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**The role of virion selective tRNA^{Lys}
packaging and the importance of the
A-rich genomic codon-bias in HIV-1
replication and assembly.**

A thesis by
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Student declaration

I certify that the material presented in this thesis is my original work. No other person's work has been used without due acknowledgement. I acknowledge I have received assistance in writing this thesis from Drs. Johnson Mak and Melissa Hill. I have received technical assistance with experimental work from Dr. Johnson Mak, Dr. Melissa Hill, Shahan Campbell, and Dr. Miranda Shehu-Xhilaga. Mutant constructs used in this study were either obtained from Drs. Gary Nabel and Yue Huang, Vaccine Research Institute, NIH, USA, or were cloned by Dr. Johnson Mak.

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III

Abstract

Human immunodeficiency virus (HIV) particles contain a pool of free tRNA molecules, and the relative abundance of specific tRNAs in the virion differs markedly from what is found in the host cell. This selective packaging of tRNA species results in a highly conserved ratio of tRNA^{Lys1,2}:tRNA^{Lys3} of 12:8 molecules in the virion. In this study, we examined the effect of altering the tRNA^{Lys} packaging ratio using plasmids containing both HIV-1 proviral DNA and a human tRNA^{Lys3} gene. We have demonstrated that the ratio of virion tRNA^{Lys} packaging is not important for genomic RNA dimerization or viral infectivity as virions with altered ratios of tRNA^{Lys} packaging contained RNA dimers of WT stability and infected both MT2 cells and peripheral blood mononuclear cells (PBMCs) with WT efficiency. HIV preferentially uses A-rich codons for viral protein synthesis in contrast to the GC-rich codons used in human protein synthesis. To investigate the importance of this conserved A-rich codon-bias in HIV-1 replication, various regions of the A-rich genome were systematically replaced with GC-rich codon sequences without changing the amino-acid sequence of expressed viral proteins. We have demonstrated that the HIV-1 codon-bias influenced virion genomic RNA packaging and viral protein processing, but was not important for virion tRNA^{Lys} packaging. Most importantly, the A-rich codon-bias of the HIV-1 genome was shown to be important for viral infectivity. In particular, the PR-RT regions of the *pol* gene were shown to be critical for viral replication in PBMCs as HIV-1 mutants with GC-rich PR-RT regions were 30-600 fold less infectious than WT.

Consequently, our data provide direct evidence that the A-rich codon-bias of the PR-RT regions in the *pol* gene is critical for viral infectivity. These findings have important implications for the clinical application of human codon-optimised (GC-rich) HIV-1 vaccine vectors, since any recombination events occurring between WT HIV-1 and vector in the host will not be advantageous for viral infection.

IV Publications and Presentations

Keating, C., M. Hill, M. Shehu-Xhilaga, S. Crowe, Y. Huang, G. Nabel, and J. Mak 2002. Introduction of Human codon-optimised sequences into the HIV-1 genome generates replication defective virions. (manuscript in preparation).

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V

List of Abbreviations

BIV	Bovine immunodeficiency virus
CA	Capsid protein
DIS	Dimerization initiation site
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ECL	Enhanced chemi-luminescence
EDTA	Ethylenediamine-tetra-acetic acid
EIAV	Equine infectious anaemia virus
EGFP	Enhanced green fluorescent protein
FIV	Feline immunodeficiency virus
GP	Glycoprotein
HBS	HEPES buffered saline
HI-FBS	Fetal Bovine serum
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IN	Integrase
LB	Luria-Bertani Medium
MA	Matrix protein
NC	Nucleocapsid protein
NERT	Natural endogenous reverse transcriptase assay
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PHA	Phytohemagglutinin
PMSF	Phenylmethyl sulfonyl fluoride
PR	Viral protease
RNA	Ribonucleic acid
RNP	Ribonucleoprotein complex
RPMI	Roswell Park Memorial Institute Medium-1640
RSV	Rous sarcoma virus
RT	Reverse transcriptase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIV	Simian immunodeficiency virus
SSC	Sodium chloride and Sodium citrate buffer
SSPE	Sodium chloride and Sodium dihydrogen phosphate EDTA
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TBS	Tris buffered saline
TCID₅₀	50% tissue culture infectious dose
tRNA	transfer RNA
UTR	untranslated region
WT	wild-type

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1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is a member of the primate lentiviruses, *Retroviridae* family (10). Retroviruses use RNA to encode their genome, which is reverse transcribed to proviral DNA prior to integration into the host cell chromosomal DNA. The HIV-1 genome is A-rich, particularly in the third codon position and this codon-bias is highly conserved (17, 27, 40). There are three major tRNA^{Lys} isoacceptors in mammalian cells (36, 37), tRNA^{Lys1,2} and tRNA^{Lys3} (collectively referred to as tRNA^{Lys}), and all three are utilised during HIV-1 protein translation and subsequently packaged into the virion (24). Amongst the hundreds of different tRNA species present in HIV-1 infected cells, tRNA^{Lys1,2} and tRNA^{Lys3} are selectively packaged and enriched ten fold in the virion core (32). tRNA^{Lys1,2} and tRNA^{Lys3} comprise 6% of total cellular tRNA, yet form 60% of the low molecular weight RNAs found in viral particles (32). Furthermore, it has been estimated that there are 20 tRNA^{Lys} molecules packaged into the virion per two molecules of genomic RNA packaged (20, 32).

The focus of this study is firstly to examine the importance of the ratio of HIV-1 virion tRNA^{Lys1,2}:tRNA^{Lys3} packaging in genomic RNA dimerization and viral infectivity. Secondly, to investigate the role of HIV-1 codon usage in virion tRNA^{Lys} packaging, and thirdly to assess the importance of the conserved A-rich genomic codon-bias for viral replication.

tRNA^{Lys1,2} and tRNA^{Lys3} molecules are packaged into the virion in a defined ratio of 12 tRNA^{Lys1,2} molecules:8 tRNA^{Lys3} molecules per 2 molecules of genomic

RNA packaged (20). The importance of the ratio of virion $\text{tRNA}^{\text{Lys1,2}}:\text{tRNA}^{\text{Lys3}}$ packaging in HIV-1 replication is unknown. Virion tRNA^{Lys} packaging is likely to be a regulated event since the total amount of tRNA^{Lys} packaged into the virion is conserved at 20 molecules even when excess $\text{tRNA}^{\text{Lys3}}$ is supplemented *in trans* (20). Excess virion $\text{tRNA}^{\text{Lys3}}$ packaging results in a corresponding decrease in $\text{tRNA}^{\text{Lys1,2}}$ packaging, which in turn leads to an altered ratio of $\text{tRNA}^{\text{Lys1,2}}:\text{tRNA}^{\text{Lys3}}$ (20). The conservation of total virion tRNA^{Lys} packaging argues for selective virion tRNA^{Lys} packaging having an important role in HIV-1 replication, however, it is unclear whether this is a critical role. Genomic RNA dimerization is essential for viral infectivity (10), and the tRNA^{Lys} molecules that are packaged into HIV-1 may act as RNA linkers between strands of genomic RNA, facilitating RNA dimerization in a model similar to that hypothesized to occur in Rous sarcoma virus (RSV) (18). We hypothesize that the ratio of virion $\text{tRNA}^{\text{Lys1,2}}:\text{tRNA}^{\text{Lys3}}$ packaging may be important for HIV-1 genomic RNA dimerization.

The mechanism of selective tRNA^{Lys} packaging into the HIV-1 virion is unclear. Selective packaging may in part be related to the preferential usage of a subset of A-rich codons and corresponding tRNAs in the translation of viral proteins. A-rich codons exhibit preferential usage of adenine in the third codon position, and the HIV-1 preference for A-rich codons contrasts with the GC-rich codons that are used in human protein synthesis (17, 27, 40). The importance of the HIV-1 codon-bias in the viral lifecycle is not understood, however, the A-rich codon-bias is known to be conserved in all lentiviruses including HIV-1 (6). We hypothesize that the A-rich codon-bias of the HIV-1 genome contributes in part

to the selective packaging of tRNA^{Lys} into the virion by determining the usage of tRNA^{Lys} during viral protein translation.

1.2 Selective virion tRNA^{Lys} packaging, genomic RNA dimerization and viral infectivity

The HIV-1 RNA genome consists of two identical copies of single-stranded RNA (9 kb) and encodes three major genes (*gag*, *pol* and *env*), as well as a number of regulatory genes (Fig. 1.1). *gag* and *pol* are initially translated as precursor polyproteins, which are subsequently processed by the viral protease (PR), in the immature viral particle, to individual proteins in the mature virion. *gag* is translated as a 55 kDA polyprotein (Pr55^{Gag}), which is cleaved by the viral PR to generate the structural proteins: matrix (MA), capsid (CA), nucleocapsid (NC), P6^{Gag}, P2 and P1. *gag* and *pol* are encoded by overlapping sequences and since *pol* lacks an initiation codon, it is synthesized as a 160 kDA polyprotein (Pr160^{gag-pol}), via an infrequent frameshifting event (23). Pr160^{gag-pol} is cleaved by viral PR to generate: MA, CA, NC, P2 and P6^{Pol}, as well as the viral enzymes PR, reverse transcriptase (RT) and integrase (IN).

The mature HIV-1 consists of a symmetrically icosahedral particle approximately 95-140 nm in diameter, containing an electron-dense cone-shaped capsid core in the centre (Fig. 1.2). CA forms the inner core of the virion and surrounds the ribonucleoprotein complex (RNP). The RNP consists of NC, two copies of genomic RNA, and a tRNA^{Lys3} molecule attached to the primer binding site (PBS) ready for transcription by RT. The PBS is located in

Figure 1.1 The HIV-1 RNA genome and translated viral proteins.

The HIV-1 RNA genome consists of two identical copies of single-stranded RNA (9 kb) and encodes three major genes (*gag*, *pol* and *env*), as well as a number of regulatory genes (*vif*, *vpr*, *rev*, *tat* and *nef*). *gag* and *pol* are initially translated as precursor polyproteins (Gag and Gag-Pol). Gag is cleaved by the viral PR to generate the structural proteins: matrix (MA), capsid (CA), nucleocapsid (NC), P6^{Gag}, P2 and P1. Gag-Pol is cleaved by viral PR to generate: MA, CA, NC, P2 and P6^{Pol}, as well as the viral enzymes PR, reverse transcriptase (RT) and integrase (IN).

Figure 1.2 A schematic representation of the mature HIV-1 virion.

The mature HIV-1 consists of a symmetrically icosahedral particle approximately 95-140 nm in diameter, containing an electron-dense cone-shaped capsid core in the centre. 19 molecules of tRNA^{Lys} (20) remain free in the virion core and a single primer tRNA^{Lys3} molecule is attached to the genome.

the non-coding leader sequence of HIV-1, immediately 5' to *gag* (Fig. 1.3). RT and PR are found in close proximity with the genomic RNA as part of the RNP (26). 19 molecules of tRNA^{Lys} (20) remain free in the virion core.

All retroviruses, including HIV-1, contain two copies of unspliced viral genomic RNA that are linked non-covalently near the 5' end of the RNA genome (10). During maturation of viral particles, viral genomic RNA undergoes rearrangement to form a more stable dimeric RNA that is important for virus replication (14, 41). The current model of genomic RNA dimerization is the "kissing loop" model, which proposes that the Dimerization initiation site (DIS) stem-loop is the primary site involved in RNA dimerization (Fig. 1.3) (29, 44). In contrast to the model that the DIS is critical for HIV-1 replication and genomic RNA dimerization, DIS mutants with either a four bp deletion (gcgc) or four bp addition (gcgc) to the DIS stem-loop formed stable dimers when replicated in a T cell line (8). Furthermore, previous work in our laboratory with DIS stem-loop deletion mutants (RNA nts 242-276) has shown that they form stable RNA dimers and are replication competent in peripheral blood mononuclear cells (PBMCs) (31). This suggests the DIS is not solely responsible for RNA dimerization and therefore it is possible other regions of genomic RNA or low molecular weight RNAs, such as tRNA^{Lys}, may be involved in RNA dimerization.

