PROTEOLYTIC CLEAVAGE EVENTS IN THE MATURATION OF HIV-1 REVERSE TRANSCRIPTASE

by

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Each of the HIV-1 *pol*-encoded enzymes, protease (PR), reverse transcriptase (RT) and integrase (IN) are released during virion maturation and are active only as dimers. Of the three, only RT comprises subunits of different mass. RT in mature infectious virions is a heterodimer of 66 kDa and 51 kDa subunits, even though its gene encodes a 66 kDa protein. The RT p51 subunit is formed by HIV-1 PR-catalyzed cleavage of RT p66, resulting in the removal of a ribonuclease H (RNH) domain. Given the existence of completely active recombinant p66/66 RT homodimers and alternative RT oligomers in other retroviruses, the apparent need for p66/51 RT heterodimers in the HIV-1 virion is unclear. To determine why the generation of active viral RT requires three processing events, we introduced mutations in the p51↓RNH and RT↓IN protease cleavage sites of an infectious HIV-1 molecular clone. Mutation of the RT↓IN cleavage site had no effect on the activity or proteolytic stability of the p98/51 RT product, although infectivity was severely attenuated. This result was similar to findings previously reported for the PR↓RT cleavage site. Surprisingly, mutation of the internal p51↓RNH cleavage site did not increase RT p66 content, but resulted in attenuated virus containing greatly decreased levels of RT that was

primarily RT p51. We further identified a compensatory second-site mutation T477A, found to restore RT activity and processing to p66/51 RT when introduced in the background of p51↓RNH cleavage site mutations. These studies demonstrate that cleavage of the internal p51↓RNH junction, not the flanking N-terminal or C-terminal junctions is essential for proteolytic stability of functional RT during virion maturation. These findings further emphasize the importance of the RNH domain in regulating proteolytic generation of p66/51 RT. The overall need for the RT heterodimer is attributable to the generation of its subunits. Formation of the 51 kDa subunit or cleavage of the p51↓RNH junction is essential for RT stability in the virion, whereas formation of the 66 kDa subunit is important for efficient viral replication.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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CHAPTER 1: INTRODUCTION

1.1 ASSEMBLY AND MATURATION OF RETROVIRAL PARTICLES

One of the most critical stages in the retroviral life cycle is the ability to transfer genomic RNA to subsequent host cells for efficient viral replication (Figure 1). This process entails the dynamic coordination of virus particle assembly, budding, and release from the host cell plasma membrane, followed by morphogenic maturation (537). An examination of the structural and functional proteins of a mature retroviral particle, such as HIV-1, does not directly provide clarification of the assembly process since these components are not individually packaged. Most viral proteins are instead translated in the cell from polycistronic unspliced viral RNA and incorporated into virions in the form of polyprotein precursors termed Gag ($Pr55^{gag}$) and Gag-Pol ($Pr160^{gag\text{-}pol}$) (Figure 2). The Gag precursor contains the structural proteins of the viral core including matrix (MA), capsid (CA), nucleocapsid (NC), $p6^{gag}$ and two spacer peptides p2 and p1. The Gag-Pol precursor contains these Gag proteins located upstream of a Pol region that encodes the functional viral enzymes protease (PR), reverse transcriptase (RT), and integrase (IN) (190). A subset of retroviruses may also encode dUTPase (DU) between RT and IN (FIV and EIAV) or N-terminal to PR (MPMV), to reduce dUTP incorporation errors during retroviral DNA synthesis in non-dividing cells (105,511). A commonly adopted model of the polyprotein

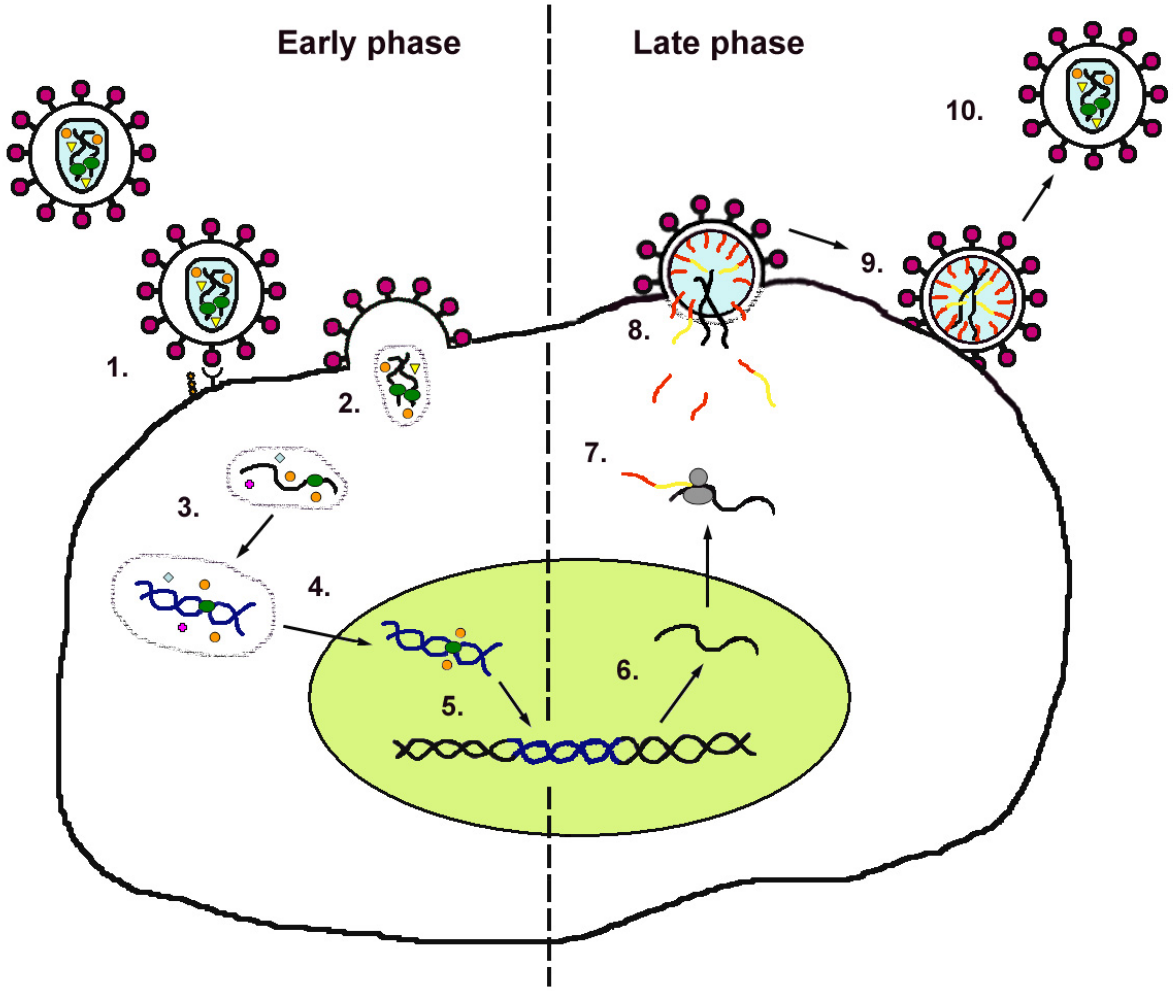

Figure 1. Replication cycle of HIV-1. The early and late phase of the HIV-1 replication cycle may be generally divided into 10 steps. The early phase begins with the binding of viral envelope SU (gp120) protein to cell surface CD4 receptor and CCR5 or CXCR4 chemokine coreceptors (step 1). This is followed by fusion, entry and uncoating of the cone-shaped viral core (step 2). Reverse transcription converts the single-stranded viral RNA genome into a double-stranded DNA copy (step 3). The preintegration complex (PIC) of viral and cellular proteins and proviral DNA is transported to the nucleus (step 4), followed by integration into the host chromosomal DNA (step 5). In the late phase of the cycle, the integrated viral DNA is transcribed by cellular RNA Pol II (step 6) forming spliced and unspliced mRNA templates used for translational synthesis of the accessory factors and polyproteins (Gag and Gag-Pol) encoding structural proteins and functional enzymes (step 7). Viral RNA, polyproteins, and envelope localize to the inner face of the plasma membrane where they are packaged into assembling viral particles (step 8). Finally, progeny virions bud from the cell surface as immature particles (step 9) and acquire infectious capacity by proteolysis-induced morphological maturation (step 10).

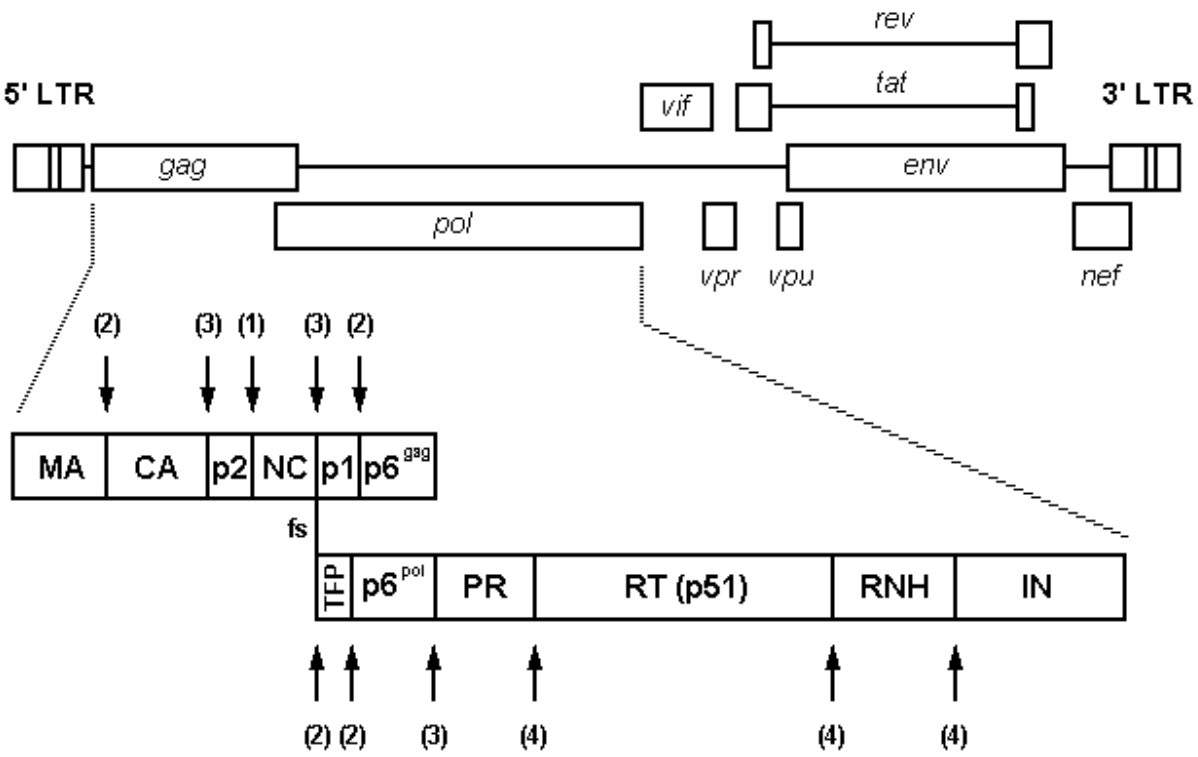

Figure 2. Schematic representation of the genomic and polyprotein organization of HIV-1. The upper diagram represents the 9.8 kbp provirus which is bordered by long terminal repeats (LTR) and contains a number of open reading frames encoding genes for structural (*gag*) and enzymatic proteins (*pol*), viral infectivity factor (*vif*), viral proteins R and U (*vpr* and *vpu*), envelope proteins (*env*), and the negative factor protein (*nef*). Proteins derived from multiply spliced mRNA include the regulator of mRNA splicing and transport (*rev*) and the transactivator of transcription protein (*tat*). The lower diagram represents the HIV-1 Gag and Gag-Pol polyprotein precursors. The proteins encoded in *pol* are synthesized at a frequency of 5-10% by a -1 translational frameshift (fs) of the unspliced genomic mRNA template to yield Gag-Pol. At the stage of virion budding and release, Gag and Gag-Pol polyproteins are proteolytically cleaved at domain boundaries by the viral protease to release their constitutive protein species and complete the maturation process. The location of these protease cleavage sites are indicated by the vertical arrows. Primary (1), secondary (2), tertiary (3), and quaternary (4) cleavage events are numbered accordingly. Gag polyproteins are processed into matrix (MA, p17), capsid (CA, p24), nucleocapsid (NC, p7), $p6^{gag}$ and two spacer proteins p2 and p1. Processing of Gag-Pol polyproteins additionally yields the transframe region proteins (TFP and $p6^{pol}$), protease (PR, p10), reverse transcriptase (RT, p66/51) which contains an RNase H (RNH) domain in its larger subunit, and integrase (IN, p32).

structure is that of a series of independently folded domains which are later excised by the action of the viral-encoded PR (31,162,262,389). This strategy of assembling polyprotein precursors is favourable for several reasons. It not only minimizes the number of components that need transporting to the site of viral particle formation, but also provides a means of regulating assembly and the proteolytic activation of viral-encoded enzymes (76,490). Furthermore, the conserved sequential order and domain interactions of precursor proteins serve to properly align structural and functional components within the virion.

One molecule of Gag-Pol is translated for every 10-20 Gag molecules (5-10% of the time), as a consequence of ribosome frameshifting or readthrough suppression of the 3' terminal end of Gag (221,222). Most retroviruses, including HIV-1 and MLV synthesize PR as part of the Gag-Pro-Pol polyprotein, otherwise referred to as Gag-Pol. In ASLV, PR is encoded as part of Gag-Pro and Gag-Pro-Pol polyproteins where only the former form is necessary and sufficient for processing (76,490). Other retroviruses such as type B (MMTV), D (M-PMV), E (BLV, HTLV) and spumaretroviruses (HFV) express Pro (PR) and Pol domains independently of Gag (221,583). Both Gag and Gag-Pol polyproteins are subjected to many modifications after translational synthesis. Phosphorylation of MA proteins may have role in proteolytic processing and nuclear localization of preintegration complexes (PICs) (42,44,139), while phosphorylation (188,344) and monoubiquitination (133,143,448) of $p6^{gag}$ may also assist in proteolytic processing and budding. Moreover, myristate fatty acids are added to N-terminal glycine residues of Gag and Gag-Pol polyproteins to direct targeting to the plasma membrane (449) along elements of the cytoskeletal system or chaperone proteins (41). Perhaps the most important post-translational modification, as discussed below, is the proteolytic processing of these polyproteins into their respective structural and functional proteins during viral maturation.

The structural proteins of Gag and Gag-Pol are of central importance to the assembly, budding and maturation of nascent viral particles. The Gag polyprotein alone is sufficient for the production of viral particles (149), since it mediates many important functions through three distinct domains (Figure 3). The membrane-binding domain (M), located at the N-terminus of MA anchors and secures Gag and Gag-Pol polyproteins to the plasma membrane by inserting a myristic acid attachment and establishing electrostatic interactions (276,365). The Gag-Gag interaction domain (I), at the C-terminus of CA and N-terminus of NC is involved in Gag multimerization, RNA binding, virus assembly, budding and reverse transcription (47,132,585). The late domain (L) is located in $p6^{gag}$ and contains a PTAP motif essential for budding.

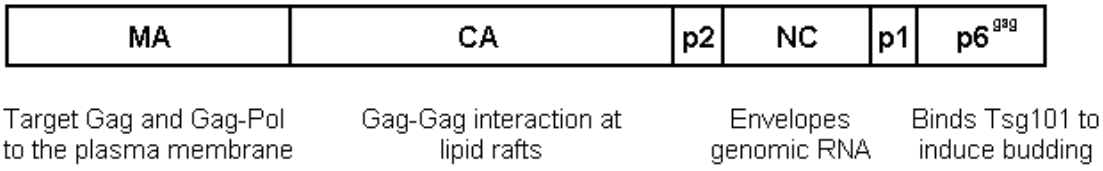

Figure 3. Primary functions of Gag-associated proteins during HIV-1 assembly and budding.

1.1.1 Retrovirus assembly

The assembly process of retroviral particles is likely to involve a number of simultaneously occurring steps. These may include: (i) multimerization and the formation of Gag-mediated complexes (Gag/Gag, Gag/Gag-Pol, and Gag-Pol/Gag-Pol), (ii) binding of genomic viral RNA to these complexes, (iii) and the formation and transportation of preassembled complexes, containing accessory viral proteins and host cell proteins to the plasma membrane (Figure 1).

Polyprotein aggregation is critical to the assembly process in that mutagenesis of Gag can diminish membrane targeting, and inhibit virus particle production, proteolytic processing, and infectivity (131,132,296,542). Dimerization of viral RNA and its interaction with the NC region and 5' splice donor site of Gag complexes are also important to ensure packaging (47,438) and the maintenance of viral particle integrity (547). A number of viral accessory proteins (Vpr, Vif, and Nef), and cellular proteins (cyclophilin A, HP68 and Tsg101) are packaged into virions, either passively or via direct interaction with Gag or Gag-Pol polyprotein precursors possessing myristolyated M-domain targeting signals (175). These proteins serve many structural and functional roles which briefly include: (i) nuclear targeting of preintegration complexes (PICs) and *trans*-activating LTR-directed expression (Vpr) (322,530), (ii) countering the antiviral activity of Apobec3G and stabilizing the viral core (Vif) (364,492), (iii) distinct roles in entry and post-entry events (cyclophilin A) (441), and (iv) the promotion of immature capsid assembly (HP68) (589) virus budding (Tsg101) (153). During assembly, these Gag complexes and components appear as electron dense patches along the plasma membrane where they induce membrane curvature to form budding viral particles (149).

1.1.2 Retrovirus budding

The packaging of Gag and Gag-Pol, 1000-2000 and 50-200 molecules, respectively (375,376,493), drives the budding of a nascent virion from the membrane of an infected host cell (Figure 1). The platform site for assembly, packaging, and budding of Gag-mediated complexes is characterized by membrane domains enriched with cholesterol, glycosphingolipids, and sphingomielin, known as lipid rafts (303). To enhance the production of high infectious virions, HIV-1 gene products Nef and Vpu down-modulate the CD4 receptor from the host cell surface

(281) and increase the incorporation of envelope (Env) proteins (8). Promotion of efficient viral particle release requires the phosphorylation (188,344) and ubquitination (153,369) of the $p6^{gag}$ L domain followed by the binding to the cellular protein Tsg101 (133,143,153).

1.1.3 Retrovirus maturation

The final stage of virus production is characterized by the release of the budding virion from the host cell membrane, followed by morphological changes associated with maturation (Figure 1) (537). Electron microscopy studies have revealed that Gag complexes assemble with genomic viral RNA to form lipid-associated spheres connected to the cell membrane by a thin stalk (202,372). Once released from the cell, viral particles are initially immature and contain a thick layer of submembrane density resembling a doughnut-shape. Maturation creates a thin layer of submembrane protein ascribed to MA, and a cone or rod-shaped electron-dense central core formed by the CA protein that in turn surrounds two strands of viral RNA bound NC (351). This morphological maturation is generally believed to be associated with dynamic structural and conformational rearrangements of the viral particle. Indeed, cross-linking experiments in MLV have suggested that some Gag-Gag protein interactions appear to change while others are maintained during the process of maturation (390). This complex transformation follows a cascade of proteolytic events mediated by a viral-encoded PR that catalytically cleaves Gag and Gag-Pol polyproteins into their respective structural proteins and functional enzymes (Figure 2). Although the exact timing of proteolytic processing is unclear, it is believed to be initiated at the plasma membrane during assembly, and completed after budding (244,245).

Pulse-chase labeling studies of infected cells were the first to provide evidence for proteolytic processing or proteolysis in a retroviral system. Full-length Gag polyprotein

precursors of ASLV ($Pr76^{gag}$) and MLV ($Pr65^{gag}$) were shown to be processed into Gag structural proteins almost immediately (226,538). Similar findings were soon observed for Gag-Pol polyprotein processing (226,367). Thereafter, the proteolytic factor responsible for both of these events was localized to the virus particle itself (541,580). Mutagenesis of this viral-encoded protease (PR) clearly indicated that processing of the Gag precursor is accompanied by morphological changes (77,488,579) and the production of infectious virus (250,562). These results were later paralleled in findings for HIV-1 (262,389).

An essential step in the viral life cycle is the proteolytic processing of Gag and Gag-Pol precursors by the viral-encoded PR. To that end, the association of HIV-1 with the AIDS epidemic has generated a considerable amount of interest to further our understanding of the mechanics of processing in the release of key structural and enzymatic proteins. In this review we summarize the current state of knowledge regarding polyprotein processing in HIV-1 with a particular focus directed towards the regulation and consequences of Pol processing, including insights into biochemical and structural aspects of RT and other Pol enzymes. For the purpose of comparison to HIV-1, related retroviral systems will be discussed perfunctorily. The general features and roles of protease (PR) will be discussed first, followed by sections on Gag and Gag-Pol polyprotein processing. Finally, important emphasis will be given to structural aspects of reverse transcriptase (RT), followed by integrase (IN).

1.2 PROTEASE

1.2.1 General features of HIV-1 PR structure and function

HIV-1 protease (PR) plays an indispensable role in the viral replication cycle (Figure 1 and 2). During the late-phase of viral replication, HIV-1 PR cleaves viral Gag and Gag-Pol polyproteins at a limited number of sites to release mature structural and functional proteins (Table 1). The role of HIV-1 PR during the early-phase remains controversial. Processing of cytoskeletal and sarcomeric proteins including vimentin, desmin, actin, myosin and tropomyosin (467,468) may be important in regulating the transport of PICs to the nucleus (41). Further processing of mature viral proteins CA (519) and NC (570) as well as accessory proteins Tat (7), Vif (255) and Nef (374,550) has also be reported to be important in efficient viral replication (Table 2)

All retroviral proteases are aspartic proteases. This class of enzymes contains a conserved active-site triplet Asp-Thr/Ser/Gly and includes such cell-derived enzymes as pepsin, renin, chymotrypsin, penicillopepsin, and cathepsin D and E (84,415). In contrast to their cellular counterparts, retroviral proteases are smaller, less efficient, and act as symmetrical homodimers with two identical subunits contributing towards the active site (415,520). Although retroviral PR generally function best at acidic pH 4.5-6.5, they display significant *in vitro* activity up to physiological pH 7.0-7.5 (268). Recombinant forms of retroviral PR also require a specific solvent composition including high ionic strength and polyethylene glycol to enhance conformational stability, substrate affinity and catalytic activity (235,268).

HIV-1 PR is a dimer of 10 kDa subunits and is well represented in the protein structural database in unliganded form and in complex with peptide and non-peptide inhibitors (544). Each

Table 1. Location, sequence and hydrolysis rates of late-phase HIV-1 PR cleavage sites

[a, b] Location and sequence of the cleavage sites in HIV-1 Gag and Gag-Pol polyprotein precursors as indicated from the LAI isolate of HIV-1 (416). The seven amino acid residues (P4-P3') that interact directly with PR flank a scissile amide bond indicated by the vertical arrow (↓). The question mark (?) signifies that this cleavage site has not been absolutely confirmed.

[c] Most cleavage site sequences were identified by N-terminal protein sequencing of HIV-1 virion proteins. The p51↓RNH cleavage site was identified by processing representative peptides or p66/66 RT.

[d] Classification of cleavage sites by type, based on sequence and residues occupying the P1 and P1' positions (170,397,520).

[e] Kinetic parameters of peptide hydrolysis: rate (K_{cat}) and efficiency (K_{cat}/K_m) at pH 5.6, where K_{cat} is the number of substrate molecules transformed per second per molecule of enzyme and K_m is the relative substrate affinity or substrate concentration at $\frac{1}{2}V_{max}$ (80,516,517)

[f] Polyprotein hydrolysis rates at pH 6.5 relative to p2↓NC (Gag) (395,396) or TFP↓p6^{pol} (Pol) (309). Bracketed numbers represent the relative order of hydrolysis, based on catalytic efficiency or rates.

Location [a]	Sequence [b] (P4-P3')	Ref ID [c]	Type [d]	Peptide Hydrolysis [e]			Polyprotein Hydrolysis Rate[f]
				K_m (mM)	**K_{cat} (s^{-1})**	**K_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)**	
Gag							
MA↓CA	SQNY↓PIV	(190,328)	1	0.15	6.8	45.3 (3)	14x (3)
CA↓p2	ARVL↓AEA	(190,328)	2	0.01	0.09	90.0 (2)	400x (5)
p2↓NC	ATIM↓MQR	(190,328)	2	-	-	-	1x (1)
NC↓p1	RQAN↓FLG	(190)	2	-	-	-	350x (4)
p1↓p6^{gag}	PGNF↓LQS	(190,534)	2	0.53	0.3	0.6 (7)	9x (2)
Pol							
NC↓TFP	RQAN↓FLR (?)	(55)	-	-	-	-	-
TFP↓p6^{pol}	DLAF↓LQG	(313,400)	2	-	-	-	1x (1)
p6^{pol}↓PR	SFNF↓PQI	(299,313)	1	<0.01	0.06	6.9 (6)	1x (1)
PR↓RT	TLNF↓PIS	(298,533)	1	0.07	1.5	24.1 (4)	-
RT p51↓RNH	AETF↓YVD	(61,169)	2	0.04	0.4	10.0 (5)	-
RT↓IN	RKVL↓FLD	(298)	2	0.006	1.2	202.0 (1)	-

Table 2. Protein substrate and sequence of early-phase HIV-1 PR cleavage sites

[a] Viral and cellular protein substrates containing HIV-1 protease cleavage sites

[b] Sequence of the seven amino acid residues (P4-P3') that interact directly with PR and flank the scissile amide bond indicated by the vertical arrow (↓). The HIV-1 sequences shown are from the LAI isolate (416). The question mark (?) signifies that this cleavage site has not been absolutely confirmed.

[c] Literature reference(s) which identified the cleavage site

[d] Classification of cleavage sites by type, based on the sequence around the cleavage site and the residues occupying the P1 and P1' positions (170,397,520)

Protein substrate [a]	Sequence [b] (P4-P3')	Ref ID [c]	Type [d]
CA (N domain)	NEEA↓AEW	(519)	2
CA (C domain)	TETL↓LVE	(519)	2
NC (N domain)	VKCF↓NCG	(570)	2
Nef	DCAW↓LEA	(374)	2
Vif	YLAL↓AAL	(255)	2
Tat	RKKR↓RQR (?)	(7)	2
Actin	SFIG↓MES	(468)	2
Vimentin	SLNL↓RET	(467)	2
EF1α	GTTL↓LEA	(370)	2
NF-κB	HYGF↓PTY	(427)	2

subunit has a flexible flap region of antiparallel β-sheets with a β-turn that extends over the substrate-binding cleft allowing it to clamp down (Figure 4) (357). Apposing aspartic acid residues, D25 and D25' in the active sites are held in position by a hydrogen-bonded network called a "fireman's grip" configuration. Stabilization of the homodimer is largely accomplished by intermolecular contacts between the antiparallel β-sheets of both amino and carboxyl termini. The dissociation constant of HIV-1 PR subunits has been largely reported in the low nanomolar range (K_d = 20-39 nM), although higher values have been cited using various solvents and methods of determination (79,168).

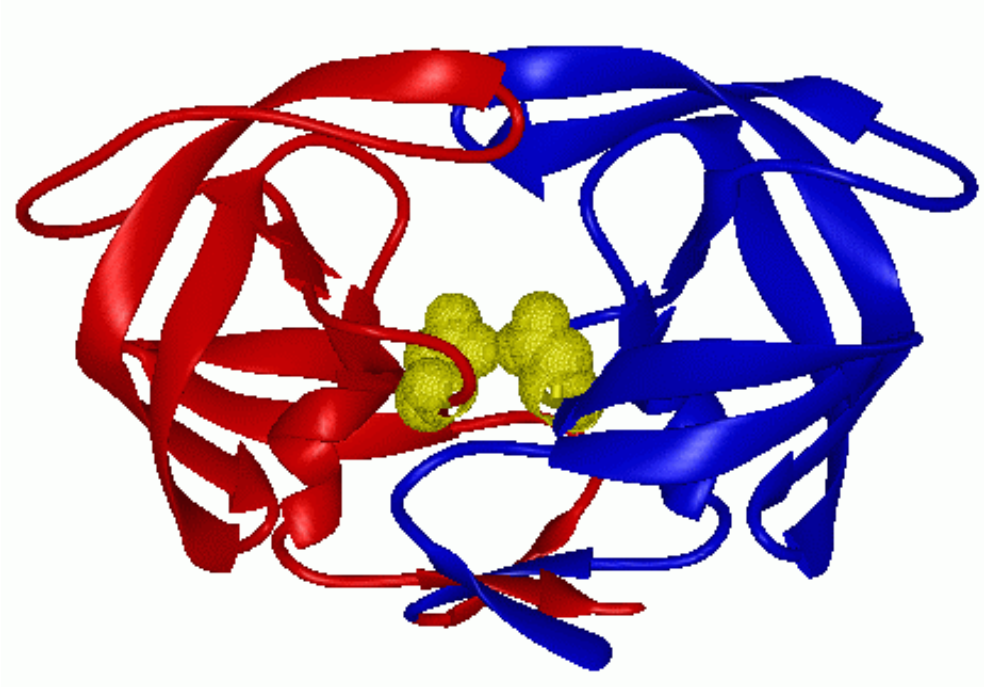

Figure 4. Molecular structure of HIV-1 protease. Representative unliganded polypeptide schematic of a tethered PR homodimer generated from the crystal coordinates of PDB file 1G6L (1.9 Å resolution) (401). HIV-1 PR is symmetrical homodimer consisting of two 10 kDa subunits (red and blue), 99 residues in length. Each subunit has a flexible flap region of antiparallel β-sheets (arrows) with a β-turn that extends over the substrate-binding cleft. Apposing aspartic acid residues D25 and D25' (spacefilled yellow) in the active sites are held in position in a hydrogen-bonded network called a "fireman's grip" configuration. Stabilization of the homodimer is accomplished by intermolecular contacts between four-stranded antiparallel β-sheets of both N- and C-termini. *(Modified from: Pillai, B. et al. 2001 Proteins 43(1):57).*

1.2.1.1 HIV-1 PR-substrate interactions

HIV-1 PR binds both peptide inhibitors and substrates asymmetrically in an extended anti-parallel β-sheet conformation, even though it is a symmetrical homodimer (406,407,548). Substrate determinants that interact with the binding cleft of HIV-1 PR lie within seven amino acid residues (P4 to P1 and P1' to P3') that flank the cleavable peptide bond (P1↓P1'), termed the scissile amide bond (Figure 5). Other retroviral forms of PR, such as FIV, EIAV, ASLV, and MLV PR are very similar in structure, but may have longer flap regions allowing them to recognize additional substrate elements (368,563). It has long been suggested that each side chain of a peptide substrate points into successive alternating subsites or pockets of the PR binding cleft, named for the corresponding substrate side chain (S4 to S3') (548). The scissile bond is held in the proper position for hydrolysis by multiple anchoring on both sides. Most of the contacts made between the substrate and enzyme are in the binding cleft or with the flaps, are not sequence specific, and involve backbone-backbone hydrogen bond interactions. These main chain interactions have been predicted to contribute more towards total binding energy than side chain interactions (172). Although substrate-specific interactions are largely hydrophobic, side chains may form up to three specific hydrogen bonds with critically important subsite residues (406,407). Each substrate residue lies in contact with at least 5 PR residues and between 1-3 molecules of water; with the most number of contacts established at the P2 and P2' positions.

After the involvement of non-specific hydrogen bond interactions, the determinants of specificity come from the shape of the substrate, not its sequence. The ability of a substrate to adopt an optimal configuration for hydrolysis is limited by *cis*, *trans*, or coupled interactions between substrate amino acids bound in adjacent enzyme subsites (i.e. P3 and P1, or P2 and P1') (397,425,516). Thus, the conformation of PR around the different substrate side chain positions

has been recently reported to comprise four primary pockets: (i) P1/P3, (ii) P2, (iii) P1'/P3' and (iv) P2'. Side chain packing between P1 and P3 or P3 and P4 results in the formation of a hydrophobic toroid and distortion of the peptide backbone around the scissile bond (P1↓P1'). This in turn may facilitate hydrolysis, by allowing the carboxyl oxygen of P1 to make an invariant hydrogen bond with the catalytic aspartate residue (D25 or D25') (406,407).

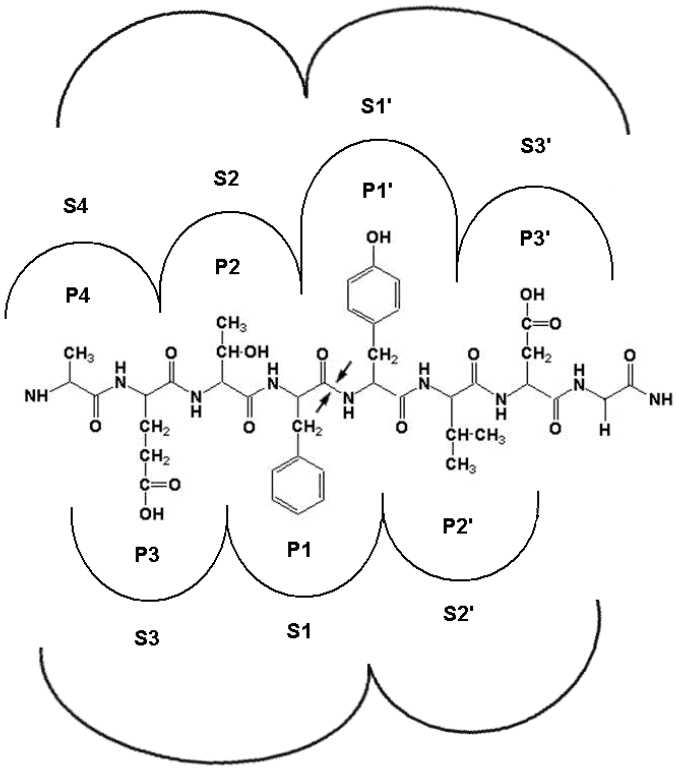

Figure 5. Schematic representation of a substrate bound to HIV-1 protease. Substrates that interact with the binding cleft of HIV-1 PR lie in an extended anti-parallel β-sheet conformation with alternating side chains (P4-P3') oriented in opposite directions, above and below the plane of the figure. The scissile amide bond to be hydrolyzed lies between P1 and P1' residues, as indicated by the apposing arrows. Most of the contacts formed in the PR-substrate complex are not sequence specific and involve many backbone-backbone interactions. Each substrate residue is in contact with at least 5 PR residues and 1-3 water molecules. Specificity is based on the shape of the substrate. Side chain packing between P1 and P3, or P3 and P4 generates a hydrophobic toroid which in turn distorts the substrate backbone about the scissile bond allowing hydrolysis. While early studies implied the existence of individual enzyme subsites, termed S4-S3' (548), more recent analyses indicate that PR conformation is more appropriately divided into four primary pockets encompassing P1/P3, P2, P1'/P3', and P2 (406,407).

1.2.1.2 Catalytic mechanism of HIV-1 PR

The catalytic mechanism of HIV-1 PR occurs by general acid-base hydrolysis and proceeds through a transient tetrahedral intermediate (220,403,469). In contrast to Ser and Cys PR, HIV-1 PR does not form a covalent acyl-enzyme intermediate with the substrate. It therefore must anchor itself to the substrate at multiple sites about the scissile bond. The two catalytic aspartate residues (D25 and D25') mediate the nucleophilic attack of a water molecule at the carbonyl of the scissile bond followed by a proton transfer to the leaving nitrogen atom of the substrate. Overall, the reaction appears to proceed in two steps, a physical closing down of the flaps onto the substrate followed by a chemical proton transfer step.

1.2.2 Determinants of HIV-1 PR cleavage site recognition

1.2.2.1 Viral substrates

The major functional role of HIV-1 PR is to process Gag and Gag-Pol polyproteins by catalyzing the hydrolysis of specific peptide bonds that separate respective protein domains (Figure 2). The exact sequence and location of these cleavage sites has been largely deduced by direct amino-terminal sequencing of viral Gag and Pol proteins (Table 1). The absence of free ribonuclease H (RNH), and the difficulty associated with carboxyl-terminal sequencing have so far prevented a determination of the RT p51↓RNH cleavage site directly from the virus. This putative cleavage site was instead identified by *in vitro* processing of recombinant p66/66 RT (61,119,203,515), a Pol polyprotein (23), and peptides spanning the p51-RNH region (169,450). The functional consequences of mutations and processing these cleavage sites are discussed later (Section 1.4.3).

1.2.2.2 Substrate specificity and stringencies

A comparison of all known HIV-1 PR-recognized cleavage sites shows that they are dissimilar with no unique consensus sequence (Table 1 and 2) (30,385,397). Recognition sequences are however, relatively hydrophobic and are expected to lie in less structured regions of the polyproteins or in exposed stretches of polypeptide connecting folded protein domains. HIV-1 PR appears to recognize a combination of substrate residues flanking the scissile bond with no one particular subsite exhibiting absolute specificity. Specificity of HIV-1 PR has been assessed by two methods. In the first approach, all known bona fide cleavage sites, both viral and non-viral, were compiled and compared (397,404). From this analysis a series of qualitative rules were deduced to define cleavage potential in terms of specific amino acid residues occupying certain substrate positions. In a second approach, substrate specificity was characterized by systematic testing and cleavage of polyproteins or oligopeptides (30,170,424,514,520). In this manner, various PR-substrate interactions could be tested through multiple amino acid substitutions and kinetic parameters of substrate binding (K_m) and catalytic efficiency (K_{cat}) could be examined. Finally, HIV-1 PR mutants have been used to establish the importance of side chain interactions between PR and substrates (305,336). While all of these approaches have yielded important information about HIV-1 PR specificity, it remains unclear how structural folding and steric interactions affect substrate specificity it the natural context of the full-length polyprotein.

Despite the diversity of cleavage site sequences, some generalizations can be made about HIV-1 PR specificity. The degree of amino acid stringency around the scissile bond appears to range from low (P1'), restricted (P4, P3, P1, P2', P3'), to high (P2) (266,383,397,518). In no instance is only a single amino acid residue tolerated at a particular substrate position. In general,

both P1 and P1' are occupied by large hydrophobic residues, P1 never contains a β-branched aliphatic side chain, P2 and P2' are typically hydrophobic or small polar residues, and P4, P3 and P3' can accommodate a variety of residues. HIV-1 PR specificity may also be influenced by the sequence context and conformation of a peptide, whereby the preference for a given subsite is strongly dependent on residues in other subsites. This structural dependence arises from *cis* and *trans* interactions between certain residues in neighboring and adjacent substrate positions (i.e. P2 and P1', P1 and P3, P4 and P1, or P1 and P1') (397,425,516).

Attempts have been made to classify diverse collections of HIV-1 PR cleavage sites into groups based on rules for specificity. The most common classification was based on Gag cleavage sites from ten different retroviruses (170,397,520). Type 1 cleavage sites were defined as having Tyr or Phe at P1 and Pro at P1', whereas type 2 cleavage sites have hydrophobic residues at both P1 and P1, excluding Pro (Table 1). In addition, certain amino acids are excluded from the P3, P2 and P2', P3' positions. A subsequent classification system was based on inferred context-dependency amongst HIV-1 PR substrates (189). Class 1 cleavage sites were cited as having an aromatic residue at P1, proline at P1', hydrophobic residue at P2', Asn at P2, and Gly or a polar residue at P3. Class 2 cleavage sites have Arg at P4, Phe at P1', Leu at P2', and residues in P3 and P2 are polar and hydrophobic, respectively. Finally, class 3 cleavage sites have Gln or Glu at P2' and hydrophobic residues at P2-P1'.

Computational algorithms have also been developed to predict cleavable sites by determining the statistical preference of amino acid residues at certain positions. Early methods of prediction were based on a cumulative specificity model to score the frequency of occurrence of amino acid residues at particular positions from a collection of substrates (404). However, this method failed to account for the interaction between subsites by assuming that each amino acid

residue acted independently in the recognition by HIV-1 PR. More recent approaches, including a vectorized sequence-coupling model (68), a discriminant function method (69), and a volume-based model (433) have shown progressive improvements in correcting for these errors with higher frequency predictions. While these methods are reasonably sufficient at scoring peptide substrates as cleavable or non-cleavable, they are deficient in providing information about cleavage efficiency. Substrate cleavage efficiencies may actually vary widely, including: (i) slow-cleaving (high K_m, low K_{cat}), (ii) low affinity, high turnover (high K_m, high K_{cat}), (iii) bind tight, cleave slowly (low K_m, low K_{cat}), and (iv) efficiently cleaved (low K_m, high K_{cat}) substrates. Finally, these prediction methods fall short in accounting for the influence of conformational folding and structural constraints that may affect accessibility and hydrolysis rates.

1.2.3 Activation and regulation of HIV-1 protease

The critical steps in virion morphogenesis include the activation of HIV-1 PR, and the cleavage of Gag and Gag-Pol polyprotein precursors. In most retroviruses, the viral-encoded PR appears to play no causal role in the assembly process since PR-inactivating mutations or protease inhibitors do not prevent immature virions from budding (250,326,488). HIV-1 may be an exception to this generalization, where proteolytic cleavage and virion assembly are invariably associated. While PR-defective virions of HIV-1 are capable of budding, an active PR may be required for this to occur with maximum efficiency (240,244,246). A role for HIV-1 PR in assembly has been proposed based on the finding that premature activation excludes processed Gag and Pol products from assembly (43,245), overexpression of PR abrogates assembly and is toxic to the cell (271,272,284), and structural attributes of HIV-1 PR affect the physical stability

of the released virion (380). While the exact timing of PR activation is unclear, it is generally believed to be delayed until the late stages of assembly, once precursor polyproteins have reached the plasma membrane, or soon after budding (118,244,245).

The molecular mechanisms that lead to PR activation and the regulation of polyprotein processing are not completely understood. Experimental findings have suggested that PR domains from two Gag-Pol polyprotein precursors must come together and dimerize for initial activation (131,140,144,318,414) and an aggregation model has been proposed to explain how this may be regulated (355). Timing and control of PR activation is critical, since linked dimers of HIV-1 PR (272) and overexpressed Gag-Pol polyprotein precursors (247,381) cause deleterious effects. The aggregation model suggests that some autoprocessing of PR may occur at the plasma membrane with occasional dimerization of Gag-Pol polyproteins. The activated Pol-associated PR would then passively diffuse away from the site of assembly. Since the majority of Gag-Pol is likely to be present in low concentration at the plasma membrane, the majority of PR would not be activated and released until after the volume is greatly reduced in the form of the budding virion. This would be followed by a cascade of intermolecular *trans* processing events to liberate the structural and functional proteins, thus making the virion infectious. While attractive, this model does not explain delayed processing in type-B and type-D retroviruses which form a compact intracytoplasmic A-type particle (ICAP) within the cell cytoplasm. In addition, other factors such as a drop in pH or an influx of Ca^{2+} ions may arguably trigger the activation of PR during budding (470,537).

Given the uncertainty of PR activation it remains equally unclear whether the morphological changes associated with maturation occur after budding (202,372) or during the late stages of budding (160). In particular, the complex series of dynamic events associated with

PR activation, processing, and virion maturation has made it difficult to draw conclusions as to when one event ends and the other begins. A role for HIV-1 PR in virion maturation is certain, since 75-100% inactivation of PR produces non-infectious virions of immature morphology (147,160,246) containing incompletely processed polyproteins (16,431). However, aside from PR, additional unknown factors appear to be essential for virion morphogenesis and infectivity. Recent evidence has suggested that early released virions appear immature and are non-infectious, but contain processed Gag proteins (40), consistent with the effect of CA (500,543) and NC mutations (28). One possible contributory factor may be protein phosphorylation or dephosphorylation at the plasma membrane, since deletions in MA cause the budding of immature viral particles into the endoplasmic reticulum (118).

While the exact mechanism of PR activation in the virion remains unclear, recombinant studies have suggested that autocatalytic intramolecular processing of its N-terminus may be important. Since both N-terminal and C-terminal ends of PR are important in dimer stability and in modulation of activity, this concept of intramolecular autoprocessing may seem difficult to geometrically perceive. The current model suggests that the N-terminal region of β strands may peel back, cause a slight transient disruption in the remaining strands, and then slide around into the active site of precursor-associated PR to be processed (73,311). Other regions of Gag and Gag-Pol may further regulate efficient proteolysis of remaining cleavage sites by promoting appropriate accessibility, order and structural recognition context (177,395,407,414). While peptide studies have reported a range of cleavage site hydrolysis efficiencies (80,274), actual values arising from polyproteins (392,393,395,396) is vastly different (Table 1). This suggests that any number of the above factors may play a significant role in regulation of ordered proteolytic processing.

