of growth tests are summarized by grouping constructs that encoded low (Figure 27A), intermediate (Figure 27B) and high (Figure 27C) abilities to establish chronic virus producers.

Growth tests of cells transfected with the NL4-3-HXB-2 recombinants revealed effects of non-LTR sequences at the 5' end of the viral genome as well as effects of 3' fragments containing defective auxiliary genes on the ability to establish chronic virus producers (Figures 27 and 28). In the presence of the 5' internal ApaI to EcoRI fragment of NL4-3 (nt 2006 to nt 5743 of NL4-3 proviral DNA), 3' HXB-2 fragment encoding defective vpr, vpu and nef did not confer high ability to establish chronic virus producers (See NLHX-4, Figures 27B and 28). Similarly, in the presence of 5' NL4-3 sequences, HXB-2 3' fragment encoding defective vpr and vpu did not confer high ability to establish chronic virus producers (see NLHX-2, Figures 27B and 28B). However, when 5' internal sequences were from HXB-2, 3' HXB-2 fragments encoding defective vpr and vpu (NLHX-3) or defective nef (NLHX-5) were able to confer high ability to establish chronic virus producers (Figure 27C). The 5' internal HXB-2 fragment on its own, however, was not sufficient to convert NL4-3 into a high chronic virus producer (see NLHX-1, Figures 27A and 28B). Thus, in the recombinants, the ability to establish chronic virus producers was context dependent and required 5'
Figure 27. Temporal analysis of H9 cells transfected with the NL4-3-HXB-2 recombinants. A) Mutants that were inefficient at the establishment of chronic virus producers; B) mutants with intermediate abilities to establish chronic virus producers; and C) mutants that were efficient at establishing chronic virus producers. H9C, uninfected H9 cells. The open symbols present data for the mutants. The closed symbols present data for NL4-3 and HXB-2.
internal HXB-2 sequences as well as 3' HXB-2 fragments containing defective auxiliary genes.

As with the NL4-3 mutants (Table 5), the overall ability to establish chronic virus producers correlated with low cytopathicity in the infected cultures (Figure 28). For example, recombinants NLHX-3 and NLHX-5 with high abilities to establish chronic virus producers, were least cytopathic for cells in culture. And recombinant NLHX-1, with low ability to establish chronic virus producers, showed high cytopathic potential for cells in culture. Two of the three recombinants with intermediate abilities to establish chronic virus producers, NLHX-4 and NLHX-6, had intermediate cytopathic potential for cells in culture. However, one exception to this overall trend was observed for the third recombinant with intermediate ability to establish chronic virus producers, NLHX-2. In NLHX-2-transfected cultures, low cell death did not correlate with the efficient establishment of chronic virus producers (Figure 28).

In contrast to the NL4-3 mutants, the NL4-3-HXB-2 recombinants were able to establish chronic virus producers that exhibited normal cell growth (Figure 27). This is shown by tests with NLHX-3 and NLHX-5, the two recombinants with the highest ability to establish chronic producers (Figure 27C). Both NLHX-3- and NLHX-5-transfected cultures had doubling times similar to those of the uninfected or HXB-2-expressing H9 cultures.

Once again, the recombinants demonstrated that a long post-transfection lag was not required for the efficient establishment of chronic virus producers.
Figure 28. Summary of data on the NL4-3-HXB-2 recombinants and schematic of their construction. A) Representation of the HIV-1 genome; B) Restriction endonuclease sites used in the construction of the recombinants, recombinant genomes and growth characteristics of cultures transfected with the recombinants; _____________________________________________, HXB-2 sequences; ____________________________, NL4-3 sequences; RI, EcoRI; (+), presence or (-) absence of auxiliary genes; H, HXB-2 5' internal sequences; N, NL4-3 5' internal sequences. The last four columns represent the extent to which the various growth profiles were observed using a scale of (-) for least to (+++++) for maximum effect.
Cultures transfected with NLHX-3 had only a one day lag over NL4-3 for the appearance of virus-expressing cells. In addition, the recombinants demonstrated that certain HXB-2 sequences may be important in the generation of the lag. Cultures transfected with NLHX-5 showed the longest lag (five days) in the appearance of virus-expressing cells. This recombinant contains the gag-pol and vif region of HXB-2 as well as the HXB-2 LTRs. HXB-2 LTRs alone did not affect the length of the lag significantly, as seen by the recombinant NLHX-6 (Figure 28B). Similarly, the gag-pol and vif region was not sufficient by itself to affect the lag significantly (see NLHX-1, Figures 27A and 28B). Thus, the gag-pol and vif region of HXB-2, in combination with the HXB-2 LTRs, was required for the long post-transfection lag.