It was proposed that RNA dimerization in RSV may involve base-pairing directly between sub-units of the RNA genome and short RNA linkers such as primer tRNA or virion associated tRNAs (18). It has also been observed that the primer binding sites of yeast retrotransposons are important for RNA dimer

Figure 1.3 Schematic of HIV-1 5'UTR.

The 5'UTR is composed of complex secondary RNA structures. The dimerization initiation site (DIS) stem-loop is the first of four stem-loop structures in the virion RNA packaging sequence at the 3' end of the 5' UTR (18), and is the proposed site for viral genomic RNA dimerization *in vitro* (29, 44). The 5' UTR consists of further stem-loops including the primer binding site (PBS).

formation (11, 15). Yeast retrotransposons are members of a large group of mobile genetic elements including retroviruses such as HIV-1. Models of genomic RNA dimerization in yeast retrotransposons have been proposed that are dependent on the binding of primer tRNA_i^{Met} to the PBS (11, 15). Opposing RNA strands are joined by tRNA-tRNA complementarity, forming a dimer complex (11, 15). An analogous model for HIV-1 may involve selectively packaged tRNA^{Lys} molecules contributing to genomic RNA dimerization via annealing to the PBS.

In B77 sarcoma virus it was hypothesized that if low molecular-weight RNAs function as linkers between RNA strands, they will be necessary in high local concentrations within the intact virion (45). tRNA^{Lys} molecules are indeed present in high local concentration in HIV-1 virions as a result of selective packaging from the cellular milieu of the host cell (32). Only a single tRNA^{Lys3} molecule is attached to the PBS and is needed to prime RT for reverse transcription of the viral genomic RNA (46). The remaining tRNA^{Lys3} molecules selectively packaged into the virion remain free in the core (32), and their function is unknown. In addition, tRNA^{Lys1,2} molecules are selectively packaged into the virion along with tRNA^{Lys3} but are unable to be used as a primer for RT (5, 30).

There are a number of interactions between the primer tRNA^{Lys3} molecule and viral genomic RNA. In addition to the PBS of the viral genome, three other binding regions have been reported. These include A-rich regions upstream and downstream of the PBS interacting with the anticodon loop of tRNA^{Lys3} (Fig.

1.4) (22, 24, 28). A third proposed interaction may occur between the T Ψ C loop of tRNA^{Lys3} with a U5 region upstream of the PBS (2, 3). The locations of these interactions between tRNA^{Lys3} and genomic RNA are at the 5' end of the genome, which is in close proximity to the proposed RNA dimerization domain. It is therefore possible tRNA^{Lys3} may act as an RNA linker via these regions to provide additional linkages for the genomic RNA dimer.

1.3 Codon usage, selective tRNA^{Lys} packaging and viral replication

The HIV-1 genome is A-rich (38.7%) and this bias is amplified in the third codon position (17, 27, 40). The A-rich genome of HIV-1 differs significantly from the human genome, which is GC-rich (6, 17, 25, 27, 40). tRNA^{Lys1,2} binds to the AAG codon and tRNA^{Lys3} binds to AAA. As tRNA^{Lys3} binds to a more A-rich codon it is preferentially used by the HIV-1 genome during viral protein synthesis.

The signals that target tRNA^{Lys} for packaging are unknown, however there is a proposed interaction between tRNA^{Lys3} and the thumb and connection domains of the RT portion of Pr160^{gag-pol} (33, 34). Indeed RT-deficient mutants don't package tRNA^{Lys3} efficiently (32, 33). Also, HIV-1 particles composed of only Pr55^{gag} do not selectively package tRNA^{Lys3} while particles composed of both Pr55^{gag} and Pr160^{gag-pol} do (32). Virion tRNA^{Lys3} packaging is unchanged in particles that are devoid of genomic RNA (32). Further, the total tRNA^{Lys} content is unchanged in virions that contain genomic RNA deleted in the PBS sequence (24, 32). Thus tRNA^{Lys3} packaging is dependent on the Pr160^{gag-pol}

Figure 1.4 Proposed regions of base pairing between tRNA^{Lys3} and the HIV-1 genome. This figure is reproduced from Mak & Kleiman (50) with the author's permission. In addition to the PBS of the viral genome, three other binding regions between tRNA^{Lys3} and the RNA genome have been reported. These include A-rich regions upstream and downstream of the PBS interacting with the anticodon loop of tRNA^{Lys3} (shown by the arrows) (22, 24, 28). A third proposed interaction may occur between the TΨC loop of tRNA^{Lys3} with a U5 region upstream of the PBS (2, 3).

precursor protein and is not directed into the virion by binding to genomic RNA. It has been hypothesized that the cytoplasmic source of viral tRNA^{Lys} selectively packaged into the virion is tRNA^{Lys} which has just left the P site on the ribosome to interact with a growing Pr160^{gag-pol} protein as it is translated (20). Consequently it is conceivable that the usage of tRNA^{Lys} during viral protein translation is a determinant of virion selective tRNA^{Lys} packaging. Since the A-rich codon-bias of the HIV-1 genome determines the usage of tRNA^{Lys} during viral protein translation, we hypothesize that the codon-bias of the HIV-1 genome may be important for virion selective tRNA^{Lys} packaging.

The A-rich codon-bias of the HIV-1 genome is conserved in all lentiviruses (48), suggesting an important role in viral replication. The A-rich codon-bias is most pronounced in the *pol* gene of HIV-1 (27). Therefore, if the codon-bias is important for viral replication, changes in codon usage made to this region of the genome should result in more significant effects on viral replication than changes made in other parts of the genome. HIV-1 codon-optimised (GC-rich) Gag/Gag-Pol expression vectors have demonstrated 10-100 fold increased viral protein production in mammalian hosts and subsequent strong host immune responses (4, 13, 19, 25, 49). HIV-1 is infamous for its ability to recombine with cellular and other viral coding sequences. If a GC-rich vaccine vector was introduced therapeutically into an HIV-1 infected patient, recombination may occur between vector and wild-type (WT) HIV-1. This could possibly result in a mutant HIV-1 with increased viral protein production and increased viral infectivity compared to WT. Therefore it is essential to understand the role of

the HIV-1 A-rich codon-bias in viral replication before clinical application of codon-optimised vectors.

The objectives of this study were (1) to examine the importance of the ratio of HIV-1 virion $tRNA^{Lys1,2}:tRNA^{Lys3}$ packaging in genomic RNA dimerization and viral infectivity. (2) To investigate the role of HIV-1 codon usage in virion $tRNA^{Lys}$ packaging, and (3) To assess the importance of the conserved A-rich codon-bias for viral replication.

Analysis of RNA dimers from HIV-1 mutants with altered ratios of virion $tRNA^{Lys1,2}:tRNA^{Lys3}$ packaging will help define the contribution of the ratio of $tRNA^{Lys1,2}:tRNA^{Lys3}$ packaging to virion RNA dimer formation. Previous work in our laboratory has found that HIV-1 DIS stem-loop mutants were able to replicate in primary cells (PBMCs) but not in T cell lines (31). This differential ability of certain HIV-1 mutants to replicate only in primary cells led us to investigate the infectivity of virions with altered $tRNA^{Lys}$ packaging in PBMCs and the T cell line; MT2. GC-rich HIV-1 sequences have been made previously and have potential uses as HIV-1 vaccines (4, 13, 19, 25, 49). However, viral codon usage has never been altered in a replicating HIV-1 sequence. Various regions of the WT A-rich HIV-1 genome have been systematically replaced with GC-rich RNA segments by Dr. Johnson Mak in our laboratory in order to assess the role of HIV-1 codon usage in selective virion $tRNA^{Lys}$ packaging and viral replication.

This study has firstly demonstrated that the ratio of virion tRNA^{Lys} packaging is not important for genomic RNA dimerization or HIV-1 infectivity in either PBMCs or MT2 cells. Secondly, codon usage in the HIV-1 genome does not play a significant role in virion selective tRNA^{Lys} packaging. Thirdly, this study has shown that the A-rich codon-bias of the HIV-1 genome is important for viral infectivity. In particular, the PR and RT regions of the *pol* gene have been implicated as being critical for viral infectivity. HIV-1 mutants with GC-rich PR-RT regions were 30-600 fold less infectious than WT. Furthermore, the codon-bias of the genome has been demonstrated to have a role in virion genomic RNA packaging and viral protein processing. Overall, this study has made several novel contributions to our understanding of the role of selective virion tRNA^{Lys} packaging in HIV-1 replication and the role of the conserved A-rich codon-bias in viral infectivity and aspects of viral assembly.

2. Materials and Methods

2.1 Mutant constructs

The full-length HIV-1 plasmids HxB2-BH10 (47) and NL 4.3 (1) were used as WT control. The virion tRNA^{Lys} packaging mutants (*BH10Lys3* and *BH10Su+*) have been described previously (20). A GC-rich Gag/Gag-Pol vector (4.3 kb) was obtained from Drs. Yue Huang and Gary Nabel, NIH, USA. Various segments of this construct were systematically engineered into the NL 4.3 plasmid by Dr. Johnson Mak to create five GC-rich RNA mutants for analysis which are described in Section 3.2.

2.2 Large scale plasmid isolation

Plasmid DNA was transformed in the HB101 *E.Coli* strain as previously described (39). A single transformed bacterial colony was inoculated into 20 ml Luria-Bertani medium (LB, Appendix I) containing 100 µg/ml ampicillin, and incubated at 37°C for 12 hrs with vigorous shaking (200 rpm). The culture was transferred into 1 L LB medium containing 100 µg/ml ampicillin and incubated for 16 hrs at 37°C. Bacterial cells were pelleted in a JA-14 rotor at 5000xg for 15 min, 4°C (Beckman J2-21M/E). DNA was isolated using the QIAGEN plasmid Maxi prep kit according to the manufacturers instructions. Plasmid DNA concentration was determined by spectrophotometry (Ultrospec 3000, Pharmacia Biotech).

2.3 Virus production

2.3.1 Transfection: calcium phosphate method

The human embryonic kidney cell line; 293T, was used in transfections for the production of WT and mutant virus. Cells were maintained in Dulbecco's Modified Eagle's medium (DMEM, JRH Biosciences) supplemented with 10% heat-inactivated fetal bovine serum (HI FBS, PA Biologicals), 100 U/ml of penicillin and 100 µg/ml of streptomycin, at 37°C, 5% CO₂. 293T cells (1.3 x 10⁶) were seeded onto 10 cm diameter plates (Nunc) 26 hrs before transfection.

DNA mixtures were introduced into 293T cells using a calcium phosphate transfection method as previously described (32). Briefly, transfection mixtures contained 10 µg of plasmid DNA, 50 µl of 2.5 M CaCl₂, and 3 µg of a reporter plasmid (pCMV[promoter]-EGFP[enhanced green fluorescent protein reporter], Clontech), made up to 500 µl with RNAase free H₂O. The transfection mixture was added dropwise to 500 µl of ice cold 2x HEPES-buffered saline (HBS, Appendix I) to form a calcium phosphate precipitate. The samples were mixed by pipetting and added dropwise to the 293T cell plate. At 14 hrs post transfection, cells were washed twice with magnesium-free phosphate-buffered saline (PBS) and maintained in fresh DMEM medium. At 36 hrs post transfection the cell lawn and supernatants were collected and centrifuged at 2000xg for 30 min, 4°C (Beckman GS-6R) to separate cells and virus-containing supernatant for analysis.