1.3 PROCESSING OF GAG

1.3.1 Ordered processing of Gag polyprotein precursors

Gag polyprotein precursors are cleaved within the nascent retroviral particle at least five times to generate mature structural proteins matrix (MA), capsid (CA), nucleocapsid (NC), $p6^{gag}$ and two smaller spacer polypeptides p2 and p1 located between CA-NC and NC-$p6^{gag}$ respectively (Figure 2) (190,368,534). Approximately 2000 Gag precursors are packaged during assembly to ensure a sufficient quantity of structural proteins are present in the virion (375,376,493). The order of Gag precursor proteins is highly conserved in all retroviruses and serves to align these structural domains within the virion, from the outside inward. Proteolytic processing of Gag induces a dramatic reorganization of the internal virion structure which is essential for morphological maturation and infectivity (537), but it not required for viral particle formation (149). Complete cleavage of the majority of Gag precursors is essential to ensure the production of viable infectious virus particles (246,395) with normal morphology (162,246,557). As a result of these events, MA protein swathes the inner surface of the membrane and CA forms the rod-shaped capsid shell which encloses the genomic RNA complexed with NC (146).

It is presumed that after autoprocessing, the remaining cleavage sites are processed by HIV-1 in *trans* by an intermolecular mechanism (91,434). Numerous *in vitro* studies have indicated that proteolytic processing of HIV-1 Gag polyprotein occurs in a time-dependent, ordered manner through a transient series of processing intermediates that precede the formation of mature structural proteins (114,395,396,522,557). Similar intermediates have also been found in infected cells and recently budded virions (163,246,269,328,481). Cleavage of each junction in Gag appears to occur independently (395). Thus, processing may be a ordered process that is

governed by the intrinsic susceptibility and relative efficiency of each site to proteolysis (516). Primary processing of $Pr55^{gag}$ (Gag) at the p2↓NC cleavage site gives rise to p43 (MA-CA-p2) and p14 (NC-p1-$p6^{gag}$) intermediates. Secondary processing at p1↓$p6^{gag}$ occurs slightly faster than at MA↓CA and produces p8 (NC-p1), $p6^{gag}$, p17 (MA) and p25 (CA-p2) intermediates and products. Final tertiary processing at NC↓p1 is slightly faster than at CA↓p2 and gives rise to p7 (NC), p1, p24 (CA) and p2 final products (Figure 2, 22, and Table 1). Interestingly, mature viral particles may contain a small portion of unprocessed $Pr55^{gag}$ as well as p41 (MA-CA) and p37 (CA-p2-NC-p1-$p6^{gag}$) intermediates (163,328,557) further suggesting that while most Gag precursors are fully processed, cleavage events are independently regulated.

1.3.2 Structural and functional consequences of Gag processing

A number of specific functions have been assigned to the individual proteins of $Pr55^{gag}$ (Gag), both before and after viral maturation. Therefore, inefficient or inaccurate processing can potentially cause profound defects in viral structure and infectivity (246). Adaptive changes in a number of these cleavage sites are possible as polymorphisms in HIV-1 PR develop with each subsequent passage of virus. The most variable Gag cleavage sites, both within and between subtypes, and before and after protease inhibitor therapy are p1↓$p6^{gag}$, p2↓NC, and NC↓p1 (74,88). Although these mutations alone may impact on processing and infectivity (158,429), in other instances they may partly compensate for the reduced catalytic activity of drug resistant mutant forms of HIV-1 PR (125). By purposely introducing mutations into Gag cleavage sites, researchers have been able to investigate the structural and functional consequences of Gag processing. Cleavage site mutations may result in (i) wild-type processing, (ii) absence of

processing at the mutated site, but efficient processing at distal sites, or (iii) no processing at the mutated site or at distal sites.

1.3.2.1 Matrix (MA)

The N-terminal myristate group and basic residues of the MA domain are important for targeting Gag and Gag-Pol precursor polyproteins to the plasma membrane (276,365) and in assisting with the incorporation of Env glycoproteins (135). MA protein in its mature trimer form lines the inner surface of the viral membrane (288). Phosphorylated MA may also have a role in the nuclear translocation of PICs in non-dividing cells by interacting with importin alpha (Rch1) (44,139), although the existence of a nuclear localization signal has been disputed (130,134). The MA↓CA cleavage site appears to play a pivotal role in core condensation during maturation, since mutations introduced here produce noninfectious particles with abnormal morphology (162,246). However, while the MA↓CA cleavage site is highly sensitive to mutagenesis (383), this does not prevent downstream processing (522).

1.3.2.2 Capsid (CA) and the p2 spacer peptide

Processing of the MA↓CA cleavage site is also structurally important for CA, as the P1' Pro residue becomes buried and establishes a putative salt bridge (151). The CA protein plays essential roles in both the early- and late-phases of infection. Mutations in the N-terminal domain of CA demonstrate its importance for infectivity by disturbing interactions with cyclophilin A (CypA) that are needed for viral uncoating (317). The C-terminal domain of CA contains the major homology region (MHR) which is essential in particle assembly (98,542) by facilitating

Gag oligomerization (585) and CA dimerization (140,543), and increasing membrane affinity through the exposure of hydrophobic residues (103,296). The conical core in mature infectious virions is formed by the association of CA proteins. Mutations introduced at the CA↓p2 cleavage site may inhibit budding in certain cell lines, in a manner unrelated to a defect in processing (162,273,395). The p2 spacer peptide itself is a 14 residue-long segment separating CA-NC. This spacer peptide negatively regulates the timing of CA↓p2 processing and is essential for ordered assembly, viral infectivity (273,395), and in excluding spliced viral RNA from packaging (439). Mutation of any or all of the cleavage sites between CA-NC results in the production of immature virions, although it does not effect the processing of other Gag cleavage sites (522,557).

1.3.2.3 Nucleocapsid (NC) and the p1 spacer peptide

Rapid cleavage of the p2↓NC site (114,275,395) is the first critical regulatory step in ordered Gag processing. Cleavage here stabilizes the viral core by triggering RNA dimerization and condensation upon binding NC (47,460,557). UV-cross linking experiments have demonstrated that the interactions between viral RNA and the NC domain of Gag differ from that of mature NC (488). NC is a basic protein with zinc fingers that specifically binds to the packaging signal (ψ) of full-length viral RNA to deliver it into the assembling viral particle (28,47,445). NC can also bind single-stranded nucleic acid, such as the genomic viral RNA in a non-specific manner to provide protection from nucleases and compactness within the viral core. This attribute for nucleic acid binding provides chaperone-like functions to promote annealing of the $tRNA^{Lys,3}$ primer, melting of RNA secondary structures, DNA strand transfer reactions during reverse transcription (54,215,229,581) and stimulation of integration (57). The p1 spacer

peptide separating NC-p6^{gag} is a 16 residue segment that is excised from p6^{gag} and NC early and late in processing, respectively. Mutational studies have shown that processing of the NC-p6^{gag} region is dependent upon the binding of viral RNA to NC; a property not found at any other cleavage site (461,462). These findings suggest that RNA binding may promote accessible processing of this region, which in turn may lead to encapsidation or condensation of the RNA.

1.3.2.4 p6^{gag}

The early release of the p1 spacer peptide from p6^{gag} appears to be one of several factors which regulate the viral budding process (212). By comparison, the N-terminal cleavage site that distinguishes p6^{gag} (p1↓p6^{gag}) from p6^{pol} (TFP↓p6^{pol}) differs by eight residues when the -1 translational frameshift is taken into account (312,393). While p6^{pol} appears to play some role in the regulation of PR activity (384,590), p6^{gag} mediates the efficient release of viral particles during budding (161,212). This essential role of p6^{gag} requires both phosphorylation (188,344) and ubquitination (153,369) followed by binding of Tsg101 (133,143,153). The p6^{gag} domain also appears to incorporate the accessory protein Vpr upon interaction with its highly conserved LXXLF motif (17,264).

1.4 PROCESSING OF POL

1.4.1 Ordered processing of Gag-Pol polyprotein precursors

The Pol region of HIV-1 Gag-Pol encodes three viral enzymes present in the polycistronic order of protease (PR), reverse transcriptase (RT), and integrase (IN). The *pol*-encoded enzymes of Gag-Pol are made in relatively small quantities compared to the structural proteins of Gag. One molecule of Gag-Pol is typically produced for every 10-20 molecules of Gag (222). The synthesis of Pol in fusion with Gag serves two purposes: first, the minimal production of Gag-Pol ensures controlled activation of the *pol*-encoded enzymes, and second, permits proper positioning of these enzymes within the virion. The initial steps in Gag-Pol polyprotein processing are very similar to that of Gag processing. However, the order of Pol proteins and the extent of processing may differ from one retrovirus to the next. Functional PR and IN are released as separate entities in all retroviruses, but the proteolytic events that generate RT appear to differ.

The order of Pol domains ensures efficient processing of its functional constituent enzymes. Of all the phylogenetically related retroviruses and LTR retrotransposable elements that encode a complete Pol region (97,574), only Ty1 and Ty2 retrotransposons of *Saccharomyces cerevisiae* (558) and copia of *Drosophilia melanogaster* (340) express IN N-terminal to RT in the order of PR-IN-RT-RNH. In HIV-1, genetic rearrangement studies have demonstrated the importance of domain dispositions in Pol region. Inefficient processing is observed with PR is inserted into the MA region, suggesting that Pol sequences outside of the PR domain are important (62,545). Others have shown that only the natural order of domains in a recombinant HIV-1 Pol polyprotein can result in efficient processing at every cleavage site (60).

This suggests that the proper context and folding of each domain in Pol warrants ordered processing of the polyprotein

Translation of full-length Gag-Pol *in vitro* has been found to result in the transitional appearance of processing intermediates as a consequence of primary, secondary, and tertiary cleavage events (Figure 2 and Table 1) (392,393,481,508). Recent evidence has suggested that the immature polyprotein form of HIV-1 PR is responsible for processing initial cleavage sites through an intramolecular (*cis*) mechanism (392). Primary processing of Gag-Pol occurs at the p2↓NC cleavage junction (395,396). This event is followed by secondary and tertiary processing of the transframe region (termed TFR or p6*) at TFP↓$p6^{pol}$ and $p6^{pol}$↓PR, respectively (309,384,399). Since the TFR region negatively regulates PR function, its removal is the concomitant with the appearance of elevated enzymatic activity characteristic of the mature PR (309,311). Subsequent quaternary processing events then occur by an intermolecular (*trans*) mechanism (384,569) to liberate the structural proteins and functional enzymes of the Gag and Pol regions, respectively. Based on these studies, the initial processing events within the Gag region of Gag-Pol give rise to p121, p114, and p107 early intermediates as a consequence of processing p2↓NC, TFP↓$p6^{pol}$, and $p6^{pol}$↓PR cleavage sites, respectively.

Despite our knowledge of HIV-1 Gag processing, there is surprisingly little information available concerning the sequence of events in processing quaternary cleavage sites in HIV-1 Pol. In particular, the events surrounding the generation of p66/51 RT and the changes that accompany the shift from precursor associated RT to this mature form remain poorly understood. Synthetic peptide cleavage studies have implied that the RT↓IN cleavage site is processed most efficiently, followed subsequently by PR↓RT then p51↓RNH (80,517). However, peptide processing efficiencies do not necessarily correlate with the order or efficiencies of polyprotein

processing (Table 1). Processing of the p107 Gag-Pol intermediate (PR-RT-IN) is likely to give rise to p76 (PR-RT) and/or p97 (RT-IN) intermediates in an ordered fashion, prior to the full release of all *pol*-encoded enzymes. Alternatively, all Pol cleavage sites could be processed with equal efficiency, resulting in the simultaneous liberation of PR, IN and both RT subunits, p66 and p51. Current evidence appears to support the first model with the demonstration that processing of recombinant PR-RT and PR-RT-IN results in a respective unimolecular and bimolecular elevation of RT activity (284). While expression of a recombinant p121 intermediate (NC-TFR-PR-RT-IN) also leads to the formation of RT, the size and identity of the two early processing intermediates was unclear (179). An extension of this work demonstrated that inhibition of the PR↓RT cleavage site *in vitro* prevented processing of RT↓IN, but not vice versa (305). This suggests that ordered processing of Pol may proceed first at the upstream PR↓RT site before cleavage at RT↓IN. Interestingly, studies using HIV-1 protease inhibitors have identified the presence of both p76 and p97 intermediates in the lysates of viral particles (246,302,481). While some have suggested that PR↓RT is the first site in Pol to be processed, based on the presence of p14 ($p6^{pol}$-PR) in infected cell lysates (2,302), the presence of the p76 (PR-RT) intermediate is sometimes overlooked (302). Thus, further analysis is needed to clarify the sequence of events in Pol processing. In particular, the ordered generation of p76 (PR-RT) or p98 (RT-IN) processing intermediates from Gag-Pol has not been investigated. Using a recombinant expression system, we and others have shown that the final step in Pol processing involves the excision of RT p66 followed by the formation of RT p51 (179,341,471), however this remains to be clarified in the virion context.

1.4.2 Limitations of model expression systems for Gag-Pol polyprotein processing

In general, the initial events of Gag-Pol processing can be recapitulated in heterologous expression systems. Regrettably, however, many of these model expression systems are inefficient at facilitating complete processing of quaternary cleavage sites from full-length Gag-Pol. Several factors may account for such inherent difficulties. First, processing of truncated forms of Gag-Pol typically excludes the involvement of extra protease domains, known to support HIV-1 PR activation and efficient processing (39,83,183,414,508,567,590). Second, ancillary factors such as cyclophilin A (325,529) or Vif (21) capable of modulating Gag-Pol precursor conformation to expose cleavage sites may be absent. And third, efficient processing of such late cleavage sites may be limited in translation expression systems by inadequate substrate concentration, conformation, or dimerization efficiency (311,355,392,393,567).

In order to circumvent such difficulties, attempts have been made to examine Gag-Pol polyprotein processing in the natural milieu of the virion. Isolated polyproteins derived from immature virus-like particles of ASLV (491) or HIV-1 (265) however, have displayed varying degrees of proteolytic susceptibility *in vitro*. While Gag cleavage sites were efficiently cleaved by exogenously added HIV-1 PR, cleavage sites in Pol were aberrantly processed, if at all (265). These studies suggest that efficient ordered processing of Gag-Pol may depend on the structure and folding context of various domains and the regulated, ordered accessibility of cleavage sites. Inhibitors of HIV-1 PR have also been used in the study Gag-Pol polyprotein processing. However, it is difficult to initiate polyprotein processing after inhibitor treatment, by washing, dialysis, or treatment with detergent or dithiothreitol (86,218,227,246,326). It therefore appears that the key difficulty limiting a direct, reliable assessment of Gag-Pol processing kinetics is our inability to synchronize the maturation of retroviral particles. Future studies should consider

avoiding the use of tight binding protease inhibitors, damaging ultracentrifugation steps, and rapidly replicating chronically infected cells.

1.4.3 Functional consequences of Pol processing

It has been suggested that the release of functional retroviral enzymes is a late, but critical event in the ordered pathway of Gag-Pol processing (267,591) whereby even the most subtle defects can profoundly attenuate viral infectivity (246). To that end, it is reasonable to speculate that ordered processing may protect against the potentially damaging consequences of premature enzyme activation. While advances have been made to explore the functional consequences of Pol processing by mutating protease-recognized cleavage sites, much work remains to be done, particularly in the virion context.

1.4.3.1 Protease (PR)

HIV-1 PR is clearly active in precursor form, when linked to downstream Pol sequences (65,91,305,341), upstream Gag sequences (275,311,508), or both (392,393). Recent evidence has suggested that Gag-Pol is initially processed by an intramolecular mechanism, carried out by an immature polyprotein-embedded form of HIV-1 PR (392,393). While HIV-1 PR also appears to be active in the cytoplasm of infected cells, as seen by the accumulation of processed viral proteins (245), overexpression of PR causes intracellular cleavage of Gag and reduces viral infectivity (321). At first, these observations appear to suggest that processing may have a minor role in the activation of PR. However, regulated autoprocessing from the Gag-Pol precursor is an important stage in the elevation of intrinsic proteolytic activity (310,567,591).

Little is known about the changes that accompany the shift from precursor-associated PR dimer to free enzyme, and the precise role of each in virion maturation. Before PR-mediated processing is observed, the PR of ASLV must be released from the Gag-Pro polyprotein by processing the NC↓PR cleavage site (48). In contrast, following the activation of PR, the initial cleavage event in HIV-1 occurs between p2 and NC (393,395,396). The subsequent series of events that lead to the complete activation of HIV-1 PR, have until recently remained unclear. The generation of the free PR dimer requires processing at two cleavage sites: the N-terminal $p6^{pol}$↓PR junction and C-terminal PR↓RT junction. Early reports, based on processing analyses of recombinant PR-fusion proteins suggested that autocatalytic excision of PR occurs first at the C-terminus followed by the N-terminus (177,341). Others subsequently reported opposing data using essentially the same bacterial expression system (310,311,313,569). Further complicating the matter were the findings that mutation of the $p6^{pol}$↓PR cleavage site did not affect autoprocessing (591) or the specific activity of PR (73,267), while mutations that block the C-terminal processing site may or may not limit the processing of other cleavage sites (305,310) or affect proteolytic activity (310). If anything, these results serve to show just how unreliable heterologous expression systems can be at mimicking the natural course of events, particularly when truncated polyproteins are used.

In an attempt to address the question whether a complete release of PR is needed to facilitate polyprotein processing, the full-length form of Gag-Pol has been studied. *In vitro* translation expression systems showed that after cleavage of p2↓NC junction, an extended form of PR completes the final processing steps by an intermolecular mechanism (392,393). Data acquired from a virus expression system further supports these findings, that PR must be expressed as an integral component of full-length Gag-Pol precursor for optimal processing

(591). It appears as well that removal of N-terminal Gag sequences does indeed elevate proteolytic activity, since mutation of the $p6^{pol}$↓PR cleavage site attenuates viral infectivity and Gag cleavage (508,591). However, the finding that $p6^{pol}$-PR fusion proteins readily exist in mature virions (2,302) suggests that the N-terminal junction may not require complete processing for sufficient quantities of active PR. Further confirming that N-terminal cleavage is needed for PR activation is the finding that mutation of the C-terminal PR↓RT cleavage site does not adversely affect the processing of viral polyproteins (65,66). While extended PR intermediates appear to have a transient function in the proteolytic cascade, fully released PR may ultimately be physiologically, if not structurally important in the virion.

1.4.3.2 Reverse transcriptase (RT)

The concept of whether processing plays a role in RT activation appears to vary amongst different retroviruses, as does the manner in which the Pol region in processed (Figure 8). It has been proposed that ordered processing of Gag-Pol is an ideal means to prevent premature reverse transcription before budding (562). While this notion holds true for ASLV, a retrovirus that shows little proteolysis before budding (489), this is not necessarily the case for MLV or HIV-1 (77,389). In studies utilizing unprocessed viral RT, the polymerase activity varies between MLV (77), ASLV (76,488), and HIV-1 (243,308,388) from 15-100% of wild-type levels.

Proteolytic processing in ASLV differs from most retroviruses in the fact that the Pol region is not completely processed. Proteolysis of ASLV Gag-Pol by the Gag-Pro-encoded PR (76) generates more PR, 63 kDa (α) and 95 kDa (β) RT subunits, an independent p32 IN domain (1,166,193), and an unstable dispensable C-terminal 4.1 kDa protein (1,164,252). Incomplete processing is demonstrated by the fact that IN forms an integral part of the RT β subunit. In this

system, viral PR activity appears to be required for complete activation of RT. Although precursor-associated forms of ASLV RT are active, processing elevates this level significantly (76,488,490). Mutational studies have revealed a need to process the N-terminal PR↓RT junction for complete activation of RT (76,488), whereas the C-terminal RT↓IN junction is dispensable (193). In MLV, the Pol region is completely processed at two cleavage sites giving rise to a single RT protein of 78 kDa. Interesting though, processing of either N-terminal PR↓RT or C-terminal RT↓IN junction is not required for activation of RT in either the virion context (77,250) or in heterologous expression systems (195,496).

Processing of HIV-1 RT is further different from either of the above systems, whereby three cleavage sites are processed: the N-terminal PR↓RT junction, the C-terminal RT↓IN junction, and an additional internal cleavage at the p51↓RNH junction. Mature HIV-1 RT is composed of 66 kDa and 51 kDa subunits, even though its gene encodes a 66 kDa protein. The smaller subunit is derived from the larger 66 kDa subunit by proteolysis at the p51↓RNH cleavage site, resulting in the release of a 15 kDa ribonuclease H (RNH) domain. While precursor associated forms of recombinant HIV-1 RT (29,211,243,388,485), and full-length Gag-Pol in immature virions (15,162,243,388) arguably exhibit some level of RT polymerase activity it is unclear how functional they are at completing reverse transcription. In order to reach a firm conclusion on what processing events are needed for full functionality of RT, additional information is required.

Of the three cleavage sites for RT, only the N-terminal PR↓RT junction has been investigated in the virion context. Inhibition of this cleavage site results in the expected 77 kDa and 62 kDa PR-RT fusion proteins with no adverse affect on either PR or RT activity, although viral infectivity is diminished (65,66). The importance of the C-terminal RT↓IN cleavage site in

the virus is currently unclear. Further complicating the matter, are disputable reports that recombinant RT-IN fusion proteins may exhibit some level of RT polymerase activity (211,293). Mutation of the p51↓RNH cleavage site generally results in stable recombinant p51-RNH (RT p66) fusion proteins, although overprocessing to RT p51 is occasionally observed (203,239,332). In spite of these observations, the necessity for each of these cleavage sites in the activation of RT remains undefined. In particular, what importance does the internal RT p51↓RNH cleavage site serve, particularly since such a junction does not exist in other retroviral forms of RT. A comparative analysis is clearly needed to examine the role of each of these Pol cleavage sites in efficient viral replication.

1.4.3.3 Integrase (IN)

Proteolytic regulation of IN activity has not been clearly investigated in all retroviruses. Both ASLV (1,193) and HTLV-I (521) generate IN as part of the β subunit of RT (αβ or ββ) and as an independent 32 kDa protein. Both forms of ASLV IN are processed at an N-terminal RT↓IN junction as well as at their C-termini to remove a non-essential 4.1 kDa fragment (1,164,252). Both forms of ASLV IN also localize to the nucleus of infected cells, indicating equal involvement in the transportation of PICs (551). Further studies have shown that IN 3'-end processing activity is present and even similar in both forms (101,155,166,292,507). However, the p32 form of IN is 30-fold more efficient in strand transfer activity (551) suggesting that it is primarily responsible for efficient integration of proviral DNA in an infected host cell. In contrast, proteolytic processing in both HIV-1 and MLV results in the complete separation of IN from Gag-Pol in the form of p32 and p46 respectively (291). Although additional cleavage sites have been predicted within HIV-1 IN (69), recombinant IN (519) and its core domain (111) are

resistant to further proteolysis. Additional studies have suggested that HIV-1 p32 IN may be released from a recombinant Pol polyprotein prior to formation of the p66/51 RT heterodimer (49,284,293,471). However, it is unclear whether the RT↓IN junction is processed before the PR↓RT junction and what effect this event has on regulating IN activities *in vitro*, or in the virion context. It therefore remains to be resolved whether extended Pol intermediates of IN are functional, as this would provide insight into the need for HIV-1 to remove the abutting RT domain.

1.5 REVERSE TRANSCRIPTASE

1.5.1 General features of reverse transcription

An essential step in the life cycle of HIV-1, as in other retroviruses is the conversion of genomic single-stranded RNA into proviral double-stranded DNA prior to integration into the genome of the infected host cell. While this conversion is highly complex, all of the chemical steps in this conversion are catalyzed by a viral-encoded multifunctional enzyme known as reverse transcriptase (RT). Since its original discovery within the context of a retroviral particle (20,505), sequence similar forms of RT has been found in a variety of organisms (574,575). Such systems include: retroviruses, hepadnaviruses, caulimoviruses, retrotransposons, retrons, and mitochondrial introns. The main enzymatic activities associated with HIV-1 RT include DNA polymerase activity on both DNA and RNA templates as well as ribonuclease H (RNH) activity, specific for RNA in RNA/DNA hybrids. By coordinating its activities, RT facilitates RNA-

dependent DNA polymerase (RDDP) synthesis of minus-strand DNA from genomic viral RNA, DNA-dependent DNA polymerase (DDDP) synthesis of plus-strand DNA from minus-strand DNA, and formation and removal of plus-/minus-strand RNA primers by both endonucleolytic and 3'-5' exonucleolytic cleavage mediated by the RNH domain (298,443). By removing the RNA template during DNA synthesis, RT can also facilitate a jump from one template to another or assist in strand transfer in order to complete reverse transcription (499).

The polymerase activities of all forms of RT are infamous for being error-prone by virtue of the fact that they lack formal proofreading editing ability and show low fidelity in incorporating correct nucleotides (411,428). Consequently, these attributes are reflected in the high mutation rate of these viruses *in vivo*, resulting in genetically diverse populations. Average mutation frequencies typically range from 10^{-4} to 10^{-3}, or about 10 base changes per retroviral genome per replication cycle (350). From an immunological standpoint, such rapid changes in the genetic makeup of HIV-1 may contribute towards the progressive and prolonged nature of infection in an individual host, thus allowing it to evade destruction (93). However, in comparison to cellular polymerase enzymes, absent proofreading activity and an ability to incorporate chemically altered nucleotide analogs have made HIV-1 RT a favourable pharmaceutical target.

In theory, reverse transcription can be completed by a single molecule of RT in coordination of its polymerase and RNH activities. Equally possible are the uncoupling of these activities on different molecules of RT to facilitate reverse transcription (94,216,503). Somewhere in between these two extremes lies the truth in what actually occurs during reverse transcription *in vivo*. Processivity of HIV-1 RT polymerization is intrinsically low on its natural heteropolymeric template in comparison to poly(rA) homopolymeric templates (216). Therefore,

to ensure completion of all of the various stages of viral DNA synthesis an excess quantity of RT may be ideal. Early estimates using SDS-PAGE or competition enzyme immunoassays predicted a 20-100-fold excess of RT molecules per particle of ASLV or MLV (375,376,493), which was also confirmed by indirect measurement of pulse-chase profiles in infected cells (182,226). Scanning transmission electron microscopy has recently demonstrated that the individual mass of RSV virions in a population is heterogeneous, and corresponds to about 1,500 Gag molecules per virion (539). Using densitometry analysis to scan immunoblots under sub-saturating conditions, we have proposed that the quantity of RT in HIV-1 may be lower than expected in virions produced from COS-7 transfected cells (data not shown). This would be consistent with the observation that 20-30 enzymatically active RT molecules in phenotypically mixed virions are sufficient to successfully complete reverse transcription upon infection (236). Further study may yet determine the absolute number of RT molecule per virion and whether cell-line dependent attributes such as translation and packaging efficiency affect the number of Gag-Pol molecules that are incorporated.

During infection, viral particles are internalized and the viral capsid is uncoated through its association with a cellular chaperone known as cyclophilin A (CypA) (317). Although limited synthesis of minus-strand viral DNA has been documented in virions (307,523), reverse transcription in its entirety, takes place within the cytoplasm of the infected cell after entry of the viral core (258). In spite of the fact that there are two copies of genomic RNA per virion, only a single provirus is generated per infection (377). After reverse transcription is complete, the newly synthesized viral DNA migrates to the nucleus in the form of a nucleoprotein complex. This preintegration complex (PIC) appears to contain viral RNA and DNA in association with viral proteins (MA, NC, RT, IN, Nef and Vpr) (46,123,329,356) and cellular proteins (LEDGF,

BAF, Ini1, HMG-I) (524), although their structural association and stoichiometry remains undefined. It appears that transportation of HIV-1 PICs to the nucleus may be mediated by MA (42,44,139), Vpr (208,358,463), and/or IN (138). While there is no evidence for catalysis of reverse transcription by any other protein but RT, a number of viral proteins appear to play important roles. It is believed that NC can provide a chaperone-like function by acting as a single-stranded binding protein, while protecting the nucleic acid template strand from nucleases (549). Current data suggests that NC may promote annealing of the $tRNA^{Lys,3}$ primer (295), enhance RT processivity (99), modulate RNH activity (386), catalyze strand transfer and template switching (54,215,386,581), and stimulate integration (57). Interestingly, the ability of NC to promote reverse transcription may require further proteolytic cleavage, suggesting a possible role for PR at the preintegration stage (349). Recent evidence has further showed that IN directly interacts with RT (185,588) and that this interaction is essential for efficient initiation (573), but not processivity (185) of reverse transcription. Finally, other reports have suggested that Nef may influence the amount of viral DNA produced (451), and that Vif may have an as of yet undefined role in reverse transcription (156,480).

1.5.2 Molecular structure of HIV-1 RT

The gene for RT encodes a 66 kDa protein, however the presumed biologically relevant form of HIV-1 RT in both virions and infected cells (298,533) is a heterodimer consisting of a 66 kDa (p66) and 51 kDa (p51) subunit in a 1:1 stoichiometry (418,485,533). While both subunits share a common N-terminus (298,533), the p51 subunit has a C-terminal truncation of its RNH domain (15 kDa) at residue F440 (178,512), suggesting that it is derived from the larger subunit by proteolytic cleavage (298,450). An extensive number of three-dimensional structures of RT

have been resolved by X-ray crystallography. These structures have provided great insight into the molecular structure of HIV-1 RT, as well as the conformational changes associated with the mechanism of DNA polymerization. The general anthropomorphic shape of HIV-1 RT may be likened to that of a "right hand", composed of fingers (residues 1-85, 118-155), palm (residues 86-117, 156-237), thumb (residues 238-318), and connection (residues 319-426) subdomains that define the polymerase domain (Figure 6). Adjoining the connection subdomain in the RT p66 subunit is the C-terminal RNH domain (residues 427-560). The HIV-1 RT heterodimer is asymmetric since the relative orientation and spatial arrangement of its respective subdomains in both p66 and p51 subunits are different. As a result, the subunit interface on p51 involves different amino acid residues than on p66. The overall picture that emerges is that of a flexible molecule, able to grasp the nucleic acid template between its fingers and thumb and thread the remainder across its putative binding cleft and in through the active site of the RNH domain (209,224,263,430,546).

The biologically relevant form of RNH resides in the C-terminal region of the RT p66 subunit, as determined by immunoaffinity analysis (178,205), activated gel analysis (333), and sequence alignment with related counterparts (97,230). Although a role for p15 RNH in the viral life cycle was once suggested (178), it very likely to be degraded in the virion. The crystal structure of the RNH domain of HIV-1 RT (85), released by proteolytic cleavage *in vitro* exhibits general folding that is quite similar to the structure of *E.coli* RNH and a model of MLV RNH (117,248,353,576) (Figure 7). This is remarkable, considering that their comparative amino acid homology is less than 30% (446). The RNH domain appears to be comprised of two subdomains separated between β1 and β4 strands. The left subdomain consists of β1, β2, β3, αE

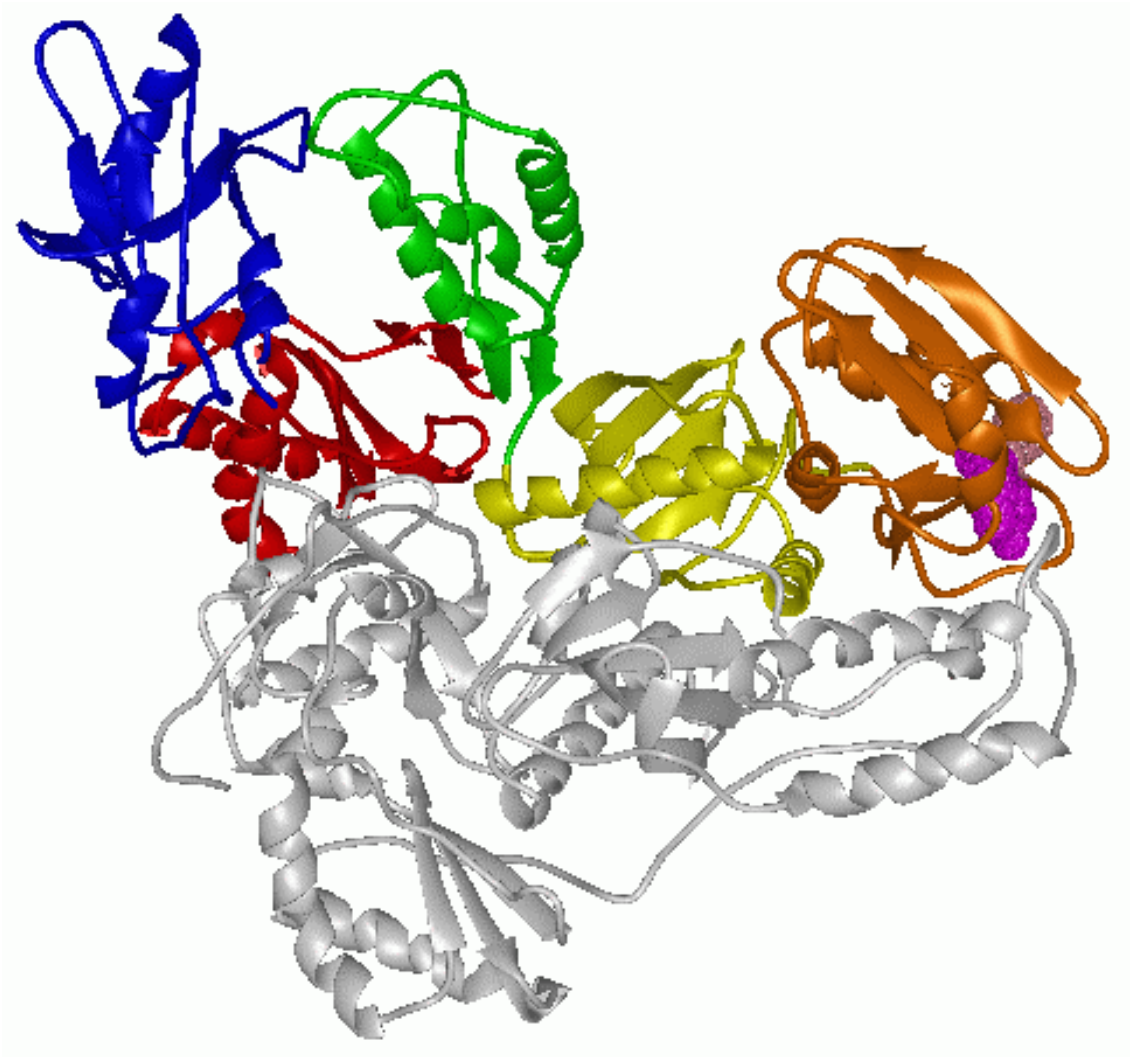

Figure 6. Molecular structure of HIV-1 reverse transcriptase. Representative unliganded polypeptide schematic of the p66/51 RT heterodimer generated from the crystal coordinates of PDB file 1LDO (2.7 Å resolution) (209). Secondary structural features, α-helices and β-strands are represented by coils and arrows, respectively. The gene for HIV-1 RT encodes a 66 kDa protein, however the presumed biologically relevant form is an obligate asymmetric heterodimer consisting of a 66 kDa (p66) and 51 kDa (p51) subunit in a 1:1 stoichiometry. HIV-1 RT is composed of fingers (blue), palm (red), thumb (green), and connection (yellow) subdomains that define the polymerase domain in the RT p66 subunit. These same subdomains adopt a different folding pattern in the RT p51 subunit (white), which is thought to have a primarily structural role. Adjoining the connection subdomain in the RT p66 subunit is the C-terminal RNH domain (orange). The protease recognized cleavage site (p51↓RNH) which generates the p51 subunit is located at residues F440↓Y441 (spacefilled pink), and is inaccessible in the p66 subunit of the mature p66/51 RT heterodimer. *(Modified from: Hsiou, Y. et al 1996 Structure 4(7):853).*

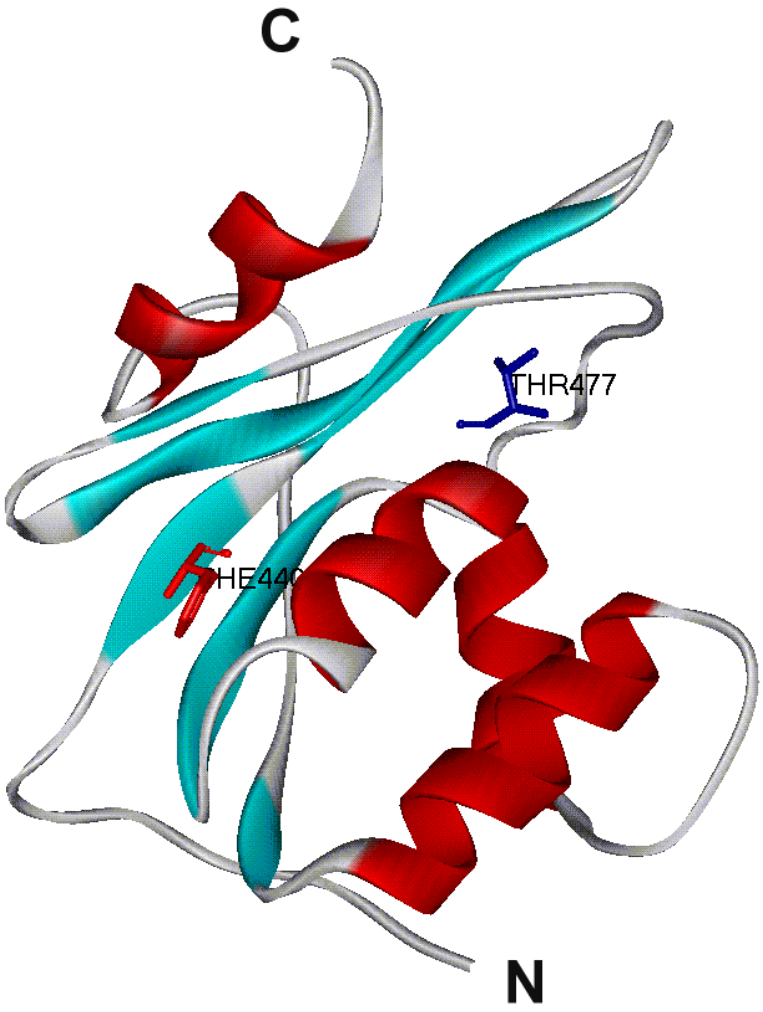

Figure 7. Molecular structure of the ribonuclease H domain of HIV-1 reverse transcriptase. Representative unliganded polypeptide schematic of RT residues 400-560 generated from the crystal coordinates of PDB file 1LDO (2.7 Å resolution) (209). The RNH domain is composed of two subdomains separated between β1 and β4 strands along a putative cleft (indicated by a forward dividing slash). The left subdomain consists of β1, β2, β3, αE and connecting loop while the right subdomain comprises β4, β5, αA, αB, αD and connecting loops. Both the polymerase domain of RT p66 and the thumb subdomain of RT p51 establish important structure-function tertiary contacts with the right subdomain of RNH in RT p66. The remaining p51↓RNH cleavage site at residues F440↓Y441 is situated in the first β strand of the left subdomain. *(Modified from: Hsiou, Y. et al 1996 Structure 4(7):853).*

and connecting loop while the right subdomain consists of β4, β5, αA, αB, αD and connecting loops. Neutron diffraction data obtained from selectively deuterated molecules of HIV-1 p66/51 RT indicate that the RNH domain has both intra- and inter-subunit contacts (286). The polymerase domain of the RT p66 subunit interacts with its RNH domain in a contact area consisting of at least β5, αB, αD and the two connecting loops (85), while the thumb subdomain of p51 establishes additional interactions with opposing RNH domain that are important for structure and function.

A number of overlapping regions within the RNH domain are of key importance in the maturation, structural stability, and function of RT. The connection subdomain (residues 319-426) plays a major role in dimer formation by establishing contacts between both p66 and p51 subunits. It also links the polymerase domain of p66 to its RNH domain (263), and has a modest role in binding and positioning the hybrid RNA-DNA substrate (237). Although sometimes described synonymously as the connection subdomain, the tether region (residues 394-423) is generally believed to be a non-functional linker region that joins the polymerase domain to the RNH domain (223,506). This region of RNH is rather malleable to mutagenic alteration (223), and composes an energy minima that serves as a hinge site to control global rotational reorientations of the RNH domain (506). One area of continued debate however, is which residue represents the actual beginning of the RNH coding region. Partial proteolytic maturation of RT results in the liberation of an RNH domain beginning at residue Y441 and the formation of the p66/51 RT heterodimer (169). The general inactivity of the free RNH domain (205) and its sequence comparison with related forms (97,230,576) would suggest that Y427 may be the start site in the p66/51 RT heterodimer, although additional N-terminal residues may be needed for substrate binding. These findings would suggest that the putative p51↓RNH cleavage site at

F440↓Y441 does not exactly delineate between the polymerase and RNH domains and may instead be relevant to the maturation of RT. While the p51↓RNH cleavage site itself is highly conserved, a number of surrounding residues are naturally polymorphic (88). Although residues 427-433 in this region loop over strands β4 and β5, and residues 430-440 form what may be a protease-sensitive region (85,354), the p51↓RNH cleavage site is actually buried within the context of β1 in the RNH domain of the p66/51 RT heterodimer (Figure 6). Further important, are residues 424-429 and 434-435 which appear to make stabilizing interactions with the thumb subdomain of p51 and other RNH residues (332,338,506).

Finally, there are two major differences between the structure of HIV-1, and *E.coli* or MLV RNH. First, the HIV-1 RNH domain is missing an 8 residue positively charged helix αC, and adjoining basic loop between αB and αD. In *E.coli*, the αC helix of RNH is crucial for binding and positioning of the nucleic acid substrate (242), while in MLV it is additionally important for RNH cleavage *in vivo* during reverse transcription (300,504). These results suggest that the absence of this helix from the RNH domain of HIV-1 RT may be why $tRNA^{Lys,3}$ primer sequences are incompletely removed during reverse transcription, as compared to other forms of retroviral RT (556). Secondly, studies have shown that the C-terminal helix αE (residues 543-553) and the preceding basic loop (residues 537-542) are disordered in the HIV-1 RNH domain (85,405). This loop contains a histidine residue (H539) that is highly conserved among the RNH family of enzymes (230), and a mutation at this position severely reduces RNH activity (442). Since this region in HIV-1 and *E.coli* RNH has been implicated in substrate binding (152,442), its correct positioning in HIV-1 RT may require interactions from either p66 or p51 polymerase domain or both. The role of the basic loop/helix αE in HIV-1 p66/51 RT appears to be in

directional processing and strand transfer of the nascent minus-strand DNA between RNA templates (150,566), as well as in stabilization of the RT-substrate interaction (327).

It is generally believed that there is a functional interdependence between both polymerase and RNH domains of HIV-1 RT that is unapparent in MLV RT (496). Mutagenesis studies have shown that some linker insertions or point mutations in the polymerase domain can affect RNH activity, and similarly vice versa (194,196,238,409). The physical interconnection of both polymerase and RNH domains and their interaction with the nucleic acid template-primer suggests that they may appear to be structurally distinct but are functionally interdependent. Specifically, a number of significant contacts are made between the RNH domain, the connection subdomains of both p66 and p51, and the p51 thumb subdomain (95,263,338). The interaction between the extended thumb of p51 and the RNH domain of p66 may be required for stability, conformational change, proteolytic maturation, and for activation of polymerase and RNH activities of the p66/51 RT heterodimer (338) and the isolated RNH domain (205). Current research has provided extensive biochemical and genetic evidence supporting both coupling (137,159,320,565) and uncoupling (94,216) of RT polymerase and RNH activities. Coupled coordination of these activities would suggest that as polymerization proceeds, the template RNA is being removed from the RNA-DNA hybrid 18-19 nucleotides away in the active site of RNH (159,249). While these studies imply that template degradation can occur in two modes, one dependent and the other independent of DNA polymerization it is unknown what happens in the virion context. The uncoupled mode may be sufficient for low level viral replication (503), while both modes could be used simultaneously to remove the template RNA prior to the first strand transfer (387).

