THE ROLE OF THE HIV NUCLEOCAPSID PROTEIN IN VIRUS PARTICLE ASSEMBLY AND RNA ENCAPSIDATION

by

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A DISSERTATION

Presented to the Department of Molecular Microbiology and Immunology and
the Oregon Health Sciences University
School of Medicine
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

July 1997

School of Medicine
Oregon Health Sciences University

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Acknowledgments

I am very grateful that I have had the opportunity to study at the Molecular Microbiology and Immunology Department of the Oregon Health Sciences University. I am especially happy that I have had the opportunity to finish my graduate research in Dr. Eric Barklis' laboratory. I came here with basic knowledge about biology. It is at the lab that I really learned how to apply molecular biology theories to true experiments. I am very grateful to Dr. Eric Barklis for his guidance and support during my thesis work. I am also grateful to my colleagues Chin-tien Wang, Mark Hansen, Marylene Mougel, Jason McDermott, Lori Farrell, Jenny Stegeman-Olsen, Sonya Karanjia, Zachary Love, Haoyu Qian for their technical assistance and helpful discussions. Finally, I want to thank my parents and my wife Limin Tian for their understanding and encouragement.

ABSTRACT

The human immunodeficiency virus (HIV) gag gene products have been shown to be necessary and sufficient for directing virus particle assembly. They are initially translated as 55 kD polyprotein precursors (Pr55gag). While or shortly after budding, Pr55gag proteins are proteolytically cleaved by a viral protease to major viral proteins: matrix (MA), capsid (CA), nucleocapsid (NC), p6, and two short peptides p2 and p1. The mechanisms of the virus assembly and genomic RNA encapsidation processes have not been clearly elucidated. This thesis focuses on the molecular basis of these two processes and attempts to provide new insights by genetic analysis of the molecular elements that are essential for the two processes.

While Gag-Gag protein interactions which affect HIV assembly occur in the CA domain of Pr55Gag, the NC domain, which functions in viral RNA encapsidation, also appears to participate in virus assembly. In order to dissect the roles of NC, and p6, the C-terminal Gag protein domain, we examined the effects of NC and p6 mutations on virus assembly and RNA encapsidation. In our experimental system, the p6 domain did not appear to affect virus release efficiency, but p6 deletions and truncations reduced the specificity of genomic HIV-1 RNA encapsidation. Mutations in the nucleocapsid region reduced particle release, especially when the p2 interdomain peptide or the amino-terminal portions of the NC region were mutated, and NC mutations also reduced both the specificity and efficiency of HIV-1 RNA encapsidation.

Although HIV-1 NC was shown to be needed for efficient virus particle assembly, in addition to its function in specific RNA encapsidation, the mechanism of NC function in the assembly process was not clearly elucidated. In order to examine how HIV-1 NC is functioning in assembly, it was substituted by polypeptide domains of known function, and the chimeric

proteins were analyzed for their abilities to direct release of virus-like particles. Our results indicate that p2 and C-terminus of CA are not necessarily toxic to assembly and that NC does not act passively by just providing a stable folded monomeric structure to restrict p2 or CA in an assembly competent conformation. In contrast, substitution of NC with polypeptides which can make interprotein contacts permitted assembly, which suggests that formation of interprotein contacts by NC is essential for normal assembly process.

We also have analyzed the roles of Gag protein NC and p6 domains on the encapsidation of retroviral RNAs into virus particles. Our data showed that NC mutations reduced both the specificity and efficiency of HIV-1 RNA encapsidation; p6 deletions and truncations reduced the specificity of genomic HIV-1 RNA encapsidation. Although assembly of all available cellular RNAs into virus particles still needs to be examined, our data suggest that for assembly, NC function can be replaced by domain which does not need to bind RNA.

Introduction

I. General introduction about retroviruses

The retroviridae comprise a large family of viruses, primarily of vertebrates. They are associated with many diseases, including rapid and long-latency malignancies, wasting diseases, neurological disorders, and immunodeficiencies, as well as lifelong viremia in the absence of any obvious ill effects. Despite the variety of interactions with the host, all retrovirus isolates are quite similar in virion structure, genome organization, and mode of replication.

The virion is enveloped and is about 125 nm in diameter. Its lipid bilayer envelope is decorated by Env proteins, products of the *env* gene. The internal nucleocapsid or core, can be a roughly spherical to conical structure made up of the products of the *gag* gene. Also included in the core are several proteins encoded by the *pol* gene that have important catalytic roles during replication: a protease that cleavages Gag and Gag-pol precursors into subunit protein products during or shortly after virus budding; the reverse transcriptase which converts the viral single -strand RNA genome to double-strand DNA; and integrase, which is necessary for covalently joining the resultant viral DNA to cell genome to for the provirus.

The viral genome consists of two usually identical molecules of single-strand RNA, ranging from 7 to 10 kb in length, capped at the 5' ends and polyadenylated at the 3' ends, reminiscent of cellular mRNAs. The order of the genes encoding structural proteins is invariably *gag-pol-env*. A number of other genes involved in regulation of virus expression are present in some virus groups.

The replication cycle can be divided in two phases. The first phase includes entry of the virion core into the cytoplasm; synthesis of double-stranded DNA using the single-stranded RNA genome as template; transfer of the double-stranded DNA to the nucleus; and integration of the DNA into the host genome. These steps are mediated by proteins found within the virion and proceed in the absence of viral gene expression. The second phase includes (i) synthesis and processing of viral mRNAs and proteins, using host-cell systems, (ii) virion assembly by Gag and Gag-pol precursors and encapsidation of viral genome, (iii) association of the viral core with the cell membrane, resulting in the release of the virion by budding, and (iv) processing of the structural precursor proteins into finished products.

Retroviruses traditionally have been divided into three subfamilies, based primarily on pathogenicity: Oncovirinae; Lentivirinae; and Spumavirinae. Viruses originally isolated as tumor-inducing agents, as well as related viruses, are traditionally classified in the subfamily Oncovirinae. Within the subfamily, retroviruses of mice and cats can be further classified by host range. The murine viruses are designated by the species distribution of their receptors: ecotropic viruses replicate only in mouse cells; xenotropic viruses use receptors found on cells of most species except mice; and polytropic and amphotropic viruses use different receptors found in both mouse and nonmurine species.

The foamy viruses of the Spumavirinae subfamily are by far the least well characterized retroviruses. They have been isolated as agents that cause vacuolation ("foaming") of cells in culture from a number of mammalian species, including monkeys, cattle, cats, and humans. Persistent infection with these viruses is not associated with any known disease.

The Lentivirinae subfamily includes exogenous viruses responsible for a variety of neurological and immunological diseases. The prototype members of this family were the " slow" viruses visna, equine infectious anemia, and caprine arthritis-encephalitis. Genomes of these viruses are characterized by a complex combination of genes in addition to *gag, pol, and env* .

Human immunodeficiency virus, HIV, a retrovirus of the lentivirus subfamily, causes the debilitating and generally fatal disease, AIDS (acquired immune deficiency syndrome). The key pathological feature of AIDS is a gradual but accelerating decline in immune competence culminating in overwhelming infection with one or more other microorganisms which exploit the immunocompromised state. AIDS was first reported in 1981 in previously healthy male homosexuals, and was suspected of being caused by a transmissible agent (98). HIV-1 was identified as the etiologic agent of epidemic AIDS in 1983 to 1984 by different research groups and named as lymphadenopathy-associated virus (LAV) (13), human T-cell lymphotropic virus type III (HTLV-III) (229) , and AIDS- related virus (ARV) (172) respectively. In early 1986, the International Committee on the Taxonomy of Viruses (ICTV) recommended human immunodeficiency virus (HIV) as an official name for AIDS virus (192). Although the first AIDS case was reported in the United States, seroepidemiological studies suggested Central Africa as the cradle of HIV-1 and the current AIDS epidemic. A second related virus, HIV-2, predominantly restricted to populations in West Africa, was identified in 1986-1987 (46, 47, 48, 135) and found to be more closely related to the simian immunodeficiency virus isolated from macaques in captivity (SIV mac) than to HIV-1. Nonetheless, HIV-1 and HIV-2 clearly shared a relatively recent progenitor (5, 53, 84, 104, 117). The major biological and genetic features are

entirely parallel. Both are associated with immunodeficiencies, although the degree of virulence may be less for HIV-2.

AIDS is a slowly developing disease and overt symptoms may take years to appear. The major target cells for viral infection are the CD4+ cells that are pivotal to the development of humoral and cell mediated immunity, and it is generally considered that virally mediated destruction of these cells is a major contributor to immune breakdown (215). How HIV-1 infection kills or injures lymphoreticular cells is unclear. Hypotheses include accumulation of unintegrated proviral DNA; increased cell permeability associated with viral budding; terminal differentiation of infected cells resulting in shortened life span; induction of cell fusion events; and production of autoimmunity (75, 118, 171, 252).

The development of AIDS is the result of a chronic progressive infection with HIV (75, 216). Initial infection with HIV may be followed by an acute disease syndrome and high levels of HIV replication; viral p24 antigen can be detected and virus can be isolated from the blood. (44, 55, 57). After initial infection, HIV-infected individuals enter a stage of clinical latency, the asymptomatic period, during which the number of CD4+ cells remains within the normal range or slowly decreases over time. Compared with the initial infection, viral replication is markedly diminished during this stage. Although evidence of viral replication in the blood is reduced during the clinical latency phase, HIV replication is not eliminated. Recent evidence suggests that significant replication persists in lymph node follicles (72, 217). In addition to an effective host immune response, virological features may also contribute to the lower levels of active HIV replication observed during the asymptomatic phase. For instance, HIV isolates derived from asymptomatic patients tend to replicate more slowly in tissue culture, to produce lower levels

of virus, and not to induce the characteristic HIV-associated cytopathic effects of syncytium induction.

The development of symptomatic HIV infection is a continuum of progressive clinical states (76). Progression to clinically defined AIDS is characterized by a dramatic loss of CD4+ lymphocytes and the development of opportunistic infections, AIDS encephalopathy, or characteristic malignancies. Accompanying the development of AIDS is a marked increase in active HIV replication detected in the blood (54, 119). According to one model, virological latency (a state of restricted viral replication within a single infected cell) plays an important role in the persistence of the asymptomatic state(clinical latency with only low levels of viral replication in an infected patient) (16). Latently infected cells containing silent proviruses may serve as reservoirs for the generation of high levels of infectious virus when appropriate activation cofactors induce HIV expression. Such cofactors could be endogenous agents, such as particular genetic traits conveying sensitivity or resistance to HIV (142, 263). Cofactors also could be exogenous agents, such as other infectious agents (209). These cofactors presumably activate virologically latent HIV. Many of the agents proposed as cofactors for the progression of HIV infection can activate HIV transcription. Activation of HIV transcription may play a direct role in activation of virologically latent HIV and could contribute to HIV disease progression.

Since the first AIDS cases were reported in male homosexuals in 1981, it is clear that the AIDS epidemic is a modern-day plague that has resulted in the tragic loss of people from a wide spectrum of society. The AIDS epidemic necessitates the study of retroviruses in the hope of developing strategies for preventive immunization or curative drug treatments. The aim of the work

presented here is to contribute to a greater understanding of the processes of retrovirus assembly and viral RNA encapsidation.

II. Retrovirus life cycle, composition and structure

A. Life cycle

1. Establishment of infection

a. Entry mechanism

One of the first breakthroughs in studies of HIV was the discovery of its major cellular receptor, the CD4 molecule. The reason for preferential growth of HIV in CD4+ lymphocytes was then explained by the binding of its envelope complex (gp120/gp41) to the CD4 protein on the cell surface (58, 155, 156). Attachment of HIV to the CD4 molecule most probably leads to some conformational changes in the gp120 and perhaps CD4. Subsequently gp120 is displaced or cleaved by cellular enzymes which causes another change in the viral envelope, permitting the interaction of gp41 with the target cell membrane; virus-cell fusion subsequently occurs (244).

However, several studies examining the role of this CD4-virus attachment have indicated that this cell receptor interaction alone is not sufficient and is not the sole means for viral entry. Some human cells expressing high levels of the CD4 protein are not productively infected by HIV (73, 151). Some animal cells, induced by molecular or somatic hybrid techniques to express human CD4 on the cell surface, can not be infected (43, 184, 269). Studies with somatic cell hybrids involving human CD4+ rodent cells suggested that a cell surface factor on human cells is needed for HIV entry. Recently, chemokine receptors CCR5 and CXCR4 have been identified as co-receptors for HIV strains that predominate in early and later stages of infection (23).

Besides entering a cell as a free infectious particle, HIV might be passed during cell-to-cell contact. In this regard, evidence that HIV can spread from one cell to another rapidly without forming fully formed particles has been presented (243). Conceivably, the transfer of nucleocapsids is involved, with subsequent de novo reverse transcription (174).

Besides entering cells via the interaction of the virus envelope with cell surface receptors, HIV can infect cells by other mechanisms. For example, HIV infection could be enhanced by antibody. This process would involve the binding of the Fab portion of nonneutralizing antibodies to the surface of the virion and transferring the virus into a cell through the complement or Fc receptor (121, 164, 233, 236, 268, 273). Another mechanism for HIV entry into cells is phenotypic mixing (24, 125, 279). By this process, a viral genome can be within the envelope of a different retrovirus and thus have the host range of that virus. Phenotypic mixing between HIV-1 and HIV-2 strains has been described (167). Moreover, cells coinfected by HIV and murine retroviruses have demonstrated pseudotype virion formation in which the HIV genome and core can be found within the envelope of different mouse retroviruses (33, 39, 40, 183, 261) . Subsequently, HIV could infect a wide variety of cells susceptible to these animal retroviruses. Whether pseudotype virus formation occurs in nature is unknown.

b. Reverse transcription and integration

Following HIV entry into the target cell, a double-stranded DNA copy of the HIV RNA genome is synthesized by the viral enzyme, reverse transcriptase. The viral DNA is transported to the nucleus while still associated with some viral Gag proteins and accessory proteins, and the viral integrase. Integrase catalyzes a concerted cleavage and ligation reaction in which the viral DNA genome becomes integrated into host DNA. Although Gag proteins

appear to be associated with the viral RNA/DNA transcription complex during the processes of reverse transcription, nuclear localization, and integration, the extent to which Gag proteins are involved in the early phases of viral replication cycle remains unclear (25).

c. Virological latency

Integrated viral DNA is the template for transcription of viral RNA in a process that is regulated by both viral and cellular factors. The integrated HIV provirus can remain in a quiescent state until it is activated (87). In general, the latency stage could be defined as any block to the complete expression of HIV proteins. It could be "absolute latency" with integrated provirus but no RNA expression, or "chronically persistent infection" with a low level of RNA expression but no detectable structural protein translation(16, 87). Several hypotheses have been presented to explain the mechanism of virological latency with HIV. It could be caused by methylation of certain portions of the integrated viral LTR needed for induction of the replicative process (17) or, by methylation of extrachromosomal viral DNA sequences (256). It could result from an inactivation of the tat or rev genes (10, 16, 67, 81, 109, 228, 250, 274). Chronically infected T cells express large amounts of multiply spliced viral mRNA, but little of the unspliced HIV genomic RNA that serves as a mRNA for translation of structural proteins (87, 228). This expression pattern is similar to the early stages of HIV-1 replication, before viral Rev protein activity becomes detectable(152). Subsequent stimulation of these cells can lead to cytoplasmic accumulation of the singly spliced and unspliced HIV-1 mRNAs that encode the structural proteins (228, 291), suggesting that post-integration viral latency may be due in part to low levels of Rev proteins (228). Other studies have suggested that the virus itself produces a protein, such as Nef, that interacts with cellular factors and establishes the silent infection (2, 38,

180, 211). The role of the nef gene in latency was proposed because deletion of this viral gene from the molecular clone of the HIV-1$_{SF2}$ strain produced a variant that replicated to high titer and was more cytopathic than the original SF2 isolate (180). Work with other HIV isolates and SIV have supported the notion that the nef gene can down-regulate virus replication in T cells and monocytes (2, 21, 211, 212, 270, 275). However, the precise function of Nef has been difficult to define. In conflict with these studies, others have observed no effect of the Nef protein on either viral replication or gene expression. (106, 153). Two reports have described HIV-1 viruses containing deletions in the Nef open reading frame isolated from selected HIV-infected long-term nonprogressors. Although infected for more than 10 years, these individuals have displayed low viral burdens or absence of CD4 decline and have been entirely free of clinical disease (63, 154). Suppression of vpu or vpr function also could be responsible (284).

d. Virus infection of quiescent cell

The initial experiments with HIV infection of lymphocytes indicated that virus replication occurred best with antigen- or mitogen-induced activation (13, 191). Some investigators have reported that resting CD4+ lymphocytes can be infected in vitro but that virus replication is arrested. Unintegrated viral cDNA forms can be detected by PCR in T cells (264, 291). Upon activation, these cells can be induced to release infectious progeny viruses. These studies suggested that HIV enters the quiescent cells but that in some cases, only limited portions of the viral gene are transcribed (291, 292), and no viral proteins are produced. However, recent studies have shown that HIV can infect nondividing monocyte-macrophages (75, 173). These studies show that, after infection, the preintegration complex of HIV-1 was rapidly transported to the nucleus of the host cell by a process that required ATP but was

independent of cell division. HIV-1 provirus integrated in the arrested cell DNA produces viral RNA and protein in a pattern similar to that in normal cells. The ability of HIV-1 to use host cell active transport processes for nuclear import of the viral preintegration complex obviates the requirement for host cell division in establishment of the integrated provirus, indicating that the completion of early viral replication events (reverse transcription, nuclear localization, and integration) of the HIV life cycle does not require a dividing host cell (30, 173).

2. Gene expression and virus assembly

a. Co-factor activation of HIV expression

Several stimuli besides the HIV Tat protein have been shown to up-regulate HIV LTR transcription and possibly activate HIV expression in vivo. HIV gene expression in infected T cells and monocytes appears to be controlled in part by the state of immune activation of these cells. The block to HIV replication in primary T cells can be removed by T-cell activation with mitogens or specific antigens (e. g., antigens, T-cell receptor stimulators, mitogens, phytohemagglutinin, or PHA, and bacterial lipopolysaccharide, or LPS, etc.)(83, 191, 197, 293). Cytokines generated during immune responses constitute a second major pathway for activation of HIV via immune stimulation. A number of cytokines (e.g., TNF-α and -β, GM-CSF, and IL-1 and -6) have been demonstrated to activate HIV gene expression, both in transient transfection assays and in the various models of latent HIV infection (16, 193). Hormones (e.g., steroids) and differentiating agents (e.g., phorbol esters, sodium butyrate, and retinoic acid) have been found to bind to HIV LTRs to induce HIV gene expression (92, 145, 165). Transcription of the HIV LTR also can be induced by a variety of physical stimuli and chemical agents that elicit intracellular stress and DNA damage. A well-studied example is ultraviolet (UV

) light (277), a potentially important cofactor for inducing HIV replication in vivo. Included among the wide array of opportunistic infections to which AIDS patients are subjected are infections involving DNA and RNA viruses (e.g., herpes simplex virus, cytomegalovirus, Epstein-Barr virus, human herpes virus type VI, varicella zoster virus, adenovirus, human hepatitis B virus, human T-cell leukemia virus, spumavirus, adeno-associated virus). These viruses may play a role in activating latent HIV chronically infected individuals. Oncogenes such as the c-rel protooncogene of the NF-κB family and ras and myb, have been shown to activate HIV-1 LTR-directed gene expression.

b. RNA transcription

HIV gene expression can be delineated into "early" and "late" phases. Early HIV gene expression features the cytoplasmic accumulation of predominantly small multiply spliced RNAs (152) that encode the viral regulatory molecules, such as Tat and Rev, which are absolutely essential for viral replication (61, 82, 271). Tat proteins, either alone or associated with cellular proteins bind to the trans-activation response element (TAR), which is a 59-nucleotide RNA stem-loop structure located downstream (positions +1 to + 60) from the site of initiation of transcription in the 5' long terminal repeat (LTR)(113, 129, 207, 237). In the absence of Tat, RNA polymerase II (pol II) terminates transcription of the HIV genome prematurely, resulting in primarily short transcripts. Interactions between Tat and TAR convert pol II into its processive form and lead to the efficient production of full-length viral transcripts (77, 136, 143, 163, 189). Along with Tat, the Rev protein is an accessory protein essential to HIV replication. The Rev protein plays a critical role in determining the relative amounts of spliced and unspliced HIV RNAs transported to the cytoplasm, for translation or packaging into the nascent

HIV particles. Rev, a nuclear protein, activates nuclear export of unspliced and singly spliced viral RNAs via an interaction with a Rev-response element (RRE) within the env gene coding region (64, 187, 207, 238), and shifts the expression to the late phase. Expression of the Tat and Rev proteins induce the cytoplasmic expression of unspliced genomic RNA and singly spliced viral mRNAs which encode the viral structural proteins (Gag, Gag-pol, and Env), while simultaneously reducing expression of the doubly spliced RNAs that encode the viral regulatory proteins.

c. Assembly, RNA packaging, budding, and maturation

Newly synthesized HIV envelope proteins are incorporated into the host cell membrane. Assembly of new viral particles with Gag and Gag-pol precursors occurs at the cell membrane with the viral RNA genome packaged into the viral core. The newly synthesized particle buds from the cell and acquires its lipid envelope, containing the HIV envelope proteins. During or shortly after budding, Gag and Gag-pol precursors are cleaved by the viral protease, and the virus particles undergo morphological maturation and acquire infectivity (158, 225). The late stages of the HIV virus life cycle are outlined above and will be discussed in detail later in this Introduction.

B. Retrovirus composition

1. RNA genome

Like all retroviruses, the HIV provirus contains two long terminal repeat (LTR) elements at each end, along with three structural genes that are essential for virus replication, *gag*, *pol*, and *env* . The LTRs contain essential cis-acting regulatory signals that specify the start sites for RNA transcription, polyadenylation, and modulate the amounts of HIV RNA synthesized. The *gag* gene encodes a polyprotein precursor that forms the viral core and packages viral genomic RNA. The *pol* gene products are synthesized as a frame-shifted

Gag fusion protein that are proteolytically processed to form the mature proteins reverse transcriptase (RT), which has associated RNAse H activity; protease; and integrase. The *env* gene encodes a glycosylated polypeptide precursor (gp160) that is processed to form the exterior glycoprotein (gp120; SU) and the transmembrane glycoprotein (gp41; TM), which are critical for viral attachment and entry into CD4+ cells. In addition to these genetic elements, HIV contains at least six accessory genes: *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* or *vpx* (86, 112), which regulates the viral replication life cycle.

2. Gene products

a. Regulatory proteins and functions.

