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The Mechanisms of Pol Expression and Assembly for Human Foamy Virus

by

David Norris Baldwin

A dissertation submitted in partial fulfillment of the requirements for the degree of

Doctor of Philoshophy

University of Washington

1999

Department of Microbiology

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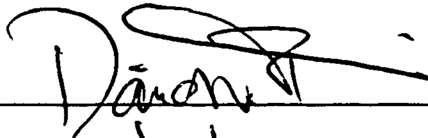
Signature 

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November 1, 1999

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Abstract

THE MECHANISMS OF POL EXPRESSION AND ASSEMBLY FOR HUMAN FOAMY VIRUS

by David Norris Baldwin

Chairperson of the Supervisory Committee: Professor Maxine L. Linial
Department of Microbiology

In this study, I have examined the role of Pol in the replication pathway of HFV. We discovered that unlike all known retroviruses, the Pol protein is expressed independently of Gag from a spliced mRNA, rather than as a Gag-Pol fusion protein. Subsequently, I compared the basic assembly strategies of foamy viruses, retroviruses and hepadnaviruses. Hepadnaviruses are the only other mammalian reverse transcriptase-encoding viruses which also express their Pol protein independently from their structural proteins. However, hepadnavirus assembly strategies are very different than those of retroviruses.

I found that virus assembly with respect to Pol in HFV is more like retroviruses than hepadnaviruses. A *pol* deletion mutant was able to assemble particles which resemble wild type particles by electron microscopy. In addition, like other retroviruses, this *pol* deletion mutant was able to package wild type leves of genomic viral RNA. In contrast, however, I found that like hepadnaviruses, there is a requirement for the Env surface glycoproteins of HFV for virus release from the cell. In the absence of Env, capsids formed by HFV Gag could be seen by electron microscopy, and quantitative analysis of capsid formation indicated that there was no difference between wild type and the *env* deletion mutant, although no extracellular virus could be detected.

The second part of this dissertation focused on mechanism by which HFV Pol is incorporated into particles. To study Pol incorporation I developed novel methods for detecting the Pol proteins in extracellular virions. By introducing a phosphorylation site

for protein kinase A (PKA) into the integrase domain of Pol, I was able to immunoprecipitate and phosphorylate the Pol proteins from transfected cells, and gradient purified virions. Using this assay, I showed that proteolytic processing by the viral protease is not required for Pol incorporation. I was able to define domains of Pol and Gag which are important for Pol incorporation into particles. I found that the primary structure of Gag around the single cleavage site in Gag is not important, but that a region of about 8-kDa upstream is essential. I also found that the protease domain of Pol is essential for Pol incorporation.

I also tested these domains of Gag and Pol in the yeast two-hybrid system and found that protein::protein interactions between Gag and PR could be detected. Since the truncation mutant of Gag which failed to package Pol was also lacking two of three basic regions known to be important for nucleic acid binding, I was interested in the ability of this virus to package RNA. I found that it was severly reduced for its ability to package RNA, beyond the limits of Pol detection using our PKA assay. I also tested a primer binding site (PBS) deletion mutant and found that it incorporated the Pol protein, implying that initiation of reverse transcription does not play a role in Pol assembly. While I found some evidence to suggest that protein::protein interactions between Gag and Pol are important for Pol incorporation, a role for RNA in this process cannot be ruled out from the work presented here.

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Chapter 1
Introduction

The family of *Retroviridae* is a very large family of viruses, members of which have been found in almost all vertebrates studied, and the ancestors of which are found in many invertebrate organisms. The study of retroviruses during the last thirty years has had a tremendous impact on many fields of basic science. Retroviruses are associated with many types of disease including malignancies, immunodeficiencies, and neuropathologies. The first descriptions of cell-free transmission of leukemia and sarcoma came from observations in 1908 and 1911 by Bang and Rous respectively, and as we now know, these diseases were caused by avian retroviruses. More recently, retroviruses have been given an enormous amount of attention due to the worldwide epidemic of human immunodeficiency virus (HIV). Retroviruses have taught us about cancer by transducing oncogenes, and by revealing the nature of molecules central to the regulation of cell division. They have also provided us with critical molecular biology tools, namely their own enzymes; reverse transcriptase and ribonuclease H, without which cloning and expressing genes of interest from eukaryotic messenger RNA might be much more difficult.

Retroviruses are enveloped viruses which typically contain two copies of single stranded, positive sense genomic RNA, which range from 7-13 kilobases in length. For comparison, various retrovirus genomes and their coding regions are outlined in (Figure 1.1). Retrovirus particles range in size from 80 to 120nm as measured by electron microscopy. The replication cycle of a retrovirus is initiated by binding to a host cell, penetration, and uncoating of the membrane from the capsid (Figure 1.2). In a process which remains poorly understood, the pre-integration complex which consists of the viral genome and a subset of viral and possibly cellular proteins, is transported to the nucleus. Concommitantly, the genome is converted from single stranded RNA to double stranded DNA with long terminal repeats (LTRs), by the viral reverse transcriptase (RT). This cDNA form of the genome is then integrated into the host cell DNA by the viral integrase

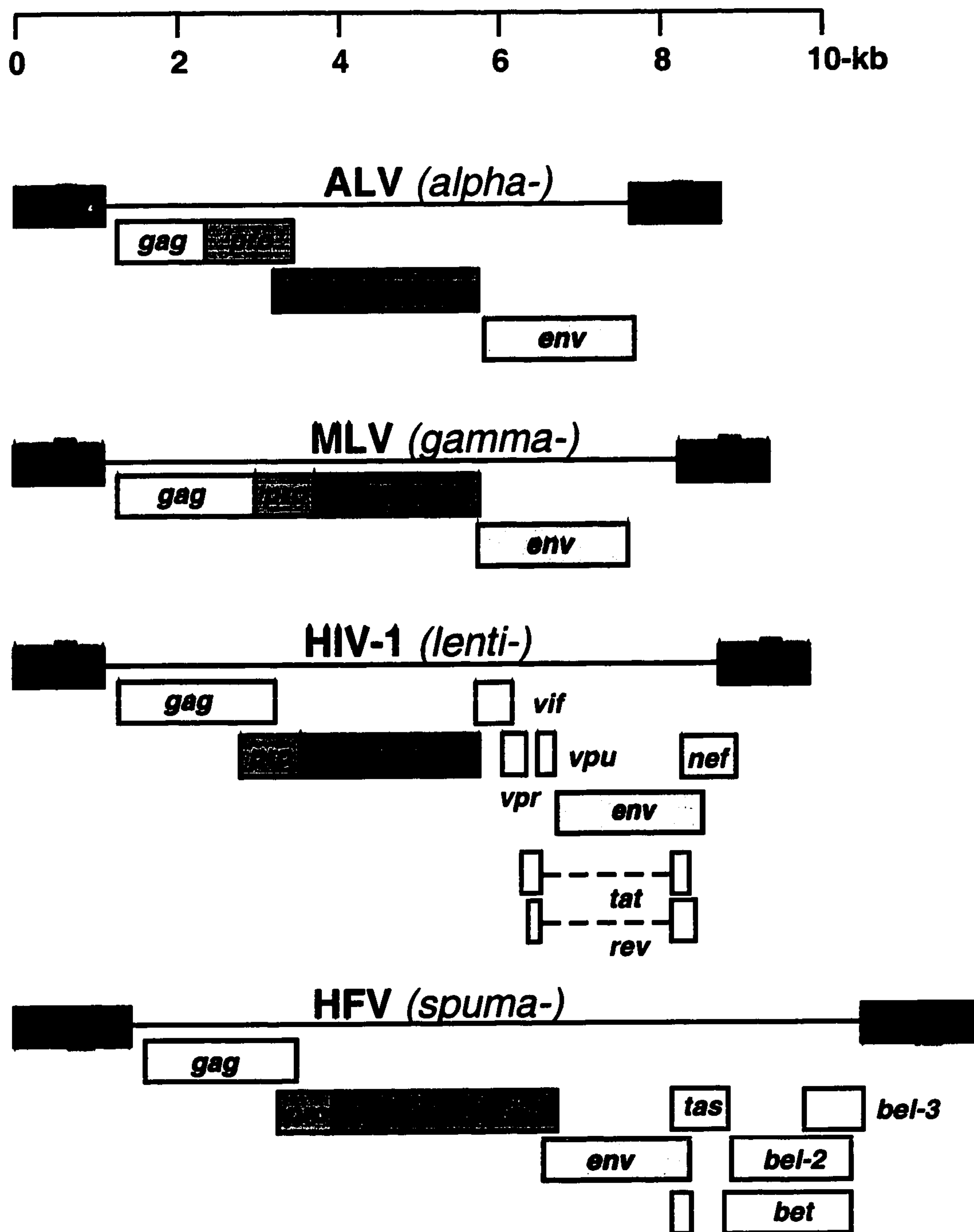


Figure 1.1 Coding regions of retroviral genomes. Adapted from Fields Virology.

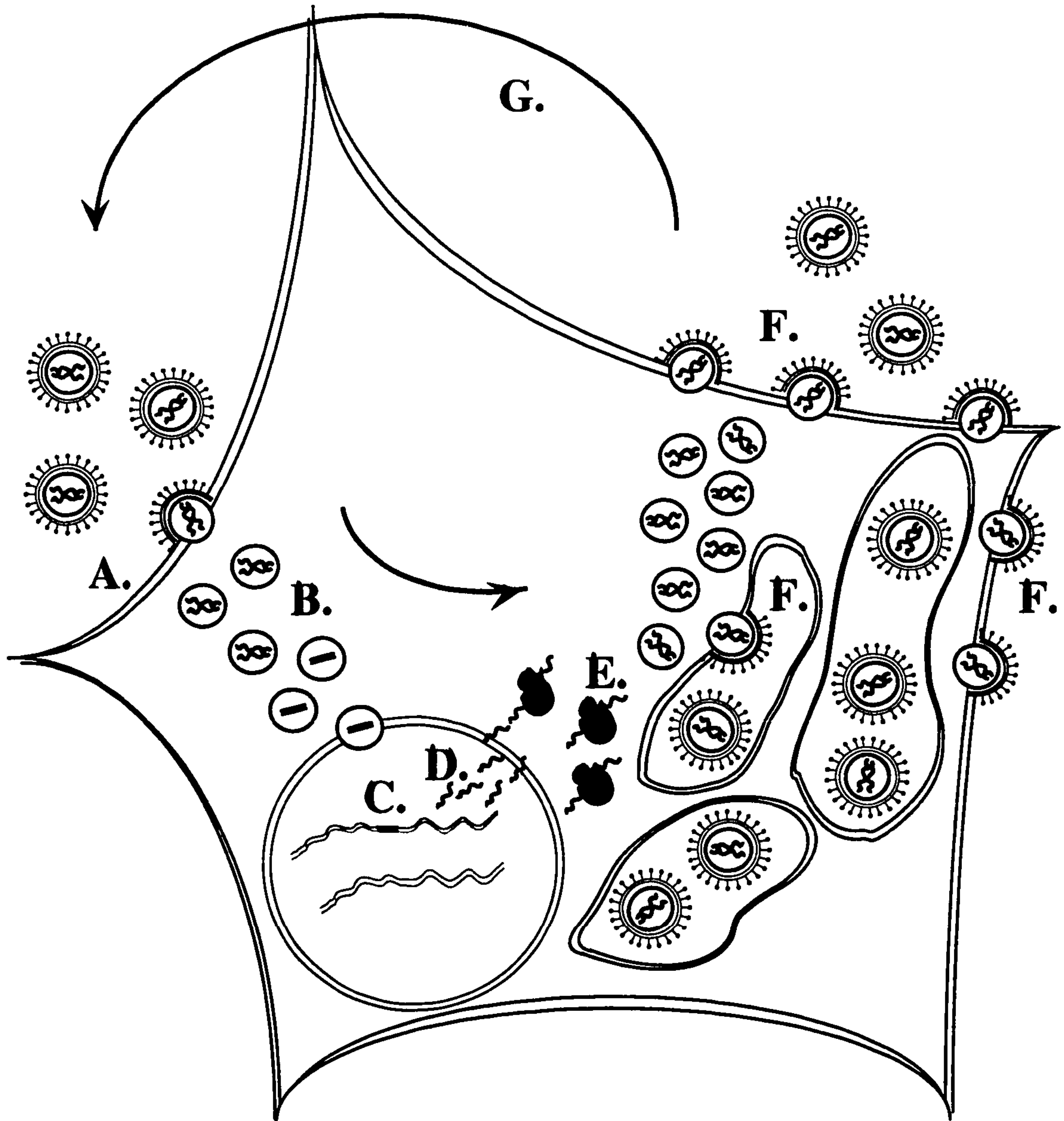


Figure 1.2 Retrovirus replication pathway.

A. Receptor binding and entry.
B. Reverse transcription and nuclear translocation.
C. Proviral integration.
D. Transcription of viral genes.
E. Translation and assembly of genome containing capsids.
F. Budding and release of virus.
G. Cell-free transmission.

(IN). Both the enzymatic processes of reverse transcription and integration have been well characterized biochemically. These stages are termed the "early" stages of the viral life cycle. The "late" stages are characterized by transcription of the proviral DNA, translation of the viral genes from genomic and subgenomic RNAs, assembly of virions, and release from the cell (Figure 1.2).

The genera of *Retroviridae* are now separated by the characteristics of their genomes, their assembly strategies, and their pathogenesis. The best studied of these genera are the *lentiviruses* such as HIV, the avian leukosis viruses (the *alpharetroviruses*), and the murine leukemia viruses (*gammaretroviruses*)(Figure 1.1). Others include the mouse mammary tumor viruses (*betaretroviruses*), the bovine leukemia viruses (*deltaretroviruses*), and the walleye dermal sarcoma viruses (*epsilonretroviruses*). The *alpha-*, *beta-* and *gamma-* genera are also referred to as the simple or *onco*-retroviruses. All retroviruses contain the three canonical genes: *gag*, *pol* and *env* (Figure 1.1). The functions of these genes are to form structural capsids, to replicate the viral genome, and to provide specificity for the host cell respectively. Three of these genera contain complex retroviruses (*delta-*. *epsilon-*, and *lenti-*), meaning that they encode additional open reading frames (ORFs) within their genomes. These ORFs are dispersed through different reading frames, both in the central and 3' regions of the genome. Accessory ORFs can be, but are not necessarily essential for the replication of the virus.

The last and least well studied of the retroviral genera are the *spumaviruses*, or "foamy viruses". They are also complex retroviruses, encoding both essential and non-essential accessory genes. Similar to the *lentivirus* genus, many species of foamy viruses have been identified in non-human primates, cats and cattle. Human foamy virus (HFV) is the prototype of the *spumavirus* genus of Retroviridae (Rethwilm et al., 1987). It was originally isolated in 1971 from tissue culture cells derived from a human cancer patient (Achong et al., 1971), but based on homology with other primate isolates, it is now thought to be of chimpanzee origin (Herchenroder et al., 1994; Schweizer and Neumann-Haefelin, 1995). The first molecular clone of the *spumaviruses*, human foamy virus

(HFV-13), became available in 1990 (Rethwilm et al., 1990). Since the molecular cloning of HFV, earlier studies correlating HFV infections with disease in humans have all been refuted, and it is now believed that foamy viruses are essentially absent from the human population (Schweizer et al., 1995; Schweizer et al., 1994). Although humans have occasionally been infected with foamy viruses, these events are not common, and there is no evidence of pathogenesis. Several other interesting observations have been made with respect to the replication pathway of HFV. The observation that Gag and Pol are expressed independently of each other, unlike all other known retroviruses, led to the work that will be the focus of this thesis.

The only other vertebrate reverse transcriptase-encoding viruses are the hepadnaviruses, such as the hepatitis B viruses (HBV). They are distantly related to retroviruses, and encode functionally similar genes (Figure 1.3A), but their replication strategies are considerably different (Figure 1.4). They are DNA viruses, meaning that reverse transcription is completed prior to virus release of virus from the cell. They do not encode the retroviral enzymes protease (PR) or integrase (IN), and they maintain their genome as a nuclear plasmid during infection. They infect only hepatacytes of the liver, and therefore are much more restricted in their host range than retroviruses, some of which can have very broad host ranges. This introduction focuses a great deal of attention to the hepadnaviruses because as we learn more about the replication of HFV, we find that there are remarkable parallels between the foamy viruses and these distant relatives. Importantly, the HBV reverse transcriptase (P protein) is expressed independently of the structural protein, core (C protein), and hepadnaviruses have evolved mechanisms very different from that of retroviruses for particle assembly and genome replication. The P protein binds to a secondary structure (ε) in the genomic RNA to initiate the assembly process (Figure 1.3B). Distinct from retroviruses, hepadnaviruses complete reverse transcription prior to egress from the cell, require their surface glycoproteins for egress, and utilize an intracellular reinfection pathway which contributes to the accumulation of nuclear genomes, and eventual hepatocellular carcinoma (Figure 1.4).

A.

B.

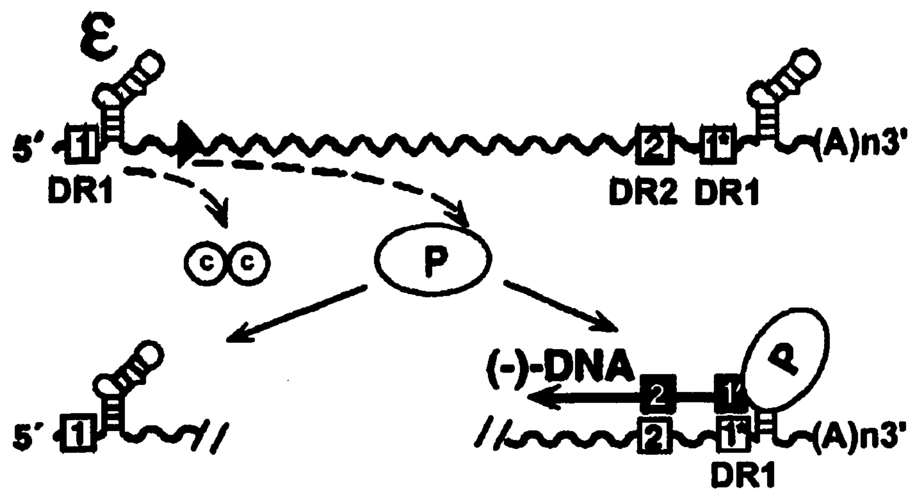


Figure 1.3 Schematic diagram of the hepadnavirus genomc and assembly strategy with respect to the P protein. Although this representation of the genome is linear to illustrate the organization of the genes (A), the template for transcription is covalently closed circular DNA (cccDNA). (B) The pregenomic RNA, and P protein binding sites (ε), illustrating a model for both RNA packaging and initiation of reverse transcription (adapted from Nassal and Reiger, 1996).

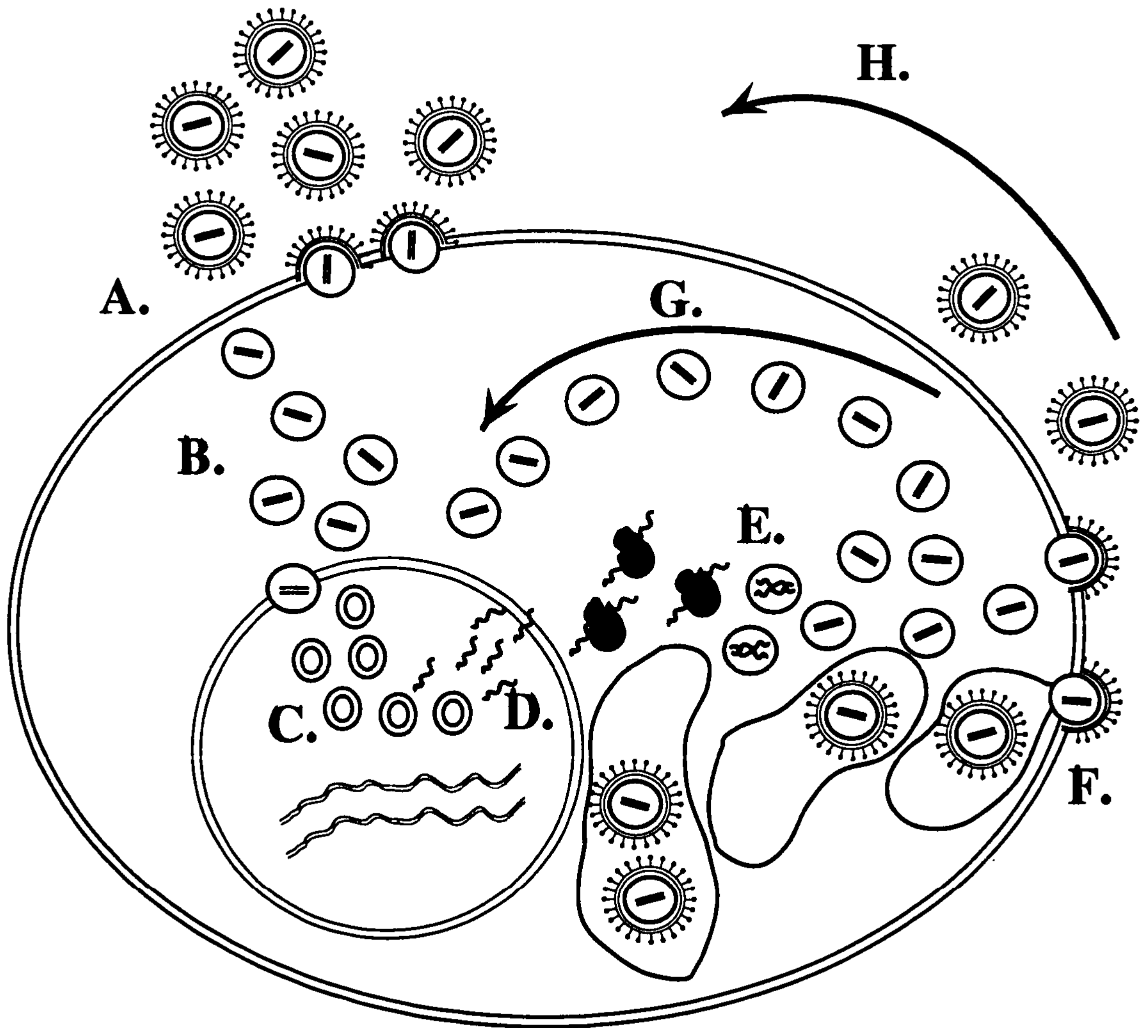


Figure 1.4 Hepadnavirus Replication Pathway.

A. Receptor mediated virus entry and uncoating.
B. Nuclear translocation.
C. DNA repair to generate cccDNA.
D. Transcription of viral genes.
E. Translation and virus assembly.
F. Budding and virus release.
G. Intracellular reinfection pathway.
H. Cell-free transmission.

In this work I examine the late stages of HFV replication, with an emphasis on how the Pol proteins are expressed and assembled into virus particles. The fact that the Pol proteins are expressed independently from the Gag structural proteins led me to compare the basic assembly strategies of retroviruses and hepadnaviruses with respect to Pol.

A. The Late Stages of Retrovirus Replication.

1. Expression of retroviral genes. Retroviruses are unique among virus families in that they specifically integrate their genomes into the DNA of their hosts. This is an essential step in the life cycle of the virus, and the integration event defines the central step in the replication pathway. The late stages of retrovirus replication begin with transcription of the viral genes from the integrated provirus. This process is mediated in large part by ubiquitous cellular proteins, such as the RNA polymerase II transcriptional machinery. Retroviruses have evolved specific mechanisms for controlling these proteins; in particular they have generated and conserved their own *cis* regulatory sequences for promoting transcription of their own genes. Since they cannot rely on a defined integration site in the host genome for acquisition of a promoter, they carry their own to ensure expression of their genes.

Retroviral promoters are encoded in repeated proviral termini called long terminal repeats (LTRs), which contain regions of the genome termed U3, R, and U5, for unique 3' region, repeated region, and unique 5' region respectively. In the provirus, the U3 region contains the viral promoter and enhancer sequences, directing transcription from the single initiation site at the 5' end of R. Initiation of transcription by RNA polymerase II is dependent on canonical TATA sequences which define the first nucleotide of the genome, and all subgenomic RNAs. The U3 regions vary in length and complexity from one retrovirus to another, and tend to reflect the relationship of the virus with its host. For example HIV, which infects T lymphocytes, contains binding sites for the NF-kB transcription factors in its LTR. These are transcription factors which play an important

role in cytokine production after T cell activation, a process which is also known to be important for activation of HIV replication. Some of the complex retroviruses also encode an accessory transactivator protein which can specifically activate their own LTR by recruiting RNA polymerase to U3. For these viruses such as HIV, HTLV, and as will be discussed later, the foamy viruses, regulation of gene expression is complicated and relies on expression of the transactivator. For all retroviruses, there are many binding sites for both transcriptional activators and repressors, often in overlapping regions of U3, indicating the complexity of interactions at these enhancer sites. Foamy viruses are unusual among retroviruses in that they contain an internal promoter located in the *env* gene, which drives the initial expression of the LTR-specific transactivator (Tas).

Retroviral genomes and consequently all subgenomic RNAs are expressed from the provirus at the beginning of R. R is duplicated at both termini of the RNA genome, and defines both the start site for transcription at its 5' end, as well as the polyadenylation processing site at the 3' end of the genome. The genomic and subgenomic RNAs both serve as messenger RNAs and are 5' capped with $m^7G5'pppGmp$. Most subgenomic RNAs are spliced from the genomic RNA, and for simple retroviruses, the only splicing event produces the *env* mRNA. Complex retroviruses, however, exhibit much more complicated splicing patterns in order to generate their accessory proteins. HIV and HTLV have also evolved genes, *rev* and *rex* respectively, to ensure efficient transport of the unspliced genome to the cytoplasm. *Cis* elements in the viral genome are important for both positive and negative regulation of splicing. Regardless of their complexity, all retroviruses must cope with regulating the splicing events such that the majority of the genomic RNA is transported intact to the cytoplasm where virus assembly occurs. For simple retroviruses, cellular factors are thought to bind to *cis* elements in the unspliced RNA and facilitate transport. This is important not only for packaging of the genome, but also because the major structural protein and most abundant viral gene product, Gag, is expressed from the genomic RNA.

Translation of the canonical retroviral genes, *gag*, *pol*, and *env* occurs both on free ribosomes and on polysomes in the rough endoplasmic reticulum (RER). Gag proteins,

which are translated from the unspliced genomic RNA, are expressed in the cytosol. The Env surface glycoproteins are thought to be translated from spliced mRNAs on polysomes in the RER where they can be transported into the secretory pathway for post-translational modification. One surprise for retrovirologists was the finding that the Pol protein was expressed as a fusion protein with Gag from the genomic RNA. Most retroviral *pol* genes are in overlapping reading frames with *gag*, with the exception of murine leukemia virus (MLV), where *gag* and *pol* are separated by a termination codon. Retroviral *pol* genes do not encode their own methionine initiation codons (with the exception of HFV, discussed later). Pol is synthesized as a Gag-Pol fusion in the cytoplasm by either ribosomal frameshifting (where reading frames are overlapping with *gag*), or stop codon suppression (where *gag* and *pol* reading frames are separated by a termination codon).

Expression of the Gag-Pol polyprotein is regulated at the level of the frameshifting or readthrough event, which occurs approximately 1-5% of the time. This is important for several reasons. Virus assembly depends on high levels of Gag expression, but the viral enzymes are required in much lower concentrations since their function is catalytic. In addition, the Gag domains of Gag-Pol fusion proteins provide a mechanism for incorporation of the viral enzymes into particles via Gag::Gag interactions.

2. Assembly of Retroviral Particles. For most retroviruses, the Gag protein encodes all of the basic assembly functions. When expressed alone from a heterologous promoter, Gag can assemble capsids and bud from the cell in the absence of viral genomic RNA, Pol, or Env. When expressed in the viral context, Gag binds to and specifically encapsidates the viral genome, and multimerizes with Gag-Pol fusion proteins, providing a mechanism for Pol incorporation into particles.

Retroviruses have evolved to assemble their capsids in several ways. Some self assemble in the cytoplasm, and then bud into membranes either at the plasma membrane or the secretory pathway, thereby accumulating Env proteins on their surface. Others

target their Gag proteins to membranes with myristylation signals, and form capsids at the membrane in a process concommitant with budding. For many years, retroviruses were classified by their virion morphology and assembly strategy, and the nomenclature to describe them remains today. A, B and D type particles are ones which form capsids in the cytoplasm, and then bud (or never bud as with type A) through the glycoprotein-containing membrane. The simple onco-retroviruses, both avian and mammalian, typically form C-type particles, meaning that they assemble capsids at the membrane.

There is evidence to suggest that Gag requires RNA to be present for proper assembly. Recombinant Gag is difficult to work with for technical reasons, but it was recently demonstrated that the CA-NC domains could multimerize *in vitro* in the presence of RNA (Campbell and Vogt, 1995). In addition, HIV cores form correctly in cell-free systems only when the proper stoichiometry of CA-NC to RNA is used (Ganser et al., 1999). In these studies, viral RNAs were used, but in the context of a cell, cellular RNAs could potentially function to nucleate or stabilize Gag multimerization. It is clear that viral RNAs are not required since retroviral packaging cell lines lacking viral packaging signals form normal particles (Linial et al., 1978). Heterologous RNAs which do contain the packaging signals can then be specifically incorporated into these virions and transfered to recipient cells (Linial and Miller, 1990). Transfer of these RNAs produced in packaging cell lines requires that the Pol proteins be specifically incorporated into particles, and that reverse transcription of the RNA can procede via normal mechanisms.