1.5.2.1 Role of HIV-1 RT subunits

The role of each subunit within the p66/51 RT heterodimer has been investigated in a variety of studies. By selectively mutating opposing active sites in either p66 or p51 subunits, HIV-1 RT has been reconstituted and studied in recombinant form (204,285,315), and *in vivo* using a Vpr-mediated *trans*-complementation approach (343). In all of these reports, it has been demonstrated that the p51 subunit cannot compensate for inactivating mutations in the p66 subunit of p66/51 RT. Thus, the catalytic residues of p51 do not directly contribute towards activity. This interpretation is supported by structural data of p66/51 RT which show that these residues are buried within the "closed" folded conformation of the p51 subunit, suggesting that this subunit largely plays a structural role in RT (4,224,263,501). Additional roles of the p51 subunit may entail positioning the $tRNA^{Lys,3}$ primer for binding and reverse transcription initiation (10,100,225,338,584), increasing the processivity of DNA synthesis (214), facilitating template-primer binding (181,323), as well as enhancing strand displacement DNA synthesis (206) and modulating RNH activity (54,225,338,453,454,474). Most mutagenic insertions into the p66 subunit of RT inactivate polymerase function, indicating that a functional p66 subunit is imperative. Additional studies employing activity gel analysis (485) or substrate analogue cross-linking (63) have verified that only the p66 subunit is labeled. The p66 subunit therefore constitutes the catalytically competent subunit of the p66/51 heterodimer and fittingly adopts a clearly defined nucleic acid binding cleft within its polymerase domain (224,263,285). The putative polymerase active site of the p66 subunit is composed of a catalytic triad of aspartic acid residues (D110, D185 and D185) positioned near the primer strand 3'OH terminus (224). These residues act to coordinate Mg^{2+} ions in the nucleophilic attack of the oxygen atom of the 3'OH

primer terminus to the α-phosphate of the incoming dNTP, resulting in the liberation of pyrophosphate (PPi) and the extension of the primer by one nucleotide.

1.5.2.2 Role of the RNH domain

The role of the RNH domain in reverse transcription has been investigated by site-specific mutagenesis and the use of defined RNA-DNA hybrids. While mutational studies have highlighted an interdependent functional relationship between DNA polymerization and template degradation, they have also identified important catalytic residues in RNH (238,334,408,442,513), and implicated this domain in the structural maintenance of RT (194,332,333). Clustered within the active site of RNH are four conserved acidic residues (D443, E478, D498, D549) which are involved in the coordination of Mg^{2+} or Mn^{2+} ions. During and after minus-strand DNA synthesis, RNH by definition, acts to degrade the plus-strand viral RNA genome of RNA-DNA hybrids through the cleavage of phosphodiester bonds. It is thought that RNH makes both specific and nonspecific cleavages to provide two important functions during reverse transcription, the removal of the RNA template strand to prepare for plus-strand DNA synthesis and both template jumps, and the formation and removal of RNA primers. While the products of template degradation are heterogeneous, several RT molecules are believed to bind after minus-strand DNA synthesis to degrade all RNA fragments except for the polypurine tracts which are used initiate plus-strand DNA synthesis. The analysis of labeled cleavage products arising from RNA-DNA hybrids have indicated that RT-associated RNH may utilize a combination of endonuclease and 3'-5' exonuclease activities to carry out its responsibilities (137,443). Based on these types of studies, it appears that the internal structure of the RNA-DNA hybrid, and the structure extending beyond the RNA 5' end are what determine the site and

efficiency of cleavage (371,561). To permit cleavage of the RNA strand, the hybrid duplex adopts a change in structure from A-form to B-form between the polymerase and RNH active sites (9). A unique stepwise mechanism of RNH-directed cleavage has been proposed, in accordance with the rates of each cut. To remove the RNA left after polymerization, RT positions the RNH active site approximately 18 nucleotides from the RNA 5'-end before making the primary cut. The enzyme then rebinds or slides to make a secondary cut, 8 nucleotides from the RNA 5'-end. Afterwards, RT binds the new 5'-end of the RNA created by the first primary or the secondary cuts in order to make the next primary cut (559-561).

1.5.2.3 Recombinant RT

Evidence that RT in mature infectious HIV-1 virions is a heterodimer comes from immunoaffinity (307,314,512,533) and sequential ion exchange-affinity-adsorption purification studies (174,178,568). Virus particles were shown to contain a roughly equal proportion of 66 kDa and 51 kDa protein subunits, suggesting that this stoichiometry may be important for infectivity (485,533). However, recombinant HIV-1 RT can be prepared in multiple active oligomeric forms, including p66/51 heterodimers as well as p66/66 and p51/51 homodimers (Table 3). It should be noted, that in their immunoaffinity purification of p66/51 RT, most studies make the assumption that the epitopes of all possible oligomeric forms are equally accessible. Interestingly, some studies with HIV-1 (18,178), FIV (361), SIV (270,525) and chimeric SIV/HIV-1 (319,525) have reported the immunoaffinity isolation of more RT p66 than RT p51. Using several anti-RT monoclonal antibodies developed in our laboratory, we have shown that epitope binding in solution can differ between homodimeric and heterodimeric forms of HIV-1 RT (294). While we are not disputing the fact that the RT heterodimer may be the most

predominant form of RT in the virion, the existence of alternative oligomeric forms during virion maturation has not been fully investigated.

Table 3. Activities and stabilities of alternative oligomeric forms of recombinant HIV-1 reverse transcriptase.

[a] Specific RNA dependent DNA polymerase (RDDP) activity from a poly(rA)-oligo$(dT)_{12-18}$ template-primer (128)
[b] Present (+) or absent (-) of RNH activity as determined from a poly(dC)-poly([^{3}H]G) DNA-RNA hybrid (128).
[c] Dissociation constant as determined from urea denaturation isotherms (472)
Values chosen for [a, b, c] are from studies in which all three oligomeric forms of recombinant RT were simultaneously examined. Data not determined is represented by N/D

RT form	RDDP activity [a] (pmol/min/μg)	Relative to WT	RNH activity [b] (pmol/min/μg)	Present (+)/ Absent (-)	K_D [c]	Relative to WT
p51/51	8.5 ± 1	53-fold	N/D	-	6.7×10^{-4}	4467-fold
p66/66	150 ± 10	3-fold	N/D	+	2.7×10^{-6}	18-fold
p66/51	450 ± 50		N/D	+	1.5×10^{-7}	

1.5.2.3.1 Monomeric HIV-1 RT

Since RT p66 contains all of the sequence information needed for activity, monomeric forms of RT should be active, but are not (22,52,418,419). One proposal suggests that the conformation of HIV-1 RT monomer is closed, similar to the catalytically inactive p51 subunit in the p66/51 RT heterodimer (204,263,314,546). Dimerization of RT monomers can essentially activate RT by providing the free energy required to convert one of the subunits to an open conformation, similar to the active p66 subunit in p66/51 RT (546). Under dilute conditions, recombinant RT

p66 is monomeric in solution, resistant to processing, and exhibits low specific activities (92). Monomeric forms of recombinant RT p66 can also be generated by the introduction of dimerization inhibiting mutations, such as L234A or W236A (471,494). In virions however, these mutations diminish Gag-Pol incorporation and cause aberrant processing of RT (582), suggesting that formation of RT may not be as simple as the association of two separate monomeric subunits. Linker insertions and substitution of the connection subdomain or RNH domain from MLV (196,331,373,409) or FLV (457) into HIV-1 RT has also been found to result in the formation of active chimeric monomers of HIV-1 RT that are resistant to proteolytic cleavage. These studies suggest that interactions between the β7-β8 loop of p51 and the palm subdomain of p66 during dimerization are essential for activation of RT by inducing formation of the polymerase binding cleft. In this regard, the added length between the polymerase and RNH domain in the chimeric MLV/HIV-1 RT monomer could permit movement of the latter domain to support the former (373).

1.5.2.3.2 p66/66 RT

Recombinant p66/66 RT homodimers can be readily generated and purified in the absence of HIV-1 PR (128,283). *In vitro*, p66/66 RT homodimers are catalytically active, and exhibit significant polymerase (23,128,204,206) and RNH activities (128,178) comparable to the p66/51 RT heterodimer (Table 3). Although p66/66 HIV-1 RT could equally be active in virions, this has not been demonstrated. The only evidence that p66/66 RT may be functional *in vivo* was demonstrated in *Saccharomyces cerevisiae,* with the substitution of the Ty1 retrotransposon (359,360). Although it was originally suggested that the p66/51 RT heterodimer is far more stable that the p66/66 RT homodimer (418), recent evidence has confirmed a difference in

stability of 18-fold (Table 3) (128,472). Due to practical difficulties associated with subunit-selective mutagenesis of p66/66 RT, and the lack of a reliable crystal structure it remains unclear whether both p66 subunits are functional. Current evidence appears to suggest that either subunit in p66/66 RT is capable of primer binding (22), and that switching of active subunits may be possible (458). This would suggest that p66/66 RT may adopt a quasi-stable structure. Alternatively, it has been proposed that p66/66 RT may adopt an asymmetric structure similar to the p66/51 RT heterodimer whereby one RNH domain is partially unfolded to permit proteolytic cleavage (203,223,458). Similarities in RT polymerase and RNH activities between recombinant p66/51 RT and p66/66 RT would tend to support such a scenario.

1.5.2.3.3 p51/51 RT

Recombinant RT p51 can be expressed and purified in much the same manner as other oligomeric forms. However, conflicting studies exist as to the extent to which this RT p51 active, ranging from completely inactive (178,204,285,485), weakly active (29,128,199,346,409,418), or substantially active (100,104,308,509,512). The contradictory nature of these reports can be attributed to oligomeric state of RT p51 studied. While p51/51 RT homodimers are believed to exhibit polymerase activity far lower than that of p66/66 RT or p66/51 RT, monomeric forms may be inactive (Table 3). Furthermore, DNA-dependent DNA polymerase activity of recombinant p51/51 RT is 13-50-fold more efficient than its RNA-dependent DNA polymerase activity (128,217,509). Recent evidence has indicated that p51 RT subunits do not readily dimerize (26,418), and that p51/51 RT homodimers are on average about 4500-fold less stable in solution than p66/51 RT heterodimers (Table 3) (128,472). In this regard, the use of low concentrations of p51 RT and thus p51/51 RT homodimers could account for low DNA

polymerase activity. However, significant DNA polymerase activity has been associated with the isolated p51 RT subunit under dilute subunit concentration conditions ranging from 20 to 200 nM (24,100,128). We have recently demonstrated by radiation inactivation analysis, that while RT p51 is a monomer even at high concentrations, a homodimeric form of the enzyme is induced in the presence of a nucleic acid template-primer substrate (473). Finally, since active p51/51 RT homodimers displays no significant difference in substrate affinity compared to p66/51 RT (24), both enzymes may adopt similar conformations. While p51/51 RT-catalyzed polymerization is possible in the absence of an RNH domain (443), it is clear that the presence of this domain in p66/51 RT enhances processivity (24).

1.5.2.3.4 Recombinant RNH

Unlike the isolated RNH domain of MLV and *E.coli*, most isolated forms of recombinant HIV-1 RNH are inactive (25,85,484) or exhibit extremely low levels of activity (174,176,179,450). It is hypothesized that inactivity associated with the isolated RNH domain of RT is attributable to (i) the absence of critical binding residues (N-terminal tryptophan-rich region of p51 or lack of basic helix αC/loop), (ii) dynamic differences or (iii) structural differences from the RT-associated RNH domain (C-terminal basic loop/helix αE region).

In effort to study the structure of HIV-1 RNH, attempts have been made to reconstitute the activity of the isolated RNH domain of HIV-1 RT. Sequences N-terminal to the putative p51↓RNH cleavage site have been introduced with mixed results (residues 427-560 or 400-560). In general, it appears that natural N-terminal sequences are unable to restore activity (25), while non-HIV-1 purification tags work well (115,116,475,484). Reconstitution of RNH activity has

also been observed by the addition of purified RT p51 (115,205,474), or introduction of the basic helix αC/loop structure of *E.coli* RNH into the corresponding position of the isolated HIV-1 RNH domain (253,482). Although the exact mechanism of compensation is presently unclear, it is believed that the basic nature of these additions may restore RNH activity by enhancing substrate binding affinity. Early reports have further indicated that inactivity associated with the isolated RNH domain of HIV-1 RT may be due to its extreme dynamic behavior (254,324). However, a recent study using innovative NMR relaxation techniques and a physiological pH found that the statistical differences in dynamic behavior are marginal (342). Finally, the invariant H539 residue in the basic loop/helix αE region of RNH, important for substrate binding, appears to be disordered in crystals of the isolated RNH domain (152,442). While this region can be stabilized by the presence of divalent metal ions (379), it is unknown whether this alone is enough to restore activity to the isolated RNH domain.

1.5.2.4 Other retroviral forms of RT

A comparative examination of other retroviral forms of RT may permit some comprehension of what have necessitated the generation of the p66/51 RT heterodimer in HIV-1. A number of groups have investigated the origins and phylogenetic relationship among retroviruses, by comparing amino acid sequence divergency in such regions as reverse transcriptase. Although functionally similar, all known RT enzymes are architecturally diverse and have low to moderate primary sequence identity and similarity (97,230,402,574). Lentiviruses appear to possess the most sequence similar forms of RT, while progression from type B to D retroviruses coincides with a divergence in oligomeric subunit composition and structure to include monomers and homodimers (Figure 8). Retroviral RTs of different vertebrate

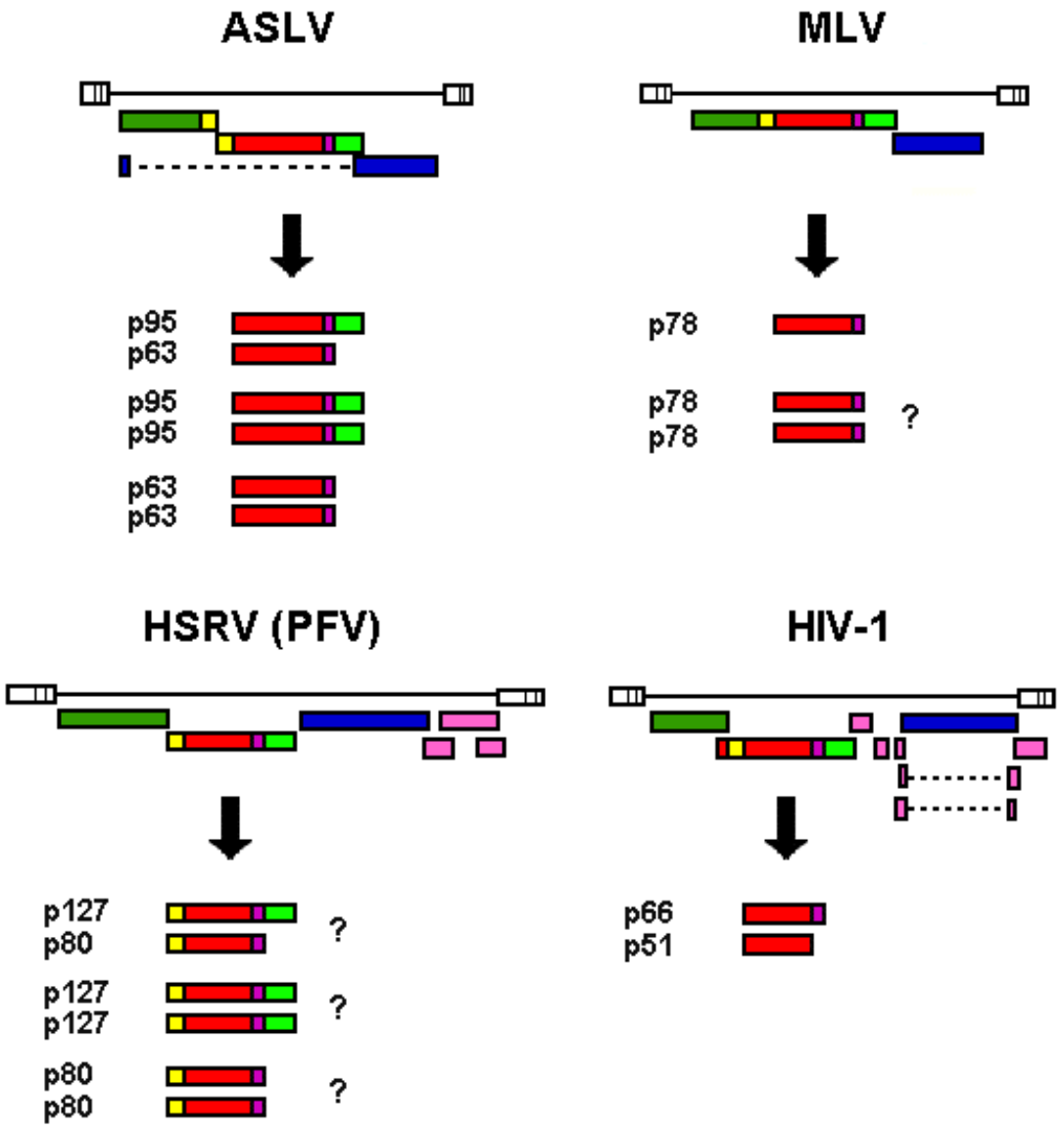

Figure 8. Biosynthesis and subunit relationship of various retroviral reverse transcriptase enzymes. Pol enzymes are colour coded for clarification of RT subunit composition: PR (yellow), RT polymerase (red), RNH domain of RT (liliac), and IN (lime green). ASLV RT is principally a heterodimer, consisting of an incompletely processed RT-IN subunit (β) and a smaller RT subunit (α). Up to 45% of ASLV RT is additionally comprised of completely processed RT homodimers (αα) and unprocessed RT-IN (ββ) homodimers (165,197). The fully processed form of MLV RT is a monomer is solution, although it may dimerize upon binding to a nucleic acid substrate (148). While the oligomeric composition of HSRV (PFV) RT remains unclear, recombinant RT includes homodimeric or heterodimeric forms with PR-RT-IN and PR-RT polypeptides (261). In lentiviruses, such as HIV-1, the biologically prevalent form of RT is a p66/51 heterodimer, generated by proteolytic processing of three cleavage sites. The smaller RT p51 subunit is derived from p66 and has a C-terminal truncation of its RNH domain (512).

origin generally exhibit distinctive properties *in vitro* with respect to metal requirement, template preferences, processivity, and error rates. However, consistent with their evolutionary relatedness, the structures of RT active sites are highly conserved and retain the YXDD catalytic motif in their polymerase domain. Chimeric studies have further provided structure-function insights into different species-specific forms of RT (4,13,455,501), while also demonstrating that they can substitute for each other to facilitate reverse transcription (11) and viral replication (432,477).

1.5.2.4.1 Lentiviral RT

Aside from HIV-1, the lentiviral class of retroviruses consists of immunodeficiency viruses HIV-2, FIV (feline), SIV (simian), infectious anemia virus EIAV (equine), arthritis-encephalitis virus CAEV (caprine) and visna virus. The predominant form of RT in these viruses is a heterodimer, and like HIV-1, is composed of a catalytic subunit with a polymerase and RNH domain and a smaller subunit truncated at its C-terminal end.

HIV-2 RT has been purified from virions as a p68/55-58 RT heterodimer (90), although homodimeric forms of these subunits have been characterized in recombinant heterologous systems (200,345). A crystal structure of this enzyme revealed that its overall folding is generally similar to that of HIV-1 RT (417). Closer examination shows that the putative p51↓RNH cleavage site in HIV-2 RT lies in the same structural region as HIV-1, however processing occurs at residue M484 instead of F440 (119). While both enzymes exhibit the same catalytic activities, there are significant differences in their enzymatic properties. The RNH activity of the

HIV-2 RT heterodimer is 10 times lower than that of HIV-1, and its template affinity during polymerization is 15-fold higher (201,454).

Recombinant FIV RT is enzymatically active as homodimers as well as a p66/51 RT heterodimer (3,362). Although it was reported that virion-purified FIV RT consists of a single 66 kDa polypeptide (361), immunoprobing of viral lysates has demonstrated that both p66 and p51 RT subunits are indeed present. Furthermore, cleavage of synthetic peptides *in vitro* with FIV PR indicates that the putative p51↓RNH cleavage site may lie at residue W440, the same primary sequence position as in HIV-1 RT (106). Comparative analyses with HIV-1 RT have further demonstrated that that FIV RT and its subunits exhibit similar catalytic functions and structural roles (4,78,501).

EIAV RT purified from virions is a heterodimer. Although some researchers have indicated that the size of EIAV RT subunits are 66 kDa and 51 kDa subunits (89), other estimates place them as being closer to 70 kDa and 59 kDa (89,510). In addition, recombinant forms of EIAV p51/51 RT homodimers have been studied (479). Both HIV-1 RT and EIAV RT exhibit comparable specific RNH activity (437,510) and fidelity of polymerization (19,89). Noticeable differences between these enzymes include specific DNA polymerase activity and sensitivity to inhibition (437), implying some aspect structural divergency.

Finally, due to its ancestry, SIV RT has lower sequence homology to HIV-1 RT than any other lentiviral form. Most closely related to HIV-1 is SIV from chimpanzees (SIVcpz), while SIV from macaques (SIVmac), sooty mangabeys (SIVmn), African green monkey (SIVagm), and

mandrill (SIVmnd) are closely related to HIV-2 (459). SIV RT has been purified from virions as a p64/48-50 heterodimer and exhibits discernable polymerase and RNH activities (270,319). Although these activities have not been extensively studied *in vitro*, it is clear that SIV RT exhibits a similar sensitivity to polymerase inhibition compared to the HIV-1 (410).

1.5.2.4.2 Monomeric forms of RT

Murine leukemia virus (MLV), also called Moloney murine leukemia virus (abbreviated MMLV, Mo-MLV, or M-MuLV) are type C retroviruses that possess a 70-80 kDa monomeric form of RT (148,210,335,531). Other monomeric retroviral RT enzymes have been characterized biochemically from porcine endogenous retrovirus (14), bovine leukemia virus (BLV) (391), feline leukemia virus (FLV) (361), mouse mammory tumor virus (MMTV) (502), and Mason-Pfizer monkey virus (MPMV) (180). While both HIV-1 and MLV RT display polymerase and RNH activities (210,436) they exhibit different cation requirements (199,531) and abilities to define RNH cleavage sites amongst mismatched segments in RNA-DNA hybrids (141). A continued area of dispute is that monomeric MLV RT may dimerize upon binding to a nucleic acid substrate (331,504). Interestingly though, BLV RT remains enzymatically active as a monomer, even after substrate binding (391). A further difference from HIV-1 RT is the finding that the polymerase and RNH domains of MLV RT act independently to complete viral DNA synthesis during infection (503). As such, physical separation of these domains maintains functionality (496). These unique attributes of MLV RT may be attributable to the length of its tether region, and the presence of non-conserved motifs in the connection subdomain and RNH domain, such as the basic loop/helix αC (141,331). The absence of such features in HIV-1 RT may explain the need for dimerization to maintain the active open conformation of the p66

subunit (181,373). The recently resolved full-length structure of MLV RT has indicated that there are a number of striking differences from the HIV-1 RT (81). The relative positions of the thumb, connection, and RNH domains, and the angles between the fingers and palm subdomains are different in the two structures. Consequently, the palm subdomain of MLV RT is larger, and the clamp-like shape of its binding cleft requires that the trajectory of the template-primer be bent by 90^{o}, as opposed to 45^{o} when exiting the polymerase active site. Finally, the tether region linking the polymerase and RNH domains in MLV RT exits the connection subdomain in a completely opposite direction, suggesting that it may permit conformational flexibility of the RNH domain.

1.5.2.4.3 Homodimeric and alternative heterodimeric forms of RT

Avian sarcoma and leukosis viruses (ASLV) form the genre of type C retroviruses which includes Rous sarcoma virus (RSV) and avian myeloblastosis virus (AMV). The putative form of ASLV RT is a αβ heterodimer, with 63 and 95 kDa subunits respectively. However, gel filtration and sedimentation velocity experiments have since shown that while the αβ form is favoured at 4^{o}C, $(\alpha\beta)_2$ tetramers predominate at 20^{o}C (301). The β subunit (p95) contains polymerase, RNH, and IN domains, while the smaller α (p63) subunit contains only polymerase and RNH domains (155,165,292,421). Interestingly, ASLV virions are also comprised of 8% completely processed p63 (α) RT monomers/homodimers, and 37% unprocessed p95/95 (ββ) RT homodimers (165,197). Recombinant studies suggest that all oligomeric forms of ASLV RT may be organized in the same asymmetric manner as HIV-1 p66/51 RT (553,554), and that interestingly, processing of Pol does not require dimerization (1). Compared to the HIV-1 p66/66 RT homodimer, the ASLV αα RT homodimer is very unstable and sensitive to heat inactivation

(478,555). Furthermore, a single β subunit of ASLV RT (αβ or ββ) is proposed to play a comparable structural role to the p51 subunit of HIV-1 p66/51 RT (164,553). While the RNH domain of ASLV RT does not exhibit directional processing (443,555), it does have a fairly long tether region and a basic loop/helix αC. However, unlike MLV RT, physical separation of RT polymerase and RNH domains results in an inactive polymerase suggesting some degree of functional interdependence (280). Finally, the catalytic activities of the different ASLV RT oligomers can be comparatively summarized as: (i) relative RDDP activity is 1:3:5 for αα, ββ, and αβ respectively, (ii) αβ and ββ are more processive than αα, (iii) αβ has the greatest affinity for RNA templates, (iv) strand transfer efficiency follows αα = αβ > ββ and (v) RNH activities of αβ are greater than ββ (198,532,552,555).

Other retroviruses that possess alternative oligomeric forms of RT include human T-cell leukemia viruses type I and II (HTLV-I and II), and spumaretroviruses or foamy viruses which include FFV (feline), and HFV or HSRV (human) now called PFV (prototype). In HTLV-I, the Gag-Pro-Pol fusion protein is expressed by double ribosomal frameshifting (222), whereas the Pro-Pol domain of PFV is expressed independently of Gag at its own initiation site (304,583). Originally purified from virions as a 95 kDa protein (422), recent characterization of HTLV-I RT has shown that it has both α and β subunits similar to ASLV RT, but that they associate to form an active α_3/β tetramer *in vitro* (521). PFV RT is further different, being as that virions are comprised of a completely processed 80 kDa PR-RT subunit and an unprocessed 127 kDa PR-RT-IN subunit (398). Although the oligomeric composition of PFV RT remains unclear, both polypeptides exhibit functional *in situ* polymerase and RNH activities (261), and may likely adopt homodimeric and heterodimeric forms. It has also been demonstrated that polymerase

activity of PFV RT is greater and more processive than HIV-1 RT (35,426). These results could account for the extremely low number of PFV RT molecules per virion (186), and further suggest that only a few molecules of PFV RT can accomplish the same tasks as a greater number HIV-1 RT molecules.

1.5.3 Assembly and generation of HIV-1 p66/51 RT

HIV-1 RT is not synthesized *de novo,* but instead processed by HIV-1 PR from a fully translated Gag-Pol polyprotein precursor. This proteolytic maturation of HIV-1 RT is partial in the sense that half of the available p51↓RNH cleavage sites are processed to produce an equal proportion of p66 and p51 RT subunits (485,533). In the final mature p66/51 RT heterodimer the remaining p51↓RNH cleavage site is accessibly buried, making the structure refractory to further proteolytic attack. Although the mechanism of RT generation has not been determined in a virion context, as few possibilities exist. In the first scenario, concerted independent processing of both p66 and p51 RT subunits may be followed by their assembly into p66/51 RT heterodimers. Alternatively, a sequential form of processing would entail the liberation of RT p66 from Gag-Pol, assembly into p66/66 RT homodimers and processing of one subunit to form p66/51 RT heterodimers. An adaptation to this last scenario further suggests that p66/66 RT homodimers could be directly released from two dimerized Gag-Pol polyprotein precursors. Since monomeric forms of p66 RT are resistant to internal processing at the p51↓RNH cleavage site, dimerization of RT subunits at some stage during virion maturation is essential (92). Our laboratory has previously demonstrated, using a truncated Pol polyprotein expressed in bacteria that formation of p66/51 RT follows a sequential mechanism of processing. However, concerted assembly of separate subunits *in vitro* can similarly produce a p66/51 RT heterodimers of identical

conformation and activity (5,26). Outlined in the following section is the current state of knowledge concerning both methods of RT preparation.

Recombinant p66/51 RT has been prepared in a number of heterologous expression systems by the assembly of separate RT subunits or proteolytic processing of polyprotein-embedded RT. Host expression systems have included *E.coli* (120,199,341,346,498), *Bacillus subtilis* (282), yeast (23,104,214) and vaccinia virus (129). Dimerization of RT subunits is essentially a bimolecular mechanism, involving rapid association followed by a slower isomerization to create a functionally active enzyme (96). RT subunits have been expressed in the same host, from a biscistronic gene or from two separate plasmids (204,232,346). The p66/51 RT heterodimer can also be reconstituted by mixing equimolar amounts of recombinant RT p66 and RT p51 after purification (207,285,323,483). Alternatively, expression of HIV-1 RT and PR in *cis* (from a biscistronic gene) (120,283), or in *trans* (on two separate plasmids) (120,333,341,450) results in the formation of the p66/51 RT heterodimer. Preformed recombinant p66/66 RT homodimers can also be processed to p66/51 RT in *trans* by the addition of recombinant HIV-1 PR (61,203,450,515). Although a number of non-viral PR including *E.coli*, *Staphylococcus aureus* V8, trypsin, chymotrypsin, and papain have been used to generate the p66/51 RT heterodimer *in vitro* (126,278,314) they are generally non-specific and result in additional cleavage products and an RT p51 subunit with heterogeneous ends (126,128,225,314). Taken together, these reports demonstrate that recombinant p66/51 RT can be formed in a number of different ways.

1.5.3.1 Proposed mechanisms for processing recombinant p66/66 RT

Although it is clear that recombinant p66/51 RT can be formed from p66/66 RT by the removal of an RNH domain (61,203,314,450), the mechanism by which HIV-1 PR cleaves only one of the RT p66 subunits has not been unequivocally established. Some investigators propose that the p66/66 RT homodimer is an asymmetric dimer similar to the RT p66/51 heterodimer, but with the RNH domain of the soon-to-be p51 subunit unfolded to an extent that allow PR-mediated cleavage at the p51↓RNH junction (85,203,314,515,546). This may be the result of energy derived from RT subunit dimerization that induces strain in one of the RNH domains that is relieved by unfolding along the tether region towards the p51↓RNH cleavage site (85,169,203). Other studies, supported by circular dichroism data, suggest that p66/66 RT may be a symmetrical homodimer, but that removal of the RNH domain from one of the p66 subunits induces conformational changes in the other p66 subunit that protect this subunit from similar cleavage at its p51↓RNH junction (5,314,338).

1.5.3.2 Proteolysis of the released RNH domain of RT

Although mature p66/51 RT is refractory to proteolytic processing (61,314,515), it remains in question whether the released RNH domain is further degraded in the virion. Early reports indicated that the p15 RNH fragment could be immunoprecipitated from virions (178). However, many subsequent attempts to identify or isolate such a fragment after formation of p66/51 RT heterodimer *in vitro* or in the virion context have proven unsuccessful (61,126,314,484). It is possible that cleavage of the p51↓RNH junction produces an isolated RNH domain that cannot properly fold, having lost the initial residues needed to form the middle β1 strand in the central β sheet of RNH (Figure 7) (85). In heterologous expression systems, the

generation of p66/51 RT at neutral pH releases an RNH domain that is indeed further cleaved at Y483, N494 and Y532 (23,61,515). Further interesting is the observation that changes in environmental conditions can expose up to eighteen additional cleavage sites within RT, presumably by altering folding (69,213). It therefore appears that the RNH domain of HIV-1 RT may play an as of yet undefined, but important role in the regulated generation of RT.

1.6 INTEGRASE

1.6.1 General features of integration

Efficient replication of HIV-1 depends on the stable maintenance and efficient transcription of proviral DNA so as to produce new copies of the retroviral genome and mRNA templates that encode viral proteins (279). Following viral entry and reverse transcription, the proviral DNA copy of the full-length RNA genome is inserted into the host cell genome by a concerted mechanism known as integration. Originally called endonuclease (167), the *pol*-encoded retroviral enzyme that mediates this process is now commonly referred to as integrase (IN) (251,452). The two specific, separately occurring catalytic activities of IN are 3'-end processing, and 3'-end joining or strand transfer. In a highly coordinated manner, IN cleaves both ends of proviral DNA in a hydrolytic reaction and juxtaposes these for nucleophilic attack of the target host DNA in a subsequent transesterification reaction (50,348).

Integration of proviral DNA is essential for efficient replication of most retroviruses, bacteriophage Mu and nonviral transposons (456). In other viral systems such as parvoviruses, integration occurs infrequently and may represent an alternative salvage pathway of replication

(67). Although HIV-1 IN is vital for efficient retroviral replication, reports differ as to the extent to which unintegrated DNA can serve as a template for viral gene expression and protein synthesis. These differences appear to be attributed to the class of IN mutation examined and the lineage of the infected target cells (56,112,440,487). Class I IN mutations specifically block the integration step, whereas class II mutations cause pleiotropic defects in virion morphogenesis and reverse transcription (109). In the absence of functional HIV-1 IN, it appears that cellular recombination enzymes may facilitate illegitimate integration (145) to maintain retroviral replication in some semipermissive and permissive cell lines (56,352). As expected though, single-cycle infectivities, viral titers and DNA recombination frequencies of class I and II IN mutants are severely reduced compared to wild-type (352).

The following model for retroviral integration is supported by both *in vitro* and viral replication studies. After reverse transcription, the precursor substrate to integration remains enclosed within a specific nucleoprotein complex known as the preintegration complex (PIC). This precursor is a blunt-ended linear DNA molecule of 9.8 kbp with LTR sequences at each end, forming an imperfect inverted repeat (38,123). While it is assumed that reverse transcription proceeds to completion, most proviral DNA molecules contain a discontinuous plus-strand that is presumably repaired by RT or cellular enzymes following integration (219,330). The first step of integration occurs in the cytoplasm (33) with the coordinated removal of 3'-terminal GT dinucleotides leaving a recessed, phylogenetically conserved CA sequence with a free 3'-hydroxyl group (378,435). HIV-1 PICs are then actively transported into the nucleus (45) via MA (42,44,139) or Vpr (208,358,463) signal-mediated entry through nuclear pores. In the second step of integration, both 3'-hydroxyl groups of the viral DNA are juxtaposed to attack phosphodiester bonds on opposite strands of the target host DNA, creating a staggered cleavage

of five bases (535). As a result of this strand transfer, the recessed 3'-ends of the viral DNA are covalently joined to the 5'-phosphate ends of the target DNA creating an intermediate with unpaired dinucleotides at the viral 5'-ends and terminal single-stranded gaps of target site DNA (136). Finally, these gaps between viral and host DNA may be repaired by cellular enzymes, although a possible role of RT and IN has not been ruled out (36,578).

The overall process of integration occurs without the use of exogenous energy sources (107), and results in the permanent association of viral DNA with the genome of the host cell (123). *In vitro* studies have suggested that IN may further catalyze a disintegration reaction. Essentially, the continuity of a target DNA can be restored by releasing a viral DNA segment (71). However, since the sites of disintegration are relatively independent of the viral DNA sequence this reaction may not represent a true reversal of strand transfer (527). Under favourable conditions, integration is highly efficient and results in the insertion of up to 50% of available proviral linear DNA molecules in acutely infected, permissive cells (171). While the linear viral products of reverse transcription initially comprise the most abundant form of viral DNA in the cells, those that are not integrated, because of superinfection or IN mutation adapt to form dead-end extrachromosomal products in the nucleus (487). These products include (i) 1-LTR circles, formed by homologous recombination within the LTR region, (ii) 2-LTR circles, formed by blunt-end joining of two adjacent LTR sequences, and (iii) autointegration products, formed by intramolecular integration into the viral DNA molecule itself (466). Recent evidence suggests that viral DNA circularization results from the action of a number of host cell factors including components of the RAD50/MRE11/NBS1 nuclease to form 1-LTR circles and components of the nonhomologous DNA end-joining (NHEJ) pathway to form 2-LTR circles (122,257).

While there is no direct evidence that any other protein but IN can catalyze integration (51,75,251), the possibility remains that viral and cellular factors may play important roles (121,287,524). Insights into integration have been made by comparing *in vitro* reactions mediated by purified PICs vs. recombinant IN. Under current conditions, HIV-1 IN alone is remarkably inept at coordinating the joint integration of both ends of the viral DNA (50,51), whereas purified PICs can specifically coordinate coupled integration (58,107). Recent evidence suggests that both the NC protein (57,58) and the cellular nonhistone chromosomal protein HMG-I(Y) (121,192), present in PICs are needed to stimulate coupled integration. Metal cofactors such as Mn^{2+} and Mg^{2+} also appear to mediate assembly of the IN-DNA-metal complex (12) and contribute to substrate discrimination (577), whereas Ca^{2+} and Co^{2+} are needed to uncouple the assembly and catalysis functions of HIV-1 IN (184). Finally, the cellular protein Ini1 (hSNF5), part of the mammalian SWI/SNF complex appears to be needed during integration to remodel chromatin (337).

1.6.1.1 Determinants of IN substrate specificity and target site selection

HIV-1 IN displays an innate ability to recognize the ends of proviral DNA in a sequence specific manner, yet binds target DNA in a sequence-independent manner. The single most important feature of proviral DNA, in terms of IN binding, is the terminal CA/TG dinucleotide pair. To a lesser extent, specificity also extends up to 15 base pairs from the termini (37). A number of factors help to prevent non-specific binding of IN to internal viral DNA sequences, including sequence protection by NC (58) and HMG-I(Y) (121), the sterically controlled structure of the PIC (33), and cooperative interactions between RT and IN (185). By comparison, target site selection within the host cell genome appears to be sequence-independent and occurs

at an enormous number of locations (49,447,465,572). Integration generally favours the most accessible region of the chromatin structure where the major groove dividing phosphodiester bonds is widest, or rather highly bent DNA sites found at specific positions in nucleosomes (412,413). Interactions with cellular DNA-binding proteins such as BAF, HMG-I(Y), Ini1, Ku, and LEDGF are also believed to play a role by tethering IN to transcription factors or modified histones (363).

The dependence of integration on favourable chromosomal target sites may consequently have a decisive influence on retroviral replication. If integration occurs within a sequence unsuitable for efficient high-level transcription, this could diminish the production of progeny virions (234) and possibly cause other deleterious genetic effects (465). With the recent completion of the human genome sequence, researchers have only now begun to execute large-scale sequence-based surveys of integration events to determine target site preferences. The results of some of these studies appear to indicate that each retrovirus shows unique preferences for certain integration sites, which suggests that they each have different mechanisms of target site selection. HIV-1 integration strongly favours transcriptional units of active genes in a tissue-specific manner. MLV integration has a strong preference for regions near to transcription start sites and CpG islands. Further different is ASLV, which mildly prefers integration into active genes and exhibits no preference for transcriptional start regions (49,447,572).

1.6.2 Structural organization of HIV-1 IN

The gene for HIV-1 IN encodes as a 32 kDa (p32) protein, however the true multimeric structure and relative orientation of IN domains remains unclear. Structural and biophysical studies of full-length retroviral IN have generally been impeded by its poor solubility at low concentrations, although the use of solubilizing mutations has shown promise (228). HIV-1 IN can be divided into three independently folded domains, an N-terminal domain for zinc-binding, a catalytic core domain for polynucleotidyl transfer, and a C-terminal domain for DNA-binding (Figure 9) (110,528). Based on *in vitro* complementation and UV cross-linking experiments, IN appears to function as a multimer (70,108,110,528). Sedimentation analysis has shown that each domain exists in monomer-dimer equilibrium (191,228), while full-length IN, and its C-terminal two-thirds exist in dimer-tetramer equilibrium (228,233). Both IN dimer or tetramer models for multimerization have been proposed to account for the catalytic activities associated with integration (228,233). An IN dimer could theoretically contain separate sites for binding host DNA and viral DNA, and a single site for catalyzing IN functional activities. On the other hand, an IN tetramer may be better able at catalyzing a five base pair staggered cleavage. In this scenario, two separate IN molecules could bind to, and coordinate each of the viral ends together (50,348), and two additional IN molecules could bind to the host DNA target site to prepare it for nucleophilic attack. Irrespective of the actual oligomeric form of IN needed for integration, multimerization of subunits is essential. This is facilitated by direct contacts between the core and C-terminal domains (102,110,191) and promoted by Zn^{2+} binding to the N-terminal domain (290,586) and the presence of Mg^{2+} or Mn^{2+} (108).

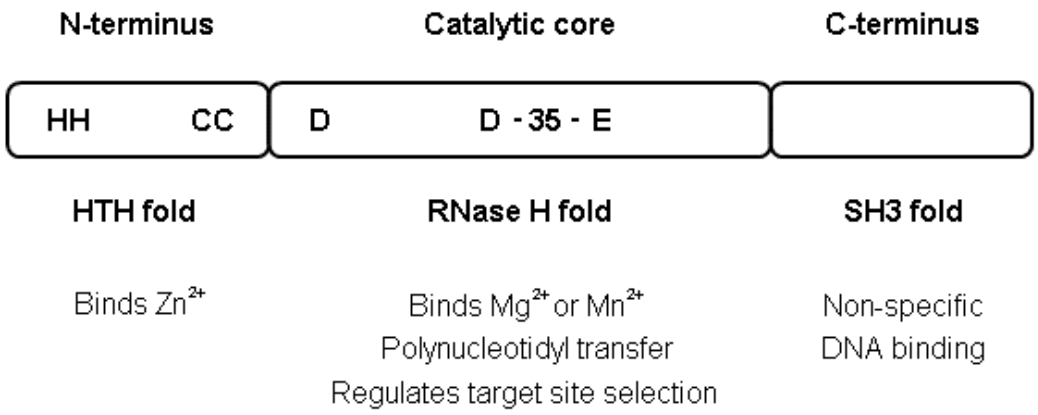

Figure 9. Schematic organization of the domain structure of HIV-1 integrase. Integrase is composed of three functional domains. The conserved central core domain contains the catalytic site, a triad of highly conserved acidic residues in the form of a D,D-35-E motif. While this domain alone can catalyze a polynucleotidyl transfer reaction (disintegration), but N- and C-terminal domains are required for 3'-end processing and strand transfer reactions. The N-terminal domain contains the conserved HHCC motif, which binds zinc. The poorly conserved C-terminal domain is capable of binding DNA non-specifically. Multimerization determinants are found in all IN domains.

1.6.2.1 Structure and roles of IN domains

Although the core domain of IN contains the active site required for chemical catalysis, the roles of the N-terminal and C-terminal domains are less well understood (423). All three domains are required to catalyze both 3'-end processing and strand transfer activities (444,536). The N-terminal domain (residues 1–50) contains a highly conserved HHCC zinc-binding site (111) and a helix-turn-helix structure similar to that found in DNA-binding proteins (53). Binding of Zn^{2+} to the N-terminal domain appears to promote multimerization, protein stabilization (290,586), and enhancement of IN activity (289,586). The core domain (residues 50–212) contains a catalytic D,D(35)E triad motif and structure characteristic of many polynucleotidyl transferases including RNH, bacteriophage Mu transposase and the *E.coli* Holliday junction resolving enzyme RuvC (102). This motif is believed to compose the active site of IN through its coordination of metal cofactors (Mg^{2+} or Mn^{2+}) and regulation of target site selection (277,464). Recent evidence further suggests that in addition to promoting dimerization (102,110,191), both the central core and C-terminal domains of IN act in connecting HIV-1 PICs with the nuclear import machinery (138). Although nonspecific DNA-binding activity has been associated with the C-terminal domain (residues 213–288), this region is not highly conserved, and its precise function is unclear (113,536,564). The C-terminal domain may play a role in associating the viral DNA ends with IN through a direct interaction with RT (185), although it remains unclear how DNA is arranged in a tertiary IN-DNA-metal ion complex.