Tat

Despite the presence of an array of upstream activation signals, maximal expression from the HIV-1 LTR is clearly dependent upon the Tat protein. Functional expression of the viral tat gene product and an intact copy of TAR, the 5' cis-acting target sequence for Tat(113, 129, 207, 237), are essential for HIV-1 replication in culture (61, 82, 237). The hypothesis that Tat functions at least in part by promoting transcription elongation has now been confirmed both in vivo and in vitro (77, 148, 190). Since transcription termination observed in the absence of Tat occurs at multiple, possibly random locations in the viral sequence, it is likely that Tat acts by increasing RNA polymerase processivity rather than by preventing a specific termination event (204). Results from several groups support the hypothesis that Tat interacts with TAR and converts pol II into its processive form; this leads to the efficient production of full-length viral transcripts (77, 136, 163, 189). The mechanism by which Tat increases the processivity of pol II is not clear. However, cellular proteins clearly play a critical role in the function of Tat. At least one of these proteins, which is encoded on human chromosome 12 (hp12), is shown to be

required to tether Tat to TAR (6, 7, 109, 110, 111). In addition, some studies suggested the involvement of other coactivators which mediate interactions between Tat and the cellular transcriptional machinery (36, 185). These include general transcription factors such as the core pol II itself (194), TATA-binding protein (141), TAF$_{II}$55 (41), and TFIIH (22), as well as the glutamine-rich activator Sp1 (131).

Rev

Although there clearly is significant control of the transcription of the HIV-1 provirus, elevated levels of transcription alone are not sufficient to ensure high levels of virus replication. A second critical control point operates post-transcriptionally. This control is effected by the virally encoded Rev protein that orchestrates the export of unspliced and partially spliced transcripts. In contrast to cellular genes, whose mRNAs undergo essentially complete mRNA splicing which remove most or all intronic sequences prior to transport to the cytoplasm, HIV must transport a variety of unspliced, singly spliced, and multiply spliced RNA to the cytoplasm. Early HIV gene expression involves the production of small multiply spliced RNAs that encode the various viral regulatory molecules, including Tat and Rev. Late gene expression is responsible for production of the unspliced and singly spliced transcripts that encode the viral structural proteins and enzymatic activities, as well as the synthesis of the new viral genomic RNAs. In the absence of functional Rev protein, only the fully spliced HIV-1 mRNAs are expressed (78, 186, 240, 259). The full-length and singly spliced mRNAs all retain a short (about 300 nucleotide) RNA sequence in the env gene coding region, the Rev response element (RRE), that is the target binding site for Rev action. In the absence of Rev, the full-length and singly-spliced mRNAs are retained in the nucleus, whereas in the presence of Rev they are exported into the cytoplasm. The

mechanisms by which Rev functions remain unclear. Rev may act primarily on the RNA transport pathway, facilitating the nucleo-cytoplasmic export of intron-containing RNAs. Rev could cause RRE-containing RNAs to be shunted out of the nucleus instead of entering into the splicing machinery of the cell (79, 187). Alternatively, Rev could act through the splicing pathway, directly inhibiting the splicing of RRE-containing RNAs (37). In either case, Rev results in the cytoplasmic transport of unspliced and singly-spliced RRE-containing RNAs.

Nef

In addition to Tat and Rev, HIV-1 encodes a third regulatory protein named Nef. Nef is translated from one of several multiply spliced mRNA species and is strongly expressed early in the infective process. The precise function of Nef has been difficult to define in part because it is dispensable for the in vitro replication of HIV in cultured cell lines. In contrast, Nef has been shown to be essential for full lentiviral replication in vivo and for lentiviral pathogenicity in humans animal models of AIDS (63, 130, 149, 154,), which implicate Nef as an important viral pathogenesis factor contributing to clinical development of immunodeficiency. Consistent with the in vivo observations, some studies performed in vitro have suggested Nef as a viral infectivity factor, conferring a replicative advantage to viruses infecting quiescent CD4+ lymphocytes subsequently activated with mitogenic stimuli (206, 262). This property of Nef was then shown to reflect enhanced infectivity of the HIV virion (42, 206). Another well-defined function of Nef is its ability to promote the endocytosis and lysosomal degradation of surface CD4 (3). Nef-mediated CD4 downregulation does not appear to be mechanistically linked with Nef-induced enhancement of viral infectivity, because Nef renders viral particles more infectious even in the presence of a CD4 molecule that is

resistant to Nef-mediated endocytosis (249). Understanding the molecular basis for Nef function will likely require identification of the host factors that interact and cooperate with Nef. So far, at least two cellular proteins were found to bind in vitro to Nef: the tyrosine protein kinase Hck, and a serine kinase (242, 245). However, the biological significance of these interactions in Nef-mediated enhancement of infectivity and downregulation of CD4 remains unclear. As has often been the case with Nef, contrasting reports have appeared regarding the effects of this regulatory protein on signal transduction and cellular activation. Several studies have revealed that expression of Nef may negatively impact on signal transduction pathways, which results in the lack of responsiveness to cell surface receptor stimulation (15, 62, 102, 182), while other studies have described Nef as an activator or enhancer of cellular activation(15, 68, 257). Nevertheless, it is now clear that Nef promotes enhanced infectivity of progeny virions and is linked with clinical progression of disease in adult rhesus monkeys infected with SIV and humans infected with HIV.

Vif, Vpr, Vpu, and Vpx

In addition to Tat, Rev, and Nef, which are the early gene products, HIV-1 also encodes the regulatory proteins Vif, Vpu, and Vpr, which are the late gene products. Vif, virion infectivity factor, may increase the virus infectivity under some circumstances. The mechanism of *vif* function is not known. Presumably, the Vif protein plays a role either in a late step in the replication cycle, such as assembly or maturation of infectious virions, or, in an early step such as virus attachment, entry, or reverse transcription. Recent in vivo and in vitro studies showed that vif-mutant viruses could enter cells normally, but were severely impaired in their ability to complete the synthesis of proviral DNA, suggesting that Vif is important for the early stages

of viral DNA synthesis. This may be due to an intrinsic effect on reverse transcription or a preceding postentry event(s), such as virion uncoating or disassembly of the virion core. (94, 280)

The Vpr protein is detectable in the virion itself, and modestly enhances HIV-1 replication rates in culture, with the most pronounced effect exerted early in infection (51, 52, 213). The presence of the Vpr protein in virions suggests that it may function at some early step in the virus replication cycle such as the formation and integration of the provirus or the initial rate of transcription from the provirus. It is also possible that Vpr helps to target the integration complex to the nucleus; recent studies have demonstrated that Vpr localizes in the nuclear matrix in the absence of other viral proteins (178).

Vpu has been shown to be associated with the cytoplasmic membranes of infected cells (267). Some studies proposed that the Vpu protein could promote the release of assembled virus particles (50, 157, 266). These studies have shown that the presence of the Vpu protein significantly increases the number of virus particles released into the supernatant of infected CD4+ T-cell lines. The increased release of virus particles from Vpu+ virus-infected CD4+ T-cells, compared with isogenic Vpu- viruses, is accompanied by a decrease in the accumulation of cell-associated protein rather than an increase in total viral proteins. The effects of the Vpu protein on increasing the rate of virus export seems to be cell type specific, it was previously observed in HeLa cells or CD4+ T-cell lines, but not in Cos7 cells. A second function of the Vpu protein is to induce the degradation of gp160/CD4 complexes in the endoplasmic reticulum of infected cells. The Vpu protein requires sequences or determinants in the cytoplasmic domain of CD4 to induce degradation of the glycoproteins (278). It was recently reported that Vpu forms cation-selective ion channels in lipid bilayers, and it was suggested that the ability of Vpu to

increase virus release from infected cells may be correlated with an ion channel activity of this protein (74, 247)

Genes *vpu* and *vif* are not present in HIV-2, but an accessory gene *vpx* is found exclusively in HIV-2 and SIV genome. Similar to Vpr, Vpx also is virus-associated (138). The function of the Vpx protein is unknown although it has been proposed to play a role in cell-specific tropism for HIV-2 (103).

b. Conserved proteins

Gag

The 5' most gene among all vertebrate retrovirus genomes is named in honor of the first recognition of the proteins encoded by it as group-specific antigens. The *gag* gene plays a central role in virus assembly. Mutations in *gag* can block virus particle formation (93, 133, 176, 251), and the gag gene products can assemble into virus-like particles without other viral components, suggesting that gag is necessary and sufficient for virus particle assembly (91, 255). The HIV-1 *gag* gene is translated from the full-length RNA to yield a precursor polyprotein, Pr55. Pr55 is contranslationally modified by removal of its N-terminal methionine and attachment of a myristic acid to the second glycine residue (248, 272, 285). Myrisylation is required for stable membrane association and assembly of Pr55 (28, 100). Some studies have suggested that myristylation is necessary but not in itself sufficient to target the Gag precursors to the plasma membrane (232, 235), where Pr55 precursors assemble into immature virus particles and bud out from cells. During or shortly after budding, Pr55 normally is cleaved by the viral protease to yield, in the order of translation, the matrix (MA, p17) protein; the capsid (CA, p24) protein; p2; the nucleocapsid (NC, p7) protein; p1; and the p6 protein (116, 168, 205, 230, 281). An incomplete cleavage product, p41, composed of MA (p17) and CA (p24), also can be found in the host cells and virus particles.

The MA protein remains myristylated at its amino terminus. This modification is characteristic of many proteins that lie on the internal faces of cell membranes and is in closest association with the membrane (28). The MA protein of HIV-1 is released on proteolytic processing of the Pr55 Gag precursor. It is associated with the innersurface of the virion phospholipid bilayer and with the core structure (88). It has been proposed that the MA protein is required for incorporation of viral envelope protein into mature virions (290). Recent studies with HIV-1 MA proteins suggest that it also may have an effect on the HIV Env stability or function through its interaction with the envelope protein complex (gp120/gp41) (282). Additionally, MA has been suggested to play a role in virus entry, and nuclear localization (29, 254, 289).

The CA protein is derived from the central domain of Pr55 Gag precursor. It constitutes the shell of the cone-shaped core structure in the mature virion that contains genomic viral RNA and viral enzyme. It appears that the CA protein contains motifs important for assembly because mutations within CA often block virus assembly and it has a strong tendency to aggregate (31, 71, 124).

The NC protein was found to be associated with genomic viral RNA within the core of the virion. In addition to the overall basic nature of the protein, HIV-1 NC contains a sequence of the form Cys-X2-Cys-X4-His-X4-Cys, presented twice in its amino acid sequence. These "Cys-His" motifs resemble the so-called "zinc-finger" domains that are well known in some DNA-binding proteins. HIV NC protein is thought to be involved in packaging of the genomic viral RNA into virion particles (69). Mutations that disrupt the Cys-His motif impair packaging of viral RNA genomes into virion particle (4, 96, 202, 203). Additionally, from studies with other retroviruses, it has been shown that the

NC protein is involved in genomic RNA dimerization (59, 69, 70, 201). Recently, NC has also been suggested to participate in the reverse transcription process (224, 287, 288)

The functions of p2 and p1 are not well defined. The C-terminal Gag cleavage product p6 has been proposed to play a role in virus budding (91, 99) , but its function in virus assembly and release remain controversial, because other investigators have observed little or no such effect (120, 123, 178, 181, 221, 239).

Gag-pol

Viral protease, reverse transcriptase, RNAse H activity, and integrase are encoded by the pol gene and are translated from the full-length RNA as part of a Gag-pol fusion protein by a -1 ribosomal frameshifting which occurs at a frequency of about 5-10% right before the gag open reading frame reaches the p6 coding region, due to a higher order structure presented on the RNA sequence there (128). This infrequent translation frameshift event leads to the more abundant production of Gag compared with Gag-pol fusion protein, and it has been shown that such a ratio of Gag versus Gag-pol in the cell is critical for virus assembly. For Moloney murine leukemia virus (M-MuLV), Gag-pol fusion proteins can not assemble into virus particle without the presence of Gag proteins, presumably due to steric hindrance by the larger Gag-pol polyproteins (80). For HIV, it has been shown that expression of Gag-pol polyprotein alone in the cell leads to efficient intracellular proteolytic processing of Gag-pol and failure of virus assembly (218). It appears that a critical amount of Gag compared to Gag-pol may serve as a buffer to prevent premature activation of the viral protease. Normally, the protease embedded in the Gag-pol polyprotein precursor remain inactivated until Gag and Gag-pol assemble into budding virus particles. Interactions among Gag-pol molecules

may create dimerization of the protease domain, which is required for activation of the viral protease (199, 208). The activated protease first cleaves itself out and then processes Gag and Gag-pol precursors. Processing of Gag and Gag-pol is not necessary for virus particle assembly or RNA encapsidation, but is essential for the budding immature particles to mature into infectious viruses (101, 158, 225). For virus particles to be infectious , cellular tRNAs have to be brought into the virion to bind to the primer binding site (PBS) on the 5' part of the genomic RNA to serve as primers for the initiation of the reverse transcription process. How the primer tRNA is selected and packaged into virus particles remain unclear, although protein sequences on Gag or Gag-pol seem to be implicated (170, 226).

Env

The HIV viral envelope is composed predominantly of a lipid bilayer and the viral envelope proteins. The HIV *env* gene product is initially translated from a singly spliced viral mRNA as a 160 kD precursor glycoprotein (gp160). gp160 is cleaved by a cellular protease at the Golgi into the gp120 external surface (SU) envelope protein and a gp41 transmembrane (TM) protein (196). After cleavage, gp41 and gp120 continue to associate with each other through noncovalent interactions. This complex is transported to the plasma membrane via the secretory pathway (286), where parts of the central and N-terminal portion of gp41 are also expressed on the outside of the virion. The central region of the TM protein binds to the external viral gp120, most probably at hydrophobic regions in the amino and carboxyl termini of gp120, in a noncovalent manner (115). Cleavage of gp160 appears to be essential for HIV infectivity (196). gp120, anchored to the membrane by noncovalent association with the transmembrane gp41, can easily shed from virus or cell membrane (198, 246). It contains the binding site for the cellular receptor CD4

(162) and is the primary determinant for cellular tropism (27, 127). The transmembrane protein gp41 appears to play a major role in the virus-cell fusion process during virus entry (85, 159).

C. Retrovirus structure

By electron microscopy, the assembling particles are first visible as crescent-shaped patches on the inner face of the cell membrane, which then appear to extend until the ends meet to form a hollow sphere as the membrane wraps around to form the envelope. During or shortly after release of the virion, processing of the Gag and Gag-pol precursors leads to a structural rearrangement of the capsid, which transforms the immature virus particles into a mature infectious viruses. Mature HIV virus particles have the characteristic of a lentivirus, with a cone-shaped core composed of the viral CA protein. Inside this capsid, are two identical genomic RNA molecules, NC proteins, and viral enzymes (reverse transcriptase, integrase, and protease). The inner portion of the viral membrane is surrounded by the myristylated MA proteins that provide the matrix for the viral structure (89, 90). Surrounding the core is the membranous lipid envelope. Complete or partial structures for the MA, NC and CA Gag proteins have been studied by magnetic resonance spectroscopy (NMR) and X-ray crystallography, and most recently by two dimensional (2D) protein crystallization of his-HIVCA on a lipid monolayer (12). By 2D crystallization, his-HIV CA has been shown to form a cage-like lattice, consisting of hexamer and trimer units, surrounding protein-free cage holes. The hexamer coordinated cage holes of 26.3A diameter are spaced at 74.2A intervals: these distances, and the hexamer-trimer arrangement are consistent with previous, lower resolution studies on immature HIV-1 virus particles produced in vivo. HIV-1 matrix (MA) protein trimer units, observed in three dimensional (3D) crystal structures, align to

the his-HIVCA trimer units of the 2D arrays. In this alignment, matrix residues previously shown to interact with the HIV-1 gp120/gp41 Envelope protein complex are oriented towards the hexamer cage holes, where they would be positioned to interact with the Env protein cytoplasmic tails.

III. Literature review

A. Assembly

Assembly of virions is a poorly understood aspect of retroviral replication. The assembly of HIV core takes place at the plasma membrane of infected cells. The HIV Env protein is initially translated as a 160 kd precursor glycoprotein (gp160). The gp160 is cleaved by a cellular protease at the Golgi into the gp120 and gp41(196). After cleavage, gp41 and gp120 continue to associate with each other through noncovalent interactions and are transported to the plasma membrane via the secretory pathway (286). The gp41 is a transmembrane (TM) protein, parts of its central and N-terminal portion of are also expressed on the out side of the virion. gp120, an external surface (SU) envelope protein, anchors to the membrane by noncovalent association with the transmembrane gp41. It contains the binding site for the cellular receptor CD4 (162) and is the primary determinant for cellular tropism (27, 127). The transmembrane protein gp41 appears to play a major role in the virus-cell fusion process during virus entry (85, 159). Incorporation of envelope glycoproteins into the virion particles may be facilitated by interaction between the TM domain of envelope glycoprotens and the MA domain of the Gag proteins. The results of studies using mutational analysis of the HIV-1 MA protein have provided evidence that an interaction between the envelope protein and the MA domain of the Gag protein is

required for the selective incorporation of the envelope protein during virion assembly. (290).

The Gag protein can assemble into virus-like particles in the absence of other viral components, suggesting that it contains all the functional elements required for particle formation (91, 105, 139, 255, 258). The HIV Gag is initially synthesized as a polyprotein precursor Pr55, which is modified cotranslationally by a cleavage of the starting methionine residue and the attachment of a myristic acid to the next glycine residue (272, 285). Myristylation is necessary for particle formation and it may help to target Gag and Gag-pol precursors to the plasma membrane (28). However, myristylation itself is not sufficient for membrane targeting, studies of other retroviruses suggested that besides the myristic acid moiety, there may be other Gag domains required for Gag transport to the plasma membrane (235).

Gag-pol are translated from the full-length RNA by a -1 ribosomal frameshifting which occurs at a frequency of about 5-10% right before the *gag* open reading frame reaches the p6 coding region, due to a higher order structure presented on the RNA sequence between NC and p6 (128). Gag-pol proteins are presumably incorporated into the assembling virus core by the interactions of its Gag sequences with the Gag precursor polyproteins. The more abundant production of Gag compared with Gag-pol fusion protein has been shown to be critical for virus assembly. For Moloney murine leukemia virus (M-MuLV), Gag-pol fusion proteins can not assemble into virus particle without the presence of Gag proteins, presumably due to steric hindrance by the larger Gag-pol polyproteins (80). For HIV, expression of Gag-pol polyprotein in the cell leads to efficient intracellular proteolytic processing of Gag-pol and failure of virus assembly (218).

Normally, Gag and Gag-pol precursors first assemble at the plasma membrane as crescent-shaped patches on the inner face of the cell membrane, which then appear to extend until the ends meet to form a hollow sphere as the membrane wraps around to form the envelope. The protease within the Gag-pol polyprotein precursor remain inactivated until Gag and Gag-pol assemble into budding virus particles. Interactions among Gag-pol molecules may create dimerization of the protease domain, which activates the protease. Activated protease first cleaves itself out and then processes Gag and Gag-pol precursors into major viral proteins MA, CA, P2 (polypeptides), NC, P1 (polypeptides), and p6. Concomitant with the processing of the Gag and Gag-pol precursors is a structural rearrangement of the capsid, which transforms the immature virus particles into a mature infectious viruses with a characteristic cone-shaped core structures. Processing of Gag and Gag-pol is not necessary for virus particle assembly or RNA encapsidation, but is essential for the budding immature particles to mature into infectious viruses (100, 158, 225).

The majority of the MA domain within the Pr55 Gag of HIV could be deleted without apparent effect on the assembly and release of virus particles (282), while the CA domain has been shown to have regions that are critical for virus particle formation. Mutations within CA domain may block virus particle assembly (124). The NC domain contains two Cys-His motifs and was implicated in viral genomic RNA packaging. Recent studies also suggest that amino sequences around the NC region may also play a role in virus assembly (32, 35, 161). The p6 domain at the carboxyl terminus of Pr55 Gag was originally thought by one research group to play a role in the budding of assembling virus particle out from the cell (101), while some subsequent studies conducted by other groups failed to identify such a effect (120, 123, 178,

181, 221, 239). The function of p6 in virus assembly remain unclear and controversial. Gag-pol fusion protein is thought to be incorporated into virions by virtue of their N-terminal Gag sequences. Although still controversial, some studies suggest that loss of the NC RNA-binding function correlates with a decreased efficiency of assembly (32).

The assembly process of retroviruses can be outlined as follow, although the precise molecular mechanism still need further investigation: Gag and Gag-pol polyprotein precursors are targeted, in part by the virtue of the myristic acid moiety at their N-terminal ends, to the plasma membrane where they self-assemble into particles; Gag-pol polyprotein precursors are incorporated into the assembling particles by the interactions of their Gag sequences with the Pr55 Gag precursors; viral RNAs are encapsidated via interaction with Gag and/or Gag-pol protein sequences; while the virions bud out from the cell membrane, viral Env protein is incorporated to the viral envelope due at least in part to an interaction of Env with the MA proteins. During or shortly after budding, Gag and Gag-pol precursors are cleaved by activated viral protease into mature products.

B. Encapsidation

1. Cis-acting factor: Psi

Despite the high levels of host RNA in infected cells, the vast majority of retroviral particles assembled in and budded out from the host cell contain a precise genomic complex consisting of two molecules of genomic RNA, rather than cellular or subgenomic viral mRNAs. The ability of the retroviral particles to choose correctly genomic RNA from the vast excess of heterologous molecules implies that specific sequences are present within the genome which direct their efficient and selective encapsidation. Analysis of

spontaneous and engineered mutants of retroviruses has in fact revealed that cis-acting sequences are involved and are present in the retroviral genome (146, 160, 169, 175, 253). For HIV, the cis-acting sequence, the packaging signal (Psi), has been localized to downstream of the 5' major splice donor site (4, 45, 169, 188). Since Psi is removed during RNA splicing, only viral genomic RNA retains this cis-acting sequence and therefore can be selectively encapsidated into the assembling virus particle.

2. Trans-acting factors

The existence of a specific cis-acting genomic segment required for highly efficient and selective genomic RNA encapsidation suggests that it is recognized by a trans-acting factor, possibly a viral protein. So far, only mutations on gag gene have been shown to impair viral RNA encapsidation, while there is not conclusive evidence to implicate either pol or env gene product to this process. The NC protein has been shown to affect viral RNA encapsidation by mutational studies (4, 26, 66, 70, 95, 96, 97, 202, 203, 231). Although some NC mutations can reduce the efficiency of viral RNA being assembled into virus particle, most previous studies have not addressed how the specificity for encapsidation of the viral genomic RNA is affected. While there was in vitro evidence suggesting both specific and non-specific binding of NC to RNA, it has been difficult to assess whether mutations in NC affected nonspecific or specific binding of NC protein to viral genomic RNA (20, 60, 140, 150, 177).

IV. Thesis rationale

While protein sequences important for virus particle assembly have been located to the CA domain, the effects of the C-terminus of Gag, including

p2, NC, p1, and p6 regions, have not been investigated extensively, although recent observations have indicated that NC and the p2 and p1 interdomain regions may play a role in virus particle formation.

Additionally, HIV NC has two cys-his, or zinc finger motifs, and it has been shown to affect genomic RNA packaging levels. However, prior to this work, it was not clear whether this effect was due to non-specific binding of this zinc finger protein to RNA, or whether the HIV NC could specifically and selectively assemble full-length HIV-1 transcripts into the virus particles. Furthermore, the effects of p6 on the process of RNA encapsidation were poorly defined. To help elucidate the process of HIV-1 assembly and RNA encapsidation, our aims were as follows: (1) To determine the roles of sequences in the HIV-1 NC domain with regard to their effects of virus particle assembly; (2) To characterize Gag sequences that can specifically encapsidate full-length viral transcripts into virus particles.