2.4 Protein

2.4.1 Isolation of cellular and viral proteins from transfected 293T cells

Following pelleting, transfected 293T cells were washed twice in PBS. Protein extraction was then performed using 500 μ l of 2xTBS lysis buffer (2xTBS, 10 μ l/ml Nonidet P-40, 20 mM phenylmethyl sulfonyl fluoride [PMSF], 1 μ M pepstatin, and 1 μ M leupeptin). Cell lysates were frozen in liquid nitrogen and thawed at room temperature three times to weaken cellular membranes. Cell debris was subsequently removed by centrifugation at 20,000 \times g for 30 min, 4 $^{\circ}$ C (Eppendorf 5417R). The supernatant containing soluble cellular proteins was collected and stored at -20 $^{\circ}$ C for analysis.

2.4.2 Isolation of virion protein

Virions were purified and concentrated by ultracentrifugation of virus-containing transfection supernatants through a 20% sucrose cushion at 150,000 \times g for 1 hr, 4 $^{\circ}$ C (Beckman L-90 model, SW 41 rotor). Virion pellets were resuspended in 50 μ l of 2xTBS lysis buffer and stored at -20 $^{\circ}$ C until analysis.

2.4.3 Determination of transfection efficiency

The inclusion of the reporter plasmid pCMV-EGFP in transfection results in the expression of green fluorescent protein alongside viral proteins, which was used to determine transfection efficiency. Serial 2-fold dilutions of 293T cellular

lysates (Section 2.4.1) were made in a 96 well flat bottom plate using 1xTBS. Relative transfection efficiency of samples was determined by measuring EGFP fluorescence using a Bio Imaging Analyser (Fuji Photo Film Co.).

2.4.4 Quantification of virion proteins

Relative amounts of isolated viral proteins in samples were determined by protein dot blot. Two-fold dilutions (in 2xTBS lysis buffer) of each protein sample were spotted onto a nitrocellulose membrane (Hybond-ECL, Amersham). The membrane was air dried for 30 min at room temperature and blocked for 1 hr in 5 ml of 1xTBS containing 3% casein. The membrane was probed with 1 μ l of HIV-1 positive pooled patients sera in 9 ml of 0.3% casein in 1xTBS-T (TBS containing 0.3% Tween 20 [BDH Chemicals]) overnight at 4°C. After 6-10 washes with 1xTBS-T, the membrane was incubated for two hours with anti-human antibody conjugated with horseradish peroxidase (HRP)(DAKO), at room temperature. A further 6-10 washes with 1xTBS-T were then performed. Viral protein dilutions were visualized via performing ECL (enhanced chemi-luminescence) according to manufacturers instructions (Amersham) and exposing the fluorescing samples to ECL hyperfilm (Amersham). The Bio Imaging Analyser (Fuji Film Co.) was used for quantification of total viral proteins in each sample.

2.4.5 Western Blot Analysis

Cellular viral protein samples were normalised by relative transfection efficiency (Section 2.4.3). Viral protein samples were normalised by virion protein dot blot (Section 2.4.4). Each sample was combined with 5 μ l of loading dye (100 mM Tris pH 6.8, 3% SDS, 33% glycerol, 0.03% bromophenol blue, and 5 mM β -mercapto-ethanol) and denatured for 5 min at 95°C. Samples were electrophoresed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% gel) in 1xTris-glycine running buffer (Appendix I) at 100V for 100 min in a Bio-Rad gel tank (Bio-Rad Laboratories). Separated proteins were electroblotted for two hours at 70 V onto a nitrocellulose membrane (Amersham) using a Bio-Rad transfer apparatus (Bio-Rad Laboratories). The membrane was washed 3 times in 1xTBS-T then blocked, probed and visualized as described in Section 2.4.4.

2.5 Assessment of viral replication kinetics

2.5.1 Micro Reverse Transcriptase (RT) assay of virus supernatants

The RT activity of each virus was determined using a micro RT assay as previously described (16). This procedure is outlined in Appendix II.

2.5.2 Infectivity assay

Peripheral blood mononuclear cells (PBMCs) are the natural target cells for HIV-1 and were isolated from buffy coats of HIV-1 seronegative blood donors (supplied by the Red Cross Blood Bank, Melbourne) as previously described by our laboratory (12). This procedure is outlined in Appendix II. Purified PBMCs (2.0×10^6 cells/ml) were stimulated with 10 μ g/ml phytohemagglutinin (PHA, Murex Diagnostics) for 3 days and cultured in RF10 media (Roswell Park Memorial Institute (RPMI) medium-1640, GibcoBRL) containing 10% HI FBS, 2 mM L-glutamine (GibcoBRL) and 10 U/ml IL-2 (Boehringer-Mannheim). MT2 cells are an HTLV-1 immortalized T-cell line, which were maintained in RF10 media containing 10% HI FBS and 2 mM L-glutamine (GibcoBRL).

An infectivity assay was performed to measure the replication kinetics of WT and mutant virions. Viral samples were normalised by relative RT activity (Section 2.4.1) and made up to 200 μ l with DMEM. Virus was stored at -70°C and thawed immediately before use. Serial 10-fold dilutions of standardized samples were made in a 96 well plate in 200 μ l of RF10 medium (MT2 cells) or in 200 μ l of RF10 medium containing 10 U/ml IL-2 (PBMCs). 1×10^5 MT2 cells or PHA-stimulated PBMCs were added to each well. The plate was incubated at 37°C and supernatants (100 μ l) were collected on days 3, 7, 10, 14 post infection for storage at -70°C . Wells were supplemented with 100 μ l of fresh media. Viral infectivity was measured by quantifying RT activity at each timepoint.

2.5.3 Quantification of infectivity (TCID₅₀)

TCID₅₀ (50% Tissue Culture Infectivity Dose) was performed as originally described (38) and was calculated from infectivity results using the formula:

$$\text{TCID}_{50} = \log \text{dilution} - \frac{(\%+ve \text{ for this dilution} - 50)}{(\%+ve \text{ for this dilution} - \%+ve \text{ for next dilution)}$$

2.6 RNA

2.6.1 Isolation of genomic RNA

Purified virions were resuspended in 100 µl of TE pH 8.0. Virions were split: 10% for protein lysis (Section 2.3.2) for normalisation of samples by viral protein dot blot (Section 2.3.3); and 90% for RNA lysis. 500 µl of RNA lysis buffer (10 mM Tris pH 7.5, 1 mM EDTA, 1% SDS, 50 mM NaCl, 125 µg/ml yeast tRNA, and 109 µg/ml Proteinase K) was added to virions for RNA lysis and samples were incubated on ice for 30 min. 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was then added to remove virion proteins and samples were centrifuged at 20,000xg for 15 min, 4°C (Eppendorf 5417R). The aqueous phase (containing RNA) was removed. This process was repeated three times in total and extracted RNA was mixed with 50 µl of 3 M sodium acetate pH 5.2 and precipitated with 1 ml of 100% ethanol overnight at -20°C.

2.6.2 Trizol extraction of virion tRNA

Purified virions were resuspended in 100 μ l of TE pH 8.0. Virions were split: 10% for protein lysis (Section 2.4.2) for normalisation of samples by viral protein dot blot (Section 2.4.4); and 90% for tRNA isolation. tRNA samples were resuspended in 500 μ l of Trizol (Gibco-BRL), and incubated on ice for 30 min. 200 μ l of chloroform was added to remove virion proteins and samples were centrifuged at 20,000 g for 30 min, 4°C (Eppendorf 5417R). The aqueous layer containing tRNA was transferred to a fresh eppendorf, mixed with 50 μ l 3 M sodium acetate pH 5.2 and precipitated with 1 ml of 100% ethanol overnight at -20°C.

2.6.3 Analysis of virion genomic RNA packaging and RNA dimer stability

Genomic RNA was initially treated the same for both genomic RNA packaging analysis and RNA dimerization analysis. Genomic RNA was isolated and precipitated overnight as described above (Section 2.6.1), then centrifuged at 20,000 g for 30 min, 4°C (Eppendorf 5417R). RNA pellets were washed with 250 μ l of 70% ethanol and centrifuged at 20,000 g for 10 min, 4°C. Cleaned RNA pellets were air dried for 15 min then resuspended in RNA dimerization buffer (10 mM Tris pH 7.5, 1 mM EDTA pH 8.0, 1% SDS, and 25 mM NaCl).

To analyse RNA dimer stability, standardised samples were heated for 11 min at the following temperatures: 4°C, 25°C, 38°C, 42°C, 48°C, 52°C, and immediately placed on ice. RNA loading dye (2 μ l) (Appendix I) was added to

each sample and dimeric and monomeric RNAs were electrophoresed at 19 V for 30 hrs (1% agarose gel in 0.5xTBE buffer) in a Quantum Scientific gel tank.

To analyse RNA packaging, a standard curve of WT RNA was prepared consisting of: 150%, 100%, 50%, and 10% of WT. 5 μ l of each normalised mutant sample was prepared and WT and mutant samples were mixed with 2 μ l of 5x Northern Running Buffer, 3.5 μ l of formaldehyde and 10 μ l of 100% deionized formamide. RNA samples were denatured by incubation for 15 min at 68°C. Formaldehyde gel loading buffer (2 μ l) (Appendix I) was added and samples were electrophoresed at 25 V for 16 hrs (1% agarose gel in 1x Northern Running Buffer) in a Quantum Scientific gel tank.

Both RNA dimer and RNA packaging gels were soaked in 50 mM NaOH for 30 min to partially hydrolyse RNA dimers and monomers to aid RNA transfer. Gels were subsequently soaked in 0.5 M Tris pH 7.5 for 30 min for neutralisation. RNA samples were transferred overnight onto a positively charged nylon membrane (Amersham) soaked in 6x SSC, by a northern blot method as previously described (39). The membrane containing RNA samples was air dried for 2 hrs then exposed to UV light for 90 sec for crosslinking. The membrane was blocked with 10 ml of RNA hybridisation buffer (Appendix I) for 1 hr at 42°C. The membrane was probed with an [α -³²P]CTP (800 Ci/mmol, NEN) labelled riboprobe, at 42°C overnight. The probe (pGEM 5'HIV) is complementary to the 5' UTR region of the HIV-1 RNA sequence. Riboprobe labelling is described in Appendix II. After probing, the membrane was washed once for 30 min with 1xSSC/ 0.1% SDS and twice for 30 min with 0.2xSSC/

0.1% SDS. RNA bands were visualized by exposing the radioactively-labelled membranes to autoradiography hyperfilm (Amersham).

2.6.4 Analysis of virion tRNA^{Lys} packaging

tRNA samples were normalised via total viral proteins (Section 2.4.4). Precipitated tRNA samples obtained via Trizol extraction (Section 2.6.2) were centrifuged at 20,000xg for 30 min, 4 °C (Eppendorf 5417R). tRNA pellets were washed with 200 µl 70% ethanol, air-dried for 15 min at room temperature, and resuspended in 20 µl of RNAase free water. Standard curves of WT virion tRNA^{Lys1,2} packaging and tRNA^{Lys3} packaging were prepared consisting of: 150%, 100%, 50%, 10% of WT. Samples were mixed with 29 µl of RNA denaturation buffer (37% formaldehyde, 60% deionized formamide and 3% 20xSSC) and incubated at 68 °C for 15 min for RNA denaturation. 78 µl of 20xSSC buffer (Appendix I) was added to each sample. Samples were loaded onto a positively charged nylon membrane (Amersham) via a dot blot apparatus (Bio-Rad Laboratories). Dotted samples were washed twice with 10xSSC and the membrane was air dried overnight at room temperature.