Retroviral Pol proteins are dispensible for virus assembly, RNA packaging, and release of virus from the cell. Normally, they are incorporated as Gag-Pol fusion proteins, and their activities are sequestered until assembly and release of the virus. While the mechanisms of expression and assembly are well characterized for the Pol proteins, the steps that lead to activation of the Pol proteins are not well understood. Most retroviruses undergo a "maturation" step which occurs during, or just after release of virus from the cell, a process dependent on PR. This is characterized by morphological changes in the capsid structure of the virion. The immature Gag and Gag-

Pol proteins are cleaved and condense around the nucleic acid as the viral protease becomes activated. This is clearly visible by electron microscopy as the capsid becomes smaller and more electron dense. It is possible that dimerization of the viral protease causes activation of the cleavage cascade, and that this can only occur when the concentration and proximity of Gag-Pol precursors is sufficient. In the case of ALV, where PR is present at the C-terminus of Gag, maturation still occurs after virus release. The question of why the virus must be released from the cell in order for this to occur remains intriguing.

Retroviral Env proteins confer specificity of the virus for a receptor on the host cell. Interactions between Env and cellular receptors determine the first layer of viral tropism. Like the Pol proteins, Env proteins are also dispensible for assembly and release of virus particles. In a few cases, expression of Env enhances particle release from the cell, but is not required. HIV-2 for example is more efficiently released in the presence of its cognate envelope proteins. Envelope proteins are translated on the RER, and transported through the secretory pathway where they are proteolytically cleaved into two domains, a transmembrane domain (TM) and an extracellular, or surface domain (SU). This cleavage is carried out by a cellular protease thought to be located in the Golgi. TM and SU remain covalently linked through disulfide bond formation, and it has been demonstrated that for some retroviruses such as HIV, these form trimeric structures through coiled-coil domains much like the HA proteins of influenza virus. In addition to transport and cleavage, which are mediated exclusively by cellular components, the Env proteins are extensively glycosylated. The pattern of carbohydrate modification is different and complex for each retroviral Env protein, and these modifications play an important role in determining receptor specificity. Envelope proteins are assembled into virions during budding as Gag either accumulates at the membrane where Env is present, or as preformed capsids bud through Env containing membranes.

For more information regarding retrovirus replication, please refer to the extensive reviews in the text books Fields Virology (Coffin, 1996), and Retroviruses (Coffin, 1997).

B. The Late Stages of Hepadnavirus Replication

1. Hepadnavirus Gene Expression. Unlike retroviruses, hepadnaviruses do not specifically integrate their genomes into that of the host. The replicative form of the genome is covalently closed circular DNA (cccDNA) which is stably maintained as a plasmid in the nucleus of infected hepatocytes. The hepadnaviral genes (C, P, S, X) are similar in function to those of retroviruses (Gag, Pol, Env, accessory gene), but the genome is much more efficiently organized (Figure 1.3). Every nucleotide in the hepadnavirus genome codes for protein, and the surface glycoproteins are expressed from a reading frame which lies entirely within the coding sequence for the P protein.

In contrast to retroviruses where transcription is always initiated at the beginning of R, transcription of the viral genes and genome can be initiated from at least four different sites in the cccDNA genome. *Cis*-acting promoter elements have been characterized for each transcript, and they are called the genomic, pre-*S*, *S*, and *X* promoters. The longest transcript which encodes the pregenomic viral RNA (pgRNA), also serves as the mRNA for both the C and P proteins. Core is expressed by canonical cap-dependent ribosomal scanning from the 5' end of the genome. The *P* gene however, is located considerably downstream of *C*, and similar to retroviruses, they are in overlapping reading frames. P protein expression relies on internal initiation at a ribosome entry site within the coding sequence of *core*. The surface glycoproteins, of which there are several (S, L, M), are expressed from a variety of transcripts with both alternative transcription and translation initiation sites. These messenger RNAs are translated on the rough ER, and the gene products are directly transported into the secretory pathway where they are post-translationally modified by glycosylation.

2. Hepadnavirus Assembly. The process of assembly for hepadnaviruses differs greatly from that of retroviruses. While both encode a reverse transcriptase, the requirement for the enzymes during assembly is very different. Hepadnavirus assembly is initiated by binding of the terminal domain (TP) of the P protein to *cis*-elements, termed epsilon (ε)

within the viral genome. This binding event serves several purposes; it provides the context for the P protein to initiate reverse transcription of the genome, it covalently couples the polymerase to the nascent DNA genome, and it facilitates packaging of both Pol and the genome into particles. Instead of recruiting a cellular RNA primer, such as a tRNA in the case of retroviruses, reverse transcription is initiated from the hydoxyl moity on a tyrosine residue within P. The P protein is thereby covalently attached to the nascent DNA genome.

The complex of RNA, the P protein, and nascent DNA is then competent to recruit dimers of the core protein. As reverse transcription proceeds, it is thought that core dimers are added until a capsid is formed around the DNA genome. This process has been difficult to study because P::C interactions have not yet been mapped. Recently it has been shown that in a cell-free system, C will assemble capsids in the absence of either Pol or RNA, but that this may be dependent on cellular chaperones (Lingappa et al., 1994). Capsids presumably bud into the ER where they acquire the surface glycoproteins (L, S, M), and enter the consitutive secretory pathway, in a manner analagous to B and D type retroviruses.

The requirement for hepadnavirus surface glycoproteins is quite different from those of retroviral Env proteins during assembly. Hepadnavirus cores cannot bud into membranes in the absence of the surface glycoproteins. Cores form in the cytoplasm, but in the absence of glycoproteins, they have an unusual fate. Cores can enter the nucleus, and bypass the extracellular pathway altogether, leading to an accumulation of cccDNA in the nucleus. This accumulation of genomes in the nuclei of infected cells is correlated with non-specific integration events, hepatocellular carcinoma, and viral persistence.

For further review, please see Fields Virology (Ganem, 1996).

C. Human Foamy Virus Replication.

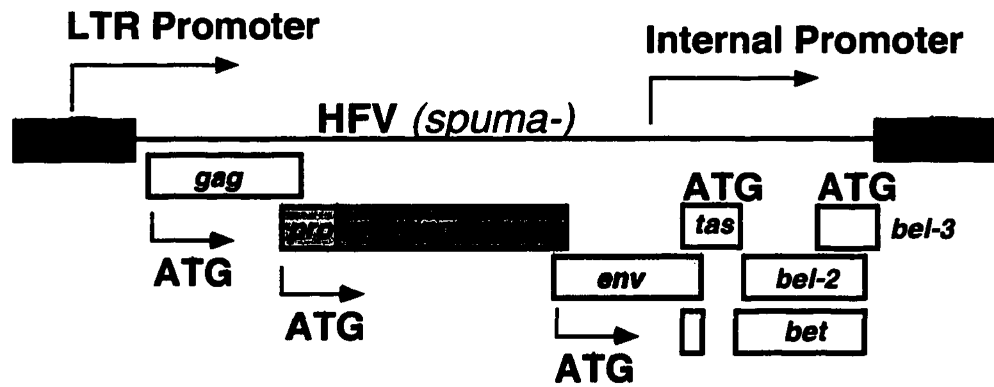
1. Foamy Virus Genes and Their Expression. As was previously mentioned, the foamy viruses are complex retroviruses (Flugel, 1991). They encode the canonical *gag*,

pol and *env* genes, and in addition, they encode two important accessory genes, *tas* and *bet* (Figure 1.5A) (Flugel et al., 1987). While Bet is one of the predominant immunogenic proteins, it is non-essential in tissue culture, and its function remains unknown (Yu and Linial, 1993). Tas, however, is an essential protein, expressed primarily from an internal promoter in the *env* region of the genome (Figure 1.5A). The *tas* gene encodes a transactivator protein required for expression of all the structural and enzymatic genes. Tas binds directly to the HFV LTR and the internal promoter (discussed below) where it presumably recruits RNA polymerase II, and other transcription factors to activate transcription (Erlwein and Rethwilm, 1993; Keller et al., 1991).

The HFV Gag protein has several unique features among members of the retrovirus family. It lacks the canonical retroviral Cys-His motifs, and instead contains glycine-arginine rich regions (GR boxes) which makes it more like the hepadnavirus C protein. In addition, HFV Gag lacks the major homology region (MHR) found in other retroviruses. In mature virions, the Gag protein is not processed into smaller subunits to form the core of the virion, but instead is cleaved at one place near the C-terminus approximately 50% of the time. This cleavage is performed by the viral protease (Konvalinka et al., 1995), and as a consequence, the capsid seen in mature HFV particles is less electron dense than other retroviruses (Figure 1.5B). As with other retroviruses, the Gag proteins are expressed from the genomic RNA in the cytosol. Much of HFV assembly occurs in the cytoplasm, with capsids both forming and budding through intracellular membranes before being translocated to the cell surface. Some budding does occur at the plasma membrane (Goepfert et al., 1997), but the majority of virus in infected cells remains cell associated. This may in part be due to the intracellular localization of Env for HFV, which is currently under investigation in several labs (Goepfert et al., 1999; Goepfert et al., 1995).

Env is expressed from a number of spliced mRNAs derived from the genomic RNA. The 5' splice site (5'-ss) is common to all LTR derived subgenomic RNAs, including *env*, and it lies 52 nucleotides from the 5' end of the genomic RNA.

A.



B.

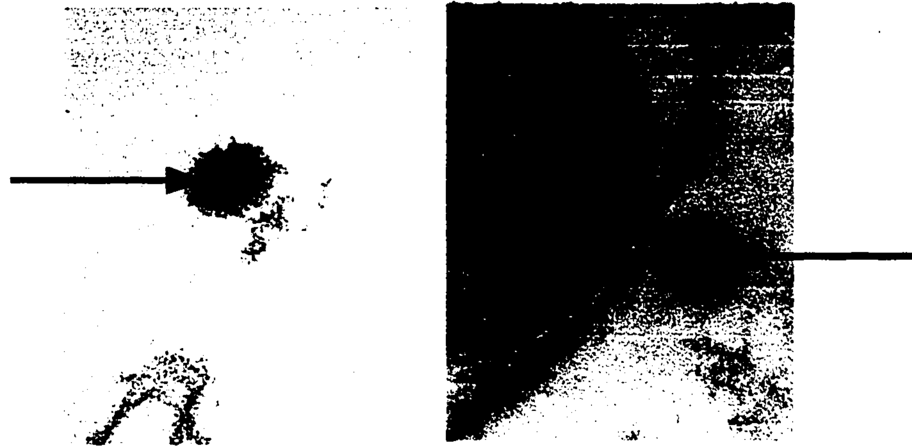


Figure 1.5 (A) The HFV proviral genome. Shown are the coding regions, the promoter elements in both the LTR and the *env* gene, and the initiation codons for all open reading frames, including *pol*. The *bet* gene is a spliced product of *tas* and *bel-2*. (B) Characteristic morphology of HFV particles, extracellular (left) and intracellular (right). Note that mature virions have a translucent core, unlike other retroviruses (Arrow). Images were produced by electron microscopy at 20,000X. Enveloped virions are approximately 120nm in diameter.

It is presumed that the spliced mRNAs are translated on polysomes in the RER like other retroviral *env* mRNAs. HFV Env surface glycoproteins are glycosylated, and then retained in the ER through a specific retention signal (Goepfert et al., 1997). As will be discussed later, there is a requirement for the Env protein in HFV assembly since particles are not released in its absence (Baldwin and Linial, 1998). The ER localization of Env presumably recruits assembled particles into these intracellular, membraned compartments, where they are slowly translocated and released.

2. The Mechanism of Foamy Virus Pol Expression. For a number of years after the molecular cloning of the first foamy virus genome (HFV-13, HSRV13), the mechanism of Pol expression was not known. All the evidence pointed to the fact that Pol is expressed independently of Gag, which is contrary to the retroviral paradigm which relies on the synthesis of Gag-Pol fusion proteins. Antibodies to Gag recognized only the 78-kDal and 74-kDal (the cleaved form of p78) structural proteins, and no higher molecular weight species which might be the Gag-Pol protein. Antibodies to the RNAse H (RH) domain of Pol recognized only 127-kDal and 80-kDal species, implying that the 127-kDal Pol precursor is also cleaved only once, and providing further evidence that no Gag-Pol fusion existed. Finally, when these analyses were performed on a protease active site mutant of HSRV13 (HFV-D/A), only the 78-kDal Gag, and 127-kDal Pol proteins were detected (Konvalinka et al., 1995), again reconfirming these observations.

The experiments described in this dissertation begin with the question of how HFV Pol is expressed. The work on the mechanism of Pol expression was carried out by several different people in the lab at the time, most notably Shuyarn Yu and Samuel Gwynn. Their experiments are the first described in this thesis because they are critical to the understanding of my first efforts, and to the subsequent directions of our work. My work continued by addressing the role that HFV Pol plays in assembly and attempting to define the mechanism by which the Pol proteins are incorporated into virions, a step crucial to the infectivity of HFV virions. I have found that like retroviruses, Pol is not required for assembly or genome encapsidation. I studied the possibilities of

protein::protein interactions and protein::RNA interactions for Pol incorporation. I found that the PR domain of Pol interacts with a basic region near but not at the Carboxy terminus of Gag, and that both of these domains are required for Pol incorporation.

Chapter 2
Materials and Methods

A. RT-PCR cloning of the spliced *pol* mRNA (performed by Samuel R. Gwynn). Total cytoplasmic RNA isolated from infected or uninfected human embryonic lung fibroblasts (HEL) was subjected to RT-PCR after treatment with RQ1 deoxyribonuclease I (Promega). The first strand complementary DNA was synthesized by Superscript II reverse transcriptase (Gibco/BRL) with the antisense primer PR2 (5'-CATGGGTACC-GTTGCCCCTGA-ATCCCAG-3') derived from the 5' region of *pol* (nucleotides 2405 to 2422; Genbank accessioin number M19427). Reaction products were subjected to PCR amplification with primers R+3 (5'-GCATCCCG-GGGCTCTT-CACTACTCG-CTGCGT-3')(nucleotides 3 to 23) and PR2, and products were analyzed by agarose gel electrophoresis. A major ~600-bp fragment was isolated from the gel using QIAEX II (Qiagen) and cloned into PCR II (Invitrogen) using the TA cloning protocol to generate pSGC5. The DNA sequence of this clone was then determined to contain a *bona fide* splice junction for the *pol* message.

B. Construction and analysis of *pol* message 3' splice site mutants (performed by Shuyarn F. Yu). The 3'-ss mutant was constructed by a two-step PCR method in which TTA (nucleotides 1844 to 1846) were mutated to CTC without changing the Leu coding sequence. In the first PCR reaction, either combinations of primers MB(5'-TACGCCCGGG-AACTGGAG-AGAGCCTCC-3')(nucleotides 1384 to 1398) and SSC_{NC}-1 (5'-CTCTGG-CATTAAGG-CCGAGAATTT-CATATACAG-CATTTAG-3') (nucleotides 1823-1862) or primers SS_{NC}-2 (5'-CTAAATGCT-GTATATGAA-ATTCTCGG-CCTTAAT-GCCAGAG-3') and MY (5'-TACGCCCG-GGGTCCCTTT-GATCTCC-3') (nucleotides 2375 to 2389) were used to amplify DNA fragments of 489 or 576 pb from pHSRV13. Both fragments were used as templates in the next round of PCR with primers MB and MY, and the resulting fragments were used to replace the

wild-type (wt) fragment in the pHSRV13 (Rethwilm et al., 1987) infectous clone. All mutations were confirmed by DNA sequence analysis.

Analysis of Pol expression from the 3'ss mutants was performed by transiently transfecting baby hamster kidney fibroblasts (BHK21) with wild-type, 3'-ss, and negative control DNAs using LipofectAmine reagent (Gibco/BRL). The cells were labeled with [^{35}S]methionine, and cells extracts were immunoprecipitated with rabbit antibody to the ribonuclease H domain of Pol (Netzer et al., 1990; Netzer et al., 1993). For details of cell culture, transfection, and radioimmunoprecipitation analysis, see the following sections.

C. Biochemical detection of the spliced *pol* mRNA by primer extension analysis. Total RNA from infected and uninfected HEL cells was isolated using Tri Reagent (Molecular Research Center, Inc.), two days after infection. For each reaction, 20μg of cell RNA or 0.2μg of control RNA was incubated with 3x10^6 cpm of [^{32}P]oligonucleotide (10ng). Annealing reactions of primers and RNA were heated to 65°C for 15 minutes, and allowed to cool slowly to room temperature. First strand synthesis salts (Gibco/BRL), 100μM deoxyribonuclotides (Promega), and Superscript II reverse transcriptase (Gibco/BRL) were added. Primer extension was carried out at 42°C for 30 minutes. Prior to electrophoresis, reactions were precipitated with ethanol, and washed to remove salts. Primers used were as follows: Primer 7, (5'-TCTCCT-CTGGCAT-TAAGGC//CA-3') (nucleotides 1866 to 1847, and 51 to 50); primer 5, (5'-ATAAA-ATATTTC-TTACCAAAC-3') (nucleotides 67 to 46); primer 4, (5'-CCCTCT-GGCATTA-AGGC//CAAAC-3') (nucleotides 1863 to 1847, and 51 to 46). Primers 7 and 4 span the splice junction site for the *pol* mRNA, whereas primer 5 is complementary to the unspliced genomic RNA at the 5'ss. Control RNAs were transcribed in vitro from *Bst XI*-digested DNA of pSGC6 (C6)(spliced control), or *Xho I*-digested pSGC10 (C10)(unspliced control), with SP6 or T3 RNA polymerase respectively.

D. Recombinant plasmid DNAs. The plasmid pSGC5 was generated by TA-cloning the RT-PCR product described above into PCR II (Invitrogen). Plasmid pSGC6 was

generated by deleting a *Hind III-Sma I* fragment from pSGC5 (described above). Plasmid pSGC10 was constructed by inserting the 361 nucleotide *Xba I-Nar I* fragment from pHSRV13 (-12 to +349) into pBluescript SKII vector (Stratagene) digested with *Xba I* and *Cla I*.

HFV deletion mutants ΔPol569 and ΔEnv190 were generated by linearizing the pHSRV13 plasmid (Rethwilm et al., 1987) at unique *Pac I* and *BspE II* restriction sites respectively, digestion with *Bal 31* exonuclease, and religation after treatment with Klenow DNA polymerase. ΔPol569 and ΔEnv190 contain 569 bp and 190 bp deletions respectively which both result in translational frameshifting. The *env* mutant ΔMN (deleted from Mro I (6957) to Nde I (8970)), was provided by Dr. Martin Lochelt.

The vector pSGC11 (Baldwin and Linial, 1998) was used to make probes for RNAse protection analysis of HFV nucleic acids. pSGC11 contains a 427 bp fragment of the HFV LTR which was amplified by PCR from the HSRV13 proviral plasmid using primers containing *BamH I* and *EcoR I* restriction sites. These were used for cloning into pBluescript SKII+ (Stratagene). The oligonucleotide primers were U3BAMP11 (5'-ACTT-GGATC-CGATAAT-GTTTTAAG-GAATACT-3') and U5ECOP11 (5'-AGCTGAATT-CTGTAT-ATTGAT-TATCC-TAAGG-3').

A shuttle vector was generated to facilitate cloning and manipulation of five unique HSRV13 (Rethwilm et al., 1987) derived restriction fragments (Figure 2.1). An annealed set of kinased linker oligonucleotides were added to the NEB193 (New England Biolabs) polylinker at the *Pac I* site to generate the plasmid HFVLink2 (L2). The insertion destroyed the existing *Pac I* site. These oligos were Linktop (5'-CGGC-CGAT-TTAAA-TTAATT-AATCCG-GAGCT-GAGCTTAAGCCT-AGGGATATC-ATGCA-TAT-3')) and Linkbot (5'-ATGCA-TGATATCC-CTAGGCTTA-AGCTCA-GCTCCG-GATTAATTA-ATTTAAAT-CGGCCGAT-3'). This insert contains all of the unique sites from the viral sequence of HSRV13. The insert was screened for orientation such that the enzyme sites were in the following order with respect to the NEB193 polylinker:*Bam HI* (NEB193), *Eag I, Swa I, Pac I, BspE I, Blp I, Afl II, Avr II, EcoR V, Nsi I, Xba I* (NEB 193), *Sal I* (NEB 193).

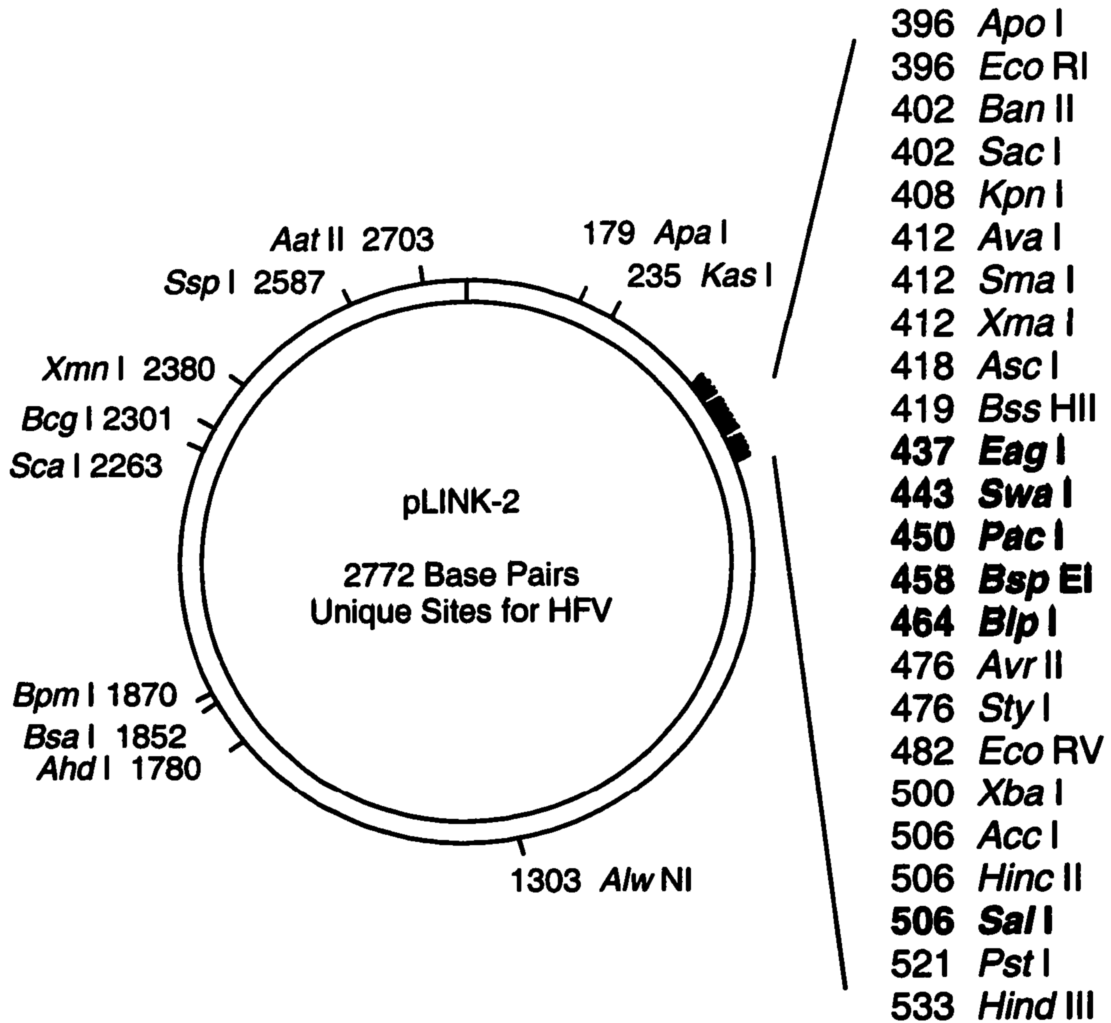


Figure 2.1 Illustration of the NEB193 derived subcloning vector pLINK2-1. Shown in bold are sites which are unique from the plasmid pHSRV-13. Not all unique sites in pLINK2-1 are shown. pLINK-2 was used to generate all Sub vectors for mutagenesis of HSRV-13 derived sequences.

Five unique fragments of the HFV genome from HSRV13 were cloned into the L2 vector. Each subclone was then named according to the position of the unique fragment in the genome. Sub1 contains *Eag I-Swa I*, Sub2 contains *Swa I-Pac I*, Sub3 contains *Pac I-BspE I*, Sub4 contains *BspE I-Blp I*, and Sub5 contains *Blp I-Sal I*. These subclones were subsequently used for PCR mutagenesis (Figure 2.2).

The CMV promoter constructs were generated such that expression of the viral RNA is initiated from the same nucleotide as for the wild type RNA transcribed from U3. To this end, the HFV LTR was eliminated from the Sub 1 and Sub 1 ΔATG constructs by digesting with *Eag I* and then partially with *Xba I*. A linker containing *Eag I* and *Xba I* overhanging ends and a unique *Xho I* site was cloned into this vector. The oligonucleotides used to create the linker were CMVLink1 (5'GGCCGTTG-CTATCCTCGAGT-3') and CMVLink2 (5'CTAGACTCGA-GGATAGCAAC-3'). CMVLink1 and 2 were kinased, annealed and ligated into the *Eag/Xba* digested Sub 1 constructs.

The CMV immediate early promoter was PCR amplified from the vector pCR 3.O (Invitrogen) using oligonucleotide primers containing an *Eag I* site in the 5' primer, and *Xho I* and *Xba I* sites in the 3' primer. The primers used were CMVPCR1 (5'-GATCGA-TCGGCCGGCGC-GCGTTGAC-ATTGATTATTG-3') and CMVPCR2 (5'-ATCT-AGACTCGA-GGCTTATA-TAGACCTCCCA-CCGTACACG-3'). The PCR products were digested with *Eag I* and *Xho I*, and ligated into the *Eag/Xho* digested CMVLink constructs. These constructs were named Sub1CMV, and Sub1CMVΔATG (see Baldwin 99). Constructs were generated by transfering the *Eag I-Swa I* fragments from these subclones into *Eag/Swa* digested HFV full length cDNA.

The two-hybrid plasmid vectors were developed by Fields (Fields and Song, 1989) and Hollenberg (Vojtek et al., 1993), and are named pBTM116 and pVP16 respectively. pBTM116 contains the LexA DNA binding domain, whereas pVP16 contains the activation domain from the herpesvirus VP16 protein. Hybrid fusions were generated to the C-terminal domains of each of these with wild type and mutant HFV Gag proteins or HFV PR.

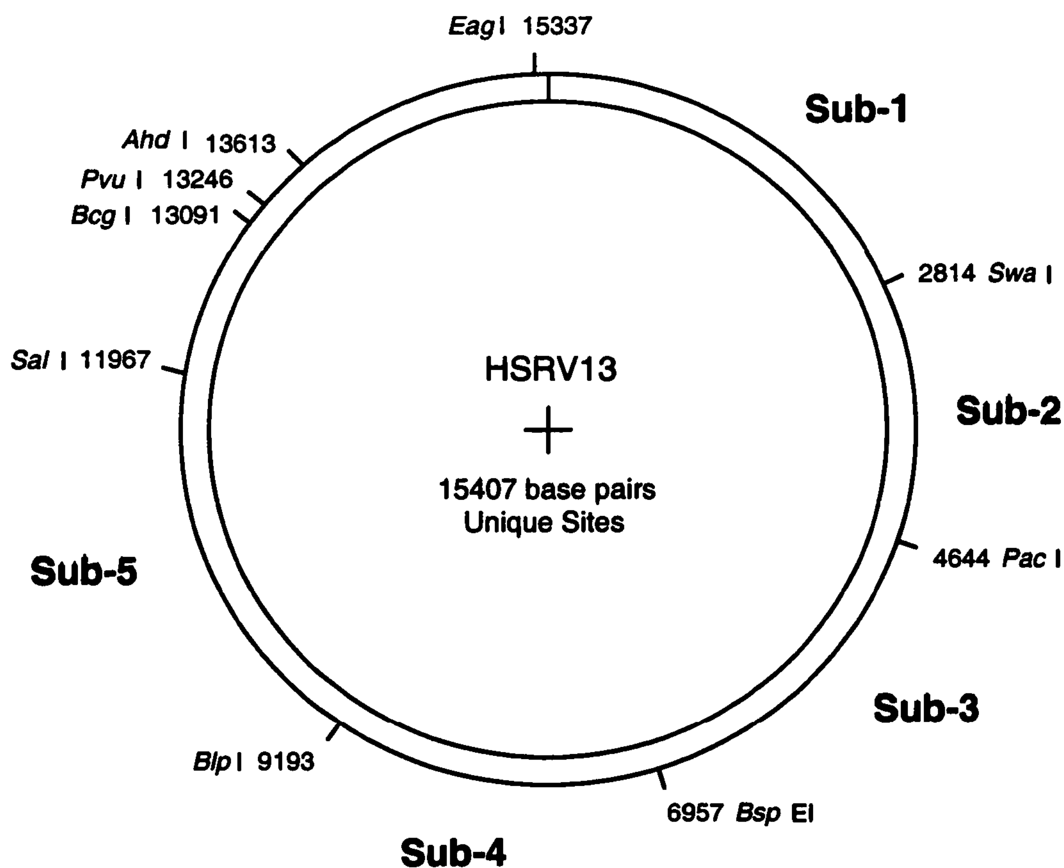


Figure 2.2 Illustration of the proviral molecular clone HSRV-13. Indicated are some of the unique restriction sites in HSRV-13, and the five independent fragments of the provirus (Sub1-5) which were subcloned into pLink-2 (NEB193 backbone containing a modified polylinker). These subclones served as templates for all site-directed mutagenesis, and are described in detail throughout Chapter 2 (Materials and Methods).