Experimental approaches and results:

A. The HIVgpt system

The parental vector HIVgpt contains the proviral sequence of HIV-1 strain HXB2, with its env gene replaced by the drug resistance gene gpt. Transfection of HIVgpt into Cos7 cells results in the production of virus particles which are Env protein deficient, but otherwise like wild type (wt). The assembly of HIVgpt mutants was measured by determination of ratios of Gag protein levels in the media versus cell samples. RNA incorporation levels normalized to Gag protein levels in released particles was used as a measure of encapsidation efficiency, while ratios of unspliced, viral genomic RNA to spliced viral RNA levels in virus particles was used as a measure of RNA packaging specificity.

B. Effects of NC mutation, deletions, and substitutions on virus particle assembly

While Gag-Gag protein interactions which affect HIV assembly occur in the capsid (CA) domain of Pr55Gag, the nucleocapsid (NC) domain, which functions in viral RNA encapsidation, also appears to participate in virus assembly. In order to dissect the roles of NC, and p6, the C-terminal Gag protein domain, we examined the effects of NC and p6 mutations on virus assembly and RNA encapsidation. In our experimental system, the p6 domain did not appear to affect virus release efficiency. Mutations in the nucleocapsid region reduced particle release, especially when the p2 interdomain peptide or the amino-terminal portions of the NC region were mutated.

The previous study showed that in addition to its function in specific RNA encapsidation, the HIV-1 nucleocapsid (NC) is required for efficient virus particle assembly. However, the mechanism by which NC facilitates the assembly process is not clear. To examine its role in the assembly process, we replaced the NC domain in Pr55^{gag} with polypeptide domains of known function, and the chimeric proteins were analyzed for their abilities to direct the release of virus-like particles. Our results indicate that NC does not mask inhibitory domains, and does not simply provide a stable folded monomeric structure. Instead, our results suggest that formation of interprotein contacts by NC is essential to the normal HIV-1 assembly process.

C. Effects of NC and p6 on the efficiency and specificity of HIV-1 RNA encapsidation.

We have analyzed the roles of the nucleocapsid and p6 regions on the encapsidation of retroviral RNAs into virus particles. We found that mutation of NC domain reduced both the specificity and efficiency of HIV-1 RNA encapsidation. p6 deletions and truncations reduced the specificity of genomic

HIV-1 RNA encapsidation. We also replaced the NC domain of Moloney murine leukemia virus (M-MuLV) with that of HIV. In encapsidation studies, we found that wt M-MuLV precursor Gag (PrGag) proteins packaged M-MuLV transcripts more efficiently than HIV RNAs. In contrast, chimeric PrGag proteins possessing M-MuLV MA, p12, and CA domains, but with the HIV-1 NC domain, encapsidated HIV transcripts to a greater extent than M-MuLV transcripts. Our results support the notion that retroviral NC domains contribute toward both the efficiency and specificity of viral genomic RNA packaging. Some of our NC substitution mutants assembled efficiently, even when RNA was not detected in the particles. These results suggest that NC can be replaced by a protein that does not appear to encapsidate RNA.

EFFECTS OF NUCLEOCAPSID MUTATIONS ON HUMAN IMMUNODEFICIENCY VIRUS ASSEMBLY AND RNA ENCAPSIDATION

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Running title: HIV-1 virus assembly and RNA encapsidation

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ABSTRACT

The human immunodeficiency virus (HIV) Pr55Gag precursor proteins direct virus particle assembly. While Gag-Gag protein interactions which affect HIV assembly occur in the capsid (CA) domain of Pr55Gag, the nucleocapsid (NC) domain, which functions in viral RNA encapsidation, also appears to participate in virus assembly. In order to dissect the roles of NC, and p6, the C-terminal Gag protein domain, we examined the effects of NC and p6 mutations on virus assembly and RNA encapsidation. In our experimental system, the p6 domain did not appear to affect virus release efficiency, but p6 deletions and truncations reduced the specificity of genomic HIV-1 RNA encapsidation. Mutations in the nucleocapsid region reduced particle release, especially when the p2 interdomain peptide or the amino-terminal portions of the NC region were mutated, and NC mutations also reduced both the specificity and efficiency of HIV-1 RNA encapsidation. These results implicated a linkage between RNA encapsidation and virus particle assembly or release. However, we found that the mutant ApoMTRB, in which the nucleocapsid and p6 domains of HIV-1 Pr55Gag were substituted with the *B. subtilis* mtrB protein domain, released particles efficiently but packaged no detectable RNA. These results suggest that, for the purposes of virus-like particle assembly and release, NC can be replaced by a protein that does not appear to encapsidate RNA.

INTRODUCTION

The human immunodeficiency virus type-1 (HIV-1) proviral DNA encodes three major genes, gag, pol, and env, as well as a number of accessory genes. Expression from the proviral long terminal repeat (LTR) promoters ultimately leads to the transcription of spliced and full-length viral RNAs. The viral Gag protein is translated from the full-length messenger RNA, and is synthesized initially as the polyprotein precursor Pr55Gag (9, 55, 65). Cellular expression of Pr55Gag has been shown to result in the formation of virus-like particles (18, 22, 35, 44, 58, 59). During or shortly after budding, Pr55Gag proteins are cleaved by the protease (PR) encoded by the pol open reading frame, to give an occasional processing intermediate (p41), and the mature Gag proteins matrix (MA), capsid (CA), nucleocapsid (NC), and p6 (26, 39, 46, 55). The interdomain peptides p2 (between CA and NC) and p1 (between NC and p6) also are generated via this event. Coincident with processing, HIV particles take on a new morphology, acquiring an electron-dense conical or cylindrical core, and becoming more sensitive to disruption by non-ionic detergents.

Several studies have indicated that protein-protein interactions required for wt levels of Pr55Gag self-assembly into virus particles map in the CA region (53, 60, 62, 66, 68), while numerous studies have implicated HIV NC in the incorporation of HIV RNA into virus particles (1, 3, 11, 16, 19, 31, 45, 71). This is not surprising, since the nucleocapsid domain consists of an N-terminal, positively charged region; a proximal cys-his finger motif; an inter-finger region; a distal cys-his finger motif; and a C-terminal section (28, 47, 50, 64). Nevertheless, while experiments have shown that NC contributes to the specificity of RNA encapsidation (7, 71), the influence of other Gag domains on this process remain unclear. Also unclear is the relationship between RNA encapsidation and HIV particle assembly. Recent observations have indicated

that NC and the p2 and p1 interdomain regions define important assembly determinants (8, 10, 38). These regions could act directly by mediating critical Gag-Gag contacts or Gag protein interactions with cellular components. Alternatively, NC might exert its effect on assembly via its association with the capsid domain, or with RNA, as has been observed *in vitro* (8).

To study the effects of the p2, NC, p1, and p6 domains of HIV-1 on virus assembly and RNA encapsidation, we have examined a series of C-terminal *gag* mutations in protease-minus (PR-) and wild-type (wt) contexts. In our experimental system, we found that PR- constructs were released more efficiently than wt, and that p1 and p6 mutations were neutral with regard to particle release. In contrast, other amino-terminal mutations, especially those in p2 or near the N-terminus of NC, produced PrGag proteins that were deficient in virus particle release. Additionally, two types of effects on encapsidation were observed: p2 and NC mutant particles packaged low levels of RNA; while p1 and p6 mutants incorporated abnormally high levels of spliced viral RNAs, indicating a loss of encapsidation specificity. We also characterized a mutant, ApoMTRB, in which the HIV p1, NC, p2 and p6 domains were replaced with the tryptophan leader RNA binding protein encoded by the *B. subtilis mtrB* gene (49). While ApoMTRB proteins efficiently assembled virus-like particles, the particles contained no detectable incorporation of spliced or unspliced viral RNA. Assuming that spliced RNA accurately assesses non-specific RNA encapsidation, our results suggest that the assembly function of HIV-1 NC can be replaced by a protein that does not encapsidate RNA.

MATERIALS AND METHODS

Recombinant constructs.

All the NC and p6 mutants were created from the parental wt construct HIVgpt (52, 66, 67), which is based on the HIV HXB2 strain (17), and the locations of all the mutations in this paper are numbered according to the numbering system of the proviral DNA sequence of HIV HXB2. HIVgpt uses the SV40 ori and early promoter to express the drug resistance guanosine phosphoribosytransferase (*gpt*) (48) gene in place of the env coding sequence, while other viral genes remain intact. Expression of HIVgpt in Cos7 cells results in the production of noninfectious (env-minus) but otherwise normal virus particles. The construct 2498T has a HpaI linker insertion at the HindII site at nucleotide (nt) 2498 of the HIV HXB2 proviral DNA sequence, stopping all three open reading frames: it expresses wt Gag, but produces immature virions since *pol* gene products are not expressed. HIVgpt A15 (1, 66, 67, 71) is a construct in which the first two cysteine residues of the C-terminal NC cys-his motif were mutated to tyrosine (1). Two other mutants, ApaI and BglII, possess linker insertions at nt 2010 and 2096 respectively, and have been described previously (66). The newly created ApoTE, MunTE, TARK, and TAM constructs cause the *gag* open reading frame (ORF) to terminate at different positions in p2 or NC, and eliminate *pol* ORF expression. The mutant sequences are as follows, where the 5' and 3' proviral nucleotide numbers are provided, wt HIV sequences are in plain font, oligonucleotides are in bold, and the termination codon is underlined: ApoTE, nt 1899 5' ACA **AAT <u>TGA</u> CCC GGG** TCA ATT C 3' nt 1906; TAM, nt 1899 5' ACA **AAT TCA GCT ACC ATA ATG <u>TGA</u> CTG** GAA TTC 3' nt 1906; TARK; nt 1899 5' ACA **AAT TCT GCT ACC ATC ATG ATG CAG AGA GGC AAT TTT AGG AAC CAA AGA AAG <u>TAG</u>** AAT TC 3' nt 1906; MunTE, nt 1962 5' TGT TTC **AAT <u>TGA</u> CCC GGG** TCA ATT G 3' nt 1972. Another

mutant, ApoMTRB, is one in which the HIV-1 NC and p6 domains have been replaced by the *B. subtilis trp* leader RNA binding protein encoded by the methyltryptophan-resistance (*mtrB*) locus (49). The ApoMTRB DNA junction sequence starting at HIV-1 proviral nt 1899 is 5' ACA **AAT TCC GGG CTG CAG GAA TTA ATT** *CAA AAG CAT TCA* 3', where HIV sequences are in plain font, linker sequences are in bold, and *mtrB* sequences, starting at *mtrB* codon number three are in italics. In addition to the above mutations, three internal deletions were created in the HIV-1 NC coding region. ΔMun deletes NC sequences from the MunI site at nt 1968 to the RsaI site at nt 2067, yielding the junction sequences of nt 1962 5' TGT TTC **AAT TCC TGC AGC CCG GGG GAT CCG CGG GGT** ACT 3' nt 2069, where linker sequences are in bold type set while the HIV sequences are in plain text. Similarly, ΔNC deletes from the ApoI site at nt 1902 to the RsaI site at nt 2067 (nt 1899, 5' ACA **AAT TCC TGC AGC CCG GGG GAT CCG CGG GGT** ACT 3', nt 2069), and Δp7bf deletes from nt 1932 to nt 2094 (nt 1920 5' ATG CAG AGA GGC **GGG GAT CGA TCC CAT C**AG ATC TGG CCT 3', nt 2105). Of these deletions, ΔMun and ΔNC retain pol gene functions, but Δp7bf, which disrupts the pol frame-shift signal, does not.

Three p6 mutations also were investigated. The p6 termination mutations p6T1 and p6T2 have linker insertions at the HIV-1 BglII site at nt 2096, resulting in the terminations of the gag ORF before the p6 coding region, while the gag-pol ORF is not terminated. The junction region for p6T1 is nt 2094, 5' AAG ATC TGA TAT CAT CGA TGA ATT CGA GCT CGG TAC CCG GGG ATC TGG CCT 3', nt 2105; while the junction region for p6T2 is nt 2094, 5' AAG ATC CCC GGG TAC CGA GCT CGA ATT CAT CGA TGA TAA CAG ATC TGG CCT 3', nt 2105 (termination codons are underlined). The internal deletion, Δp6 deletes nucleotides from the HIV-1 BglII site (nt 2096) to the compatible BclI site at 2429. With this mutation, the *pol* frame is retained, however, the protease-

coding region in the *pol* frame is partially deleted such that the mutant is protease minus.

Cell culture.

Cos7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and penicillin plus streptomycin. For calcium phosphate transfections, 20-30% confluent Cos7 cells on 10 cm plates were transfected as described previously (21, 66, 67, 68). Ordinarily, media supernatants and cells were collected at 72 hours post-transfection, but for time course experiments, at 48 hours post-transfection, plates were mock-treated or cycloheximide was added to each plate to a final concentration of 100 ug/ml. After a 15-20 min incubation, the media was aspirated, cells were washed three times with 5 ml of DMEM plus FCS, and each plate was refed with 10 ml media plus or minus 100 ug/ml cycloheximide. At subsequent time points after refeeding, cells and media supernatants were collected and processed for protein gels. For immunofluorescence experiments, Cos7 cells from confluent 10 cm plates were split 1: 40 onto coverslips 24 hours before being transfected with wt or mutant plasmids, and transfected cells were processed for immunofluorescence two days later.

Gag protein analysis.

At 72 hours post-transfection, media supernatants were collected and centrifuged at 4 degrees C for 10 min at 1,000 x g to remove cell debris. For virus release assays, the cell-free supernatants were then centrifuged through 2 ml 20% sucrose cushions in TSE (10 mM Tris hydrochloride, 100 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride) at 4 degrees C for 45 min at 274,00 x g (SW 41 rotor at 40,000 rpm; 2 ml cushion for 10 ml supernatants from each plate). The pellets were resuspended in 100 ul of IPB

(20 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, 1% Triton X-100, 0.002% sodium azide) plus 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were washed twice with 10 ml of ice-cold phosphate-buffered saline (PBS) then collected into 2 ml of PBS in a 15 ml falcon tube for each 10 cm plate. Cells then were pelleted at 4 C for 10 min at 1,000 x g. The cell pellets were lysed in 1 ml of IPB plus 0.1 mM PMSF, followed by 10 min microcentrifugation at 13,700 x g to remove debris. Cell lysate samples of 100 ul were aliquotted for virus release assays, while the rest was saved. Equal volumes of 2 x sample buffer (12.5 mM Tris hydrochloride [pH 6.8], 2% SDS, 20% glycerol, 0.25% bromophenol blue) and one tenth volumes of B-mercaptoethanol then were added to the 100 ul IPB suspensions of the virus and cell samples. After 5 min boiling, samples were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) along with an internal control recombinant HIV CA standard for Gag protein quantitation purposes. After SDS-PAGE and electroblotting onto nitrocellulose filters, Gag proteins were immunodetected with mouse anti-HIV CA monoclonal antibody from hybridoma cell line Hy183 (made by Bruce Chesebro and obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) as the primary antibody, and an alkaline phosphatase-conjugated goat anti-mouse IgG as the secondary antibody. HIV Gag proteins immunodetected on the nitrocellulose membranes were quantitated using DeskScan II 2.0 alias and NIH image 1.59/fat software, and levels were normalized using the internal control recombinant HIV CA. For analysis of proteolytic processing, viral Gag precursor, intermediate, and final processing product levels were calculated as percentages of the total amount of Gag present in a sample. For limiting antibody dilution experiments, virus pellets of wt and 2498T were resuspended and aliquoted for protein gels. The virus samples were

electroblotted in parallel onto a nitrocellulose membrane, and detected using dilutions of the primary antibody.

For sucrose density gradient fractionations (23, 32, 66), 72 hours post-transfection, supernatants were collected from three 10 cm plates of transfected Cos7 cells, and centrifuged at 4 degrees for 10 min at 1,000 x g to remove cell debris. Cell-free supernatant material was pelleted by centrifugation (4 degrees, 2 hours at 83,000 x g; 25,000 rpm for a SW 28 rotor) through 4 ml 20% sucrose cushions, resuspended in 200 ul of PBS, mixed with internal control Moloney murine leukemia virus (M-MuLV) and layered onto linear 20 to 60% sucrose gradients in TSE buffer in SW50.1 polyallomer tubes. The gradients were centrifuged at 4 degrees C for 24 hours at 240,000 x g (equilibrium for particles of 3 S or greater). After centrifugation, 400 ul fractions were collected from the top to the bottom of the gradients. Each fraction was aliquotted for measurement of density, and HIV and M-MuLV Gag protein levels.

To assay the subcellular localization of wt and mutant Gag proteins by immunofluorescence, 48 hr after transfection of cells on coverslips, cells were fixed, permeabilized and processed for indirect immunofluorescence following standard methods (32, 66). The primary antibody was a tissue culture supernatant of hybridoma cell line Hy183 used undiluted, and the secondary antibody was rhodamine-conjugated anti-mouse immunoglobulin G antibody used at 1: 300 dilution. After the final washes with DMEM plus 10% heat inactivated calf serum, penicillin, streptomycin, and 10 mM Hepes, pH 7.4, coverslips were washed three times 5 min in PBS, and mounted on slides in 50% glycerol in PBS. Cells were viewed and photographed with a Leitz Dialux 22/22 EB immunofluorescence microscope, equipped with a standard rhodamine filter.

RNA analysis.

For RNA isolation from cell-free media supernatants, particles were pelleted through 4 ml 20% sucrose cushions at 4 degrees C for 2 hours at 83,000 x g (SW 28 rotor at 25,000 rpm). The virus pellets were resuspended in 600 ul of IPB, and a 100 ul aliquot was taken for protein analysis. To the rest of each of the suspensions (500 ul), 30 ug carrier yeast tRNA was added, and samples were phenol/chloroform extracted twice, chloroform extracted twice, ethanol precipitated, and resuspended in 100 ul of TE buffer (10 mM Tris [pH 7.4], 1 mM EDTA). For cellular RNA preparation, cells from three transfection plates were washed twice with ice-cold PBS, collected into 2 ml of PBS, and pelleted at 4 degrees C at 1,000 x g for 10 min. The cell pellets were lysed with 3 ml of GTC buffer (6 M guanidium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% Sarkosyl, 100 mM B-mercaptoethanol), and loaded on a 2 ml cushion of 6.2 M cesium chloride-100 mM EDTA [pH 7.0], then centrifuged at 15 degrees C for 18 hours at 115,000 x g (SW 50.1 rotor at 35,000 rpm). The pellets were washed with 70% ethanol, air-dried, quantitated spectrophotometrically, and stored at -80 degrees C.

Anti-sense probes for RNAse protections was prepared from Blue HX 680-831 by *in vitro* transcription using T3 polymerase according to standard methods (67). The 32P-labeled probe is 183 bases in length, including 5' and 3' non-HIV sequences derived from the pBluescribe (Stratagene) vector. HIV-1 spliced and unspliced genomic RNAs are expected to yield protected fragments of 64 bases and 150 bases, respectively. For riboprobe hybridizations, 10% of the viral RNA samples or 40 ug of the cellular RNAs were mixed with 10 ug of yeast tRNA, ethanol precipitated, dried, and resuspended for use. Hybridizations, RNAse digestions, electrophoresis, and detection of protected RNA bands have been described previously (67). Protected bands on X-ray

films and Gag protein signals from corresponding Western blots were processed by DeskScan II 2.0 alias and NIH image 1.59/fat software for quantitation following previously outlined methods (71).

RESULTS

Assembly of HIV-1 p2, NC, p1 and p6 mutants.

Previous experiments have shown that the NC domain of HIV-1 is an important determinant for the efficiency of RNA packaging (1, 3, 11, 16, 19, 31, 42). *In vitro* and *in vivo* evidence also suggests that HIV-1 NC exerts some effect on the specificity of viral RNA encapsidation (6, 7, 12, 14, 33, 41, 57, 71). With regard to virus assembly, previous work has shown that the HIV-1 p2, NC, p1 and p6 regions influence particle assembly or release (10, 38), and in some systems, RNA appears to have an impact on assembly or release processes (8). To further elucidate the function of the C-terminal region of HIV Gag, we decided to examine the effects of the mutations of in the p2, NC, p1 and p6 domains of HIV-1 on particle release and RNA encapsidation. The constructs used are illustrated in Figure 1, and were based on a parental wild type (wt) construct HIVgpt, in which the HIV *env* gene was replaced by a *gpt* gene driven by SV40 ori and early promoter (52, 66, 67): when transfected into Cos7 cells, Env minus, but otherwise wt virus particles can be produced from this construct. In addition to wt HIVgpt, we also have used a *pol* ORF truncation mutant, 2498T (Figure 1), which produces non-processed immature virus particles (43). Within the p6 coding region, we created premature Gag termination mutants, p6T1 and p6T2, which retain protease (PR) function; and Δp6, which is PR-deficient (PR-). Also available were BglII, a linker insertion mutation in p1, and two directed mutations in NC: ApaI, a linker insertion between the cys-his finger motifs; and A15, possessing site-directed mutations of cysteines in the second zinc finger motif (1). Major NC mutations included internal deletions (ΔMun, Δp7bf, and ΔNC), and premature terminations (MunTE, TARK, TAM, and ApoTE). Of the internal deletion constructs, ΔMun removed the two cys-his fingers, but retained p2, p1, the amino- and carboxy-

terminal segments of NC, and the pol frame. The Δp7bf and ΔNC mutations similarly deleted almost all of the NC domain, but Δp7bf excised part of p1 and was PR-, while ΔNC excised part of p2, and was PR+. The truncation mutations also removed large portions of NC, and all of these were PR-. They differed in the placement of translation terminations: for MunTE, the *gag* ORF terminates at the start of the first cys-his motif, 17 codons into NC; TARK terminates *gag* 6 codons sooner; TAM stops translation exactly at the end of p2; while ApoTE was designed to terminate halfway through p2. The final mutation, ApoMTRB, was based on ApoTE, but replaced the HIV-1 Gag NC, p1 and p6 domains with the *B. subtilis* RNA-binding protein, MtrB (49).

The abilities of the above mutant constructs to direct virus particle formation were studied in transiently transfected Cos7 cells. At 72 hours after transfection of DNAs into Cos7 cells, media supernatant and cell lysate samples were prepared, fractionated by SDS-PAGE, and electroblotted onto nitrocellulose membranes. The HIV Gag proteins then were immunodetected with an anti-HIV-CA antibody as detailed in the Materials and Methods. A rough indication of the release efficiencies of wt and mutant constructs could be obtained by comparison of Gag protein levels in media versus cell samples, and examples of immunoblot results are shown in Figure 2. As illustrated in Panel A, lanes A and B, wt HIVgpt Gag proteins appeared to release particles efficiently, as evidenced by the relatively high levels of Gag proteins present in the media (lane A) versus the cell (lane B). Similarly, the PR- but otherwise wt construct, 2498T, also directed the efficient release of Gag proteins from transfected cells (Panel B, lanes A and B). Compared with HIVgpt or 2498T, it was apparent that certain mutants, notably ΔNC (Panel A, lanes C, D), ApoTE (Panel B, lanes C, D), and TAM (Panel B, lanes G, H) released Gag proteins inefficiently.