1.6.3 Physical association between RT and IN

The final stage of reverse transcription generates a linear proviral DNA substrate that is acted upon by IN, and a putative interaction between RT and IN within PICs could theoretically

facilitate this transfer. HIV-1 IN could be brought to the viral DNA ends by RT after DNA synthesis, and RT could repair the gapped integration intermediate and complete plus-strand DNA synthesis subsequent to nuclear transport (71,219,330). To this end, several reports have indicated precedence in retroviral (1,185,521,573) and retrotransposon (260,363,486,558) systems for a role of RT and IN domains in the proper folding and enzymatic activity of each other. HIV-1 IN is essential for efficient initiation of reverse transcription in infected cells (573), but has no influence on processivity (185). Furthermore, HIV-1 RT appears to stimulate IN-mediated strand transfer activity *in vitro,* but does not affect 3'-end processing activity (58,185,573). Associative interactions between RT and IN could be achieved in *cis*, when RT is in direct fusion with IN or in *trans*, by interactions between the individual mature proteins themselves. In ASLV (1,166) and HTLV-I (521) RT, the IN domain of the β subunit appears to play a structural role in RT activity, while the RT domain negatively regulates its contribution to integration strand transfer activity (193). In other retroviral systems such as HIV-1 and MLV, it is unclear what effect a forced end-to-end *cis* association between RT and IN would have on their respective activities. Recent reports have indicated that the close physical association between HIV-1 RT and IN is not mediated by nucleic acid bridging (573) but rather direct interaction between the C-terminal domain of IN and the fingers-palm subdomain and tether region of RT (185,588). These same functional interactions have been similarly found in the Ty3 (260,363) and Tf1 (486) LTR retrotransposon systems.

1.7 RATIONALE AND STATEMENT OF HYPOTHESIS

The complete proteolytic release of *pol*-encoded enzymes is believed to be a late event in the ordered pathway of Gag-Pol polyprotein processing during HIV-1 maturation (267,591). Surprisingly however, there is little information concerning the sequence of events and functional consequences of processing these late stage cleavage sites during virion maturation. Each of the *pol*-encoded enzymes, protease (PR), reverse transcriptase (RT) and integrase (IN) are active only as dimers. Both PR and IN are homodimers (or higher order homo-oligomers in the case of IN) which are comprised of subunits that are predicted from the size of their genes. In contrast, RT in mature infectious virions is a heterodimer of 66 kDa (p66) and 51 kDa (p51) subunits, even though its gene encodes only a 66 kDa protein. The p51 subunit is formed by HIV-1 PR-catalyzed cleavage of RT p66 during virion maturation, resulting in the removal of a ribonuclease H (RNH) domain. Thus, compared to formation of active PR or IN, the formation of mature active RT requires an additional proteolytic cleavage event. This suggests that the p66/51 RT heterodimer is essential for HIV-1 replication. However, recombinant p66/66 RT homodimers have significant enzymatic activities (23,128), and PR-RT and RT-IN fusion proteins arguably exhibit some level of RT polymerase activity (211,293). Furthermore, RT in virions of MLV is monomeric and possibly homodimeric in active form (148,531), while active RT in virions of ASLV is dimeric and comprised of at least one RT-IN subunit (155,197,292).

The p66/51 RT heterodimer of HIV-1 is formed by the proteolytic processing of three different cleavage sites: the N-terminal PR↓RT junction, the internal RT p51↓RNH junction, and the C-terminal RT↓IN junction. **The encompassing goal of the following project was to**

determine why the generation of active viral RT requires three processing events during virion maturation and as such, why formation of the RT p66/51 heterodimer is necessary for virus replication. Since inhibition of the N-terminal PR↓RT cleavage site results in stable PR-RT fusion proteins with no adverse effect on virion-associated RT activity (65,66), the focus of this project was directed at the two remaining cleavage sites. **We hypothesized that cleavage of the internal RT p51↓RNH junction is essential to the formation of stable functional viral RT, whereas cleavage of the sites flanking N-termini and C-termini are not.**

Specific Aim #1: To determine the extent that proteolytic processing of RT can be prevented or significantly diminished by mutagenesis of RT↓IN cleavage site in an infectious molecular clone of HIV-1. This aim will allow for an assessment of RT content, activity, and replication capacity of virions unable to generate RT p66.

Specific Aim #2: To determine the extent that proteolytic processing of RT can be prevented or significantly diminished by mutagenesis of RT p51↓RNH cleavage site in an infectious molecular clone of HIV-1. This aim will allow for an assessment of RT content, activity, and replication capacity of virions unable to generate RT p51.

The association of HIV-1 with the AIDS epidemic has generated a considerable amount of interest in trying to better understand the structural and functional properties of the *pol*-encoded enzymes, PR, RT and IN. The vital role that RT plays in viral replication has made it one of the most important and ideal targets of anti-retroviral therapy. While it is clear that HIV-1 RT is a heterodimer of 66 kDa and 51 kDa subunits, the apparent need for this oligomeric form

in the virion has not been clearly defined. A greater understanding of proteolytic events involved in the generation of HIV-1 RT will provide clarification of this necessity and perhaps insight into its structural differences from other retroviral forms.

The progression of work outlined in this thesis is presented in the most logical order relevant to the overall hypothesis. However, for the purpose of continuity, reference is occasionally made to work outlined in previous or subsequent chapters. Where applicable, methods are reiterated in brief form with reference to particular differences or adaptations. In Chapter 2, the effect of mutagenesis of the C-terminal RT↓IN cleavage site was investigated in the virion context. Attributes such as viral protein composition, RT activities, infectivity and viral replication capacity were examined. In Chapter 3, various mutations were introduced in the internal RT p51↓RNH cleavage site followed by an examination of the same attributes outlined above. Finally, Chapter 4 is an extension of the work initiated in Chapter 3 and involves the characterization of a second-site compensatory mutation.

CHAPTER 2: PROTEOLYTIC CLEAVAGE OF THE HIV-1 RT↓IN JUNCTION DOES NOT SERIOUSLY IMPACT RT ACTIVITY, BUT IS ESSENTIAL FOR EFFICIENT VIRAL REPLICATION

2.1 ABSTRACT

Reverse transcriptase (RT) and integrase (IN) are essential oligomeric enzymes released from the HIV-1 Gag-Pol polyprotein precursor during proteolytic maturation of virions. Mature RT is a heterodimer of 66 kDa and 51 kDa subunits. The smaller p51 subunit is derived from the larger p66 subunit by protease-catalyzed removal of a ribonuclease H (RNH) domain. Thus, generation of RT requires processing at three different cleavage sites: the N-terminal PR↓RT junction, the internal RT p51↓RNH junction and the C-terminal RT↓IN junction. To study the necessity for processing the RT↓IN cleavage site in the virion context, we selectively mutated amino acid residues at this junction in an infectious HIV-1 molecular clone. Mutation of the RT↓IN junction was found to result in the production of virions containing 98 kDa RT-IN and 51 kDa RT proteins in equivalent proportion. Interestingly, we found by inhibiting HIV-1 PR with decreasing concentrations of ritonavir that the 47 kDa RNH-IN protein, released upon cleavage of the RT p51↓RNH junction was further processed to a 35 kDa form. This result suggests putative unfolding and further proteolysis of the abutting RNH domain, consistent with

processing of recombinant p66/66 RT and the absence of RNH in virions. While RT polymerase and RNH activities were unaffected by the RT-IN fusion, viral infectivity and replication capacity were severely attenuated. Repeated passage of MT-2 cells exposed to the mutant virus eventually lead to phenotypic and genetic reversion, suggesting that IN activity may not have been completely ablated by the RT↓IN cleavage site mutation. These results suggest that proteolytic processing of the RT↓IN junction is not essential in the generation of functional RT during HIV-1 maturation, but in the context of efficient viral replication, may be important for IN activity. A *cis* association between RT/RNH and IN likely disturbs the normal functional interactions between these enzymes that are important for integration, but not reverse transcription.

2.2 INTRODUCTION

During virion assembly and budding, the 160 kDa Gag-Pol polyprotein precursor of human immunodeficiency virus type 1 (HIV-1) is proteolytically processed at discrete cleavage sites to liberate the individual components of Gag and Pol (244,262). The active enzymes encoded in the C-terminal Pol region of this polyprotein precursor include protease, reverse transcriptase and integrase (416). Each of these enzymes is responsible for an essential event in the life cycle of HIV-1. Protease (PR) cleaves polyprotein precursors into their respective components during virion maturation. Reverse transcriptase (RT) catalyzes the complex conversion of genomic RNA into a double-stranded proviral DNA through coordination of its RNA- and DNA-dependent polymerase, ribonuclease H (RNH), strand transfer and strand-

displacement activities (298,443,499). Finally, integrase (IN) acts after reverse transcription to covalently incorporate the proviral DNA into the genome of the infected host cell. The specific enzymatic reactions catalyzed by IN include: (i) 3'-end processing, the removal of two nucleotides from each 3'-end of the linear proviral DNA and (ii) strand transfer, the splicing of these processed 3'-ends into opposite DNA strands of the host chromosome (50).

Each of the *pol*-encoded enzymes must oligomerize to at least a dimer for enzymatic activity (16,110,418). Both PR and IN are homodimers with 10 kDa and 32 kDa respective subunits, predicted from the size of their genes. Mature HIV-1 PR is a symmetrical homodimer released from Gag-Pol by autoprocessing carried out by the polyprotein-embedded form of the enzyme (311). HIV-1 IN is also active in a homodimeric state, although higher order tetrameric species may facilitate integration *in vivo* (108,228,233). Functional HIV-1 IN is comprised of three independently folded domains: (i) an N-terminal domain, involved in the coordination of zinc (290,586), (ii) a catalytic core domain, for polynucleotidyl transfer (102,191) and (iii) a C-terminal domain, for DNA binding (110). The gene for HIV-1 RT encodes a protein of 66 kDa, and like PR and IN can form active homodimeric forms in heterologous expression systems (23,128,458). However, RT in mature infectious virions is a heterodimer with 66 kDa (p66) and 51 kDa (p51) subunits (512). The smaller RT p51 subunit is derived from the larger RT p66 by proteolytic cleavage at an internal p51↓RNH junction, resulting in the removal of a C-terminal RNH domain (61,119,203,515). Although both p66 and p51 subunits have identical amino acid sequences, their folding within the active RT heterodimer differs, resulting in an asymmetric structure (263,546). Consequently, p66 forms the catalytic subunit of the p66/51 RT heterodimer, while p51 is presumed to play largely a structural role (4,224,285).

Reverse transcription generates linear proviral DNA that is acted upon by IN. Thus, a potential interaction between RT and IN in the nucleoprotein or preintegration complex (46,124,210) could facilitate the transfer of reverse transcription product to integration substrate. It has been further suggested that RT and IN may intimately affect each others activity. IN appears to stimulate the initiation of reverse transcription (573), while RT dramatically enhances the strand transfer activity of IN (58,185,573). Since most proviral DNA molecules contain a discontinuous plus-strand it is also possible that RT is required for polymerization subsequent to nuclear transport or integration (219,330). An associative interaction between RT and IN could be achieved in *cis*, when RT is in direct fusion with IN, or in *trans*, by interactions between the individual mature proteins. In both ASLV (166,198,292) and HTLV-I (521), IN forms an integral part of the β subunit of active RT in a *cis* association. In contrast, the RT and IN proteins of HIV-1 are fully separated by proteolytic cleavage (187,537) and appear to directly interact in *trans* (185,573,588).

The generation of mature p66/51 RT in HIV-1 virions requires processing at three cleavage sites, the N-terminal PR↓RT and C-terminal RT↓IN junctions as well as within RT p66 at p51↓RNH. Our previous studies suggest that p66/51 RT is generated from a truncated Pol polyprotein in an ordered manner through a p66/66 RT homodimer intermediate (471). This series of events may be preceded by PR-RT and/or RT-IN processing intermediates which have themselves, been observed in heterologous (239,293) and viral expression systems (302,481). In the virion context, mutation of the PR↓RT cleavage site produces 77 kDa and 62 kDa PR-RT fusion proteins and has no adverse affect on PR or RT activities, although infectivity is diminished (65,66). We have recently demonstrated that cleavage of the internal RT p51↓RNH junction is necessary to provide proteolytic stability of active RT during virion maturation

(Chapter 3). These results suggests that a heterodimeric form of HIV-1 RT is essential for virus replication. However, recombinant HIV-1 RT is active in p66/66 homodimers (23,128), and disputably active in fusion with IN (211,293). Furthermore, functional RT in virions of ASLV are comprised of at least one RT-IN subunit (155,292). Thus, it is unclear why HIV-1 RT requires processing of its C-terminus in the generation of a functional heterodimeric enzyme during virion maturation. By mutating the RT↓IN junction in molecular clones of HIV-1, we attempted to determine the functional consequences of processing this cleavage site. We found that blockage of the RT↓IN cleavage site produced the expected 98 kDa RT-IN and 51 kDa RT products, however viral infectivity was severely attenuated. Our data show that processing of the C-terminal RT↓IN junction is not essential for RT activity, since neither RT polymerase or RNH activities were affected by the fusion of RT and IN. However, in the context of efficient viral replication, cleavage of the RT↓IN junction is essential. While our findings lead us to believe that diminished IN activities may have been the source of the replication defect, this remains to be determined.

2.3 MATERIALS AND METHODS

2.3.1 Reagents

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAD, NIH: anti-HIV-1_{SF2} p24/25 IgG mAb (76C) from Dr. Kathelyn Steimer, Chiron Corporation; and anti-HIV-1_{HXB2} IN (2C11 and 8G4) IgG mAb

from Dr. Dag Helland. Rabbit anti-HIV-1 PR polyclonal serum directed against PR residues 86-108 (284,341) was obtained from Dr. Stuart Le Grice, NCI-Frederick (Frederick, MD). Anti-HIV-1_{IIIB} RT and anti-HIV-1_{IIIB} RNH (2F2) IgG mAb were previously generated in our laboratory against recombinant p66/51 RT (294). Homopolymeric template/primer poly(rA)-oligo$(dT)_{12-18}$, [^{3}H]-TTP, goat anti-mouse-HRP and donkey anti-rabbit secondary mAb were all purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The SuperPico ECL Substrate System for detection of peroxidase-labeled antibody was obtained from PIERCE (Rockford, IL). 4-MUG (4-methylumbelliferyl-β-D-galactopyranoside), a β-galactosidase fluorescent substrate was obtained from Sigma-Aldrich (St. Louis, MO). HIV-1 p24 antigen ELISA kits were obtained from SAIC-Frederick (Frederick, MD). Sequencing, PCR amplification and mutation-containing oligonucleotide primers were purchased from Invitrogen (Carlsbad, CA). Oligonucleotides purchased from TriLink Biotechnologies (San Diego, CA) included 5'-GAU CUG AGC CUG GGA GCU-fluorescein-3' and 5'-Dabcyl-AGC TCC CAG GCT CAG ATC-3' provided as an annealed RNA/DNA hybrid. COS-7, 293T, and CD4+ MT-2 and MT-4 lymphocytoid cell lines were obtained from the American Type Culture Collection (Rockville, MD).

2.3.2 Cell lines

The human T-lymphocytoid MT-2 and MT-4 cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Human 293T and monkey COS-7 fibroblast cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS. P4R5 HeLa fibroblast cells, a generous gift of Dr. John Mellors at the University of Pittsburgh (originally obtained from Dr. Ned Landau, The Salk Institute for Biological Studies, La Jolla, CA) were maintained in DMEM/10% FBS additionally supplemented with Puromycin

(0.5 μg/mL). These cells express endogenous CXCR4 and are stably transfected to express CD4, CCR5, and a β-galactosidase reporter gene under the control of an HIV LTR promoter (347).

2.3.3 HIV-1 molecular clone mutagenesis and transfection

Plasmid pSVC21-BH10 encodes an infectious molecular clone of the IIIB (HxB2) strain of HIV-1 and carries an SV40 origin of replication for expression in 293T and COS-7 cells (127). A double amino-acid substitution (F1I/L2K) was introduced into the region corresponding to the RT-IN cleavage site ($RKIL^{560}\downarrow F^{1}LD$) using the Quick Change™ Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). To assess $Pr160^{gag\text{-}pol}$ incorporation into virions a catalytic inactive mutation (D25A) was introduced into the PR coding region of wild-type and mutant molecular clones. The presence of the expected mutations was verified by sequencing. Virus was prepared by transfection of 293T cells by calcium phosphate co-precipitation. Virus-containing culture supernatants were harvested 60 h post-transfection, clarified by centrifugation (3000 x g, 1 h at $4^{o}C$), and quantified by analyzing the levels of HIV-1 p24 antigen. Aliquots of virus preparations were stored at $-80^{o}C$ until use.

2.3.4 Infectivity and viral spread

Infectivity of virus particles produced from transfection of 293T cells was determined by using normalized quantities of HIV-1 p24 antigen content and P4R5 or MT-2 cells as targets. Single-cycle viral infectivity was assessed using P4R5 cells in a fluorescence-based microplate β-galactosidase detection assay (5 or 25 ng of viral p24 per 5 x 10^3 cells per well). Briefly, virus-containing culture supernatants were removed at 48 h post-infection. Cells were lysed in 100 μL

lysis buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4 [pH 7.2], 1 mM $MgSO_4$, 100 mM β-mercaptoethanol, 2% [v/v] triton X-100) for 1 h at 37°C. Reactions were initiated by addition of 50 μL 4-MUG to a final concentration of 0.5 mM, incubated for 1 h at 37°C and quenched with 150 μL 0.2 M Na_2CO_3, pH 11.2. Fluorescence intensity was assessed on a SPECTRAmax GEMINI XS dual-scanning microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) using an excitation wavelength of 355 nm and an emission wavelength of 480 nm, with cutoff filter set to 475 nm.

Multiple-round viral replication was assessed by inoculation of MT-2 lymphoblastoid cells in 96-well microplates (2.5 or 20 ng of viral p24 per 6.5 x 10^4 cells per well) followed by daily microscopic observation of HIV-1 induced syncytium formation, as previously described (32,339). In a concurrent manner, the median tissue culture infective dose ($TCID_{50}$/mL) of each virus was determined after seven days of culture, as described elsewhere (231).

2.3.5 Molecular cloning of an RT-IN$^{F1I/L2K}$ revertant

Cytopathology appeared in infected MT-2 cells maintained in culture for 14 days. At this time virus-containing culture fluids were expanded briefly for 4 days by infection of fresh MT-2 cells and collected for subsequent analysis. RNA was extracted from 0.5 mL of supernatant containing revertant virions by ultracentrifugation (22,500 x g, 1 hr at 4°C) and incubation in the presence of 20 mg/mL proteinase K (1 h at 55°C), 5.8 M guanidinium isothyocyante (5 min at 22°C) and ethanol precipitation. RNA was reverse transcribed for 50 min at 42°C using the SuperScript™ First-Strand Synthesis kit (Invitrogen, Carlsbad, CA). The region encoding HIV-1 RT-IN was then PCR amplified from the cDNA reaction mixture and directly cloned into pTrcHIS/TOPO (Invitrogen, Carlsbad, CA) for sequencing analysis.

2.3.6 Immunoblotting analysis of viral proteins

Clarified virus-containing cell culture supernatants (1 μg of viral p24) were purified through 20% (w/v) sucrose cushions (175,000 x g, 1.5 h at 4^{o}C) and pellets lysed in 16 μL RIPA-lysis buffer (20 mM Tris-Cl [pH 8.0] containing 120 mM NaCl, 2 mM EDTA, 0.5% [v/v] DOC, 0.5% [v/v] NP-40 as well as 2 μg/mL PMSF, 10 μg/mL apoprotein and 10 μg/mL pepstatin A). Virion particle protein composition was assessed by subjecting the lysate proteins to SDS-10% PAGE resolution and Western blotting. Western blots were incubated with either anti-HIV-1 RT (6 μg/mL), anti-HIV-1 RNH (2F2, 6 μg/mL), anti-HIV-1 IN (2C11 and 8G4, 1:40), anti-HIV-1 PR (1:40) or anti-HIV-1 p24 (3 μg/mL) antibodies followed by incubation with the appropriate horse radish peroxidase-conjugated secondary antibody (1:1000). To minimize non-specific binding Western blots were blocked with 7% [w/v] skim milk/0.05% [v/v] tween 20 in PBS as well as normal goat or donkey serum (1:100) where appropriate. Immunoreactive protein bands were visualized by enhanced chemiluminescence (PIERCE, Rockford, IL) on a VersaDoc Imaging System, and quantitated by densitometry under sub-saturating exposure conditions using Quantity One v4.3.0 software (Bio-Rad, Hercules, CA). To assess the level of Gag-Pol ($Pr160^{gag\text{-}pol}$) incorporation, viruses containing inactivated PR (D25A) were pre-normalized by Gag ($Pr55^{gag}$) content prior to sucrose-cushion purification and Western blot analysis.

2.3.7 Analysis of intravirion processing of Gag and Gag-Pol polyprotein precursors

The accumulation of Gag-Pol processing intermediates during proteolysis was assessed by immunoprobing Western blots of viral protein derived from ritonavir-treated transfected COS-7 cells. Briefly, COS-7 cells (1.6 x 10^5 cells/well) were transfected with 3 μg of proviral

plasmid DNA (pSVC21-BH10) using LipofectAMINE Plus (Invitrogen, Carlsbad, CA) following which the transfection medium was replaced with cell culture medium containing varying concentrations of the HIV-1 PR inhibitor ritonavir (RTV), courtesy of Dr. John Mellors, University of Pittsburgh. Virus-containing culture supernatants were harvested at 48 hr post-transfection, clarified and purified through 20% [w/v] sucrose cushions (175,000 x g, 1.5 h at 4^{o}C). Virion particle protein composition was assessed by subjecting the lysate proteins to SDS-10% PAGE resolution, Western blotting and immunoprobing against IN and p24, as described above. In the absence of a readily procurable cross-specific anti-p24/Pr55^{gag} IgG mAb, previous studies suggested (240,246) as was shown herein (Figure 12-B) that particle production was relatively equivalent at each concentration of ritonavir. Furthermore, ritonavir concentrations were kept below cytotoxic levels that would inhibit COS-7 cell growth, as determined by MTS cytotoxicity analysis (Promega, Madison, WI).

2.3.8 Assay of virion-associated RT activity

Virion-associated RT RNA-dependent DNA polymerase (RDDP) activity was determined as follows. Briefly, virus-containing culture supernatant (250 ng HIV-1 p24) was sucrose cushion-purified and lysed for 15 min on ice in 48 μL reaction-lysis buffer (50 mM Tris-Cl [pH 7.9], 5 mM $MgCl_2$, 150 mM KCl, 0.5 mM EGTA, 0.05% [v/v] triton X-100, 2% [v/v] ethylene glycol, 5 mM DTT, 0.5 mM reduced GSH and 50 μg/mL poly(rA)-oligo$(dT)_{12-18}$). Reactions were initiated by the addition of 20 μCi [^{3}H]-dTTP, incubated for 1.5 h at 37^{o}C and quenched with 250 μL of ice-cold 10% trichloroacetic acid containing 20 mM $NaPP_i$. Following filtration through glass fiber Type C filter multi-well plates (Millipore Corporation, Bedford,

MA) and sequential washing with 10% [v/v] trichloroacetic acid and ethanol, the extent of radionucleotide incorporation was determined by liquid scintillation spectrometry.

2.3.9 Assay of virion-associated RNH activity

A previously described fluorescence-based microplate assay for HIV-1 RT-associated RNH activity (382) was adapted for determination of virion-associated RNH activity. Twenty five microliters of virus-containing culture supernatants (normalized to 25 ng HIV-1 p24 with conditioned DMEM/10% FBS) were added to individual wells of a 96-well microplate (CoStar black). Viral particles were then lysed for 10 min at 22^{o}C upon addition of an equal volume of 100 mM Tris-Cl [pH 8.0] containing 120 mM KCl, 20 mM $MgCl_2$ and 1% [v/v] triton X-100. Reactions were initiated by addition of 50 μL of a 0.5 μM solution of RNA/DNA hybrid in 50 mM Tris-Cl [pH 8.0], containing 60 mM KCl and allowed to proceed at 37^{o}C. Fluorescence intensity in each well was assessed in kinetic mode on a SPECTRAmax GEMINI XS dual-scanning microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) using an excitation wavelength of 490 nm and an emission wavelength of 528 nm, with cutoff filter set to 515 nm. The increase in fluorescence arising from hydrolysis of the substrate was measured over 15 min time intervals and was linear up to 45 min at which point reactions were quenched with 50 μL of 0.5 M EDTA, pH 8.0. Under these conditions, background fluorescence values using the same quantity of conditioned media were typically less than 10% of the test wells.

2.4 RESULTS

2.4.1 Effect of RT↓IN cleavage site mutation on viral phenotype of RT and IN proteins

The sequence of the RT↓IN cleavage site is RKIL^{560}↓F^{1}LD. To investigate the functional consequences of processing the RT↓IN cleavage site, by preventing its cleavage, we introduced mutations at the P1' (F1I) and P2' (L2K) positions in this PR-recognized sequence. Under normal wild-type conditions, proteolytic maturation of the Pol region of Gag-Pol generated completely processed 10 kDa PR and 32 kDa IN proteins, and an incompletely processed RT protein, composed of 66 and 51 kDa subunits (Figure 10-A). Based on the molecular mass of these proteins, it was expected that inhibition of the RT↓IN cleavage site would result in the formation of a 98 kDa RT-IN fusion protein, a 51 kDa RT protein and an incompletely processed 47 kDa RNH-IN fusion protein. Immunoprobing for RT revealed the presence of 98 kDa RT-IN and 51 kDa RT proteins in the RT-IN$^{F1I/L2K}$ cleavage site mutant virus (Figure 10-B, panel i-iii). The stability of these two immunoreactive RT bands was confirmed in replicate analyses (n = 3) and indicated a total average RT content of 69.2 ± 0.1 percent of the wild-type. Similarly, total immunoreactive RNH content averaged 62.8 ± 37.0 percent of the wild-type. Compared to the wild-type RT p66:p51 content ratio (0.9 ± 0.1), the relative ratio of RT-IN$^{F1I/L2K}$ RT p98:p51 was occasionally higher (1.7 ± 0.8). While this result would suggest a partial interference with processing the RT p51↓RNH cleavage site, it was statistically insignificant. Surprisingly, the processing of this junction in the RT-IN$^{F1I/L2K}$ mutant did not result in the expected formation of a stable 47 kDa RNH-IN fusion. Immunoprobing for IN revealed instead, the more prominent appearance of a 35 kDa intermediate RNH-IN protein. The relatively high ratio of p98:p35 IN

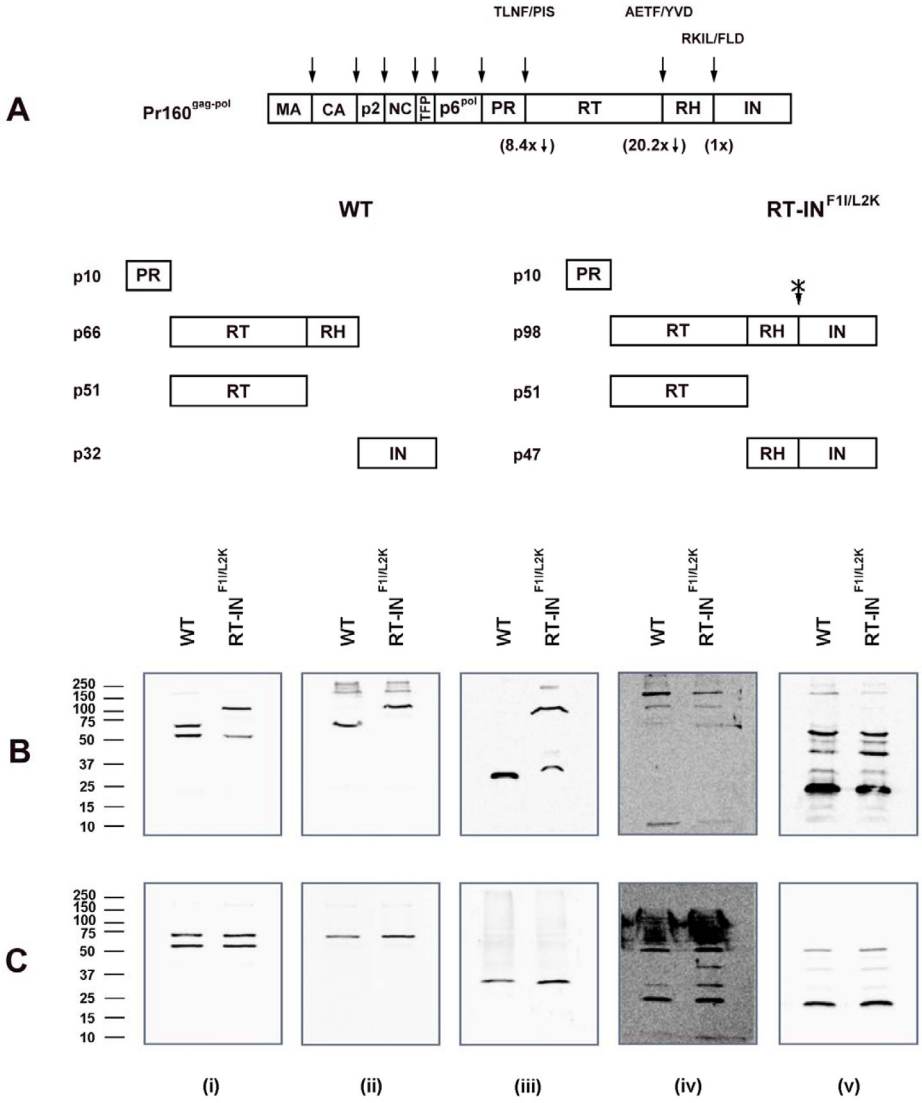

Figure 10. Processing products of the Pol region of wild-type and RT-IN$^{F1I/L2K}$ cleavage site mutant Gag-Pol. (A) Schematic representation of the Pr160$^{gag-pol}$ precursor with the nine major processing sites shown as vertical arrows. The rates of cleavage of the three cleavage sites in Pol, relative to RT↓IN cleavage site are shown below, as predicted by peptide cleavage *in vitro* with recombinant PR (80,517). In the lower panel are the expected processed products of Pol, with their estimated polyprotein sizes under wild-type (WT) conditions and when the RT↓IN cleavage site is mutated (RT-IN$^{F1I/L2K}$). (B) Western blots of WT and RT-IN$^{F1I/L2K}$ viruses (1 μg viral p24), generated by transfection of 293T cells and probed with (i) anti-RT, (ii) anti-RNH, (iii) anti-IN, (iv) anti-PR and (v) anti-p24 antibodies, followed by ECL exposure. The position of molecular size markers are shown to the left of each panel. With the exception of IN (ca. p35), immunoreactive Pol products of the RT-IN$^{F1I/L2K}$ mutant line up with their expected sizes. (C) Western blots of WT and RT-IN$^{F1I/L2K}$ viruses (1 μg viral p24), generated after multiple rounds of MT-2 cell infection (18 d). Immunoprobing and lane assignments were the same as those described above, before MT-2 cell infection.

content in the RT-IN$^{F1I/L2K}$ mutant (3.9 ± 1.3), combined with the low overall content of IN (20 ± 13 percent of wild-type) suggests that the 47 kDa RNH-IN fusion product may have been degraded upon formation. Our inability to detect either the 47 kDa or 35 kDa RNH-IN fusion protein on anti-RNH probed blots suggests that the epitope was lost. Further analysis using a PR-inactivated RT-IN$^{F1I/L2K}$ mutant indicated that this degradation of the 47 kDa RNH-IN fusion protein in the analogous PR-active mutant was due to HIV-1 PR activity and not diminished incorporation of Pr160$^{gag\text{-}pol}$ (Figure 11). Finally, as expected, the RT-IN$^{F1I/L2K}$ cleavage site mutation did not appear to effect the viral content level of immunoreactive PR or Gag proteins (Figure 10-B, panel iv and v) or viral particle/p24 production (Table 4).

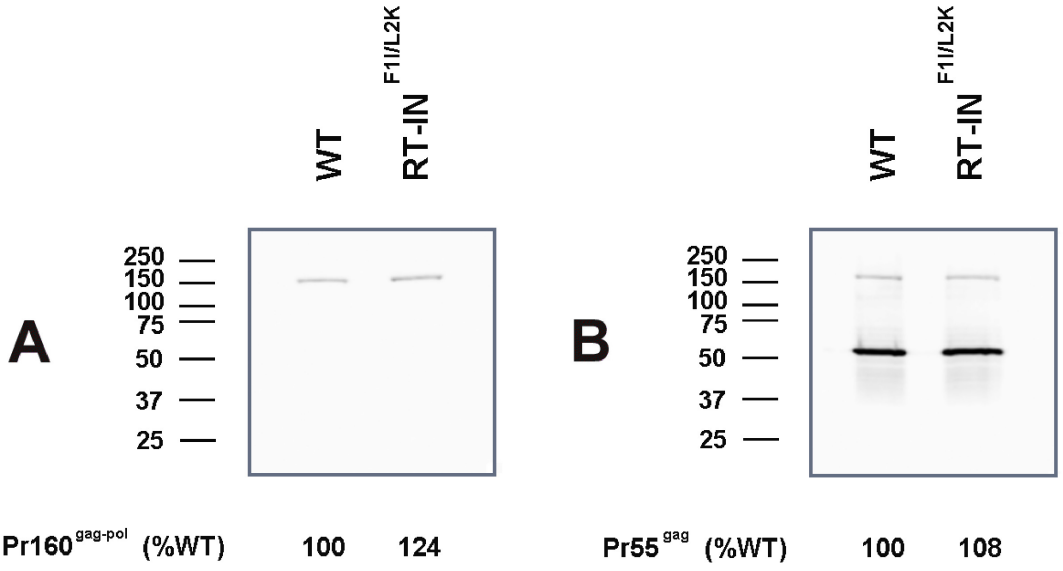

Figure 11. Effect of RT-IN$^{F1I/L2K}$ cleavage site mutation on Gag-Pol incorporation in immature virions. Immature virions containing inactive HIV-1 PR (D25A) and previously standardized by Pr55^{gag} content were ultracentrifuged, lysed and resolved by SDS-10% PAGE and Western blot analysis. Relative viral content of (A) Pr160$^{gag\text{-}pol}$ and (B) Pr55^{gag} were determined by probing separate blots with anti-RT and anti-p24 monoclonal antibodies respectively, followed by densitometry scanning analysis of ECL exposed blots under sub-saturating conditions.

2.4.2 Effect of RT↓IN cleavage site mutation on intravirion processing of Gag-Pol

Using an inducible bacterial expression vector we previously showed that the RT-IN$^{F1I/L2K}$ cleavage site mutation has no impact on the formation, or kinetics of appearance of RT p51 from a 90 kDa Pol polyprotein (Appendix, Figure 29) (471). However, in the absence of the terminal 28 kDa portion of IN, this expression system was not ideal to study the formation of full-length RT-IN and RNH-IN fusion proteins. A similar study showed upon expression of a full-length Pol polyprotein, that mutation of the RT↓IN cleavage site (L560I) resulted in the stable formation of 47 kDa RNH-IN (239). To investigate the effect of an RT-IN$^{F1I/L2K}$ mutation on the accumulation of Gag-Pol processing intermediates in the natural viral milieu, we used the protease inhibitor ritonavir (RTV). Virions isolated from COS-7 cells that were transfected in the presence of varying concentrations of RTV exhibited a dose-dependent diminution in the extent of Gag and Gag-Pol processing when probed for p24 and IN, respectively (Figure 12). Although the accumulative pattern and molecular weights of immunoreactive Gag-Pol intermediates was consistent with expected cleavage events (302,392,481), it was unfeasible to verify their identity by protein sequencing. At progressively elevated levels of PR activity, the disappearance of the higher molecular weight 98 kDa RT-IN intermediate was appropriately followed by the appearance of 47 kDa RNH-IN and 32 kDa IN. Introduction of the RT-IN$^{F1I/L2K}$ cleavage site mutation lead to the sustained presence of both 98 kDa RT-IN and 47 kDa RNH-IN intermediates to higher activity levels of PR. Between 0.05 and 0 μM RTV, however, the immunoreactive 47 kDa RNH-IN fusion protein diminished as a lower molecular weight 35 kDa IN protein became more prominent. The results of Figure 10 and 12 combined show that while an RT-IN fusion protein is stable to further proteolysis, an RNH-IN fusion protein is not.

2.4.3 Viral infectivity and replication kinetics of the RT↓IN cleavage site mutant

Single-cycle infectivity was assessed by infecting P4R5 cells with equivalent amounts (25 ng viral p24) of virus-containing cell-free supernatants derived from transfected 293T cells. After 48 h postinfection, cells were lysed and β-galactosidase expression was quantitated by fluorescence. The infectivity of the RT-IN$^{F1I/L2K}$ mutant virus was found to be severely attenuated, relative to the wild-type virus (Figure 13-A). This result did not correlate with the minor changes in virion RT and IN content. To determine whether the RT-IN$^{F1I/L2K}$ mutant virus could support productive infection we assessed viral replication kinetics during long-term propagation in MT-2 cells. MT-2 cells were inoculated with 293T-derived virions (20 ng viral p24) and HIV-1 induced cytopathic effect was monitored over time (Figure 13-B). In the wild-type infected culture, virus replication peaked after 5 days. In contrast, virus production in the RT-IN$^{F1I/L2K}$ infected culture was considerably delayed, and peaked 7 days later. An assessment of end point dilution infectivity in MT-2 cells further showed that the infectious capacity ($TCID_{50}$/p24) of the RT-IN$^{F1I/L2K}$ mutant virus was substantially lower than the wild-type virus (Table 4). Nevertheless, fusion between RT and IN in the RT-IN$^{F1I/L2K}$ mutant did not completely inhibit viral spread.

2.4.4 Virion-associated RT polymerase and RNH activities

To investigate the basis for the attenuated infectivity of the RT-IN$^{F1I/L2K}$ mutant virus we examined the virion-associated activities of the affected Pol enzymes. Both RT polymerase (RDDP) and RNH activities were assessed as described in Materials and Methods using appropriate substrates and equivalent amounts of virus. Interestingly, fusion of IN to the C-

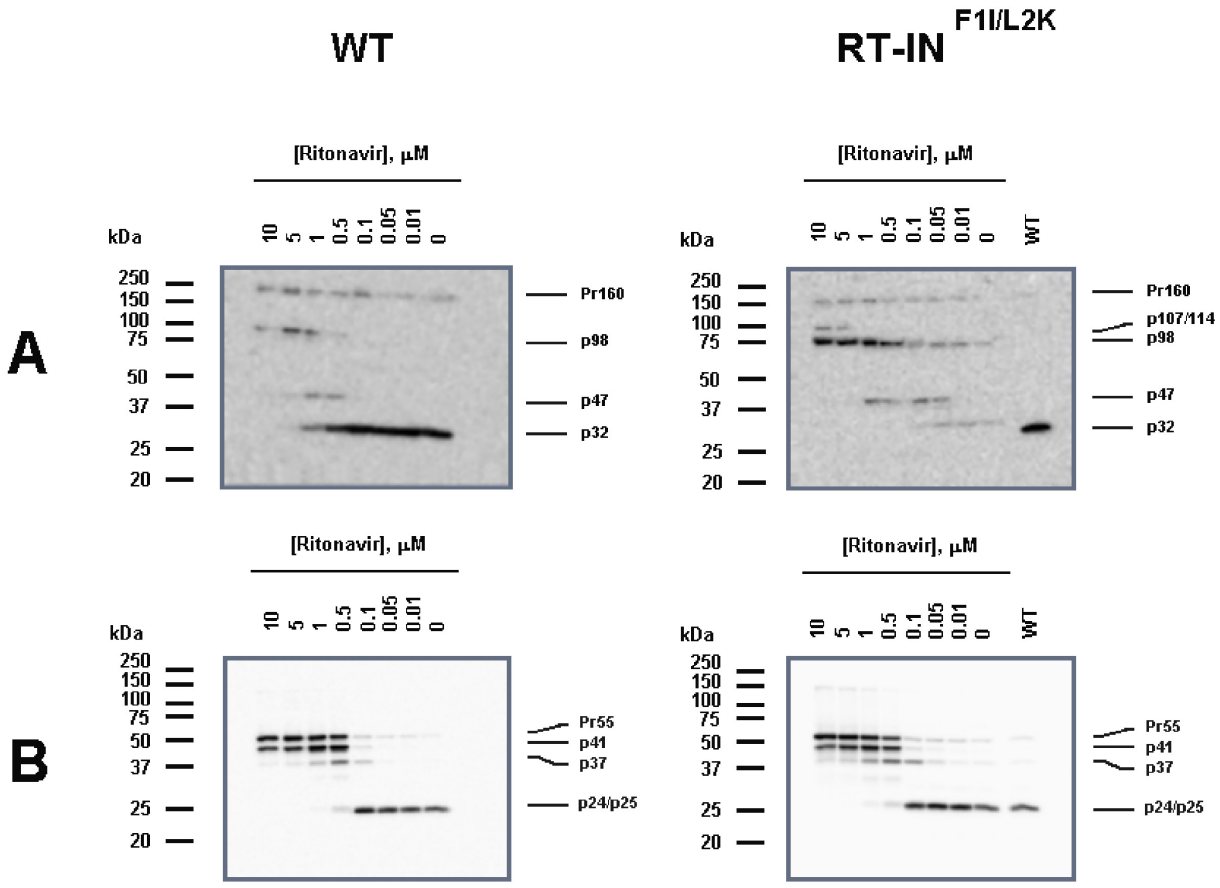

Figure 12. Effect of RT-IN$^{F1I/L2K}$ cleavage site mutation on ordered intravirion processing of Gag and Gag-Pol polyproteins. WT (left panel) and RT-IN$^{F1I/L2K}$ (right panel) virus-containing culture supernatants derived from COS-7 cells, transfected in the presence of decreasing concentrations of ritonavir were subjected to SDS-10% PAGE resolution and Western blotting analysis. Ordered accumulation of immunoreactive (A) Gag-Pol and (B) Gag polyprotein processing intermediates were observed by probing separate Western blots with anti-IN and anti-p24 antibodies respectively, followed by ECL exposure. The position of molecular size markers are shown to the left of each panel. Lines to the right of each panel indicate the positions and estimated molecular masses of expected polyprotein processing intermediates (302,392).