For the LexA fusions, pBTM116 was digested with *BamH I* and *Sal* or *Pst I*, sites which are present in the polylinker such that in-frame fusions were created. Gag and PR were then amplified with primers containing these restriction sites such that they could be digested and ligated into the vector. Primers for amplifying PR were 5PRBAM (5'-CTAGGGA-TCCTGA-TGAATCC-TCTTCA-GCTGTTA-3') and 3PRPST (5'-CTAGC-TGCAGCT-ATTATAACT-GAAGTGGT-TGCTGTGT-3'). Primers for amplifying Gag were 5MABAM (5'-CTAGGG-ATCCTGAT-GGCTTC-AGGAA-GTAATGTT-3') and 3NCSAL (5'-CTAGG-TCGACCT-ATTAGT-CCCTTTGAT-CTCCGCC-3'). The VP16 fusions were created by amplifying with the same 5' primers (5PRBAM and 5MABAM), but with 3' primers which contain *Not I* sites present in the VP16 polylinker. The 3' primer for PR was 3PRNOT (5'-CTAGGC-GGCCGCCT-ATTATAAC-TGAAGTGGT-TGCTGTGT-3') and for Gag was 3NCNOT (5'-CTAGC-GGCCG-CCTATTATTC-ATTTTT-TTCCA-AATG-3'). All Gag mutants described elsewhere were amplified with the same primers.

E. PCR mutagenesis. The following general strategy for mutagenesis was used throughout. Two oligos corresponding to sequences outside the NEB 193 polylinker were used in all mutagenesis reactions. The external primers were 193 (+), corresponding to positions 21-40 in NEB 193 (5'-GGTGA-AACCTCT-GACACAT-3'), and 193 (-) corresponding to positions 577-558 (5'-CCCAG-GCTTTAC-ACTTTATG-3'). Internal primers were designed to contain mutations in viral sequences and a unique *Nhe I* site for ligation of PCR products. Ligated PCR products were digested with enzymes unique to the L2 polylinker, and subcloned into L2.

A protein kinase A (PKA) phosphorylation site was introduced in the C-terminus of the integrase (IN) domain of Pol. The consensus PKA sequence is Arg-Arg-X-Ser-X. (RRxSx), where x is preferably a small hydrophobic amino acid (Bartenschlager et al., 1992; Bartenschlager and Schaller, 1992; Whitehorn et al., 1995). Nucleotide positions 6262-6265 were changed from ATT to CGT, converting isoleucine to arginine. Positions 6269-6275 were changed from ACTTCT to GCTAGC converting a threonine to alanine

such that the amino acid sequence IRTSL was changed to RRASL. The oligos for PCR were Nhe1TOP (5'-TTACA-GGAAC-GTCGTGCT-AGCTT-ATACC-ATCCA-TCCACCC-CTCCA-GCC-3') with 193 (-), and Nhe1BOT (5'-ATGG-TATAA-GCTAGCA-CGACGTT-CCTGTAA-AAGAGAA-AGTTCT-TCTTC-3') with 193 (+). 5ng of Sub3 was used as a template for PCR. PCR products were digested with *Nhe I*, and ligated to each other. The ligation product was digested with *Bam HI* and *Sal I* and subsequently cloned into *Bam HI/Sal I* digested L2 vector. The *Pac I/BspE I* fragment containing the PKA (Sub3-PKA) site was then cloned back into HSRV13 and named HFV-PKA. The same *Pac I/BspE I* fragment was then cloned into different mutant backgrounds.

The protease active site mutant HFV-D/A contains an aspartic acid to alanine mutation in the active site of the viral protease (Konvalinka et al., 1995). Sub3-PKA was cloned into the D/A background as described above, and named HFV-D/A-PKA. The negative control for assembly, ΔATG, was generated by digesting the Sub1 vector with *Mfe I* and *Nco I*, filling in with Klenow, and religating. This was then transfered to the PKA and the D/A-PKA backgrounds and the resultant constructs were called ΔATG-PKA and ΔATG-D/A-PKA respectively. Three Gag mutants were constructed in a similar fashion using the Sub2 vector. These are called 78T/A, 74Stop, and 68Stop. For each mutant two separate PCR reactions were performed using mutagenic oligos, and the vector based oligos 193 (+/-). Both PCR products contained an *Nhe I* site in the region downstream of the desired mutation in Gag. PCR products were digested with *Nhe I*, ligated together, and redigested with enzymes unique to the polylinker flanking the HSRV unique sites. The Sub2 mutants were then cloned back into the viral background with the enzymes *Swa I* and *Pac I.* The oligos used for these reactions were as follows. 78T/A-1 (5'-AGCCTTGCTAGCCAGAGTGCCACGTCCTCCACAGATC-3'), 78T/A-2 (5'-CAGTTCGCTAGCTGCGGCGACAGCGCGTGAGTCACCAGC-3'), 74STOP-2 (5'-GTACGCTAGCTTACTAATTGACAGCGCGTGAGTCACCAGC-3'), 68STOP-1 (5'-AGCCTTGCTAGCCGCGGAGGAAGAGGTAACCACAACCG-3') 68STOP-2 (5'-GTACGCTAGCTTACTAAGCTGGTCTGGGAGTTTGTGACTG-3'). Primer pairs

were as follows for the initial reactions. 78T/A; (193(+) and 78T/A-2), (193(-) and 78T/A-1). 74STOP; (193(+) and 78T/A-2), (193(-) and 74STOP-1). 68STOP; (193(+) and 68STOP-2), (193(-) and 68STOP-1). For the STOP mutants, the coding sequence was unaltered prior to the TAA insertion. The cleavage site mutant 78T/A contains four mutations at and near the cleavage site, changing the amino acid sequence from NTVT to AAAS. The amino acids AS correspond to the coding sequence of the *Nhe I* site (GCTAGC) used to ligate the PCR products. These mutants were generated twice; once in the wild type HFV-PKA background and once in the D/A-PKA background.

The primer binding site was deleted from Sub1 and subsequently recloned into various viral backgrounds. The PBS sequence (5'-TGGC-GCCC-AACG-TGGGG-3')(position 1124-1142 of the proviral DNA) was replaced with an *Nhe I* site (AGCGCT) using the PCR strategy described above. The oligos used for the PBS deletion were PBS-NHE-1 (5'-AGTGA-TGCTA-GCCTC-GAATATA-AGTCGG-GTTTA-TTTG-3') and PBS-NHE-2 (5'-TCAGA-TGCTA-GCATT-GTCATGG-AATTTTG-TATATTG-3'). Primer sets were (193(+) and PBS-NHE-2), (193(-) and PBS-NHE-1).

The ΔPR-PKA (in frame protease deletion) mutant was constructed in analagous fashion in Sub2. The oligos used were NHE-PRO-1 (5'-CATAG-CTAGCT-TTAGTCC-CTTTGAT-CTCCG-CCGG-3') and NHE-PRO-2 (5'-GATAG-CTAGCA-CAATTTT-AGTTCCTC-TTCAAG-3'). Primer pairs for PCR were (193(+) and NHE-PRO-1), (193(-) and NHE-PRO-2).

A C-terminal Gag truncation mutant, p60-D/A-PKA was also generated by the same method, using Sub1+2 (*Eag I-Pac I* subclone of HFV). The oligonucleotides used were 60Stop1 (5'-TAGCTA-GGCTA-GCTTA-TTATCC-TCCTT-GATT-TAAAT-TATC-3') and 60Stop2 (5'-CCAGA-TGCTAG-CCCCCGT-ACTTACCA-ACCTCAA-AGG-3'). Primer pairs were (193(+) and 60Stop1), and (193(-) and 60Stop2). The *PflM I* fragment containing the mutation was exised and placed into the D/A-PKA background to minimize the amount of PCR product for sequencing. This construct was also generated in both HFV LTR and CMV promoter containing proviruses.

F. Cells and culture. FAB indicator cells expressing β-galactosidase from the HFV long terminal repeat (LTR) have been described elsewhere (Yu and Linial, 1993), and were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 5% fetal bovine serum (FBS). Human embryonic lung fibroblasts (HEL), COS and 293T cells were maintained in DME+10% FBS.

G. Transient transfection. FAB cells were transfected with proviral plasmid constructs derived from integrated viral sequences using lipfectamine reagent (Gibco-BRL). Liposome complexes were generated by diluting 5μg of plasmid DNA in 750 μl of DME lacking penicillin and streptomycin (P/S-), and adding 750 μl DME P/S- containing 25 μl of lipofectamine reagent. This mixture was allowed to stand for 30 minutes while FAB cells were rinsed with DME P/S-. The mixture was added to a 10 cm plate with cells at 75% confluency (approx. $1x10^6$ cells per plate) and rocked gently before adding 6 mls of additional DME P/S-. Transfections were incubated for 2-3hrs at 37°C with 6% CO_2, washed twice with isotonic buffer, and 8-10 mls DME with P/S and 5% FBS were added to each plate.

H. Expression and purification of recombinant proteins. The central domain of HFV Gag and the RNAse H domain of Pol were cloned into pGM484 for overexpression in *E. coli.* Nucleotide sequences 1494-2658 and 4635-5978 of HSRV13 were amplified by PCR using primers containing 5'-*BamH I* or 3'-*EcoR I* restriction sites and three in-frame CACCAT (6xHis tag) repeats at the 5' end of the target sequence. Protein expression was induced in *E. coli* strain JDBE3 (BL21) by addition of 1mM IPTG (isopropyl-ß-D-thiogalactopyranoside) during log phase growth for 4 hrs. Both recombinant proteins were recovered from the insoluble fraction of the cells after lysis by sonication. Denatured proteins were partially purified using Ni+ column chromotography as recommended by the column manufacturer (QIAGEN), and further purified by denaturing polyacrylamide gel electrophoresis.

I. Antibodies. New Zealand White rabbits were immunized with recombinant proteins corresponding to the central region of the HFV Gag protein and the RNAse H domain of the Pol protein for the generation of polycolonal monospecific antisera (see Expression and purification of recombinant proteins). Pure proteins were isolated by polyacrylamide gel electrophoresis, and homogenized in Freunds incomplete adjuvent for injection. Antiserum against this domain of Gag recognizes the 78kD Gag precursor, and the 74kD cleavage product. Antiserum against the RNAse H domain of Pol recognizes the 127kD Pol precursor, and the 80kD product from which integrase has been cleaved by the viral protease.

J. Radioimmunoprecipitation assay (RIPA). Transiently transfected FAB cells were washed with DME 24 hrs post transfection, and labeled with [^{35}-S] methionine (NEN Research Products) at 50μCi/ml in met-/cys- DME and 5% dialysed fetal bovine serum for 12 hrs. Supernatants were passed through 0.45μm Nalgene syringe filters, and virus was pelleted through 20% sucrose cushions. Virus pellets were resuspended directly in antibody buffer (20 mM Tris pH 7.5, 50mM NaCl, 0.5% NP40, 0.5% SDS, 0.5% DOC, 0.5% Aprotinin, and *ex tempora* 10mM iodacetamide) for RIPA. 10 cm plates of cells were disrupted in 1ml of Ab buffer, and chromosomal DNA was sheared by passing the extract through a 23 gauge needle. Cell debris were pelleted by centrifugation, and incorporation was measured by TCA precipitation. Lysates were incubated in the presence of protein A sepharose (Pharmacia) and 2μl of rabbit antiserum (either anti-Gag or anti-Pol) for 2hrs at room temp. Protein A beads were washed twice with high stringency RIPA buffer (10mM Tris pH 7.4, 150mM NaCl, 1% NP-40, 1% DOC, 0.1% SDS, 0.5% aprotinin), once with HIGH SALT buffer (10mM Tris pH 7.4, 2M Nacl, 1% NP-40, and 1% DOC), and then once with TE. Beads were boiled for five minutes in 2X denaturing SDS-PAGE Laemmli sample buffer, and samples were electrophoresed through 10% acrylamide gels. Quantitation was performed using a PhoshorImager and ImageQuant software.

K. Ribonuclease protection assay (RPA). Total nucleic acids from concentrated virions or whole cells were detected using a Direct Protect kit (Ambion). Purified nucleic acids were treated with RNAse-free DNAse or DNAse-free RNAse, and then diluted in lysis buffer for further analysis. Radiolabeled RNA probe was generated using pSGC11 (see recombinant plasmid DNA) for *in vitro* transcription with T7 RNA polymerase and [^{32}-P]UTP (Dupont; 3000 Ci/mmol) after digestion with *Eag I*. Quantitation was performed using a PhoshorImager and ImageQuant software.

L. Intracellular capsid analysis. Transiently tranfected FAB cells were washed 36 hrs post-tranfection with isotonic buffer, and harvested for lysis and analysis of intracellular particles. Transfected cells were divided into two pools, and either lysed gently with Triton-X lysis buffer (0.25M sucrose, 0.5% Triton-X, 10mM Tris pH 7.5, 1mM EDTA, 140mM NaCl) or completely in RIPA Ab buffer to solublize all remaining intracellular structures. Lysates were cleared of cellular debris at 2000 rpm for 30 minutes, and then placed on top of a 20% sucrose cushion and centrifuged at 24K rpm in an SW28 rotor (Beckman) for 3hrs. Pellets were analysed for the presence of intracellular particles by western blotting with Gag antiserum. The Ab buffer generated pellets were treated with DNAse prior to SDS-PAGE.

M. Western blot analysis. Cell lysates and virus were typically prepared for either capsid analysis by RIPA, or IP-PKA analysis of Pol. In each of these cases, a fraction of each preparation was mixed with 5X or 2X SDS-PAGE sample buffer for western blot analysis. Samples ranging from 10-25μl, corresponding to 1-2.5/100 of a 10 cm tissue culture plate of confluent, transfected cells, were separated on 10-12% SDS-PAGE gels. Prestained molecular weight markers, "Benchmark protein ladders", were obtained from Gibco/BRL. Gradient purification and analysis of virus is described below. Proteins were transfered onto PVDF membranes (Immobilon-P, Millipore) using an Ellard Instruments semi-dry blotting apparatus. PVDF membranes were wetted with methanol, then rinsed with ddH_2O prior to blotting. Blotting was performed in 15% methanol,

192mM Glycine, and 25mM Tris base. After blotting, membranes were blocked with phosphate buffered saline (PBS), 0.2%Tween-20, 5% nonfat dry milk for at least one hour. All subsequent steps were performed at 4°C. Anti-Gag antibody was added in the block solution at 1:2000, and incubated for at least two hours. Blots were washed 3X for 10 minutes, then probed with secondary antibody (anti-rabbit HRP conjugate-Amersham) for at least two hours at 1:8000 in block solution. Finally, blots were washed 3X for at least 10 minutes per wash, then rinsed 3X with PBS. Blots were developed using ECL reagent (Amersham) according to the manufacturer's instructions.

N. Electron microscopy. Thin section transmission electron microscopy was performed on FAB cells which were transiently transfected with ΔPol569, ΔEnv190, and wild type HFV (HSRV13). 36hrs post transfection, cells were fixed in Karnofsky's reagent for sectioning and further staining. Sections were analyzed using a JOEL 100SX transmission electron microscope at 80 kV.

O. Gradient purification of HFV particles. Transfected cell supernatants were clarified of cell debris by low speed centrifugation (2,000 rpm, IEC clinical HN-SII table-top centrifuge), and filtered through Nalgene 0.45μm syringe filter. Virus was pelleted through standard buffer (SB; 50mM Tris pH 7.5, 1mM EDTA, 140mM NaCl) containing 20% sucrose by ultracentrifugation in a SW28 rotor (Beckman instruments) at 24,000rpm for 2 hours. Virus was resuspended in SB, and placed on a 10-40% step gradient of iodixanol (Optiprep; Nycomed Pharma). The 5ml gradients were formed by sequencially underlaying 1ml aliquots of increasing concentrations of iodixanol at 10%, 20%, 30%, and 40%. Gradients were centrifuged from 4-12 hours at 36,000rpm in a Ti55 rotor (Beckman instruments). Gradients were made in 5ml Beckman Ultraclear tubes (13x51mm). 0.7ml fractions were collected from the top of the gradient and proteins were precipitated from each by adding trichloroacetic acid (TCA) to a final concentration of 10%. The precipitates were pelleted at 14K rpm in a microcentrifuge (Eppendorf), washed once with 10% TCA to remove the iodixanol, and finally with

acetone to remove the TCA. Pellets were resuspended in TE containing 1% SDS and boiled to solubilize the precipitate. 10% of each fraction was then analyzed by western blotting for viral Gag protein (Baldwin and Linial, 1998), and the remaining 90% was diluted with TE pH 8.0 1:10 (0.1%SDS final) for IP/PKA analysis of the Pol protein (see below).

P. Protein kinase assay for detection of Pol. The catalytic subunit of protein kinase A (PKA, Sigma) was used to phoshorylate viral and cellular Pol proteins containing the recognition sequence RRxSx (Bartenschlager et al., 1992; Bartenschlager and Schaller, 1992; Whitehorn et al., 1995). Pol proteins were immunoprecipitated (IP'd) from cell lysates or viral pellets using either anti-RH (Baldwin and Linial, 1998) or anti-IN serum (generously provided by Martin Löchelt, Heidelberg). Cellular Pol protein was IP'd from one transfected plate of FAB cells. Lysates for IP were prepared by resuspending cells or virus in antibody buffer (20 mM Tris pH 7.5, 50mM NaCl, 0.5% NP40, 0.5% SDS, 0.5% DOC, 0.5% Aprotinin), cellular nucleic acids were sheared with a 23 guage needle, and insoluble materials were pelleted and discarded. 2-4μl of antiserum was added to the lysate and vortexed. The immune complexes were precipitated with protein A sepharose for 3 hours at 10°C, washed twice with high stringency RIPA buffer (10mM Tris pH 7.4, 150mM NaCl, 1% NP-40, 1% DOC, 0.1% SDS, 0.5% aprotinin), once with HIGH SALT buffer (10mM Tris pH 7.4, 2M Nacl, 1% NP-40, and 1% DOC), and finally with 1X PKA buffer (20mM Tris pH 7.5, 100mM NaCl, 12 mm $MgCl_2$, 4mM Dithiothreitol). PKA was added in 1X PKA buffer (20 units/reaction) in the presence of 10-25μCi ^{32} or ^{33}P γ-ATP. Reactions were carried out at 37°C for 30-60min. The complex was then washed 2x with RIPA to remove most of the unincorporated label. A second IP was performed by boiling the complex in TE+1% SDS, removing the supernatant, and diluting it 1:10 in TE (pH 8.0). Fresh anti-Pol antiserum and protein A sepharose were added for 3 hours. This complex was again washed 3X with RIPA buffer, and 1X with TE. 1X SDS-PAGE sample buffer was added directly, and the samples were boiled for

5-10min. Proteins were separated by SDS-PAGE, dried onto Whatman 3MM, and exposed to film or a PhophorImage screen.

Q. Two-hybrid analysis. The yeast two-hybrid system (Fields and Song, 1989; Vojtek et al., 1993) was used to study the interaction of PR with Gag. HFV Gag was cloned into both the DNA binding domain vector (LexA, LexNA) and the VP16 activation domain vector as described in the recombinant DNA section, and used as a positive control for protein::protein interaction. The PR domain was expressed as a C-terminal fusion to LexA. These plasmids were prepared from XL-1Blue cells (Stratagene), and transformed into the L40 strain of *Saccharomyces cerevisiae* (MATa, his3D200, trp1-901, leu2-3, 112ade2, LYS2::9lexAop)$_4$-HIS3, URA3::(lexAop)$_8$-LacZ). Yeast cells transformed with both plasmids were selected by plating on Leu-Trp- (W-L-) plates. Two-hybrid interactions were assayed directly by omitting histidine in addition to leucine and tryptophan. Quantitative comparison of two-hybrid interactions was performed by omitting uracil (URA) which stimulates expression of ß-galactosidase. Enzymatic activity was either assayed visually by freeze-thawing colonies and adding XGAL substrate, or by measuring the activity present in lysates. For liquid culture ß-galactosidase assays, yeast cells were grown to mid-log (0.6-1.2 ODU) phase in (W-L-) media. Cell numbers were normalized by measuring the O.D.$_{600}$ prior to resuspension in Hepes buffer(0.1M Hepes, 154mM NaCl, 2mM hemi-Mg Aspartate, 0.05% Tween 20, 1% BSA, pH 7.2) at 5 ODU/ml in 800μl total volume. Lysis was initiated by adding 55 μl of Chloroform, and 55μl 0.1% SDS. Tubes were vortexed at high speed for 5min (VWR platform vortexer), and incubated at 30°C for 15-30min. Cell debris and chloroform was pelleted by centrifugation and lysate was collected from the top. Lysates were aliquoted into microtiter plates (180μl per well), and ß-galactosidase substrate (Chlorophenolred-ß-D-galactopyranoside (CPRG)) was added to a final concentration of 5mM. Plates were incubated at 37°C until visible color change (orange to dark red) could be monitored by eye, and then by spectrophotometry at 574nm or by a

spectrophotometric plate reader with a filter for 560nm or 590nm. Under these growth and lysis conditions, incubations were typically overnight.

Chapter 3
Detection and Characterization of the Spliced *pol* mRNA for Human Foamy Virus

A. Background.

Foamy viruses are members of the family *Retroviridae*, but they have been poorly studied in comparison with their relatives the onco- and lentiviruses. It has been assumed that foamy virus replication strategies would resemble those of their related genera. Indeed like other retroviruses, their genomes encode the canonical *gag*, *pol* and *env* genes, a primer binding site (PBS) at the 5' end of the genome, and the provirus is flanked by two LTRs (Rethwilm et al., 1987). In the case of conventional retroviruses (onco- and lenti-), mechanisms of replication are well understood. The Gag structural protein is expressed from genomic RNA, and contains all the information necessary for assembling capsids, genome packaging, and budding from the cell. Pol is expressed as a Gag-Pol fusion protein from the genomic RNA by one of two different mechanisms; stop codon suppression or -1 ribosomal frameshifting. Gag-Pol fusions are incorporated into capsids at the frequency of Gag-Pol expression which is approximately 5% that of Gag. After assembly, the PR domain of Pol becomes active and capsid condensation occurs whereby both Gag and Pol are cleaved into their mature forms (Coffin, 1996; Coffin, 1997). While these characteristics had been demonstrated for all retroviruses examined to date, it has recently become clear that HFV expresses the Pol protein independently of Gag, implying unique mechanisms for expression, assembly and activation of the Pol protein.

In a protease active site mutant of HFV (HFV-D/A), no high molecular weight proteins have been detected by a combination of anti-Gag and anti-Pol antibodies, implying that HFV Pol contains no Gag domains. The largest *pol* gene product is 127-kD which is presumably initiated at a start codon within *gag*. We have considered possible mechanisms for *pol* expression in HFV. The *pol* reading frame is +1 with respect to *gag*, making well studied mechanisms of frameshifting unlikely. mRNA splicing however, is used extensively to express other HFV genes such as *env*, and the accessory genes *tas* and

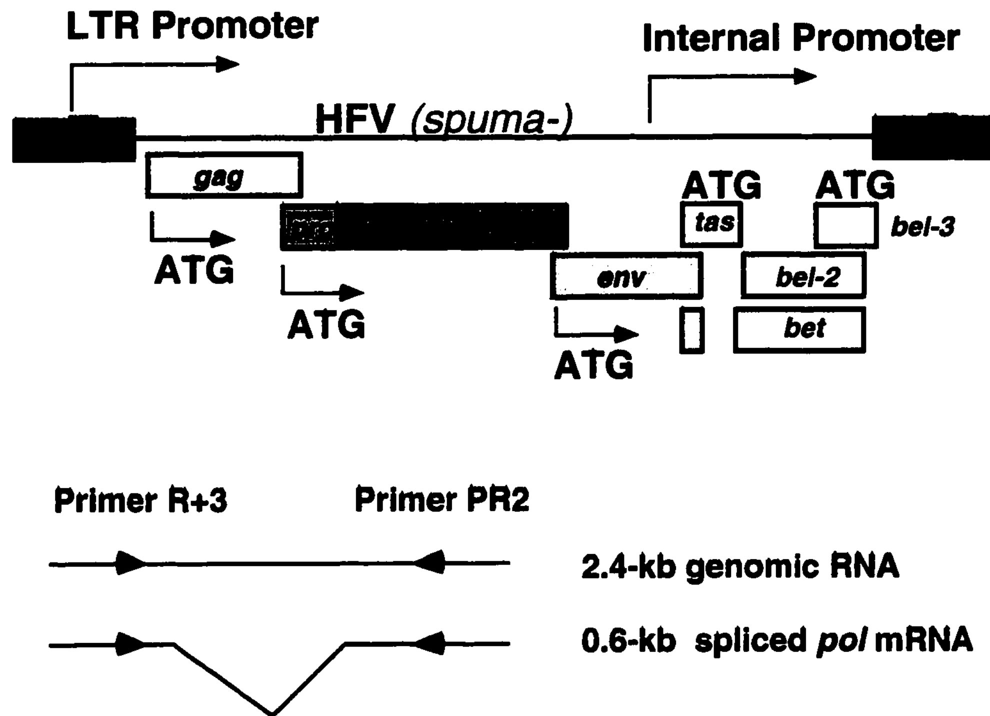
bet. Here we demonstrate that unlike other known retroviruses, HFV uses a spliced *pol* mRNA which is essential for expression of Pol.

B. Results.

1. Discovery of the Spliced *pol* Message (Samuel R. Gwynn). We investigated the possibility of a spliced *pol* mRNA by using the reverse-transcriptase polymerase chain reaction (RT-PCR) to amplify viral RNA from infected cells. Primers were chosen to produce PCR products including the major 5' splice site and part of the protease domain of *pol* (Figure 3.1A). These primers should yield a ~2.4-kb product from genomic-length RNA, and a considerably shorter product from a spliced *pol* mRNA. Total RNA was extracted from human embryonic lung (HEL) fibroblast cells acutely infected with HFV derived from the molecular clone pHFV13. A RT-PCR product of 0.6-kb was detected from infected but not uninfected cells (data not shown). DNA from this band was cloned, and the splice junction sequenced (Figure 3.1B). There is a juxtaposition of the major 5'ss and a consensus 3'ss, creating a splice junction at nucleotides 51 and 1848 at the 3' end of the CA domain of *gag*. The AUG at nucleotide 2340 (of the genome) in this RNA is the start codon for the *pol* open reading frame.

2. Biochemical detection of the spliced *pol* mRNA. As further confirmation of the existence of a spliced pol mRNA, a primer extension assay was developed in which two oligonucleotide primers spanning the pol 5'ss and 3'ss were used to produce extension products from a control spliced RNA but not unspliced RNA (Figure 3.3, lanes 3, 4, 11, and 12). The expected products from our assay are described in Figure 3.2. We could detect the spliced *pol* mRNA in HFV-infected cells (Figure 3.3, lanes 1 and 9). Although this assay is not quantitative, comparison of the intensity of the extension products obtained with primers 4 and 7 (Figure 3.3, lanes 1 and 9), which are specific for spliced RNA, with that from the unspliced genomic RNA primer 5 (lane 5), suggests that

A.



B.

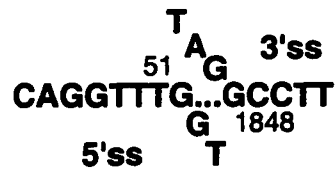


Figure 3.1 RT-PCR strategy for detection and cloning of the HFV spliced *pol* mRNA. (A) HFV genome depicting location of the ATG in *pol*. Primers R+3 and PR2 were designed to detect all RNAs initiated at R, and containing the *pol* ATG. Observed RT-PCR products correspond to genomic and spliced *pol* mRNAs. (B) Sequence of the splice junction for the *pol* mRNA, and surrounding genomic RNA.