In the process of quantitating Gag protein release values, a disparity was observed for the release of proteolytically processed versus unprocessed Gag proteins. In particular, when release levels of wt and 2498T were compared, it appeared that release from the PR- construct (2498T) was more efficient than that of wt (compare Figure 2, Panels A and B, lanes A and B). There are several potential reasons for the apparent difference in PR- and PR+ virus assembly and release. Released, unprocessed virus particles could be more stable than processed ones. Alternatively, the antibody used in detection might react better to the precursor Gag than the cleavage product CA, leading to an underestimation of the Gag proteins of the processed virus particles in the medium. Or, in transfected cells, if Gag proteins from the PR- construct were less stable than Gag proteins from the wt construct, it could show an artificially high ratio of Gag protein levels outside versus inside cells. Finally, the Gag protein release rate for our transfection system might be higher for PR- than PR+ constructs. We performed several experiments to distinguish between these possibilities (see Figure 3). The results in Figure 3E showed that the extracellular stabilities of Gag proteins from PR+ and PR- constructs were similar, and antibody dilution experiments (Figure 3F) suggested that our antibody reacts equally well to precursor, partially processed, and mature forms of Gag. These results implied that the observed release differences derived from differences in the cellular handling of the proteins. To examine cellular processes, media supernatant and cell samples were collected at various time points from Cos7 cells transfected with wt (PR+) and 2498T (PR-) constructs (Figure 3 A, B). Although intracellular Gag protein levels remained roughly steady during the time course, the relative amount of virus release for the PR- construct exceeded that for the PR+ construct by 3 to 10 fold, suggesting more efficient release for the PR- construct. In Figures 3C and 3D,

experiments were performed similarly, except that transfected cells were treated with cycloheximide prior to and during time course collections to assess intracellular Gag protein stabilities. As shown, cycloheximide greatly reduced Gag release from both PR+ and PR- construct-transfected Cos7 cells, suggesting that active synthesis might be required for efficient particle assembly and release. However, results also showed that under cycloheximide treatment, Gag protein stabilities were approximately 165 min for wt and 280 min for the PR- construct. These results indicate that the observed apparent higher level of release for PR- Gag is not due to a low intracelluar PR- Gag stability. Rather, wt virus particles seem to be released from the transfected cells less quickly, and the non-released Gag proteins appear to be degraded more quickly. This observation will be discussed (see below) in the context of previous studies (5, 29, 34, 54).

The results shown in Figure 3 indicated that PR- virus particles were released more efficiently than wt viruses in our system. Consequently, for evaluation of assembly efficiencies, it was necessary to compare PR+ mutants with wt, and PR- mutants with 2498T (PR-). The results of such comparisons are shown in Figure 4, in which the relative assembly efficiency levels of the PR+ constructs are shown by white bars, and those of the PR- constructs are shown by black bars. With the exception of the p6T1 and p6T2 constructs, all mutants appeared to assemble less efficiently than their wt counterparts. We believe the release levels for p6T1 and p6T2 appear anomalously high because they are processed less well than wt HIVgpt (see below). Insofar as other mutants were concerned, there was considerable variation in Gag protein release levels. Some mutations, such as the linker insertions (ApaI and BglII), the site-directed cys-his motif mutation (A15), and the PR- p6 deletion (Δp6), reduced the efficiency for virus assembly about two-fold or less. However,

major deletions and truncations of the NC region significantly reduced the assembly levels. Furthermore, it appeared that p2 and the very amino-terminus of NC were important to Gag protein assembly and release, as evidenced by the extremely low release ratios of ΔNC, ApoTE, and TAM constructs, in which deletions extended to the very N-terminus of NC or into p2. However, it is not clear how p2 and NC impact particle assembly: their contributions to this process may or may not be sequence specific. An argument against a sequence specific requirement is the fact that ApoMTRB, in which p2, NC, p1 and p6 are replaced by an unrelated sequence, directed the efficient release of chimeric Gag proteins.

As illustrated above (Figures 2, 4), some of our mutant proteins were not assembled and released efficiently, even though Gag proteins were readily detected within cells. It is possible that these mutant Gag proteins were blocked during transport to the cell surface, as has been observed previously for some mutant Gag proteins (23, 32, 66, 68). Alternatively, plasma membrane-localized proteins might have been defective in the processes of assembly or release. To find where Gag proteins resided in cells, we examined the subcellular localizations of wt and release-impaired truncation mutant Gag proteins (ApoTE, TAM, TARK, MunTE) by immunofluorescence light microscopy. Our results indicated that, wt Gag proteins stained a perinuclear ring plus a heterogeneous pattern through the cell periphery, while ApoTE, TAM, TARK, and MunTE proteins showed heterogeneous non-nuclear staining, with staining along cell edges (data not shown). These results did not support the notion that ApoTE, TAM, TARK or MunTE proteins were trapped at intracellular membranes, but suggested that they were delivered to the cell surface, as has been reported for other NC mutant retroviruses (38).

Characterization of wild type and mutant virus particles.

In our virus assembly and release assays, collection of cell-free media supernatant samples involved pelleting through 2 ml 20% sucrose cushions for 45 min at 274,000 x g. Based on centrifugation clearing rates, the minimum particle size to pellet would be 165S, and over 90% of our media HIVgpt Gag protein was recoverable by this method in control experiments (data not shown).

These data suggest that wt and mutant Gag proteins were released from cells in particle forms. Although a complete analysis of each mutant virus awaits electron microscope analysis, biochemical characterization of the particles also was of interest. For Gag proteins produced from PR+ constructs, one avenue of analysis was to examine whether released Gag proteins were processed by the viral protease. Consequently, CA, p41, and PrGag levels in pelleted media supernatant samples for PR+ constructs were determined (Table 1). Not surprisingly the site-directed and linker insertion mutants (A15, ApaI, BglII) were processed at approximately wt levels. The p6T1 and p6T2 proteins also were processed, albeit at lower efficiency than wt . More dramatic were results for the mutants which had major deletions in NC (Δ NC, Δ Mun). Seventy percent or more of the Gag proteins synthesized by these two constructs remained unprocessed, suggesting that mutant Gag-pol proteins do not assemble into virions or are impaired for PR activity, or that Gag proteins are packed in such a way that they are not accessible for processing.

Another method for analysis of released particles is by density gradient fractionation. Retrovirus particles have densities of 1.140-1.180 g/ml, and while evidence has suggested that retroviral densities are not grossly dependent on RNA encapsidation levels, aberrant densities might be an indication of different packing arrangements of the Gag proteins within virus particles (5). To measure the densities of wt and mutant particles, virus

resuspensions recovered from the transfected Cos7 medias were mixed with an internal control mouse retrovirus stock (Sup1 Moloney murine leukemia virus; M-MuLV; 56) and fractionated by sucrose density gradient centrifugation. Collected fractions were assayed for density and Gag protein content (HIV and M-MuLV), and results are shown in Figure 5. The fractionation profiles of the PR+ constructs are shown in the left two columns for comparison with wt HIVgpt, while the profiles of PR- constructs are shown in the right two columns for comparison with 2498T, since we found that processed wt HIVgpt particles came to equilibrium at a density of 1.148 g/ml (equal to that of M-MuLV), while the PR- 2498T particles came to a density equilibrium of 1.168 g/ml(greater than that of M-MuLV). Relative to wt HIVgpt, the p6 terminators (p6T1, p6T2) showed higher densities, but since the p6T1 and p6T2 proteins were incompletely processed (Table 1), it was not surprising that they had a densities similar to that of 2498T. ApaI, A15, BglII, TARK, MunTE, ΔMun and Δp6 had densities similar to their wt counterparts, while ΔNC, ApoTE, TAM, and Δp7bf showed lower densities than the wt versions. These results suggest that p2 and the very amino terminal portion of NC are necessary for packing Gag proteins into wt-like virus: critical residues apparently map between the fourth (Δp7bf) and the eleventh (TARK) residues of NC, although the results with the ΔMun construct are not in strict agreement with this assessment. Interestingly, while ApoMTRB particles assemble and are released at a reasonable efficiency, the particles are of low density, at least compared with the PR- 2498T particles. This result is reminiscent of some murine leukemia virus Gag fusion proteins (32), and suggests that high density packing mediated by NC domains is not essential to efficient Gag protein release from cells.

Encapsidation of retroviral RNA.

Efficient and selective encapsidation of viral genomic RNA into virus particles requires incompletely defined interactions between virus core proteins and the viral encapsidation signal, which appears to be localized near the 5' portion of the HIV-1 RNA (1, 11, 25, 40). To examine viral RNA packaging into wt and mutant particles, Cos7 cells were transiently transfected with wt or mutant constructs, and cellular and viral RNAs were isolated 72 hr post-transfection, as described in the Materials and Methods. Aliquots also were taken from virus preparations prior to RNA isolation, for Gag protein quantitation. A quantitative RNAse protection assay (67, 71) was employed to measure the viral RNAs present in cells and particles, using an antisense riboprobe, designed to span the major spliced donor site, allowing differentiation of full-length viral genomic RNA and spliced viral transcripts. Examples of the protection gels are shown in Figure 6, panels A, B, and C. The probe of 183 bases long is shown in lanes 16, 26, and 42, and little background protection was detected with the control yeast tRNA samples (lanes 15, 25, and 41). As expected, wt HIVgpt was expressed well in the transfected Cos7 cells (lanes 8, 21, and 35), showing bands corresponding to viral full-length (150 nt) and spliced (64 nt) RNAs. Full-length viral RNA was detected in particles released from wt HIVgpt-transfected cells, but little spliced viral RNA could be detected in the particles (lanes 1, 17, and 28), indicating that full-length genomic RNA was efficiently and specifically packaged into wt virus particles. Similar results were obtained with the PR- construct, 2498T (lanes 18, 22). By comparison, all mutants showed high levels of full-length genomic and spliced RNAs in cellular RNA samples (lanes 8-14, 21-24, and 34-40). However, the constructs with major NC deletions, truncations or replacements showed no RNA in the particle samples (lanes 2-7, 30, and 33). As seen previously (71), A15, in which cysteines of the second cys-his motif of NC were mutated to

tyrosines, showed reduced HIV RNA in the virus particles, and a lower ratio of genomic versus spliced RNA, suggesting that the zinc finger affects both packaging efficiency and specificity. The other constructs, ApaI (lanes 32, 39), BglII (lanes 31, 38), and the three p6 mutants (lanes 19, 23; 27, 34; 29, 36) all appeared to show efficient genomic RNA encapsidation, but reduced specificity, as evidenced by high spliced RNA levels in virus particles.

To evaluate encapsidation results, cell and virus protection band signals and their corresponding Gag proteins signal from Western blots were quantitated using DeskScan II 2.0 alias and NIH image 1.59/fat programs. From these data, ratios were calculated for virus RNA versus cell RNA; virus RNA versus virus Gag protein; and virus genomic RNA versus virus spliced RNA. Values, normalized to wt HIVgpt results, are given in Table 2. As shown, the PR- 2498T construct gave no clear-cut indication of a loss of encapsidation efficiency (see first two columns), but appeared to show a somewhat increased tendency to package spliced transcripts (rightmost column). However, less subtle results were obtained for other mutants. Notably, all deletions or truncations that removed the HIV-1 NC cys-his fingers failed to package viral RNA. This result also applied to ApoMTRB, in which NC, p1 and p6 domains were replaced with the unrelated *trp* leader RNA binding protein encoded by the *B. subtilis* methyltryptophan-resistance (*mtrB*) gene. Results for A15, the site-directed double cys-his finger mutation, showed a reduced packaging efficiency and specificity, as observed previously (71). The situation was slightly different for the linker insertion between the two NC fingers (ApaI), the insertion in p1 (BglII) and the p6 deletions and truncations. All of these appeared to package RNA efficiently, with virus to cell RNA ratios of at least 80% wt levels, and particle RNA to Gag ratios at least half that of wt. However, the encapsidation specificities for these mutants were clearly reduced as

indicated by particle associated unspliced to spliced RNA ratios 8-25% that of wt. These results indicate that both NC and p6 domains contribute to the specificity of HIV RNA encapsidation, although p6 may act indirectly, by influencing NC folding.

DISCUSSION

It has been shown that mutations affecting retrovirus NC cys-his motifs can reduce levels of genomic RNAs packaged into retrovirus particles (1, 3, 11, 16, 19, 31, 45), although the extent to which NC contributes to encapsidation specificity has not been elucidated completely (6, 7, 12, 33, 41, 57, 71). In addition to its function in RNA encapsidation, NC and the C-terminal portion of retroviral Gag proteins appear to possess a region involved in increasing the efficiency of virus particle assembly, this region has been referred to as an assembly domain (AD) (5), although its exact contribution to virus assembly is not clear (10, 15, 18, 20, 27, 38). Our current study focused on the evaluation of the effects of p2, NC, p1 and p6 domains of HIV-1 on virus assembly and RNA encapsidation.

In accord with previous observations (33, 37), major deletions, truncations, or replacements of the NC domain of HIV-1 *gag* were found to eliminate the encapsidation of HIV genomic or spliced RNAs into virus particles. Although theoretically possible, we do not believe these results are due to a defect of mutant construct cis-active encapsidation (Psi) signals, since the mutations occurred away from the known Psi signals (4, 24, 25, 42, 57). We have not measured the content of non-viral RNAs in these mutant particles, but if packaging of spliced viral RNA is an indication of non-specific RNA encapsidation, we would expect little RNA of any kind in these virus particles, although this assumption has yet to be proven. While major NC mutations apparently eliminated encapsidation, other mutants showed more subtle effects. Linker insertion between the two NC zinc fingers in the mutant ApaI, reduced the specificity of encapsidation, while the total amount of RNA packaged was normal. This observation supports the previously described model that NC contributes to the specificity of RNA encapsidation (7, 71).

Perhaps more surprisingly, mutants of p1 or p6 maintained wt levels of encapsidated RNA, but the specificity of RNA encapsidation was reduced (Figure 6, Table 2). This strongly suggests that PrGag determines the packaging specificity, and implicates the entire C-terminal region of HIV-1 Gag in the process.

The PR- construct, 2498T, seemed to release particles more efficiently than wt HIVgpt in our experimental system. This result differs somewhat from results with avian (63) and murine (13, 36) retroviruses in which protease activity does not appear to affect release levels, and contrasts with one report on HIV, in which PR- particles were released less efficiently than PR+ particles (34). It is unclear how the PR+ phenotype might impair Gag protein transport or establishment of interprotein contacts required for assembly and budding, although a number of studies have shown that perturbation of Gag protein to PR activity ratios can alter virus release efficiencies (29, 34, 44, 53, 54). Because of the differences observed with wtHIVgpt and 2498T, we compared mutant particle release efficiencies with either wt HIVgpt or 2498T, depending on the PR phenotype of the mutant construct (Figure 4). In performing such comparisons, we observed that mutants without p6 were released at reasonable efficiencies, which is consistent with the notion that the effects of p6 on virus release may be affected by PR or cell-type specific (29, 34, 44, 61). However, constructs with mutations in p2 and the amino-terminal portion of NC were released much less well than wt, supporting the notion that this region defines an assembly domain which appears to function after Gag protein delivery to the plasma membrane (see Figure 3) (10, 38). Still, it is not evident how this region contributes to the efficiency of HIV-1 assembly, since substitution of this region with the unrelated sequence in ApoMTRB permitted efficient release of virus-like particles. This result is

reminiscent of the abilities of some M-MuLV Gag fusion proteins to direct particle assembly (23, 32), and the ability of ApoMTRB proteins to release particles at reasonable efficiency might be interpreted in several ways. One possibility is that the MTRB domain may prevent the C-terminus of CA from folding into an assembly-incompatible conformation. Alternatively, the MTRB domain may substitute for an active assembly function of p2/NC. One might favor the first of these alternatives since, in addition to the assembly and release of HIV Gag-MTRB particles, we have observed that MuLV Gag fusions to B-galactosidase also form virus-like particles. However, both MTRB and B-galactosidase proteins form higher order oligomers (2, 30), so it may be that the essential assembly function provided by NC is a nonspecific ability to make interprotein contacts. Although the MTRB domain was fused to HIV-1 *gag* because it potentially acts as an RNA binding protein (49), no evidence of specific or non-specific RNA incorporation into ApoMTRB particles was observed in our experiments using either HIV RNA (Figure 6) or MTRB target RNA (data not shown). Thus, it appears that the NC assembly function can be replaced by a protein that does not encapsidate detectable levels of RNA in our system. We believe that further analysis of the mechanism(s) by which the NC assembly domain acts will be of basic and practical interest.

ACKNOWLEDGMENTS

We thank Marylene Mougel, Jason McDermott, Sonya Karanjia, Zachary Love, Chin-tien Wang and Mark Hansen for help and advice throughout the course of this work. The anti-M-MuLV-CA monoclonal antibody was a gift from Bruce Chesebro, who also made the anti-HIV CA Hy183 hybridoma cell line that was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. A molecular clone encoding the MTRB protein was kindly provided by Paul Gollnick, and the A15 clone originally was from Anna Aldovini. This work was supported by grant 2RO1CA47088-07 from the National Cancer Institute.

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TABLE AND FIGURE LEGENDS

Table 1. Proteolytic processing of HIV-1 Gag proteins.

Media supernatant pellets from Cos7 cells transfected with the indicated constructs were fractionated by SDS-PAGE, electroblotted, and immunodetected as described in Figure 2. The particle-associated PrGag, partially processed p41, and CA protein signals were quantitated and are expressed in the table as percentages of the total Gag protein signals of the respective samples. Multiple experiments were carried out to obtain the averages and the standard deviations; the numbers of experiments averaged to obtain values were as follows: wt, 30; ΔNC, 5; ΔMun, 2; ApaI, 8; A15, 13; BglII, 3; p6T1, 3; p6T2, 6.

Table 2. RNA encapsidation into virus particles.

Genomic and spliced RNA signals from cell and virus samples of Figure 7, and corresponding Gag protein levels from virus particles detected by Western blots, were quantitated using DeskScan II 2.0 alias and NIH image 1.59/fat software. From these data, for each indicated construct the following ratios were calculated: the total levels of viral spliced and unspliced RNAs in particle samples divided by cell samples; the total particle-associated viral RNA signals divided by the particle Gag protein signals; and the particle genomic RNA signals divided by the particle spliced viral RNA signals. Calculated ratios were normalized to wt HIVgpt ratios and are listed as percentages of wt levels (wt = 100%). For mutants which showed no particle-associated viral RNA signal above background, values in the first two columns are given as less than (<) a given level, while values for the right-most column were not applicable (NA). Note that although some mutants released particles very inefficiently, experiments were scaled up as necessary to obtain the Gag-normalized levels in the middle column of the table.

Figure 1. Mutant HIV Gag constructs.

The parental construct HIVgpt (55, 66, 67) is based on HIV HXB2 (17), and is diagrammed to show the C-terminal portion of the gag gene and the beginning of the pol gene. Only the C-terminus of CA, p2, NC, p1 and p6 of the *gag* open reading frame (ORF) are shown in the diagram: CA is in black, p2 and p1 have diagonal bars, NC is in white with cys-his motifs indicated as diamonds, p6 is in white, and HIV-1 proviral nt numbers are designated. An arrow indicates when the *pol* frame is intact. The construct 2498T has a terminator oligonucleotide insertion at the Hind II site at nt 2498, stopping all three ORFs: it expresses wt Gag, but not pol gene products (43). The p6 terminator mutations p6T1 and p6T2 have linker insertions at the BglII site at nt 2096, resulting in the termination of Gag ORFs before the p6 coding sequences, while the gag-pol ORF is not terminated. In Δp6, the deleted HIV sequence from the BglII site at nt 2096 to the BclI site at nt 2429 is indicated by the thin lines in the diagram: the gag-pol frame is retained, although the PR coding region is disrupted, so the mutant is PR-. BglII is a linker insertion mutant with a linker coding for four amino acid residues inserted at the BglII site at nt 2096 in p1, between the NC and p6 domains; ApaI is a mutant with a linker inserted at the ApaI site at nt 2010, adding six codons between the two cys-his motifs of the NC domain (66), and in A15, the first two cysteine residues of the C-terminal NC cys-his motif were mutated to tyrosine (1). Of the NC deletions, ΔMun and ΔNC retained the pol frame, while Δp7bf was *pol*-minus. Specific deletions were as follows: ΔMun from the MunI site (nt 1968) to the RsaI site (nt 2067), removed the two zinc fingers of NC; ΔNC deleted from the ApoI site (nt 1900) in p2 to the RsaI site at nt 2067; and Δp7bf deleted nt 1928 to nt 2096, leaving only four amino-terminal and four carboxy-terminal codons of the nucleocapsid domain. The terminator mutants MunTE, TARK, TAM, and ApoTE

have oligonucleotide insertions, which cause the Gag ORF to stop at different positions in p2 or NC, and neither p6 of the *gag* gene nor the *pol* gene are expressed. MunTE has an oligonucleotide at the MunI site (nt 1968) and terminates translation 17 codons after the beginning of NC, before the two zinc fingers. TARK has an oligonucleotide inserted at the nt 1900 ApoI site, and should terminate translation 11 residues into NC. TAM and ApoTE also have sequences inserted at nt 1900, and TAM terminates precisely at the end of p2, while ApoTE causes the *gag* ORF to end in p2, 5 codons before the beginning of NC. Instead of terminating the *gag* ORF at the nt 1900 ApoI site as in ApoTE, in ApoMTRB, the NC and p6 domains of HIV are swapped for a bacterial RNA binding protein MtrB (49), which is indicated by a gray bar. MtrB (8 kDa) is encoded by the methyltryptophan-resistance (*mtr*) locus of *B. subtilis*, which is a two-gene operon consisting of *mtrA* and *mtrB*. MtrB has been shown to bind specifically to *trp* leader RNA in a tryptophan-dependent manner. Constructs with active *pol* frames have them indicated by arrows, and the precise sequences of the junctions of these constructs are provided in the Materials and Methods.

Figure 2. Gag protein levels in cell lysate and media samples.

Media supernatants (V) and cell (C) samples were collected 72 hours after transfection of Cos7 cells with the indicated constructs. Particles were pelleted from media samples and resuspended: half of each pelleted media preparation was used for loading on SDS-PAGE gels. Cell pellets were lysed and centrifuged to remove debris, and one twentieth of each cell lysate sample was prepared for SDS-PAGE. Gag proteins in samples were separated by SDS-PAGE, electroblotted onto nitrocellulose filters and immunodetected using a mouse anti-p24 monoclonal antibody from hybridoma cell line Hy183 as the primary antibody. Precursor (white triangle), partially processed (grey triangle), and mature (black triangle) Gag proteins were identified by antibody reactivity and comparison of gel migration mobilities to known standards. For each of the two panels (panels A and B), media samples are in lanes A, C, E, G, I, K, M, and O, and cell samples correspond to lanes B, D, F, H, J, L, N, and P. Lane designations are as follows. Panel A (PR+ constructs): wt, lanes A, B; ΔNC, lanes C, D; ΔMun, lanes E, F; ApaI, lanes G, H; A15, lanes I, J; BglII, lanes K, L; p6T1, lanes M, N; p6T2, lanes O, P. Panel B (PR- constructs): 2498T, lanes A, B; ApoTE, lanes C, D; ApoMTRB, lanes E, F; TAM, lanes G, H; TARK, lanes I, J; MunTE, lanes K, L; Δp7bf, lanes M, N; Δp6, lanes O, P.