The membrane was exposed to UV light for 2 mins for crosslinking and blocked with 10 ml RNA hybridisation buffer at 42 °C for 1 hr. To detect tRNA^{Lys1,2} and tRNA^{Lys3}, samples were incubated with probes synthesized from 18-mer oligonucleotides complementary to the 3' 18 nucleotides of tRNA^{Lys1,2} (5'-TGGCGCCCAACGTGGGGC-3') or tRNA^{Lys3} (5'-TGGCGCCCAACAGGGAC-3'). The DNA oligomers were 5' end labelled with T4 polynucleotide kinase

(New England Biolabs) and [γ - ^{32}P]ATP (3,000 Ci/mmol, Amersham). End labelling is described in Appendix II. Membranes were incubated with the [γ - ^{32}P]ATP labelled probes at 42°C overnight, washed once for 30 min with 1xSSC/ 0.1% SDS and twice for 30 min with 0.2xSSC/ 0.1% SDS. tRNA samples were visualized by exposing the radioactively-labelled membranes to autoradiography hyperfilm (Amersham). Quantification of virion tRNA^{Lys1,2} and tRNA^{Lys3} packaging in each sample was performed using the Bio Imaging Analyser (Fuji Film Co.)

3. Results

3.1 THE IMPORTANCE OF THE RATIO OF VIRION $\text{tRNA}^{\text{Lys1,2}}:\text{tRNA}^{\text{Lys3}}$ PACKAGING IN RNA DIMERIZATION AND VIRAL INFECTIVITY

3.1.1 Mutant constructs

This study utilised HxB2-BH10 (47) as WT control, and two HIV-1 mutants that package excess WT $\text{tRNA}^{\text{Lys3}}$ (*BH10Lys3*) or mutated $\text{tRNA}^{\text{Lys3}}$ (anticodon TTT changed to CTA) (*BH10Su+*) molecules into the virion (Fig. 3.1.1). These HIV-1 mutants were obtained from Dr. Yue Huang, NIH, USA, and have been described previously under different experimental conditions (20).

3.1.2 Confirmation of excess virion $\text{tRNA}^{\text{Lys3}}$ packaging in mutant constructs

Dot blot analysis of virion $\text{tRNA}^{\text{Lys1,2}}$ and $\text{tRNA}^{\text{Lys3}}$ packaging in HIV-1 mutants was performed to confirm excess $\text{tRNA}^{\text{Lys3}}$ packaging in *BH10Lys3* and *BH10Su+*. Virion particles were produced by transfecting 293T cells with WT and mutant proviral DNA along with an EGFP-reporter plasmid to measure transfection efficiency. Similar levels of EGFP protein were expressed in all cell lysates (data not shown), suggesting that proviral WT and mutant DNA transfected with equal efficiency. tRNA molecules within the virion core were isolated by lysing purified virion particles in Trizol, and extracted with chloroform. 10% of the purified viral particles were removed before tRNA

Figure 3.1.1 A schematic representation of WT and mutant proviral DNA with excess virion tRNA^{Lys3} packaging. The WT plasmid HxB2-BH10 has been described previously (47). Two HIV-1 mutants that package excess WT or mutant (anticodon TTT changed to CTA) tRNA^{Lys3} molecules into the virion were analysed. These constructs have been previously described under different experimental conditions (20) and were obtained from Dr. Yue Huang, NIH, USA. The number of tRNA^{Lys} molecules packaged into mutant virions was described in Huang *et al.* (20) and is shown in Fig. 3.1.1.

extraction, in order to standardise samples by measuring total viral proteins. Standardised amounts of tRNA samples were then spotted onto a nylon membrane and probed with a radioactive probe specific for tRNA^{Lys1,2} or tRNA^{Lys3}. The radioactively-labelled tRNA samples were visualized by phosphor imaging and quantitative densitometry was performed.

A standard curve of WT virion tRNA^{Lys1,2} or tRNA^{Lys3} packaging was constructed using four dilutions: 150%, 100%, 50%, and 10% (Fig. 3.1.2). *BH10Lys3* packaged 70% of WT levels of tRNA^{Lys1,2} and 198% of WT levels of tRNA^{Lys3}. *BH10Su+* packaged tRNA^{Lys1,2} at 80% of WT levels and tRNA^{Lys3} at 165% of WT levels. Analysis of tRNA^{Lys} packaging confirmed that *BH10Lys3* and *BH10Su+* both packaged excess tRNA^{Lys3} molecules and less tRNA^{Lys1,2} molecules into the virion compared to WT, as previously reported (20). The values described in Fig. 3.1.2 approximate previously reported tRNA^{Lys} packaging results of *BH10Lys3* and *BH10Su+* (20), within the range of variability of this assay.

3.1.3 Virion tRNA^{Lys} packaging mutants had similar viral protein profiles to WT

Virion particles were produced by transfecting 293T cells with WT and mutant proviral DNA along with EGFP-reporter plasmid DNA. Cellular and virion proteins were isolated and normalised for transfection efficiency and viral protein expression, respectively. Virion tRNA^{Lys} packaging mutants had similar levels of viral protein expression to WT, when analysed by protein dot blot (data

Figure 3.1.2 Confirmation of excess virion tRNA^{Lys3} packaging in HIV-1 mutants. Confirmation of excess virion tRNA^{Lys3} packaging was determined using a tRNA dot blot method. Virions produced by transfected 293T cells were lysed in trizol and tRNA molecules were extracted with chloroform. Similar amounts of tRNA samples were heat-denatured for 15 min at 68°C and spotted onto a nylon membrane. Samples were probed with a radioactive probe specific for tRNA^{Lys1,2} or tRNA^{Lys3} and visualized by phosphor imaging.

Comment:

not shown). Standardised samples were resolved by SDS-PAGE and probed with HIV-1 positive pooled patient sera and HRP-conjugated anti-human antibody.

Mock contains cellular proteins isolated from 293T cells transfected with EGFP DNA only and it demonstrates the specificity of the pooled anti-HIV antibody to viral proteins in lanes 2-4 (Fig. 3.1.3i). For WT, Pr160^{gag-pol}/ gp 160, p66 RT, Pr55 Gag, p39 MA-CA/ gp 41, and p24 CA viral proteins were visualized. *BH10Lys3* and *BH10Su+* had similar cellular protein profiles to WT (Fig. 3.1.3i). In the virion protein profile of WT, the p98 RT-IN, p66 RT, Pr55 Gag, p51 RT, p39 MA-CA/ gp 41, and p24 CA viral proteins were identified (Fig. 3.1.3ii). *BH10Lys3* and *BH10Su+* had similar virion protein profiles to WT.

3.1.4 Virion tRNA^{Lys} packaging mutants and WT displayed similar genomic RNA dimer stability

Genomic RNA was extracted from WT and mutant purified viral particles with phenol:chloroform. 10% of the purified viral particles were removed before genomic RNA extraction, in order to standardise samples by measuring total viral proteins. Standardized amounts of RNA samples were heated at the indicated temperatures. As temperature increases, dimeric RNA dissociates to monomeric form allowing a comparison of dimeric RNA stability relative to WT. Dimers and monomers were electrophoretically separated in a 1% native agarose gel. Separated RNA was transferred to a nylon membrane by northern

Figure 3.1.3 Virion tRNA^{Lys} packaging mutants and had similar viral protein profiles to WT. Virion particles were produced by transfecting 293T cells with mutant and WT proviral DNA along with EGFP-reporter plasmid DNA. i) Cellular lysates were prepared by collecting transfected 293T cells, lysing them, and removing cellular debris. ii) Virions were isolated, purified and lysed. Cellular viral proteins and virion proteins were electrophoresed in a 10% agarose gel and electroblotted onto a nitrocellulose membrane. Membranes were probed with HIV-1 positive pooled patient sera and HRP-conjugated anti-human antibody.

blotting and probed with a radioactive riboprobe that is complementary to the 5' end of the HIV-1 genomic RNA sequence.

WT genomic RNA dimers were stable at temperatures up to 42°C but completely dissociated into monomers when heated at 48°C and 52°C (Fig. 3.1.4). *BH10Lys3* and *BH10Su+* had similar RNA dimer stability to WT. Although monomers were not clearly visible in any sample at 48 °C and 52°C, the fact that the overall profile of the mutants was similar to that of WT suggests there is no difference in dimer stability.

3.1.5 tRNA^{Lys} packaging mutants displayed similar infectivity to WT in MT2 cells and PBMCs

The *BH10Lys3* and *BH10Su+* mutants were utilised in a previous study to infect MT4 cells over 7 days, and no differences in infection efficiency compared to WT were reported (20). As previous work in our laboratory has found that HIV-1 DIS stem-loop mutants were able to replicate in primary cells (PBMCs) but not in T cell lines (31), we analysed the infectivity of mutant constructs in PBMCs and MT2 cells (a different clone of the same T cell line as MT4). Viral supernatants were standardised by measuring relative RT activity. WT and mutant virus was used to infect MT2 cells or PBMCs in a 96 well plate (Day 0), and at day 3, 7, 10, and 14, supernatants were collected and fresh media was added to the ongoing infection. The RT activity of all collected samples was measured via RT assay and plotted to generate an infectivity profile for each virus.

Figure 3.1.4 tRNA^{Lys} packaging mutants displayed similar genomic RNA dimer stability to WT. The impact of altered tRNA^{Lys} packaging on genomic RNA dimerization was determined using melting curve and electrophoretic analysis. Genomic RNA was heat denatured for 10 min at the indicated temperatures. Dimers and monomers were electrophoresed in a 1% native agarose gel and probed with an HIV-1 riboprobe.

Figure 3.1.5 Virion tRNA^{lys} packaging mutants had similar infectivity to WT in MT2 cells and PBMCs. i) MT2 cells were infected with either WT or mutant virus. ii) Freshly isolated PBMCs were PHA-stimulated for 3 days and then infected with either WT or mutant virus. This figure is representative of infectivity assays conducted in 3 PBMC donors. For both PBMCs and MT2 cells, supernatants were collected 3, 7, 10, and 14 days after infection, and the RT activity in each sample was measured. The results represent the mean and standard deviation of duplicate samples. Symbols: *WT* - ■ ; *BH10Lys3* - ○ ; *BH10Su+* - □.

Virus production in MT2 cells infected with WT increased from day 3, peaked at day 10, and decreased between day 10 and day 14 (Fig. 3.1.5i). The decrease in virus production occurring between day 10 and 14 reflects the lysis of infected MT2 cells, which occurs during this time. *BH10Lys3* and *BH10Su+* had similar infectivity profiles to WT.

Virus production in PBMCs infected with WT increased from day 3, peaked at day 10, and decreased between day 10 and 14. *BH10Lys3* and *BH10Su+* again had similar infectivity profiles to WT. Infectivity was determined in 3 different PBMC donors and Fig. 3.1.5ii is representative of the replication kinetics observed in these assays.

3.2 THE IMPORTANCE OF CODON-USAGE IN VIRION tRNA^{LYS} PACKAGING AND VIRAL REPLICATION

3.2.1 Mutant constructs

NL 4.3 (1) was used as WT control. Five HIV-1 mutants were created with altered genomic RNA base sequences that retain WT amino acid sequences of expressed viral proteins (Fig. 3.2.1). This was achieved by systematically replacing WT A-rich codons in the NL 4.3 plasmid (performed by Dr. Johnson Mak) with segments of a GC-rich Gag/Gag-Pol expression vector (4.3 kb) obtained from Drs. Yue Huang and Gary Nabel, NIH, USA. For example, in *NL-h-Gag-PR-RT*, the majority of the Gag, PR and RT regions were replaced with humanised GC-rich RNA segments (Fig. 3.2.1). The WT Gag/Gag-Pol overlapping region was not altered in mutant constructs to allow normal frameshifting to occur.