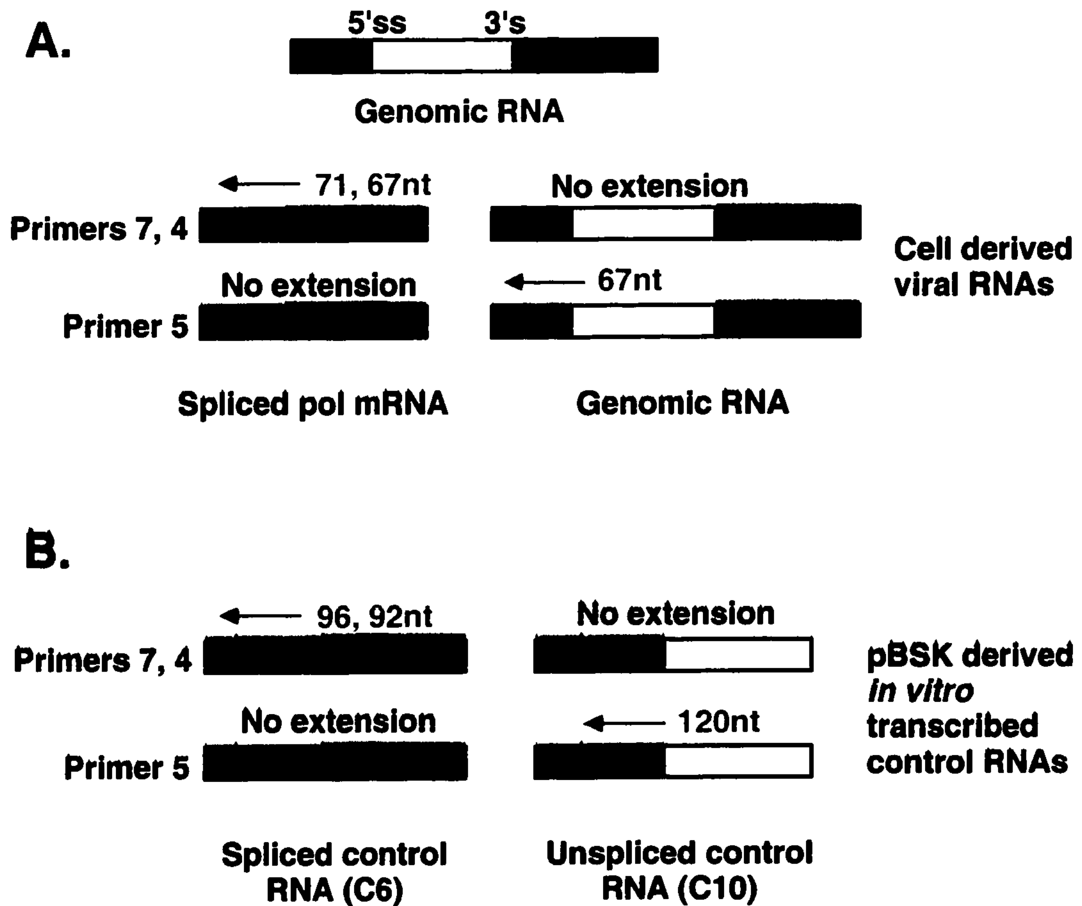


Figure 3.2 Expected primer extension products from HFV-infected cellular RNAs (A) and *in vitro* transcribed control RNAs (B). Red arrows indicate extensions products and their respective sizes. Primers 7 and 4 span the splice junction site for the pol mRNA, whereas primer 5 is complementary to the unspliced genomic RNA at the 5' splice site. Green boxes indicate sequences derived from the RNA expression plasmid pBSKII+. Purple boxes indicate the 52nt leader of HFV, white boxes indicate sequences within *gag*, and the blue boxes correspond to the region downstream of the 3' splice site in *pol*.

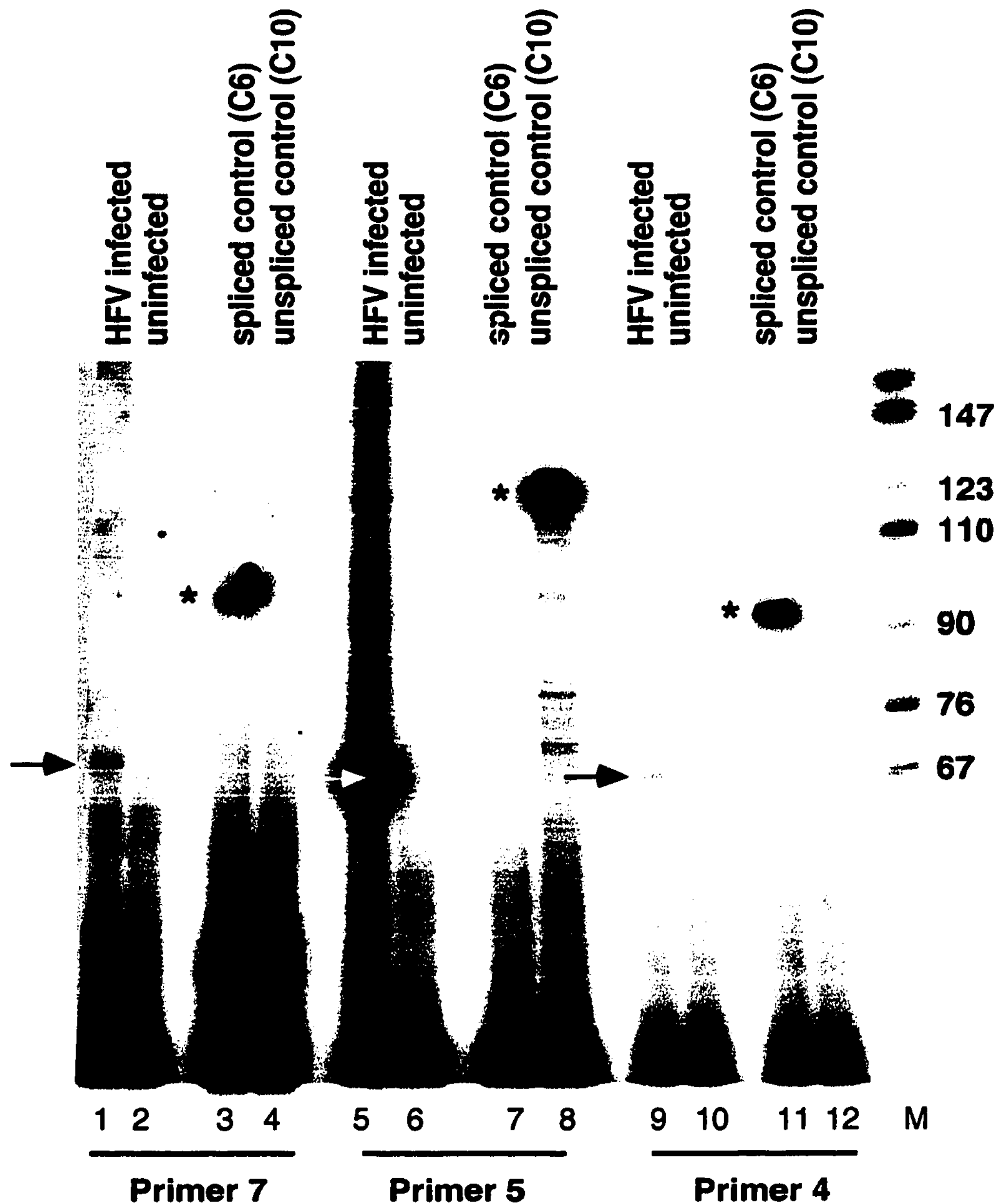


Figure 3.3 Results from the primer extension assay. Each lane contained 20μg of cellular RNA from infected or uninfected HEL cells collected 48 hours post-infection, or 0.2μg of control RNA mixed with 3×10^6 cpm of [^{32}P] oligonucleotide (10ng). Lane M shows molecular size markers (in nucleotides, ssDNA) prepared from *Msp I*-digested pBR322. Black arrows indicate extension products from spliced *pol* mRNA, and the white arrow indicates those from unspliced genomic RNA. Expected extension products from control RNA are indicated by asterisks.

the *pol* mRNA is not abundant in infected cells. In addition, we were unable to detect the spliced *pol* mRNA in total nucleic acid isolated from virions (data not shown).

3. Characterization of *pol* 3' Splice Site Mutants (Shuyarn F. Yu). To determine whether this putative pol mRNA is used for Pol translation, we introduced two mutations into the infectious clone that altered the 3'ss upstream of nucleotide 1851, but did not change the reading frame of the Gag protein. DNA encoding this 3'ss mutant was transfected into baby hamster kidney cells (BHK21) and viral supernatants were tested in the foamy activation of ß-galactosidase (FAB) assay (Yu and Linial, 1993), which measures the ability of a virus to transactivate the HFV LTR present in the FAB cell genome. The HFV 3'ss mutant could not produce infectious virions. Western blotting and radioimmunoprecipitation analysis with anti-Pol antibodies failed to detect any Pol proteins in cells transfected with the 3'ss mutant although levels of Gag expression were similar to those of wild type after 48 hours (data not shown). In wild type transfected cells, both the p78 Gag precursor and the p74 cleaved form of Gag could be detected, whereas for the 3'ss mutant, only the p78 precursor could be detected, implying that PR activity contained in Pol was absent (data not shown). These data demonstrate that the 3'ss in *gag* is absolutely required for Pol expression.

We next asked whether the 3'ss mutants could complement two mutant defective in the *gag* gene (GagHI, and GagHIII). These mutants contain substitutions in the glycine-arginine-rich domain of the nucleocapsid domain and are non-infectious, although they produce both Gag and Pol proteins. We found that cotransfection of the 3'ss mutant DNAs yielded infectious virus. The two gag mutants could not complement each other, demonstrating that we were not detecting DNA recombination. In contrast, conventional retroviruses, Gag and Pol mutants cannot complement each other because the Gag portion of Gag-Pol cannot subsitute for Gag in viral assembly. We also created a double stop codon in the *gag* gene 3' of the nucleocapsid domain, in the region cleaved from p78Gag. This mutation allows synthesis of p74Gag and a truncated version of p78Gag. If a frameshift were required for synthesis of Pol, then this mutant should

prevent replication. However, tansfection of the GagStop plasmid into cells allowed production of infectious virus (data not shown). Because termination before the normal gag UAG in the pol overlap region allows viral replication, this result argues against a frameshift mechanism for Gag-Pol synthesis. These data are published in (Yu et al., Science, 1996).

C. Discussion.

In this chapter, we have demonstrated that unlike conventional retroviruses, expression of HFV Pol requires a spliced mRNA. In many respects the HFV genome is similar to the well-studied retroviruses, such as the presence of a PBS complementary to $tRNA_{1,2}^{lys}$ at the 5'end of the genome, and the order of the *gag*, *pol*, and *env* genes. However, in other retroviruses, Pol is assembled into particles through Gag domains in the Gag-Pol fusion protein. After assembly and maturation (PR activation), Pol binds to the PBS in complex with tRNA to initiate reverse transcription.

There are marked similarities between HFV and the hepadnaviruses such as hepatitis B virus (HBV). In both, the viral structural protein is not cleaved in virions and its carboxyl end contains stretches of basic amino acids that interact with DNA. In HBV, reverse transcriptase (P protein) is translated from genomic-length RNA by an internal initiation mechanism. Once sythesized, P binds in *cis* to its own mRNA through a specific region call ε, possibly cotranslationally. P itself contains a priming domain, and the DNA product is covalently linked to the reverse transcriptase protein. Reverse transcription is the completed in the context of the core protein during assembly, and the majority of DNA synthesis is completed prior to virus release. In experiments not presented here, we have also found that HFV synthesizes full-length dsDNA within virions, and that this DNA is infectious (Yu et al., Science, 1996). Both retroviral and hepadnaviral assembly pathways ensure Pol incorporation, as well as sequestration of Pol activity until assembly occurs.

Our data suggests that the HFV assembly pathway is distinct from both retroviruses and hepadnaviruses. It will be interesting to determine the requirement for Pol during assembly since it is required for RNA packaging in hepadnaviruses, but not for retroviruses. The HFV assembly pathway and mechanism of Pol incorporation will be the focus of the following chapters in this thesis. One possible mechanism for Pol incorporation is that Pol forms a complex with genomic RNA and tRNA at the PBS. Genome encapsidation by Gag would then coassemble Pol. Another possibility is that high affinity protein::protein interactions between Gag and Pol are responsible for recruiting Pol to the assembling capsid. Despite similarities in genome structure to other retroviruses, it will be interesting to uncover the unique features of HFV assembly with respect to the Pol protein.

Chapter 4
The Roles of Pol and Env in the Assembly Pathway of Human Foamy Virus

A. Background.

In chapter 3, we demonstrated that HFV Pol is expressed from a spliced, subgenomic mRNA. This observation has been confirmed by others for HFV (Enssle et al., 1996; Lochelt and Flugel, 1996), and more recently for foamy viruses isolated from other species such as cats and cattle (Bodem et al., 1996; Holzschu et al., 1998). This mechanism of *pol* expression is unique among all known retroviruses and implies that the HFV Pol assembly pathway must also be unique. HFV Pol is a 127kD polyprotein consisting of protease (PR), reverse transcriptase (RT), RNAse H (RN), and integrase domains (IN) (Netzer et al., 1993). Expression of Pol as a Gag-Pol fusion protein, in the case of other retroviruses, provides mechanisms for both expression of Pol and assembly of the viral enzymes into particles through Gag::Gag interactions (Hatfield et al., 1992; Jacks, 1990). In addition, for most retroviruses Pol is completely dispensable for virus assembly and RNA packaging, both processes depending entirely upon Gag (Oertle and Spahr, 1990). While the Env protein determines tissue tropism for the mature virion, it is also dispensable for assembly, packaging and budding (Shields et al., 1978).

Pararetroviruses have evolved very different mechanisms for packaging viral RNA and for budding. Hepadnaviruses such as human hepatitis B (HBV) require their reverse transcriptase (P protein) for encapsidation of genomic RNA (Bartenschlager et al., 1990; Bartenschlager and Schaller, 1992; Pollack and Ganem, 1993; Pollack and Ganem, 1994). Assembly is initiated by binding of the nascent P protein to a secondary structure in the viral RNA called epsilon (ε), after which capsid protein dimers are recruited to complete capsid formation (Birnbaum and Nassal, 1990). Capsids form in the cytoplasm and are thought to be transported to the RER where they acquire the surface glycoproteins during budding into the lumen of the ER. In contrast to

retroviruses, HBV requires expression of the surface glycoproteins for release of extracellular particles (Bruss and Ganem, 1991).

While HFV shares features with both retroviruses and the other mammalian reverse transcriptase encoding-viruses such as hepadnaviruses, its replication pathway is distinct from both. As in the case of HBV, cell-free virions contain significant amounts of DNA (Yu et al., 1996; Yu et al., 1999), indicating that reverse transcription is either initiated prior to assembly, or that the Pol protein is highly active inside virions. However, unlike HBV, where a majority of virions contain nicked, circular dsDNA (Nassal and Schaller, 1993), only about 10% of HFV particles contain full-length dsDNA (Yu et al., 1996). While both HBV and HFV express their Pol proteins independently of their structural proteins, they initiate reverse transcription by different mechanisms. In the case of HBV, initiation of reverse transcription is coupled to assembly which is initiated by Pol binding to HBV RNA (Bartenschlager and Schaller, 1992; Pollack and Ganem, 1994; Wang and Seeger, 1993). HFV RNA contains a primer binding site for $tRNA^{Lys}$, and all evidence suggests that reverse transcription proceeds by a retroviral pathway (Kogel et al., 1995). The roles of Gag, Pol and Env in HFV assembly are not known. One recent study suggested that expression of Gag alone did not lead to particle formation (Fischer et al., 1997).

In this chapter, I have examined the roles of Pol and Env in the assembly of human foamy virus. I have shown that as is the case of other retroviruses, the Pol protein is not required for particle assembly, or encapsidation of genomic RNA. In contrast, I have found that like hepadnaviruses, the foamy virus Env protein is required for production of extracellular virions but not for intracellular particle formation.

B. Results.

1. The Env glycoprotein is required for efficient budding from the cell, but not particle formation. I was interested in the role of the Env glycoprotein in the HFV assembly pathway. A deletion mutant (ΔEnv190) was used to analyze virus assembly

and RNA packaging. I predicted that such a mutant would assemble and bud normally from the cell, being wild type for Gag and Pol proteins, and therefore contain RNA as is the case of murine leukemia virus and other retroviruses. Originally, we hoped that this construct would serve as a positive control for assembly with respect to Gag, Pol, and RNA, and as a negative control for viral spread. Transient expression was used to compare assembly of virus particles and RNA packaging in the presence or absence of Env. Transfections were performed in FAB cells which contain an HFV LTR driving ß-galactosidase expression (Yu and Linial, 1993), thus only cells containing the Bel 1 (Tas) transactivator protein will turn blue after staining with the chromogenic substrate X-GAL (5-bromo-4-chloro-3-indoyl-5-ß-D-galactopyronoside). This allowed comparison of transfection efficiency prior to virus particle and protein analysis. Equivalent transfection efficiencies were observed using wild type and mutant proviruses, usually in the range of 10-20%.

In order to look at intra- and extracellular viral protein expression, radioimmunoprecipitation analysis (RIPA) was performed using Gag antiserum. The major products detected with this serum are of 74 and 78kD. Unlike other retroviruses, no cleavage to mature Gag proteins is detected (Hahn et al., 1994). Intracellular expression levels of Gag (Figure 4.1, lanes 1 and 3) were similar for wild type and ΔEnv190 as measured by RIPA. However, no detectable Gag protein was present in the supernatant of ΔEnv190 transfected cells (Figure 4.1, lane 8), indicating that extracellular virus was not produced in the absence of the Env glycoprotein. Quantitative analysis of the wild type bands corresponding to extracellular Gag indicated a 20-fold difference between wild type and ΔEnv190 at 36hrs post-transfection (Figure 4.1, lanes 6 and 8), which was the limit of the sensitivity of the assay. I also looked at Pol protein expression. The major proteins detected by our RNAse H antiserum are a 127kD precursor and a 80kD Pro-Pol (PR-RT-RN) product from which the 40kD integrase domain has been cleaved (Netzer et al., 1993). I found no difference in intracellular Pol expression between HFV (wt) (Figure 4.2, lane 1) and ΔEnv190 (Figure 4.2, lane 3). Since no particles were detected in the supernatant, it was not surprising that no RNA

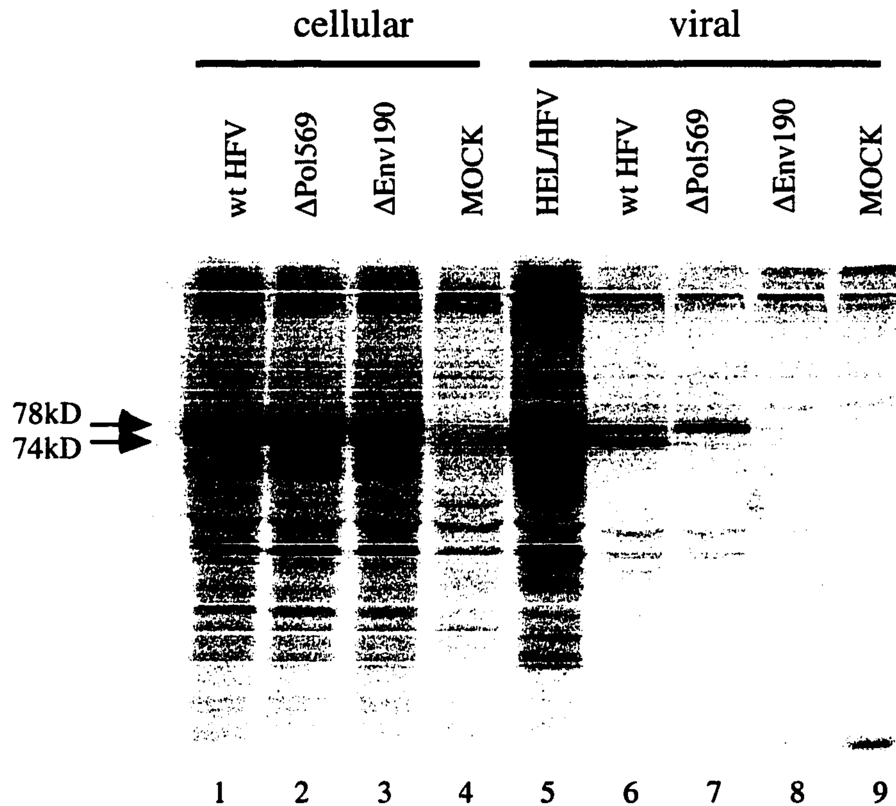


Figure 4.1 Radioimmunoprecipitation analysis (RIPA) of cellular and viral Gag and Pol proteins. Assay conditions and antibodies are described in the Materials and Methods section. Lanes 1-4 represent cellular expression levels of Gag from HFV (wt), ΔPol569, ΔEnv190, and untransfected FAB cells. Lane 5 contains virus from the supernatant of labelled, acutely infected HEL cells, and lanes 6-9 represent the extracellular virus from the supernatants of the transfections. Lanes 6 and 7 were used to normalize the number of HFV (wt) and ΔPol569 particles for the packaging experiments.

cellular

wt HFV | ΔPol569 | ΔEnv190 | MOCK

127kD →

80kD →

1 2 3 4

Figure 4.2 Results of radioimmunoprecipitations using anti-Pol antiserum. Cellular lysates for this figure were identical to those used with the anti-Gag antiserum. Lane 1 contains wild type, lane 2 was prepared from cells transfected with ΔPol569, and lane 3 contains ΔEnv190.

could be detected (Figure 4.7, lane 5). A different *env* mutant ΔMN (provided by Dr. Martin Lochelt, Heidelberg), was also tested for comparison. This mutant, whose deletion spans a much larger portion of the *env* gene, yielded the same result (data not shown). The ΔMN construct had also been previously characterized for correct expression of the other major HFV genes, and does not interfere with the essential internal promoter for Bel 1 transcription (Lochelt et al., 1995). At very late timepoints after transfection of these mutants, a very small amount of viral Gag could be detected in the culture supernatant (data not shown). However, some of this signal could be Gag released from dying cells.

I next examined the impact of the Env deletion on intracellular particle formation. The strategy was to lyse WT or ΔEnv190 transfected cells in different detergent-containing buffers. Lysis buffer containing Triton-X should lyse cells membranes but not disrupt intracellular capsids, whereas lysis with SDS should disrupt the integrity of virions as well as membranes. Methods similar to this have been used to quantitate intracellular retroviral D-type capsids (M-PMV) as well as HBV nucleocapsids (Lavine et al., 1989; Rhee and Hunter, 1987). Figure 4.3A indicates the amount of viral Gag present in pellets after ultracentrifugation of both Triton-X and SDS lysates. I found similar levels of pelletable Gag from Triton-X lysates from both wild type (Lane 1) and from ΔEnv190 (Lane 2) transfected cells. No Gag was recovered in pellets after complete lysis in SDS containing antibody buffer (Lanes 4 and 5), or from mock transfected cells (Lane 3). Conversely, little or no Gag protein was detectable in the ultracentrifugation supernatants of Triton-X lysed cells (Figure 4.3B, Lanes 1-3), whereas wild type and ΔEnv190 Gag were detected at similar levels in the SDS lysis supernatants, in which capsid structures are disrupted (Figure 4.3B, Lanes 4 and 5). These results indicate that the intracellular Gag seen in ΔEnv190 transfected cells, like wild type, is present in assembled particles and not as free protein. Thus I conclude that Env is not required for assembly of intracellular capsids, but is required for release of virions from the cell. Transmission electron microscopy (TEM) was performed in order to verify that particles assembled in the absence of Env resemble wild type. I was able to detect

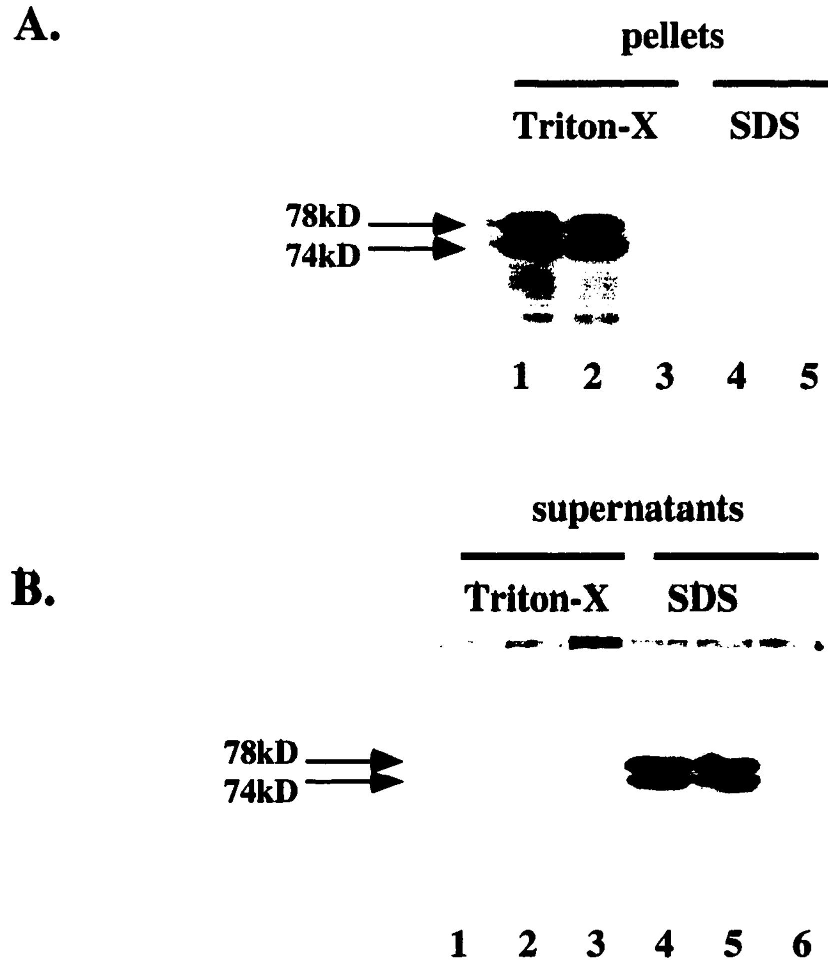


Figure 4.3 Comparison of intracellular particle formation by wild type and ΔEnv190 using Western blot analysis. Cell lysates were prepared as discussed in Materials and Methods. (A) Pellets. Lanes 1-3, Triton-X lysed cells, Lanes 4 and 5, SDS lysed cells. Lanes 1 and 4, wild type; lanes 2 and 5, ΔEnv190; Lane 3, mock transfected. (B) Supernatants. Lanes 1- 3, Triton-X lysed cells. Lanes 4-6, SDS lysed cells. Lanes 1 and 4, wild type; Lanes 2 and 5, ΔEnv190; Lanes 3 and 6, mock transfected.

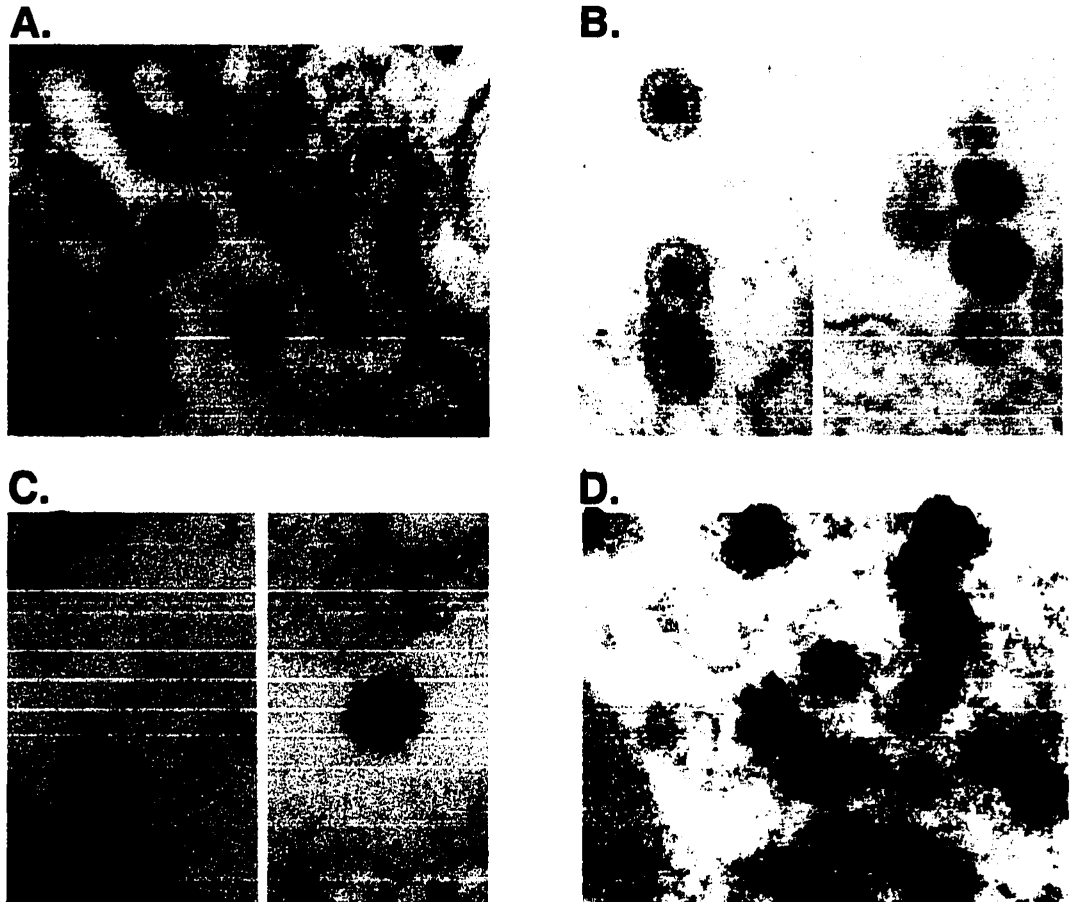


Figure 4.4 Transmission Electron Microscopy of HFV particles at 20,000X. For scale, see 100nm bar on Figure 4.5A. (A) Intracellular compartment of FAB cells transfected with ΔEnv190. (B) Wild type HFV; left, intracellular compartment; right, extracellular virus. (C) ΔPol569 virus particles; left, intracellular compartment; right, extracellular virus. (D) Wild type HFV cytoplasmic capsids.