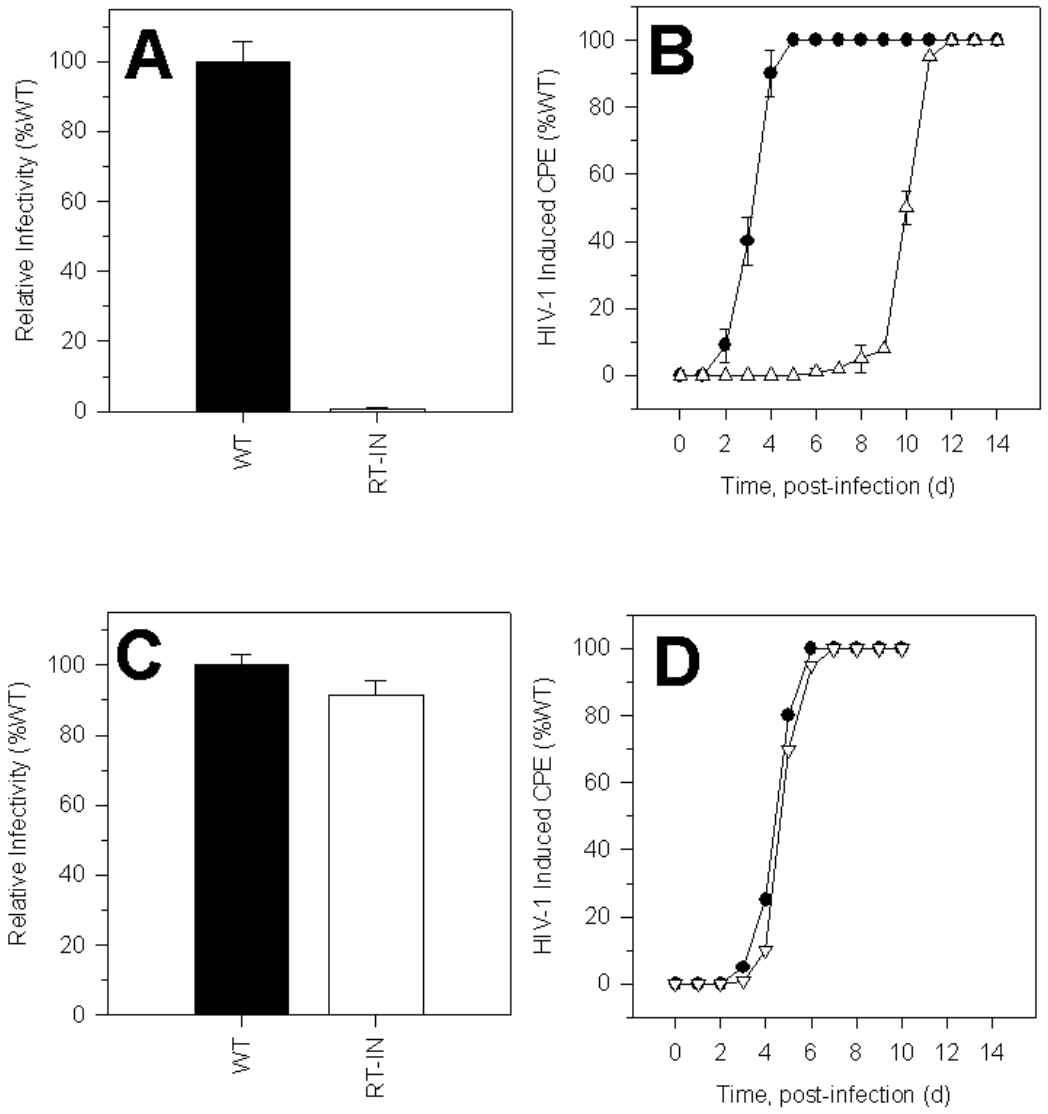

Figure 13. Effect of RT-IN$^{F1I/L2K}$ mutation on viral infectivity and replication. Left: (A, C) Single-cycle viral infectivity. P4R5 cells (5 x 10^3 cells) were infected in replicate (n = 5) with WT or RT-IN$^{F1I/L2K}$ mutant viruses (A) generated by transfection of 293T cells (25 ng viral p24) or (C) generated after 18 d of multiple rounds of MT-2 cell infection (5 ng viral p24). Infectivity was determined, relative to WT after 48 h of culture by fluorescent measurement of β-galactosidase gene expression, as described in Section 2.3.4. Right: (B, D) Viral replication kinetics. (B) MT-2 lymphocytoid cells (6.5 x 10^4 cells) were infected in duplicate with WT or RT-IN$^{F1I/L2K}$ mutant viruses generated by transfection of 293T cells (20 ng viral p24). (D) Virus stocks generated after 18 d of multiple rounds of MT-2 cell infection (B), were used to infect fresh MT-2 cells (5 ng viral p24). Cultures were split 1:2 every 3 d to prevent overgrowth and examined daily for HIV-1 induced cytopathic effect (syncytium formation). Symbol legend: WT (□), RT-IN$^{F1I/L2K}$ (▽).

Table 4. Absolute infectivity of WT and RT-IN$^{F1I/L2K}$ mutant HIV-1 before and after long-term replication in MT-2 cells.

[a] Median tissue culture infective dose ($TCID_{50}$/mL) of virions generated from transfected 293T cells

[b] Median tissue culture infective dose ($TCID_{50}$/mL) of replication-recovered viruses from MT-2 cells

[c] $TCID_{50}$/p24, expressed as percent wild-type represents the relative infectivity per virion. Data represent the means ± S.D. from three separate experiments.

Mutant	$TCID_{50}$/mL	p24 (ng/mL)	$TCID_{50}$/p24 (%WT) [c]
[a]WT	$9.2 \times 10^6 \pm 3.0 \times 10^6$	1222 ± 606	100 ± 18
[a]RT-IN$^{F1I/L2K}$	$2.2 \times 10^2 \pm 7.7 \times 10^1$	637 ± 252	$1.6 \times 10^{-3} \pm 6.3 \times 10^{-4}$
[b]WT	$1.5 \times 10^8 \pm 3.7 \times 10^7$	278 ± 0	100 ± 24
[b]RT-IN$^{F1I/L2K}$	$1.3 \times 10^8 \pm 2.3 \times 10^7$	285 ± 0	83 ± 15

terminal end of RT did not affect either the polymerase or RNH activity of RT. No significant differences were found with the RT-IN$^{F1I/L2K}$ mutant virus as compared to wild-type (Figure 14).

2.4.5 Characterization of viral revertants of the RT↓IN cleavage site mutant phenotype

Although infectivity was severely attenuated (Figure 13-A), repeated passage of MT-2 cells exposed to RT-IN$^{F1I/L2K}$ mutant virus eventually resulted in the appearance of virus with improved replication kinetics (Figure 13-B). This result was observed in separately conducted experiments. To determine if the increase in peak cytopathic effect was due to the emergence of

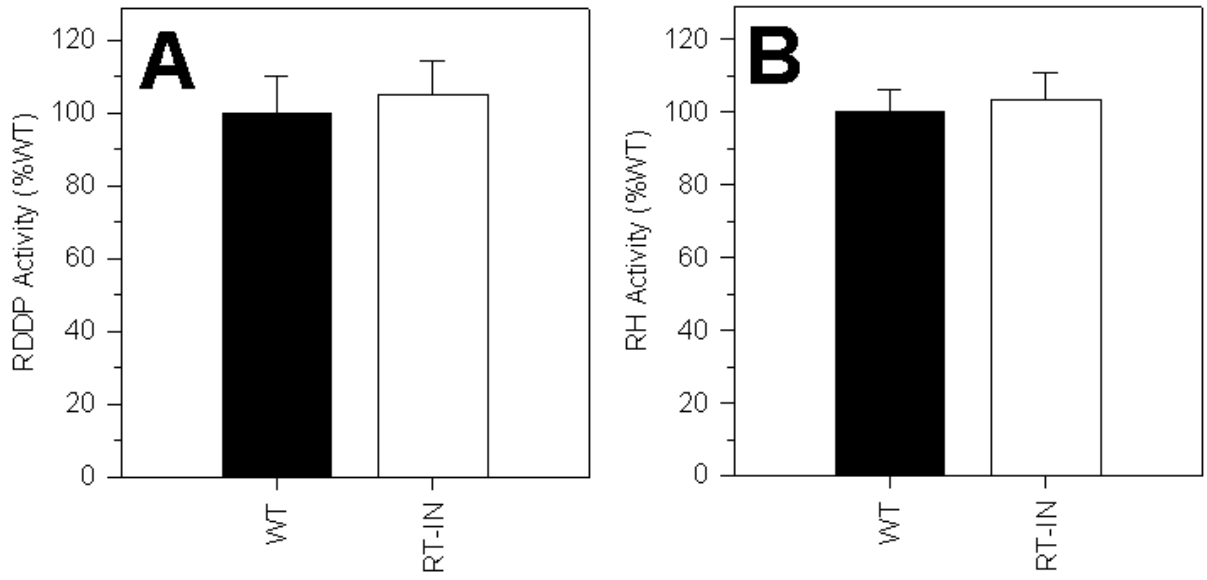

Figure 14. Effect of RT-IN$^{F1I/L2K}$ mutation on virion-associated RT polymerase and RNH activities. (A) RNA-dependent DNA polymerase (RDDP) activity. Virus-containing culture supernatants (250 ng viral p24) generated by transfection of 293T cells were purified through sucrose cushions, lysed and resuspended in reaction mixtures. The extent of [^{3}H]-dTTP incorporation into poly(rA)-oligo(dT)$_{12-18}$ was measured in replicate (n = 3) by liquid scintillation spectrometry after incubation for 1.5 h at 37^{o}C, as described in Section 2.3.8. (B) RNH activity. Virus-containing culture supernatants (25 ng viral p24) generated by transfection of 293T cells were assayed in replicate (n = 3) for RNH activity, as described in Section 2.3.9. Fluorescence intensity derived from hydrolysis of a fluorophore-quencher end-labeled RNA/DNA hybrid was monitored periodically and quenched after 45 min at 37^{o}C, during the linear phase of the reaction.

a phenotypic revertant, supernatant from this 14 d cell culture was briefly expanded and used to infect fresh MT-2 cells. Figure 13D shows that this long-term passaged RT-IN$^{F1I/L2K}$ mutant virus replicated in MT-2 cells almost as well as the wild-type. Consistent with this finding was a noticable increase in single-cycle infectivity (Figure 13-C) to near wild-type levels. Analysis of protein composition of the replication-recovered RT-IN$^{F1I/L2K}$ mutant virus revealed a normal wild-type RT p66/51 ratio and content of p32 IN (Figure 10-C), suggesting that a phenotypic reversion had occurred. Sequencing analysis of the proviral RT-IN coding region, extracted from the recovered RT-IN$^{F1I/L2K}$ mutant showed that the mutated RT↓IN cleavage site had reverted to the wild-type sequence (data not shown).

2.5 DISCUSSION

The complete proteolytic release of *pol*-encoded enzymes is believed to be a late event in the ordered pathway of Gag-Pol polyprotein processing during HIV-1 maturation (267,591). To date however, only a few studies have attempted to resolve the functional consequences of processing these PR-recognized cleavage sites in the context of the virion. Formation of the RT p66/51 heterodimer appears to require processing at three different sites, the N-terminal PR↓RT junction, the internal p51↓RNH junction and the C-terminal RT↓IN junction. The PR domain flanking the N-terminus of RT does not appear to influence RT activity (65,66), while processing of the internal RT p51↓RNH junction is essential for ensuring adequate virion levels of active RT that are resistant to further proteolysis (Chapter 3). However, in the context of the virion, the necessity for processing the C-terminal RT↓IN junction in the generation of functional RT

p66/51 heterodimers has remained unclear. In the current study, we have shown that mutation of the RT↓IN cleavage site generates RT p51 and a stable 98 kDa RT-IN fusion protein in the virion. Our data clearly show that processing of the C-terminal RT↓IN junction does not seriously impact on RT activity, since neither RT polymerase or RNH activities were affected by the fusion between RT and IN. Our results further suggest that proteolytic cleavage of the RT↓IN junction may be essential for IN activity, although the effect of RT-IN$^{F1I/L2K}$ cleavage site mutation on reverse transcription and other post-entry events, such as integration remains to be defined.

It was interesting to find that the 47 kDa RNH-IN fusion protein, released upon cleavage of the RT p51↓RNH junction in the RT-IN$^{F1I/L2K}$ mutant was further processed by HIV-1 PR to a 35 kDa form. This is in contrast to the observation that a full-length Pol polyprotein, containing an RT-IN^{L560I} cleavage site mutation and expressed in bacteria does not lead to further proteolysis, but rather the appearance of the expected 98 kDa RT-IN and 47 kDa RNH-IN fusion products (239). Moreover, recombinant HIV-1 IN is exceptionally resistant towards further proteolysis (111,519). By using decreasing concentrations of ritonavir we observed that the 47 kDa RNH-IN fusion product was indeed reasonably stable until higher activity levels of HIV-1 PR were present. This instability of the RNH-IN fusion protein thus seems to be a function of the intravirion milieu. Late stage processing of the RNH-IN fusion protein to the 35 kDa form may be due to the recognition of cleavage sites within a putative unfolded RNH domain that is released from RT p51. Indeed, RNH released in the formation of recombinant p66/51 RT from p66/66 RT is further cleaved at residues Y483, N494 and Y532 (23,61,515) and several attempts to identify or isolate the p15 RNH fragment from virions have proven unsuccessful (61,126,314).

In both retroviral and retrotransposon systems there is increasing evidence for the existence of an RT-IN processing intermediate in the maturation of the RT (1,521,558) and for functional interactions between RT and IN (185,363,573). Precedence for a *cis* association between RT and IN proteins can found in ASLV (166,198,292) and HTLV-I (521), where IN forms an integral part of the β subunit of active RT. In both αβ and ββ dimeric forms of ASLV RT, the IN domain of the β subunit serves to stabilize the enzyme (165,166,198). Interestingly, the ββ form of ASLV RT exhibits significant RT polymerase and RNH activities (164,553), implying that one of the β subunits must adopt a catalytic conformation. While cleavage of the RT↓IN junction in ASLV may be partial or not at all, it is not surprising that mutation of this site has little effect on RT polymerase or RNH activities (193). Similar observations of RT↓IN cleavage site mutation have also been documented in the MLV system (195,496,497), where Gag-Pol processing appears to have no role in the activation of RT (77,250). We now have extended these findings by demonstrating that processing of the C-terminal RT↓IN junction is not critical in the generation of active HIV-1 RT.

It is unclear whether there is significant RT activity (polymerase and RNH) associated with unprocessed forms of Gag-Pol to facilitate reverse transcription (15,211,243,388,485). The current state of evidence, based on polymerase activity alone, suggests that proteolytic processing is indeed necessary to achieve complete activation of RT. Our findings, in combination with those of the PR↓RT cleavage site (65,66) indicate that processing of either flanking RT cleavage site (N-terminal or C-terminal) has no adverse effect on viral RT activities. The caveat in either scenario is that the internal RT p51↓RNH cleavage site continues to be processed. As mentioned earlier, we have recently shown that this internal cleavage event is necessary in the generation of a stable form of active RT that is refractory to further proteolysis

(Chapter 3). Moreover, mutation of both RT↓IN and RT p51↓RNH cleavage sites results in the same instability of RT (Appendix, Figure 30). Thus, at this stage, it is difficult to determine whether viral PR-RT or RT-IN fusion proteins alone would be capable of facilitating efficient postinfection reverse transcription. While recombinant RT-IN exhibits a low level of RT polymerase activity *in vitro* (211,284), complementation with RT p51 in the virion may serve to acheive complete activation and proteolytic stability of the final enzymatic form.

Partial cleavage within the RT subunit appears to be unique to lentiviruses such as HIV-1, whereas partial cleavage of the RT↓IN junction is common to ASLV and HTLV-I. We have shown that an end-to-end *cis* association between HIV-1 RT and IN proteins in virions does not seriously impact on RT activities. This result is consistent with the report that RT activity and processivity is unaffected by IN when provided in *trans* (185,573). Our finding that p98 RT-IN and p51 RT were present in near equal proportion in RT-IN$^{F1I/L2K}$ mutant virions suggests that p98/51 RT heterodimers may have been formed. Although p98/98 RT homodimers may also have been present, it is unlikely that this oligomeric form alone could account for the observed wild-type levels of RT activity (211,284,293). In considering that the RT-IN (β) subunit of ASLV αβ RT is not the catalytic subunit (164,553), further biochemical and biophysical study of a fully functional p98/51 RT heterodimer could certainly prove very interesting.

The final stage of reverse transcription generates a linear proviral DNA substrate that is acted upon by IN. Both RT and IN are present within the nucleoprotein complex during reverse transcription and are retained within the nuclear preintegration complex prior to integration (46,124,210). Recent evidence suggests that an association between RT and IN is mediated through direct interaction of the C-terminal region of IN with the fingers-palm and connection subdomain of RT (185,573,588). Furthermore, this *trans* association between RT and IN not

only stimulates the initiation of reverse transcription (573), but also dramatically enhances the strand transfer activity of IN (58,185,573). It therefore seemed reasonable to ask whether prevention of this *trans* association would have an adverse effect. While mutation of the RT↓IN cleavage site was found to have no serious affect on virion-associated RT activity, infectivity and replication capacity were significantly attenuated. Taken together, these results suggest that processing of the RT↓IN cleavage site is not essential for RT activity, but in the context of viral replication may be important for IN activity.

Two forms of viral IN were present in RT-IN$^{F1I/L2K}$ mutant virions, RT-IN and RNH-IN. It was interesting, that attenuated RT-IN$^{F1I/L2K}$ mutant virus was able to replicate and eventually cause genetic and phenotypic reversion. This suggests that IN activity may not have been completely ablated by the RT↓IN cleavage site mutation. Alternatively, host-mediated illegitimate DNA recombination could have taken place until such time that the virus could recover; a phenomenon which has been documented in a subset of transformed T-lymphocytoid cell lines (145,316,352). Whether the RT-IN or RNH-IN form of IN was more active remains unclear. However, it is likely that our HIV-1 RT-IN$^{F1I/L2K}$ mutant resembles the ASLV system where αβ and ββ forms of RT are 30-fold less efficient than p32 IN in strand transfer activity (193). This begs the question then, why would an abutting RT or RNH domain negatively regulate IN activity, and how? A timely regulation of IN activity, by cleavage of the RT↓IN junction during virion maturation may simply reflect its position in the polyprotein and attributes of folding and assembly, since there is no rational reason to delay its activation in the virion context. Functional IN is clearly not required until after reverse transcription in the infected host cell. Possible factors that might account for inefficient intracellular integration, when the RT↓IN cleavage site is mutated include structural constraints of preintegration complexes, defects in

nuclear import or the timing of initiation of IN activities. Moreover, the RT or RNH domain in the RT-IN$^{F1I/L2K}$ mutant could have created a structural barrier hindering accessibility to the DNA LTR ends, or caused disturbances in multimerization and the highly conserved functional motifs of the adjoining IN domain.

In summary, formation of functional viral RT does not appear to require processing of either N- or C-termini cleavage sites (PR↓RT and RT↓IN). However, processing of the internal RT p51↓RNH cleavage site is critical, since mutation here was shown to lead to aberrant processing and loss of activity (Chapter 3). Our finding that processing of the RT↓IN junction may be necessary for IN activiation suggests that while HIV-1 RT can functionally exist in fusion with IN, this form of association is deterimental to efficient viral replication. While a physical *trans* association between RT and IN has been reported to be biologically relevant (185,573,588), we now suggest that a *cis* association between these enzymes may disturb those functional interactions essential for integration, but not reverse transcription.

CHAPTER 3: VIRION INSTABILITY OF HIV-1 RT MUTATED AT THE RT p51↓RNH PROTEASE CLEAVAGE SITE

3.1 ABSTRACT

Each of the HIV-1 *pol*-encoded enzymes, protease (PR), reverse transcriptase (RT) and integrase (IN) is active only as a dimer (or higher order oligomer in the case of IN). Of the three, only RT comprises subunits of different mass. RT is a heterodimer of 66 kDa and 51 kDa subunits. The latter is formed by HIV PR-catalyzed cleavage of p66 during virion maturation, resulting in the removal of the ribonuclease H (RNH) domain of a p66 subunit. In order to study the apparent need for RT heterodimers in the context of the virion, we introduced a variety of mutations in the RT p51↓RNH protease cleavage site of an infectious HIV-1 molecular clone. Surprisingly, rather than leading to virions with increased RT p66 content, most of the mutations resulted in significantly attenuated virus that contained greatly decreased levels of RT that in many cases was primarily RT p51. This finding was in direct contrast to the effect that these mutations had on the processing of recombinant p66/66 RT and a truncated Pol polyprotein expressed in bacteria. Most mutant virions had wild-type IN levels; only a few showed somewhat diminished incorporation of the $Pr160^{gag\text{-}pol}$ precursor polyprotein. Mutant virion p24 Gag levels were equivalent to wild-type virus, suggesting that the incorporation and processing

of Gag was not compromised by the RT p51↓RNH site mutations. Repeated passage of MT-2 cells exposed to mutant viruses led to the appearance of virus with improved replication capacity; these virions contained normally processed RT at near wild-type levels. These results imply that additional proteolytic processing of RT to the p66/p51 heterodimer is essential to provide proteolytic stability of RT during HIV-1 maturation.

3.2 INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) genome encodes a variety of different proteins including the essential viral enzymes protease (PR), reverse transcriptase (RT) and integrase (IN). These viral enzymes are translated, not as discrete units, but rather as segments of a much larger polyprotein termed Gag-Pol ($Pr160^{gag\text{-}pol}$), and nascent virions assemble using these polyprotein precursors. The individual active enzymes are subsequently formed by proteolytic cleavage at specific sites on Gag-Pol during virion assembly and budding, a "maturation" process catalyzed by PR (187,244).

The monomeric subunits of the HIV-1 enzymes are inactive; each enzyme must oligomerize to at least a dimer for enzymatic activity (16,110,418). PR and IN are homodimers with subunits of the size predicted from their genes. The mature active form of HIV-1 PR is a symmetrical homodimer released from Gag-Pol upon auto-processing carried out by the polyprotein form of the enzyme (311). RT and IN are formed after activation of PR, but it is still unclear whether the PR-mediated proteolytic processing of these Pol proteins occurs in *cis*, in *trans*, or some combination of these. Like HIV-1 PR, active HIV-1 IN is at least a homodimer,

although higher order homo-oligomers may play a role in the multiple activities of this enzyme (228,233). In contrast, RT in mature infectious virions is a heterodimer with subunits of 66 kDa (p66) and 51 kDa (p51) (512), even though the gene for HIV-1 RT encodes only a protein of 66 kDa. The smaller p51 subunit is derived from the larger p66 subunit by proteolytic cleavage between RT amino acid residues F440↓Y441 during virion maturation (61,119,203,515). Although both p66 and p51 subunits have identical amino acid sequences, their folding in the context of the active RT heterodimer differs, resulting in an asymmetric dimer structure (263,546). The catalytic activities of HIV-1 RT, namely DNA polymerase and ribonuclease H (RNH), are carried out solely by the p66 subunit of the RT heterodimer (285). The function of the p51 subunit is not entirely clear, but it may play a primarily structural role (4).

Thus, formation of mature active RT from the Gag-Pol polyprotein precursor in HIV-1 virions requires an additional PR-catalyzed cleavage event compared to that for the formation of active IN or PR. This suggests that the heterodimeric form of HIV-1 RT is essential for virus replication. However, recombinant RT p66/p66 homodimers have significant enzymatic activities (DNA polymerase and RNH) compared to RT p66/p51 heterodimers (23,128). Furthermore, the RT in virions of other retroviruses such as Moloney murine leukemia virus (Mo-MuLV) consists of a single subunit (210), suggesting that the catalytically active enzyme is a monomer or perhaps homodimer. In addition, chimeras constructed from the first 425 amino acid residues of HIV-1 RT and the last 200 amino acid residues of Mo-MuLV RT have a single subunit composition and possess substantial enzymatic activity (331).

So why does maturation of HIV-1 RT require an additional proteolytic processing step to convert p66 to p51 to form an active heterodimeric enzyme? To address this question, we introduced into molecular clones of HIV-1 a variety of mutations in the proteolytic cleavage site

for the conversion of RT p66 to p51, in an attempt to block this PR-dependent processing step. Unexpectedly, we found that all amino acid substitutions tested resulted in much reduced levels of virion RT. As well, when present, the major antibody-reactive RT in the mutant virions was almost exclusively the p51 form. Our data suggest that the additional proteolytic processing step for RT during virion maturation may be needed to stabilize the conformation of this viral enzyme to optimize its essential function in viral replication.

3.3 MATERIALS AND METHODS

3.3.1 Reagents and plasmid mutagenesis

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAD, NIH: HeLa-CD4-LTR/β-Galactosidase cells (HCB) obtained from Dr. Michael Emerman (259); human T-lymphocytoid MT-2 cells from Dr. Douglas Richman; HIV-1_{SF2} p24/25 Gag and anti-HIV-1_{SF2} p24/25 IgG mAb (76C) from Dr. Kathelyn Steimer, Chiron Corporation; and HIV-1 PR antiserum from DAIDS, NIAID by Biomolecular Technologies. The p6HRT-PROT vector (283) for expression of poly(histidine) affinity labeled recombinant HIV-1_{IIIB} p66/51 RT was a generous gift of Dr. Stuart Le Grice, NCI-Frederick (Frederick, MD). Anti-HIV-1_{IIIB} RT and anti-HIV-1_{IIIB} RNH (2F2) IgG mAb were previously generated in our laboratory against recombinant p66/51 RT (294), as was anti-IN polyclonal mouse Ab against recombinant IN. Homopolymeric template/primers (T/P), poly(rA)-oligo$(dT)_{12\text{-}18}$, poly(dC)-oligo$(dG)_{12\text{-}18,}$ [^{3}H]-TTP, [^{3}H]-dGTP, restriction enzymes, and goat anti-mouse-HRP secondary mAb were all purchased from Amersham Pharmacia Biotech

(Piscataway, NJ, USA). The SuperPico ECL Substrate System for detection of peroxidase-labeled antibody was obtained from PIERCE (Rockford, IL, USA). Sequencing primers and mutation-containing oligonucleotides were purchased from Invitrogen (Carlsbad, CA, USA). COS-7 and human T-lymphocytoid MT-2 cell lines were obtained from the American Type Culture Collection (Rockville, MD). HIV-1 p24 antigen ELISA kits were obtained from SAIC-Frederick (Frederick, MD). *E.coli* strains TOP10, BL21(DE3)pLysS and JM109 were obtained from Invitrogen and Promega (Madison, WI), respectively.

3.3.2 HIV-1 molecular clone preparation, transfection and virus culture

Plasmid pSVC21-BH10 encodes an infectious molecular clone of the IIIB (HxB2) strain of HIV-1 and carries an SV40 origin of replication for expression in COS-7 cells (127). Mutations in the region corresponding to the RT p51↓RNH cleavage site (residues 437 - 443) were introduced using the Quick Change™ Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). For the purpose of studying Gag-Pol ($Pr160^{gag\text{-}pol}$) incorporation a catalytic inactivating mutation (D25A) was similarly introduced into the PR coding region of each of these clones. The presence of the expected mutations was verified by sequencing.

All cell lines were maintained in either DMEM (COS-7 and HCB) or RPMI 1640 (MT-2) medium containing 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, penicillin G (100 U/mL), and streptomycin (100 U/mL, Gibco-BRL/Life Technologies, Gaithersburg, MD). Culture medium for HCB cells was additionally supplemented with Gentemicin (G418; 200 μg/mL) and hygromycin B (100 μg/mL). Virus was prepared by transfection of COS-7 cells using LipofectAMINE Plus (Invitrogen, Carlsbad, CA). Virus-containing culture supernatants were harvested 60 h post-transfection, clarified by centrifugation (3000 x g, 1 h at 4^{o}C), and

quantified by analyzing the levels of HIV-1 p24 antigen. Aliquots of virus preparations were stored at -80^{o}C until use.

Infectiousness of virus particles produced from transfection of COS-7 cells was determined by using normalized quantities of HIV-1 p24 antigen content and HCB or MT-2 cells as targets. Single-cycle viral infectivity was assessed using HCB cells in a MAGI assay (1 μg or 100 ng HIV-1 p24 per 4 x 10^4 cells), as previously described (259). Multiple-round viral replication was assessed by inoculation of MT-2 lymphoblastoid cells (1 μg or 50 ng of HIV-1 p24 per 1 x 10^5 cells) followed by daily microscopic observation of HIV-1 induced syncytium formation, as previously described (32,339). In a concurrent manner, the median tissue culture infective dose ($TCID_{50}$/mL) of each mutant virus was determined after seven days of culture, as described elsewhere (231).

Cytopathology appeared in infected MT-2 cells maintained in culture for 30 days. At this time virus-containing culture fluids were expanded briefly for 5 days by infection of fresh MT-2 cells and collected for subsequent analysis. Chromosomal DNA was extracted from these infected MT-2 cells using QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA). The HIV-1 Pol-encoding region was then PCR amplified and cloned into pCR-T7/CT TOPO (Invitrogen, Carlsbad, CA) for sequencing analysis.

3.3.3 Immunoblotting analysis of viral proteins

Clarified virus-containing supernatants (1 or 5 μg viral p24) were purified and concentrated by ultracentrifugation through 20% (w/v) sucrose cushions (175,000 x g, 1.5 h at 4^{o}C). Virus pellets were lysed in 35 μL RIPA-lysis buffer (20 mM Tris-Cl, pH 8.0 containing 120 mM NaCl, 2 mM EDTA, 0.5% DOC, 0.5% NP-40 as well as 2 μg/mL PMSF, 10 μg/mL

apoprotein and 10 μg/mL pepstatin A). Virion particle protein composition was assessed by subjecting the lysate proteins to SDS-10% PAGE resolution and Western blotting. Western blots were incubated with either anti-HIV-1 RT (5 μg/mL) or anti-HIV-1 p24 (5 μg/mL) monoclonal antibodies, or mouse anti-IN polyclonal serum (1:50) followed by incubation with secondary horse radish peroxidase-conjugated goat anti-mouse antibody (1:3000). Immunoreactive protein bands were visualized by enhanced chemiluminescence on a VersaDoc Imaging System, and quantitated by densitometry under sub-saturating exposure conditions using Quantity One v4.3.0 software (Bio-Rad, Hercules, CA). To assess the level of Gag-Pol ($Pr160^{gag\text{-}pol}$) incorporation, RT p51↓RNH mutant viruses containing inactivated PR (D25A) were pre-normalized by Gag ($Pr55^{gag}$) content prior to ultracentrifugation and Western blot analysis.

3.3.4 Assay of virion-associated RT activity

Virion-associated RT RNA-dependent DNA polymerase (RDDP) activity was measured using [^{3}H]-TTP and poly(rA)-oligo$(dT)_{12\text{-}18}$ as template-primer and RT DNA-dependent DNA polymerase (DDDP) activity was measured using [^{3}H]-dGTP and poly(dC)-oligo$(dG)_{12\text{-}18}$ as template-primer. Reaction mixtures (50 μL total volume) contained 50 mM Tris-Cl (pH 7.9), 5 mM $MgCl_2$, 150 mM KCl, 0.5 mM EGTA, 0.05% (v/v) triton X-100, 2% (v/v) ethylene glycol, 5 mM DTT, 0.5 mM GSH, 50 μg/mL poly(rA)-oligo$(dT)_{12\text{-}18}$ or poly(dC)-oligo$(dG)_{12\text{-}18}$, and 20 μCi [^{3}H]-TTP or 10 μCi [^{3}H]-dGTP. Reactions were initiated by the addition of virus-containing culture supernatant (8 ng HIV-1 p24), incubated at 37^{o}C for 5 h, then quenched with 250 μL of ice-cold 10% trichloroacetic acid containing 20 mM $NaPP_i$. Quenched samples were left on ice for 30 min, filtered through glass fiber Type C filter multi-well plates (Millipore Corporation, Bedford, MA, USA) and washed sequentially with 10% trichloroacetic acid containing 20 mM

$NaPP_i$ followed by ethanol. The extent of radionucleotide incorporation was determined by liquid scintillation spectrometry.

3.3.5 Analysis of proteolytic processing of RT from recombinant p66/66 RT and a Pol polyprotein expressed in bacteria

These experiments used the pol-pET28a(+) vector that we previously used to study wild-type Pol polyprotein processing (471), and the p6HRT-PROT vector to express recombinant p66/51 RT. The pol-pET28a(+) vector encodes for a truncated 90 kDa Pol polyprotein precursor encompassing the last four amino acid residues of NC, the native transframe region or TFR (TFP + $p6^{pol}$), PR, RT, and the first 46 amino acid residues of IN. The p6HRT-PR vector encodes for the PR and RT genes (283). We introduced the same RT p51↓RNH cleavage site mutations as used in preparation of mutant virions into both vectors, as well as the catalytic inactivating D25R mutation into the PR gene of duplicate constructs.

To examine proteolytic processing of the truncated Pol polyprotein from pol-pET28a(+), overnight transformed cultures of *E.coli* BL21(DE3)pLysS were diluted 1/150 in minimal media (containing 35 μg/mL kanamycin and chloramphenicol), grown at 37°C to an OD_{600nm} of 0.05, and induced with 1 mM IPTG. At various time intervals post-induction (0, 40, and every 10 min to 200 min), 0.5 mL aliquots were removed, briefly centrifuged (13,000 *g* for 30 s) and pellets resuspended in 1X SDS loading buffer. Induced proteins from total cell lysates were resolved by SDS-10% PAGE followed by Western blot analysis and probing with anti-HIV-1 RT IgG mAb (5 μg/mL). The kinetics of Pol polyprotein expression and processing was assessed by visualizing immunoreactive RT protein bands by enhanced chemiluminescence on a VersaDoc Imaging System (Bio-Rad, Hercules, CA).

To examine proteolytic processing of RT p66, *E.coli* JM109 was transformed with p6HRT-PROT, and pDPM1.I (to control for basal expression from the lac gene) (59,283). Overnight cultures were diluted 1/100 in minimal media (containing 100 μg/mL ampicillin and 35 μg/mL kanamycin), grown at 37^{o}C to an OD_{600nm} of 0.6 and induced with 1 mM IPTG. After 4 h post-induction, RT protein content in total cell lysates derived from 0.5 mL aliquots was examined in the same manner as described above.

3.4 RESULTS

3.4.1 Selection of mutations in the p51↓RNH cleavage site in HIV-1 RT

Previous work has shown that while HIV-1 PR does not recognize a consensus sequence, the specificity of HIV-1 PR-catalyzed cleavages depends strongly on sequence context (27,383,385,397,406,407,516). The scissile peptide bonds recognized by HIV-1 PR lie within a seven amino acid segment, and the specificity of cleavage depends on the structural and conformational context of this segment rather than the primary sequence itself (537). The sequence of the RT p51↓RNH cleavage site is AETF^{440}↓Y^{441}VD. Our choice of mutations to introduce into this sequence (Table 5) was based on PR context-dependent specificity, stringency around PR-recognized scissile bonds, ranging from low (P1'), restricted (P4, P1, P2'), to high (P2), and the type of amino acid substitution (conservative or non-conservative).

Table 5. Mutations introduced into the RT p51↓RNH cleavage site.

[a] Amino acid substitutions introduced at various positions flanking the RT p51↓RNH scissile bond are underlined

[b, c] Abbreviation and substrate position(s) of the mutation(s)

[d] Conservative or radical mutation based Dayhoff's ranking of functionally conserved amino acid groups, whereby chemical similarity is not necessarily as valuable as size (87)

[e] Basis for the mutation with respect to known commonalities and determinants of HIV-1 PR context-dependent specificity (170,383,397,516)

Abbrev. [b]	Sequence [a] (P4-P3')	Substrate position [c]	Classification [d]	Comments [e]
WT	AETF↓YVD	N/A	N/A	Wild-type sequence
A437I	***I***ETF↓YVD	P4	Radical	Longer P4 to disrupt interactions with PR flap regions
V442S	AETF↓Y***S***D	P2'	Radical	Polar residue substitution
F440W	AET***W***↓YVD	P1	Conservative	Maintain hydropathicity and scissile bond aromatic symmetry
F440V	AET***V***↓YVD	P1	Radical	Non-preferred β-branched residue
T439S/V442G	AE***S***F↓Y***G***D	P2/P2'	Conservative	Diminish side chain interactions of these corroborative positions
Y441I/V442K	AETF↓***IK***D	P1'/P2'	Radical/ Conservative	Disrupt hydropathicity / Longer positive residue
F440A	AET***A***↓YVD	P1	Radical	Non-preferred small side-chain
F440A/Y441A	AET***A***↓***A***VD	P1/P1'	Radical	Non-preferred small side-chain
F440W/Y441W	AET***W***↓***W***VD	P1/P1'	Conservative	Maintain hydropathicity and scissile bond aromatic symmetry
E438N	A***N***TF↓YVD	P3	Conservative	Removal of γ-carbonyl to diminish H-bond interactions with PR

3.4.2 RT content of mutant virions

We expected that the mutations chosen would provide HIV virions containing varying ratios of RT p66 and p51, depending on the contribution of the specific mutated residue to PR recognition of the cleavage sequence (Table 5). Surprisingly, most of the RT p51↓RNH cleavage site mutations significantly diminished virion RT levels (Figure 15-A1). Different mutations provided different perturbations of virion RT p66/p51 heterodimer content, ranging from an increased p51 to p66 ratio relative to wild-type (A437I, V442S), to only p51 (F440A/Y441A), to virtually complete loss of all immunoreactive RT protein (F440V, Y441I/V442K). All mutant virions with detectable immunoreactive RT possessed dramatically increased levels of low molecular weight RT fragments (Figure 15-A2).

3.4.3 Effect of RT p51↓RNH cleavage site mutations on HIV virion content of other Pol proteins

The appearance of small molecular weight RT fragments in many of the mutant virions suggested that aberrant PR activity might be a factor in the observed phenotype. However, it was also possible that the mutations affected virion incorporation of Gag-Pol ($Pr160^{gag\text{-}pol}$). To test this, we probed mutant virions for IN p32. Mutant virions with the most pronounced defects in RT p66/p51 content also showed reduced IN p32 levels (Figure 15-B), although IN levels were not reduced to nearly the same extent as that of RT. We were unable to probe for levels of virion PR as the anti-PR polyclonal antibody on hand had insufficient specificity. However, the mutants showed wild-type levels of Gag ($Pr55^{gag}$) processing (Figure 15-C), suggesting near normal levels of PR activity in the virions. To better evaluate whether RT p51↓RNH cleavage site mutations affected virion incorporation of Gag-Pol during virus assembly, we inactivated HIV-1

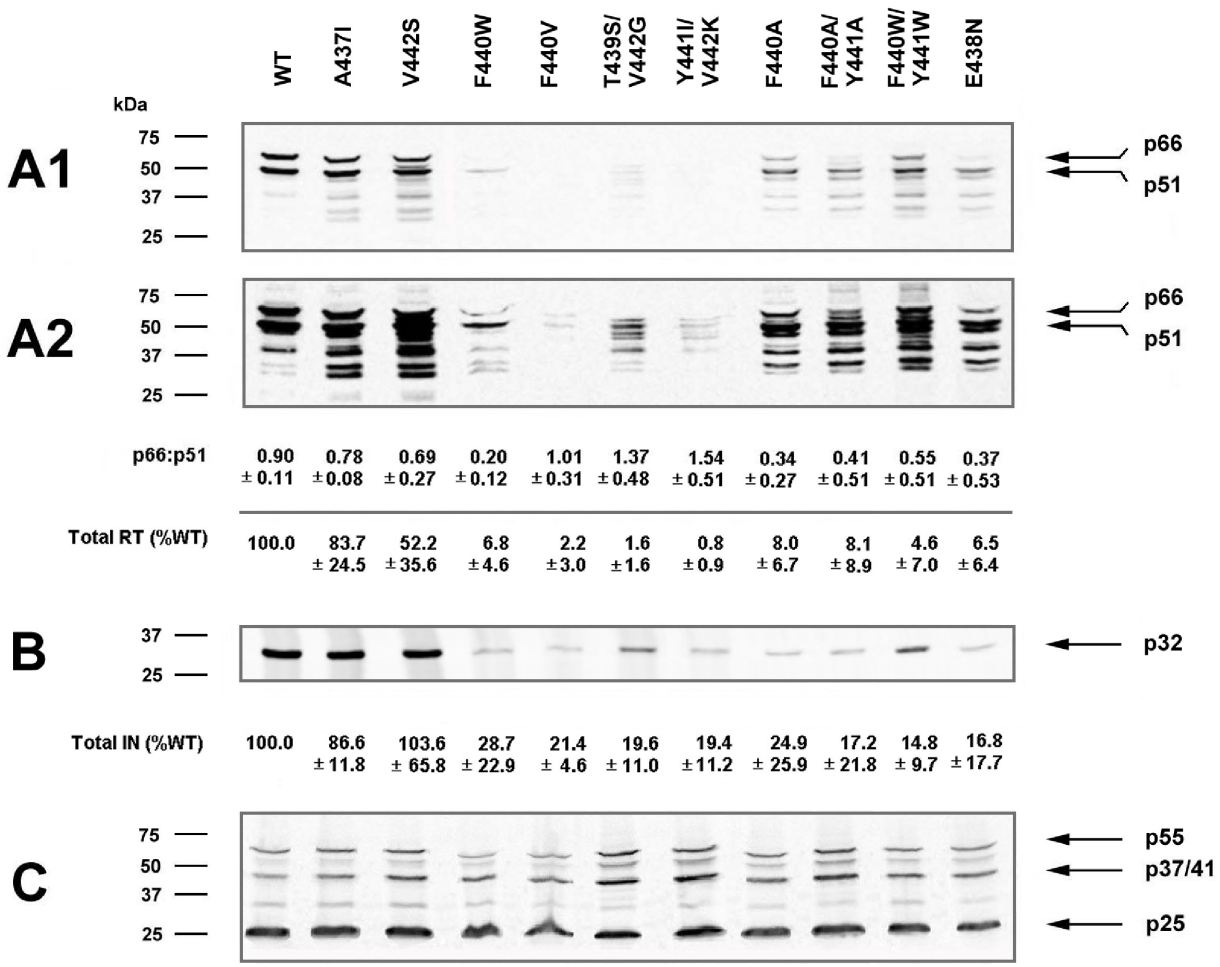

Figure 15. Effect of p51↓RNH cleavage site mutations on viral particle protein composition. Western blot analysis of wild-type and p51↓RNH cleavage site mutant viruses (5 μg viral p24) generation by transfection of COS-7 cells and probed with (A1-2) anti-RT, (B) ant-IN, and (C) anti-p24 antibodies, respectively. A1 and A2 represent under and over-exposures of viral RT content, respectively. Quantitation of viral proteins, from three independent trials was performed by densitometric scanning analysis of ECL exposed blots under sub-saturating conditions.

PR by introducing the D25A mutation into the protease gene (284,305) in our RT p51↓RNH cleavage site mutants. As shown in Figure 16, relative levels of $Pr160^{gag\text{-}pol}$ and $Pr55^{gag}$ in most mutants (with the exception of E438N and T439S/V442G) were similar to those of wild-type HIV-1 particles. The lack of degradation products in the PR-inactivated RT p51↓RNH cleavage site mutants suggests that the degradation of RT noted in the analogous PR-active mutants was due to HIV-1 PR activity, and not to any cellular proteases that may have been carried into the nascent virion particles.

3.4.4 Virion-associated RT activity, infectivity, and viral replication kinetics of the RT p51↓RNH cleavage site mutants

Equivalent amounts (8 ng of HIV-1 p24) of virion-containing cell-free supernatants of transfected COS-7 cells were assessed for RT RDDP and DDDP activities. All mutants showed substantially decreased RT polymerase activity levels, ranging from 1-60% of of the wild-type virus (Figure 17). This diminution of RT activity correlated well with the severity of the loss of p66/p51 heterodimeric RT in the various mutant virions (Figure 15-A). Similarly, mutant virus infectivity in single cycle MAGI cell infectivity assays was attenuated to different extents (Figure 18-A), and correlated well with virion RT content, composition and activity.

We also assessed virus replication kinetics during long-term propagation in MT-2 cells. MT-2 cells were inoculated with high input (1 μg HIV-1 p24 per 1 x 10^5 cells) of COS-7 derived virions and HIV-1 induced cytopathic effect was monitored over time (Figure 18-B). Mutants with RT p66/p51 heterodimer content of at least 50% that of wild-type virus (A437I, V442S) showed replication kinetics similar to wild-type when cells were exposed to a high virus inoculum. However, these same mutants showed delayed replication kinetics compared to wild-

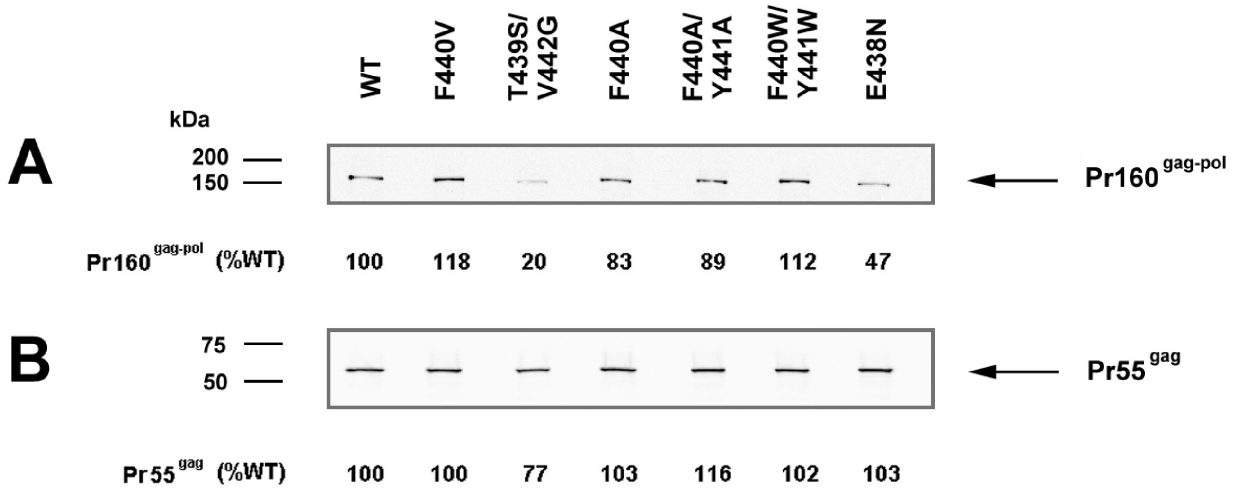

Figure 16. Effect of p51↓RNH cleavage site mutations on incorporation of Gag-Pol ($Pr160^{gag\text{-}pol}$) into immature viral particles. Western blot analysis of immature viruses, previously standardized for Gag ($Pr55^{gag}$) content by densitometry. Relative viral content of (A) $Pr160^{gag\text{-}pol}$ and (B) $Pr55^{gag}$ were determined by probing separate blots with anti-RT and anti-p24 monoclonal antibodies, respectively. Quantitation of viral polyproteins was performed by densitometric scanning analysis of ECL exposed blots under sub-saturating conditions.