DISCUSSION

Involvement of HIV-1 sequences in the establishment of chronic virus producers. We have demonstrated that multiple regions of the HIV-1 genome determine the ability to generate chronic virus producers. For NL4-3 infections, defective vpr and nef genes were sufficient to change the efficiency of the generation of chronic virus producers from low to high (Figure 26 and Table 5). For infections with NL4-3-HXB-2 recombinants, 5' internal HXB-2 sequences as well as 3' fragments containing defective auxiliary genes were required for the efficient establishment of chronic virus producers (Figures 27 and 28).
Context-dependent requirement for 5' internal sequences. Tests of the recombinants of NL4-3-HXB-2 demonstrated that 5’ internal sequences play an important role in the establishment of chronic virus producers. The role of 5’ sequences was context dependent, with 5’ internal sequences of HXB-2 requiring the presence of 3’ internal HXB-2 fragments for the efficient establishment of chronic producers (Figures 27 and 28). In the case of NLHX-3, the 3’ EcoRI to XhoI fragment contained defective vpr and vpu genes (Figure 28). In the case of NLHX-5, the 3’ fragment contained a defective nef gene (Figure 28). In addition to defects in auxiliary genes, these 3’ internal fragments also contained a number of other sequences that were different from those in the NL4-3 genome (Table 3). Thus, requirement for these 3’ fragments suggests, but does not prove, that intact auxiliary genes limit the ability of NL4-3-HXB-2 recombinants to generate chronic virus producers.

Role of gag sequences in the establishment of chronic virus producers. The 5’ fragment of HXB-2 required for the generation of chronic virus producers includes the last half of gag, all of pol and vif, and the 5’ end of vpr. Two regions of this 3737 bp 5’ fragment have been previously implicated in cytopathic potential. The first of these involves the 3’ end of gag and most of pol. This region may contribute to the highly virulent phenotype of HIV-1-NDK (Spire et al., 1990; Hirsch et al., 1992).

Role of vif in the establishment of chronic virus producers. The second region in the 5’ HXB-2 fragment that has been implicated in determining
cytopathicity is \textit{vif}. Vif enhances viral infectivity during virus production in a cell-line dependent manner (Fisher et al., 1987; Strebel et al., 1987; Sakai et al., 1991; Fan and Pedan, 1992; Gabuzda et al., 1992). Delays in virus expression due to deletions in \textit{vif} can increase the ability to establish chronic virus producers (Sakai et al., 1991). However, in the NL4-3 mutants and NL4-3-HXB-2 recombinants, delayed virus expression was not a prerequisite for the efficient establishment of chronic virus producers (Table 5 and Figure 28). Also, in cultures that did exhibit delayed virus expression, the length of the delay did not correlate with whether \textit{vif} was from HXB-2 or NL4-3 (see NLΔARΔN in Figure 26C and NLHX-5 in Figure 28B). Thus, if \textit{vif} affects the ability of NL4-3 and HXB-2 to establish chronic virus producers, these effects are context dependent and not necessarily associated with delays in the appearance of virus expressing cells.

\textbf{Low cytopathic potential is not a direct correlate of the ability to establish chronic virus producers.} These studies once again demonstrate that low cytopathic potential is not sufficient, by itself, for the efficient establishment of chronic virus producers. This is shown by the data for cells transfected with NLHX-2. NLHX-2 had low cytopathic potential but poor ability to generate chronic virus producers (Figures 27 and 28).