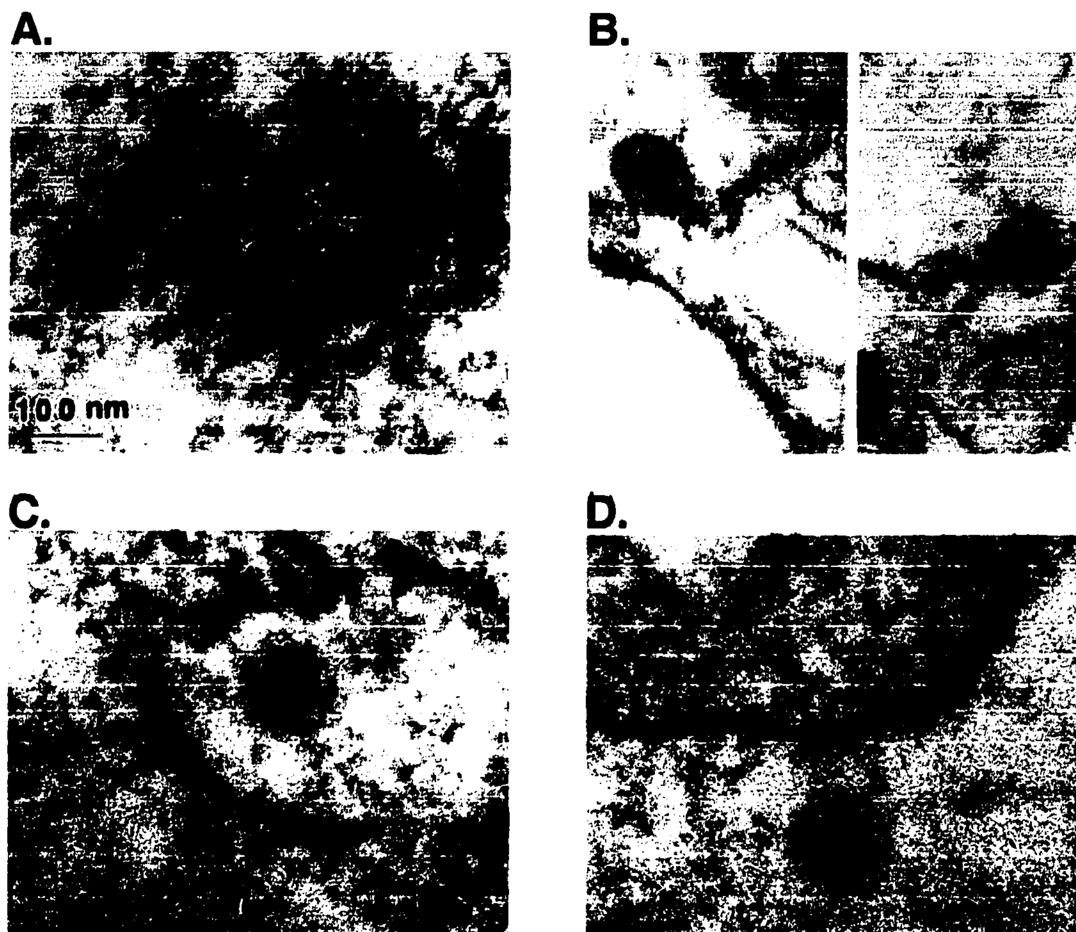


Figure 4.5 Transmission Electron Microscopy of HFV particles at 20,000X. For scale, see 100nm bar panel A. (A) Cytoplasmic capsids from cells transfected with ΔEnv190. (B) Budding virus particles in cellular compartments from FAB cells transfected with ΔEnv190. (C) and (D) Virus particles in cellular compartments of untransfected FAB cells.

intracellular particles in FAB cells transiently transfected with both wild type and ΔEnv190 DNA. Virus-like particles were seen in intracellular compartments (Figure 4.4A) which resemble wild type particles (Figure 4.4B), and occasionally could be found budding from intracellular membranes (Figure 4.5B). No particles were detected at or near the cell surface, and none were found budding from the plasma membrane. Preformed cytoplasmic capsids could also be found in both wild type (Figure 4.4D) and ΔEnv190 transfected cells (Figure 4.5A). Since publishing this work, I have learned of an endogenous retrovirus which can be seen in BHK21 cells, from which FAB cells are derived. When I looked in untransfected FAB cells I was occasionally able to see budded viruses in the intracellular compartments (Figure 4.5C) which resemble those that were previously thought to be budded ΔEnv190 virions (Figure 4.4A). Conversely, I was never able to see capsids in untransfected controls, indicating that endogenous viral proteins are not abundant, and/or cannot assembly capsids in the cytoplasm. Therefore, BHK endogenous viruses do not interfere with analysis of HFV capsids in the cytoplasm. I can conclude from this that the phenotype of the ΔEnv190 mutant is complete. In the absence of Env, HFV Gag will form capsids in the cytoplasm, but these capsids cannot bud through membranes which do not contain Env (Wang et al., 1999).

2. The Pol protein is not required for RNA packaging. Unlike other retroviruses, HFV Pol is expressed independently of Gag. I was interested to know whether the HFV assembly pathway is dependent on the Pol protein as is the case for HBV. A *pol* deletion mutant (ΔPol569) was used to evaluate whether the Pol protein was required for RNA packaging. The viral protease, encoded within the Pol precursor, cleaves the 78kD Gag precursor near the C-terminus to release 74kD and 4kD products. I used RIPA to analyze Gag and Pol protein expression in HFV(wt) and ΔPol569 transfected cells. As expected, I did not see any cleavage of intracellular Gag (Figure 4.1, lane 2) or virion associated Gag from supernatants (Figure 4.1, lane 7) of ΔPol569 transfected cells. Also as expected, no Pol protein could be detected in ΔPol569 transfected cells (Figure 4.2, lane 2).

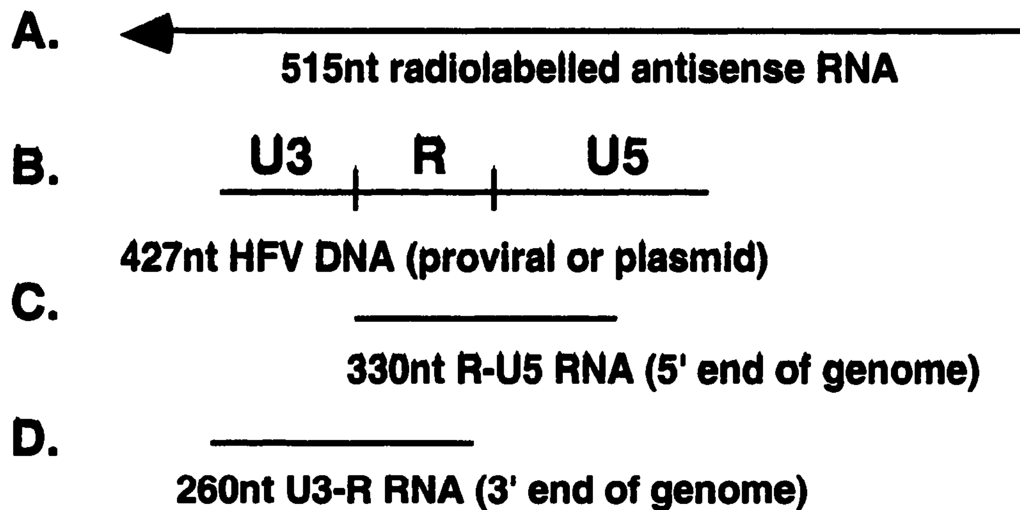


Figure 4.6 Expected sizes for protected RNA and DNA fragments with probe generated from pSGC11. (A) In vitro transcribed probe RNA corresponding to the (-) sense of HFV genomic RNA. (B) Protection of probe by LTR containing DNA, either proviral or plasmid. (C) Protection of probe by the 5' end of the genomic RNA (R-U5). (D) Protection of probe by the 3' end of genomic RNA (U3-R).

Viral RNA was quantitated using RNAse protection analysis (RPA). The probe (pSGC11) used in these experiments distinguishes RNA from DNA since it spans part of U3, all of R and part of U5 (Figure 4.6). DNA (either endogenous viral DNA or plasmid from the transfection) protects an RNA probe fragment corresponding to 427nt. Genomic viral RNA protects two fragments of the riboprobe: the 5' end of the genome protects a 330nt fragment of the probe corresponding to R-U5, and the 3' end of the genome protects a 260nt fragment corresponding to U3-R. Under the conditions used for hybridization in this assay, RNA-RNA hybrids are detected more readily than RNA-DNA hybrids (data not shown). The signal in lanes 3 and 4 (Figure 4.7) is from RNA, as determined by comparison to the molecular weight markers, although virion DNA is easily detected in virus isolated from the media of acutely infected cells (data not shown). Virus used for RPA assays was isolated as soon as it was detectable by RIPA using anti-Gag antiserum in the supernatants (36hrs post transfection) in an effort to compare assembly prior to spread of wild type virus. I found that similar levels of RNA were detected in wild type HFV and ΔPol569 particles (Figure 4.7, lanes 3 and 4). To verify the finding that genomic RNA is packaged by the Pol mutant, nucleic acids were purified from virus particles and subjected to treatment with RNAse-free DNAse (Figure 4.7, lanes 11 and 16) or DNAse-free RNAse (Figure 4.7, lanes 10 and 15) prior to analysis by RPA. Both wild type and ΔPol569 signals disappeared after treatment with RNAse but not DNAse. This demonstrates that the probe is discriminating between RNA and DNA in the RNAP reactions, and that RNA is packaged by the ΔPol569 mutant.

As a control in these studies, I compared the ΔPol569 mutant to a protease point mutant (D/A) since it was shown that it assembles particles and expresses full length Pol protein, but is not replication competent (Konvalinka et al., 1995). I considered that if the Pol protein was required for packaging, this mutant would serve as a positive control in the absence of viral spread. I found that the assembly, RNA packaging, and budding of D/A particles were identical to those for ΔPol569 (data not shown).

In order to quantitate the amount of RNA packaged/virion, particles were normalized to viral Gag protein using RIPA (Figure 4.1, lanes 6 and 7). PhosphorImage

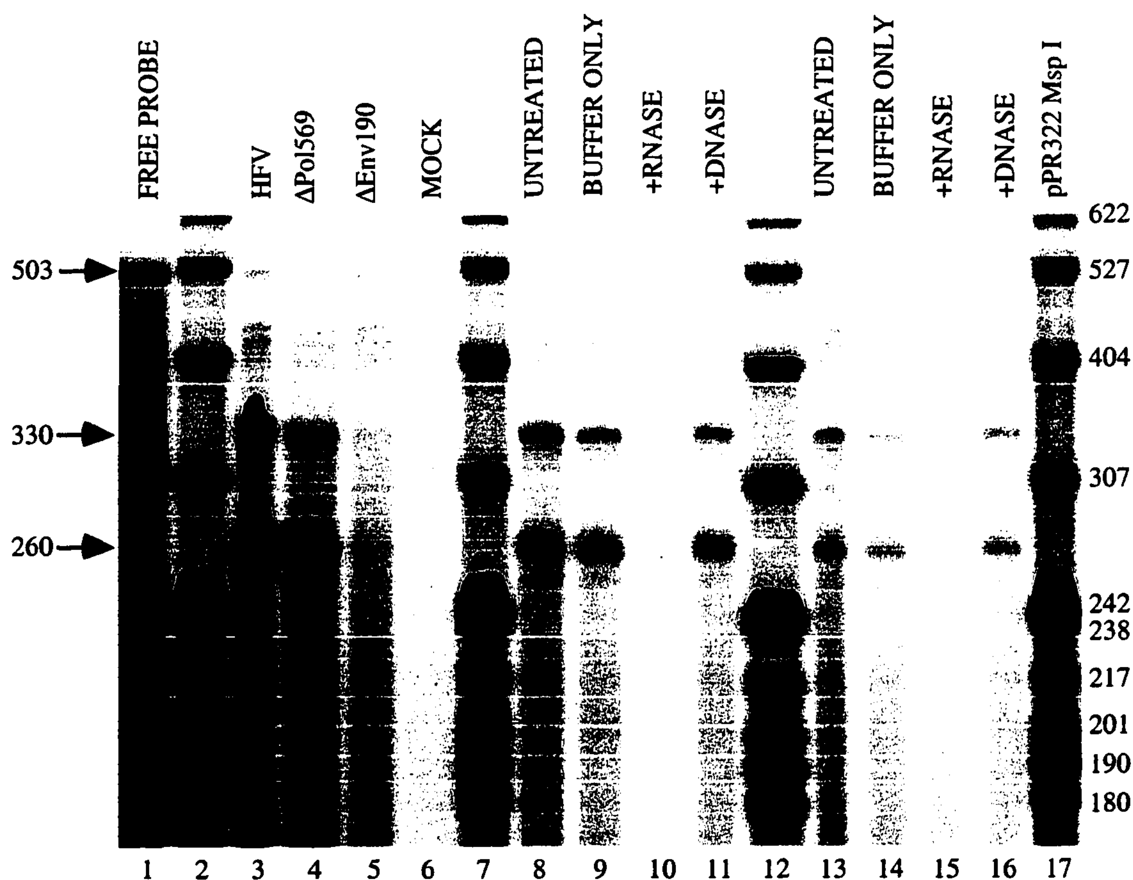


Figure 4.7 Ribonuclease protection analysis (RNAP) of wild type and mutant HFV viral nucleic acids. Shown in Fig. 4.6 are the predicted fragments of the pSGC11 RNA probe which should be protected by viral nucleic acids. Radiolabeled pBR322/*Msp I* digested DNA was used as a marker (lanes 2, 7, 12, 17). 1% of total undigested free probe is represented in lane 1. Lanes 2-5 were used to quantitate WT HFV, ΔPol569, and ΔEnv190 nucleic acids. Lanes 8-11 depict untreated or nuclease treated WT HFV nucleic acids prior to RNAP analysis. Lanes 13-17 depict untreated or treated ΔPol569 nucleic acids prior to RNAP analysis.

analysis was used to determine the efficiency of RNA packaging for ΔPol569 relative to wild type. Ratios of RNA (Figure 4.7, lanes 3 and 4) to Gag (Figure 4.1, lanes 6 and 7) indicate that the ΔPol569 mutant packages genomic RNA with wild type efficiency. This experiment was repeated four times, each time determining the ratio of Gag/RNA for both HFV and ΔPol569, and then calculating the efficiency of packaging by comparing HFV(Gag/RNA)/ΔPol569(Gag/RNA) values. The average packaging efficiency of HFV/ΔPol569 was 0.96 with a standard deviation of ±0.31.

3. The Pol protein is not required for virus assembly or budding. Transmission electron microscopy (TEM) was used to analyze particle formation in the absence of the Pol protein. Pol mutant particles were detected by TEM in both intracellular compartments as well as at the plasma membrane (Figure 4.4C). The intracellular (left) and extracellular (right) particles formed by the ΔPol569 mutant (Figure 4.4C) are very similar to wild type (Figure 4.4B). The *pol* mutant particles are detected in the extracellular media at wild type levels indicating that budding is not impaired by the lack of Pol protein. This data is consistent with results obtained with a protease active site mutant of HFV (D/A) and indicates that cleavage or partial cleavage of the Gag protein is not required for virus assembly or budding. These results demonstrate that virus assembly occurs in the absence of the Pol protein, as in other retroviruses.

C. Discussion.

While assembly of conventional retroviruses occurs without either the Pol protein or the Env protein, I have shown here that HFV assembly is unique. Transient expression of proviral HFV *pol* and *env* mutants in FAB cells has permitted me to evaluate the roles of Pol, and Env during the late stages of foamy virus infection. I have found that like all other retroviruses studied, HFV Pol is not required for assembly, RNA packaging, or budding. Particles produced by a mutant which is lacking the Pol protein resemble wild type HFV (Figure 4.4). This finding confirms previous data that the activity of the viral

protease is not required for particle formation or release from the cell. However, it is in contrast to a previous report, in which it was suggested that a protease point mutant (D/A) assembles aberrantly, and that cleavage of the Gag precursor is required for normal virion morphology (Konvalinka et al., 1995). Although it is possible that our mutant (ΔPol569), which retains a portion of the protease open reading frame, has some protease activity, no Gag cleavage could be detected using anti-Gag antiserum (Figure 4.1, lanes 2 and 7). ΔPol569 packages wild type levels of RNA as measured by RNAse protection analysis of viral nucleic acids isolated from extracellular particles.

Analysis of the *env* deletion mutant produced an unexpected result. Although Gag and Pol expression from the *env* mutant were equivalent to wild type, extracellular particles were undetectable using RIPA (Figure 4.1, lane 8) or RNAse protection (Figure 4.7, lane 5). Intracellular particles were however, detected using transmission electron microscopy. Preformed capsids were found in the cytoplasm (Figure 4.5A), some budding was seen (Figure 4.5B), and some particles appeared within membraned cytoplasmic structures such as the lumen of the rough endoplasmic reticulum, the golgi, or secretory vesicles (Figure 4.4A). Comparison of intracellular particle levels demonstrated that Env is not required for wild type levels of capsid assembly, and that this function is mediated by Gag alone (Figure 4.3). While the role of Gag in assembly seems to be retrovirus-like, intracellular particle retention in the absence of Env is atypical for most retroviruses which do not require the Env protein for budding or particle release from the cell (Shields et al., 1978). However, this finding is reminiscent of the hepadnavirus assembly pathway in which there is a strict requirement of surface glycoproteins for particle release, but not nucleocapsid formation (Bruss and Ganem, 1991).

One possible explanation for this observation is that a signal in the Env protein interacts with the secretory machinery and actively induces transport of enveloped virions to the cell surface. In the absence of Env, virions could be retained in cytoplasmic secretory vesicles. Most cells utilize a constitutive secretory pathway for transport of membrane components, and glycoproteins of the extracellular matrix. More specialized

secretory cells can utilize an additional pathway which is regulated. These cells are able to store soluble proteins and other substances in secretory vesicles for regulated release (reviewed in (Hong and Tang, 1993)). If this were the case I might have expected to see a greater accumulation of *env* mutant virions within intracellular compartments. Also, this type of mechanism would be highly cell-type specific and could have implications for both cytopathicity and virus production. These mutants have not been analyzed in cells other than fibroblasts.

Additionally, as in the case of other retroviruses such as HIV-1, there may be a definable interaction between the cytoplasmic tail of the Env protein and the N-terminus of Gag (Bugelski et al., 1995; Cosson, 1996; Dorfman et al., 1994; Freed and Martin, 1996; Freed and Martin, 1995). It may be that such interactions enhance Gag multimerization, and thus particle assembly. If the interaction between Gag and Env is required for efficient particle formation, then it is also possible that expression, modification, and localization of mature Env protein may be the rate limiting steps in mature particle formation. In the case of human immunodeficiency virus type 2 (HIV-2), the cytoplasmic tail of the Env glycoprotein has been to be reported to be important in enhancing particle release (Bour et al., 1996; Ritter et al., 1996). However, there was only about a 10 fold effect, comparable to the enhancement of particle release by the Vpu protein of human immunodeficiency virus type 1 (HIV-1) (Strebel et al., 1989; Terwilliger et al., 1989). The foamy virus *env* mutant phenotype is therefore more similar to that of HBV envelope protein mutants which are not released in the absence of their surface glycoproteins (Bruss and Ganem, 1991).

Multimerization of Gag is likely to be concentration dependent (Welker et al., 1997), and if interaction with Env at a specific membrane occurs, it could enhance particle formation at that membrane. It has been demonstrated that the matrix (MA) domain of Gag can target the structural polyproteins to specific locations in the cell and direct the site of assembly. In one case, a single point mutation in MA changed a type D retrovirus to type C which was able to bud from the plasma membrane without preforming capsids (Rhee and Hunter, 1990). Recently it has been reported that non-

budding, immature intracellular A type particles (IAPs) can be directed to the plasma membrane for assembly and release by altering the ER targeting signal within Gag (Welker et al., 1997). Although nuclear localization of HFV Gag has been reported, it does not seem to be required for replication (Schliephake and Rethwilm, 1994), and no other targeted localization has been described.

In addition, it has been observed that both HFV (Goepfert et al., 1995) and HBV (Kuroki et al., 1989) surface glycoproteins contain ER retrieval signals, while other retroviral Env proteins do not. The biological relevance of this observation for HFV remains to be determined, although it has been reported that the HFV ER retrieval signal is not required for intracellular budding, or budding from the plasma membrane. Mutants in the ER retrieval signal bud more often from the plasma membrane, but release fewer particles with respect to wild type (Goepfert et al., 1997). These data are consistent with the idea that co-localization of Gag and Env may occur more efficiently at the membranes of the ER, enhanced by the ER signal in Env, but does not explain why particles which still form intracellularly without Env are not released from the cell. More recently, it has been shown that assembly is more restricted to these membranes (Goepfert et al., 1999).

There appears to be an evolutionary relationship between foamy viruses, and other retroviruses, as well as hepadnaviruses such as HBV. Several features of HFV replication are of particular interest in this regard. Like HBV, HFV expresses reverse transcriptase independently of the structural proteins. HFV lacks extensive proteolytic processing of the Gag structural protein (Hahn et al., 1994), and contains GR box nucleic acid binding motifs instead of the canonical retroviral Cys-His boxes (Flugel, 1991). In addition, infectious double stranded DNA can be extracted from a small percent of extracellular HFV virions (Yu et al., 1996). However, the HBV assembly pathway is initiated and completed very differently than that of retroviruses. The P protein (reverse transcriptase and ribonuclease H) initiates the assembly process and is required for genome encapsidation (Bartenschlager et al., 1990; Bartenschlager and Schaller, 1988; Pollack and Ganem, 1994), and surface glycoproteins are required for release of virus

from the cell (Bruss and Ganem, 1991). Thus while HFV may represent an evolutionary bridge between retroviruses and hepadnaviruses, its replication is distinct from both.

In the next chapter I focus on developing methods for detecting the Pol proteins in extracellular virions. This has been technically impossible with traditional methods such as western blotting or radioimmunoprecipitation analysis. With a Pol incorporation assay available, I study the roles of proteolysis, protein::protein interactions, and reverse transcription in the assembly pathway for Pol.

Chapter 5
Proteolytic Processing, the Carboxy Terminus of Gag, and the Primer Binding Site are not Required for Pol Incorporation into Foamy Virus Particles.

A. Background.

In chapter 4 I showed that the requirement for HFV Pol during assembly is more like retroviruses than hepadnaviruses. As with other retroviruses, abrogation of HFV Pol expression has no effect on assembly of particles, packaging of viral genomic RNA, or release of virus from the cell (Baldwin and Linial, 1998). While these data suggest an HFV assembly pathway which is initiated like other retroviruses, they give no hint of how the Pol protein might be incorporated into the particles.

The pathway of HFV reverse transcription also appears to follow the retroviral paradigm in which the initiation at the primer binding site (PBS) requires complex formation with a specific tRNA. The HFV PBS contains 18 nucleotides of perfect homology to the 3' end of both rat and human tRNA-$Lys_{1,2}$, and there is evidence for synthesis of strong stop DNA (Kogel et al., 1995; Kogel et al., 1995). In contrast, the HBV P protein binds in *cis* to a secondary structure (ε) in the genomic RNA (Bartenschlager et al., 1990; Bartenschlager and Schaller, 1992; Pollack and Ganem, 1993; Pollack and Ganem, 1994), an event which initiates both reverse transcription and assembly, processes which are intimately coupled (Pollack and Ganem, 1994). Priming of HBV reverse transcription uses a tyrosine residue on the N-terminal domain of the P protein (Wang and Seeger, 1993). A HFV Pol deletion mutant still packages RNA (Baldwin and Linial, 1998), demonstrating a clear difference from the assembly pathway of HBV.

The activities of the HFV Pol domains have been studied *in vitro*. The reverse transcriptase (RT) activity from a foamy virus (FV) was first demonstrated in 1971 (Parks et al., 1971), and later template-primer sets for endogenous RT activity were

optimized for simian foamy virus-1 (SFV-1) (Benzair et al., 1982), and the FV "strain H4188" (Liu et al., 1977). The HFV RT domain has been expressed in *E. coli* and has been shown to have DNA polymerase activity using *in situ* RT gel assays (Kogel et al., 1995; Kogel et al., 1995). The ribonuclease H (RH) domain has also been shown to be active (Benzair et al., 1983; Kogel et al., 1995). An unusual feature of HFV is that Pol is activated prior to or during viral assembly and release. About 25% of HFV particles contain full length DNA (Yu et al., 1996; Yu et al., 1999), and experiments with RT inhibitors such as 3'-azido-3'-deoxythymidine (AZT) are consistent with the fact that DNA is the infectious genome (Moebes et al., 1997; Yu et al., 1996). It is unclear exactly when HFV reverse transcription begins. While we know that the RNA genome is packaged by Gag (Baldwin and Linial, 1998), this does not rule out the possibility that reverse transcription is initiated early in the assembly process. Therefore, it is also possible that complex formation between Pol, tRNA, and the PBS is required for packaging Pol protein into virions.

Although HFV Pol contains a protease domain (PR), the proteolytic processing of Gag and Pol by HFV PR is different from other retroviruses. Only two cleavage events are known to occur (Konvalinka et al., 1995). The 78kD HFV Gag protein is processed once at its C-terminus, to release a 4kD peptide, an event which occurs in approximately 50% of the Gag precursor molecules. Recent work suggests that this cleavage is required for efficient replication, since mutants which lack cleavage site replicate less well and revert to wild type in culture. Mutants lacking the C-terminal p4 protein can also replicate, but at very low levels (Enssle et al., 1997). The exact Gag cleavage site has been identified biochemically using recombinant PR (Pfrepper et al., 1998). PR also cleaves the 127 kD Pol polyprotein once to release a 45 kD integrase protein (IN), and the exact site of cleavage between the reverse transcriptase (RT/RH) and IN has been identified *in vitro* (Pfrepper et al., 1998). This cleavage is probably essential, as a protease active site mutant (HFV-D/A) is not replication competent (Konvalinka et al., 1995). Nothing has been published regarding the role of proteolytic processing for packaging of Pol into virions.

In this chapter, I have investigated the role of proteolytic cleavage, complex formation of RT at the PBS, and initiation of reverse transcription with respect to their importance for the incorporation of the Pol protein into particles. I have demonstrated that neither PR activity nor the PBS are required for Pol assembly. In addition, the primary sequence of Gag surrounding the cleavage site at the C-terminus of Gag is not important.