Figure 3. Stability and release of Gag proteins.

Panels A, B: At 48 hours after transfection of Cos7 cells with wt HIVgpt (panel A) or 2498T (panel B), cells were washed and refed. At time points thereafter, cell (thin line) and media supernatant (thick line) samples were collected and processed for immunodetection. Gag protein signals were quantitated for each time point and are plotted as percentages of the zero time cellular Gag value versus collection time. **Panels C, D:** Experiments were performed and results plotted as in panels A and B, respectively, except that 100 ug/ml cycloheximide (final concentration) was present in the wash, during a 20 min preincubation step, and throughout each time course. **Panel E:** Media supernatants from wt- and 2498T-transfected Cos7 cells were collected and incubated 0, 24 or 48 h at 37 degrees C, after which virus particles were pelleted and processed for immunodetection of Gag proteins. Results are plotted as percentages of zero time Gag protein levels versus collection times and are given for PrGag proteins from 2498T particles and p41 and CA proteins from wt particles. **Panel F:** Equal aliquots of wt or 2498T particles were electrophoresced, electroblotted and immunodetected in parallel, using varying dilutions of the mouse Hy183 anti-CA monoclonal antibody. Gag protein signals were quantitated and are plotted as percentages of the maximum values of PrGag (white circles), p41 (gray circles), or CA (black spots) versus antibody dilutions. The p41 and CA results derive from wt particles, while PrGag results were from 2498T particles.

Figure 4. Levels of Gag protein release from transfected cells.

Gag proteins in matched cell and media supernatant samples were detected as in Figure 2, and quantitated using the programs DeskScan II 2.0 alias and NIH image 1.59/fat. After normalization of Gag protein levels using a bacterially expressed HIV CA protein standard run on each gel, ratios of the total Gag protein levels in the medias versus cells were calculated. The ratios of the PR+ constructs (white bars) were normalized to that of wt HIVgpt, and the ratios of the PR- constructs (black bars) were normalized to that of 2498T. Thus, the higher the release ratio, the greater the level of Gag protein release. Note that the release ratio of 2498T was 2.6 times that of HIVgpt, and that standard deviations are shown, when available.

Figure 5. Sucrose density gradient fractionation of wild type and mutant HIV-1 particles.

Virus pellets prepared from 30 ml cell-free supernatants from transfected Cos7 cells were resuspended in 200 ul of PBS, mixed with mouse M-MuLV (Moloney murine leukemia virus) suspensions, and layered on top of the linear 20-60% sucrose gradients. Gradients were centrifuged 24 h at 240,000 x g such that particles with a sedimentation coefficient of 3S or greater would come to equilibrium. After centrifugation, 400 ul fractions of were collected from the top to bottom, and each fraction was monitored for density, and for HIV-1 and M-MuLV Gag protein content. Gag protein bands were quantitated using DeskScan II 2.0 alias and NIH image 1.59/fat programs, and HIV (diamonds) or M-MuLV (squares) Gag protein levels in fractions are expressed as percentages of the respective peak fraction value. The x axes indicate the density gradient fractions from top (left, low numbers) to bottom (right, high numbers). The peak densities of each gradient were: wt, 1.152 g/ml; ΔNC, 1.137 g/ml; ΔMun, 1.131 g/ml; ApaI, 1.150 g/ml; A15, 1.148 g/ml; BglII, 1.161 g/ml; p6T1, 1.164 g/ml; p6T2, 1.177 g/ml; 2498T, 1.168 g/ml; ApoTE, 1.155 g/ml; ApoMTRB, 1.155 g/ml; TAM, 1.140 g/ml; TARK, 1.180 g/ml; MunTE, 1.160 g/ml; Δp7bf, 1.146 g/ml; Δp6, 1.182 g/ml.

Figure 6. Genomic and spliced RNA levels in cells and virus particles.

RNA samples were prepared from transfected cells and virus pellets, as described in the Materials and Methods. RNAs (10% of viral samples, or 40 ug of the cellular samples) were mixed with 10 ug of yeast tRNA, ethanol precipitated, dried, and processed for RNAse protection assays using an antisense probe of 183 nt (lanes 16, 26, 42; indicated by the letter P) capable of detecting both spliced viral transcripts at a fragment size of 63-64 nt (indicated by a letter S), and unspliced, genomic RNAs at a protected fragment size of 150 nt (indicated by a letter G). In each panel, results from mock reactions using yeast tRNA samples also were used (lanes 15, 25, 41). Lane designations for experimental virus (listed first) and cell (listed second) samples are as follows: wt, lanes 1, 8; MunTE, lanes 2, 9; TAM, lanes 3, 10; TARK, lanes 4, 11; ApoTE, lanes 5, 12; ΔNC, lanes 6, 13; ΔMun, lanes 7, 14; wt, lanes 17, 21; 2498T, lanes 18, 22; Δp6, lanes 19, 23; A15, lanes 20, 24; p6T1, lanes 27, 34; wt, lanes 28, 35; p6T2, lanes 29, 36; Δp7bf, lanes 30, 37; BglII, lanes 31, 38; ApaI, lanes 32, 39; ApoMTRB, lanes 33, 40. Note that viral RNA signals were normalized for total Gag protein content to yield encapsidation efficiencies as described in the Materials and Methods.

Table 1. Proteolytic processing of HIV-1 Gag proteins.

	percentage of total viral Gag		
CONSTRUCT	PrGag	p41	CA
wild type	20.8±7.6	20.9±8.6	58.3±12.9
ΔNC	69.9±17.8	15.9±14.9	14.2±15.8
ΔMun	92.2±0.8	6.1±1.1	1.7±1.8
Apa	20.2±10.6	23.6±9.0	56.2±14.6
A15	14.9±7.4	31.4±7.5	53.7±11.1
BglII	37.8±8.6	9.7±3.7	52.5±11.5
P6T1	33.5±1.0	26.2±1.2	40.3±0.6
P6T2	47.2±6.8	32.4±6.2	20.4±11.9

Table 2. RNA encapsidation into virus particles.

CONSTRUCT	Total Viral RNA Virus/Cell	Total Viral RNA Viral Gag protein	Viral Genomic RNA Viral Spliced RNA
wild type	100.0	100.0	100.0
2498T(PR-)	328.6	67.4	51.8
ApaI	84.4	55.7	17.3
A15	14.3	31.7	39.1
BglII	84.4	52.9	25.0
P6T1	118.8	52.1	12.8
P6T2	106.3	62.1	8.7
Δp6	89.8	51.6	19.8
ΔNC	<1.0	<1.0	NA
ΔMun	<1.0	<2.0	NA
ApoTE	<1.0	<1.0	NA
ApoMTRB	<1.0	<1.0	NA
TAM	<1.0	<1.0	NA
TARK	<1.0	<1.0	NA
MunTE	<9.5	<8.0	NA
Δp7bf	<1.0	<1.0	NA

Fig.1

MUTANT HIV GAG CONSTRUCTS

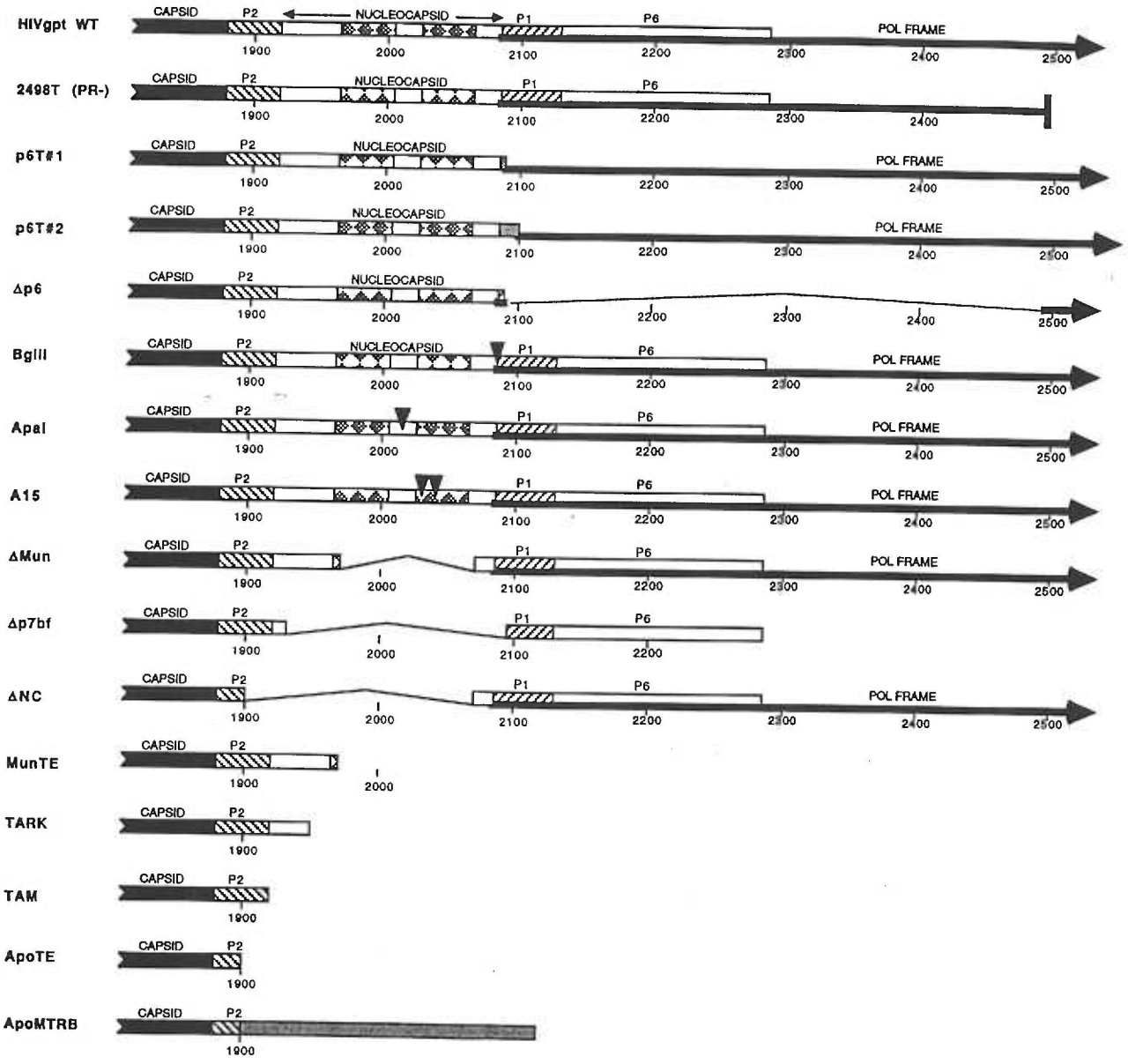

Fig.2A

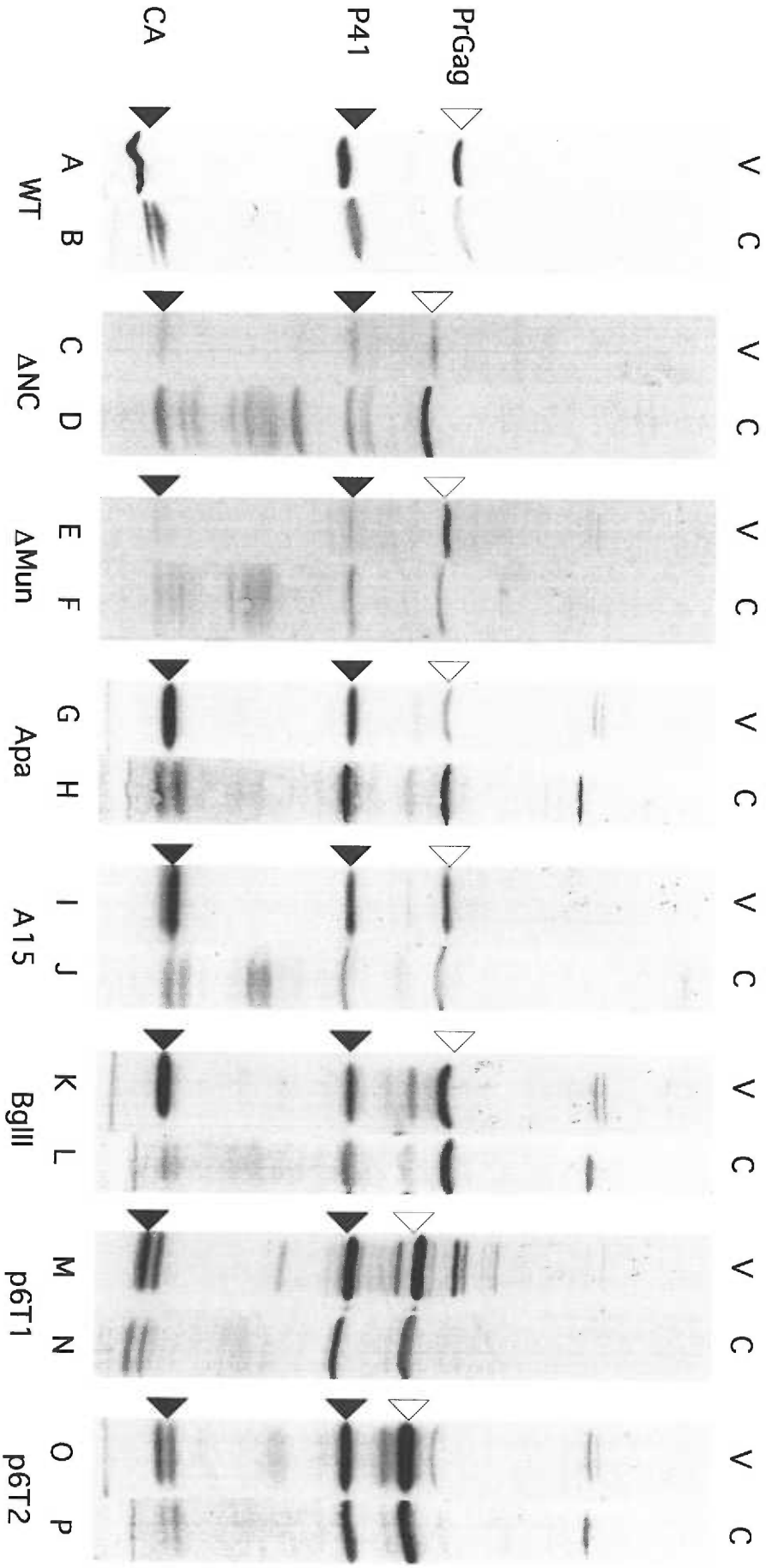
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Fig.2B