3.2.2 GC-rich RNA mutants had similar levels of viral protein expression to WT

As HIV-1 codon-optimised (GC-rich) expression vectors have demonstrated 10-100 fold increased viral protein production in mammalian models (20). The viral protein expression of replicating GC-rich RNA mutants was assessed. Virion particles were produced by transfecting 293T cells with WT and mutant proviral DNA along with an EGFP-reporter plasmid. Similar levels of EGFP protein were expressed in all cell lysates (data not shown), suggesting that proviral WT

Figure 3.2.1 A schematic representation of GC-rich codon-optimised HIV-1 proviral DNA used in the study. Various segments of a GC-rich Gag/Gag-Pol expression vector (4.3 kb) obtained from Drs. Yue Huang and Gary Nabel, NIH, USA, were systematically cloned into the NL 4.3 proviral DNA by Dr. Johnson Mak. The A-rich and GC-rich sequences in HIV-1 mutants are indicated. The amino acid sequence of expressed viral proteins were the same as WT.

and mutant DNA transfected with equal efficiency. A 2 fold serial dilution of isolated virion proteins was performed and samples were spotted onto a nitrocellulose membrane and probed with HIV-1 positive pooled patient sera to measure total virion proteins.

All five GC-rich RNA mutants had similar signal intensity to WT at non-saturating dilutions eg 1/16 dilution (Fig. 3.2.2). GC-rich RNA mutants and WT had similar viral protein dilution profiles and thus similar viral protein expression.

3.2.3 HIV-1 mutants containing GC-rich Gag-PR-RT regions had protein processing defects

Western blot analysis was performed on viral proteins in 293T cellular lysates and isolated virion proteins using HIV-1 positive pooled patient sera.

Fig. 3.2.3i shows the cellular protein profiles of the WT and GC-rich RNA mutant virus. For WT the Pr160^{gag-pol}/ gp 160, p98 RT-IN, p66 RT, Pr55 Gag, p39 MA-CA/ gp 41, and p24 CA viral proteins were resolved. *NL-h-Gag* and *NL-h-Gag-PR-RT* had similar protein profiles to WT. *NL-h-CA-NC*, *NL-h-CA-NC-PR-RT*, and *NL-h-PR-RT* demonstrated slightly increased p66 RT compared to WT but other viral protein levels were similar to WT.

Fig. 3.2.3ii shows the virion protein profiles of WT and GC-rich RNA mutants. For WT, the Pr160^{gag-pol}/ gp160, p98 RT-IN, p66 RT, Pr55 Gag, p51 RT, and p24 CA virion proteins were visualized (Fig. 3.2.3ii). In *NL-h-Gag*, *NL-h-CA-NC*,

Figure 3.2.2 GC-rich RNA mutants had similar levels of virus expression to WT. Virion particles were produced by transfecting 293T cells with WT and mutant proviral DNA. Virions were isolated and lysed, and a serial 2-fold dilution of virion proteins was made. Samples were spotted onto a nitrocellulose membrane and probed with HIV-1 positive pooled patient sera and HRP-conjugated anti-human antibody.

Figure 3.2.3 HIV-1 mutants containing GC-rich Gag-PR-RT regions had protein processing defects. Virion particles were produced by transfecting 293T cells with mutant and WT proviral DNA along with EGFP-reporter plasmid DNA. i) Cellular lysates were prepared by collecting transfected 293T cells, lysing them, and removing cellular debris. ii) Virions were isolated, purified and lysed. Cellular viral proteins and virion proteins were electrophoresed in a 10% agarose gel and electroblotted onto a nitrocellulose membrane. Membranes were probed with HIV-1 positive pooled patient sera and HRP-conjugated anti-human antibody.

and *NL-h-CA-NC-PR-RT* there was a decrease in a 130 kDA protein, thought to be an intermediate cleavage product, compared to WT. *NL-h-Gag-PR-RT* had less Pr160^{gag-pol}/ gp 160 and less of the 130 kDA protein than WT. In addition, *NL-h-Gag-PR-RT* displayed significantly more p39 MA-CA/ gp 41 but significantly less p24 CA than WT. The remaining virion proteins in *NL-h-Gag-PR-RT* were similar to WT. *NL-h-PR-RT* had less 130 kDA protein and less p98 RT-IN than WT but other virion proteins were similar to WT.

3.2.4 GC-rich RNA mutants packaged similar levels of tRNA^{Lys1,2} and tRNA^{Lys3} into the virion compared to WT

Dot blot analysis of virion tRNA^{Lys1,2} and tRNA^{Lys3} packaging in GC-rich RNA mutants was performed to assess the importance of HIV-1 codon usage for virion tRNA^{Lys} packaging.

A standard curve of WT virion tRNA^{Lys1,2} or tRNA^{Lys3} packaging was constructed using four dilutions: 150%, 100%, 50%, and 10% (Fig. 3.2.4). *NL-h-Gag* packaged 98% of tRNA^{Lys1,2} and 129% of tRNA^{Lys3} compared to WT. *NL-h-Gag-PR-RT* packaged 103% of tRNA^{Lys1,2} and 154% of tRNA^{Lys3} compared to WT. *NL-h-CA-NC* packaged 250% of WT levels of both tRNA^{Lys1,2} and tRNA^{Lys3}. However, this result was only seen in one of two experiments. It is likely that this result was due to an error in standardising samples by total viral proteins. Since both virion tRNA^{Lys1,2} and tRNA^{Lys3} packaging are increased by the same amount (250% of WT), and according to the paradigm of conserved virion tRNA^{Lys} packaging, an increase in virion packaging of either tRNA^{Lys}

Figure 3.2.4 GC-rich RNA mutants packaged similar levels of tRNA^{Lys1,2} and tRNA^{Lys3} into the virion compared to WT. The impact of CG-rich genomic RNA sequences on virion tRNA^{Lys1,2} and tRNA^{Lys3} packaging was determined using a tRNA dot blot method. Virions produced by transfected 293T cells were lysed in trizol and tRNA molecules were extracted with chloroform. Similar amounts of tRNA samples were heat-denatured for 15 min at 68°C and spotted onto a nylon membrane. Samples were probed with a radioactive probe specific for tRNA^{Lys1,2} or tRNA^{Lys3}.

isospecies would result in a corresponding and complementary decrease in the virion packaging of the other tRNA^{Lys} isospecies (20). *NL-h-CA-NC-PR-RT* packaged 86% of WT levels of tRNA^{Lys1,2} and 103% of WT levels of tRNA^{Lys3}. *NL-h-PR-RT* packaged 133% of tRNA^{Lys1,2} and 158% of tRNA^{Lys3} compared to WT. The virion tRNA^{Lys} packaging values of *NL-h-Gag*, *NL-h-Gag-PR-RT*, *NL-h-CA-NC-PR-RT*, and *NL-h-PR-RT* range between 86-158% of WT tRNA^{Lys} packaging, which can be described as approximating 100% of WT due to assay variability. Therefore all GC-rich RNA mutants packaged virion tRNA^{Lys} at levels similar to WT.

3.2.5 Codon usage in the PR-RT regions of the *pol* gene is critical for viral infectivity

To determine the impact of codon usage in HIV-1 replication, WT and mutant virus was used to infect PBMCs, as described in Section 3.1.4. Infectivity was determined in 3 different PBMC donors and Fig. 3.2.5i is representative of the replication kinetics observed in these assays. To directly assess the relative infectivity of WT and mutant HIV-1, TCID₅₀ calculations were performed for day 10 of infection.

Virus production from PBMCs infected with WT virus increased from day 3, peaked at day 10, and decreased between day 10 and 14 (Fig. 3.2.5i). *NL-h-Gag-PR-RT* and *NL-h-CA-NC-PR-RT* were non-infectious. The third HIV-1 mutant with a GC-rich PR-RT region, *NL-h-PR-RT*, had significantly less virus production than WT but virus production did rise gradually from day 3 to 14.

Figure 3.2.5 Codon usage in the PR-RT regions of the *pol* gene is critical for viral infectivity. i) Freshly isolated PBMCs were PHA-stimulated for 3 days and then infected with either wild-type or mutant virus. Supernatants were collected 3, 7, 10, and 14 days after infection, and the RT activity in each sample was measured. The results represent the mean and standard deviation of duplicate samples. Infectivity was determined in 3 different PBMC donors and Fig. 3.25i is representative of the replication kinetics observed in these assays. Symbols: *WT* - ■ ; *NL-h-Gag* - ○ ; *NL-h-Gag-PR-RT* - □ ; *NL-h-CA-NC* - ▽ ; *NL-h-CA-NC-PR-RT* - ✱ ; *NL-h-PR-RT* - ◇ . ii) TCID₅₀ was calculated as described in Materials and Methods.

HIV-1 mutants with WT A-rich PR-RT regions: *NL-h-Gag* and *NL-h-CA-NC*, had less virus production than WT between day 3 and 10 but more virus production than mutants with GC-rich PR-RT regions. The virus production of *NL-h-Gag* and *NL-h-CA-NC* increased between day 3 and 14 and reached a similar peak to WT at day 14 as opposed to day 10.

TCID₅₀ data showed that WT had 1780×10^2 infectious particles/ml on day 10 of infection in PBMCs (Fig. 3.2.5ii.). In comparison, HIV-1 mutants with GC-rich PR-RT regions: *NL-h-Gag-PR-RT*, *NL-h-CA-NC-PR-RT*, and *NL-h-PR-RT* had 30-600 fold less infectious particles than WT. HIV-1 mutants with WT A-rich PR-RT regions: *NL-h-Gag* and *NL-h-CA-NC*, had 5 fold less infectious particles than WT. Therefore GC-rich mutants with WT (A-rich) PR-RT regions were less infective than WT but mutants with GC-rich PR-RT regions were non-infectious or had severe replication defects compared to WT.

3.2.6 GC-rich RNA mutants had similar RNA dimer stability to WT

The dimeric conformation of HIV-1 genomic RNA is critical for viral replication (10). Since dramatic alterations in viral infectivity were found for GC-rich RNA mutants, the stability of mutant RNA dimers was investigated.

WT genomic RNA dimers were stable at temperatures up to 42°C but completely dissociated into monomers when heated at 48°C and 52°C (Fig. 3.2.6). For *NL-h-Gag-PR-RT*, RNA dimers or monomers were not visualized at any temperature and this result was consistently seen in 5 experiments. Overall, *NL-h-Gag*, *NL-h-CA-NC*, *NL-h-CA-NC-PR-RT*, and *NL-h-PR-RT* had

Figure 3.2.6 GC-rich RNA mutants had similar RNA dimer stability to WT.

The impact of GC-rich RNA sequences on genomic RNA dimerization was determined using melting curve and electrophoretic analysis. Genomic RNA was heat denatured for 10 min at the indicated temperatures. Dimers and monomers were electrophoresed in a 1% native agarose gel and probed with an HIV-1 riboprobe.

similar RNA dimer stability profiles to WT. However, there was a potential slight increase in RNA dimer stability in these mutants compared to WT as mutant RNA dimers heated at 48°C did not fully dissociate, in contrast with WT RNA dimers, which had fully dissociated into monomers at this temperature.

3.2.7 GC-rich RNA mutants packaged varying levels of genomic RNA into the virion

The GC-rich RNA mutant: *NL-h-Gag-PR-RT*, consistently packaged insufficient genomic RNA into the virion for RNA dimerization analysis. Virion RNA packaging was assessed to determine the potential deficiency of virion RNA packaging in *NL-h-Gag-PR-RT*, and evaluate the importance of HIV-1 codon usage for virion RNA packaging. Genomic RNA was extracted from WT and mutant purified viral particles with phenol:chloroform. 10% of the purified viral particles were removed before RNA extraction, in order to standardise samples by measuring total viral proteins. Standardized amounts of each sample were heated at 68°C to completely dissociate all RNA dimers to monomeric form. Samples were electrophoresed in a 1% native agarose gel and transferred to a nylon membrane by northern blotting. Samples were then probed with a riboprobe complementary to the 5' end of HIV-1.