\textit{Env} sequences do not determine the ability to establish chronic virus producers. \textit{Env} is a frequent determinant of the cytopathic potential of HIV-1 isolates (Sodroski et al., 1986a; Cheng-Mayer et al., 1990; Koga et al., 1990; Stevenson et al., 1990a; York-Higgins et al., 1990; Hoxie et al., 1991; Kowalski et
125

al., 1991; de Jong et al., 1992). Interestingly, the ability of the NL4-3-HXB-2 recombinants to generate chronic virus producers did not correlate with the presence of HXB-2 env sequences (Figure 28). The env sequences of NL4-3 and HXB-2 are both derived from the HIV-1-IIIB/LAV family of viruses (Bare-Sinoussi et al., 1983; Gallo et al., 1984; Adachi et al., 1986). Env proteins of both of these viruses cause syncytia. Thus, the presence of syncytium-inducing Env glycoproteins did not prevent the efficient establishment of chronic virus producers. This is in agreement with results of Spire et al., 1990 who found the syncytium-inducing env of HIV-1-NDK not to be the major determinant of the cytopathic potential of this virus.
SUMMARY AND FUTURE DIRECTIONS
SUMMARY AND FUTURE DIRECTIONS

Studies presented in this thesis have revealed the complex nature of the ability of HIV-1 to establish chronic virus producers. Mapping of genetic determinants for the ability to establish chronic virus producers revealed that multiple HIV-1 genes and sequences affect this ability. In the case of NL4-3 mutants, defects in two auxiliary genes, vpr and nef, were sufficient for the efficient establishment of chronic virus producers. However, tests of the NL4-3-HXB-2 recombinants revealed the additional influence of a 5' internal region. This internal fragment contained gag, pol, vif, and vpr sequences.

The complexity of these results shows that the ability to establish chronic virus producers is a multi-gene, multi-step phenomenon. Whether vpr, nef, and the 5' internal sequences act together or independently is not known. It is possible that the mechanism responsible for the establishment of chronic virus producers requires certain steps which in one viral strain may be met by one set of factors (like defects in both vpr and nef in NL4-3) and in another viral strain by yet a different set of factors (like 5' internal sequences in combination with defective auxiliary genes in NL4-3-HXB-2 recombinants).

Roles of vpr, nef, and the 5' internal sequences in the establishment of chronic virus producers. Our studies have suggested that the establishment of chronic virus producers was affected both by the cytopathic potential of the virus and the growth potential of the infected cells. Viruses that had high cytopathic
potential with adverse effects on the growth potential of the host cells were poor chronic virus producers, while viruses with low cytopathic potential that did not adversely affect the growth potential of the cells were good chronic virus producers. Although a decrease in cytopathicity was not sufficient, by itself, to increase the ability of a virus to establish chronic virus producers, it was a prerequisite. Thus, lowering the cytopathic potential, and thereby influencing the survival of the infected cells, may be the first step towards the establishment of chronic virus producers.

The fact that a minimum of two defective proteins were required for the enhancement of the ability of NL4-3 to establish chronic virus producers suggests that a second function may be involved in allowing a virus to become a chronic virus producer. With low cytopathic potential being the first of these functions, the second may relate to factors that control cell growth such as those involved in signalling pathways.

**Cytopathic potential.** The cytopathic potential of a virus can be affected by many factors including: products encoded by the virus being directly toxic to the cell (Stevenson et al., 1988; Terwilliger et al., 1989), products encoded by the virus sequestering or disrupting host cell functions (Hoxie et al., 1986; Koga et al., 1990), or the budding of large amounts of progeny virions disrupting the integrity of host cell membranes (Leonard et al., 1988).

Both Vpr and 5' internal sequences have been noted for effects on cytopathicity. Vpr has been shown to increase the rate of replication and
cytopathicity in T-cells (Cohen et al., 1990b). 5' internal viral sequences have also been shown to increase the cytotoxicity of HIV-1 in T-cells (Hirsch et al., 1992). Thus, either of these could affect chronic virus production by affecting the cytopathic potential of the virus. By contrast, most reported effects of Nef have affected cell signalling rather than viability.

**Infected cell growth.** The second step towards the establishment of chronic virus producers, good growth of infected cells, could be modulated by the action of viral proteins on cell signalling pathways. Such activities could prevent the virus from establishing chronic virus producers by limiting the growth of infected cells. For example, Nef has been shown to down regulate surface CD4 molecules (Garcia and Miller, 1991), IL-2 mRNA (Luria, Chambers, and Berg, 1991), and NFkB (Niederman et al., 1992) -- factors important for T-cell activation and growth. In addition, Nef has been shown to have phosphorylation-dephosphorylation activities whereby it could activate or deactivate important signalling pathways (Guy et al., 1987; Poulin and Levy, 1992). Very limited studies have been done on the effects of Vpr on cell signalling pathways. Vpr could affect cell signalling pathways through its reported ability to affect factors that trans-activate the HIV-1-LTR (Cohen et al., 1990b). At this point, it is not clear how 5' internal sequences might affect pathways that control cell growth.