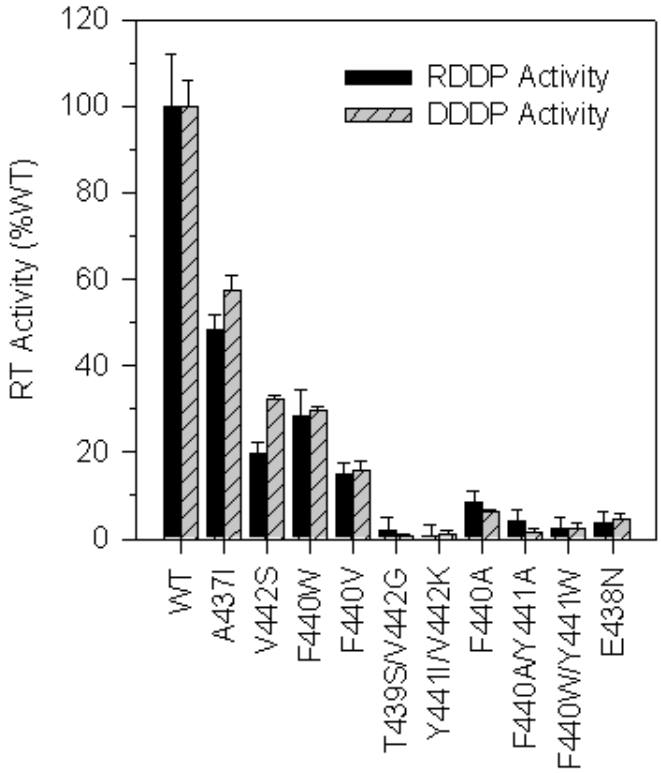

Figure 17. Virus particle-associated RT activities. Clarified virus-containing culture supernatants (8 ng viral p24) were assayed separately for RNA-dependent DNA polymerase (RDDP) activity and DNA-dependent DNA polymerase (DDDP) activity by the incorporation of [^{3}H]-dTTP into poly(rA)-oligo(dT)$_{12\text{-}18}$ and [^{3}H]-dGTP into poly(dC)-oligo(dG)$_{12\text{-}18}$, respectively. Reaction mixtures were prepared as described in Section 3.3.4, incubated at 37^{o}C for 5 h, and quenched with 10% TCA/NaPP$_i$. Values are expressed as a percentage relative to WT virus. Each bar represents the average of three separate measurements.

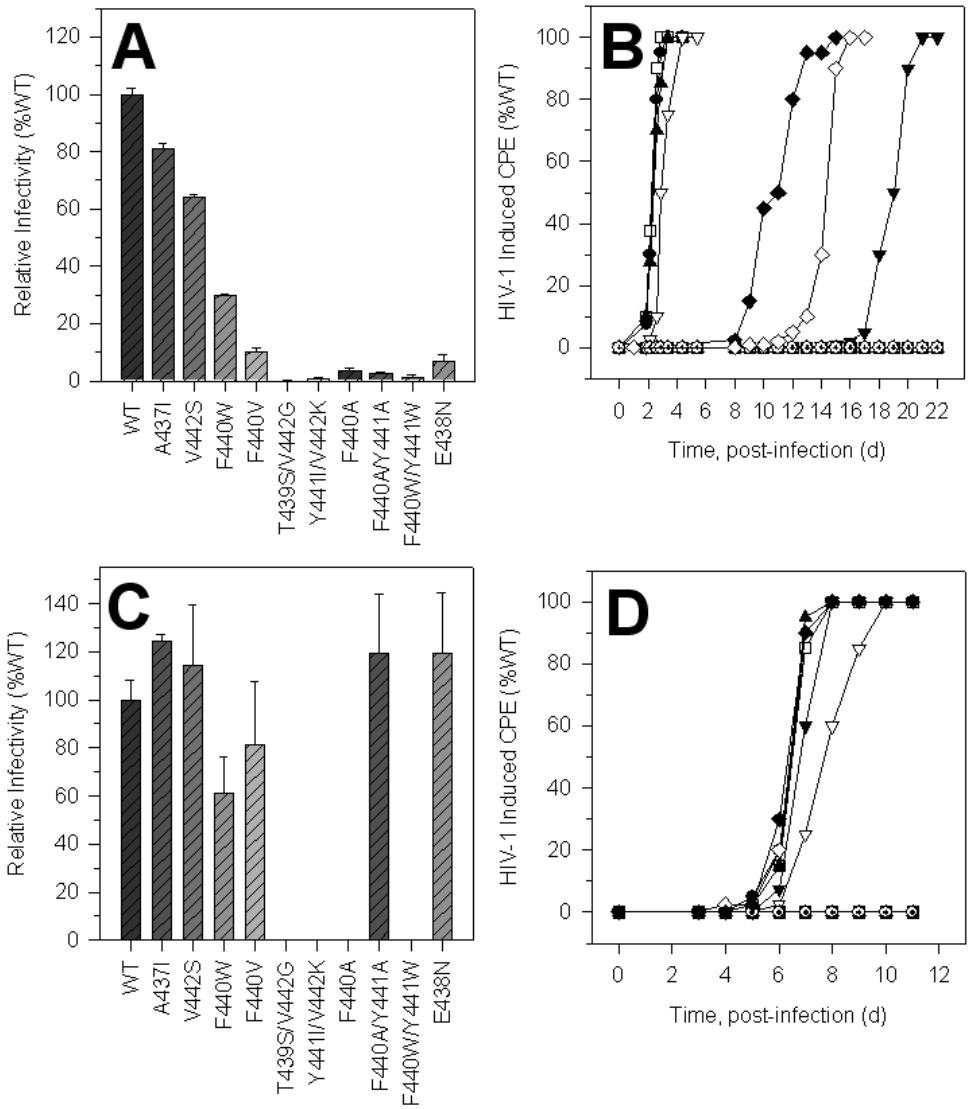

Figure 18. Replication analysis of p51↓RNH cleavage site mutant viruses. (A, C) Single-cycle (MAGI) viral infectivity. HeLa-CD4-LTR/β-galactosidase (MAGI) cells (4 x 10^4) were infected separately with (A) COS-7 generated mutant virions (1 μg viral p24) or (C) MT-2 cell generated mutant viruses derived after 35 d of culture (100 ng viral p24). MAGI cells were stained and quantitated for β-galactosidase gene expression at 48 h post-infection. Percent infectivity is expressed relative to WT with each bar representing the average of two separate measurements. (B, D) Viral replication kinetics analysis. MT-2 lymphocytoid cells (1 x 10^5) were (B) infected with COS-7 generated mutant virions (1 μg viral p24) or (D) re-infected with MT-2 cell-generated mutant viruses derived after 35 d of culture (50 ng viral p24). Cultures were split every 3 d to prevent overgrowth and HIV-1 induced cytopathic effect (CPE) was scored daily as percent syncytium formation. WT (□), A437I (G), V442S (▲), F440W (▽), F440V (◆), T439S/V442G (○), Y441I/V442K (□), F440A (△), F440A/Y441A (▼), F440W/Y441W (⊙), E438N (∀).

type when lower viral inocula were used (data not shown), consistent with the lower $TCID_{50}$/p24 values of these mutants relative to wild-type (Table 6). Interestingly, the F440W mutant showed only a minimal delay in replication, despite significantly decreased virion RT composition and content (Figure 15-A). All other mutants showed significantly delayed (F440V, E438N, F440A/Y441A) or no detectable replication even after 35 days of passage.

Repeated passage of MT-2 cells exposed to mutant viruses resulted in the appearance of virus with improved replication capacity. Viruses inducing discernable cytopathic effect were harvested 30 days post-infection (12 cell passages), briefly amplified for 5 days and then assessed in both single cycle MAGI cell infectivity assays and in virus spread (multiple round replication assays) assays. All long-term passaged mutant viruses except F440A, T439S/V442G and Y441I/V442K mutants (none of which replicated during long term passage) showed near wild-type replication kinetics both in single cycle MAGI cell infectivity assays (Figure 18-C) and in virus spread assays in MT-2 cells (Figure 18-D).

Analysis of the protein composition of the "recovered" mutant viruses showed near normal RT content and RT p66/p51 ratio (Figure 19-A) as well as wild-type levels of IN (Figure 19-B). Sequencing of the proviral RT gene produced by the "recovered" mutants (E438N, F440A/Y441A) indicated that the mutated RT p51↓RNH cleavage site had reverted to the wild-type sequence. The other "recovered" mutants retained the cleavage site mutations. We are presently carrying out detailed sequencing analysis of these mutants in an attempt to identify potential compensatory mutations that might contribute to the restoration of normal RT processing and viral infectivity (Chapter 4).

Table 6. Infectivity of recombinant WT and mutant HIV-1.

Median tissue culture infective dose ($TCID_{50}$/mL) of COS-7 cell-generated virions assayed as described in Materials and Methods. Data represent the means ± S.D. from four separate experiments. The ratio $TCID_{50}$/p24 represents the relative infectivity per virion, calculated upon dividing the mean $TCID_{50}$/mL by the amount of produced viral p24 in units of ng/mL. Virus-containing supernatants that were unable to be titered due to low infectivity are indicated by ND (not determined).

Mutant	$TCID_{50}$/mL	p24 (ng/mL)	$TCID_{50}$/p24 (%WT)
WT	$2.2 \times 10^4 \pm 4.0 \times 10^3$	300 ± 58	100 ± 14
A437I	$4.7 \times 10^3 \pm 1.7 \times 10^3$	176 ± 90	31 ± 11
V442S	$3.3 \times 10^3 \pm 4.6 \times 10^2$	146 ± 83	25 ± 3
F440W	$5.0 \times 10^2 \pm 5.3 \times 10^1$	204 ± 69	3 ± 0
F440V	ND	241 ± 34	ND
T439S/V442G	ND	178 ± 1	ND
Y441I/V442K	ND	208 ± 7	ND
F440A	ND	182 ± 19	ND
F440A/Y441A	ND	182 ± 60	ND
F440W/Y441W	ND	219 ± 36	ND
E438N	ND	183 ± 69	ND

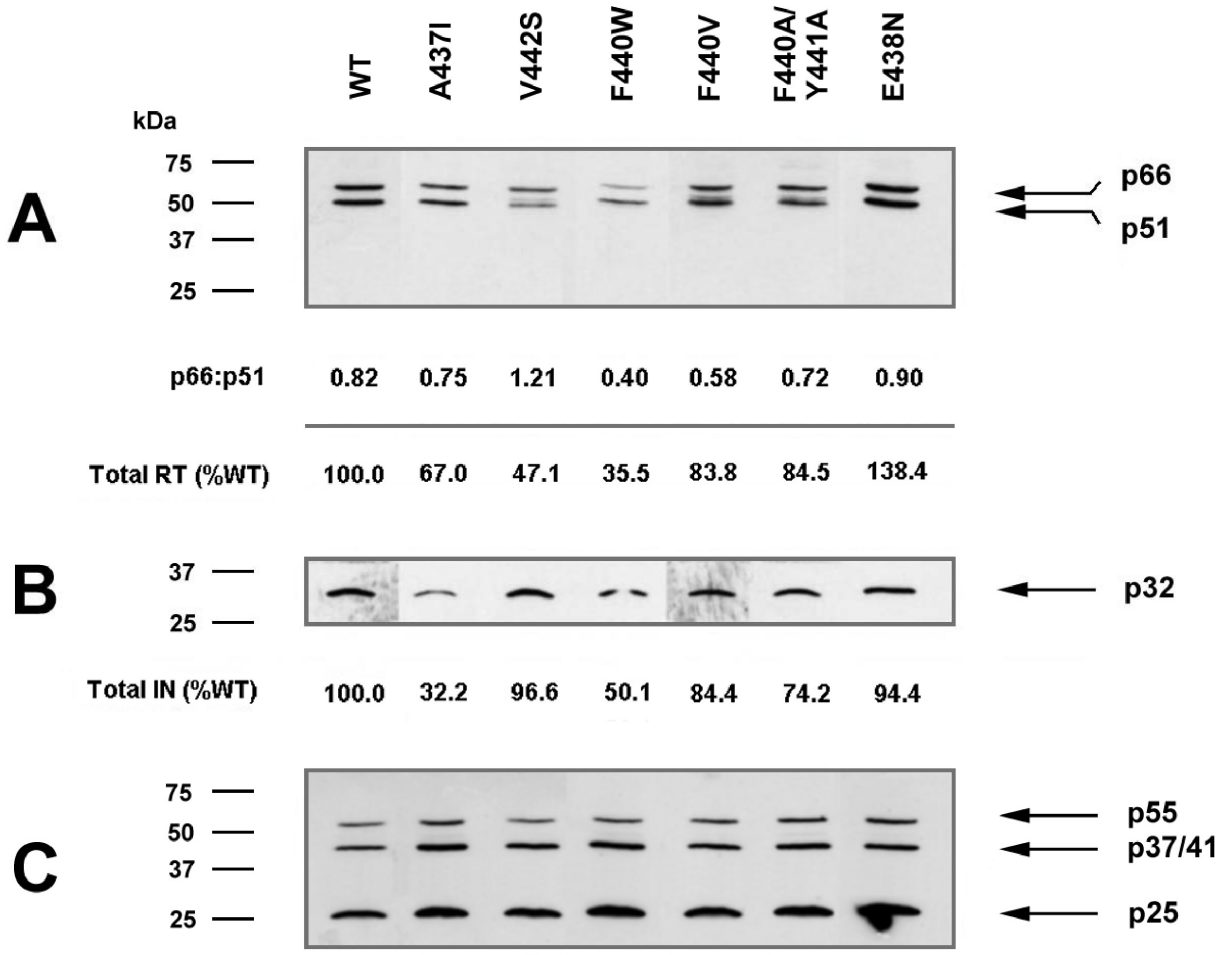

Figure 19. Western blot analysis of viral protein expression of p51↓RNH cleavage site mutant viruses after multiple rounds of MT-2 cell infection. Wild-type and replication-recovered p51↓RNH mutant viruses (100 ng viral p24), generated after long term culture in MT-2 cells were probed with (A) anti-RT, (B) anti-IN, and (C) anti-p24 antibodies, respectively. Quantitation of viral proteins was performed by densitometric scanning analysis of ECL exposed blots**.** The position of molecular size markers are shown to the left of each panel.

3.4.5 Effect of p51↓RNH cleavage site mutations on RT content in bacterial expression systems

As we have previously seen (471), and repeated in the following study, the kinetics of wild-type RT processing follow an ordered sequential pathway from a 90 kDa Pol polyprotein expressed in bacteria. After a buildup of RT p66, RT p51 begins to appear. The levels of RT p51 increase over time concomitant with a decrease in RT p66, until the levels of the two RT subunits are approximately equal (Figure 20-A). Inhibition of HIV-1 PR with the D25R mutation indicated that this course of events was not due to the action of bacterial proteases (Figure 20-E, F and Figure 21-B). In contrast to our findings in the virion context (Figure 15-A), the introduction of p51↓RNH cleavage site mutations into pol-pET28a(+) resulted in the formation of vastly different RT proteins. The E438N mutation resulted in the eventual formation of RT p51, as per the viral expression system, however F440A/Y441A and F440V mutations resulted only in the formation of RT p90 and RT p66, respectively (Figure 20-D, B and C). Similarly, expression of cleavage site mutant HIV-1 RT p66 and PR from p6HRT-PROT resulted in minimal processing to RT p51 (Figure 21-A).

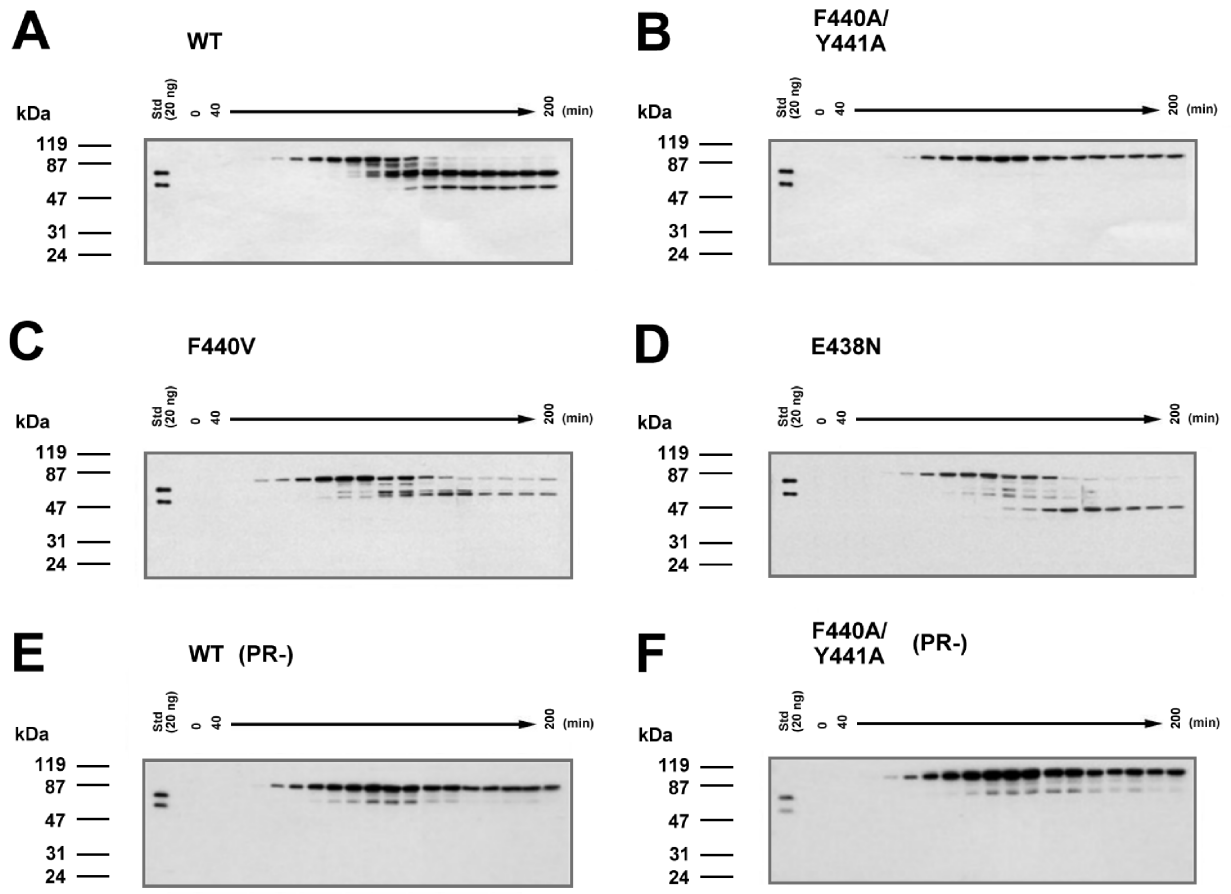

Figure 20. Effect of p51↓RNH cleavage site mutations on the synthesis and processing of a 90 kDa Pol polyprotein *in vitro*. (A) Western blot analysis of the synthesis and processing of RT products following IPTG induction of a wild-type 90 kDa Pol polyprotein. Aliquots were taken every 10 min for analysis (from 0, 40 to 200 min post-induction) and probed with anti-RT monoclonal antibodies. Western blot of the synthesis and processing of RT products following IPTG induction of a 90 kDa Pol polyprotein containing (B) F440A/Y441A, (C) F440V, and (D) E438N p51↓RNH cleavage site mutations. As a negative control, synthesis and processing of (E) wild-type and (F) F440A/Y441A Pol polyproteins was examined upon inactivation of HIV-1 PR (D25R). The position of molecular size markers are shown to the left of each panel.

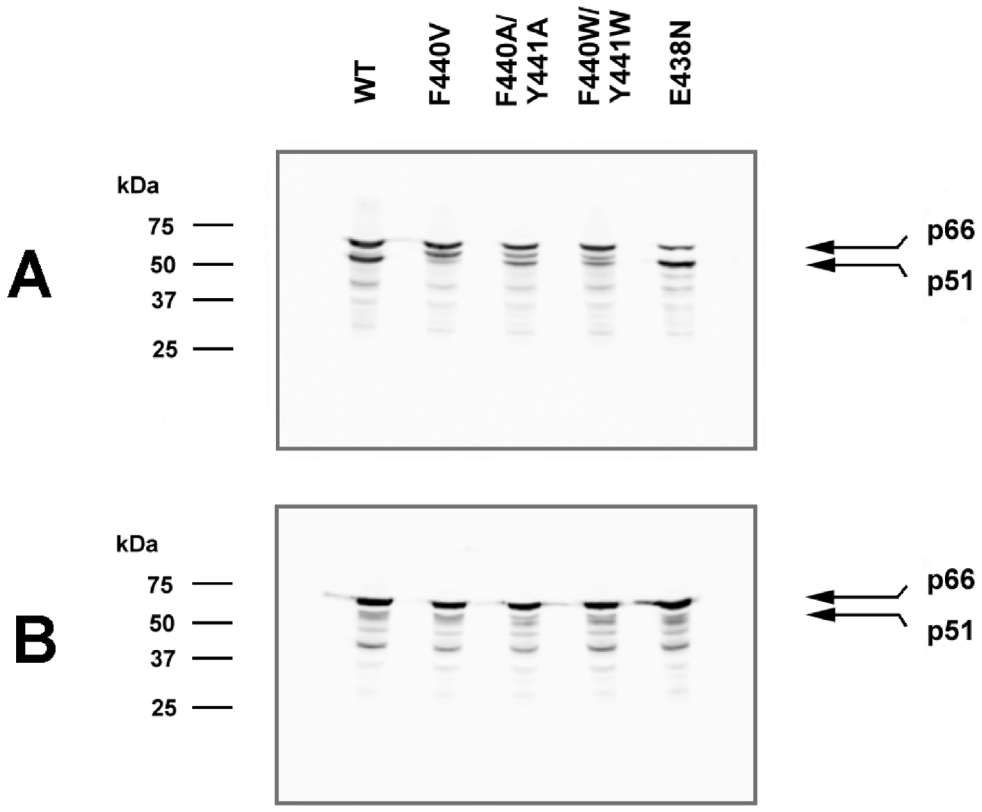

Figure 21. Effect of p51↓RNH cleavage site mutations on the synthesis of p66/51 RT *in vitro*. (A) Western blot analysis of RT subunit composition, following IPTG induction of RT p66 in the presence of HIV-1 PR. (B) Western blot analysis of RT subunit composition, following IPTG induction of RT p66 in the presence of inactive HIV-1 PR. Blots were probed with a pool of anti-RT monoclonal antibodies, followed by ECL exposure. The position of molecular size markers are shown to the left of each panel.

3.5 DISCUSSION

Each of the three HIV-1 Gag-Pol-derived enzymes, PR, RT and IN, are active only as dimers (or possibly higher order oligomers in the case of IN). However, of the three, only RT is a heterodimer. The formation of the p66/p51 RT heterodimer thus requires an additional proteolytic cleavage event during virion maturation. We have previously demonstrated that the kinetics of RT processing follow an ordered sequential pathway from a truncated Pol polyprotein expressed in bacteria (471). HIV-1 PR inhibitor studies suggest that similar processing kinetics may occur in the HIV-1 virion (471) (Chapter 4). However, because isolated recombinant HIV-1 p66/66 RT homodimers possess similar enzymatic activity *in vitro* as recombinant p66/51 RT heterodimers (23,128,178,206), the need for the RT heterodimer structure in HIV-1 virions is unclear. One possibility is that the initially processed RT homodimer is in a quasi-stable conformation, unlike that of purified recombinant RT homodimers, and that formation of p66/p51 RT heterodimers proceeds through this quasi-stable p66/66 RT homodimer intermediate (471).

Our data clearly show that mutations within the seven amino acid protease recognition sequence that defines the p51↓RNH cleavage site in the RT p66 subunit lead to defects in virion RT processing such that the mutant virions contain primarily RT p51 and/or much reduced levels of RT. Since Gag-Pol incorporation in the mutant virions was in most cases similar to that of wild-type virions, it seems that the p66/66 RT homodimer (or perhaps the RT p66 monomer) may not be proteolytically stable in the virion. Proteolytic removal of one of the RNH domains may thus be essential for providing a conformation of RT that is refractory to further proteolytic events within the virion, thereby stabilizing the protein. Our data also clearly show that the

diminution of mutant virion RT content is due to over-processing by HIV-1 PR, since no aberrant processing of RT was noted in when HIV-1 PR was inactivated (Figure 16) in mutant constructs that showed severe processing defects with active PR.

The mechanism by which HIV-1 PR cleaves only one of the RT p66 subunits to form the p66/51 RT heterodimer has not been unequivocally established. Numerous crystal structures of the p66/p51 RT heterodimer show that the seven amino acid p51↓RNH cleavage sequence in the p66 subunit is buried (263,314,546) and therefore presumably inaccessible to PR. Some investigators propose that the p66/66 RT homodimer is an asymmetric dimer similar to the p66/51 RT heterodimer, but with the RNH domain of the soon-to-be p51 subunit unfolded to an extent that allows PR-mediated cleavage at the p51↓RNH junction (85,203,314,515,546). This may be the result of energy derived from RT subunit dimerization that induces strain in one of the RNH domains that is relieved by unfolding along the tether region towards the p51↓RNH cleavage site (85,169,203). Other studies, supported by circular dichroism data, suggest that RT p66/p66 may be a symmetrical homodimer, but that removal of the RNH domain from one of the p66 subunits induces conformational changes in the other p66 subunit that protect this subunit from similar cleavage at its p51↓RNH junction (5,338). Unfortunately, no structure for the p66/p66 RT homodimer is yet available, so it is unclear whether the p51↓RNH cleavage sequence is accessible in only one or in both subunits of the homodimer, or whether the homodimer possesses different inter- or intra-subunit contacts with this region. It is possible that the p51↓RNH cleavage site mutations may have altered surface accessibility of this region to allow processing of alternative nearby cleavage site(s) in both RT p66 subunits, leading to extensive destabilization and subsequent degradation of the protein.

While our present studies do not allow us to unequivocally differentiate between these models, we favour a model in which the virion p66/p66 RT homodimer initially exists in a "quasi-stable" symmetrical conformation, and that it is this form of the RT homodimer that can undergo proteolytic processing to the p66/p51 RT heterodimer. We have been unable to generate significant amounts of p66/p51 RT heterodimer by *in vitro* treatment of purified recombinant p66/p66 RT with HIV-1 PR in *trans* under physiological pH and enzyme-substrate concentrations (Appendix, Figure 31). This suggests that the conformation of the purified p66/p66 RT homodimer may be very different from that formed during HIV-1 PR-mediated processing of the Gag-Pol polyprotein in the virion. Interestingly, introduction of certain RT p51↓RNH cleavage site mutations into RT p66 or a truncated Pol polyprotein followed by expression in bacteria did not generally lead to aberrant proteolytic cleavage RT p51 (Figure 20 and 21). Instability of RT mutated in the p51↓RNH cleavage sequence thus seems to be a function of the intravirion milieu. The observation that many of the p51↓RNH cleavage site mutations resulted in primarily RT p51 in the virions is intriguing. The predominance of RT p51 may be due to cleavage at proposed alternative site(s) (e.g. G436↓A437) near the p51↓RNH junction (23,61,515) that do not induce conformational changes in the other p66 subunit to protect this subunit from similar cleavage at its p51↓RNH junction. Alternatively, the mutations may not in themselves prevent cleavage at the p51↓RNH junction, but instead prevent the attainment of a stable form of the RT p66/p51 heterodimer that is refractory to additional PR cleavage. Our attempts to identify the C-terminus of the RT p51 in mutant virions have been unsuccessful, due to the limited amounts of virion protein attainable. Furthermore, efforts to mutate possible alternative cleavage sites in combination with p51↓RNH mutations have resulted in the same instability of RT (Appendix, Figure 32).

Virion RT content has been estimated at 20 to 100 molecules of RT heterodimer (375,376,493), but in theory a single molecule of p66/p51 RT could complete reverse transcription. Certain p51↓RNH cleavage site mutants (e.g. A437I, V442S) showed relatively equivalent levels of virion RT p66 and p51 subunits, although the level of RT was reduced relative to wild-type virus (Figure 16-A). The infectivity of these mutants was significantly attenuated (Table 6). This implies that the virion needs a substantial quantity of the RT heterodimer for efficient viral replication. Our data are consistent with previous studies using phenotypically mixed virions (236) that showed virus infectivity correlated with the amount of active RT, especially RT DNA polymerase activity, in the virus inoculum. The apparent "excess" of RT in the virion may be important to compensate for the relatively low processivity of HIV-1 RT, thereby facilitating completion of viral DNA synthesis.

The complete proteolytic release of *pol*-encoded enzymes is believed to be a late event in the ordered pathway of Gag-Pol polyprotein processing (267,591). Formation of the p66/p51 RT heterodimer requires proteolytic cleavage at three different sites, the N-terminal PR↓RT junction, the C-terminal RT↓IN junction, and the internal p51↓RNH junction. However, few studies have attempted to define the functional consequences at the virus level of mutating these PR-recognized cleavage sites in the Pol polyprotein. Mutation of the PR↓RT junction prevented cleavage at this site, and resulted in virions containing RT subunits of 77 kDa (corresponding to the PR-RT p66 fusion) and 62 kDa (corresponding to the PR-RT p51 fusion) (65,66). The mutant virions showed wild-type levels of RT activity, and were only slightly attenuated in infectivity. Other retroviruses such as prototype foamy virus (PFV), a human spumaretrovirus, contain PR-RT fusions. RT in PFV virions consists of a completely processed 80 kDa PR-RT subunit and an unprocessed 127 kDa PR-RT-IN (Pro-Pol) subunit (398). RT activity appears to be associated

with both homodimer forms (p80/p80 and p127/p127) as well as the p127/p80 heterodimer (261).

We have recently mutated the RT↓IN junction and found that the ensuing virions contain RT subunits of 98 kDa (corresponding to the expected RT p66-IN fusion) and 51 kDa (RT p51), as well as IN subunits of 98 kDa (RT p66-IN) and approximately 35 kDa (corresponding to an RNH-IN fusion) (Chapter 2). Mutant virions containing the RT-IN fusions retain wild-type levels of RT activity. RT-IN fusions are found in virions of avian sarcoma leucosis virus (ASLV) (526). ASLV RT exists as subunits of 63 kDa (α subunit, corresponding to the mass defined by the RT gene) and 95 kDa (β subunit, corresponding to an RT-IN fusion). RT activity is present in both αα and ββ homodimers as well as the αβ heterodimer form of ASLV RT (64,553).

Thus, mutations in the cleavage sites defining the N- and C-termini of HIV-1 RT p66 result in stable fusion proteins of the expected mass, with relatively normal RT DNA polymerase activity. However, cleavages within the RT subunit seem to be unique to lentiviruses such as HIV-1. We examined the effect of a large number of conservative and non-conservative mutations throughout the seven amino acid p51↓RNH cleavage site in HIV-1 RT. None of these resulted in the expected virion p66/p66 RT homodimer phenotype, but instead resulted in dramatic alterations in PR processing of the RT protein, suggesting that the phenotype arising from this internal cleavage is essential for ensuring adequate virion levels of active RT.

CHAPTER 4: THE SECOND-SITE MUTATION T477A IN HIV-1 REVERSE TRANSCRIPTASE RESTORES NORMAL PROCESSING OF GAG-POL MUTATED IN THE p51↓RNH CLEAVAGE SITE

4.1 ABSTRACT

The gene for HIV-1 reverse transcriptase (RT) encodes a 66 kDa protein, but mature HIV-1 RT is a p66/51 heterodimer. RT p66 is translated as part of a 160 kDa Gag-Pol polyprotein, and the RT p51 subunit is derived by proteolytic cleavage of the RT p66 subunit C-terminal ribonuclease H (RNH) domain during virus maturation. We have previously shown that p51↓RNH cleavage site mutations potentiate pleiotropic defects on the intravirion protein levels of RT resulting in a disproportionately greater composition of RT p51 over RT p66 and diminished viral replication capacity. Repeated passage of MT-2 cells exposed to the mutant viruses however, eventually led to the appearance of peak cytopathic levels. In the following study, we identified and characterized a viral revertant of the p51↓RNH cleavage site mutant phenotype bearing the second-site compensatory mutation T477A. We report that T477A is sufficient to improve viral replication competency and restore RT subunit protein content ratios when introduced into the context of molecular constructs bearing a variety of RT p51↓RNH cleavage site mutations. To determine the compensatory effect of T477A on the mechanics of

RT processing we compared the accumulation of intravirion Gag-Pol processing intermediates inhibited by decreasing concentrations of ritonavir. In the context of p51↓RNH cleavage site mutations we found that early Gag-Pol intermediates accumulated, indicating inefficient processing of quaternary cleavage sites in Pol. The introduction of T477A elevated the proportion of RT p66, resulting in improved processing to p66/51 RT. Although the structural basis for the compensatory role of T477A is presently unclear, we hypothesize that it may impact on protein folding of RT mutants altered in the p51↓RNH cleavage site, thereby promoting proteolytic processing at, or close to the normal cleavage site. Our data suggest that the compensatory effect of the T477A second-site mutation was to restore proteolytic stability of p51↓RNH cleavage site mutant RT during HIV-1 maturation. Taken together, these findings underline the importance of the RNH domain in regulating proper proteolytic processing and the transition of Gag-Pol intermediates towards formation of the p66/51 RT heterodimer.

4.2 INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) contains a multifunctional reverse transcriptase (RT) enzyme responsible for the complex conversion of genomic RNA into double-stranded proviral DNA through coordination of its DNA polymerase and RNase H (RNH) activities. RT in mature infectious virions is a heterodimer of 66 kDa (p66) and 51 kDa (p51) subunits (512). The smaller p51 subunit is derived from the larger p66 RT by proteolytic cleavage of an internal p51↓RNH junction, resulting in the removal of a C-terminal ribonuclease H (RNH) domain (61,119,203,515). The folding of each subunit within the active RT

heterodimer differs, resulting in an asymmetric structure (263,546). In addition, the proportion of these RT subunits is relatively equivalent in viral particles, suggesting that this may be essential for efficient viral replication (485,571). Many studies involving extensive protein mutagenesis and structure-function analysis (154,223,408) have provided great insight into the roles of each subunit of RT. While initial reports identified important catalytic residues and highlighted the interdependent functional relationship of RT polymerase and RNH activities (196,200,334,409,442,513), more recent studies have explored the importance of the RNH domain in the structural maintenance of RT (194,332,333).

HIV-1 RT, like other viral proteins, is not synthesized de novo, but rather translated as part of a larger polyprotein precursor. HIV-1 expresses the structural and enzymatic functions required for assembly of infectious viral particles within two polyprotein precursors, termed Gag ($Pr55^{gag}$) and Gag-Pol ($Pr160^{gag\text{-}pol}$). The HIV-1 Gag precursor contains the structural proteins of the viral core including matrix (MA), capsid (CA), nucleocapsid (NC), $p6^{gag}$ and two spacer peptides p2 and p1 (522). The HIV-1 Gag-Pol precursor contains, in addition to these Gag proteins the functional viral enzymes protease (PR), reverse transcriptase (RT), and integrase (IN) within its Pol domain (368,537). These individual proteins of HIV-1 are subsequently released from their respective polyproteins by proteolytic cleavage at specific sites during virion assembly and budding, a "maturation" process catalyzed by the viral-encoded PR (244,262). Recent evidence has suggested that the polyprotein-embedded immature form of HIV-1 PR is responsible for processing initial cleavage sites (395,396) by an intramolecular *cis* mechanism (392). Removal of the transframe region (TFP and $p6^{pol}$), N-terminally collinear to PR subsequently results in a significant elevation in proteolytic activity. Secondary and tertiary cleavage events (114,395,396,557) have been shown *in vitro*, to follow a time-dependent,

ordered transition of polyprotein intermediates as a consequence of intermolecular *trans* processing (310,384,569). Finally, quaternary processing of cleavage junctions within the Pol region of Gag-Pol result in the complete release of the essential viral enzymes, PR, RT and IN (Figure 22-A).

Despite the critical role that HIV-1 PR plays in the processing of Gag and Gag-Pol polyproteins during viral maturation, there is surprisingly little information concerning the events surrounding the release of *pol*-encoded enzymes (Figure 22-B). Many *in vitro* expression systems have proven inefficient at exhibiting late stage processing of full-length Gag-Pol due to limitations in substrate concentration, conformation, and dimerization efficiency (311,392,393). It is clear that p66/51 RT is formed by the processing of three cleavage sites within the Pol region of Gag-Pol: the N-terminal PR↓RT junction, the internal p51↓RNH junction, and the C-terminal RT↓IN junction. We have previously shown that the kinetics of wild-type RT processing follow an ordered sequential pathway from a truncated Pol polyprotein expressed in bacteria (471). After formation of the polyprotein, RT p66 appears first then decreases with the concomitant appearance of RT p51, until both subunits are approximately equal. This suggests that RT processing may proceed through a quasi-stable p66/66 RT homodimer intermediate; although the contributions of higher order RT processing intermediates arising from full-length Gag-Pol is presently unclear.

In an earlier study we described the effect that a panel of quaternary p51↓RNH cleavage site mutations has on viral replication capacity and the intravirion phenotype of RT (Chapter 3). Since other retroviruses, such as MuLV (210) and ASLV (526) possess different oligomeric forms of RT, and recombinant HIV-1 p66/66 RT exhibits significant catalytic activity (178,204,314) our objective was to understand the need for proteolytic generation of an HIV-1

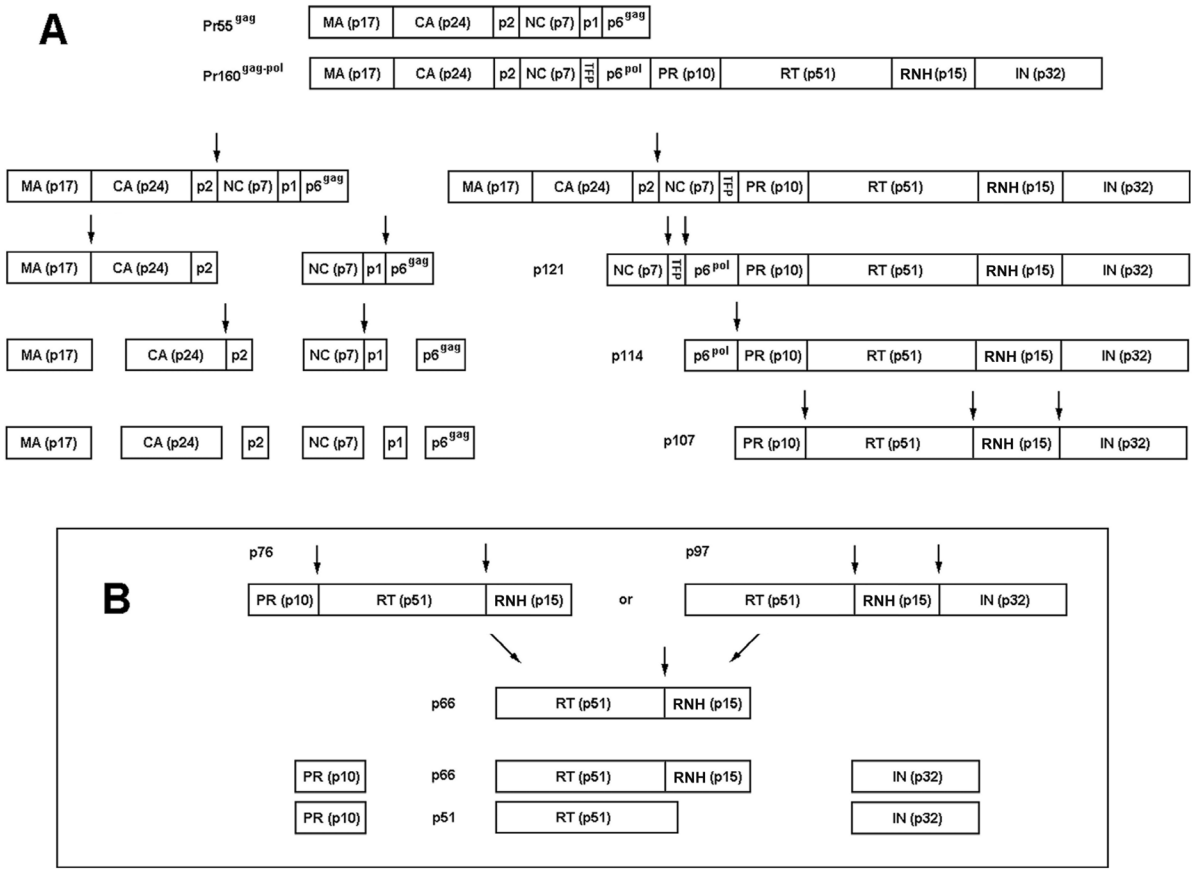

Figure 22. Schematic of sequential processing of HIV-1 Gag and Gag-Pol polyprotein precursors. (A) Gag and Gag-Pol precursors are presented as boxes with processing cleavage sites, hydrolyzed by HIV-1 PR as vertical arrows (11 in total). Ordered processing of Gag and Gag-Pol based on the proteolytic rates of primary, secondary, and tertiary cleavage sites (114,302,392-394,396). Polyprotein intermediates and processed components are represented with their estimated molecular masses and accepted nomenclature (291). (B) Gag-Pol polyprotein RT intermediates and functional retroviral enzymes (PR, RT, IN) arising from the processing of quaternary cleavage sites. Schematic does not reflect the oligomeric nature of these intermediates or products.

p66/51 RT heterodimer. We surprisingly found that mutations in the RT p51↓RNH cleavage site resulted in the generation of attenuated viruses containing greatly decreased levels of RT that in many cases was primarily RT p51. However, in contrast, these same mutations largely permitted the expected generation of RT p66 from our truncated Pol polyprotein expressed in bacteria. It thus appeared that proteolytic instability of RT containing p51↓RNH cleavage site mutations was a function of the viral milieu. We further found that repeated passage of MT-2 cells exposed to p51↓RNH cleavage site mutant viruses eventually lead to the appearance of viruses with normally processed RT and improved replication capacity.

In the following study, we describe the isolation and characterization of a phenotypic revertant virus of one of these p51↓RNH cleavage site mutations (F440V). We report that a seemingly innocuous second-site mutation of a Thr to Ala at residue 477 was compensatory and sufficient to alleviate the defects imposed by F440V and several other p51↓RNH cleavage site mutations. To investigate the compensatory role of T477A on RT processing, we compared the accumulation of intravirion Gag-Pol processing intermediates arising by inhibiting HIV-1 PR with decreasing concentrations of ritonavir (RTV). In the presence of p51↓RNH cleavage site mutations, the second-site T477A mutation appeared to elevate the relative proportion of RT p66, resulting in improved generation of p66/51 RT. While the structural basis for the compensatory role of T477A is presently unclear, these results suggest that the compensatory effect of the T477A second-site mutation was to restore proteolytic stability of p51↓RNH cleavage site mutant RT during HIV-1 maturation. Furthermore, these findings underline the importance of the RNH domain in regulating proper proteolytic generation of RT.