B. Results.

1. Proteolytic processing of Gag or Pol is not required for assembly of Pol into HFV particles. To better understand the role of the viral protease (PR) during assembly, I wanted to test whether the HFV protease active site mutant (HFV-D/A) was capable of assembling Pol into virions. However, there are inherent difficulties in detecting the small amount of Pol protein present in virions from transiently transfected cells. Since traditional methods such as western blot analysis and radioimmunoprecipitation analysis failed to convincingly detect Pol, I introduced a consensus recognition sequence for the catalytic subunit of protein kinase A (HFV-PKA) into the *pol* gene. This method had previously been used to detect the the HBV P protein (Bartenschlager et al., 1992; Bartenschlager and Schaller, 1992). The PKA site was introduced near the C-terminus of integrase (IN) using site-directed PCR mutagenesis, by changing the amino acid sequence IRTSL to RRASL (Figure 5.1A). Using antibodies raised against the RH domain of Pol, I was able to immunoprecipitate (IP) and specifically phosphorylate the 127kD Pol precursor from disrupted transfected cells (Figure 5.1B, lane 3; Figure 1C lane 1). Although I used anti-RH serum, I also detected the cleaved form of IN (45kD) by SDS-PAGE. This suggests that either cleavage can occur *in vitro* after immunoprecipitation, or that the complex between the cleaved Pol proteins is stable under the conditions used for the IP. Indeed this has been demonstrated for other retroviral Pol proteins (Hu et al., 1986; Wu et al., 1999). In the wild type HFV transfected cells (Figure 5.1B, lane 2), no specific bands were seen, indicating that phosphorylation of Pol requires the engineered

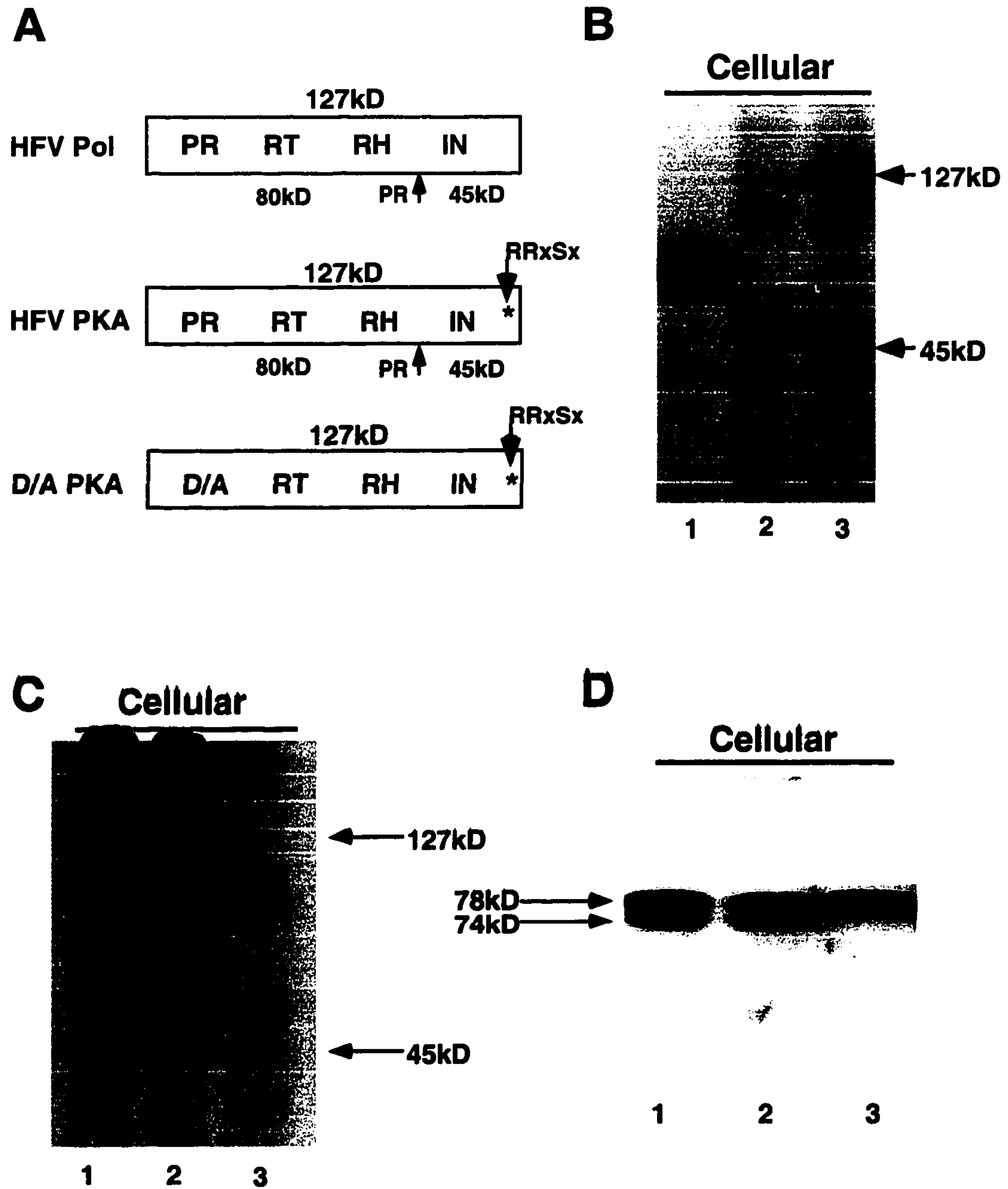


Figure 5.1 IP/PKA and western blot analysis of HFV-PKA and HFV-D/A-PKA. Assay conditions are as described in Materials and Methods. (A) Schematic diagram of HFV (wt) and HFV-PKA Pol proteins. (B) PKA analysis of HFV-PKA. Lanes: 1, Positive control for PKA phosphorylation; purified interleukin-1 receptor 2, HFV (wt) transfected cells; 3, HFV-PKA transfected cells. (C) PKA analysis of cell lysates transfected with HFV-PKA and HFV-D/A-PKA. Lanes: 1, HFV-PKA; 2, HFV-D/A-PKA; 3, Mock. (D) Western blot analysis of HFV-PKA Gag proteins. Lanes: 1, HFV (wt); 2, HFV-PKA; 3, HFV-D/A-PKA.

PKA site. Analysis of the Gag proteins demonstrated that cleavage of Gag by the HFV-PKA Pol is similar to that seen for wild type (Figure 5.1D, lanes 1 and 2). While the expression and processing of Gag and Pol appear normal for HFV-PKA, the infectivity was reduced by 1000-fold with respect to wild type (data not shown). Although I presume that this is due to a defect in integration, this has not been examined. All of my analyses are done during the first round of replication, before reinfection can occur, so that the integration status is not important.

I next subcloned the PKA mutation into the protease active site mutant background, HFV-D/A, in order to study the role of proteolysis during assembly. As expected for HFV-D/A-PKA, I no longer detected cleavage of either Pol [using the IP/kinase assay (Figure 5.1C, lane 2)] or Gag [by western blot analysis (Figure 5.1D, lane 3)]. As a negative control for virus assembly, I constructed a mutant with a deletion spanning the initiation codon for Gag. This mutant, ΔATG-D/A-PKA (Figure 5.2A), contains a deletion of 1240 bases beginning at position 1120 of the HFV sequence and ending at position 2368, upstream of the 3'splice site for *pol*. ΔATG-D/A-PKA makes no detectable Gag by western blot analysis (Figure 5.3C, lane 1), but expresses Pol at higher levels than Gag expressing proviral constructs such as HFV-D/A-PKA (Figure 5.2B, lanes 1 and 2).

I then purified HFV-D/A-PKA virus particles of the correct density from cell supernatants in order to determine whether Pol is encapsidated. To this end, viral pellets were resuspended and centrifuged to density equilibrium on gradients of iodixanol (Optiprep). Iodixanol gradients have previously been used to study the incorporation of Vif into human immunodeficiency virus type I virions (Dettenhofer and Yu, 1999). I found that HFV-D/A-PKA particles sedimented at the appropriate density of 1.12-1.15 g/cm^3 (Figure 5.2C, lanes 4 and 5) as detected by Gag western blot analysis of the gradient fractions. When I analyzed the same fractions for the presence of Pol, I was able to detect HFV-D/A-PKA Pol co-sedimenting with Gag (Figure 5.2D, lane 2). Importantly, extracellular Pol was not detected in gradient fractions of comparable density from cells expressing Pol but no Gag (Figure 5.2E, lanes 1-4). Thus, I conclude

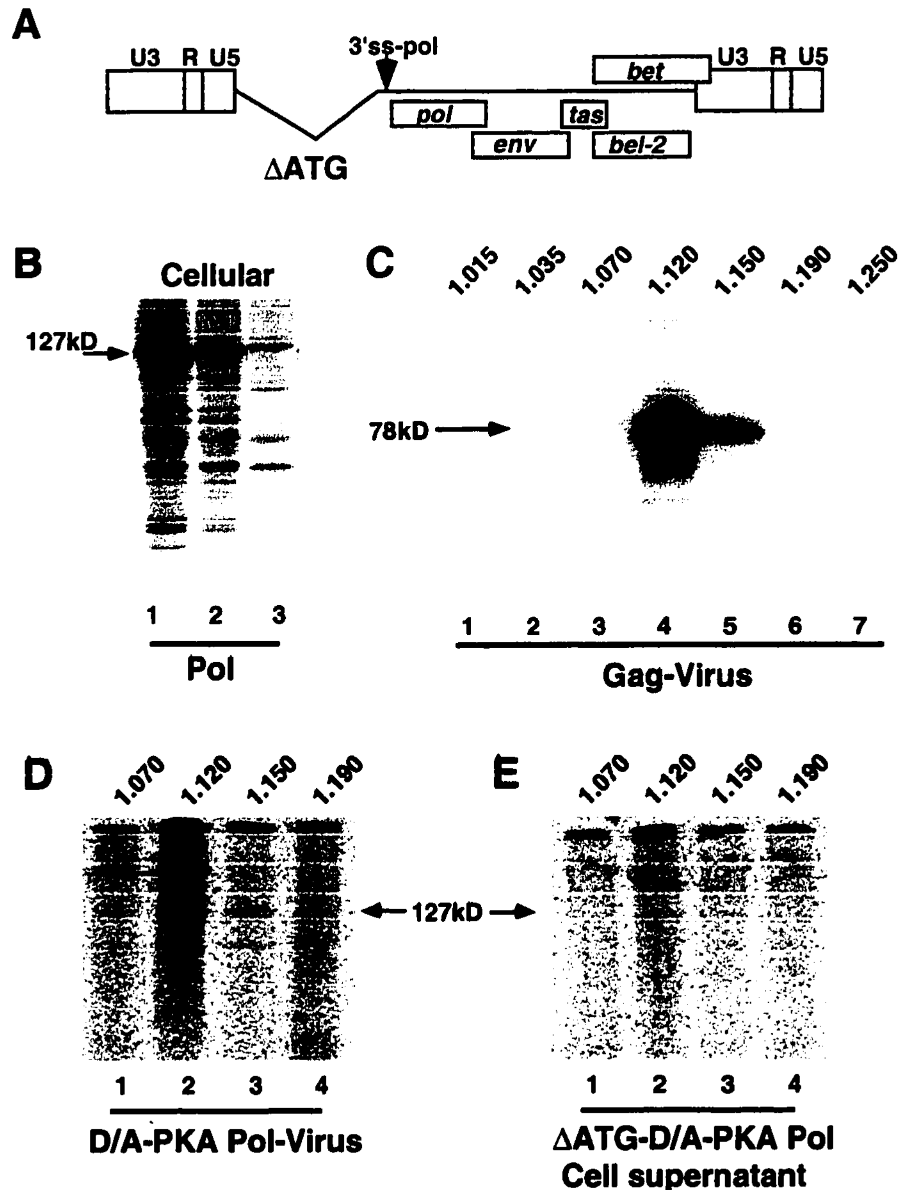


Figure 5.2 Optiprep gradient purification and analysis of Gag and Pol proteins from HFV-D/A-PKA virus particles. (A) Schematic of negative control for virus assembly and Pol incorporation, ΔATG-PKA. (B) Western blot analysis of purified D/A-PKA particles using anti-Gag antiserum. Fraction densities (g/cm3) are listed above each lane. Lanes 1-7 correspond to fractions 1-7 from the gradient. 78kD is the expected size for unprocessed Gag from HFV-D/A expressing constructs (see Fig. 3A). (C) IP/PKA analysis of cell associated Pol proteins from ΔATG-PKA and HFV-D/A-PKA. Lanes: 1, ΔATG-PKA; 2, HFV-D/A-PKA; 3, Mock. (D) IP/PKA analysis of gradient purified HFV-D/A-PKA virus particles. Lanes 1-4 correspond to fractions 3-6 from the gradient. Fraction densities (g/cm3) are listed above each lane. (E) IP/PKA analysis of gradient fractions from the negative control, ΔATG-PKA. Lanes 1-4 correspond to fractions 3-6.

that detection of Pol in fractions at 1.12-1.15 g/cm^3 requires particle formation. Taken together, these data indicate that proteolytic activity is not necessary for the specific incorporation of HFV Pol into virions.

2. The primary structure at the cleavage site in Gag is not important for Pol incorporation into particles. I was interested to see if the binding event which initiates the single cleavage of Gag by PR might be responsible for recruiting Pol into particles. I tested several mutations at or near the cleavage site (Figure 5.3A). The Gag cleavage site was mutated to block cleavage (78T/A), and two Gag truncation mutants were also generated. 74Stop was truncated exactly at the cleavage site, and 68Stop was truncated 25 amino acid residues upstream of the cleavage site. I introduced these mutations into the HFV-PKA (wild type PR) background. I found that Gag proteins of the expected sizes were expressed by all of these clones after transfection (Figure 5.3B, lanes 3, 4, and 5).

Detection and quantitation of HFV-PKA Pol in virions proved difficult due to HFV protease activity either prior to, or during, the IP/kinase assay. Although the signal to noise ratio was greatly improved by the use of a second IP after the IP/kinase reaction, cleaved forms of IN might be lost during the second IP, preventing quantitative comparison of the Gag mutants to the protease active site mutant. As protease activity was not required for Pol incorporation, the same Gag mutations were generated in the D/A-PKA background. When the resultant mutant viruses were analyzed by western blotting, the correct sized Gag proteins were detected (Figure 5.3C, lanes 4, 5, and 6). No Gag was synthesized by the Gag deletion mutant, ΔATG-D/A-PKA (Figure 5.3C, lane 1). Using the IP/kinase assay, Pol proteins were expressed at similar levels in cell extracts after transfection with the Gag mutant constructs (Figure 5.3D, lanes 2-4).

Virus particles produced after transfection with the Gag mutants were purified on iodixanol gradients and probed for Gag by western blotting (Figure 5.4A, lanes 4 and 5). All of the mutants analyzed sedimented at a similar density to the D/A-PKA parental virus (Figure 5.2B). When the corresponding fractions were analyzed for Pol, I was able

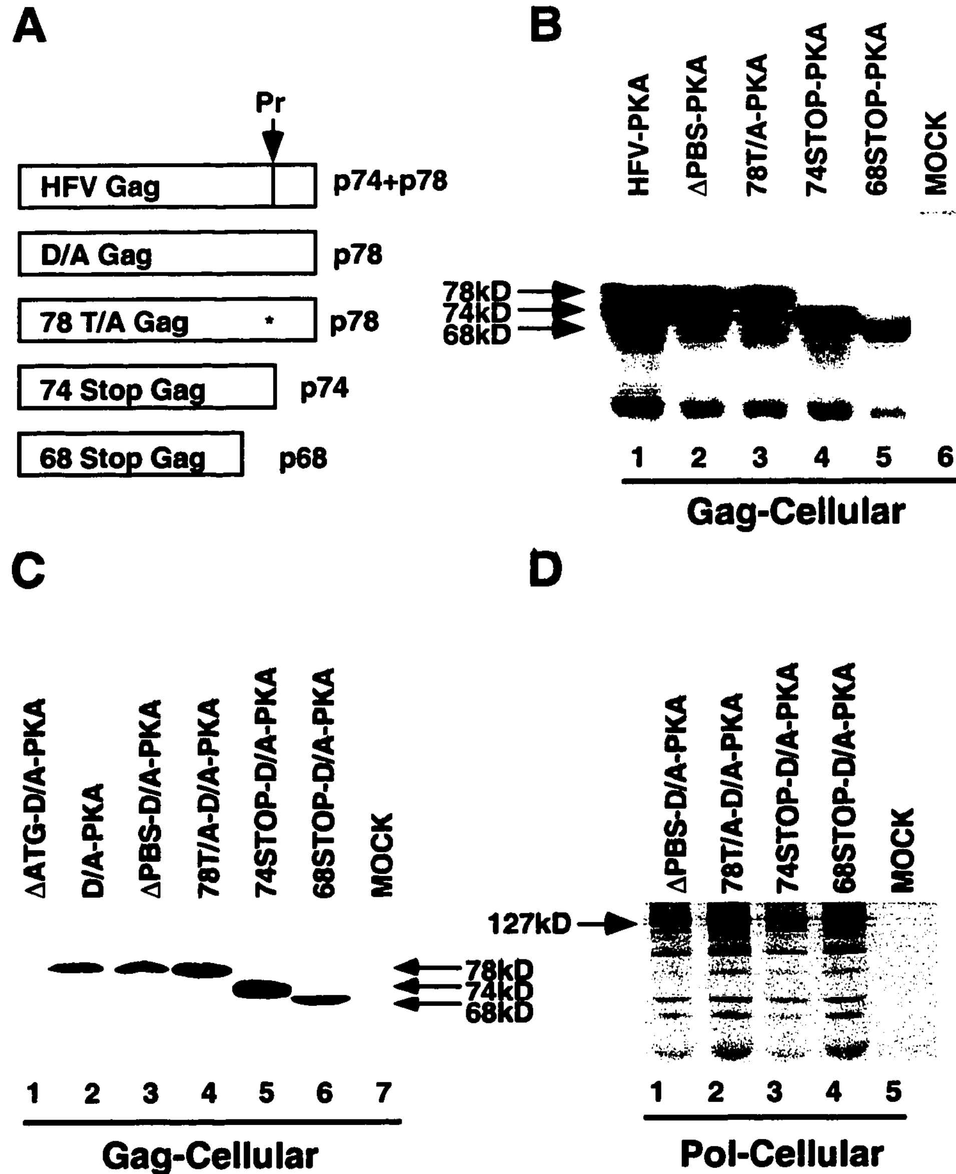


Figure 5.3 Analysis of cellular Gag and Pol expression from HFV-PKA and HFV-D/A-PKA mutants. (A) Schematic of expected Gag protein products from mutant proviral constructs. (B) Western blot analysis of Gag proteins from cells transfected with HFV-PKA mutants. Lanes: 1, HFV-PKA; 2, ΔPBS-PKA; 3, 78T/A-PKA; 4, 74Stop-PKA; 5, 68Stop-PKA; 6, ΔPr-PKA; 7, Mock. (C) Western blot analysis of Gag proteins from cells transfected with HFV-D/A-PKA expressing constructs. Lanes: 1, ΔATG-PKA; 2, HFV-D/A-PKA; 3, ΔPBS-D/A-PKA; 4, 78T/A-D/A-PKA; 5, 74Stop-D/A-PKA; 6, 68Stop-D/A-PKA; 7, Mock. (D) IP/PKA analysis of cellular Pol proteins. Shown are the mutants in the D/A-PKA background which were tested for Pol incorporation into virus particles. Lanes: 1, ΔPBS-D/A-PKA; 2, 78T/A-D/A-PKA; 3, 74Stop-D/A-PKA; 4, 68Stop-D/A-PKA; 5, ΔPr-PKA; 6, Mock.

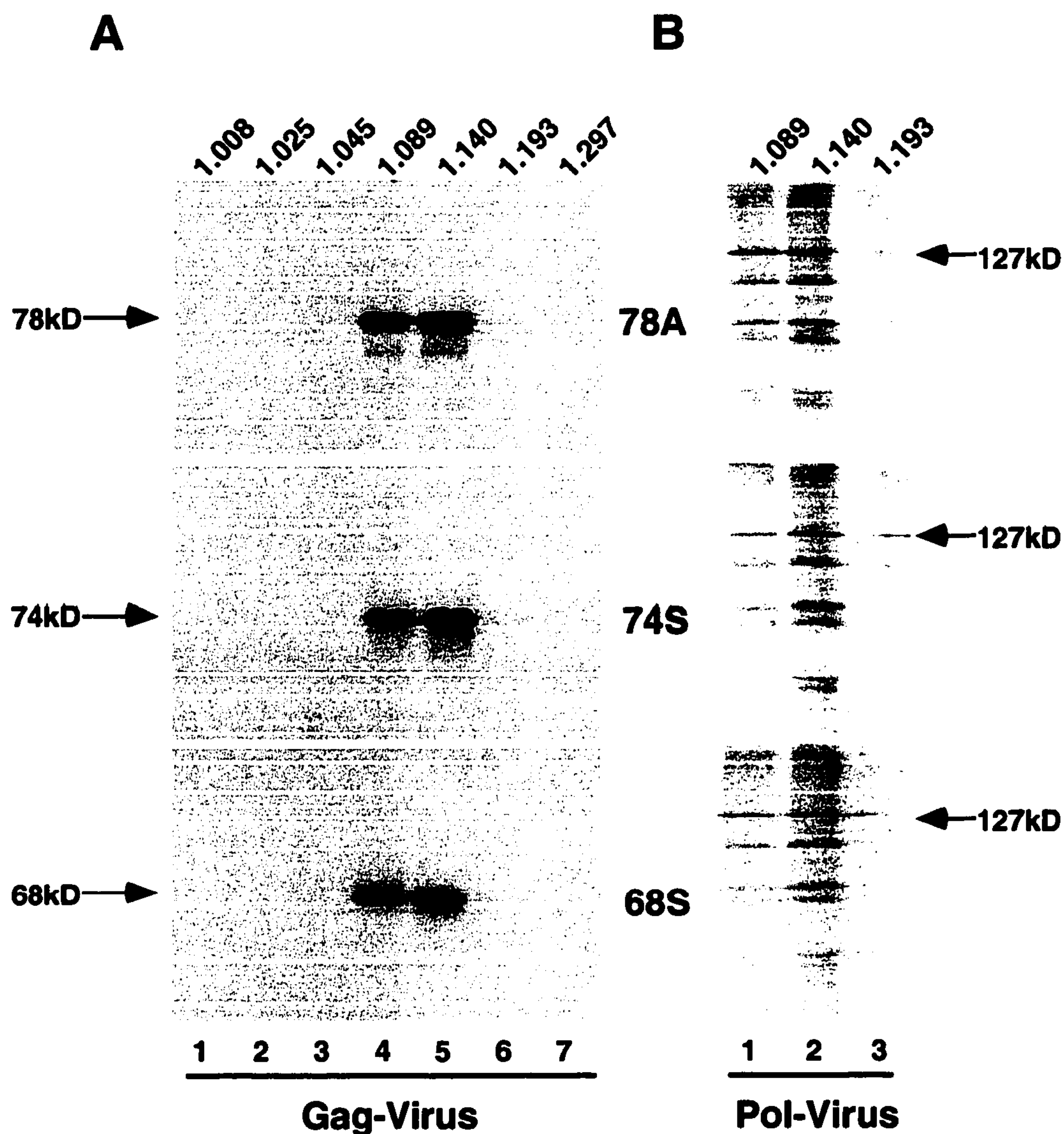


Figure 5.4 Gradient purification and analysis of Gag mutant viruses. (A) Western blot analysis of gradient fractions for viral Gag. Lanes 1-7 correspond to gradient fractions 1-7. (B) IP/PKA analysis of fractions 4-6 from the same gradients shown in (A). Lanes 1-3 correspond to fractions 4-6. For both panels, the top set are 78T/A, the middle set are 74Stop, and the bottom set are 68Stop (See Fig. 3B for schematic). Fraction densities (g/cm3) are listed at the top of each lane.

to detect Pol in each of the Gag mutants (Figure 5.4B, lanes 1 and 2). No Pol could be detected in other fractions of the gradient (data not shown). These results demonstrate that while the cleavage of Gag seems to be an important step in the replication cycle of HFV (Enssle et al., 1997), neither the Gag cleavage site itself, nor the primary sequence immediately surrounding it, are required for incorporation of Pol into virus particles.

3. The primer binding site is not essential for Pol incorporation. Binding of Pol to the PBS occurs at an early stage of HFV assembly, and it is possible that this step is responsible for its incorporation into particles. Such a scenario would be similar to what is found for HBV, where P protein binds to (ε) and initiates both reverse transcription and assembly (Bartenschlager et al., 1990; Bartenschlager and Schaller, 1992; Pollack and Ganem, 1993; Pollack and Ganem, 1994). Since HFV Pol is active during assembly, rather than at an early stage after infection, the mechanism of HFV Pol assembly could involve a specific stage of reverse transcription. I deleted the PBS using PCR mutagenesis (Figure 5.5A). In the ΔPBS constructs, Gag was expressed as expected. Gag was processed in the HFV-PKA background (Figure 5.3B, lane 2) and not processed in the D/A-PKA background (Figure 5.3C, lane 3). ΔPBS-D/A-PKA Pol was expressed in transfected cells at similar levels to other D/A-PKA expressing mutants (Figure 5.3D, lane 1). Gradient purified particles from the ΔPBS-D/A-PKA transfected cells sedimented at 1.12-1.15 g/cm^3, as seen for other mutant viruses (Fig. 5.5B, lanes 4 and 5), and Pol was detected in the same fractions (Figure 5.5C, lanes 2 and 3) demonstrating that Pol was indeed present in these particles. Thus, the PBS is not required for Pol assembly.

C. Discussion.

During the course of these studies on the mechanisms of Pol expression, and assembly, I have noticed that HFV Pol expression is quite low, both at the mRNA (Yu et al., 1996) and protein level (Baldwin and Linial, 1998; Baldwin and Linial, 1998). I was

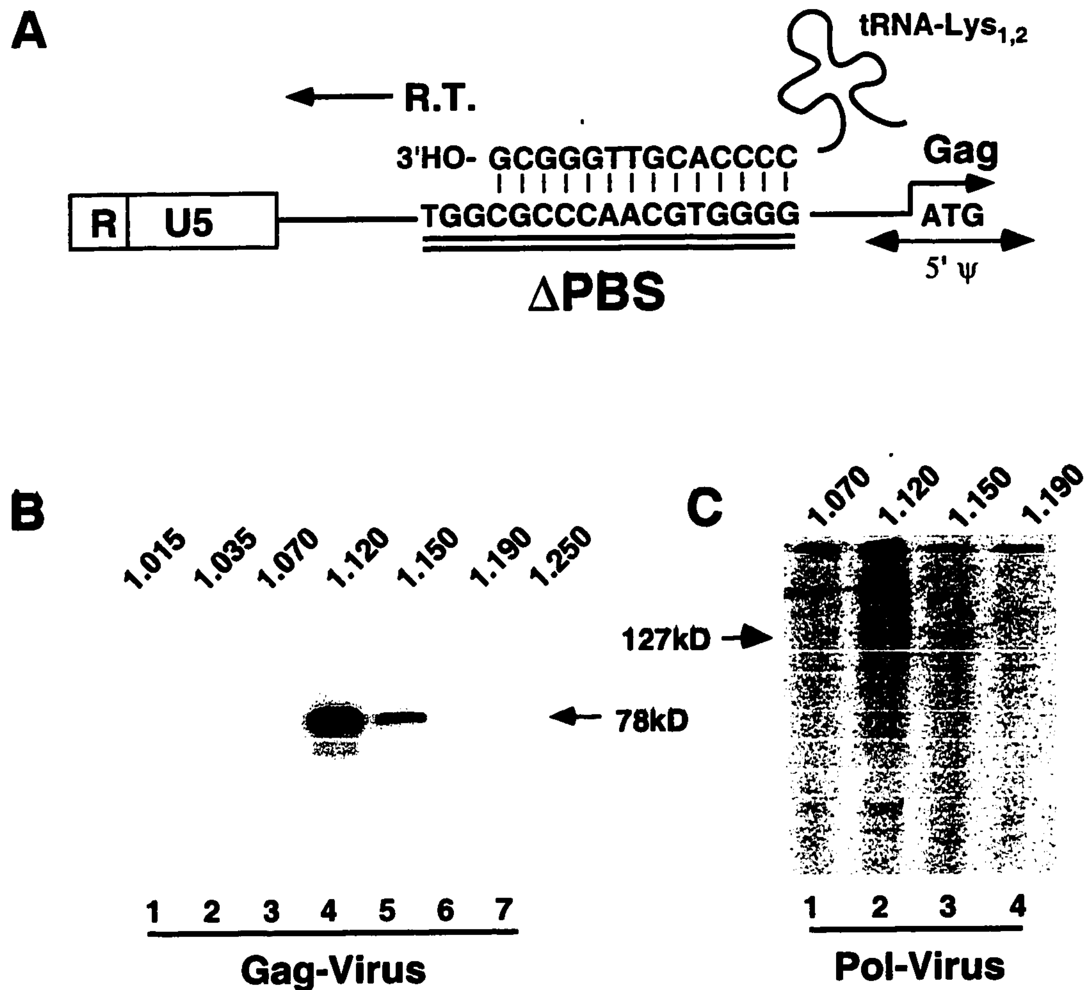


Figure 5.5 Gradient purification and analysis of ΔPBS-D/A-PKA. (A) Detailed schematic of the PBS deletion on the RNA genome. Shown is the homology with the 3' end of tRNA-Lys1,2 from which reverse transcription is initiated. Deleted nucleotides are underlined. Putative 5' encapsidation signal is labelled ψ. (B) Western blot analysis of Gag. Lanes 1-7 correspond to fractions 1-7. Fraction densities are listed above each lane. (C) IP/PKA analysis of Pol proteins. Lanes 1-4 correspond to fractions 3-6 shown in (B).

unable to detect Pol in virus particles without radiolabelling *in vitro*. The regulation of Gag and Pol stoichiometry is an important aspect of retroviral replication. Conventional retroviruses have evolved sophisticated mechanisms to regulate both Pol expression and activation [reviewed in Refs. (Jacks, 1990), (Craven et al., 1991)]. Synthesis of Pol as a Gag-Pol fusion protein keeps the level of Pol protein low, allows incorporation of Pol into particles via Gag domains, and prevents activation of Pol until a subsequent round of infection. It could be detrimental to the host cell to have active RT in the cytoplasm where mRNA substrates are abundant. In the case of conventional retroviruses, overexpression of Gag-Pol and the resulting activation of PR in the cytoplasm has been shown to negatively affect virus assembly (Karacostas et al., 1993). While on average, retroviruses contain two copies of their genomic RNA per virion, they contain one Gag-Pol fusion for every twenty Gag molecules (Jacks, 1990), or about 100 Pol proteins per virion (Vogt and Simon, 1999). Hepadnaviruses have a much lower ratio of P protein to core protein per virion. Since P binds to the ε signal in the RNA, there are only one or two P proteins per virion (Bartenschlager and Schaller, 1992). It is not known how many Pol molecules there are per HFV particle. If Pol-RNA interactions are required, then the ratio of Pol to Gag in virions could be as low as 1-2 Pol proteins per virion, which is consistent with difficulty in detecting Pol in particles. However, even if Gag::Pol interactions are responsible for assembly, low levels of Pol synthesis could be the limiting factor. Quantitative analyses of the ratio of Gag and Pol in cells and particles have not yet been done.