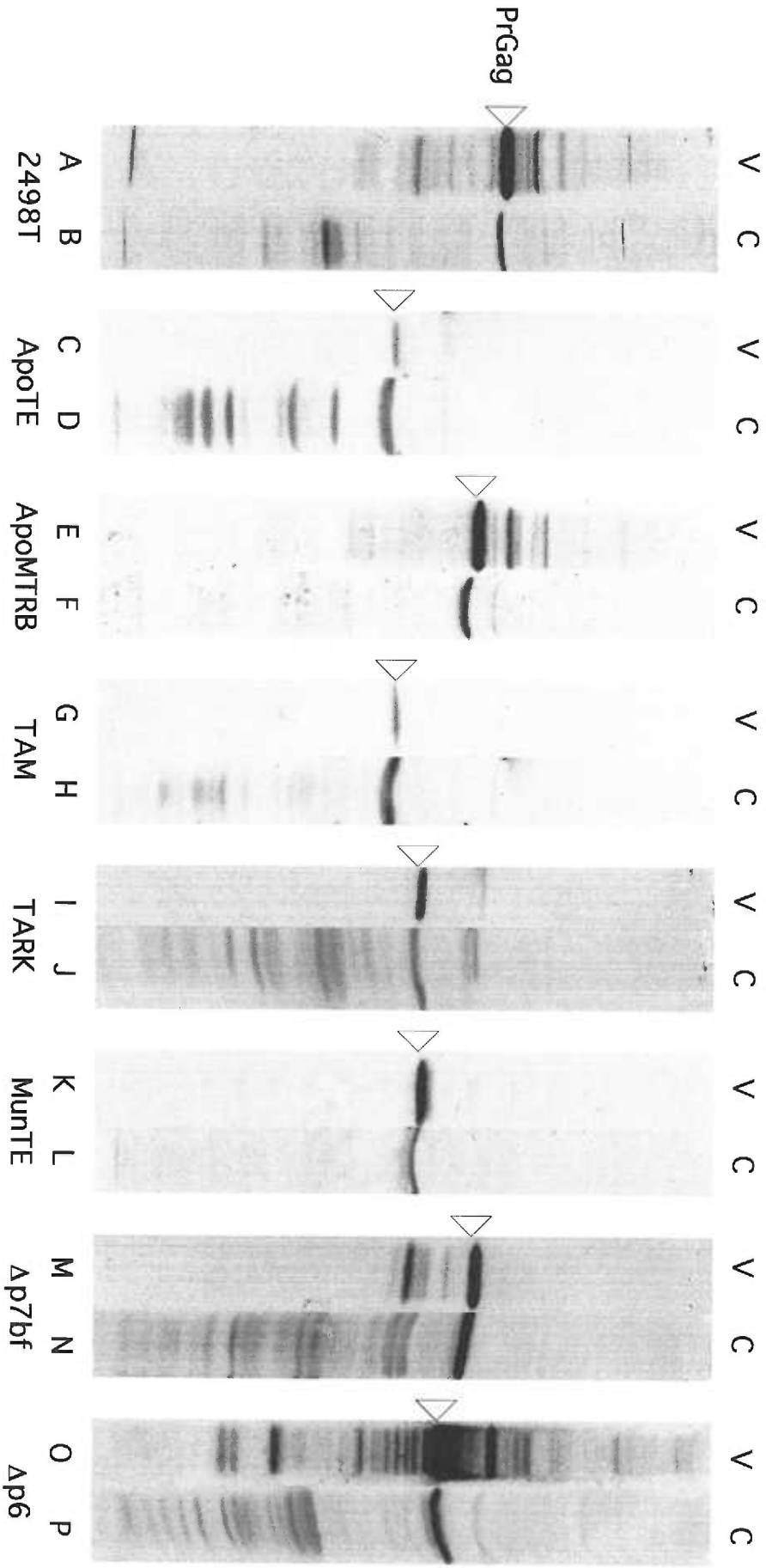
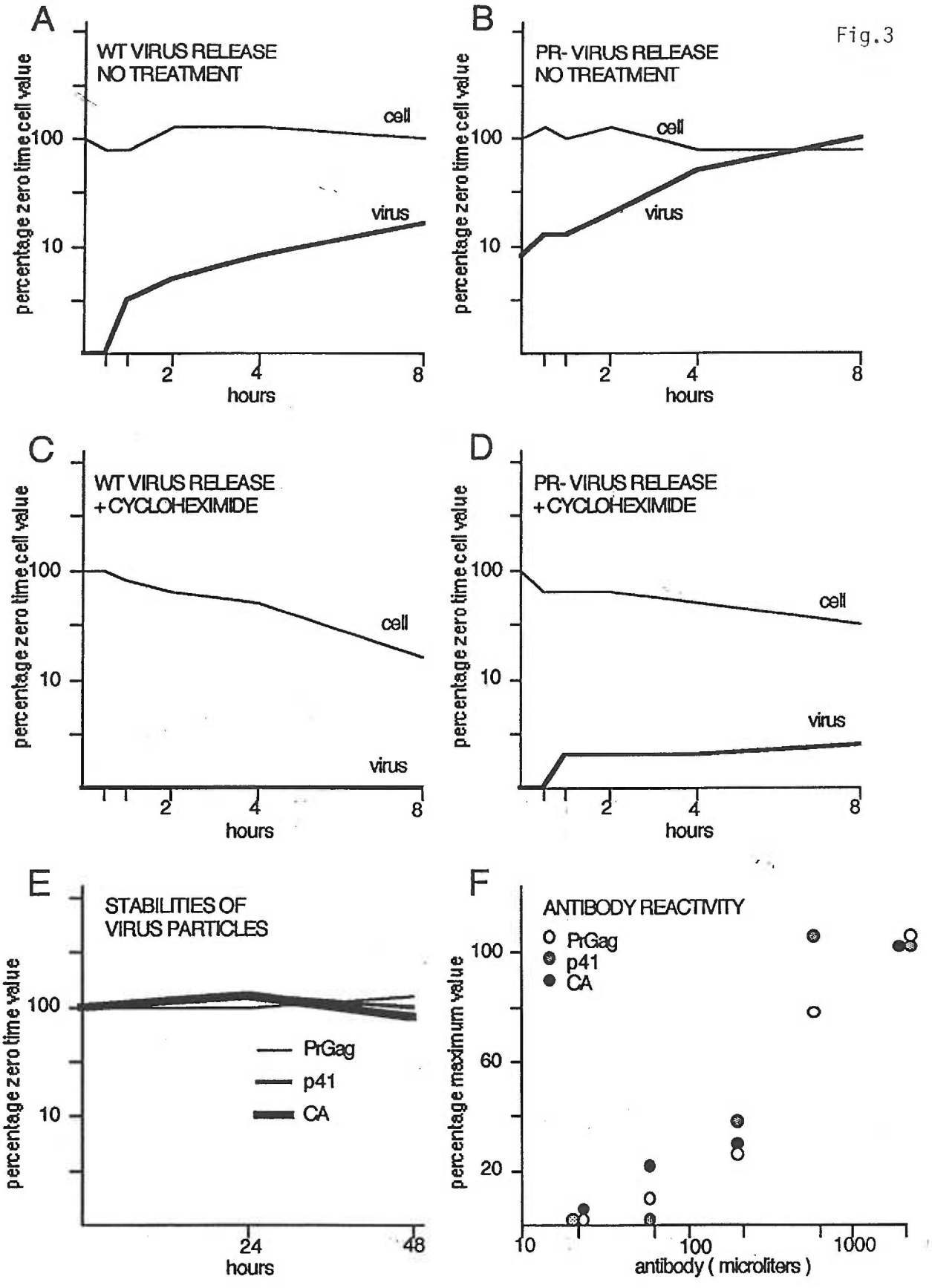
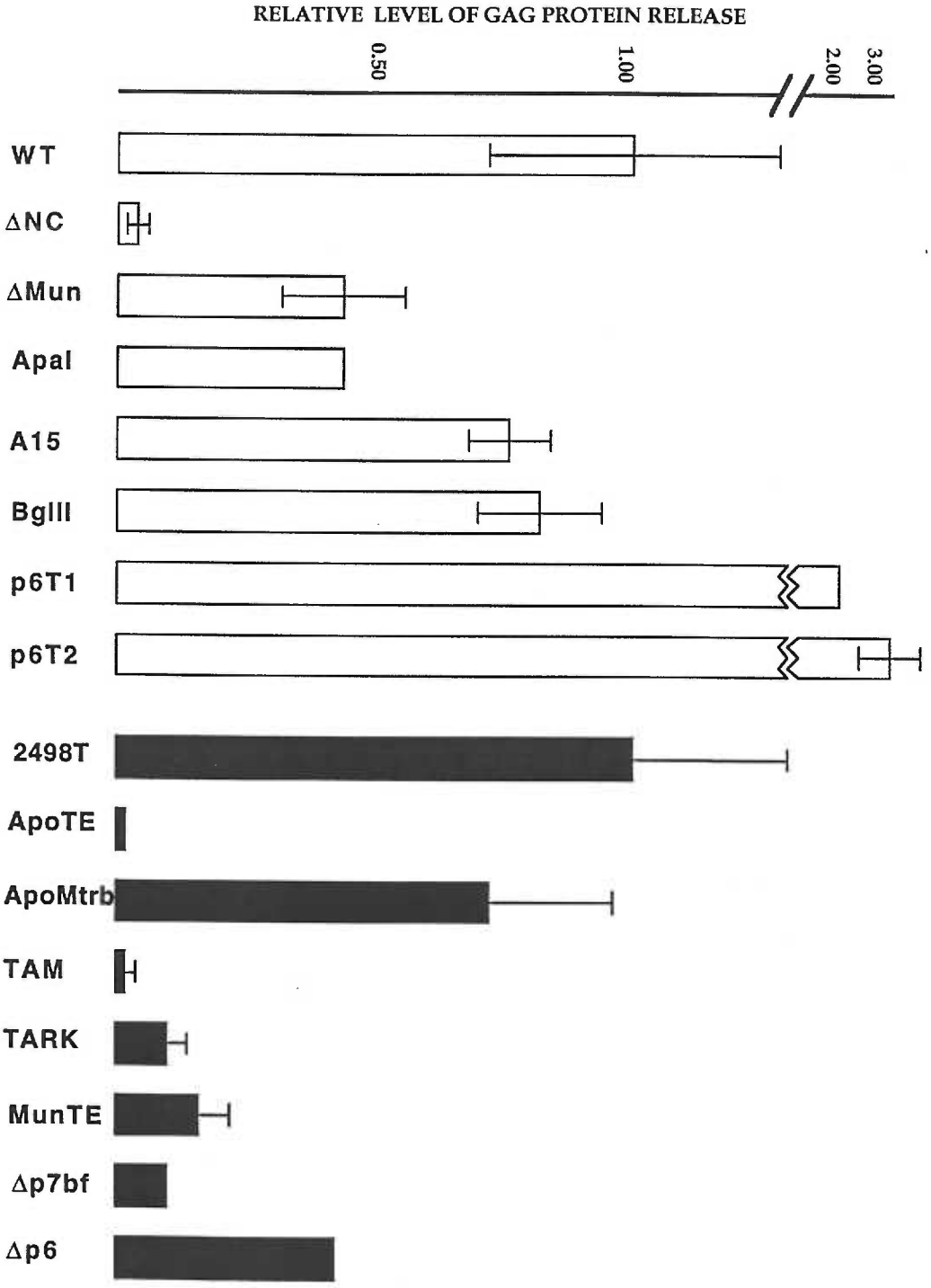
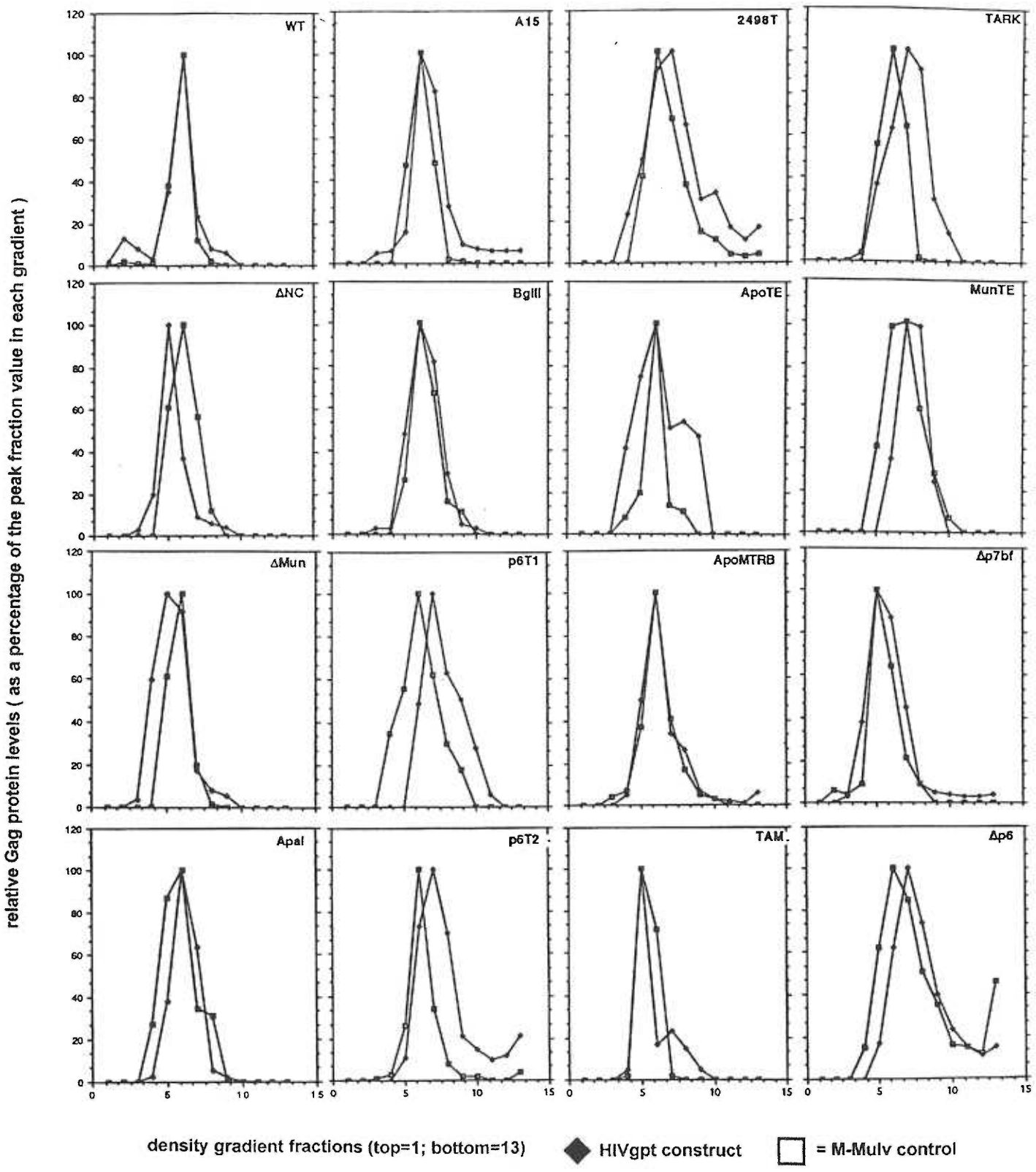
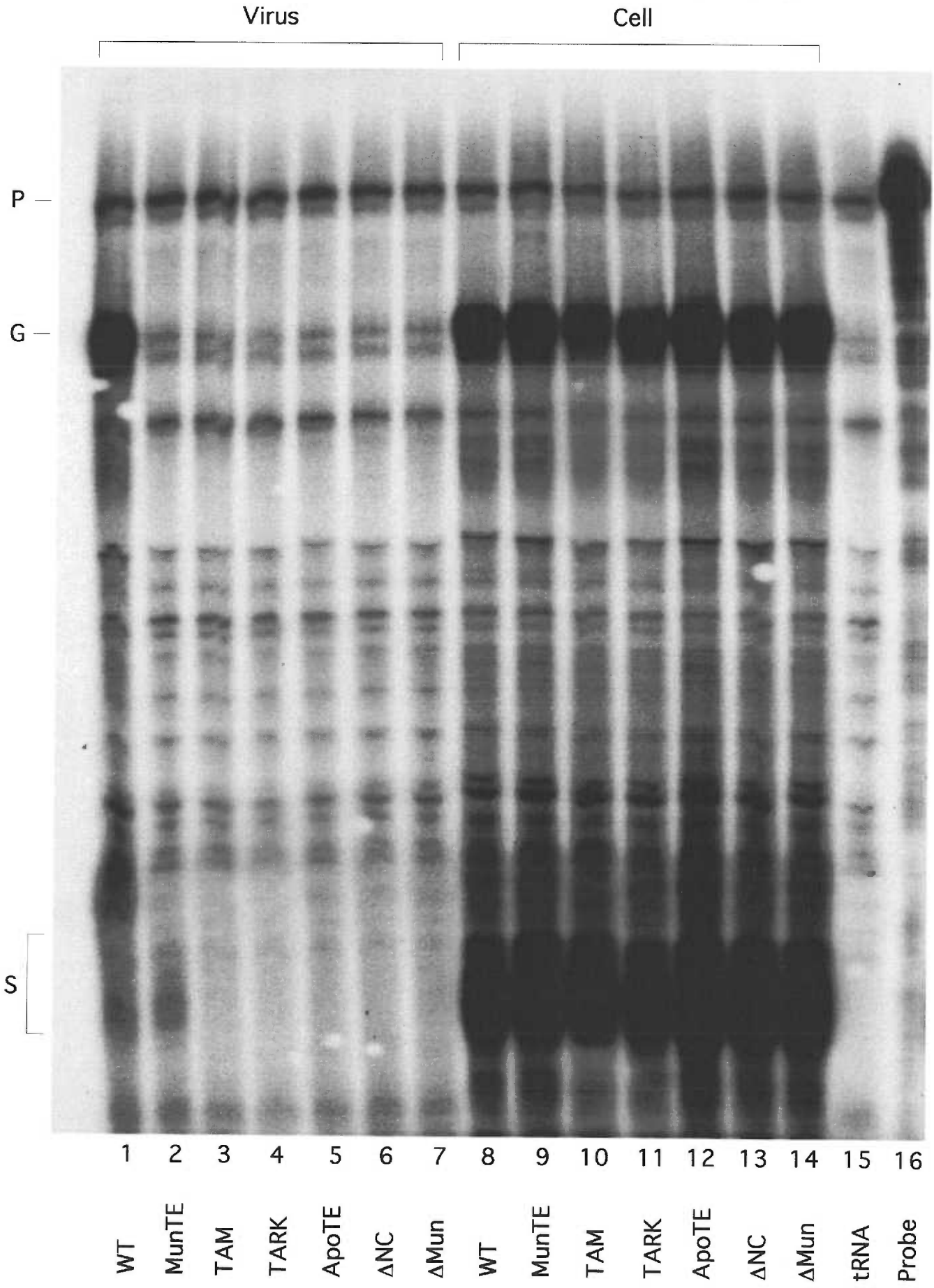

PrGag
A
B
2498T
V
C
C
D
ApoTE
V
C
E
F
ApoMTRB
V
C
G
H
TAM
V
C
I
J
TARK
V
C
K
L
MunTE
V
C
M
N
Δp7bf
V
C
O
P
Δp6
V
C

Fig.3


A
WT VIRUS RELEASE
NO TREATMENT
percentage zero time cell value
100
10
cell
virus
2
4
8
hours
B
PR- VIRUS RELEASE
NO TREATMENT
percentage zero time cell value
100
10
cell
virus
2
4
8
hours
C
WT VIRUS RELEASE
+CYCLOHEXIMIDE
percentage zero time cell value
100
10
cell
virus
2
4
8
hours
D
PR- VIRUS RELEASE
+CYCLOHEXIMIDE
percentage zero time cell value
100
10
cell
virus
2
4
8
hours
E
STABILITIES OF
VIRUS PARTICLES
percentage zero time value
100
10
PrGag
p41
CA
24
48
hours
F
ANTIBODY REACTIVITY
PrGag
p41
CA
percentage maximum value
100
60
20
10
100
1000
antibody (microliters)


RELATIVE LEVEL OF GAG PROTEIN RELEASE
0.50
1.00
2.00
3.00
WT
ΔNC
ΔMun
ApaI
A15
BglII
p6T1
p6T2
2498T
ApoTE
ApoMtrb
TAM
TARK
MunTE
Δp7bf
Δp6


WT
A15
2498T
TARK
ΔNC
BglII
ApoTE
MunTE
ΔMun
p6T1
ApoMTRB
Δp7bf
ApaI
p6T2
TAM
Δp6
relative Gag protein levels (as a percentage of the peak fraction value in each gradient)
density gradient fractions (top=1; bottom=13)
HIVgpt construct
= M-Mulv control


Virus
Cell
P
G
S
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
WT
MunTE
TAM
TARK
ApoTE
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WT
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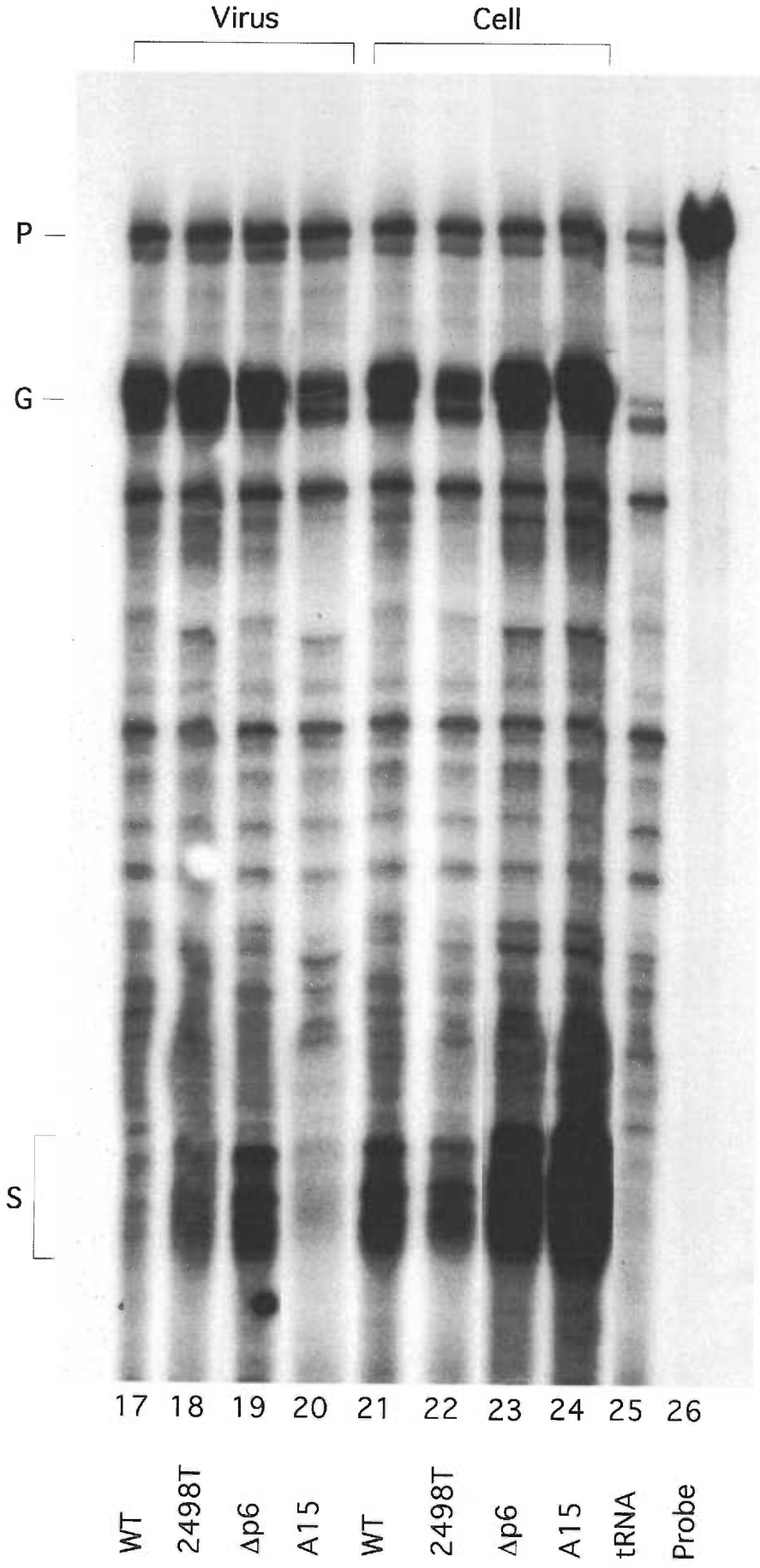
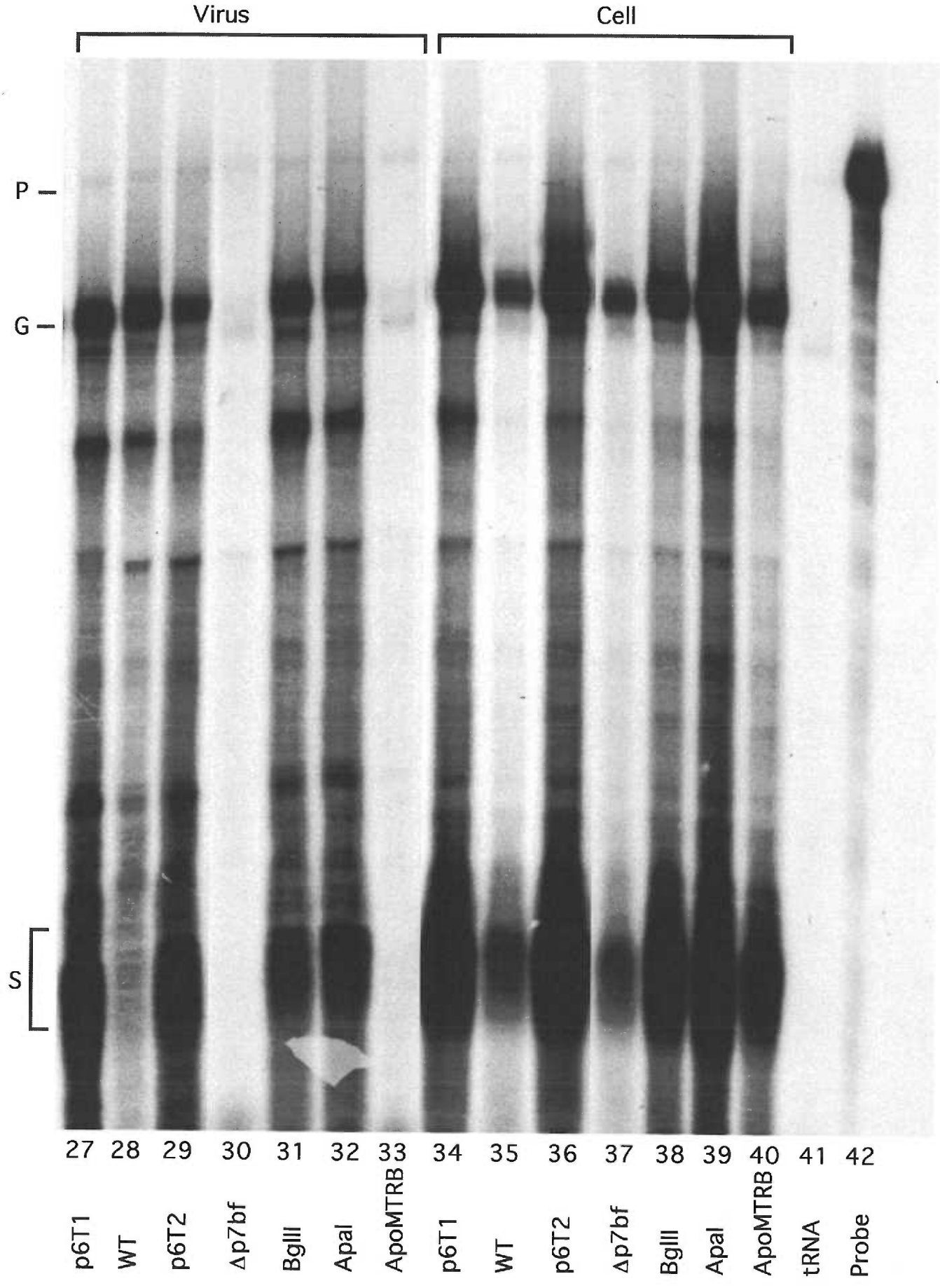
ΔNC
ΔMun
tRNA
Probe


Virus
Cell
P
G
S
17
18
19
20
21
22
23
24
25
26
WT
2498T
Δp6
A15
WT
2498T
Δp6
A15
tRNA
Probe


Virus
Cell
P
G
S
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p6T1
WT
p6T2
Δp7bf
BglII
ApaI
ApoMTRB
p6T1
WT
p6T2
Δp7bf
BglII
ApaI
ApoMTRB
tRNA
Probe

ANALYSIS OF THE ASSEMBLY FUNCTION OF THE HIV-1 NUCLEOCAPSID PROTEIN

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Running title: HIV-1 virus assembly

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ABSTRACT

Previous studies have shown that in addition to its function in specific RNA encapsidation, the HIV-1 nucleocapsid (NC) is required for efficient virus particle assembly. However, the mechanism by which NC facilitates the assembly process is not clearly established. Formally, NC could act by constraining the $Pr55^{gag}$ polyprotein into an assembly-competent conformation, or by masking residues which block the assembly process. Alternatively, the capacity of NC to bind RNA or make interprotein contacts might affect particle assembly. To examine its role in the assembly process, we replaced the NC domain in $Pr55^{gag}$ with polypeptide domains of known function, and the chimeric proteins were analyzed for their abilities to direct the release of virus-like particles. Our results indicate that NC does not mask inhibitory domains, and does not act passively, by simply providing a stable folded monomeric structure. However, replacement of NC by polypeptides which form interprotein contacts permitted efficient virus particle assembly and release, even when RNA was not detected in the particles. These results suggest that formation of interprotein contacts by NC is essential to the normal HIV-1 assembly process.

INTRODUCTION

HIV-1 encodes three major structural genes, *gag*, *pol*, and *env*, which are commonly found in all mammalian retroviruses. It also encodes accessory genes whose protein products are important for regulation of its life cycle (6, 30, 35). However, of all the genes encoded by HIV-1, only the protein product of the *gag* gene has been found to be necessary and sufficient for the assembly of virus like particles (11, 13, 17, 22, 32, 33). The HIV-1 Gag protein initially is expressed as a 55 kD polyprotein precursor (Pr55gag), but during or shortly after particle release, $Pr55^{gag}$ ordinarily is cleaved by the viral protease. The products of the protease action are the four major viral proteins matrix (MA), capsid (CA), nucleocapsid (NC), and p6, and the two spacer polypeptides p2 and p1, which represent sequences between CA and NC, and between NC and p6 respectively (15, 19, 23, 30).