**Future directions.** Now that we have an appreciation for the viral proteins and sequences involved in the establishment of chronic virus producers, in order to understand the mechanism, two approaches should be undertaken. The first is...
to further map the genetic determinants that are important in the establishment of chronic virus producers. The second is to carry out functional studies on Vpr and Nef to test how these proteins directly affect cytopathicity and cell growth.

**Genetic studies:** Tests of the NL4-3 mutants revealed the importance of Vpr and Nef in the establishment of chronic virus producers. Tests of the NL4-3-HXB-2 recombinants revealed the importance of 5' internal sequences (3' gag, pol, vif, and 5' vpr) plus 3' fragments encoding defective auxiliary genes in the establishment of chronic virus producers. Defining the critical sequences in 5' and 3' fragments in the NL4-3-HXB-2 recombinants should allow a better understanding of the mechanism of chronic virus production, especially in complementation with what we already know for NL4-3. In addition, it would be helpful to better define the auxiliary gene requirements of both NL4-3 and HXB-2 for chronic virus production. Experiments designed to define the genetic determinants would include:

a) Further mapping of the 5' internal sequences of HXB-2 to identify sequences that determine the ability to establish chronic virus producers

b) Further mapping of the 3' HXB-2 fragments to identify genes/sequences that determine the ability to establish chronic virus producers

c) Further testing of the role of mutations in the vpr and nef genes of NL4-3 in chronic virus producers by mutating the initiator codons of these genes to eliminate possible effects of truncated translation products on the establishment of chronic virus producers
**Functional Studies:** Since we have identified two genes that are important in the establishment of chronic virus producers in NL4-3, these can be tested for effects on cytopathicity and cell growth. Our data reveal that the cytopathic potential of the virus and the growth potential of infected cells are both important for the generation of chronic virus producers. Thus, expression of Vpr, Nef, and Vpr plus Nef proteins in H9 and Jurkat cells (cells that can establish chronic virus producers) should allow testing for the cytopathic effects of these proteins and the effects of these proteins on cell growth.

Similarly, Vpr, Nef, and Vpr plus Nef can be tested for cytopathic effects and for effects on growth potential in cells that do not establish chronic virus producers (such as A3.01). How these proteins interact with T-cell lines that differ in their ability to establish chronic virus producers may provide important insights into the roles of these genes in chronic virus production.

Several lines of evidence suggest that Nef affects the expression of cell surface signalling proteins. Thus, in order to understand the role of Nef (and perhaps Vpr) in the establishment of chronic virus production, it would be valuable to study the effects of Vpr and Nef on the expression of cell surface markers as well as other signal transducing molecules important in T-cell growth. These would include molecules such as: CD4, CD3, TCR, CD45, IL-2R, and NFkB.

Along the same lines, defining the cell surface markers on populations of cells that are found in the acute and the chronic phases of infection may yield
important information on the mechanism of how chronic virus producers are generated. Preliminary data have already suggested interesting differences between these two cell populations (see Discussion to Chapter I).

**Relevance of the phenomenon of chronic virus production to infected hosts.**
The observation that defective auxiliary genes are important in the generation of chronic virus producers may have some relevance to disease progression. Due to the infidelity of the viral reverse transcriptase, mutant viruses are constantly generated in the infected individual (Goodenow et al., 1989). Thus, over the course of the immunodeficiency syndrome, perhaps chronic virus producers are also continuously generated by cells harboring appropriate mutants. Since people infected with HIV-1 mount a significant immune response to the initial virus infection, one would predict that chronic virus producers would be cleared by hosts with functioning immune systems. However, with disease progression, severe defects in the immune surveillance mechanisms of the infected individual develop. Therefore, in the later stages of the disease the immune system may not be able to clear virus-expressing cells. In such patients, chronic virus producers could act as factories of virus, exacerbating the onset and severity of disease.
BIBLIOGRAPHY


73) Folks, T., S. Benn, A. Rabson, T. Theodore, M.D. Hoggan, M. Martin, M.
susceptible to the cytopathic effects of the acquired immunodeficiency
syndrome (AIDS)-associated retrovirus. Proc. Natl. Acad. Sci., USA
82:4539-4543.


1991. Lymphoid germinal centers are reservoirs of human
immunodeficiency virus type 1 RNA. J. Infect. Dis. 164:1051-1057.