A standard curve of WT genomic RNA packaging was constructed from four dilutions: 150%, 100%, 50%, and 10% (Fig. 3.2.7). *NL-h-Gag-PR-RT* packaged genomic RNA at 10% of WT levels. *NL-h-PR-RT* packaged genomic RNA at 460% of WT levels. Genomic RNA packaging in *NL-h-Gag* occurred at 85% of WT levels. *NL-h-CA-NC* packaged 153% of WT levels of genomic RNA. *NL-h-*

Figure 3.2.7 GC-rich RNA mutants packaged varying levels of genomic RNA into the virion. The impact of GC-rich genomic RNA sequences on virion genomic RNA packaging was determined using northern analysis. Genomic RNA was heat denatured for 15 min at 68°C. Samples were electrophoresed in a 1% native agarose gel and probed with an HIV-1 riboprobe.

CA-NC-PR-RT packaged genomic RNA at 116% of WT levels. The genomic RNA packaging values of *NL-h-Gag*, *NL-h-CA-NC*, and *NL-h-CA-NC-PR-RT* ranged between 85-153% of WT RNA packaging, which can be described as approximating 100% of WT due to assay variability. Therefore, *NL-h-Gag-PR-RT* packaged 10 fold less genomic RNA than WT and *NL-h-PR-RT* packaged 5 fold more than WT, whilst the other GC-rich RNA mutants packaged genomic RNA at levels similar to WT.

4. Discussion

The objectives of this study were (1) To examine the importance of the ratio of HIV-1 virion $\text{tRNA}^{\text{Lys1,2}}:\text{tRNA}^{\text{Lys3}}$ packaging in genomic RNA dimerization and viral infectivity. (2) To investigate the role of HIV-1 codon usage in virion tRNA^{Lys} packaging, and (3) To assess the importance of the conserved A-rich genomic codon-bias for viral replication. Firstly, this study demonstrated that the ratio of virion tRNA^{Lys} packaging is not important for genomic RNA dimerization or HIV-1 infectivity in either PBMCs or MT2 cells. Secondly, codon usage in the HIV-1 genome does not play a significant role in virion selective tRNA^{Lys} packaging. Thirdly, this study has shown that the A-rich codon-bias of the HIV-1 genome is important for viral infectivity, virion genomic RNA packaging, and viral protein processing. The codon-bias in the PR and RT regions of the *pol* gene have been implicated as critical for viral infectivity.

tRNA^{Lys} molecules are packaged into the virion core in concentrations ten fold greater (60% of total virion tRNA) than the concentrations they are found at in the infected host cell (6% of total cellular tRNA)(32). Whilst only a single $\text{tRNA}^{\text{Lys3}}$ molecule is required to prime viral RT for reverse transcription, it is unclear what is the function of the remaining 19 $\text{tRNA}^{\text{Lys1,2}}$ and $\text{tRNA}^{\text{Lys3}}$ molecules (20, 32) packaged into the virion. Furthermore, the mechanism of virion selective tRNA^{Lys} packaging is unknown.

In contrast to the current model of RNA dimerization, there is evidence to suggest the DIS is not the only critical domain. DIS stem-loop mutants with a four bp [gcgc] addition or deletion to the loop formed stable RNA dimers when

replicated in a T cell line (8), while DIS stem-loop mutants with larger deletions (RNA nts 242-276) formed stable RNA dimers and replicated successfully in PBMCs (31). Following from a model established in RSV where tRNA^{Lys} was proposed to act as a linker in RNA dimer formation (18), we hypothesized that the ratio of virion selective tRNA^{Lys1,2}:tRNA^{Lys3} packaging may play a role in HIV-1 genomic RNA dimerization.

This study utilised HIV-1 mutants that package excess WT tRNA^{Lys3} (*BH10Lys3*) or mutated tRNA^{Lys3} (*BH10Su+*) into the virion. The results of RNA dimerization analysis suggest that the WT ratio of virion tRNA^{Lys1,2}:tRNA^{Lys3} packaging does not contribute to the initiation of RNA dimerization, since RNA dimers were formed in mutant constructs (Fig. 3.1.4). Similarly, the WT ratio of virion tRNA^{Lys1,2}:tRNA^{Lys3} packaging does not contribute to the maintenance of RNA dimer conformation, since RNA dimer stability was unaffected in mutant constructs. Virus containing an excess of WT or mutant tRNA^{Lys3} (*BH10Lys3* or *BH10Su+*) infected PBMCs (Fig. 3.1.5i) and MT2 cells (3.1.5ii) with the same efficiency as WT virus. Therefore the ratio of virion tRNA^{Lys1,2}:tRNA^{Lys3} packaging is not important for viral infectivity. These findings are in agreement with and expand upon previous studies in which the same HIV-1 mutants were shown to infect MT4 cells for 7 days with the same efficiency as WT (20).

Virion tRNA^{Lys1,2} packaging is deficient in both *BH10Lys3* and *BH10Su+* compared to WT, however no changes in RNA dimer formation or stability were seen. Therefore it is unlikely that tRNA^{Lys1,2} molecules inside the virion play a role in HIV-1 RNA dimer linkage as hypothesized. However, this does not

exclude a role for tRNA^{Lys3} in RNA dimer formation. The primer tRNA^{Lys3} molecule attached to the PBS may mediate RNA dimer linkage in a model analogous to RNA dimerization in yeast retrotransposons (11, 15). In both *BH10Lys3* and *BH10Su+* it is likely that a WT tRNA^{Lys3} molecule was able to prime RT as mutant virus was as infectious as WT in PBMCs and MT2 cells (Fig. 3.2.5i and 3.2.5ii), and this event has been shown to be required for viral infectivity (30). Furthermore, suppressor tRNA^{Lys3} molecules (produced along with WT tRNA^{Lys3} by *BH10Su+*) are unable to act as a primer for RT in an *in vivo* system (21).

The coding regions of the HIV-1 genome exhibit a strong A-rich bias particularly in the third codon position, in contrast to human codons, which are GC-rich (17, 27, 40). The codon-bias of the HIV-1 genome is conserved in all lentiviruses (6, 7, 9, 48) and has been used to determine the evolutionary relationships of this family to other retroviruses (6). However, the importance of the codon-bias in HIV-1 assembly and replication is unknown. We hypothesized that the codon-bias of the genome contributes in part to virion selective tRNA^{Lys} packaging by determining the usage of tRNA^{Lys} molecules during viral protein translation.

Altering the A-rich codon-bias of the HIV-1 RNA genome in various sections of Gag and/or Gag-Pol did not affect the normal virion packaging of either tRNA^{Lys1,2} or tRNA^{Lys3} in four out of five GC-rich RNA mutants (*NL-h-Gag*, *NL-h-Gag-PR-RT*, *NL-h-CA-NC-PR-RT*, and *NL-h-PR-RT*) (Fig. 3.2.4). The A-rich WT HIV-1 RNA genome preferentially utilises the AAA codon to code for the amino acid lysine (tRNA^{Lys3}). In contrast, GC-rich HIV-1 RNA mutants

preferentially code for lysine using the AAG codon (tRNA^{Lys1,2}). Therefore GC-rich RNA mutants code for viral proteins using a different subset of codons and tRNA^{Lys} isospecies than WT HIV-1. However, virion tRNA^{Lys1,2} or tRNA^{Lys3} packaging was not altered in GC-rich RNA mutants, which does not support our hypothesis that the usage of tRNA^{Lys} during viral protein translation is a determinant of what ratio of tRNA^{Lys} molecules are selectively packaged into the virion. The RT portion of Pr160^{gag-pol} has been implicated in virion tRNA^{Lys3} packaging (32, 33). However, HIV-1 mutants with a GC-rich RT segment (*NL-h-Gag-PR-RT*, *NL-h-CA-NC-PR-RT*, and *NL-h-PR-RT*) all packaged virion tRNA^{Lys3} at WT levels. This result emphasizes that the codon-bias of the HIV-1 genome is unlikely to play a major role in determining the tRNA^{Lys} molecules selectively packaged into the virion.

This study also gave insight into the relationship between genomic RNA packaging and selective tRNA^{Lys} packaging. *NL-h-Gag-PR-RT* packaged genomic RNA into the virion at 10% of WT levels and *NL-h-PR-RT* packaged RNA at 460% of WT levels (Fig. 3.2.7). However, both constructs packaged similar levels of virion tRNA^{Lys1,2} and tRNA^{Lys3} to WT (Fig. 3.2.4). Our mutants confirm that the mechanism of virion tRNA^{Lys} packaging is independent of genomic RNA packaging. This finding is in agreement with previous work which demonstrated that virion tRNA^{Lys} packaging was unchanged in virions devoid of genomic RNA (32), or in virions that contained genomic RNA with a PBS deletion (24).

This study provides the first evidence that the conserved A-rich genomic codon-bias in the PR and RT regions of the *pol* gene is critical for viral infectivity. HIV-1 mutants with GC-rich PR-RT regions (*NL-h-Gag-PR-RT*, *NL-h-PR-RT*, and *NL-h-CA-NC-PR-RT*) were either non-infectious or replicated at extremely low levels in PBMCs compared to WT (Fig. 3.2.5i), and had between 30-600 fold less infectious particles than WT (Fig. 3.2.5ii). Mutant constructs with WT A-rich PR-RT regions (*NL-h-Gag* and *NL-h-CA-NC*) were also less infectious than WT (5 fold less infectious particles, Fig. 3.2.5ii) but were more infectious than mutant constructs containing GC-rich PR-RT regions. This suggests that the A-rich codon-bias in the entire HIV-1 Gag/Gag-Pol RNA sequence is important for viral infectivity. The A-rich bias of the HIV-1 genome is most pronounced in the *pol* gene (27). Consequently changes made to the codon-bias in this region were expected to produce more dramatic changes to viral replication than changes made elsewhere in the RNA genome, which is indeed the case. Altering the codon-bias of HIV-1 genomic RNA may impair viral infectivity by altering the conformation of genomic RNA incorporated into the virion. The most obvious implication being that altered genomic RNA folding may impair the accessibility of the RT enzyme to the RNA genome for reverse transcription in the newly infected host cell. Indeed, RNA folding predictions of *NL-h-PR-RT* and *NL-h-Gag-PR-RT* show significant differences compared to WT (Appendix III). To test this hypothesis, a cDNA synthesis assay in the infected host cell could be performed to determine the level of HIV-1 proviral DNA transcribed from mutant GC-rich RNA genomes. If this hypothesis is correct, lower levels of proviral DNA would be expected in host cells infected with GC-rich RNA mutants compared to WT. Another way to examine this hypothesis would be to

perform a natural endogenous RT (NERT) assay. This tests reverse transcription within the virion. Again, a decreased ability to reverse transcribe the RNA genome would be expected in GC-rich RNA mutants if this hypothesis is correct. In order to specifically determine what regions of codon usage in the PR-RT regions of the *pol* gene are critical for viral infectivity, HIV-1 mutants with smaller GC-rich segments of PR alone and RT alone could be cloned and analysed in future studies.