The HIV-1 nucleocapsid proteins have two Cys-X2-Cys-X4-His-X4-Cys (Cys-His) motifs, reminiscent of the zinc finger motifs found in many DNA binding proteins, and NC has been shown to facilitate the specific encapsidation of HIV-1 genomic RNAs. In addition to its encapsidation function, NC also influences virus particle assembly (7, 10, 17, 21, 40). In particular, Gag proteins lacking the NC domain fail to assemble virus particles efficiently. Nevertheless, some chimeric Gag proteins which carry foreign sequences in place of NC have been shown to assemble and release virus particles at wild type (wt) levels (2, 37, 40). Thus, it appears that, in some circumstances, the function NC plays in virus particle assembly can be replaced. To date, it is not clear how NC affects particle assembly, although several possibilities might be envisioned. One possibility is that deletion of NC unmasks inhibitory sequences in p2 or the C-terminus of CA. Alternatively, NC may simply provide a stable monomeric folded structure which locks CA or other Gag domains into an

assembly-competent conformation. Another possibility is that NC facilitates assembly by forming essential protein-protein contacts between neighbor PrGag molecules. Finally, the assembly role of NC may stem from its RNA-binding capabilities.

To distinguish among possible mechanisms by which NC facilitates HIV-1 assembly, we replaced NC with polypeptides having known structural characteristics, and examined particle assembly directed by these chimeric proteins. Using this approach, we have found that NC does not play a passive role in HIV-1 assembly as either a mask to assembly inhibitor domains or a non-specific, stably folded structure. Rather, sequences known to form strong interprotein contacts were observed to enhance assembly, suggesting a similar role for the nucleocapsid domain itself. With several assembly-competent chimeric proteins, we detected no particle-associated RNAs. These results suggest that while RNA may be essential to virus assembly in the context of the wt $Pr55^{gag}$ protein, it is dispensible for formation of virus-like particles from chimeric proteins.

MATERIALS AND METHODS

Recombinant DNA constructs.

The NC mutants used in this study are based on a wt parental construct HIVgpt (27, 36, 38). In HIVgpt, viral sequences derive from HIV-1 strain HXB2, and the *env* gene has been replaced by the *E. coli* drug resistance guanosine phosphoribosytransferase (*gpt*) gene (25), transcribed from the simian virus 40 early promoter. Mutations are numbered according to the HIV HXB2 proviral sequence. The constructs 2498T, TARK, TAM and ApoTE have been described previously (21, 40). Briefly, 2498T is a protease minus (PR-) version of wt HIVgpt, which produces wt Gag proteins but no *pol* open reading frame (ORF) protein products upon transfection into Cos-7 cells. TARK, TAM and ApoTE are Gag C-terminal truncation mutants. In TARK, the *gag* ORF is terminated within the NC region, six residues before the first zinc finger motif; in TAM, the *gag* ORF terminates at the junction of p2 and NC; in ApoTE, the *gag* ORF terminates midway through p2. In the constructs PstTARK, PstTAM, and PstApoTE, portions of CA, P2 and NC were duplicated. For PstTARK, this was achieved by fusing the C-terminal portion of the TARK construct gag coding region (from the *Pst* I site at nt 1419) to the nt 2096 *Bgl* II site of wt HIVgpt. The nucleotide junction sequence at the fusion site is nt 2096 5' AG ATC **CCC GGG TAC CGA GCT CGA ATT CAT CGA TCC TCT AGA GTC GAT CGA C***CT GCA GAA TGG GAT* 3' nt 1432, where the normal *gag* gene sequences are in plain font, linker sequences are in bold, and the duplicated CA sequences derived from TARK are in italics. PstTAM and PstApoTE were created similarly by using sequences from TAM or ApoTE in place of TARK. The junction sequences at the fusion sites for PstTAM and PstApoTE are identical as those in PstTARK, but the ORFs terminate sooner, respectively at either the C-terminus or the middle of the duplicated p2 regions.

For the constructs UPRT HGXPRT, the NC region was replaced with *Toxoplasma gondii* monomeric enzymes Uracil Phosphoribosyltransferase (UPRT) and Hypoxanthine-Xanthine-Guanine Phosphoribosyltransferase (HXGPRT), which catalyze the phosphoribosylation of pyrimidine and purine bases to the nucleotide level (8, 9, 31). For constructs, UPRT and HXGPRT sequences (8, 9; kindly provided by Dr. Buddy Ullman) were inserted at the p2 region of *gag*. The junction sequences for both constructs are nt 1899 5' ACA **AAT TCC TGC AGC CCT** *ATG* 3', where the HIV-1 sequences are in plain font, the linker sequences are in bold, and the UPRT or HGXPRT ATG start codons in italics: UPRT and HGPRT use their own stop codons to terminate ORFs. In other constructs, NC regions were replaced with wt or mutant leucine zipper domains from human CREB protein (20). In the construct wtzip, the wt CREB leucine zipper domain, from CREB residue 284 to its C-terminus was fused to *gag* : the juncture sequence is HIV-1 nt 1899 5' ACA **AAT TCC TGC AGC CCG GGG GAT** *CGA GAG TGT CGT* 3', where HIV-1 sequences are in plain font, the linker sequences are in bold, and the sequences derived from the human CREB protein encompassing the leucine zipper domain, starting from amino acid residue 284, are in italics. The constructs Ezip and Kzip have the same juncture sequences as wtzip. However, in Ezip, Arg300, Gln307, Ile312, Lys319, and Leu321 were mutated to Glu, while in Kzip, Glu298, Arg300, Glu305, Gln307, Ile312, Glu314 Leu321 were mutated to Lys, and Asn308 was mutated to His(20). In a seperate construct, the *Escherichia coli* bacteriophage MS2 coat protein coding region(1, 24; kindly provided by Dr. Marvin Wickens) was used to replace the HIV-1 NC domain. Two similar constructs were made with different junction sequences. For MS2BglII, the junction sequence is HIV-1 nt 1899 5' ACA **AAT TCC TGC AGC CCG GGG GAT CCG CGG GGT ACT GAG AGA CAG GCT AAT TTT TTA GGG AAG ATC CAT** *ATG GCT TCT AAC TTT ACT* 3', where the HIV-

1 sequences are in plain font, the linker sequences are in bold, and the sequences derived from the bacteriophage MS2 coat protein starting from its first amino acid residue are in italics. For MS2Sma, the junction sequence is HIV-1 nt 1899 5' ACA **AAT TCC TGC AGC CCG GGG ATC CAT** *ATG GCT TCT AAC TTT ACT* 3'. The bacteriophage MS2 coat protein has been shown to bind to a short hairpin in its genomic RNA (1). For testing possible RNA encapsidation, a short Eco RI fragment, gaatt ccggc tagaa ctagt ggatc ccccg ggcag cttgc atgcc tgcag gtcga ctcta gaaaa catga ggatc accca tgtct gcagg tcgac tctag aaaac atgag gatca cccat gtctg caggt cgact ctaga ggatc ggaat tc, containing two such hairpins was gratefully received from Dr. Marvin Wickens and inserted at different sites along the MS2BglII proviral genome. As a control, an EcoRI fragment from HIV nt 4648 to nt 5743 from MS2BglII was deleted to make the construct MS2ΔEco. The MS2 binding site then was inserted at this EcoRI site, creating MS2BSEco. In MS2BSPsi, the EcoRI binding site fragment (above) was inserted into the compatible ApoI site at HIV nt 757 of MS2BglII. Finally, in MS2BSBcl, the binding site fragment ends were converted to BamHI sites (ggatc ccccg ggcag cttgc atgcc tgcag gtcga ctcta gaaaa catga ggatc accca tgtct gcagg tcgac tctag aaaac atgag gatca cccat gtctg caggt cgact ctaga ggatc ggaat tc**ctgcagcccgggggatcc;** sequences derived from the origional EcoRI fragment are in plain font, and the modifying sequences are in bold), and inserted into the MS2BglII nt 2429 BclI site.

Cell culture.

Cos-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and penicillin plus streptomycin. For calcium phosphate transfections, 20-30% confluent Cos-7 cells on 10 cm plates were transfected as described previously

(12, 36, 37, 38). Media supernatants and cells were collected at 72 hours post-transfection.

Gag protein analysis.

Detailed procedures for virus release assays have been described (40). Briefly, at 72 hours post-transfection, media supernatants were collected and centrifuged at 4 degrees C for 10 min at 1,000 x g to remove cell debris. Cell-free supernatants then were centrifuged through 2 ml 20% sucrose cushions to pellet virus particles. Cells were washed twice with 10 ml of ice-cold phosphate-buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 1.47 mM KH_2PO_4, 8.05 mM $NaHPO_4$, PH 7.4), then pelleted at 4 C for 10 min at 1,000 x g. The cell pellets were lysed, and collected by 10 min microcentrifugation at 13,700 x g. Aliquots of virus pellet resuspensions and cell lysates were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) along with an internal control recombinant HIV CA standard (21) for Gag protein quantitation purposes. After SDS-PAGE and electroblotting onto nitrocellulose filters, Gag proteins were immunodetected with mouse anti-HIV CA monoclonal antibody from hybridoma cell line Hy183 (made by Bruce Chesebro and obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) as the primary antibody, and an alkaline phosphatase-conjugated goat anti-mouse IgG as the secondary antibody. HIV Gag proteins immunodetected on the nitrocellulose membranes were quantitated using DeskScan II 2.0 alias and NIH image 1.59/fat software, and levels were normalized using the internal control recombinant HIV CA.

Detailed procedures for sucrose density gradient fractionations also can be found in previous publications (14, 16, 36, 40). In short, 72 hours post-transfection, supernatants were collected from transfected Cos7 cells, and centrifuged to remove cell debris. Cell-free supernatant material was pelleted

by centrifugation through 20% sucrose cushions, resuspended in 200 ul of PBS, mixed with internal control Moloney murine leukemia virus (M-MuLV) and layered onto linear 20 to 60% sucrose gradients in SW50.1 polyallomer tubes. Gradients were centrifuged at 4 degrees C for 24 hours at 240,000 x g (equilibrium for particles of 3 S or greater). After centrifugation, 400 ul fractions were collected from the top to the bottom of the gradients. Each fraction was aliquotted for measurement of density, and HIV and M-MuLV Gag protein levels.

RNA analysis.

Isolation and detection of viral and cellular RNA samples was performed according to previous methods (40). After transfections, aliquots of virus resuspensions were used for protein analysis, while the remainder of the virus preparations were used for viral RNA isolations by multiple phenol/chloroform extractions, and ethanol precipitation. Total cellular RNAs were prepared by guanidium thiocyanate-cesium chloride equilibrium centrifugation, and were quantitated spectrophotometrically.

Anti-sense 183 base ^{32}P-labeled probes for RNAse protections were prepared from Blue HX 680-831 by *in vitro* transcription using T3 polymerase as described before(38). For protection assays, probes were hybridized to aliquots of the viral and cellular RNA samples, which were mixed with carrier *Saccharomyces cerevisiae* RNA. Hybridizations, RNAse digestions, electrophoresis, and detection of protected RNA bands proceeded according to published methodology(38). Protected bands on X-ray films and Gag protein signals from corresponding Western blots were processed by DeskScan II 2.0 alias and NIH image 1.59/fat software for quantitation following previously outlined methods (39).

RESULTS

Release of nucleocapsid substitution mutants.

Besides its function in the specific encapsidation of the viral genomic RNA, the HIV-1 nucleocapsid protein appears to influence the assembly phase of the virus life cycle (3, 4, 7, 10, 17, 21, 39, 40). In particular, deletions or major mutations in NC or p2 have been shown to inhibit virus particle assembly (40). However, little is known about the mechanisms by which NC exert its effects on assembly. To investigate what role(s) NC might play during assembly, we replaced it with polypeptides with known structural characteristics. Our assumption (see discussion section) was that the requirements for particle assembly by chimeric Pr^{gag} proteins are similar to those for wt $Pr55^{gag}$. Thus, it might be possible to infer NC function from analysis of chimeras.

Initially, we tested the hypothesis that NC deletion or mutation exposes regions of p2 or CA that inhibit virus particle assembly and/or release. As a positive control for these experiments, we used 2498T, a PR- construct which efficiently (44, 77; Fig. 1a) produces unprocessed immature virus particles. Other control constructs were NC deletions ApoTE and TAM, which have been shown to be release-defective, and the partial NC deletion construct TARK, which assembles and releases virus-like particles, but at reduced efficiency relative to 2498T (77; Fig. 1a). Our experimental constructs PstApoTE, PstTAM and PstTARK, all have wt *gag* sequences through NC and into p1. However, these constructs have C-terminal *gag* sequence duplications such that PstApoTE terminates after a partial duplication of CA and p2; PstTAM after CA and a complete duplication of p2; and PstTARK after a duplication of CA, p2, and eleven residues of NC. Our rationale was that the duplicated C-terminal CA and

p2 sequences of PstApoTE and PstTAM ought to block virus particle release from cells if they were inhibitory.

To assess assembly and release levels, cell lysate and virus-like particle-associated Gag protein levels were measured after transfection of Cos7 cells with experimental and control constructs. As expected, the 2498T Gag protein was detected in cells (Fig. 1b, lane B) and was released well from the cells (Fig. 1b, lane A). In contrast, and as observed previously, the ApoTE and TAM proteins did not direct release of virus-like particles efficiently (Fig. 1b, lanes E-H). Also expected were results with TARK (lanes C, D), showing Prgag release at higher levels than those of ApoTE and TAM, but reduced compared to 2498T. When the duplication proteins PstApoTE, PstTAM and PstTARK were tested in the same assay, all three of them appeared to be released at reasonably high efficiencies (lanes I-N). For quantitative purposes, experiments were repeated several times, cellular and particle-associated Gag protein levels were determined, and release levels were compiled (Fig. 1c). As illustrated, 2498T released well from cells, ApoTE and TAM released poorly, and TARK was released at a reduced efficiency compared with 2498T. All duplication constructs released virus-like particles at considerably higher levels than ApoTE and TAM and 45-58% as well as 2498T (Fig. 1c). These results do not support the notion that the free C-termini of p2 or CA actively inhibit particle assembly by ApoTE and TAM proteins.

While the above experiments suggest that CA or p2 residues in NC deletion mutants do not actively inhibit assembly, NC might be required simply because it non-specifically restricts p2 and/or CA into an assembly-competent conformation. To test this possibility, NC was replaced with monomeric proteins which form stable structures. The protein sequences used to replace

NC were uracil phosphoribosyltransferase (UPRT) and hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) enzymes from *Toxoplasma gondii* (8, 9; Fig. 2a). UPRT and HXGPRT are 26-27 kD polypeptides, and UPRT behaves as a monomer in solution (8), while HXGPRT is a monomer at concentrations lower than 4 uM, but can form weak dimers with a dissociation constant of 40 uM(9, 31). As shown in Fig. 2b lane D and F, chimeric Gag proteins which have the HIV-1 NC regions replaced by UPRT and HXGPRT were expressed well in transfected Cos7 cells. When protein samples from the transfected Cos7 cell lysates were assayed for UPRT and HXGPRT enzymatic activities (8, 9, 31), we observed specific activities of approximately 0.16nmol/minug and 0.14nmol/minug for UPRT and HXGPRT, respectively (data not shown). These specific acivities correspond to 36% and 0.2% of the acitivities observed for the purified enzymes, suggesting that the enzyme domains retain intact folded structures. However, while 2498T proteins were released efficiently from cells (lane A versus B), the UPRT and HXGPRT versions were not (lanes C, E). Quantitation of serveral independent transfections (Fig. 2c) showed that release levels of UPRT and HXGPRT were only 4-5% 2498T levels, and only marginally higher than levels for the negative control ApoTE and TAM constructs. These data suggest that NC does not enhance virus assembly simply because it forms a stable structure.

To test whether it might function as an active assembly domain by making interprotein contacts, NC was replaced by proteins with known abilities to form protein-protein interactions. One such construct was wtzip, in which NC was replaced by 44 residues comprising the wt leucine zipper domain of human CREB DNA binding protein (20; Fig. 3a). Two control constructs were the mutant zipper constructs Ezip and Kzip (Fig. 3a), which form homodimers inefficiently, but readily form leucine zipper heterodimers

(20). As shown in Fig. 3b (lanes C and D), the wtzip construct directs release of virus-like particles similar to that seen for the positive control 2498T (lanes A, B), and quantitation of independent transfections showed wtzip release levels to be over half that of the control (Fig. 3b). In contrast with wtzip, we observed that the mutant Ezip and Kzip chimeric proteins did not direct particle release efficiently (Fig. 3b, 3c). Because we found that the Ezip chimeric protein consistently showed a higher mobility than the wtzip or Kzip proteins in SDS-PAGE gels, it was possible to distinguish the Kzip and Ezip chimeras in cotransfections. Interestingly, when the Kzip and Ezip constructs were cotransfected into Cos7 cells, virus-like particles were released from cells at higher levels than with either construct alone. As shown in Fig. 3b, cotransfection of Ezip and Kzip constructs into cells (lane J) resulted in a relative increase in Ezip protein release, and a marked increase in Kzip protein release (lane I). Quantitation (Fig. 3c) showed that chimeric protein release was increased over five-fold in cotransfection relateive to individual transfections. While cotransfection release levels were only 18% that of wtzip release levels, part of this difference may be attributable to cotransfection efficiency, difference in Ezip and Kzip chimeric protein expression, and reduced dimerization of mutant versus wt proteins. Taken together, our results with wt and mutant zipper chimeras strongly indicated that the assembly function of HIV-1 NC can be replaced by polypeptides which form interprotein contacts.

As an additional test, we replaced NC with the *Escherichia coli* bacteriophage MS2 coat protein (24)(Fig. 3a), which functions as a multimer(24). Ordinarily, this protein binds and encapsidates the bacteriophage RNA, and also acts as translational repressor of the phage replicase by binding to an RNA hairpin structure in the phage RNA genome

(1, 18, 26, 28, 29, 34). Since the MS2 coat protein tolerates N-terminal fusions, we reasoned that it might function in place of NC. As shown in Fig. 3b (lanes K, L), cells transfected with MS2 constructs release high levels of virus-like particles--nearly comparable to 2498T release levels (Fig. 3c). These results substantiate the hypothesis that NC assembly domain can be replaced by protein domains known to make interprotein contacts.

Characterization of wtzip and MS2 virus-like particles.

Previous work has implied a correlation concerning the presence of the NC domain and assembly of tightly-packed virus-like particles which have characteristic densities (16, 40). To assay the densities of well-released NC substitution mutants, virus particles were pelleted from cell-free supernatants from transfected Cos-7 cells and mixed with internal control Moloney murine leukemia virus (M-MuLV), then sedimented by equilibrium centrifugation through linear 20 to 60% sucrose gradients. Fractions were collected from the top to the bottom of the gradients after centrifugation, and each fraction was aliquotted for measurement of HIV and M-MuLV Gag protein levels by SDS-PAGE and immunoblot. As shown in Fig. 4 and as seen previously (40), 2498T particles are slightly more dense than the internal control M-MuLV particles, suggesting that they are tightly packed. In contrast, the NC deletion mutant TAM (Fig. 4, second panel) comes to equilibrium at a slightly lower density than the M-MuLV control. The chimeric Gag proteins wtzip and MS2 (Fig. 4) show sedimentation patterns similar to that of 2498T, suggesting that these domains help mediate tight packing of virus particles.

Although wt zipper portion of CREB protein has no known RNA binding function, it was of interest to ascertain whether spliced or full-length viral RNA might be incorporated into wtzip particles. Similarly, because the MS2 coat protein is known to bind RNA, it also was of interest to assay the RNA

content of MS2 chimeric particles. In this regard, since the MS2 coat protein preferentially binds a target RNA hairpin structure, we introduced this element into the MS2BglII construct at three different sites, creating the construts MS2BSPsi, MS2BSBcl, and MS2BSEco (Fig. 5). For detecting viral RNAs in cells and virus-like particles produced from Cos7 cell transfections, an antisense RNA probe that crosses the HIV-1 major splice donor site was used, so that full-length and spliced viral RNAs could be detected simultaneously (39, 40). Using this probe and RNA samples from cells and virus-like particles, we followed previous protocols for quantitation of spliced and full-length viral RNAs, and normalization of virus yields by Gag protein quantitation (39, 40; see Materials and Methods).

As shown in Fig. 6a, genomic and spliced viral RNAs are readily detected in both wtzip and 2498T transfected cells (lanes 3 and 4). Additionally, 2498T specifically encapsidates large levels of genomic RNA and low levels of spliced RNAs into virus particles (Fig. 6a, lane 2; Table 1). In contrast, wtzip does not appear to encapsidate either genomic or spliced RNAs (Fig. 6a, lane 1; Table 1). Similar results were observed when NC was replaced by the MS2 coat protein (see Fig. 6b). Once again, 2498T was observed to encapsidate high levels of full-length viral RNAs and lower levels of spliced RNAs (Fig. 6, lanes 9, 12). In contrast, the virus-like particles formed by MS2 chimeric proteins failed to package RNAs transcribed from MS2ΔEco construct (Fig. 6, lanes 8, 11; Table 1). Even insertion of two MS2 RNA hairpin binding sites at the EcoRI site in MS2BSEco, the BclI site in MS2BSBcl or the HIV-1 5'non-coding region in MS2BSPsi (Fig. 5) failed to facilitate detectable RNA encapsidation into virus-like particles produced by MS2 chimeric proteins (Fig. 6, lanes 7, 10; data not shown for MS2BSBcl and MS2BSPsi). While it is possible that non-viral RNAs may have been encapsidated into wtzip or MS2 particles, these results are

consistent with the interpretation that the assembly function of the HIV-1 nucleocapsid protein can be replaced by a polypeptide which does not bind RNA (see discussion).

DISCUSSION

Previous studies have shown that NC is essential to assembly of mammalian or avian retrovirus (7, 10, 17, 21, 40). Although it is clear that deletions of NC inhibit virus assembly, previous studies have not elucidated the mechanism(s) by which NC influences assembly. Our approach has been to assess the abilities of polypeptides to substitute for the assembly function of NC, and to infer nucleocapsid's role from the known characteristics of the replacement sequences. This method certainly can be employed to examine how protein sequences can enhance virus-like particle assembly or release in the context of the amino-terminal portions of the HIV-1 Pr^{gag} protein (MA-CA). However, it should be obvious that inferences regarding NC function are dependent on the number of possible ways a polypeptide might influence particle assembly or release.

Subject to the above qualification, our results support the hypothesis that NC is an active assembly domain (2), which can be replaced by heterologous domains which form interprotein contacts. Duplication of CA and p2 at the C-terminus of Gag did not inhibit virus assembly (Fig. 1), suggesting that NC does not simply mask sequences which are toxic to virus assembly. Replacement of NC by monomeric proteins UPRT and HGPRT did not facilitate virus-like particle release (Fig. 2), which suggests that NC does not act passively, by forming a stable structure that restricts p2 or CA in an assembly-competent conformation. However, MS2 coat protein, wt CREB zipper domain both form interprotein contacts, and replacing NC with either of them increased release levels, relative to the NC deletion proteins ApoTE and TAM (Fig. 3). Additionally, replacement NC with mutant Ezip or Kzip leucine zippers which are incapable of forming homodimers did not facilitate assembly of virus-like particles. In

contrast, in cotransfection experiments, which permit the formation of heterodimers, release levels were increased (see Fig. 3).