4.3 MATERIALS AND METHODS

4.3.1 Reagents

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAD, NIH: anti-HIV-1_{SF2} p24/25 IgG mAb (76C) from Dr. Kathelyn Steimer, Chiron Corporation; and anti-HIV-1_{HXB2} IN (2C11 and 8G4) IgG mAb from Dr. Dag Helland. Rabbit anti-HIV-1 PR polyclonal serum directed against PR residues 86-108 (284,341) was obtained from Dr. Stuart Le Grice, NCI-Frederick (Frederick, MD). Anti-HIV-1_{IIIB} RT mAb were previously generated in our laboratory against recombinant p66/51 RT (294). Goat anti-mouse-HRP and donkey anti-rabbit secondary mAb were all purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The SuperPico ECL Substrate System for detection of peroxidase-labeled antibody was obtained from PIERCE (Rockford, IL). 4-MUG (4-methylumbelliferyl-β-D-galactopyranoside), a β-galactosidase fluorescent substrate was obtained from Sigma-Aldrich (St. Louis, MO). HIV-1 p24 antigen ELISA kits were obtained from SAIC-Frederick (Frederick, MD). Sequencing, PCR amplification and mutation-containing oligonucleotide primers were purchased from Invitrogen (Carlsbad, CA). COS-7, 293T and CD4+ MT-2 lymphocytoid cell lines were obtained from the American Type Culture Collection (Rockville, MD).

4.3.2 Cell lines

The human T-lymphocytoid MT-2 cell line was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). The human 293T and monkey COS-7 fibroblast cell lines

were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS. P4R5 HeLa fibroblast cells, a generous gift of Dr. John Mellors at the University of Pittsburgh (originally obtained from Dr. Ned Landau, The Salk Institute for Biological Studies, La Jolla, CA) were maintained in DMEM/10% FBS additionally supplemented with Puromycin (0.5 μg/mL). These cells express endogenous CXCR4 and are stably transfected to express CD4, CCR5, and a β-galactosidase reporter gene under the control of an HIV LTR promoter (347).

4.3.3 Molecular cloning of p51↓RNH phenotypic revertants

Molecular cloning of replication-recovered p51↓RNH cleavage site mutants was described previously (Section 3.3.2). Cytopathology appeared in infected MT-2 cells maintained in culture for 30 days. At this time virus-containing culture fluids were expanded briefly for 5 days by infection of fresh MT-2 cells, followed by extraction of chromosomal DNA using QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA). The HIV-1 Pol-encoding region was then PCR amplified and cloned into pCR-T7/CT TOPO (Invitrogen, Carlsbad, CA) for sequencing analysis.

4.3.4 HIV-1 molecular clone mutagenesis and transfection

Plasmid pSVC21-BH10 encodes an infectious molecular clone of the IIIB (HxB2) strain of HIV-1 and carries an SV40 origin of replication for expression in 293T and COS-7 cells (127). The construction of ten different derivatives of pSVC21 BH10 containing various amino acid substitutions about the p51↓RNH cleavage site (amino acid residues 437-443) was previously described (Section 3.3.2). In brief, these mutant proviral clones included: A437I,

V442S, F440W, F440V, T439S/V442G, Y441I/V442K, F440A, F440A/Y441A, F440W/Y441W, and E438N. A second-site mutation, T477A, identified in the background of the p51↓RNH cleavage site mutation F440V (Figure 23) was subsequently introduced into the RT coding region of all pSVC21 BH10 derivatives using the Quick Change™ Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). To assess $Pr160^{gag\text{-}pol}$ incorporation into virions a catalytic inactive mutation (D25A) was introduced into the PR coding region of wild-type and mutant molecular clones. The presence of the expected mutations was verified by sequencing. Virus was prepared by transfection of 293T cells by calcium phosphate co-precipitation. Virus-containing culture supernatants were harvested 60 h post-transfection, clarified by centrifugation (3000 x g, 1 h at 4^{o}C), and quantified by analyzing the levels of HIV-1 p24 antigen. Aliquots of virus preparations were stored at -80^{o}C until use.

4.3.5 Infectivity and viral spread

Infectiousness of virus particles produced from transfection of 293T cells was determined by using normalized quantities of HIV-1 p24 antigen content and P4R5 or MT-2 cells as previously described (Section 2.3.4). Briefly, single-cycle viral infectivity was assessed by using P4R5 cells in a fluorescence-based 96-well microplate β-galactosidase detection assay (25 ng viral p24 per 5 x 10^3 cells per well). Multiple-round viral replication was assessed by inoculation of MT-2 lymphoblastoid cells in 96-well microplates (25 ng of viral p24 per 6.5 x 10^4 cells per well) followed by daily microscopic observation of HIV-1 induced syncytium formation (32,339). Median tissue culture infective dose ($TCID_{50}$/mL) was determined after seven days of culture (231).

4.3.6 Immunoblotting analysis of viral proteins

Virion particle protein composition was assessed by subjecting the viral lysate (equivalent to 1 μg viral p24) to SDS-10% PAGE resolution and Western blotting, as previously described (Section 2.3.6). Separate Western blots from replicate trial experiments (n = 3) were probed for *pol*-encoded enzymes (PR, RT and IN) and p24 Gag. Immunoreactive protein bands were visualized by enhanced chemiluminescence (PIERCE, Rockford, IL) on a VersaDoc Imaging System, and quantitated by densitometry under sub-saturating exposure conditions using Quantity One v4.3.0 software (Bio-Rad, Hercules, CA). To assess the level of Gag-Pol ($Pr160^{gag\text{-}pol}$) incorporation, viruses containing inactivated PR (D25A) were pre-normalized by Gag ($Pr55^{gag}$) content prior to sucrose-cushion purification and Western blot analysis.

4.3.7 Analysis of intravirion processing of Gag and Gag-Pol polyprotein precursors

The accumulation of Gag-Pol processing intermediates during proteolysis was assessed by immunoprobing Western blots of viral protein derived from ritonavir-treated transfected COS-7 cells, as previously described (Section 2.3.7). Briefly, virion particle protein composition was assessed by subjecting viral lysate proteins to SDS-10% PAGE resolution, Western blotting and immunoprobing against RT and p24, as described above. Immunoreactive protein bands visualized by enhanced chemiluminescence were quantitated by densitometry under sub-saturating exposure conditions, followed by graphical representation versus the concentration of ritonavir.

4.4 RESULTS

4.4.1 Identification of a second-site mutation in the background of a p51↓RNH cleavage site mutation

We previously showed that p51↓RNH cleavage site mutations result in the generation of significantly attenuated virus containing greatly diminished levels of RT that in many cases is primarily RT p51. Over the course of subsequent passage in MT-2 cells, some of these viruses eventually became replication-competent with improved content and proportion of RT subunits (Chapter 3). To determine the genetic basis for this phenotypic reversion, the entire RT coding region of each putative revertant virus was sequenced. One group of these "replication-recovered" p51↓RNH cleavage site mutant viruses (F440A/Y441A and E438N) had reverted to the wild-type sequence as well as contained a number of second-site mutations within the RNH-coding region (Figure 23). Another group of viruses (F440V) retained the original p51↓RNH cleavage site mutation, but also possessed an Ala to Thr amino acid substitution at position 477 (codon ACT to GCT). Although three additional second-site mutations (Q340R, Q367R, I393T) were identified in the some of these clones, the prevalence of T477A in all clones suggested that this may have been important for the observed recovery of RT content.

4.4.2 Effect of p51↓RNH ± T477A mutations on infectivity and viral spread

To determine if the T477A second-site mutation was responsible for the revertant phenotype, we introduced this amino acid substitution into proviral DNA constructs possessing a variety of p51↓RNH cleavage site mutations, including wild-type, F440V, and mutation sets that

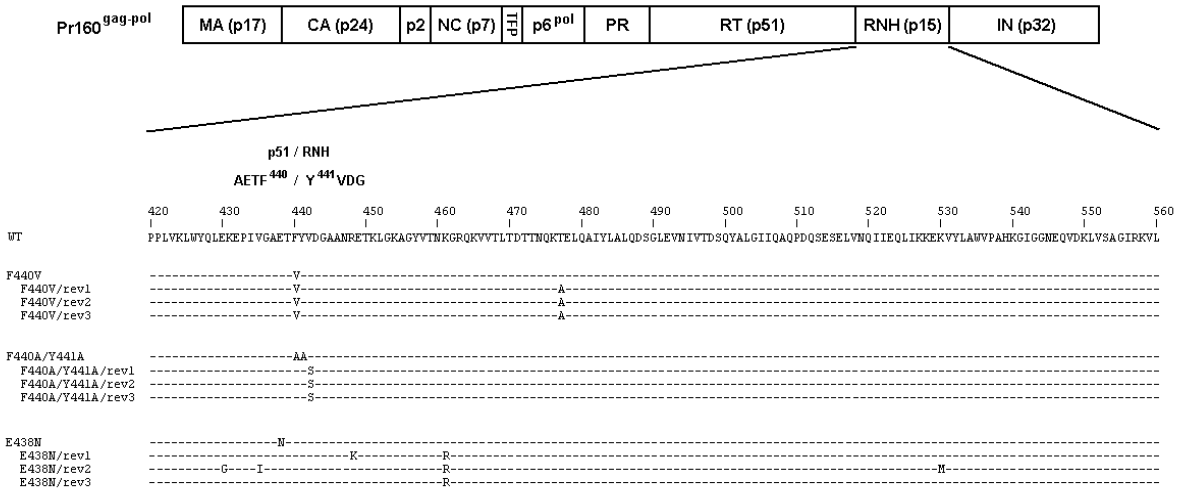

Figure 23. Sequence analysis of revertants of the p51↓RNH cleavage site mutant phenotype. The schematic represents the Gag-Pol precursor polyprotein with the highlighted amino acid sequence below indicating the region that encompasses the p51↓RNH cleavage site and the entire cleaved RNH domain of wild-type (WT) HIV-1_{IIIB} RT (amino acids 420-560). Below that sequence are the sequences of the phenotypic revertant-derived clones with the original p51↓RNH cleavage site mutant presented at the top of each set. A dash indicates amino acid identity with WT. Changes relative to the WT sequence is indicated in the single-letter amino acid code.

had reverted to wild-type during recovery of virus infectivity. Consistent with our previous findings (Section 3.4.4), most p51↓RNH cleavage site mutant viruses exhibited severe attenuations in single-cycle viral infectivity (Figure 24-A) and replication capacity (Figure 25-A). Introduction of T477A in the context of the p51↓RNH cleavage site mutation F440V was found to result in a significant 28% elevation of viral infectivity (Figure 24-B) and a considerable acceleration in viral spread (Figure 25-B). This finding indicates that the acquisition of T477A by this virus was indeed compensatory. Interestingly, this improvement in viral

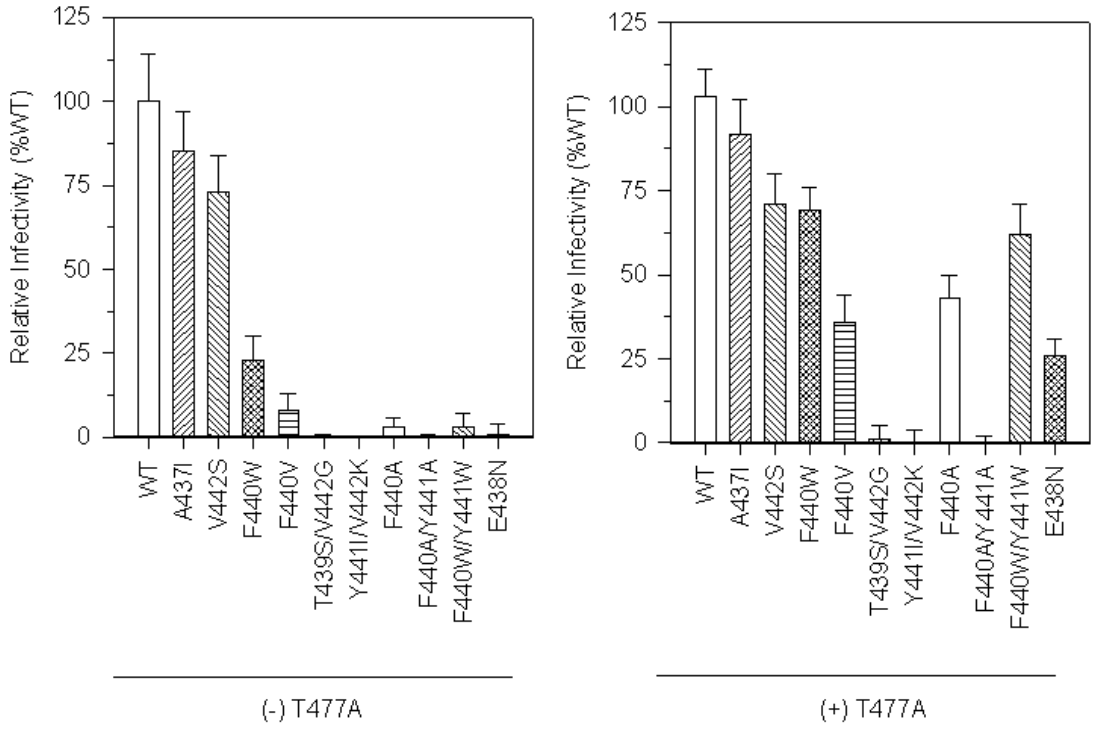

Figure 24. Relative infectivity of WT and p51↓RNH ± T477A mutant viruses after a single-round infection. P4R5 cells ($5x10^3$) were infected in replicate (n = 16) with (A) p51↓RNH - T477A or (B) p51↓RNH + T477A mutant viruses derived from transfected 293T cells, normalized to 25 ng viral p24. Infectivity was determined after 48 h of culture by fluorescent measurement of β-galactosidase gene expression, as described in Section 2.3.4. Results are presented as an average percentage of WT virus infectivity. Asterisks (*) indicate a statistically significant compensatory effect of T477A relative to its non-substituted counterpart ($p < 0.05$), as calculated using a one-tailed Student's *t*-test assuming equal variance.

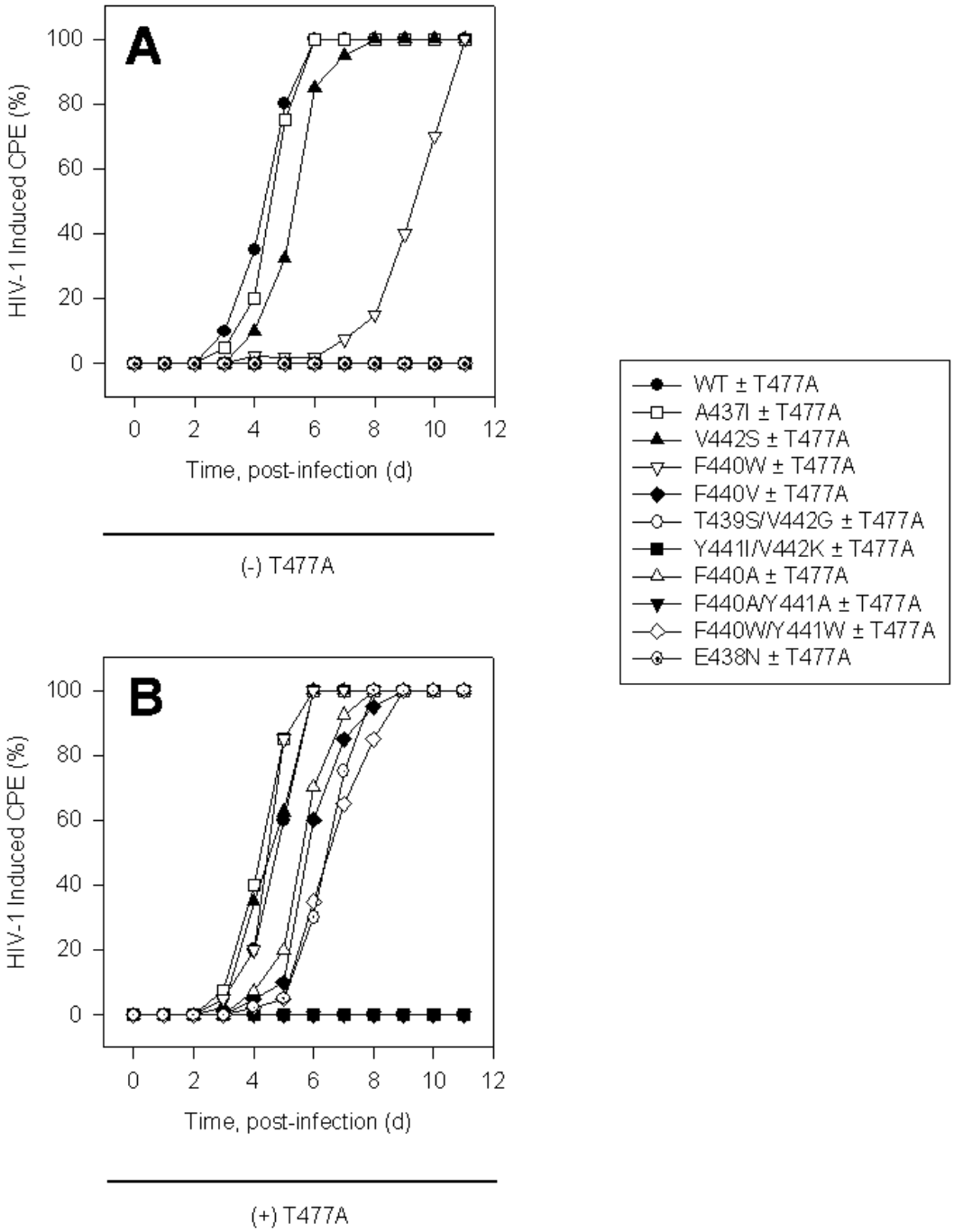

Figure 25. Virus replication kinetics of p51↓RNH ± T477A mutant viruses. MT-2 lymphocytoid cells ($1x10^5$) were infected in duplicate with (A) p51↓RNH – T477A or (B) p51↓RNH + T477A mutant viruses derived from transfected 293T cells, normalized to 25 ng viral p24. Cultures were split 1:2 every 3 d to prevent overgrowth and HIV-1 induced cytopathic effect (CPE) was scored daily, post-infection as percent syncytium formation.

replication was not specific to F440V. Other p51↓RNH cleavage site mutant viruses were found to exhibit a much greater improvement in single-cycle infectivity and viral replication upon addition of T477A. Most notably, these T477A-substituted mutant viruses included: F440W (46% increase), F440A (40% increase), F440W/Y441W (58% increase), and E438N (25% increase). An assessment of end point dilution infectivity in MT-2 cells further showed that the infectious capacity ($TCID_{50}$/mL) of these viruses was significantly increased in the presence of the T477A second-site mutation (Table 7). Finally, T477A appeared to exhibit no discernable effect on either the infectivity or replication capacity of the wild-type virus, suggesting that it was sufficient to correct for the defects imposed by most p51↓RNH cleavage site mutations.

4.4.3 Effect of p51↓RNH ± T477A mutations on viral protein content and Gag-Pol incorporation

Consistent with our previous findings (Section 3.4.2), most p51↓RNH cleavage site mutations significantly diminished virion RT levels and adversely affected the RT p66 to RT p51 ratio in favour of RT p51 (Figure 26-B). Introduction of T477A, significantly improved both the total RT content and the RT p66 to RT p51 ratio in a number of p51↓RNH cleavage site mutant viruses, namely those mutants which had displayed enhanced infectivities and replication kinetics. These improvements however, were not complete, as observed by the continual presence of low molecular weight RT fragments and RT content levels that were not equivalent to the wild-type. In most cases, T477A also increased the total IN content (Figure 26-C) but did not significantly affect the level of PR (Figure 26-A).

Although near normal levels of PR activity appeared to be present in all mutant viruses, based on the wild-type levels of $p24/Pr55^{gag}$ products we proceeded to confirm for certain that

Table 7. Absolute infectivity of T477A complemented viral particles.

[a] Absolute infectivity per virion ($TCID_{50}$/p24) was calculated by dividing $TCID_{50}$/mL by the normalized amount of viral p24 used in units of ng/mL, as described in Materials and Methods

[b] Results are presented as an average percentage of WT virus infectivity with standard deviation (S.D.) derived from four separate experiments.

[c] *P* values were calculated using a one-tailed Student's *t*-test assuming equal variance to determine the statistical significance of compensatory effect inferred by T477A, relative to its non-substituted counterpart

Mutant		$TCID_{50}$/p24 (%WT) [a,b]	*P* value [c]
WT	- T477A	100.0 ± 10.0	0.494
	+ T477A	100.4 ± 30.2	
A437I	- T477A	40.0 ± 18.2	0.075
	+ T477A	68.9 ± 24.5	
V442S	- T477A	23.5 ± 10.0	0.053
	+ T477A	56.8 ± 27.5	
F440W	- T477A	0.9 ± 0.3	< 0.01
	+ T477A	28.2 ± 6.7	
F440V	- T477A	0.1 ± 0.1	< 0.01
	+ T477A	10.4 ± 3.0	
T439S/V442G	- T477A	0.0 ± 0.0	0.178
	+ T477A	0.0 ± 0.0	
Y441I/V442K	- T477A	0.0 ± 0.0	0.199
	+ T477A	0.0 ± 0.0	
F440A	- T477A	0.1 ± 0.1	< 0.01
	+ T477A	10.2 ± 4.6	
F440A/Y441A	- T477A	0.1 ± 0.1	0.181
	+ T477A	0.5 ± 0.9	
F440W/Y441W	- T477A	0.1 ± 0.0	< 0.01
	+ T477A	31.9 ± 14.3	
E438N	- T477A	0.1 ± 0.2	< 0.05
	+ T477A	4.4 ± 3.7	

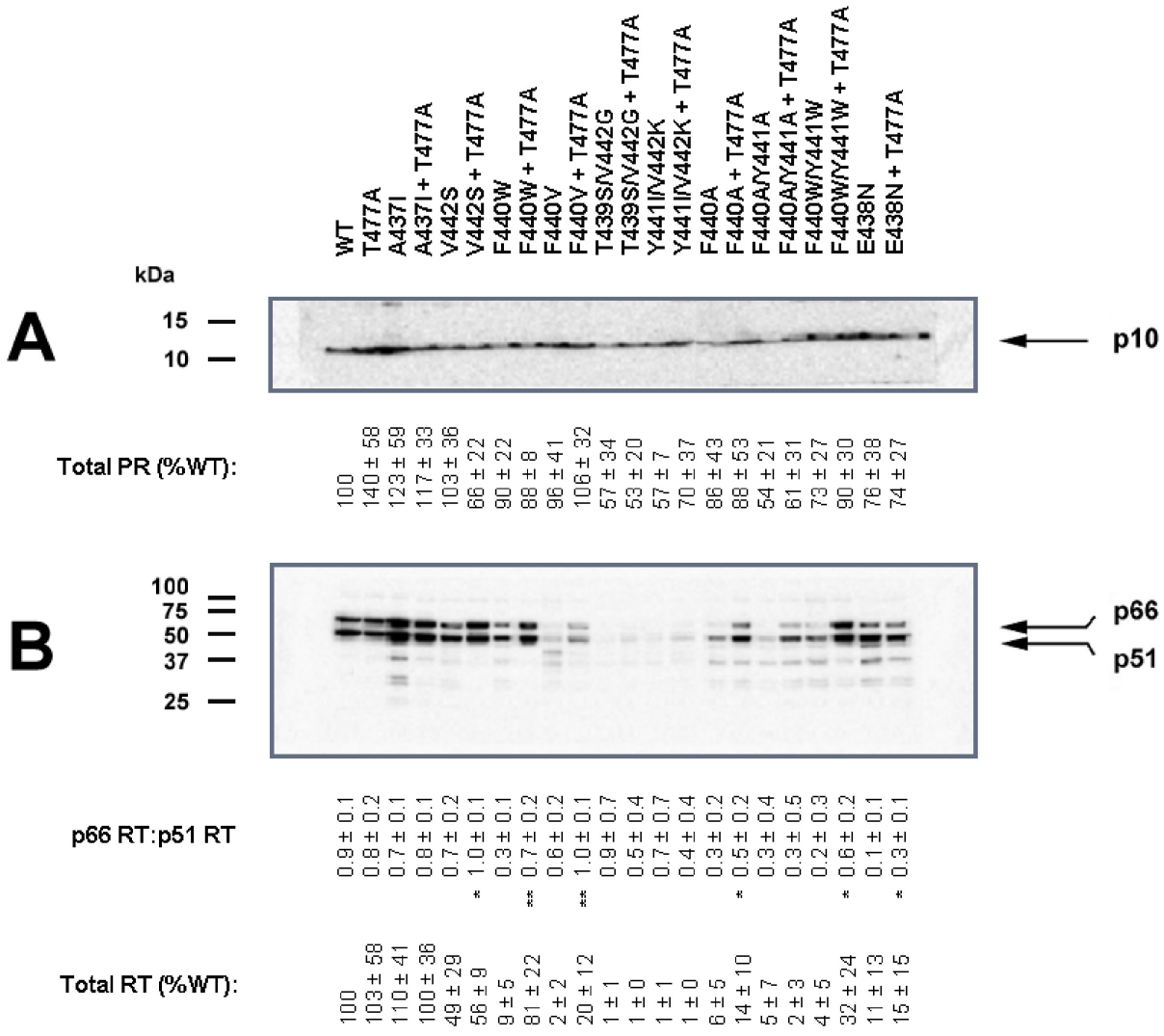

Figure 26 (Part 1 of 2)

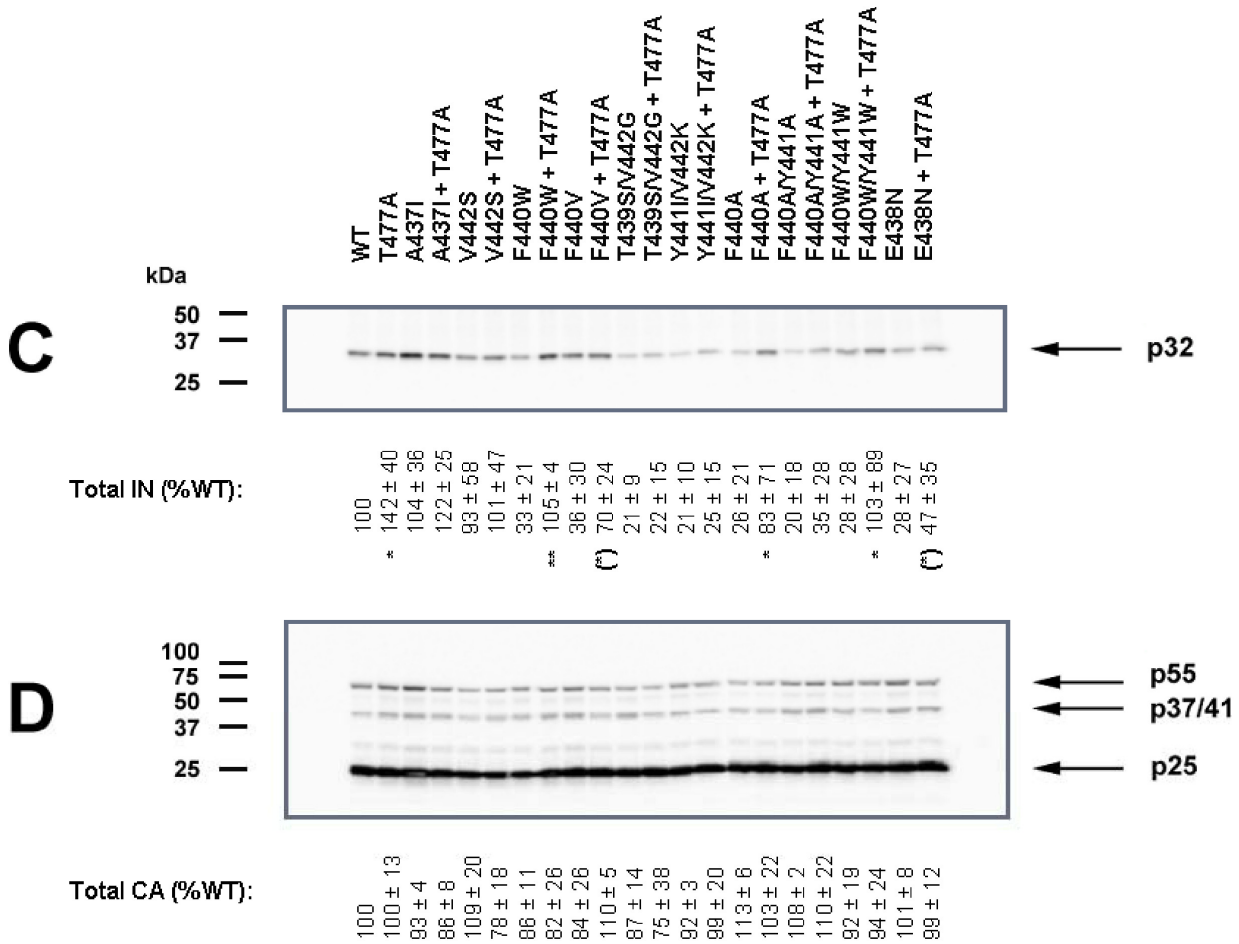

Figure 26. Effect of p51↓RNH ± T477A mutations on viral particle protein composition. Western blots of wild-type (WT) and p51↓RNH ± T477A mutant viruses (1 μg viral p24) generated by transfection of 293T cells and probed with (A) anti-PR, (B) anti-RT, (C) anti-IN, and (D) anti-p24 antibodies. The position of molecular size markers are shown to the left of each panel. Arrows to the right of each panel indicate the positions and molecular masses of immunoreactive viral proteins. Quantitation of viral proteins, from three independent trials was performed by densitometric scanning analysis of ECL exposed blots under sub-saturating conditions. Statistical significance of the T477A compensatory effect was determined for each individual mutant virus relative to its non-substituted counterpart using a one-tailed Student's *t*-test assuming equal variance. Asterisks indicates the degree of statistical significance in relation to the size of the type I error: (*)$p < 0.10$, *$p < 0.05$, **$p < 0.01$.

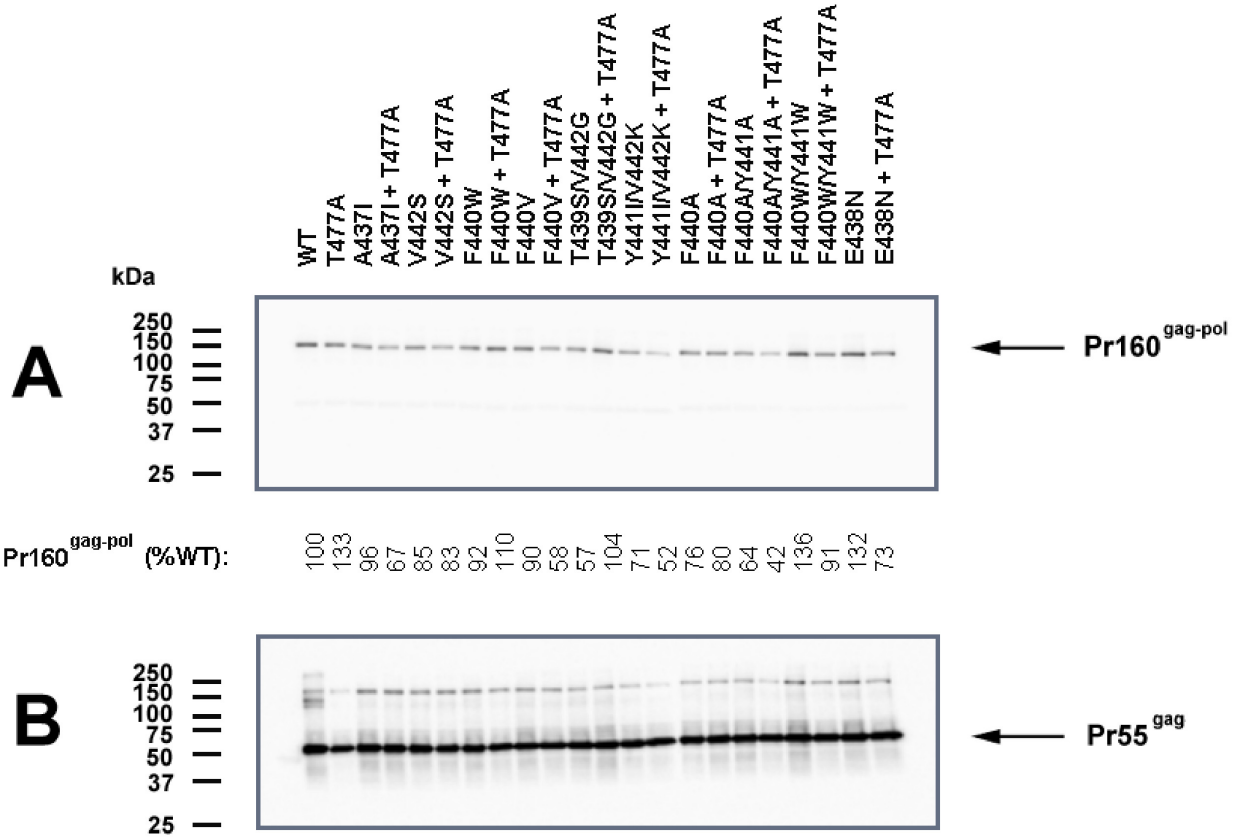

Figure 27. Effect of p51↓RNH ± T477A mutations on Gag-Pol incorporation in immature virions. Immature virions containing inactive HIV-1 PR (D25A) and previously standardized by $Pr55^{gag}$ content were ultracentrifuged, lysed and resolved by SDS-10% PAGE and Western blot analysis. Relative viral content of (A) $Pr160^{gag\text{-}pol}$ and (B) $Pr55^{gag}$ were determined by probing separate blots with anti-RT and anti-p24 monoclonal antibodies respectively, followed by densitometry scanning analysis of ECL exposed blots under sub-saturating conditions. The position of molecular size markers are shown to the left of each panel.

Gag-Pol incorporation was not enhanced by the T477A mutation. Elevated levels of viral p24 were occasionally detected in 293T cell-derived virus preparations, suggesting that T477A may have had a positive influence on viral packaging and budding (data not shown). While the level of PR inactive (D25A) Gag-Pol polyprotein ranged from 57-136% of the wild-type in p51↓RNH cleavage site mutant viruses, introduction of T477A did not appreciably affect this level (Figure 27). Taken together, these results suggest that the improved RT phenotype in T477A-substituted p51↓RNH cleavage site mutant viruses was due to an increase in proteolytic stability.

4.4.4 Effect of p51↓RNH ± T477A on intravirion processing of Gag-Pol

As we (Section 3.4.5) and others (311,355,392,393,567) have shown, there are inherent limitations in using heterologous expression systems for investigating late-stage Gag-Pol polyprotein processing. Therefore, we chose instead to compare the accumulation of Gag-Pol processing intermediates in the natural viral milieu by titrating HIV-1 PR activity with the inhibitor ritonavir (RTV). While this is not a kinetic analysis by any means, it is advantageous over previous attempts to synchronize viral maturation by treatment and removal of protease inhibitors (86,218,227) in that the full range of PR activity can be examined. Wild-type virions, isolated from COS-7 cells transfected in the presence of varying concentrations of RTV exhibited a dose-dependent diminution in the extent of Gag and Gag-Pol processing when probed for RT and p24, respectively (Figure 28, panel i, upper and lower blots, respectively). Although the accumulative pattern and molecular weights of immunoreactive polyprotein intermediates were consistent with the expected cleavage events (302,392,481), it was unfeasible to verify their identity by protein sequencing. As PR activity was progressively elevated, higher molecular weight RT intermediates disappeared as lower molecular weight intermediates became

evident. Most notably, after a build up of RT p66, RT p51 began to appear, consistent with our previous findings in a bacterial expression system (Section 3.4.5). At the highest concentration of RTV examined, both full-length Gag-Pol ($Pr160^{gag\text{-}pol}$) and the first processing intermediate, p121 were present. This result complements previous observations that polyprotein-embedded PR exhibits a different mechanism of action and sensitivity to inhibition than the mature free form (302,392,481). The exact positions of other higher molecular weight RT intermediates were difficult to estimate due to their migration pattern. However, based on their expected molecular weight (Figure 22), and previous resolution of longer resolved SDS-10% PAGE gels, we predicted these intermediates to be p114/p107, p97 and p76 respectively.

The most noticeable effect of p51↓RNH cleavage site mutations on Gag-Pol processing was the severe loss of RT subunits with increasing levels of PR activity (Figure 28, panels iii, v and vii). Between 10 and 1 μM RTV, the intensity of the p107 (PR-RT-IN) Pol intermediate was elevated in all mutants. This suggests that quaternary cleavage events were more sensitive to proteolytic inhibition, and possibly occurred with decreased efficiency. Between 0.05 to 0 μM RTV, intermediate RT products were also observed in a number of mutants (ca. 56/57 kDa and 61 kDa), likely due to cleavage of normally unexposed junctions within the RNH domain (23,61,515). Minor detection of 55 kDa and 24 kDa Gag proteins in the panel of F440W/Y441W ± T477A mutants were the consequence of using exceeding high concentrations of anti-RT antibodies in this particular trial.

In the context of the same p51↓RNH cleavage site mutations, the addition of T477A was found to have to no compensatory effect on the accumulation of the p107 Pol intermediate as the level of PR activity was increased (Figure 28, panels iv, vi and viii). However, between 1 and 0 μM RTV, the T477A second-site mutation appeared to primarily elevate the proportion of RT

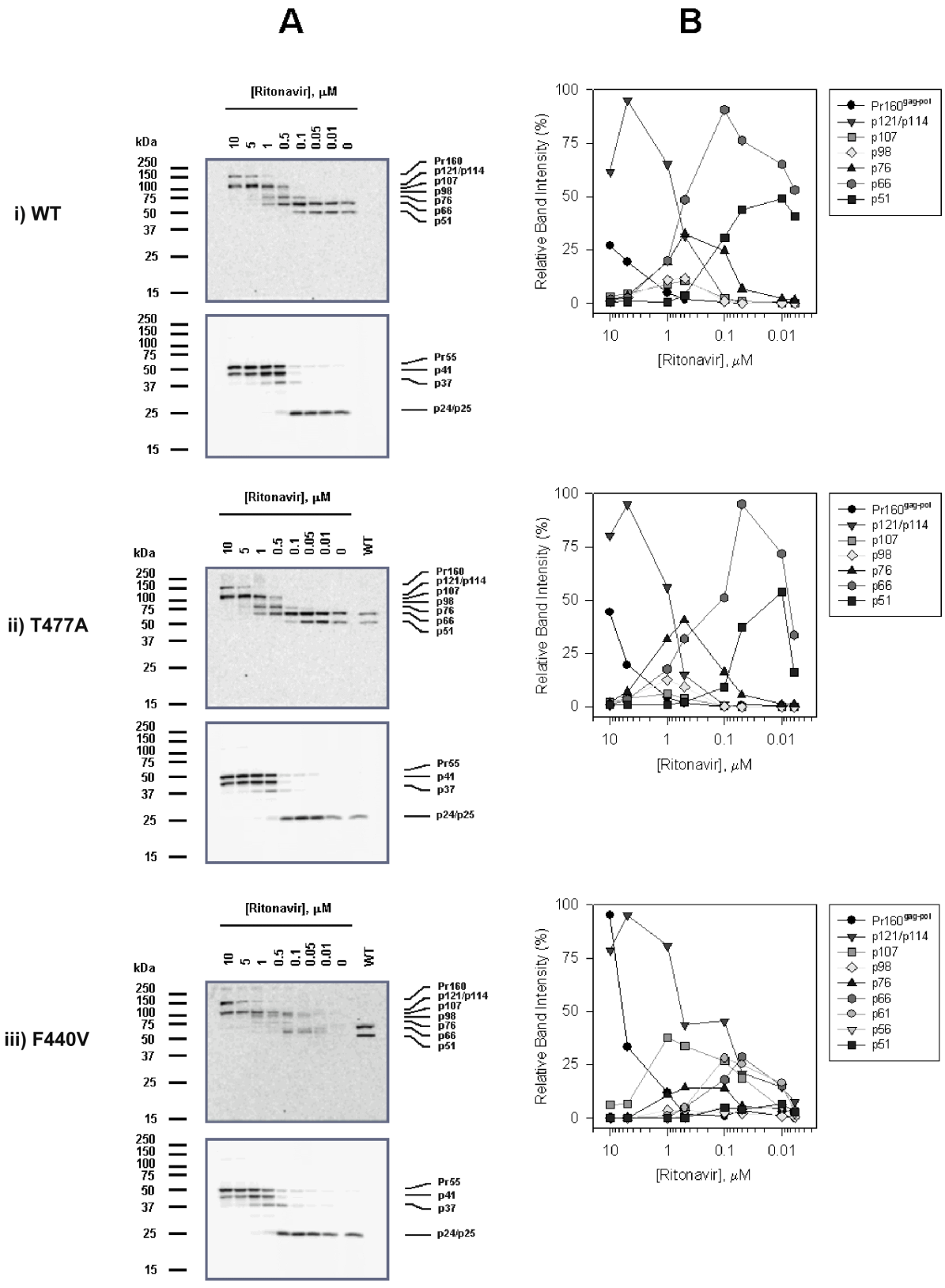

Figure 28 (Part 1 of 3)

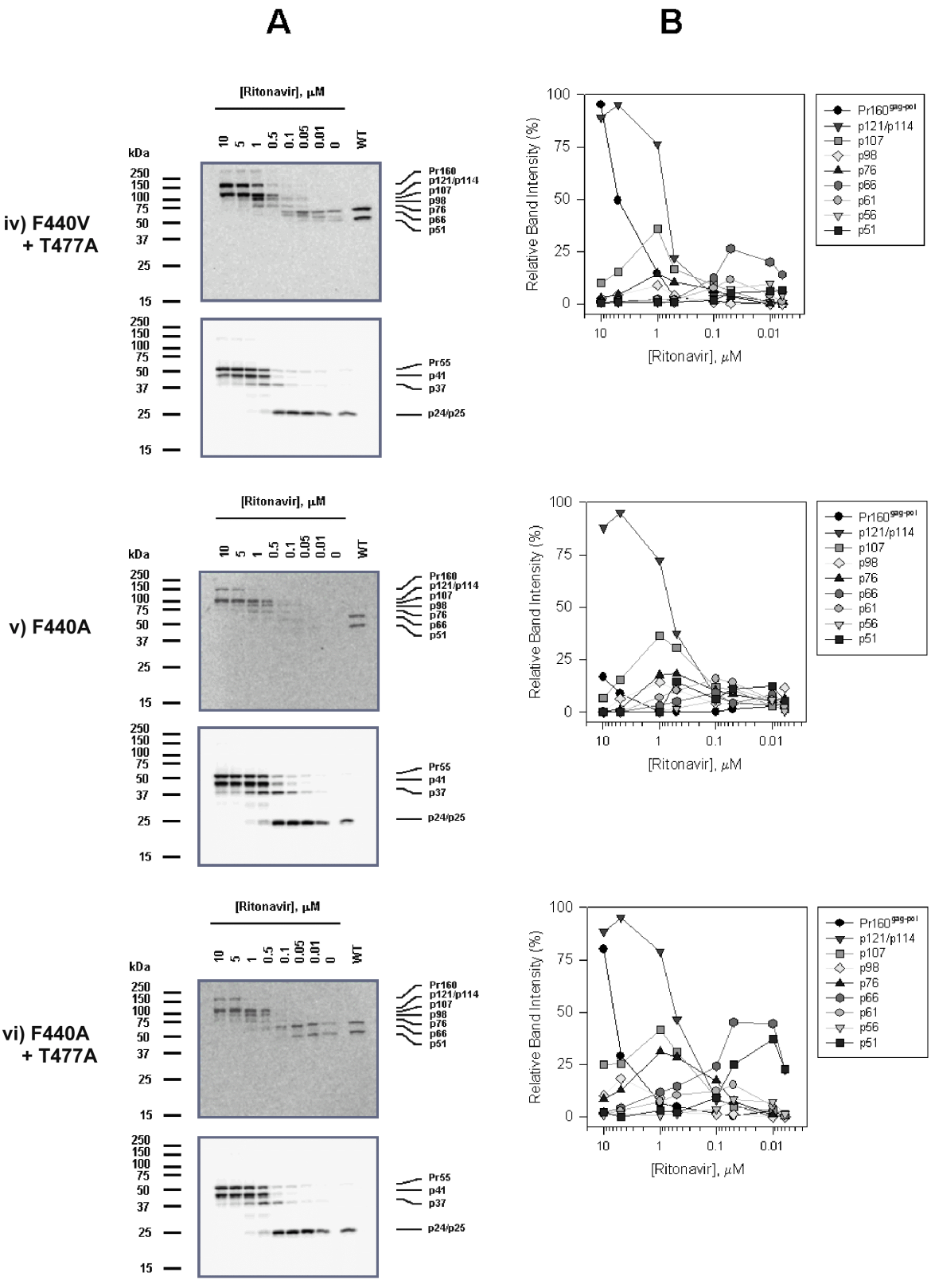

Figure 28 (Part 2 of 3)

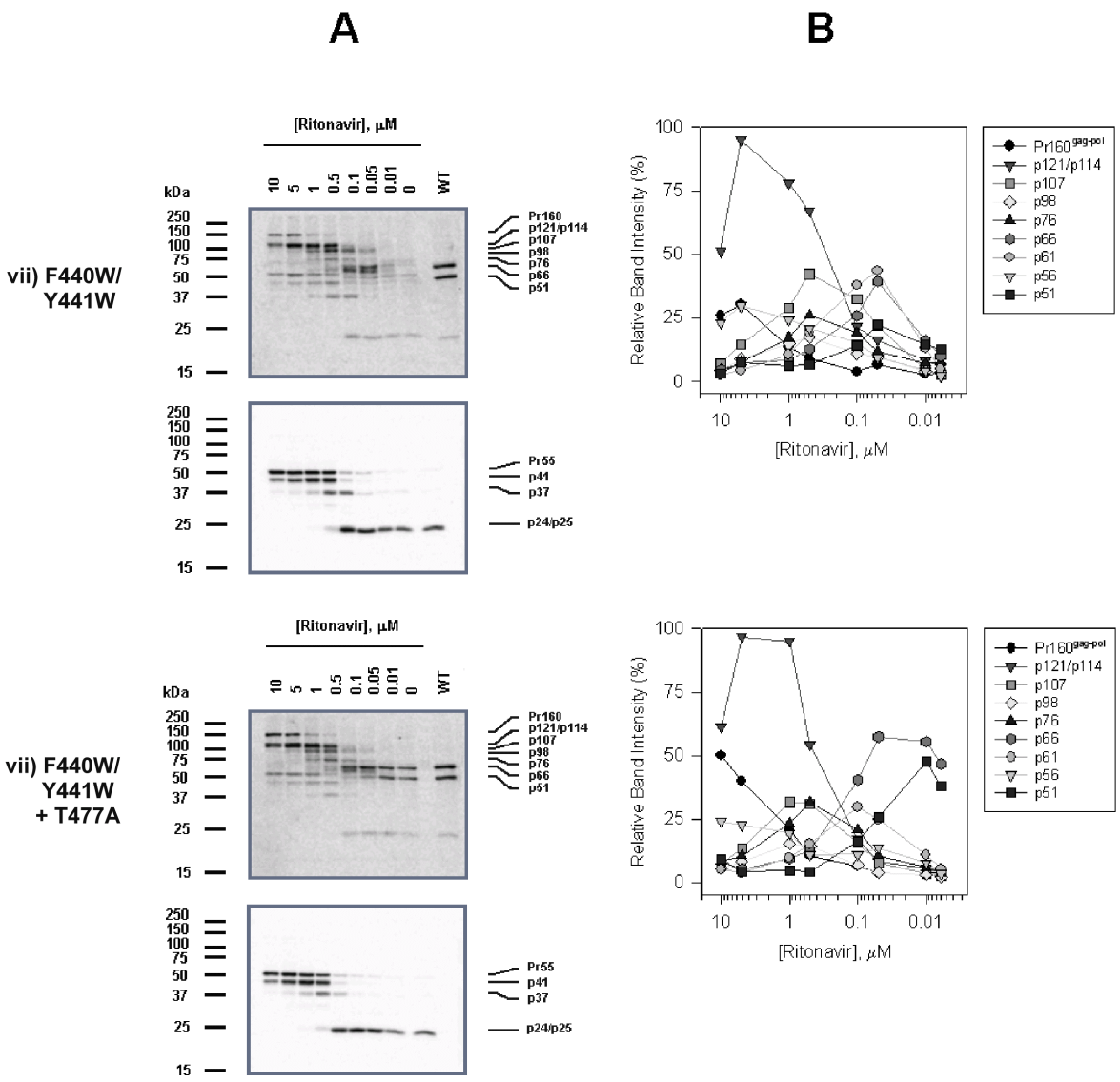

Figure 28. Effect of p51↓RNH ± T477A mutations on ordered intravirion processing of Gag and Gag-Pol polyproteins. Wild-type (i) and p51↓RNH ± T477A mutant (ii-viii) virus-containing culture supernatants derived from COS-7 cells, transfected in the presence of decreasing concentrations of ritonavir were subjected to SDS-10% PAGE resolution and Western blotting analysis. Ordered accumulation of immunoreactive Gag-Pol (A, upper panel) and Gag (A, lower panel) polyprotein processing intermediates were observed by probing Western blots with anti-RT and anti-p24 monoclonal antibodies respectively, followed by ECL exposure. The position of molecular size markers are shown to the left of each panel. Lines to the right of each panel indicate the positions and estimated molecular masses of expected polyprotein processing intermediates (302,392). Immunoreactive RT processing intermediates from (A) were analyzed by densitometry and graphically represented in (B). Relative band intensity of each intermediate was normalized relative to the most intense band, set to 95% in arbitrary units. For graphical purposes, intermediates detected in the absence of ritonavir (0 μM) were assigned a ordinate value of greater than zero.

p66 in most mutants, leading to improved levels of p66/51 RT. This result was generally concomitant with a reduction in intermediate RT products of 56/57 kDa and 61 kDa. In the case of F440A, the introduction of T477A also slightly elevated the proportion of the preceding p76 PR-RT intermediate. Finally, as expected, T477A alone had no discernible effect on the accumulation of any of the RT processing intermediates (Figure 28, panel ii), suggesting that it was sufficient to correct for defects imposed by most p51↓RNH cleavage site mutations.