Another possible explanation of why altered genomic codon usage affects viral infectivity may involve increased RNA dimer stability. The RNA dimers of GC-rich RNA mutants appear to be slightly more stable (5°C) than WT dimers (Fig. 3.2.6). It has been suggested that regions outside the DIS, such as the Gag and Gag-Pol coding sequences are involved in RNA dimerization (35). This may occur through hydrogen bonding between nucleic acid base pairs on monomer strands, possibly at multiple sites on the RNA (35). Therefore the slight increase in stability of mutant RNA dimers may be due to increased hydrogen bonding strength between monomer strands of the GC-rich mutants. For RT to reverse transcribe genomic RNA into proviral DNA the RNA dimer must dissociate to allow RT access to either monomer. Therefore if the monomer strands are bonded more tightly in GC-rich RNA mutants, dimer dissociation may occur less readily, impairing reverse transcription. However, there does not seem to be a correlation between the extent of the replication defect of GC-rich RNA mutants and the increase in stability of mutant RNA dimers of the corresponding mutants. *NL-h-Gag*, *NL-h-CA-NC*, *NL-h-CA-NC-PR-RT*, and *NL-h-PR-RT* all appear to have a similar and small (5°C) increase

in dimer stability, yet there were distinct differences between the infectivity profiles of these mutants. However, it may simply be the case that the temperature gradations used in RNA dimerization analysis were too large to pick up small differences in RNA dimer stability between different GC-rich RNA mutants. Therefore, in order to further investigate the possibility that RNA dimers from GC-rich RNA mutants have increased stability compared to WT, RNA dimerization analysis could be performed with smaller temperature gradations (eg 3°C instead of 5°C).

The A-bias of the HIV-1 genome is a feature of all members of the lentiviral family (HIV, SIV, BIV, FIV, CAEV, EIAV, visna), the human spuma retrovirus, and bovine papillomavirus (48). Therefore it is an excellent drug target for multiple viruses. Also, the fact that all GC-rich RNA mutants in this study demonstrated decreased viral infectivity compared to WT augurs well for the clinical application of GC-rich human codon-optimised HIV-1 vaccine constructs. Codon-optimised Rev-independent Gag/Gag-Pol expression vectors have demonstrated 10-100 fold increased viral protein production in mammalian hosts and subsequent strong host immune responses (4, 13, 19, 25, 49). However, retroviruses including HIV-1 commonly recombine with cellular and other viral coding sequences. Therefore if a GC-rich vaccine vector was introduced therapeutically into an HIV-1 infected human host, recombination may occur between vector and WT HIV-1. This study has clearly demonstrated that GC-rich RNA sequences introduced into WT HIV-1 do not result in increased viral protein expression (Fig. 3.2.2). This is likely to be due to continued Rev-dependency in the replicating GC-rich HIV-1 mutants in

contrast to the Rev-independent non-replicating GC-rich vaccine vectors. Furthermore, this study has shown that GC-rich RNA sequences introduced into WT HIV-1 substantially decrease viral infectivity and hence are not advantageous changes for the virus. It would be of interest to perform infectivity studies over a longer timecourse to determine if *NL-h-PR-RT* has delayed replication and to see whether GC-rich RNA mutants eventually revert back to A-rich codons and WT infectivity levels.

The results of this study demonstrate that HIV-1 codon usage is not important for virion selective tRNA^{Lys} packaging but can influence other aspects of viral assembly including viral protein processing and genomic RNA packaging. GC-rich RNA mutants packaged varying amounts of genomic RNA into the virion compared to WT, however, different lengths of GC-rich optimised sequences in WT HIV-1 have different effects on virion RNA packaging. For example, *NL-h-Gag-PR-RT* packaged genomic RNA at 10% of WT levels, while *NL-h-PR-RT* packaged genomic RNA at 460% of WT levels (Fig. 3.2.7). The remaining GC-rich RNA constructs packaged genomic RNA at levels similar to WT. It has been previously hypothesized that the A-rich HIV genome may have evolved to accommodate selective packaging of the HIV genomic plus strand into retroviral particles (7). However, our data have not been able to identify a specific region of the RNA genome that can account for the observed changes in RNA packaging. Again, the impact of RNA folding may explain these results as the overall folding of virion RNA could influence RNA packaging, the ultra structure of virion core formation and ultimately viral infectivity. It would be useful to look

at the conformation of the virion core by electron microscopy in GC-rich RNA mutants in future studies.

The virion protein profile of *NL-h-Gag-PR-RT* showed increased p39 MA-CA but decreased p24 CA (Fig. 3.2.3ii) and indicates that normal cleavage of p39 MA-CA by the viral protease is not occurring. This deficiency in p24 CA has been confirmed by p24 assay (not performed by the author). Western blot analysis utilising monoclonal antibodies to p24 would be useful in exploring the viral protein processing deficiency of *NL-h-Gag-PR-RT*. The significant decrease in p24 CA processing in *NL-h-Gag-PR-RT* may also account for the inability of this mutant to replicate in PBMCs as a direct result of altered virion structure (Fig. 3.2.5i). All five GC-rich RNA mutants (*NL-h-Gag*, *NL-h-Gag-PR-RT*, *NL-h-CA-NC*, *NL-h-CA-NC-PR-RT*, and *NL-h-PR-RT*) had less of the 130 kDA protein (assumed to be an intermediate cleavage product) in viral protein profile compared to WT (Fig. 3.2.3ii). This change in protein profile may be further evidence of protein processing defects caused by changing HIV-1 codon usage. Western blot analysis of virion protein profiles utilising monoclonal antibodies would need to be performed to identify the unknown 130 kDA protein. An explanation of why altering genomic codon-bias affects protein processing may lie in the anticipated change to RNA folding. It has been shown that the presence of RNA is required for efficient cleavage of HIV-1 p15NC protein in the virion (42, 43). Since genomic RNA and the viral protease may interact for efficient cleavage of viral proteins, changing the RNA codons and thereby altering the folding of genomic RNA may subsequently affect the proper RNA-viral protease interaction that is required for viral protein processing.

Overall, this study has made several novel contributions to our understanding of the role of selective virion tRNA^{Lys} packaging in HIV-1 replication and the role of the conserved A-rich codon-bias in viral infectivity and aspects of viral assembly. Codon usage in the HIV-1 genome has been shown to have a role in virion genomic RNA packaging and viral protein processing but not to be important for virion selective tRNA^{Lys} packaging. This study has also expanded on work performed by Huang *et al.* (20) and has demonstrated that the ratio of virion tRNA^{Lys} packaging is not important for genomic RNA dimerization or HIV-1 infectivity in either PBMCs or MT2 cells. Notably, this study provides the first evidence that the conserved A-rich codon-bias of the HIV-1 genome in the PR and RT regions of the *pol* gene is critical for viral infectivity. These findings have important implications for the clinical application of human codon-optimised (GC-rich) HIV-1 vaccine vectors, since any recombination events occurring between WT HIV-1 and vector in the host will not be advantageous for viral infection.

5. References

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone *J Virol.* **59**:284-91.
2. **Aiyar, A., D. Cobrinik, Z. Ge, H. J. Kung, and J. Leis** 1992. Interaction between retroviral U5 RNA and the T psi C loop of the tRNA(Trp) primer is required for efficient initiation of reverse transcription *J Virol.* **66**:2464-72.
3. **Aiyar, A., Z. Ge, and J. Leis** 1994. A specific orientation of RNA secondary structures is required for initiation of reverse transcription *J Virol.* **68**:611-8.
4. **Andre, S., B. Seed, J. Eberle, W. Schraut, A. Bultmann, and J. Haas** 1998. Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage *J Virol.* **72**:1497-503.
5. **Arts, E. J., M. Ghosh, P. S. Jacques, B. Ehresmann, and S. F. Le Grice** 1996. Restoration of tRNA³Lys-primed(-)-strand DNA synthesis to an HIV-1 reverse transcriptase mutant with extended tRNAs. Implications for retroviral replication *J Biol Chem.* **271**:9054-61.
6. **Berkhout, B., A. Grigoriev, M. Bakker, and V. V. Lukashov** 2002. Codon and amino acid usage in retroviral genomes is consistent with virus-specific nucleotide pressure *AIDS Res Hum Retroviruses.* **18**:133-41.
7. **Berkhout, B., and F. J. van Hemert** 1994. The unusual nucleotide content of the HIV RNA genome results in a biased amino acid composition of HIV proteins *Nucleic Acids Res.* **22**:1705-11.
8. **Berkhout, B., and J. L. van Wamel** 1996. Role of the DIS hairpin in replication of human immunodeficiency virus type 1 *J Virol.* **70**:6723-32.
9. **Bronson, E. C., and J. N. Anderson** 1994. Nucleotide composition as a driving force in the evolution of retroviruses *J Mol Evol.* **38**:506-32.

10. **Coffin, J. M., S.H. Hughes, and H.E. Varmus** 1997. Retroviruses. Cold Spring Harbor Laboratory Press.
11. **Cristofari, G., D. Ficheux, and J.-L. Darlix** 2000. The Gag-like protein of the Yeast Ty1 retrotransposon contains a nucleic acid chaperone domain analogous to retroviral Nucleocapsid proteins J. Biol. Chem. **275**:19210-19217.
12. **Crowe, S., J. Mills, and M. S. McGrath** 1987. Quantitative immunocytofluorographic analysis of CD4 surface antigen expression and HIV infection of human peripheral blood monocyte/macrophages AIDS Res Hum Retroviruses. **3**:135-45.
13. **Deml, L., A. Bojak, S. Steck, M. Graf, J. Wild, R. Schirmbeck, H. Wolf, and R. Wagner** 2001. Multiple effects of codon usage optimization on expression and immunogenicity of DNA candidate vaccines encoding the Human Immunodeficiency Virus Type 1 Gag protein J. Virol. **75**:10991-11001.
14. **Fu, W., R. J. Gorelick, and A. Rein** 1994. Characterization of human immunodeficiency virus type 1 dimeric RNA from wild-type and protease-defective virions J Virol. **68**:5013-8.
15. **Gabus, C., D. Ficheux, M. Rau, G. Keith, S. Sandmeyer, and J. L. Darlix** 1998. The yeast Ty3 retrotransposon contains a 5'-3' bipartite primer-binding site and encodes nucleocapsid protein NCp9 functionally homologous to HIV-1 NCp7 Embo J. **17**:4873-80.
16. **Goff, S., P. Traktman, and D. Baltimore** 1981. Isolation and properties of Moloney murine leukemia virus mutants: use of a rapid assay for release of virion reverse transcriptase J Virol. **38**:239-48.
17. **Grantham, R. a. P. P.** 1986. *Nature*. **319**:727-728.
18. **Haseltine, W. A., A. M. Maxam, and W. Gilbert** 1977. Rous sarcoma virus genome is terminally redundant: the 5' sequence Proc Natl Acad Sci U S A. **74**:989-93.
19. **Huang, Y., W.-p. Kong, and G. J. Nabel** 2001. Human Immunodeficiency Virus Type 1-specific immunity after genetic immunization is enhanced by modification of Gag and Pol expression J. Virol. **75**:4947-51

20. **Huang, Y., J. Mak, Q. Cao, Z. Li, M. A. Wainberg, and L. Kleiman** 1994. Incorporation of excess wild-type and mutant tRNA(3Lys) into human immunodeficiency virus type 1 J Virol. **68**:7676-83.
21. **Huang, Y., A. Shalom, Z. Li, J. Wang, J. Mak, M. A. Wainberg, and L. Kleiman** 1996. Effects of modifying the tRNA(3Lys) anticodon on the initiation of human immunodeficiency virus type 1 reverse transcription J Virol. **70**:4700-6.
22. **Isel, C., J. M. Lanchy, S. F. Le Grice, C. Ehresmann, B. Ehresmann, and R. Marquet** 1996. Specific initiation and switch to elongation of human immunodeficiency virus type 1 reverse transcription require the post-transcriptional modifications of primer tRNA3Lys Embo J. **15**:917-24.
23. **Jacks, T., M. D. Power, F. R. Masiarz, P. A. Luciw, P. J. Barr, and H. E. Varmus** 1988. Characterization of ribosomal frameshifting in HIV-1 gag-pol expression Nature. **331**:280-3.
24. **Jiang, M., J. Mak, A. Ladha, E. Cohen, M. Klein, B. Rovinski, and L. Kleiman** 1993. Identification of tRNAs incorporated into wild-type and mutant human immunodeficiency virus type 1 J Virol. **67**:3246-53.
25. **Kotsopoulou, E., V. N. Kim, A. J. Kingsman, S. M. Kingsman, and K. A. Mitrophanous** 2000. A Rev-Independent Human Immunodeficiency Virus Type 1 (HIV-1)-based vector that exploits a codon-optimized HIV-1 gag-pol gene J. Virol. **74**:4839-52.
26. **Krausslich, H. G.** 1996. Morphogenesis and Maturation of Retroviruses. Springer-Verlag, Berlin.
27. **Kypr, J. a. J. M.** 1987. Nature. **327**:20.
28. **Lanchy, J. M., C. Ehresmann, S. F. Le Grice, B. Ehresmann, and R. Marquet** 1996. Binding and kinetic properties of HIV-1 reverse transcriptase markedly differ during initiation and elongation of reverse transcription Embo J. **15**:7178-87.
29. **Laughrea, M., L. Jette, J. Mak, L. Kleiman, C. Liang, and M. A. Wainberg** 1997. Mutations in the kissing-loop hairpin of human immunodeficiency virus type 1 reduce viral infectivity as well as genomic RNA packaging and dimerization J Virol. **71**:3397-406.