The mechanisms for regulation of expression and activation of HFV Pol are not known. Splicing to generate *pol* mRNA is one step where regulation could occur (Enssle et al., 1996; Lochelt and Flugel, 1996; Yu et al., 1996). Translation of the spliced mRNA may also be regulated, as the *pol* gene contains a very long 5' untranslated region (UTR). I was unable to get Pol expression from CMV promoter constructs lacking the *bona fide* splice junction (data not shown), consistent with a role for the UTR in protein expression. It has recently been shown for HBV that the dicistronic message which results in P protein expression, is regulated at the translational level (Hwang and Su, 1998). The

ΔATG mutant, however, leads to higher levels of Pol expression than Gag expressing proviruses (compare Figure 5.2C, lanes 1 and 2). Perhaps for the ΔATG mutant, Pol can be translated from both the spliced and unspliced mRNAs, whereas for wild type HFV, *gag* translation downmodulates *pol* expression during viral replication. Another outstanding question is how how Pol is activated. My data suggest (Figures 5.1 and 5.2) that PR activity is not important for Pol incorporation, but how are the activities of PR and RT temporally controlled? It is likely that cleavage of Pol plays a role in activation, a step which appears to be initiated after Pol incorporation. This is the case for other retroviruses where RT activity is dependent on cleavage by PR during virion maturation. For avian leukosis virus (ALV), Gag-Pol precursors have very little RT activity until processed by PR *in trans* after assembly and during maturation (Stewart and Vogt, 1991). While the mechanisms regulating retroviral maturation remain a mystery, the regulation of RT activation is clearly an important issue to both the virus and its host. Perhaps a molecular chaperone, such as one of the Hsp family members, sequesters the Pol protein until it can find its binding partners. In the case of HBV reverse transcriptase, the chaperone Hsp90 binds to and is essential for the activity of the enzyme during assembly (Hu and Seeger, 1996; Hu and Seeger, 1996; Hu et al., 1997).

In this chapter, I examined the mechanism of Pol assembly in HFV. I have considered both Pol::protein and Pol::RNA interactions, as well as the role of proteolysis. I have found that neither the activity of the viral protease nor the PBS is required for assembly of Pol into particles. My studies to date, therefore, do not answer the question of whether Pol incorporation occurs through protein::protein, or protein::RNA interactions. If protein::protein interactions are important, the PR domain remains a likely candidate. Delineation of a region in Gag critical for Pol incorporation is an important next step.

If Pol::RNA interactions are important, the region of the HFV genome located at the 3' end of the *pol* gene which has been reported to be critical for HFV vector transfer (Erlwein et al., 1998; Heinkelein et al., 1998), is a good candidate. While the role that this genome region plays in vector transfer is not known, it might contain sequence

information or secondary structure which directs Pol binding. If the *pol* region of the genome is important for Pol assembly, the spliced *pol* mRNA might interfere, but this mRNA should not be packaged since it does not contain the putative Ψ region required for vector transfer. Alternatively, the presence of ribosomes on the *pol* message might block the ability of the RNA to form the appropriate secondary structure for Pol recognition. Additionally, post-translational targeting of Pol protein would also be required. Pol proteins would then have a much higher probability of encountering the abundant viral genome than the spliced *pol* message in the cytoplasm. If Pol-RNA interactions are important for assembly, then genome dimerization could also play a role in secondary structure formation, and hence Pol assembly. Dimerization of Pol proteins and viral genomes might even act in concert to assemble Pol and RNA. It is also possible that Env might play a role in the assembly of Pol since Gag-Env interactions are required for very late stages of assembly (Baldwin and Linial, 1998).

While these studies do not reveal the actual mechanism of Pol assembly, they have ruled out two essential processes in the replication pathway. In the following chapter of this thesis, I examine domains of Gag and Pol involved in Pol assembly, and try to uncouple RNA packaging and Pol incorporation in Gag mutants. Delineation of a mechanism could come from Gag mutants lacking only one of these functions.

Chapter 6
The Mechanism of Pol Incorporation

A. Background.

In chapter 5, I developed methods to study the incorporation of Pol into virions. I showed that neither the primer binding site (PBS) nor PR activity are required to assemble Pol into particles. Despite important similarities with hepadnaviruses, I have previously shown that the foamy virus assembly pathway with respect to Pol is more like that of retroviruses than hepadnaviruses (Baldwin and Linial, 1998). Foamy virus Pol is not required for assembly, packaging of viral RNA, or virus egress. The important question of how Pol is packaged has remained elusive. Pol packaging must occur by a mechanism unique to foamy viruses, and distinct from other retroviral family members since HFV Pol has no Gag domains. The mechanism of Pol incorporation must also be distinct from that of the hepadnaviruses since HFV Pol is dispensible for assembly and genome packaging.

HFV Pol packaging may involve complex interactions between Gag and genomic RNA. I have shown that reverse transcription is not coupled to Pol packaging (Baldwin and Linial, 1999), but this does not rule out a role for RNA. *Cis*-acting sequences in the genomic RNA required for virus replication have been identified, and at least two distinguishable regions are essential for vector transfer. One region is near the 5' end of the genome, in a similar location to retroviral packaging signals, and the other resides at the 3' end of *pol* (Erlwein et al., 1998; Heinkelein et al., 1998; Wu et al., 1998). While the region at the 5' end of the genome is probably important for genome encapsidation, it is not yet clear what role the 3' *cis* element in *pol* plays. It could play a role in reverse transcription since it contains polypurine rich regions, it could play a role in RNA packaging, or it is possible that the requirement for this region in vector transfer is related to Pol packaging. The *trans*-acting regions of Gag important for RNA packaging have been studied with recombinant proteins, but have not been carefully analyzed in the viral

context (Yu et al., 1996). In this chapter I study the effects on RNA packaging of various Gag mutants, hoping to understand what role RNA may play in Pol incorporation. To date, I have not found Gag mutants whose ability to incorporate RNA and Pol proteins can be separated.

In this chapter, I have defined regions in both Gag and Pol which are required for Pol incorporation. I have found that viruses lacking the PR domain of Pol, or a basic region in the carboxy terminus of Gag no longer assemble Pol into virions. I have also found that these regions in Gag and Pol important for Pol incorporation are also important for protein::protein interactions by demonstrating specific interactions with the yeast two-hybrid system (Fields and Song, 1989). Lastly, I attempted to evaluate the role of genomic RNA in Pol incorporation. My truncation mutant of Gag which no longer incorporates Pol is also defective in RNA packaging, to the limit of the sensitivity of the assay for Pol incorporation, so I cannot rule out the possibility that RNA is involved.

B. Results.

1. HFV PR is required for assembly of Pol into virions. Although protease activity and the primary sequence surrounding the cleavage site in Gag are not important for incorporation of Pol into virions (Baldwin and Linial, 1999), the PR domain might still be required. To test this, an in-frame 88 amino acid deletion including the active site of PR was generated by PCR mutagenesis using HFV-PKA as a template. The resulting Pol protein could be detected in transfected cells at similar levels to other Gag expressing proviruses (Figure 6.1A, lane 1). Gag was expressed normally, but was not cleaved, as expected (Figure 6.1B, lane 2). When the ΔPR mutant virus was gradient purified, the Gag proteins were detected at the appropriate density of 1.12-1.15g/cm^3 (Figure 6.2A, lanes 2 and 3). In contrast, I found that no Pol protein could be detected in these virions (Figure 6.2B, lanes 6 and 7). This experiment was performed several times, each time using the HFV-D/A-PKA construct as a positive control for Pol incorporation (Baldwin and Linial, 1999). These results suggest that while the proteolytic activities of HFV PR

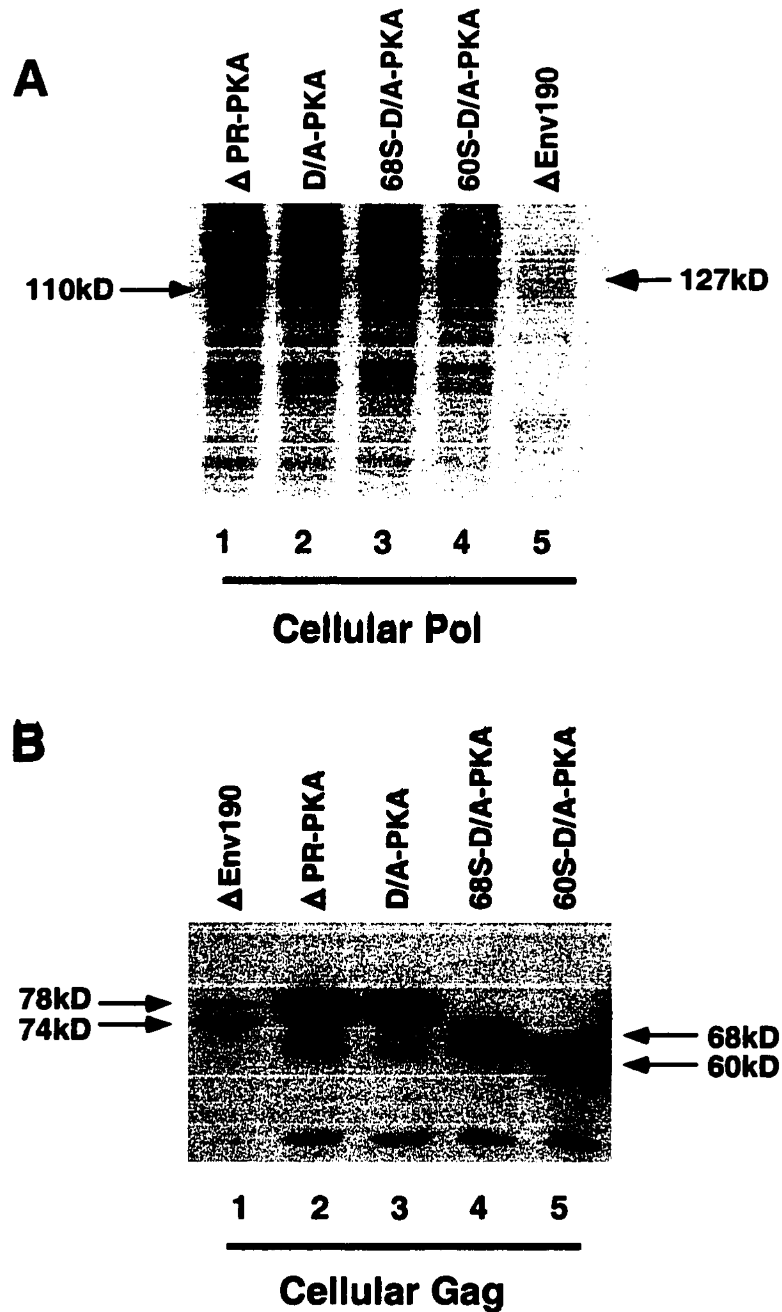


Figure 6.1 Analysis of cellular Pol (A) by IP-PKA analysis and cellular Gag (B) by western blot analysis. (A) Lanes: 1, ΔPR-PKA; 2, D/A-PKA; 3, 68S-D/A-PKA; 4, 60S-D/A-PKA; 5, ΔEnv190. Arrows indicate PR deleted (110-kD) and full-length (127-kD) Pol proteins. (B) Lanes: 1, ΔEnv190; 2, ΔPR-PKA; 3, D/A-PKA; 4, 68S-D/A-PKA; 5, 60S-D/A-PKA. Arrows indicate wild type HFV Gag proteins (78-kD and 74-kD), and truncated forms (68-kD and 60-kD).

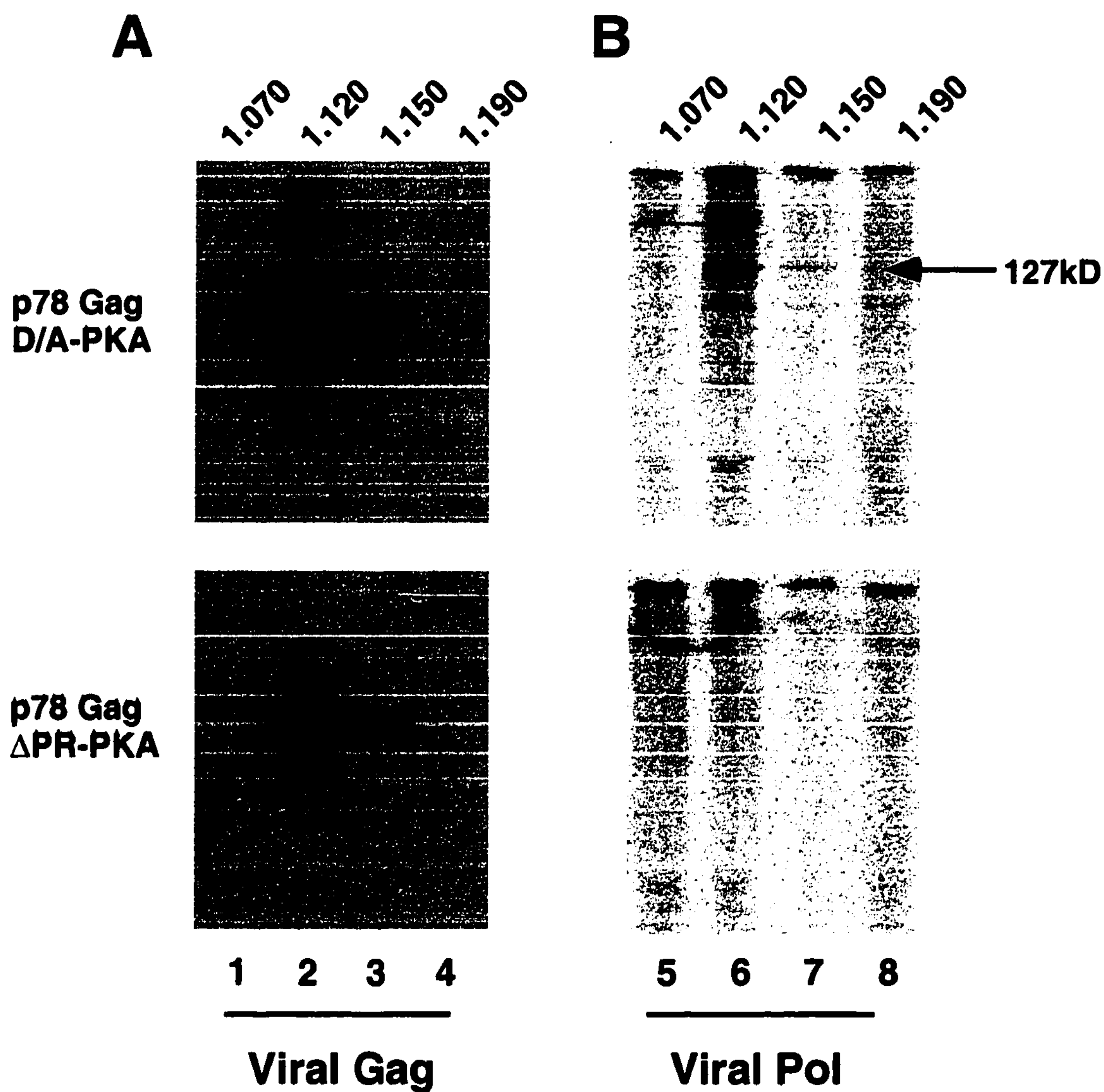


Figure 6.2 Gradient purification and analysis of D/A-PKA and ΔPR-PKA. (A) Western blot analysis of gradient fractions for viral Gag. Lanes 1-4 correspond to gradient fractions 3-6. (B) IP/PKA analysis of fractions 3-6 from the same gradients shown in (A). Lanes 5-8 correspond to fractions 4-6. For both panels, the top set are D/A-PKA, and the bottom set are ΔPR-PKA. Fraction densities (g/cm3) are listed at the top of each lane.

are not required for assembly of Pol into virions, the PR domain itself is essential for Pol incorporation.

2. A p60 Gag truncation mutant no longer incorporates Pol. In the previous chapter, I demonstrated that a C-terminal truncation mutant of Gag (p68 Gag) is capable of incorporating the Pol precursor protein. To further address which domains of Gag are required for Pol incorporation, I have tested a larger C-terminal truncation mutant of Gag which expresses a 60-kD protein. p60-Gag is deleted for two of the three glycine-arginine rich regions of Gag (GR boxes) including the nuclear localization signal of unknown function (NLS-GR box II). In the context of the protease active site (D/A) and PKA mutations, it is referred to as 60S-D/A-PKA. It should be noted that the Gag truncation mutants are named with respect to their migration pattern in comparison with Benchmark prestained molecular weight markers (Gibco-BRL), and not according to their calculated mass (see Materials and Methods, Recombinant DNAs).

Cells transfected with 60S-D/A-PKA express Gag at levels similar to other Gag mutants (Figure 6.1B, lane 5). p60-Gag migrates as expected when compared with wild type Gag (p78), a mutant truncated at the cleavage site (p74), and the p68 mutant when analyzed by western blot analysis (Figure 6.1B, lane 5). The Pol protein expressed from this provirus is also expressed at levels similar to other PKA mutants (Figure 6.1A, lane 4). Cells expressing 60S-D/A-PKA form particles which bud normally from the cell. These particles band on a density gradient of iodixanol with wild type density (Figure 6.3A, lanes 1-3). I found that the gradient fractions which contain p60 Gag do not contain the Pol protein (Figure 6.3B, bottom, lanes 6-8), whereas in the case of the positive control, HFV-D/A-PKA, the Pol protein is readily detectable in the same fractions as the 78kD Gag (Figure 63B, top, lanes 2 and 7). A Gag deletion mutant, ΔATG-D/A-PKA (Baldwin and Linial, 1999), was repeatedly tested as a negative control to show that extracellular Pol proteins do not band on gradients in the absence of Gag (Baldwin and Linial, 1999).

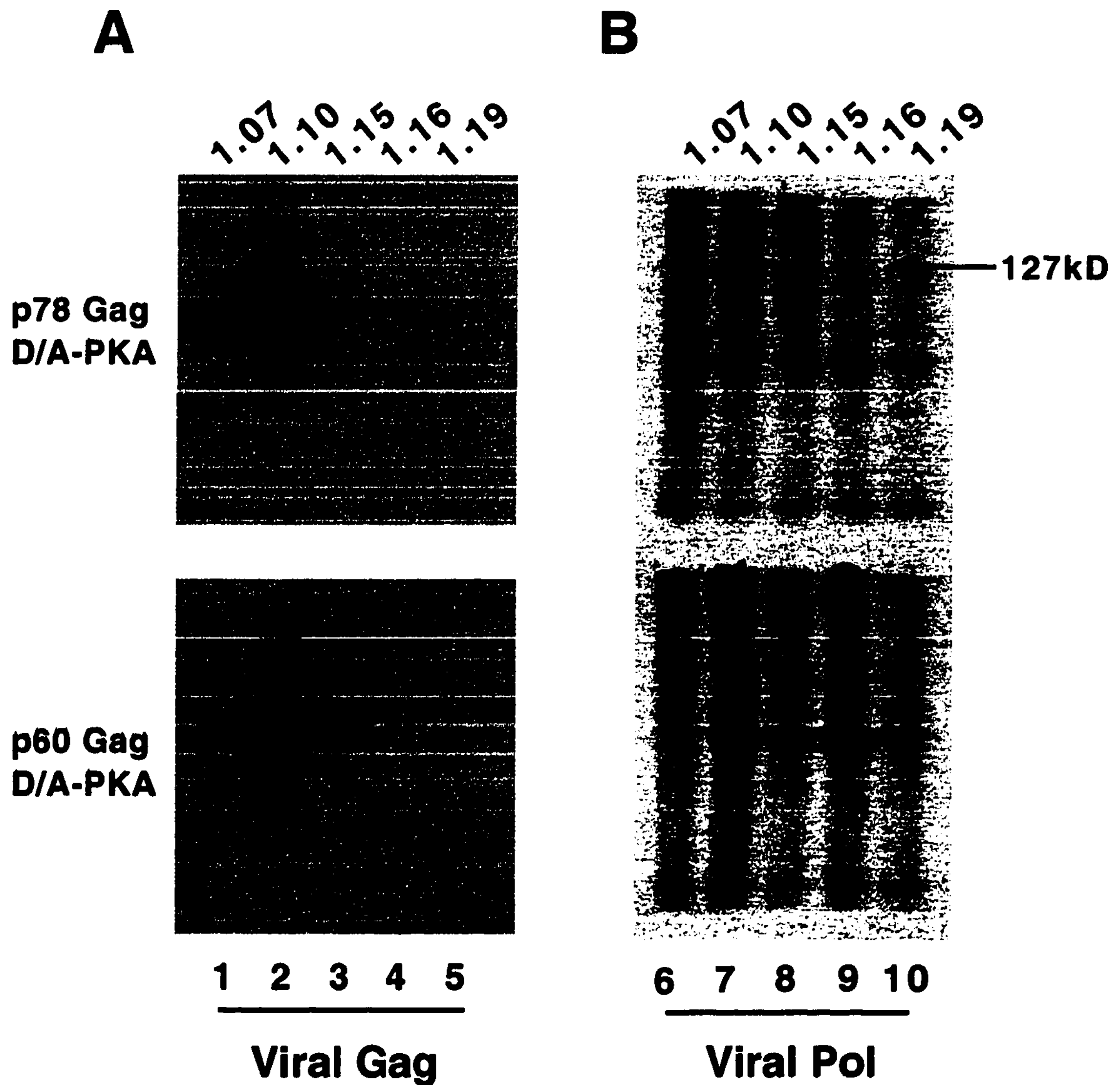


Figure 6.3 Gradient purification and analysis of D/A-PKA and 60S-D/A-PKA. (A) Western blot analysis of gradient fractions for viral Gag. Lanes 1-5 correspond to gradient fractions 2-6. (B) IP/PKA analysis of fractions 2-6 from the same gradients shown in (A). For both panels, the top set are D/A-PKA, and the bottom set are 60S-D/A-PKA. Fraction densities (g/cm3) are listed at the top of each lane.

3. Virus encoding p60 Gag is deficient in both genome and Pol packaging. I was interested to know whether the p60 mutant contained genomic RNA. I used RNAse protection analysis (RPA-described in (Baldwin and Linial, 1998)) to study the RNA packaging of p60-Gag virions. One glycine-arginine (GR) rich region of Gag known to be important for nucleic acid binding (GR box I) is still contained in p60 Gag (Yu et al., 1996). I have found however, that GR box I is not sufficient for wild type levels of genome packaging. When similar amounts of virus were assayed for the presence of genomic RNA, the packaging was reduced by ten fold (Figure 6.4B, lane 3) with respect to the protease active site mutant of Pol which is wild type for assembly and packaging (Figure 6.4B, lane 1) (Baldwin and Linial, 1998). Dilutions of viral Gag were used to verify that virus quantities were similar. Western blot signals were equivalent for each virus at all dilutions tested (Figure 6.4A, lanes 2-4). Since an *env* deletion mutant (ΔEnv190, (Baldwin and Linial, 1998)) does not release particles, it serves as a control for the presence of viral RNA in transfected cell supernatants lacking viral particles. 60S-D/A-PKA packaged RNA less than two fold above the background of Mock, or ΔEnv190 tranfected cell supernatants (Figure 6.4B, lanes 4 and 5).

4. A region between p60 and p68 of Gag is critical for protein::protein interactions between Gag and PR. I used the yeast two-hybrid system to test for possible protein::protein interactions between PR and Gag. This system has been used both to discover, and to further characterize a wide range of protein::protein interactions (Fields and Song, 1989; Vidal et al., 1996; Vojtek et al., 1993). The principle is depicted in Figure 6.5. The basic idea evolved from the knowledge that transcription factors such as LexA or Gal4 contain separable domains for DNA binding (DB; DNA binding) and activation of transcription (AD; activation domain). Heterologous proteins, in this case viral proteins, can be fused to the C-termini of these domains, and tested for their ability to interact. If interaction does occur, this "two-hybrid" complex will bind to target sequences in the chromosomal DNA (UAS; upstream activating sequence), and recruit RNA polymerase via the AD to drive expression of a downstream marker. The L40

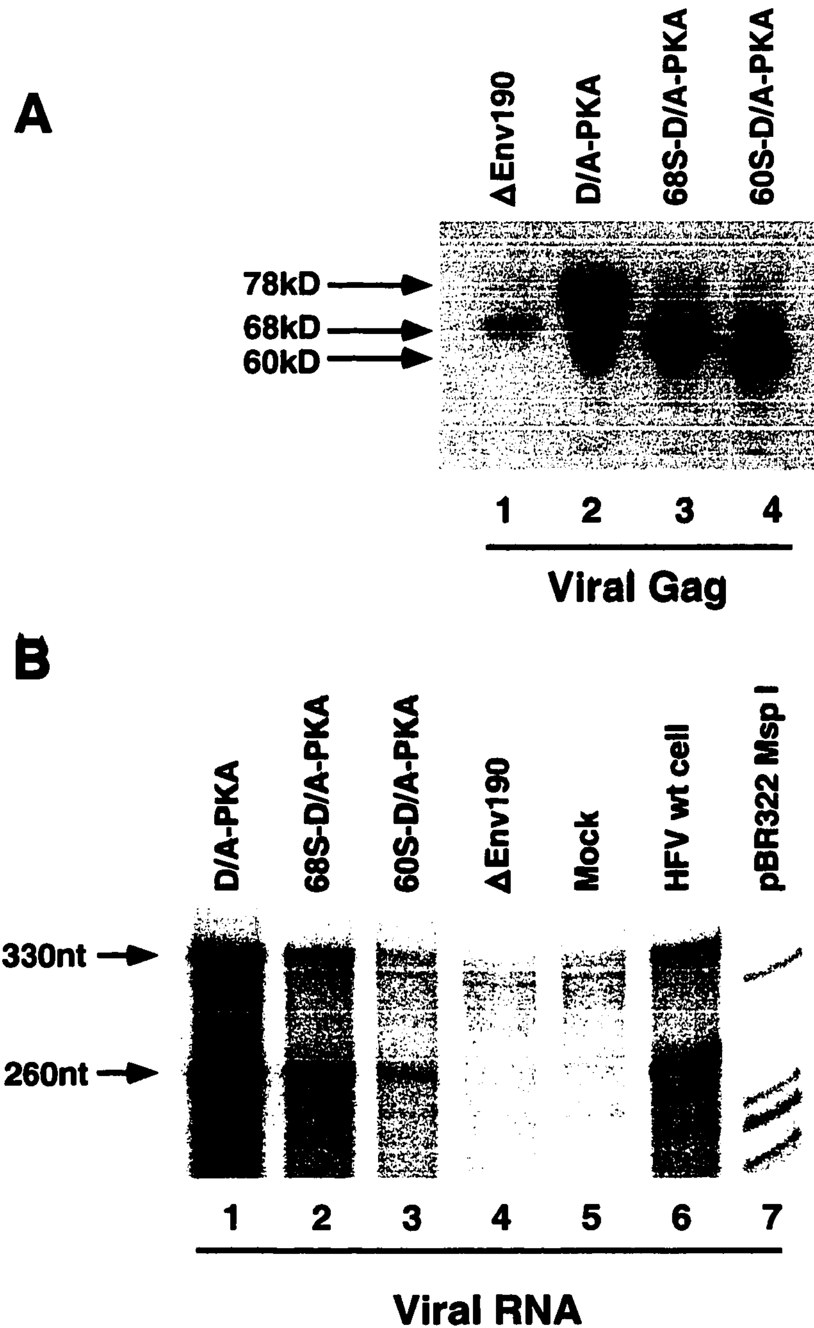


Figure 6.4 RNA packaging analysis of Gag truncation mutants. (A) Western blot analysis of viral pellets with Gag anti-serum. Lanes: 1, ΔEnv190; 2, D/A-PKA; 3, 68S-D/A-PKA; 4, 60S-D/A-PKA. (B) Ribonuclease protection of viral RNA. Lanes: 1, D/A-PKA; 2, 68S-D/A-PKA; 3, 60S-D/A-PKA; 4, ΔEnv190; 5, Mock; 6, WT-HFV cellular RNA; 7, pBR322 Msp I digested marker.

strain, described in Chapter 2, contrains separate markers for either growth on His- plates (HIS3), or ß-galactosidase.

Gag and PR were both C-terminally fused to the activation domain of VP16, and to the DNA binding domain of LexA. These plasmids contain auxotrophic markers for selection on Leu-(L-) and Trp-(W-) respectively. Wild type Gag (p78), and each truncation mutant of Gag tested (p74, p68, p60) interacted with p78Gag efficiently, and displayed growth kinetics under His selection (H-)(Figure 6.6A) for interaction which were similar to those of Ras::Raf interactions (data not shown) (Vojtek and Hollenberg, 1995). Ras::Raf interactions were discovered using this system, and are known to be relatively strong under growth selection, and also for ß-galactosidase activity in the absense of selection. When the filter ß-gal assay was used, blue color developed in the yeast colonies expressing Gag::Gag, and Ras::Raf, but not for Gag::PR. Gag::PR interactions could be measured under His selection for the protein::protein interaction but the growth was reduced as compared with Gag::Gag (Figure 6.6B). PR::PR interactions were also detected with growth kinetics similar to Gag::PR interactions (data not shown). This was not unexpected since PR is known to be active as a dimer in vitro (Pfrepper et al., 1997).