76) Franchini, G., M. Robert-Guroff, J. Ghrayeb, N. T. Chang, and F. Wong-
Staal. 1986. Cytoplasmic localization of the HTLV-III 3' orf protein in
cultured cells. Virology 155:593-599.

proteins that interact with specific regulatory genetic elements, p. 159-174.
In B. R. Franz, Jr., B. R. Cullen, and F. Wong-Staal (ed), The control of
human retrovirus gene expression. Cold Spring Harbor Laboratory, Cold
Spring, NY.


phosphorylated GTP-binding protein resembling an oncogene product.


107) **Hattori, N., F. Michaels, K. Fargnoli, L. Marcon, R. C. Gallo, and G. Franchini.** 1990. The human immunodeficiency virus type 2 *vpr* gene is
Acad. Sci. USA 87:8080-8084.

the conserved basic domain of human immunodeficiency virus tat protein.

by PCR. Oncogene 6:491-493.

110) Hirsch, V., N. Ridel, and J. I. Mullins. 1987. The genome organization of
STLV-3 is similar to that of the AIDS virus except for a truncated

cytopathogenicity: genetic differences between direct cytotoxic and

immunodeficiency virus type 1 in the blood of infected persons. N. Engl. J.


127) **Kanner, S. B., J. P. Deans, and J. A. Ledbetter.** 1992. Regulation of CD3-induced phospholipase C-gamma 1 (PLC gamma 1) tyrosine phosphorylation by CD4 and CD45 receptors. Immunology **75**:441-447.


maintenance of high virus loads and for development of AIDS. Cell 65:651-662.


is linked to the last stage of virus infection. Proc. Natl. Acad. Sci. USA 85: 3570-3574.


153) **Li, L. X., T. Moudgil, H. V. Vinters, and D. D. Ho.** 1990. CD4-independent, productive infection of a neuronal cell line by human immunodeficiency virus type 1. J. Virol. 64: 1383-1387.


168) **Martins, L. P., N. Chenciner, B. Asjo, A. Meyerhans, and S. Wain-Hobson.**

169) **McDougal, J. S., M. Kennedy, J. Sligh, S. Cort, A. Mowie, and J. Nicholson.**


infection of H9 cells: an immunoelectron microscopic study. AIDS 6:1105-1116.


208) Rusche, J. R., K. Javaherian, C. McDanal, J. Petro, D. Lynn, R.
Grimala, A. Langois, R. C. Gallo, L. O. Arthur, P. J. Fischinger, D. P.
fusion of human immunodeficiency virus-infected cells binds a 24-amino
acid sequence of the viral envelope, gp 120. Proc. Natl. Acad. Sci. USA 85:
3198-3202.

209) Saag, M. S., M. J. Crain, W. D. Decker, S. Campbell-Hill, S. Robinson, W.
High-level viremia in adults and children infected with human
immunodeficiency virus: relation to disease stage and CD4(+) lymphocyte

210) Saag, M. S., B. H. Hahn, J. Gibbons, Y. Li, E. S. Parks, W. P. Parks, and
G. M. Shaw. 1988. Extensive variation of human immunodeficiency virus

211) Sadaie, M. R., J. Rappaport, T. Benter, S. F. Joseph, R. Willis and F.
immunodeficiency viral genome: functional mapping of tat and
85:9224-9228.


216) Samuel, K. P., D. R. Hodge, Y. M. Chen, and T. S. Papas. 1991. Nef proteins of the human immunodeficiency viruses (HIV-1 and HIV-2) and simian immunodeficiency virus (SIV) are structurally similar to leucine


245) **Stevenson, M.C., C. Meier, A. M. Mann, N. Chapman, and A. Wasiak.** 1988. Envelope glycoprotein of HIV induces interference and cytolysis resistance in CD4+ cells: mechanism for persistance in AIDS. Cell 53: 483-496

246) **Stevenson, M., T. L. Stanwich, M. P. Dempsey, and C. A. Lamonica.** 1990c. HIV-1 replication is controlled at the level of T-cell activation and proviral integration. EMBO J. 9:1551-1560.


250) **Subramanian, T., M. Kuppuswamy, L. Venkatesh, A. Srinivasan, and G. Chinnadurai.** Functional substitution of the basic domain of the HIV-1 trans-activator, tat, with the basic domain of the functionally heterologous rev. Virology 176:178-183.