30. **Li, X., J. Mak, E. J. Arts, Z. Gu, L. Kleiman, M. A. Wainberg, and M. A. Parniak** 1994. Effects of alterations of primer-binding site sequences on human immunodeficiency virus type 1 replication J Virol. **68**:6198-206.
31. **Mak, J., Hill, M.K., Shehu-Xhilaga, M., Campbell, S.M., Poupourios, P., and Crowe S.M.** 2002. The Dimer Initiation Sequence Stem-Loop of Human Immunodeficiency Virus Type 1 is Dispensable for Viral Replication in Peripheral Blood Mononuclear Cells Submitted to J Virol.
32. **Mak, J., M. Jiang, M. A. Wainberg, M. L. Hammarskjold, D. Rekosh, and L. Kleiman** 1994. Role of Pr160gag-pol in mediating the selective incorporation of tRNA(Lys) into human immunodeficiency virus type 1 particles J Virol. **68**:2065-72.
33. **Mak, J., A. Khorchid, Q. Cao, Y. Huang, I. Lowy, M. A. Parniak, V. R. Prasad, M. A. Wainberg, and L. Kleiman** 1997. Effects of mutations in Pr160gag-pol upon tRNA(Lys3) and Pr160gag-plo incorporation into HIV-1 J Mol Biol. **265**:419-31.
34. **Mishima, Y., and J. A. Steitz** 1995. Site-specific crosslinking of 4-thiouridine-modified human tRNA(3Lys) to reverse transcriptase from human immunodeficiency virus type I Embo J. **14**:2679-87.
35. **Ortiz-Conde, B. A., and S. H. Hughes** 1999. Studies of the genomic RNA of Leukosis viruses: Implications for RNA dimerization J. Virol. **73**:7165-7174.
36. **Raba, M., K. Limburg, M. Burghagen, J. R. Katze, M. Simsek, J. E. Heckman, U. L. Rajbhandary, and H. J. Gross** 1979. Nucleotide sequence of three isoaccepting lysine tRNAs from rabbit liver and SV40-transformed mouse fibroblasts Eur J Biochem. **97**:305-18.
37. **Ratner, V. A., L. V. Omel'ianchuk, A. A. Zharkikh, and N. A. Kolchanov** 1985. Theoretical analysis of the structural characteristics and evolution of transfer RNAs Zh Obshch Biol. **46**:732-42.
38. **Reed, L., and H. Muench** 1938. A simple method for estimating 50 percent endpoint Am J Hyg. **27**:493-97.
39. **Sambrook, J., Fritsch, E.F. & Maniatis, T.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.
40. **Sharp, P. M.** 1986. Nature. **324**:114.

41. **Shehu-Xhilaga, M., S. M. Crowe, and J. Mak** 2001. Maintenance of the Gag/Gag-Pol ratio is important for human immunodeficiency virus type 1 RNA dimerization and viral infectivity J Virol. **75**:1834-41.
42. **Sheng, N., and S. Erickson-Viitanen** 1994. Cleavage of p15 protein in vitro by human immunodeficiency virus type 1 protease is RNA dependent J Virol. **68**:6207-14.
43. **Sheng, N., S. C. Pettit, R. J. Tritch, D. H. Ozturk, M. M. Rayner, R. Swanstrom, and S. Erickson-Viitanen** 1997. Determinants of the human immunodeficiency virus type 1 p15NC-RNA interaction that affect enhanced cleavage by the viral protease J Virol. **71**:5723-32.
44. **Skripkin, E., J. C. Paillart, R. Marquet, B. Ehresmann, and C. Ehresmann** 1994. Identification of the primary site of the human immunodeficiency virus type 1 RNA dimerization in vitro Proc Natl Acad Sci U S A. **91**:4945-9.
45. **Stoltzfus CM FAU - Snyder, P. N., and S. P. L.-e. P.-J. Article.** 1975. Structure of B77 sarcoma virus RNA: stabilization of RNA after packaging. J Virol. **5**:1161-70.
46. **Telesnitsky, A. G., S.P.** 1997. Reverse Transcriptase and the generation of retroviral DNA, p. 121-161, Retroviruses. Cold Spring Harbor Laboratory Press.
47. **Terwilliger, E., J. G. Sodroski, C. A. Rosen, and W. A. Haseltine** 1986. Effects of mutations within the 3' orf open reading frame region of human T-cell lymphotropic virus type III (HTLV-III/LAV) on replication and cytopathogenicity J Virol. **60**:754-60.
48. **van Hemert, F. J., and B. Berkhout** 1995. The tendency of lentiviral open reading frames to become A-rich: constraints imposed by viral genome organization and cellular tRNA availability J Mol Evol. **41**:132-40.
49. **zur Megede, J., M.-C. Chen, B. Doe, M. Schaefer, C. E. Greer, M. Selby, G. R. Otten, and S. W. Barnett** 2000. Increased expression and immunogenicity of sequence-modified Human Immunodeficiency Virus Type 1 gag gene J. Virol. **74**:2628-35.
50. **Mak, J., Kleiman, L.** 1997. Primer tRNAs for Reverse Transcription J. Virol. **71**: 8087-95

Appendix I

Composition of Media and Buffers

Media and Buffers	Composition
Luria-Bertani Medium (LB)	1% Bacto Tryptone 0.5% Yeast extract 0.5% NaCl 1 mM NaOH
Formaldehyde gel loading buffer	50% glycerol 1 mM EDTA 0.25% bromophenol blue 0.25% xylene cyanol
RNA loading dye	10% 5x Northern running buffer 17.5% formaldehyde 50% formamide
5x Northern running buffer	0.1 M MOPS pH 7.0 40 mM Sodium acetate 5 mM EDTA pH 8.0
RNA hybridisation buffer	5% 5xSSPE 50% deionized formamide 4g dextran sulfate 400 µl salmon sperm DNA (10 mg/ml) 13% SDS 8% ddH ₂ O
20xSSC Stock pH 7.0	175.3 g NaCl 88.2 g Sodium citrate ddH ₂ O to 1 L
Tris Buffered Saline (TBS) pH 7.4	12.2 g Tris-HCl 29.2 g NaCl ddH ₂ O to 1 L
TE pH 8.0	10 mM Tris-HCl 1 mM EDTA

Media and Buffers	Composition
Tris-Borate-EDTA (TBE)	45 mM Tris-borate 1mM EDTA
5x Tris-glycine Stock	15 g Tris-HCl 72 g glycine ddH ₂ O to 1 L
Tris-glycine running buffer	20% 5x tris-glycine 0.1% SDS 79.9% ddH ₂ O
Transfer Buffer	20% 5x Tris-glycine 20% methanol 60% ddH ₂ O
2x HEPES buffered saline (HBS) pH 7.1	280 mM NaCl 50 mM HEPES 1.5 mM Na ₂ HPO ₄

Appendix II

Additional Methods

Micro Reverse Transcriptase (RT) assay of virus supernatants (Section 2.5.1)

Briefly, 10 μ l of culture supernatant was added to 10 μ l of 0.3% Nonidet P-40 (NP-40, Sigma) and 40 μ l of RT reaction mix (50 mM Tris pH 7.8, 7.5 mM KCl, 5 mM MgCl₂, 2 mM dithiothreitol [Sigma], 5 μ g/ml of template-primer Poly(rA).p(dT)12-18 [Pharmacia], and 3 μ Ci ³³P-dTTP [Amersham]) in a 96 well plate. Samples were incubated for 2 hrs at 37°C. 7.5 μ l of the reaction products were then spotted onto DE81 chromatography paper (Whatman) and air-dried overnight. Dry filters were washed 5 times with 2xSSC buffer (Appendix I) to remove unincorporated ³³P-dTTPs, and air-dried. Meltilex scintillant (Wallac) was melted onto filters and quantification of β radiation emitted from samples was performed using a liquid scintillation counter (LKB micro beta counter, Wallac).

Isolation of PBMCs (Section 2.5.2)

Whole blood was diluted 1:2 with PBS (GibcoBRL). Diluted blood (25 ml) was layered over 15 ml of Ficoll-Paque (Amersham) in 50 ml conical tubes and centrifuged at 700xg for 20 min, room temperature, no brake (Beckman Allegra 6R). PBMCs were collected from the interface of the Ficoll and plasma using a plastic transfer pipette (Fullerton), washed twice in PBS and pelleted by

centrifugation at 400xg for 10 min, 10°C, followed by two further washed in PBS and centrifugation at 140xg for 10 min, 4°C to remove contaminating platelets.

Labelling of the genomic RNA Riboprobe (Section 2.6.3)

The Riboprobe reaction mix contained 1 µl of template (pGem7z-5'HIV-1), 0.5 µl 20 mM ATP, 0.5 µl 20 mM GTP, 0.5 µl 20 mM UTP, 20 µl 5x T7 buffer (Promega), 1 µl T7 RNA polymerase (Promega), 1.5 µl of [α -³²P]CTP (800 Ci/mmol, NEN), and 300 µl of TE pH 8.0. The reaction mix was incubated for 1.5 hrs at 37°C. Unincorporated nucleotides were removed using a sephadex G-50 column as previously described (39).

End labelling the tRNA^{Lys1,2} and tRNA^{Lys3} specific oligomers (Section 2.6.4)

The end label reaction mixes contained 2 µl of oligomer (tRNA^{Lys1,2} or tRNA^{Lys3}, 68 ng), 1.5 µl 10x T4 kinase buffer (New England Biolabs), 1.5 µl T4 kinase (New England Biolabs), 10 µl of [γ -³²P]ATP (3000 Ci/mmol, Amersham), and 300 µl of TE pH 8.0. The reaction mix was incubated for 1 hr at 37°C. Unincorporated nucleotides were removed using a sephadex G-50 column as previously described (39).

Appendix III

The RNA folding pattern of *NL 4.3*, *NL-h-PR-RT* and *NL-h-Gag-PR-RT* was estimated using a program by M.Zuker and D. Stewart of Washington University School of Medicine, with the assistance of D. Purcell at The University of Melbourne.