To further study these interactions, a lysate-based assay was developed to quantitate the ß-galactosidase activity in cells where the interaction could be detected under growth selection, but not with the filter ß-galactosidase assay. Cotransformed cells were grown in the absense of Trp and Leu, and harvested during log phase. Cells were gently lysed (see Chapter 2), and CPRG substrate for ß-galactosidase was added to normalized amonts of lysate. Again, activity was detected for all Gag::Gag interactions, but not the weaker interactions of Gag::PR or PR::PR (data not shown).

When cotransformed cells were plated on selective media (WHL-), the number of colonies for Gag::PR, and PR::PR were approximately 50-fold reduced with respect to Gag::Gag. This was not due to differences in transformation efficiency because when the same transformations were plated on media which selected for the two plasmids (WL-) and not the two-hybrid interaction (WHL-), the numbers of colonies were identical.

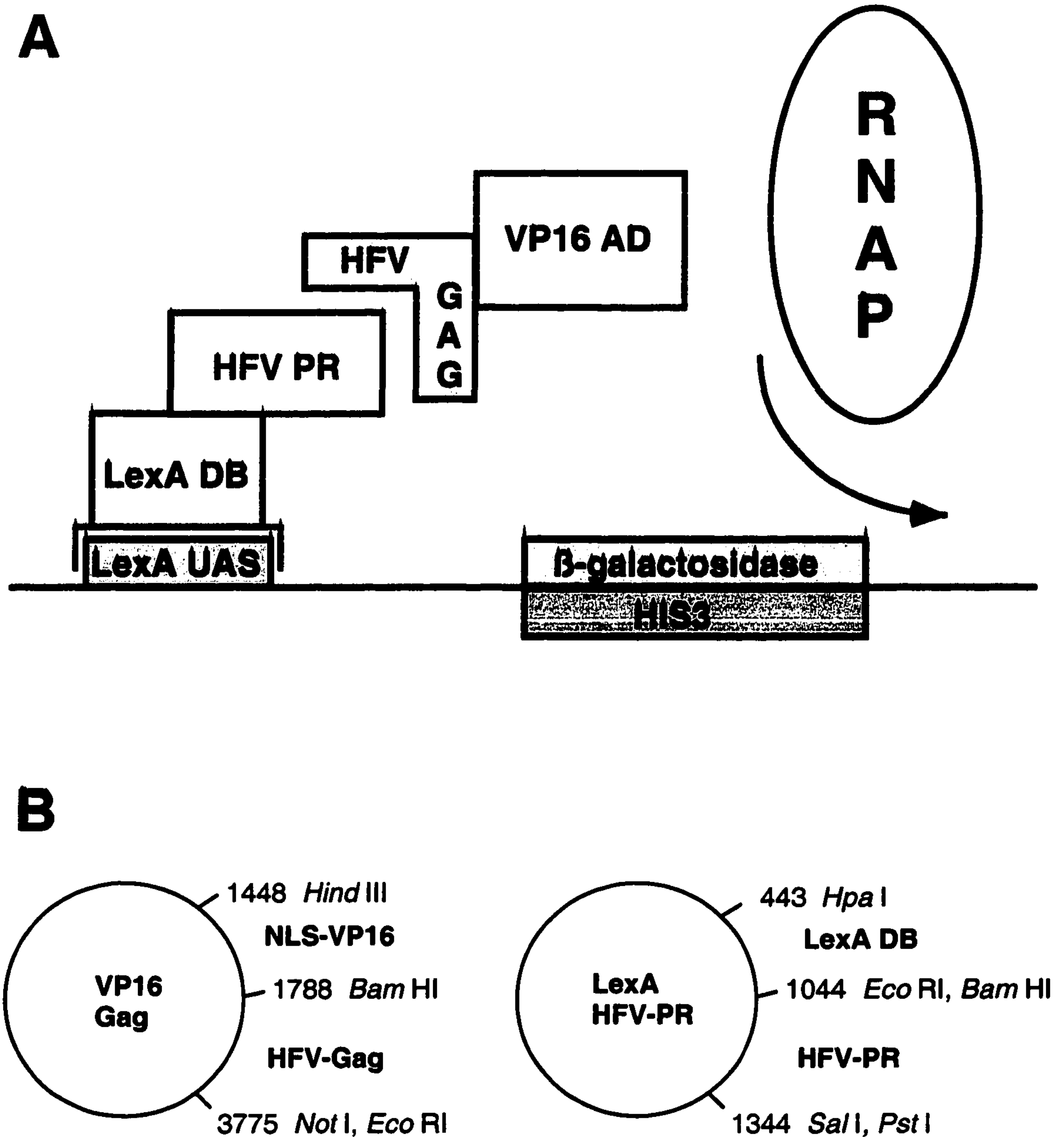


Figure 6.5. The two-hybrid system. (A) Schematic of two-hybrid interaction. HFV Gag::Gag, Gag::PR, or PR::PR interactions position the VP16 activation domain at the LexA promoter (UAS), and recruit RNA Polymerase II to express either ß-galactosidase, or HIS3 in the absense of histidine. These integrated markers are on different chromosomes. (B) Simplified maps of the two-hybrid plasmids showing restriction sites used for cloning HFV proteins in-frame with either LexA or VP16.

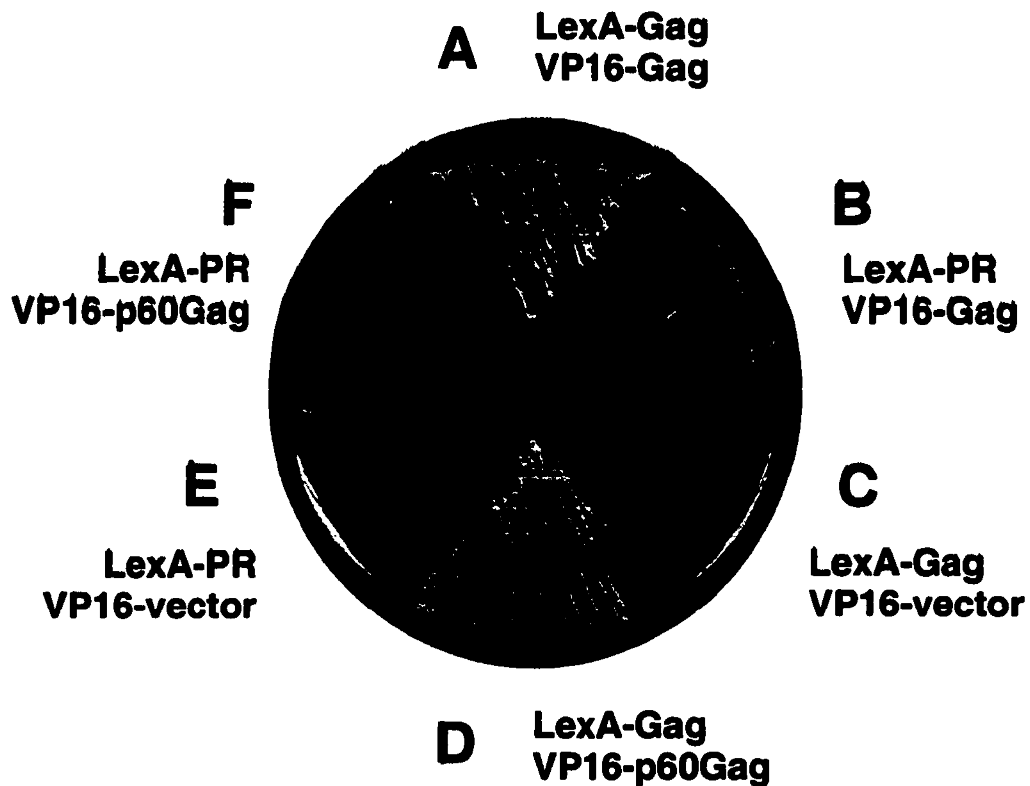


Figure 6.6 Two-hybrid analysis; His (-) selection for protein::protein interactions. (A) Wild type Gag fused to both the LexA DNA binding domain and the VP16 activation domain. (B) HFV PR fused to LexA-DB and HFV Gag fused to VP16-AD. (C) LexA-Gag and VP16 (-). (D) LexA-HFVGag and VP16-p60Gag. (E) LexA-HFVPR and VP16 (-). (F) LexA-PR and VP16-p60Gag. For each section of the plate, growth indicates positive two-hybrid interaction.

Neither Gag nor PR permitted growth on selective media when cotransformed with the empty LexA or VP16 vectors (Figure 6.6C, E).

C. Discussion.

In this chapter I have further defined the components required for HFV Pol incorporation. I have found that protein::protein interactions between Gag and Pol can be detected, and that the domains required for these interactions are critical for assembly of Pol into virions. It is perhaps not surprising that these interactions are between PR and the carboxy terminus of Gag, in a region not far from the only cleavage site known to occur in mature virions (Bartholoma et al., 1992). I have previously shown that the 4-kD product of Gag cleavage, which is released from approximately 50% of virion associated Gag molecules, is not required for Pol incorporation. Indeed, approximately 10-kD of primary Gag sequence surrounding the cleavage site is not required (Baldwin and Linial, 1999).

PR presumably interacts with Gag in such a way that the cleavage site in Gag is positioned near the active site of PR, but the protein::protein interaction which establishes the initial contact could take place at a distal site in Gag or PR. I have found that a region between p60 and p68 of Gag is important for Pol incorporation, and that this region is also required for protein::protein interactions with PR in a yeast two-hybrid assay. I do not yet know where in these regions of Gag and PR that contacts are made, but it will be interesting to screen for mutants within these regions. One important goal of this screen would be to identify mutants which are no longer capable of incorporating Pol, but are still able to package genomic RNA. To fully understand the mechanism of Pol incorporation, it will be important to characterize mutants which are defective in either Pol incorporation, or RNA packaging, but not both.

PR may be required because Pol is incorporated through Gag::PR or Pol::RNA interactions. In a model where RNA is required for Pol assembly, PR might be important for the dimerization of Pol such that the RT or IN domains can efficiently recognize their

RNA target. The only Gag mutant I have isolated which buds normally, but does not contain Pol protein, is also deficient in genome encapsidation. If it did contain RNA, then I could conclude that Pol assembly is mediated by protein::protein interactions alone, and that these occur between PR and Gag. However, since the p60Gag mutant did not contain wild type levels of RNA, one can propose a model where Pol::RNA interactions might still be required for Pol incorporation.

If the PR domain is simply required for dimerization of the Pol precursor, and this dimerization is required for proper recognition of secondary structure in the genome, then it is possible that I have mapped a region in Gag which is recognized by PR during the cleavage of Gag, but only after assembly and RNA packaging is complete. If RNA is a critical component for Pol incorporation, then very low levels of Pol would be incorporated into particles since the typical retrovirus packages an average of two RNA genomes per virion. It is not yet known how many copies of HFV genome are encapsidated per particle. Hepadnaviruses replicate efficiently by incorporating very low levels of RT into particles as a consequence P protein::RNA interactions initiating assembly, however, retroviruses typically contain at least 100 Pol proteins per particle since Gag-Pol fusions are incorporated via Gag::Gag interactions (Jacks, 1990; Vogt and Simon, 1999). I have had great difficulty in detecting Pol proteins in virions, and this may be due to the mechanism of Pol assembly not permitting high levels of Pol incorporation.

In addition, I have previously shown that the Pol protein can be incorporated as an uncleaved precursor (Baldwin and Linial, 1999). This suggests that Pol is not active until after assembly, despite high levels of reverse transcription in late stages of the replication pathway. This observation may or may not reflect the natural assembly pathway of HFV. Others have shown that when Pol is overexpressed alone in the cell, cleavage can occur to release the IN domain from some of the precursors (Martin Heinkelein, personal communication). Perhaps like other retroviruses, dimerization of Pol (and presumably PR) is the critical step for the activation of PR, and this can occur prior to assembly. This would fit the data from work on recombinant HFV PR being active as a dimer (Pfrepper

et al., 1997), and also the work on other retroviral aspartic acid proteases. For all of these proteases, dimer formation is critical for generating an proteolytic active site. If the conformation of PR does not differ between states of Pol cleavage, then both forms of Pol may be assembly-competent.

If the cleaved form of Pol can be assembled, then the models for Pol incorporation become more complex. Either tight association of the cleaved IN domains with PR-RT would be required, or IN must have an assembly pathway independent of PR-RT. If RT is active prior to assembly, then perhaps IN recognizes regions of the DNA genome during late stages of replication and prior to assembly. Some evidence suggests that PR-RT::IN interactions may be quite strong. After immunoprecipitating Pol-PKA proteins with anti-RH antibodies, and performing PKA analysis which includes stringent washes, I have found that both labeled precursor and the cleaved IN proteins can be detected when the complex is dissociated during SDS-PAGE (Baldwin and Linial, 1999). This implies that the complex between PR-RT and IN is in fact very stable. It has also been shown for the retrotransposon Tf1 of *Schizosaccharomyces pombe* that RH::IN interactions can be detected in the two hybrid system (Steele and Levin, 1998).

Most retroviruses complete reverse transcription during the early stages of replication, after infection of the new cell. During the infection process, the capsid is disassembled and the preintegration complex is transported to the nucleus while the genome is converted from RNA to DNA. In the case of MLV, reverse transcription is completed prior to nuclear translocation. This preintegration complex (PIC) has been purified biochemically from newly infected cells, and this complex which is capable of *in vitro* integration contains both the RT and IN domains (Brown et al., 1987; Miller et al., 1997), implying that other retroviral *pol* gene products remain associated at least until reverse transcription is completed. The interactions responsible for this complex association of capsid proteins, nucleic acid, and the viral enzymes have been difficult to study since the PIC must be biochemically purified from infected cells, but some evidence suggests that the RT and IN proteins remain associated in the PIC after the capsid proteins are dissociated (Miller et al., 1997). These PICs were isolated from

newly infected cells, because reverse transcription takes place after infection for HIV and MLV. For HFV, cleavage and complex formation of RT and IN might take place prior to assembly if PR is in fact activated prior to assembly.

It would also be interesting to know the structure of the foamy virus Pol proteins. There is a putative linker region between the PR and RT domains of Pol, and this domain may have a specific structure involved in the assembly pathway. This linker region has no homology to known retroviral Pol proteins. By deleting the eighty two amino acids of PR (ΔPR-PKA), I may have disrupted some important structure in this linker region, rather than PR itself, thereby preventing Pol incorporation. It would be interesting to make deletions in the region between PR and RT and study the effects on PR activity and Pol incorporation. Perhaps this linker permits the activites of both PR and RT in the PR-RT protein by separating their folded domains. For studying the evolutionary relationship between foamy viruses and their RT-encoding relatives, it might useful to compare the structures of the PR and RT domains. Some effort has been made to crystallize the carboxy terminus of Gag (Strong, R., Yu, S., and M. Linial, unpublished results), without success, but it would be very informative to crystallize the complex of PR with the carboxy terminus of Gag. At the very least, it should be possible to crystallize the PR domain which can be purified in an active recombinant form.

Perhaps learning more about the interactions which govern assembly will shed light on the steps required for Pol activation. One group has reported that a *cis*-acting sequence in the 5' region of the genome is important for proteolytic processing of Gag. These data suggest that the genomic RNA may be promoting the activation of PR in the viral context. Another region in *pol* is critical for vector transfer of HFV RNA (Erlwein et al., 1998; Heinkelein et al., 1998), and could imply a role for this part of the genome in Pol packaging. With mutations which abolish HFV Pol incorporation, RNA packaging, PR dimerization, or combinations of these, we may be able to dissect the pathways of both assembly and activation for HFV RT.

Chapter 7
Conclusions and Future Directions

In this study, I have examined the role of Pol in the replication pathway of HFV. We discovered that unlike all known retroviruses, the Pol protein is expressed independently of Gag from a spliced mRNA, rather than as a Gag-Pol fusion protein (Yu et al., 1996). Subsequently, I compared the basic assembly strategies of foamy viruses, retroviruses and hepadnaviruses. Hepadnaviruses, the only other mammalian reverse transcriptase-encoding viruses, also express their Pol protein independently from their structural proteins, but their assembly strategies are very different than those of retroviruses. I found that assembly with respect to Pol in HFV is more like retroviruses than hepadnaviruses (Baldwin and Linial, 1998). In contrast, however, I found that like hepadnaviruses, there is a requirement for the surface glycoproteins of HFV for virus release from the cell (Baldwin and Linial, 1998). Lastly I developed novel methods for detecting the Pol proteins in extracellular virions (Baldwin and Linial, 1999), and defined domains of Pol and Gag which are important for Pol incorporation into particles (Baldwin and Linial, 1999).

One goal of this work has been to try to determine if there is a functional evolutionary relationship between retroviruses, hepadnaviruses, and the foamy viruses. Although the foamy viruses are clearly retroviruses, an interesting question is whether they might be a common ancestor to both retroviruses and hepadnaviruses, whether they might represent an evolutionary bridge between them. Sequence information cannot tell us this, but perhaps functional relationships could provide insight. Retroviruses and their relatives are very old in evolutionarily terms. They are found in almost all vertebrates studied, and have very diverse tropisms. Retroviruses have ancestors in many invertebrate organisms such as Ty elements in yeast, and *copia* in *Drosophila*, suggesting that they have evolved over a very long period of time. However, hepadnaviruses are in some ways more highly evolved. Every nucleotide of their genome codes for protein, and their genomes are considerably smaller and more efficient than those of retroviruses.

Since they infect cells which do not divide very often, they have evolved an intracellular reinfection pathway which permits amplification of the genome without cell division (Seeger and Mason, 1996). They do not encode either of the retroviral enzymes protease or integrase. In addition, they have evolved an assembly strategy which couples P protein incorporation with replication of the genome (Bartenschlager et al., 1990; Bartenschlager and Schaller, 1988; Hirsch et al., 1990; Kochel et al., 1991; Pollack and Ganem, 1994; Wang and Seeger, 1992).

Alternatively, hepadnaviruses may have evolved independently from a distinct primordial reverse transcriptase. This theory is strengthened by the existence of plant para-retroviruses which are clearly distant relatives of the retrovirus family, and have genomic organizations more similar to those of hepadnaviruses. Hepadnaviruses, however, have evolved genes for surface glycoproteins in a similar region of their genome to the *env* gene of retroviruses. While it is possible that an ancient herbivore found itself infected with a plant pararetrovirus (which then evolved into the hepadnavirus family), I favor an evolutionary tree which contains the mammalian RT-encoding viruses being closely related, and plant reverse transcriptases being quite distant. When the *pol* genes of reverse transcriptase-encoding elements are compared phylogenetically, the HBV RT clusters with the retroviruses and not the plant, or *Drosophila* relatives (Doolittle et al., 1989; Doolittle et al., 1990). However, HBV RT is nevertheless distantly related to the retroviral RTs. In principle, an insect vector could have transmitted a mammalian virus to plants, but I consider this less compelling.

In the course of these studies, we have found that the mechanism of HFV Pol incorporation is unique to foamy viruses, and distinct from the rest of their reverse transcriptase-encoding relatives. Protein::protein interactions are important for assembling Pol into virions, but I still cannot rule out the possibility that the genomic RNA plays a role, as is the case for hepadnaviruses. If foamy viruses are indeed the precursor to both families of RT-encoding viruses, then retroviruses have since evolved the mechanism of Gag-Pol fusion protein expression. This is plausible since translational regulation of the genomic RNA is rarer and mechanistically more highly evolved than

RNA splicing, which all eukaryotes use for gene expression, and all retroviruses use for expression of Env and accessory proteins. If foamy viruses represent an evolutionary link between retroviruses and hepadnaviruses, then the divergent spliced mRNA mechanism for Pol expression may have led to other mechanisms of independent Pol expression (translational regulation for HBV), and novel assembly strategies. Understanding the role of RNA in HFV Pol assembly may shed light on this possibility. If a domain of HFV Pol does specify Pol::RNA interactions which are required for Pol packaging but not reverse transcription, then this would support an evolutionary model in which foamy viruses are predecessors to both retroviruses and hepadnaviruses. The N-terminal domain (TP) of HBV RT contains information for RNA recognition. While TP does not have sequence homology to any known protein, perhaps the foamy virus protease, or linker between PR and RT could have evolved an RNA binding function. We and others have had great difficulty detecting the Pol proteins in virions, indicating a low copy number and suggestive that association of Pol and RNA may be important for Pol encapsidation. Hepadnaviruses encapsidate one copy of genomic RNA, and retroviruses on average encapsidate two copies of their genome. If the ratio of Pol:RNA is 1:1 in HFV virions as is the case for hepadnaviruses, that might explain the difficulty we have detecting Pol in virions.

This work has answered a few important questions regarding the replication strategies of HFV, but several things remain unknown. The most important question is whether there is a role for RNA in the assembly of Pol into HFV particles. Perhaps protein::protein and protein::RNA interactions are both required for foamy virus Pol incorporation. This can be addressed in several ways. One way is to coexpress the viral proteins in the absence of any viral RNA packaging signals (complementation analysis). While this is technically possible, it might be difficult to engineer since there are thought to be RNA packaging signals in the Gag open reading frame. In addition, it is possible that the roles of protein::protein and protein::RNA interactions contribute equally to Pol incorporation, in which case neither alone might be sufficient. Ruling out RNA sequences present in the Gag coding region could be difficult without reconstructing a

Gag gene with entirely new codons which would change the structure of the RNA, but not the amino acid sequence of Gag. Little is known about the HFV RNA packaging signal. We do know that minor changes in minimal retroviral packaging signals can have a major impact on the efficiency of RNA packaging (Banks and Linial, 1999), so this approach is not out of the question. Another possibility is to look for mutants in Gag which are no longer capable of packaging either the Pol protein or genomic RNA. So far, I have only been able to isolate mutants which are defective for both, but it should be feasible to map the regions in Gag which are critical for one function or the other.

Complementation analyses to study the role of RNA in HFV Pol incorporation have been inconclusive. I have attempted to express Gag, Pol, and Env from heterologous promoters (CMV) where no genomic RNA is present, but have failed to recover enough extracellular virus to analyze for it various components. These expression constructs all contain wild type HFV sequences, but the expression of each gene is not temporally regulated as it would be during the course of infection. Pol expression is very high when expressed from the CMV promoter, and if protein::protein interactions govern Pol packaging, then Pol overexpression could have a detrimental effect on assembly and virus release. I am currently trying to demonstrate whether this is a possibility by comparing quantities of virus from Gag/Env and Gag/Pol/Env cotransfections. If virus is produced from Gag/Env but not Gag/Pol/Env cotransfections, one might speculate that overexpression of Pol can have a dominant negative effect on assembly by interfering with Gag multimerization, and implying that protein::protein interactions are critical for Pol incorporation.

Another intriguing idea is that RNA may play a role in the activation of HFV PR, and subsequently RT. It was recently reported at the International Foamy Virus Conference (Paris, 1999) that a region in the 5'-UTR of Gag is critical for PR activity in extracellular virions. It is not clear whether this observation is due to a defect in Pol incorporation or PR activation, since they are unable to detect Pol proteins or RT activity in virus particles which are not replication competent. They were unable to provide any evidence to distinguish between the models for the role of RNA in either Pol

incorporation or PR activation, but Gag was not processed in these virions. RNA does not appear to be involved in PR activation, and subsequent RT activation for other retroviruses (Craven et al., 1991; Karacostas et al., 1993; Stewart et al., 1990; Stewart and Vogt, 1991). In the case of avian retroviruses however, which encode both Gag-PR and well as Gag-PR-Pol, it is clear that the Gag-PR molecule must be present and active for the cascade of events which lead to maturation and RT activation. It is impossible to rule out a role for generic RNA in this process, but genomic RNA is not essential for PR or RT activity in most retroviruses. Retrovirus particles produced from packaging cell lines for example are active for both PR and RT.

Another question is how Pol incorporation and activation are temporally regulated. It should be possible to dissect the cascade of assembly-related binding events and their relationship with PR and RT activation. Using the two-hybrid system, one could perform mutagenesis on the viral protease with two goals in mind. First one could search for mutants which block the ability of HFV PR to self-dimerize. These could then be tested for association with wild type Gag. From these experiments, one might learn whether dimerization of PR is required or is inhibitory for Gag::PR interaction. One could make a library of PR mutants in the LexA vector, and cotransform this library with both VP16-Gag and VP16-PR. It would be interesting to see if mutants which block interaction with PR could actually increase the affinity of PR for Gag. This result would support a model for Pol incorporation in which Pol is incorporated as a monomer, and then activated by dimerization after assembly. This screen could be performed by looking for colonies which do not replica plate in the PR::PR cotransformation, indicating that dimerization has been lost. In turn, cotransformations of Gag::PR could be plated on XGAL plates to screen for blue colonies, indicating that the Gag::PR interaction has been enhanced relative to wild type Gag::PR in the two hybrid context. Conversely, it is possible that blocking dimerization of PR could abolish Gag::PR interactions altogether, in support of a model for dimerization and activation which can occur prior to assembly. Finally, any mutants could be tested in the viral context for Pol incorporation.

Two-hybrid studies of PR dimerization could lead to the study of PR activation, and eventually RT activation. It has been demonstrated that when HFV Pol is overexpressed in the cell by itself, that some cleavage of the 127-kD precursor can be detected (Martin Heinkelein, personal communication). This implies that the functions necessary for PR activation are contained entirely within Pol, and the obvious thing to test is the dimerization function. All retroviral PRs are active only as dimers, and there are likely to be other domains important for the dimerization of Pol, but PR is a logical domain to examine. An important feature of HFV Pol is that the PR domain is not cleaved from the RT domain. Since dimerization is essential for PR activity, PR-RT-RH may be active as a dimer as well. Different retroviral RTs are active in different conformations. MLV-RT for example is active as a monomer (and perhaps a dimer) of RT-RH. HIV-RT is active as a heterodimer of RT-RH/RT, and it both activities are derived from the p66 RT-RH domain. ALSV is also heterodimeric, but the large subunit contains RT-RH-IN, and the small subunit contains RT-RH. Many others have not been studied for their subunit composition, but it is possible that there are dimerization motifs in HFV RT in addition to HFV PR. To study the effects of PR dimerization on RT activation, we need to be able to detect the activity of RT in extracellular virions.

The critical assay which has eluded us is the classic retroviral RT assay to examine the activity of virion associated RT. While others have published repeatedly that this assay works for replication competent viruses, we have been unable to repeat it with wild type virus, making the study of replication-incompetent viruses impossible. It is striking that while others have published such assays, it has not been used to study Pol incorporation. Despite this puzzling technical roadblock, we should be able to capitalize on the assays we have developed to study the cascade of events which lead to Pol incorporation and Pol activation. We have both Pol packaging and RNA packaging assays at our disposal, and vectors for mutagenesis of sequences in both the viral context and the two-hybrid system. An RT assay would be essential for studying the effects of PR and Gag mutations on RT activation in extracellular virions.

With a sensitive RT assay for replication incompetent viruses, one could address many interesting questions related to the sequence of events which lead to Pol incorporation and activation. It would be interesting to know whether proteolytic processing of HFV Pol is required for the activity of RT, as is the case for other retroviruses. It would also be interesting to know whether the PKA mutants I have used to study Pol incorporation are active for RT, and are defective in different processes such as integration. With PR dimerization mutants, it would also be interesting to uncouple the activities of PR and RT. Based on the biochemical studies of HFV PR, and retroviral RTs, the prediction would be that PR dimerization is required for PR activity, and consequently that PR activity is required for RT activation.

Another question is whether Pol is assembled after reverse transcription is initiated. I have shown that reverse transcription is not an obligate step in the assembly of Pol, but I have not ruled out the possibility that Pol can be assembled after genome replication has begun. The Pro-Pol protein is not cleaved, and some evidence suggests that Pro-Pol can form a stable complex with the cleaved IN domain (see Chapters 3 and 4). I have not rigorously tested this idea using the two-hybrid system because preliminary studies with full-length Pol proteins were not successful. I do not yet know whether this is due to protein expression levels, or the inability to translocate these rather large proteins to the yeast nucleus. It might be possible to detect interactions between IN and Pro-Pol using the two-hybrid system. Perhaps the cleaved complex of PR-RT::IN is active prior to assembly. The majority of virus in an infected cell culture is cell-associated, and it is clear that reverse transcription occurs in the late stages of replication, prior to release from the cell. It would be interesting to know when the genome replication begins relative to assembly, and whether Pro-Pol can be assembled in the absence of IN. For our studies, the IN domain facilitated detection of Pol in extracellular virus, so these experiments have not been done.

In summary, I have established methods for studying Pol in the viral context as well as in the yeast two hybrid system, and I have learned several important things about the mechanisms of Pol expression and incorporation into virions. I have shown that Pol

is not required for HFV assembly or RNA packaging, and that Pol can be incorporated as a precursor. While the C-terminus and cleavage site in Gag are not important for Pol assembly, the basic regions II and III (GR boxes II and III) are important for recognition by HFV PR. I have only begun to understand the role of PR in assembly and it will be interesting to know what role this interaction plays in the activation of RT. In the future, it will be interesting to uncouple the various functions of the Pol proteins, and to learn the details of HFV assembly with respect to both Pol and RNA packaging, perhaps providing insight into the evolution of mammalian reverse transcriptase-encoding viruses.

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Publications

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