4.5 DISCUSSION

Processing of Gag and Gag-Pol precursor polyproteins into their respective structural proteins and functional enzymes is a critical, carefully controlled event in the life cycle of HIV-1. However, the forces that regulate ordered late-stage processing of Gag-Pol leading to mature p66/51 RT remain poorly defined. We have previously shown that p51↓RNH cleavage site mutations potentiate pleiotropic detrimental effects on the intravirion protein levels of RT and IN resulting in a disproportionately greater content of RT p51 over RT p66 and diminished viral replication capacity (Chapter 3). In this study, we identified and characterized a viral revertant of the p51↓RNH cleavage site mutant phenotype of F440V bearing the second-site compensatory mutation T477A. Introduction of T477A into a context of varying p51↓RNH cleavage site mutations, including the original resulted in the production of replication-competent viruses with restored protein content ratios of RT p66 RT to RT p51. By titrating the activity of HIV-1 PR over a range of inhibitor concentrations we were able to investigate the compensatory effect of T477A on the mechanics of RT processing affected by p51↓RNH cleavage site mutations. These

studies yielded several new insights into the transition of Gag-Pol processing intermediates which lead to the formation of RT and the importance of the RNH domain in regulating this process.

Many studies have documented the analysis of viral revertants as a powerful tool to identify second-site changes which compensate for structural and functional defects imposed by mutation (72,256,366,495). To that end, it was not entirely surprising that continued passage of p51↓RNH cleavage site mutant viruses would eventually select for reversionary or second-site changes to overcome the phenotypic defect in RT content. Our identification of the compensatory nature of the second-site mutation T477A is not only a credit to the selective pressure presented by p51↓RNH cleavage site mutations, but additionally highlights the importance of this residue in the maintenance of a proteolytically stable form of RT. Like many polymorphic residues in RT, it should be noted that amino acid residue 477 exhibits a 3.8% frequency of variation of Thr to Ala (420), suggesting that this second-site mutation may naturally arise due to adaptation. Nevertheless, our findings with respect to T477A complement previous reports that other C-terminal RNH residues are important for maintaining proper folding and stabilizing interactions within RT. Of particular note, N494 appears to be important in stabilizing interactions with A437 and I434 (85,540) and D443N compensates for structural instability caused by D498N (332,334).

Although the second-site mutation T477A conferred a selective advantage in the presence F440V, in the context of other p51↓RNH mutations, T477A was equally if not more compensatory in the restoration of RT and IN content and improvement of viral infectivity. One of our most intriguing findings was that while both F440W/Y441W and F440A/Y441A mutant viruses contained primarily RT p51, only the former was compensated by T477A. We have

recently demonstrated that RT is sequentially processed from a Pol polyprotein through a possible quasi-stable p66/66 RT homodimer intermediate (Section 3.4.5). Unfortunately, no structure for the p66/66 RT homodimer is yet available and forced stabilization during crystallization is unlikely to ever mimic the true form of this enzyme in the virion context. Therefore, in the absence of a structural understanding of proteolytic instability caused by p51↓RNH cleavage site mutations, the structural basis for the restored stability inferred by T477A remains unclear.

We have previously suggested that the predominance of RT p51 in p51↓RNH cleavage site mutant viruses may be due to the cleavage at alternative cleavage sites (169,515). If RT is indeed formed through a p66/66 RT intermediate, then in the context of p51↓RNH mutations, cleavage of one subunit may not induce structural changes in the other p66 subunit to protect it from similar cleavage at its p51↓RNH junction. The presence of T477A could have in turn improved processing of this region in RT by permitting restored proteolytic stability. While T477A is located within the same domain as the original p51↓RNH mutations it could be (i) distant in the primary sequence but close in the tertiary structure, (ii) reverse a global or local conformational change induced by the original mutation(s), or (iii) potentially remove or establish intramolecular or intermolecular interactions. In the p66/51 RT heterodimer, residue T477 lies in close proximity (3.6-4.8 Å) to G444 and A445 of the p51↓RNH cleavage site. The p51↓RNH cleave-site (F440↓Y441) itself is situated in a putative β1-sheet less than 15 residues away from the proposed start (Y427) of the RNH domain as well as the inter-domain linker or tether region (K424-L429) which separates the p51 polymerase domain from RNH (Figure 7). The RNH domain is divided into left and right subdomains with β1, β2 opposing αA, β4 along a separating cleft (85). Coincidently, residue T477 lies within a short polypeptide chain which

connects these two subdomains between β3 and αA in the form of a loop motif. In considering the topology of amino acid contacts, a number of tertiary contacts likely participate in the stabilization of the tether region and right subdomain as well as in the maintenance of this cleft in the final form of RT (26,85,546). Therefore, local perturbation or interference of such interactions could potentially disrupt conformation, hydrodynamic hydration, functional motions, or even dimeric stability. If any of these attributes are at all similar in the p66/66 homodimeric form of RT, then it is possible that T477A may stabilize structural alterations(s) induced by p51↓RNH cleavage site mutations to prevent overprocessing to RT p51. Replacement of a hydroxyl side chain with a shortened alkyl group at position 477 (Thr to Ala) could have caused a slight disruption in the local hydrogen binding or electrostatic network, which did not in itself, appreciably affect the processing of wild-type RT from Gag-Pol. We suspect that in the context of p51↓RNH cleavage site mutations, T477A may have provided sufficient conformational flexibility of the would be β3-αA loop motif to accommodate folding defects and promote processing at 440↓441 or nearby alternative cleavage sites.

Analysis of ritonavir-treated viral protein content revealed that processing intermediates of Gag and Gag-Pol accumulated sequentially as a function of increasing HIV-1 PR activity. These results were consistent with previous reports that polyprotein cleavage sites are differentially sensitive towards inhibition (246,271,392,481) and the relative order of primary, secondary and tertiary cleavage events (392,393,481,508). Inherent limitations of heterologous expression systems have to date precluded a reliable determination of the sequence of events leading to the formation of p66/51 RT from full-length Gag-Pol (311,355,392,393,567). Our present work extends these studies by demonstrating for the first time in the virion context, the spectrum of Gag-Pol processing intermediates that lead towards p66/51 RT. We note that a

disappearance of the p107 Pol intermediate (PR-RT-IN) is followed by the appearance of both p76 (PR-RT) and p97 (RT-IN) intermediates, suggesting relatively similar cleavage efficiencies of RT↓IN and PR↓RT junctions. Sequencing analysis of these intermediates will yet conclusively confirm their identity. Diminution of these intermediate(s) is followed by the appearance of RT p66 and later RT, consistent with our recent findings in a bacterial expression system (Section 3.4.5). In conjunction with previous studies on the early events of Gag-Pol processing, we suggest that the order of cleavage events may follow: p2↓NC > TFP↓$p6^{pol}$ > $p6^{pol}$↓PR > RT↓IN ≥ PR↓RT > p51↓RNH

While the exact mechanics and intricacies of ordered Gag-Pol polyprotein processing has not been resolved, it is reasonable to speculate that a number of factors may be important. After cleavage of the initial p2↓NC junction (395,396), the dynamics and efficiencies of subsequent processing events may be influenced by concentration and conformational folding of accumulative Gag-Pol intermediates and the accessibility and complementarily of cleavage sites to HIV-1 PR (183). Indeed, extra PR domains have been reported to affect dimerization and subsequent processing events (39,83,393,414,508,567), and the order of Pol domains themselves is important for efficient processing at all cleavage sites (60). After translation, the domains of the Gag-Pol polyprotein form by global folding and multimerization so as to compactly bury hydrophobic residues (34,542), and the cleavage sites, which separate these domains are expected to occur in less structured or exposed regions. Since peptide cleavage efficiencies do not mimic those seen in the polyprotein context (Table 1), efficient ordered processing of Gag-Pol appears to depend on the structural context and accessibility of the cleavage sites in processing intermediates, as well as the adaptability of HIV-1 PR itself (183,395,407). Based on our findings of the detrimental effect of p51↓RNH cleavage site mutations on the accumulation

of Gag-Pol processing intermediates, and the compensation thereof by T477A, progressive processing may be important in structurally influencing the efficiency of quaternary cleavage events. The accumulation of the p107 Pol intermediate in particular, suggests that forces promoting processing to RT may include contributions from sequences outside the RT region. Furthermore, putative unfolding of the RNH domain may lead to diminished proteolytic stability of subsequent intermediates.

In summary, we have demonstrated that defects imposed by p51↓RNH cleavage site mutations can be compensated to various extents by the intragenic second-site mutation T477A. Our major finding was that this second-site mutation restored processing to p66/51 RT during virion maturation by elevating the proportion of RT p66 in the context of p51↓RNH cleavage site mutations. This suggests improved proteolytic stability of RT and a significant positive impact of the RNH domain on progressive transition of Gag-Pol intermediates towards the p66/51 RT heterodimer. While T477A was not completely compensatory in its capacity to restore RT content for all p51↓RNH cleavage site mutations, our data indicate that it alone was sufficient in most instances. These findings should lead to further investigation of the effect of C-terminal RNH residues on the structural formation RT during virion maturation.

CHAPTER 5: SUMMARY AND CONCLUSIONS

An essential step in the life cycle of HIV-1 is the conversion of genomic single-stranded RNA into proviral double-strand DNA; a complex procedure catalyzed by the viral-encoded enzyme known as reverse transcriptase (RT). Since its initial characterization nearly 20 years ago (298,533), the notion that the biologically relevant form of HIV-1 RT is a heterodimer of 66 kDa and 51 kDa subunits has generally gone uncontested. However, the apparent need for this oligomeric form of RT in the context of the virion has not been clearly defined. This is in light of the fact that recombinant p66/66 RT homodimers have comparable enzymatic activities to the p66/51 RT heterodimer (23,128), and internal processing of the RT subunit at p51↓RNH is unique to lentiviruses such as HIV-1. The goal of the following studies was to determine why the generation of active HIV-1 RT requires three proteolytic cleavage events for efficient viral replication. Since inhibition of the N-terminal PR↓RT cleavage site has been previously reported to have no adverse effect on virion-associated RT activity (65,66), our focus was directed at the remaining cleavage sites. We hypothesized that cleavage of the internal RT p51↓RNH is essential in the formation of functional viral RT, whereas cleavage of the flanking C-terminal RT↓IN junction is not.

5.1 Major Findings and Conclusions

5.1.1 Chapter 2: Proteolytic cleavage of the HIV-1 RT↓IN junction does not seriously impact RT activity, but is essential for efficient viral replication

While precursor-associated forms of recombinant HIV-1 RT (29,211,388,485) as well as PR-defective or inhibited virions (15,162,243,388) arguably exhibit some level of RT polymerase activity, it is generally believed that proteolytic processing is necessary to obtain completely functional RT (284,293). Furthermore, there is increasing evidence in both retroviral and retrotransposon systems for the existence of an RT-IN processing intermediate in the maturation of RT (1,521,558), and for functional interactions between RT and IN proteins (185,363,573). Physical association between RT and IN could be achieved in *cis*, when RT is in direct fusion with IN, or in *trans*, through interaction of the individual mature proteins.

In the following study, mutation of the RT↓IN cleavage site resulted in the expected generation of stable 98 kDa (RT-IN) and 51 kDa (RT) proteins in virions. Furthermore, virion-associated RT polymerase and RNH activities were unaffected by the blockage of this cleavage event. These findings represent the first demonstration in the virion context of the importance of the C-terminal RT↓IN cleavage. Our findings, in combination with those of the PR↓RT cleavage site (65,66) indicate that processing of either flanking RT cleavage site (N-terminal or C-terminal) during virion maturation does not seriously impact on RT activity. The caveat in these findings however, was that the internal p51↓RNH junction continued to be processed, which suggested that this cleavage site was unique and possibly essential. This premise set the stage for the investigation outlined in Chapter 3. Interestingly, while RT activities were unaffected by the RT-IN fusion, viral infectivity and replication capacity were severely attenuated. Although this

defect may have been due to diminished IN activities and integration of proviral DNA, this was beyond the scope of the study, and thus remains unclear. Further investigation may demonstrate, for the first time in the virion context that RT or RNH negatively regulate IN activation until late stage processing of the RT↓IN cleavage site. Overall, the results of this study further our recent understanding of functionally important interactions between RT and IN (90,476,573) by demonstrating that a *cis* association between these enzymes does not disturb RT activities.

5.1.2 Chapter 3: Virion instability of HIV-1 RT mutated at the RT p51↓RNH protease cleavage site

The p66/51 RT heterodimer has been prepared in a number of heterologous expression systems through the assembly of separate subunits or proteolytic processing of RT-containing polyprotein substrates such as the p66/66 RT homodimer (23,120,129,282). Recent studies from our laboratory have indicated that the kinetics of RT processing follow an ordered sequential pathway from a truncated Pol polyprotein expressed in bacteria, whereby RT p66 begets onto RT p51 until both are in equal proportion. Thus, formation of p66/51 RT heterodimers may proceed through a quasi-stable p66/66 RT homodimer intermediate (471).

In order to study the apparent need for RT heterodimers in the context of the virion, we introduced a variety of mutations in the RT p51↓RNH cleavage site with the expectation of generating p66/66 RT homodimer-containing viral particles. We found that surprisingly, most of the mutations resulted in the generation of significantly attenuated virus containing greatly decreased levels of RT that in many cases was primarily RT p51. This was in direct contrast to what was observed in the bacterial expression system, where most of the p51↓RNH cleavage site mutations prevented proteolytic processing to RT p51. Catalytic inhibition of HIV-1 PR showed

that the defect was attributable to processing by HIV-1 PR and not diminished incorporation of Gag-Pol.

This is the first report to address the necessity for processing the internal RT p51↓RNH cleavage site in the context of the virion, and as such, that p51-RNH (RT p66) fusion proteins or the p66/66 RT homodimer may not be proteolytically stable in the virion. Our results imply that additional proteolytic processing, by removal of one of the RNH domains may be essential for providing a conformation of RT that is refractory to further proteolytic events within the virion, thus ensuring adequate levels of functional RT. Unfortunately, it remains to be determined how processing of the p51↓RNH cleavage site specifically stabilizes RT. Limiting further exploration in this field continues to be the lack of an unequivocal mechanism to explain how HIV-1 PR cleaves only one of the RT p66 subunits to form the p66/51 RT heterodimer, and a reliable crystal structure of the p66/66 RT homodimer, to explore key molecular interactions. Our observance of contrasting p51↓RNH cleavage site mutant phenotypes between bacterial and viral expression systems succinctly indicates for the first time, that the study of RT processing in any other milieu except the virus is essentially erroneous. The results presented in Chapter 2, 3 and 4 collectively suggest the existence of alternative cleavage site(s) near the RT p51↓RNH junction that are capable of being processed efficiently only in the virion context. It is regrettable that our efforts to sequence the C-terminus of RT p51 in mutant virions proved unsuccessful, due to the limited amounts of virion protein attainable as well as the sensitivity and sequence coverage of various methods of mass spectroscopy. Significant advancement in either of these areas is certain to prompt a re-initiation of efforts to identify the p51↓RNH cleavage site directly from the virus, for the first time.

5.1.3 Chapter 4: The second-site mutation T477A in HIV-1 RT restores normal processing of Gag-Pol mutated at the RT p51↓RNH protease cleavage site

A number of reports have highlighted the interdependent functional relationship between HIV-1 RT polymerase and RNH activities (196,409,513). Given the structural importance of the RNH domain in RT (194,332,333), it is reasonable that it may equally be important in the proteolytic generation of the p66/51 RT heterodimer. Furthermore, despite the resolution of events and intermediates involved in the processing of the Gag polyprotein (163,396,522), the sequence of events surrounding the liberation of RT from the full-length Gag-Pol polyprotein have remained unclear.

Repeated passage of MT-2 cells exposed to p51↓RNH cleavage site mutant viruses were found to eventually lead to the appearance of viruses with normal RT content and improved replication capacity (Chapter 3). In the following study, we identified and characterized a viral revertant of the p51↓RNH cleavage site mutant phenotype bearing a second-site compensatory mutation in the RNH domain. We found that a seemingly innocuous change of a Thr to an Ala at position 477 was able to restore the viral content of RT subunits in several p51↓RNH cleavage site mutant viruses. We proposed that the T477A change might disrupt structural interactions to accommodate folding defects of the mutated p51↓RNH cleavage site and allow regulated processing of this region, perhaps at alternative nearby sites. However, in the absence of a structural understanding of proteolytic instability caused by p51↓RNH cleavage site mutations, the structural basis for the restored stability inferred by T477A remains unclear. Previous reports have suggested the existence of interactions between residues of the RNH domain and the p51↓RNH cleavage site (85,332,334,540). Thus, the identification of T477A in itself extends these findings by emphasizing the importance of the RNH domain in regulating processing of the

p51↓RNH cleavage site region. The incredible adaptive potential of HIV-1 to revert the RT phenotype further exemplifies the essential need for the p66/51 RT heterodimer and cleavage of the internal p51↓RNH junction for efficient viral replication.

In attempt to investigate the compensatory effect of T477A on the mechanics of RT processing, we analyzed the accumulation of intravirion processing intermediates arising from full-length Gag-Pol by inhibiting HIV-1 PR with decreasing concentrations of ritonavir. As proteolytic activity was elevated in the wild-type virus, higher order RT intermediates appeared, followed by RT p66 and RT p51, consistent with our previous findings in the bacterial expression system (471). This represents the first extensive examination in the virion context of the transition of Gag-Pol processing intermediates that give rise to p66/51 RT. While p51↓RNH cleavage site mutations resulted in the expected diminution of RT p66, higher order processing intermediates appeared more accentuated at greater levels of active HIV-1 PR. This result suggested a reduced efficiency of late processing events. The introduction of T477A elevated the proportion of RT p66, resulting in improved processing to p66/51 RT. Overall, these findings suggest that the compensatory effect of the T477A second-site mutation was to restore proteolytic stability of p51↓RNH cleavage site mutant RT during HIV-1 maturation.

5.2 Significance and Future Studies

Our studies show why the generation of functionally active RT requires three proteolytic cleavage events during HIV-1 maturation. In the context of the virion, processing of either N-terminal PR↓RT or C-terminal RT↓IN cleavage site is not essential for the generation of active

RT. Recombinant forms of PR-RT and RT-IN arguably exhibit some level of RT activity far lower than the mature p66/51 RT heterodimer (29,211,293,388,485). However, mutation of either N-terminal (65,66) or C-terminal (Chapter 2) RT-flanking cleavage sites results in wild-type levels of virion-associated RT activities. The generation of virions, solely containing these fusion forms of RT is seemingly not possible, due to the continued processing of the p51↓RNH cleavage site. We show that preventing cleavage of this internal p51↓RNH junction, the unique cleavage event associated with HIV-1, significantly reduces the stability of the RT in the virion. This is a significant contribution to knowledge and the take home message of this dissertation. Processing of the internal RT p51↓RNH cleavage site is unequivocally essential to ensure proteolytic stability of functional RT in the virion.

Without question, the biologically relevant form of RT is a heterodimer of 66 kDa and 51 kDa subunits. However, it appears that this particular heterodimeric form of viral RT is not absolutely required for wild-type levels of activity since p77/62 PR-RT and p98/51 RT-IN fusion forms are just as active. Whether all of these RT heterodimers are equally efficient at facilitating reverse transcription remains to be seen. Additional biochemical and biophysical studies on recombinant versions of these enzymes has the potential to yield a wealth of useful and interesting information. We have demonstrated that cleavage of the internal p51↓RNH junction is essential for activity and proteolytic stability of viral RT, whereas cleavage of the flanking junctions is not. So why then, is formation of the p66/51 RT heterodimer itself necessary for efficient viral replication? The answer lies, not in the effect of an abutting PR or IN domain on RT activity *per se*, but rather the effect of such oligomeric structures on other aspects of the viral life cycle. Inhibition of the PR↓RT cleavage site has no effect on the proteolytic processing of Gag and Gag-Pol polyproteins, however infectivity is diminished 20-fold (65,66). Fusion of RT

to PR may have enhanced the activity of PR (6,157), which in turn may have increased intracellular cleavage of Gag (321) or important cellular factors such as actin (468), vimentin (467) or NF-κB (427). Mutation of the C-terminal RT↓IN cleavage site similarly had no affect on RT activity, yet infectivity was significantly attenuated. Our results appear to indicate that this defect may have been attributable to an inhibition of IN activity by RT or RNH. Further study in this area will produce important information on the proteolytic regulation of IN activity and the functional interactions that exist between RT and IN.

Our demonstration that mutagenesis could not produce stable p51-RNH (RT p66) fusion protein in virions by mutagenesis directly implies the critical importance of this internal RT p51↓RNH cleavage site during HIV-1 maturation. While the number of possible mutagenic combinations have not been exhausted, our results suggest that any future study should take into account the structural context of this entire region, including intra- and intermolecular interactions and the existence of alternative cleavage sites. However, in the absence of a reliable crystal structure of the p66/66 RT homodimer it remains a difficult task to plan a rational set of next generation p51↓RNH cleavage site mutations. Although other methods were initially considered, mutagenesis was judged the most straightforward means of addressing the importance of this internal cleavage junction. If the specific goal of future studies is to assess the replication capacity of viruses containing p66/66 RT homodimers, consideration could be given to altering the substrate specificity of HIV-1 PR. Recent reports suggest that certain amino acid substitutions can be introduced into HIV-1 PR to prevent the hydrolysis of specific cleavage sites in Gag-Pol (82,306,336). Finally, our finding that the second-site mutation T477A improved the proteolytic stability of RT containing p51↓RNH cleavage site mutations warrants further exploration on what effect of other substitutions in the RNH domain would have on the

generation of p66/51 RT. These studies could provide new insight into molecular interactions within the p66/66 RT homodimer that are important during virion maturation.

It is interesting to speculate why an additional, internal RT p51↓RNH cleavage site is unique to lentiviruses such as HIV-1. The tether region and various residues leading up to the p51↓RNH cleavage site at F440 serve a number of important roles in the HIV-1 p66/51 RT heterodimer, including: (i) coordination of RT polymerase and RNH active sites (223,506), (ii) dimerization and interdomain interactions (332,338,506), and (iii) control of global rotational reorientations of the RNH domain (506). At this stage, it is unclear whether the functional attributes of this region are at all applicable in other retroviral forms of RT. Although the structure of full-length MLV RT has been recently solved (81), the absence of other retroviral forms of RT from the protein structural database precludes a detailed comparative analysis. As discussed in Chapter 1, the monomeric nature of MLV RT may be attributable to the length of its tether region and the presence of non-conserved motifs in the RNH domain (141,331). While the absence of such features in HIV-1 RT would explain the need for dimerization to support the catalytic subunit, they do not explain why this supporting RT subunit must be 51 kDa and not 66 kDa in the virion. The results of our studies suggest that an additional important role of the p51↓RNH cleavage site region is to regulate proteolytic stability of RT during virion maturation to ensure generation of adequate levels of functional RT. The internal RT p51↓RNH cleavage site, and thus p51 subunit may be unique to HIV-1 for the simple reason that an extra RNH domain may serve a structural, if not functional deterrence in the confines of the viral particle.

The past two decades have seen remarkable progress in elucidating the order of polyprotein processing and the maturation of retroviruses such as HIV-1. There is now a wealth of information about the structure and functions of HIV-1 *pol*-encoded enzymes and their roles

in the retroviral life cycle. With this basic framework in hand, efforts should continue towards new frontiers in the field of HIV-1 biology in an attempt to fill in the remaining gaps of knowledge. The interactions between trafficking Gag and Gag-Pol polyproteins are of central importance to the assembly and maturation of viral particles. How do such interactions affect domain arrangements, multimerization, particle budding and the regulation of ordered polyprotein processing? Defining the molecular details of specificity and control for any of these steps will represent major advancement.

There is still much to learn about the mechanics of late stage Gag-Pol polyprotein processing. Although our work using decreasing concentrations of ritonavir represents a first step towards resolving the transition of processing intermediates, further study is needed. We caution against the use of *E.coli* or other heterologous expression systems to investigate polyprotein processing, due to their unreliable nature in mimicking the true effect of mutations. Formation of HIV-1 RT does not appear to be as simple as mixing together 66 kDa and 51 kDa subunits. Our results suggest that efficient ordered processing of RT intermediates is mediated, not by the availability of cleavage sites *per se*, but by appropriate conformation and domain constraints. This is consistent with a recent report that the order of Pol domains is important for efficient processing at all cleavage sites (60). Future study may resolve the contribution of molecular chaperone proteins and other interacting viral factors in the regulation of folding and efficient proteolysis of Gag-Pol intermediates during virion maturation. Also worthy of consideration, is the need to dissect the role of the proteosome-ubiquitin system in the release and maturation of infectious HIV-1 particles (448), particularly given its involvement in the degradation of misfolded proteins in other viral systems (142).

It is likely that ongoing and future work will further our understanding of how cleavage events lead to molecular, biochemical and biophysical changes in the *pol*-encoded enzymes of HIV-1 Gag-Pol. As discussed earlier in Chapter 1, there are a number of limitations in synchronizing retroviral particles. One key difficulty is initiating proteolysis after treatment with protease inhibitors. Future studies may consider producing immature virions in the presence of loose-binding protease inhibitors that exhibit high K_m and low K_{cat} values. Membrane permeabilization with β-octylglucoside (72) or cholesterol-depleting agents such as β-cyclodextrin (173,297) followed by dialysis could allow for a kinetic analysis of polyprotein processing. Further in need of resolution, is an analysis of the structural and conformational context of intermediates which transition towards p66/51 RT. Biophysical analyses are likely to be important contributors in these efforts, including crystallography, NMR or circular dichroism spectroscopy of full-length Gag-Pol and its processing intermediates. Collectively, the above approaches will provide a view of viral assembly and maturation that is certain to be intellectually satisfying.

In summary, we have demonstrated the importance of proteolytic processing in the generation of functionally active HIV-1 reverse transcriptase. It is our hope that this work will form an integral part of our global understanding of HIV-1 reverse transcriptase and other enzymatic proteins regulated by proteolysis in other systems. Our observation that the internal RT p51↓RNH cleavage site is essential to provide proteolytic stability of RT during virion maturation presents a novel target for therapeutic intervention. The recent advent of a betulinic acid inhibitor of the CA↓p2 cleavage site (241,587) suggests that a similar small-molecule could be designed to specifically bind to the RT p51↓RNH cleavage site. Certainly, in the face of

possible resistance to common HIV-1 inhibitors, complete obliteration of RT during virion maturation would be a favourable scenario.

APPENDIX: MISCELLANEOUS FIGURES

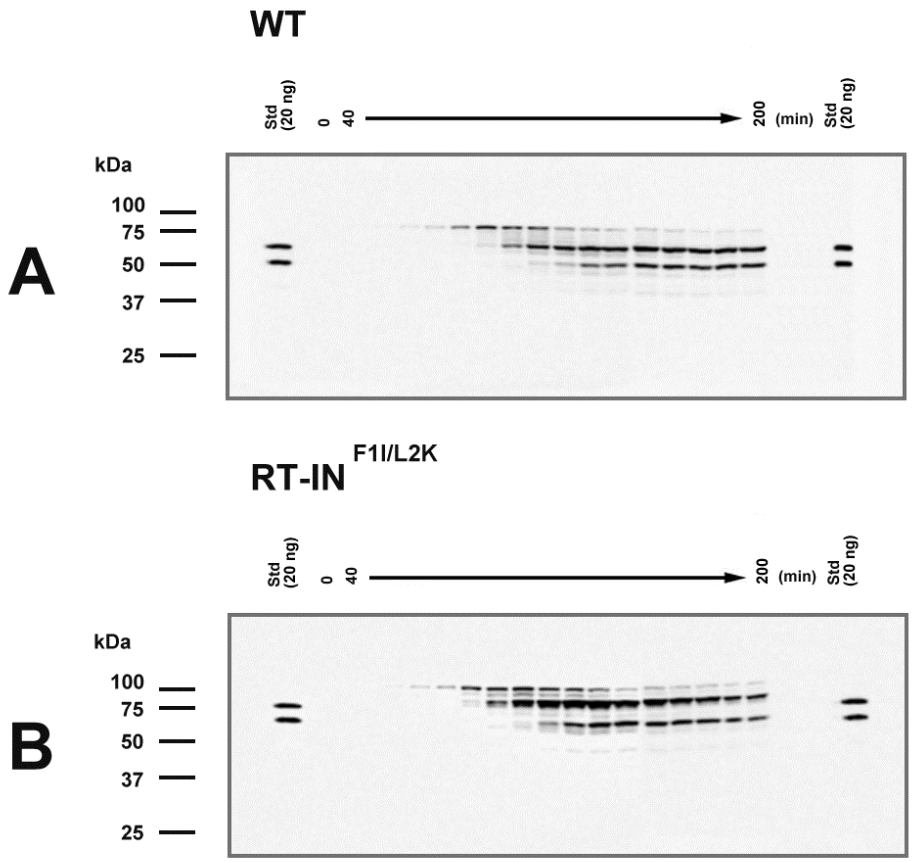

Figure 29. Effect of RT-IN$^{F1I/L2K}$ cleavage site mutation on the synthesis and processing of the 90 kDa Pol polyprotein *in vitro*. (A) Western blot of the synthesis and processing products following IPTG induction of the wild-type 90 kDa Pol polyprotein, detected by a pool of monoclonal antibodies specific for HIV-1 RT. (B) Western blot of the synthesis and processing of products following IPTG induction of the 90 kDa Pol polyprotein mutated in the RT↓IN junction, detected by a pool of monoclonal antibodies specific for HIV-1 RT. Preparation and analysis of Pol polyprotein processing was performed as previously described (Section 3.3.5). The mutations F1I/L2K completely prevented cleavage of the RT↓IN junction, leading to the formation of the 71 kDa RT-IN N-terminus fusion protein. Formation of RT p51 is unaffected. *(Modified from: Sluis-Cremer, N. et al. 2004 Int. J. Biochem. Cell. Biol. 36(9):1836).*

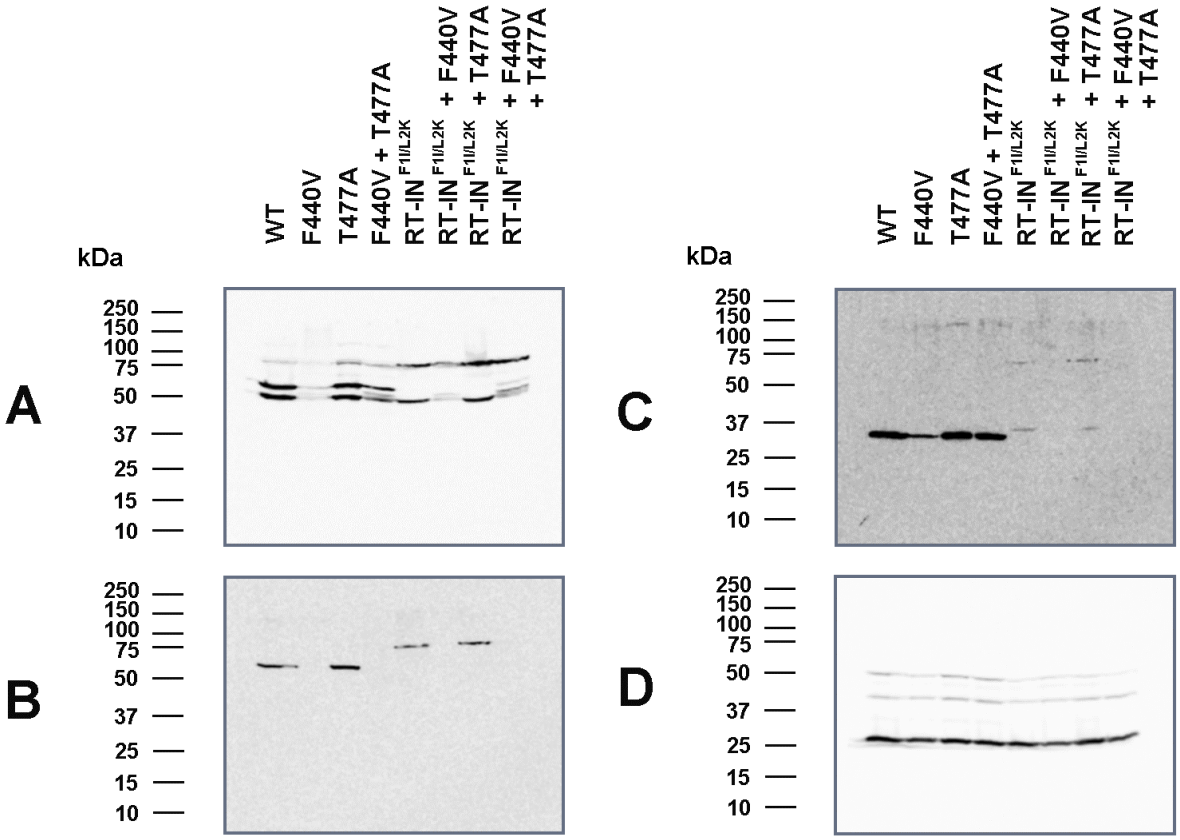

Figure 30. Effect of p51↓RNH ± RT↓IN ± T477A mutations on viral particle protein composition. 293T cells were transfected with 10 μg of each indicated proviral DNA construct. Virus containing supernatants were harvested at 60 h post-transfection, normalized by p24 (1 μg), purified, and analyzed by Western blotting as previously described (Section 2.3.6). HIV-1 Gag-Pol proteins (A) RT, (B) RNH, (C) IN and HIV-1 Gag proteins (D) $Pr55^{gag}$, p41, p37, and p24 CA were probed on separate blots with appropriate primary and secondary antibodies followed by enhanced chemiluminescence (ECL). The position of molecular size markers are shown to the left of each panel.

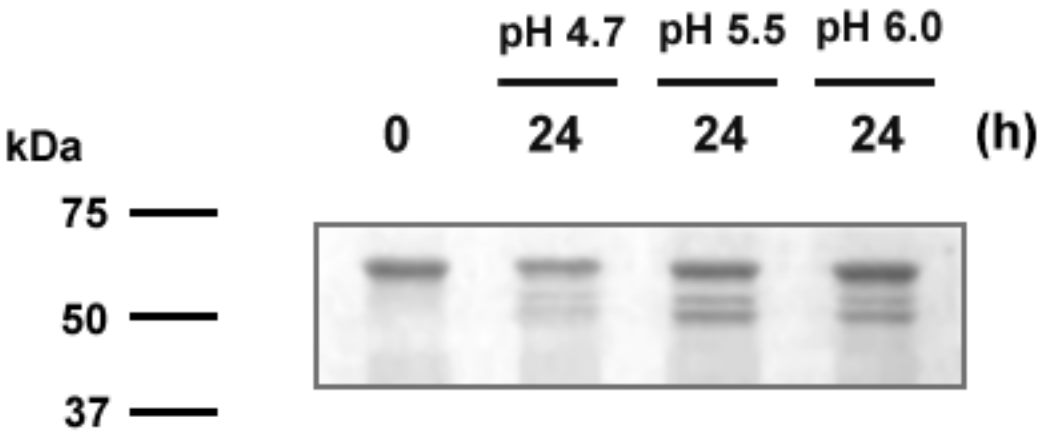

Figure 31. ***In vitro*** **processing of purified recombinant p66/66 RT homodimer with HIV-1 PR in** ***trans*****.** SDS-10% PAGE analysis of RT subunit composition (6 μg per lane), following incubation (0, 24 h at 37^{o}C) of 1 mg recombinant p66/66 RT in the presence of 11 μg HIV-1 PR. Various pH conditions tested included: lane 2, pH 4.7 (0.1 M sodium acetate, 1 M NaCl, 1 mM EDTA, 0.1% BSA); lane 3, pH 5.5 (0.1 M sodium citrate, 1 M NaCl, 1 mM EDTA, 0.1% BSA); lane 3, pH 6.0 (50 mM MES ,1 M NaCl, 1 mM EDTA, 0.1% BSA). The position of molecular size markers are shown to the left of each panel.

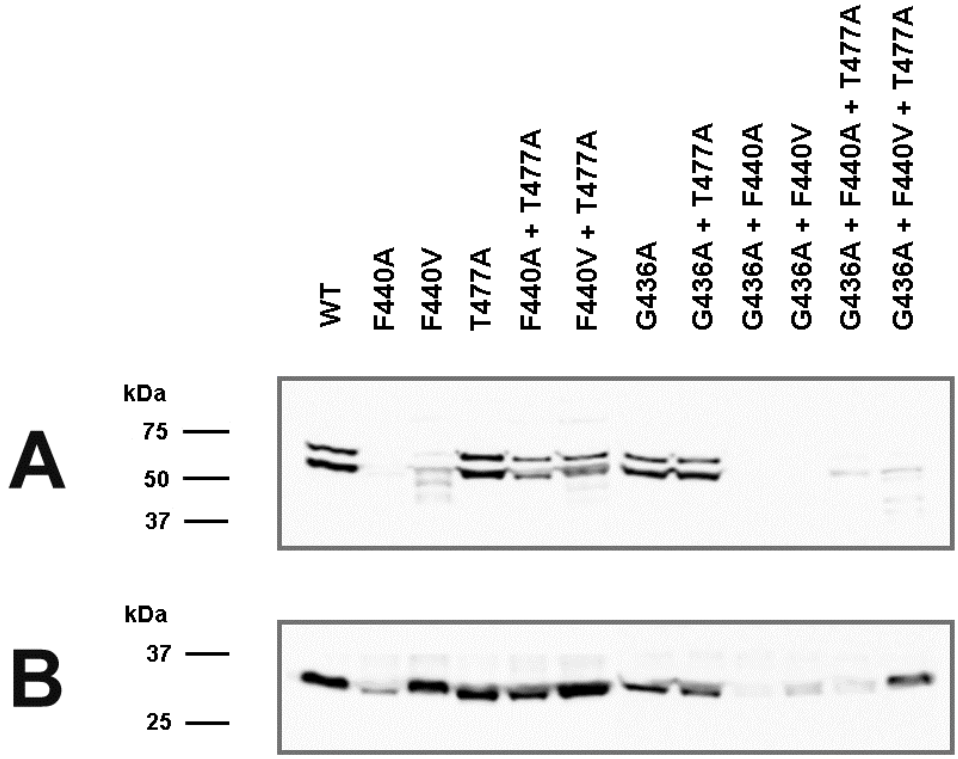

Figure 32. Effect of G436A ± p51↓RNH ± T477A mutations on viral particle protein composition. 293T cells were transfected with 10 μg of each indicated proviral DNA construct. Virus containing supernatants were harvested at 60 h post-transfection, normalized by p24 (1 μg), purified, and analyzed by Western blotting as previously described (Section 2.3.6). HIV-1 Gag-Pol proteins (A) RT and (B) IN were probed on separate blots with appropriate primary and secondary antibodies followed by ECL exposure and analysis. The position of molecular size markers are shown to the left of each panel.

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