The wt human CREB zipper domain is not known to bind RNA. Thus, when it replaced NC, it was not surprising that no viral genomic or spliced RNA appeared to be encapsidated into the wtzip virus particles (Fig. 6, Table 1). Although the bacteriophage MS2 coat protein can bind to its own RNA genome at a specific hairpin structure (1, 18, 26, 28, 29, 34), in the HIV-1 Gag fusion proteins, it did not appear to bind spliced or unspliced HIV-1 viral RNA sequences, even when MS2 binding sites were present in the viral genoms (Fig. 6, Table 1). Although we have not exhaustively tested assembly of all cellular RNAs into virus particles, if spliced RNA encapsidation is indicative of non-specific RNA incorporation, as we have seen previously (40), we would expect little RNA in these particles. This implies that NC can be replaced by domain which does not need to bind RNA (40). While evidence suggests that fusion proteins of Gag with MS2 coat protein and wt zipper domain do not need to bind RNA for assembly purposes, a number of studies suggest that loss of the NC RNA-binding function correlates with a decreased efficiency of assembly (5). It is possible that NC interprotein contacts might be mediated indirectly by RNA; or RNA might be required for assembly in the context of NC. Insofar as specific RNA encapsidation is concerned, it is clear that the wt $Pr55^{gag}$ protein can efficiently and specifically encapsidate genomic-length HIV-1RNA (40; Fig. 6; Table1), In contrast, even in the presence of its binding sequence at different sites in the HIV-1 genome, the Gag-MS2 chimera (MS2) did not appear to bring viral RNA into virus particles (Fig. 6; Table 1; data not shown). What might be the reasons for this lack of RNA binding? Possibly, the MS2 coat protein when fused to Gag is non-functional for RNA binding. Alternatively, the RNA binding site may not be folded appropriately, or might

be masked by cellular or viral factors. Yet another explaination is that the viral RNAs may not localize to the assembly sites of the chimeric proteins. Investigation of these and other possibilities may provide further insights to HIV-1 assembly and encapsidation mechanisms.

ACKNOWLEDGMENTS

We thank Jason McDermott, Marylene Mougel and Sonya Karanjia for help and advice throughout the course of this work. The anti-M-MuLV-CA monoclonal antibody was a gift from Bruce Chesebro, who also made the anti-HIV CA Hy183 hybridoma cell line that was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. Two molecular clones encoding the UPRT and HXGPRT proteins used in this study were generously provided by Buddy Ullman, and Darrick Carter kindly performed enzyme assays. The clones encoding the wt and mutant E and K leucine zippers were the gifts of Richard Goodman, Marc Loriaux, and Jim Lundblatt, and the bacteriophage MS2 coat protein and binding site clones were generously provided by Marvin Wickens. This work was supported by grant 2RO1 CA47088-07 from the National Cancer Institute.

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FIGURE AND TABLE LEGENDS

Table 1. RNA encapsidation into virus particles.

Genomic and spliced RNA signals from cell and virus samples of Figure 6, and corresponding Gag protein levels from virus particles detected by Western blots were quantitated using DeskScan II 2.0 alias and NIH image 1.59/fat software. From these data, for each indicated construct the following ratios were calculated: the total levels of viral spliced and unspliced RNAs in particle samples divided by cell samples; the total particle-associated viral RNA signals divided by the particle Gag protein signals; and the particle genomic RNA signals divided by the particle spliced viral RNA signals. Calculated ratios were normalized to 2498T ratios and are listed as percentages of 2498T levels (2498T = 100%). For mutants which showed no particle-associated viral RNA signal above background, values in the first two columns are given as less than (<) a given level, while values for the right-most column were not applicable (NA).

Figure 1. HIV Gag truncation and capsid duplications.

A. All the constructs used in this study are based on a parental construct HIVgpt (30, 36, 38) which has the viral sequences of HIV HXB2. Both HIVgpt and its protease minus version 2498T have been described in previous publications (21, 40). Since NC deletion and substitution mutants are all protease minus, they are compared with 2498T. In this figure, 2498T is diagrammed to show the C-terminal portion of the *gag* gene and the beginning of the *pol* gene. Only the C-terminus of CA, p2, NC, p1 and p6 of the *gag* open reading frame (ORF) are shown in the diagram: CA is in black, p2 and p1 have diagonal bars, NC is in white with Cys-His motifs indicated as diamonds, p6 is in white, and HIV-1 proviral nt numbers are designated. 2498T expresses the wt $Pr55^{gag}$ polyprotein, but not *pol* gene products due to a termination codon inserted on the *pol* frame at the *Hind* II site at nt 2498 (21, 40). NC deletion mutants TARK, TAM and ApoTE have translation terminators in the *gag* gene, causing the *gag* ORF to terminate 11 residues after (TARK), precisely at (TAM), or 5 residues before (ApoTE) the junction of p2 and NC. For PstTARK, PstTAM, and PstApoTE, sequences from TARK, TAM and ApoTE, starting from the *Pst* I site at nt 1419 in CA, and ending beyond the *gag* coding sequences of these constructs, were joined to the wt *gag* sequence at the nt 2096 *Bgl* II site in the p1 coding region of HIVgpt through a short linker sequence. Consequently, these constructs encode duplications of most of CA plus part of p2 (PstApoTE), all of p2 (PstTAM), or all of p2 plus 11 residues of NC. The precise junction sequences of these constructs are provided in the Materials and Methods.

B. Media supernatant (V) and cell (C) samples were collected 72 hours after transfections of Cos7 cells with the indicated constructs. Particles were pelleted from cell free media samples, and half of the resuspended pellets were

separated by SDS-PAGE. Cell pellets were lysed and centrifuged to remove debris, and one twentieth of the cell lysate samples were fractionated by SDS-PAGE. After electrophoresis, Gag proteins were electroblotted onto nitrocellulose filters and immunodetected using a mouse anti-p24 monoclonal antibody from hybridoma cell line Hy183 as the primary antibody. Precursor Gag proteins, identified by antibody reactivity and comparison of gel migration mobilities to known standards, are indicated with black triangles. Media samples are in lanes A, C, E, G, I, K, and M, and cell samples correspond to lanes B, D, F, H, J, L, and N. Lane designations are as follows: wt, lanes A, B; TARK, lanes C, D; TAM, lanes E, F; ApoTE, lanes G, H; PstTARK, lanes I, J; PstTAM, lanes K, L; PstApoTE, lanes M, N.

C. Gag proteins in matched cell and media supernatant samples from several experiments were detected as in **B**, and quantitated using the programs DeskScan II 2.0 alias and NIH image 1.59/fat. Gag protein levels were normalized using a bacterially expressed HIV CA protein standard run on each gel, and ratios of the total Gag protein levels in the medias versus cells were calculated. The ratios of the Gag truncation and capsid duplication constructs were normalized to that of 2498T. Thus, the values of the ratios indicate relative levels of Gag protein release. Note that standard deviations are shown with the mean values.

Figure 2. Monomeric enzyme substitutions.

A. 2498T has been described and diagrammed in Figure 1a. The constructs UPRT and HGPRT were constructed from the coding regions of the uracil phosphoribosyltransferase (UPRT) and hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) enzymes from *Toxoplasma gondii* (8, 9, 31). Starting from their first Met residues, the enzyme coding regions were fused to *gag* at nt 1899 in the p2 region through a short linker. The precise sequences of the fusion sites of these constructs are provided in the Materials and Methods.

B. Particle-associated media supernatant (V) and cell (C) samples from Cos7 cell transient transfections with the indicated constructs were prepared, electrophoresced, and electroblotted, and Pr^{gag} proteins (black triangles) were detected as described in Fig. 1b. As shown, media supernatant samples correspond to lanes A, C, and E, and cell samples correspond to lanes B, D, and F. Lane designations are as follows: 2498T, lanes A, B; UPRT, lanes C, D; HGPRT, lanes E, F.

C. Gag proteins in matched cell and media supernatant samples from several transfections were detected, quantitated, and normalized as in Figure 1c. Ratios of the total Gag protein levels in the particle-associated media supernatant versus cell samples were calculated and normalized to that of 2498T. Values shown thus indicate relative levels of Gag protein release. Standard deviations are shown with the mean values.

Figure 3. Oligomerization domain substitutions

A. 2498T is described and diagrammed as in Figure 1a. In the construct wtzip, the wt leucine zipper domain from the human CREB protein (20) starting from amino acid residue 284 was fused to HIV-1 nt 1899 in the p2 region of *gag* through a short linker sequence. The constructs Ezip and Kzip were created similarly to wtzip, but possess zipper region mutations: in Ezip, Arg300, Gln307, Ile312, Lys319, and Leu321 were converted to Glu, while in Kzip, Glu298, Arg300, Glu305, Gln307, Ile312, Glu314 Leu321 were converted to Lys, and Asn308 was converted to His(20). In the construct MS2, the bacteriophage MS2 coat protein (1, 18, 19, 24, 26, 28, 29, 34), starting from its first amino acid residue, was fused to HIV-1 nt 1899 through a short linker sequence. Precise sequences of the fusion sites of these constructs are available in the Materials and Methods.

B. Gag proteins in particle-associated media supernatant (V) and cell (C) samples from Cos7 cell transfected with the indicated constructs were detected as described in Fig. 1b. Media samples correspond to lanes A, C, E, G, I, and K, and cell samples correspond to lanes B, D, F, H, J, and L. Lane designations are as follows: 2498T, lanes A, B; wtzip, lanes C, D; Ezip, lanes E, F; Kzip, lanes G, H; E+Kzip, lanes I, J; MS2, lanes K, L.

C. Gag proteins in matched cell and media supernatant samples from several transfections were detected, quantitated, and normalized as in Figure 1c. Ratios of the total Gag protein levels in the medias versus cells were calculated and normalized to that of 2498T. Standard deviations are shown with the mean values.

Figure 4. Sucrose density gradient fractionation of virus and virus-like particles.

Virus pellets prepared from cell-free supernatants from transfected Cos7 cells were resuspended in PBS, mixed with mouse M-MuLV (Moloney murine leukemia virus) suspensions, and layered on top of the linear 20-60% sucrose gradients. Gradients were centrifuged 24 h at 240,000 x g such that particles with a sedimentation coefficient of 3S or greater would come to equilibrium. After centrifugation, a total of thirteen fractions were collected from the top (left)to bottom (right). Each fraction was monitored for density, and HIV-1 and M-MuLV Gag protein (black and gray triangles) were visualized after SDS-PAGE by immunoblotting. As illustrated, the top panel shows immunoblot detection of Gag proteins from the 2498T gradient; the second panel correspond to TAM gradient; the third panel correspond to wtzip; and the bottom panel shows results with the MS2 construct.

Figure 5. MS2 binding site constructs.

The bacteriophage MS2 coat protein has been shown to bind to a short hairpin in its genomic RNA (1). For testing possible RNA encapsidation, a short Eco RI fragment containing two such hairpins was inserted at different sites along the MS2BglII (see Materials and Methods; Fig. 3a) proviral genome. As a control, an EcoRI fragment from HIV nt 4648 to nt 5743 from MS2BglII was deleted to make the construct MS2ΔEco. The EcoRI fragment containing the MS2 binding sites then was inserted at this EcoRI site, creating MS2BSEco. In MS2BSPsi, the EcoRI binding site fragment was inserted into the compatible ApoI site at HIV nt 757 of MS2BglII. Finally, in MS2BSBcl, the binding site fragment ends were converted to BamHI sites and inserted into the MS2BglII nt 2429 BclI site. Precise sequences of the junction sites of these constructs are available in the Materials and Methods.

Figure 6. Genomic and spliced RNA levels in cells and virus particles of wtzip and MS2 HIV-1 constructs.

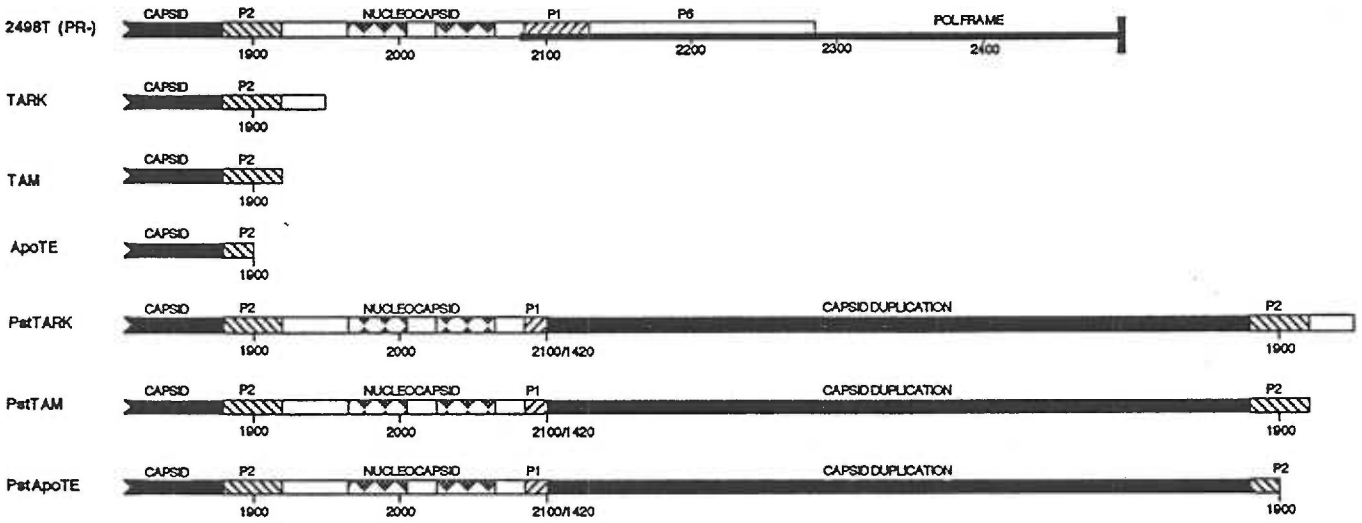
Aliquots of cellular and viral RNA samples prepared from transfected cells and virus pellets, as described in the Materials and Methods, were mixed with 10 ug of yeast tRNA, ethanol precipitated, dried, and hybridized to an HIV-1 antisense probe of 183 nt (lanes 6 and 14; indicated by the letter P), which crosses the HIV-1 major splice donor site. Following the RNAse protection described in the Materials and Methods, the probe is capable of detecting both spliced viral transcripts at a fragment size of 63-64 nt (indicated by a letter S), and unspliced, genomic RNAs at a protected fragment size of 150 nt (indicated by a letter G). In each panel, results from mock reactions using yeast tRNA samples alone were used (lanes 5 and 13), and lane designations for virus (listed first) and cell (listed second) samples from transfection samples are as follows: wtzip, lanes 1, 3; 2498T, lanes 2, 4; MS2BglIIBSR1, lanes 7, 10; MS2BglIIΔR1, lanes 8, 11; 2498T, lanes 9, 12. Note that viral RNA signals were normalized for total Gag protein content to yield encapsidation efficiencies as indicated in Table 1.

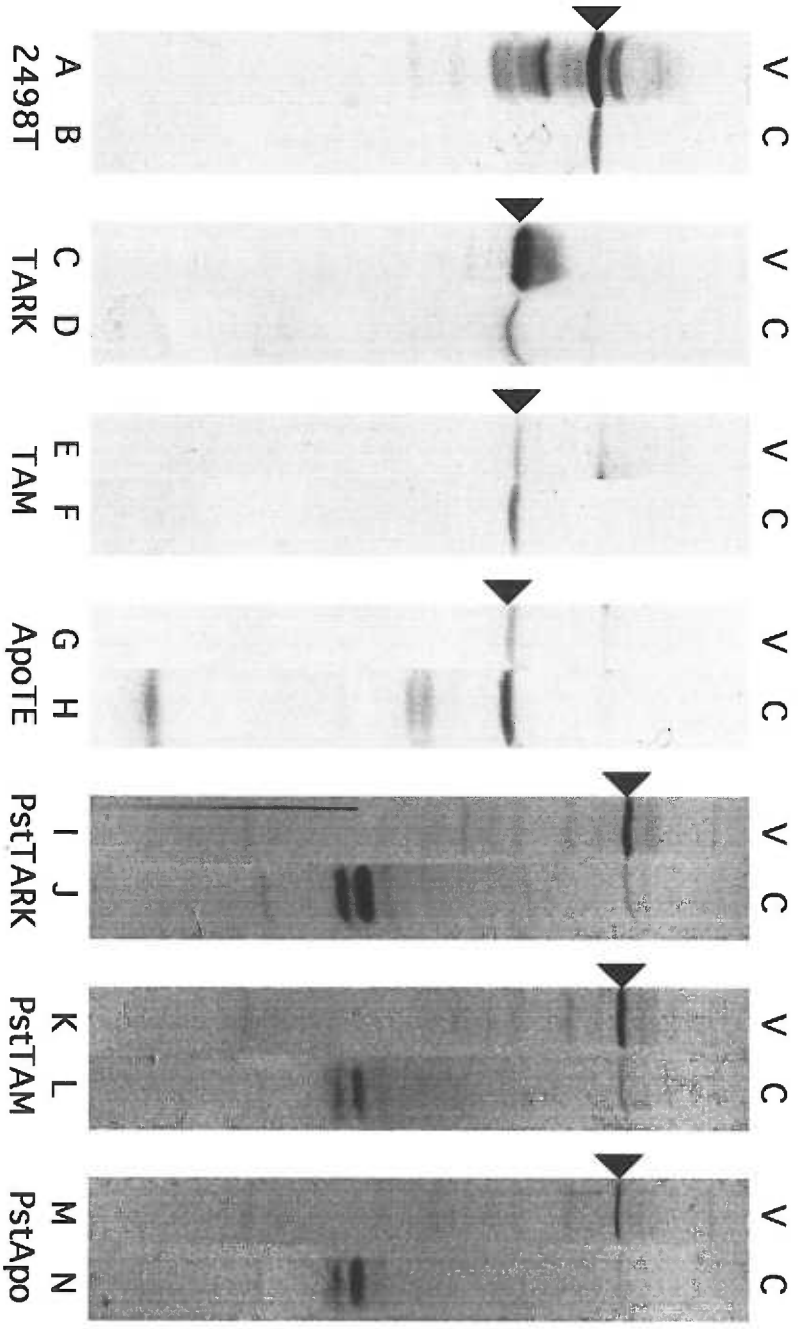
Table 1. RNA encapsidation into virus particles.

CONSTRUCT	Total Viral RNA Virus/Cell	Total Viral RNA Viral Gag protein	Viral Genomic RNA Viral spliced RNA
2498T (PR-)	100	100	100
wtzip	<1.0	<1.0	NA
MS2BglIIBSRI	<1.0	<1.0	NA
MS2BglIIΔRI	<1.0	<1.0	NA

Fig.1A

HIV GAG TRUNCATIONS AND CAPSID DUPLICATIONS

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2498T
V C
C D
TARK
V C
E F
TAM
V C
G H
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V C
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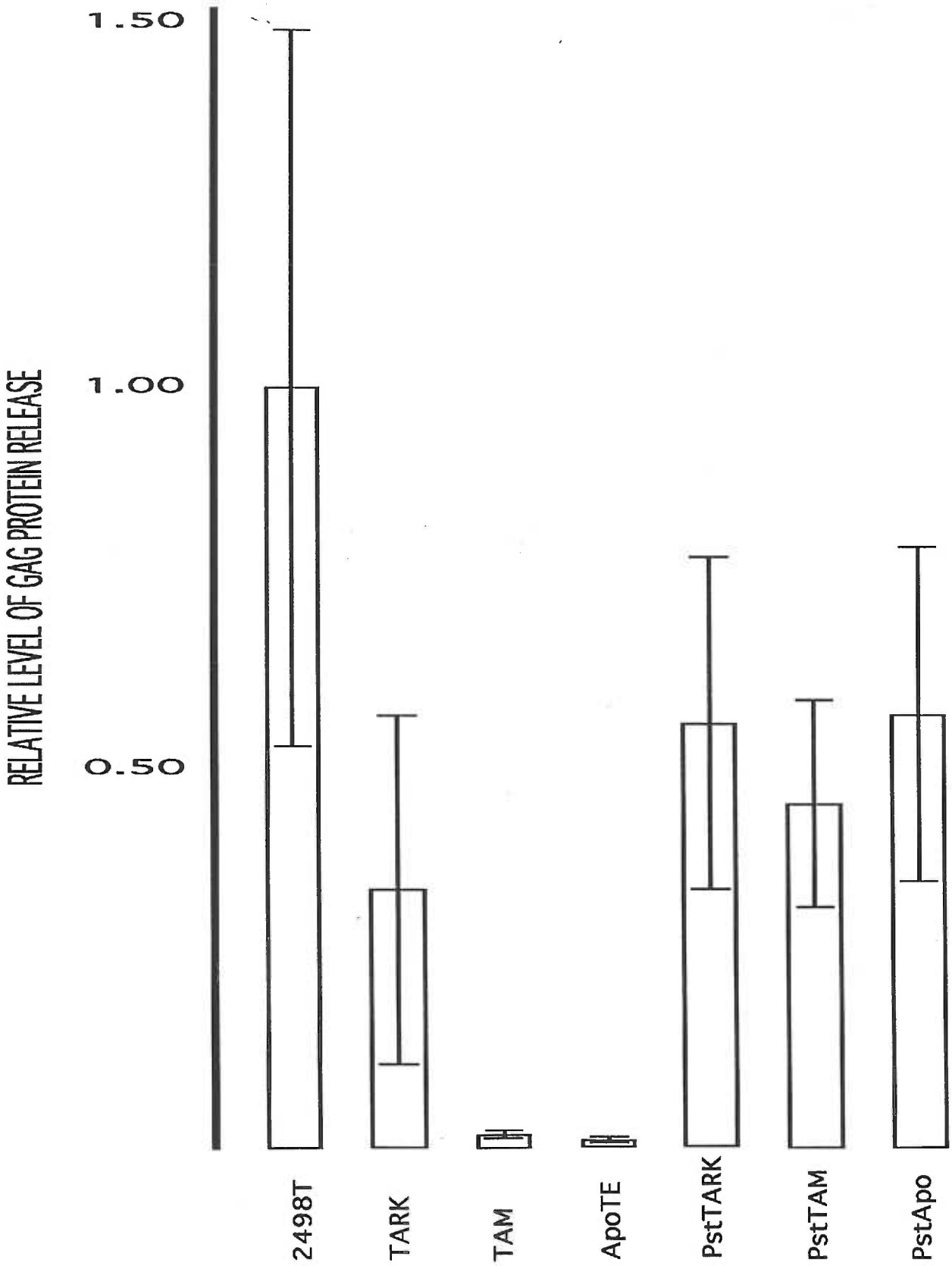
V C
K L
PstTAM
V C
M N
PstApo
V C

Fig.1C


RELATIVE LEVEL OF GAG PROTEIN RELEASE
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1.00
0.50
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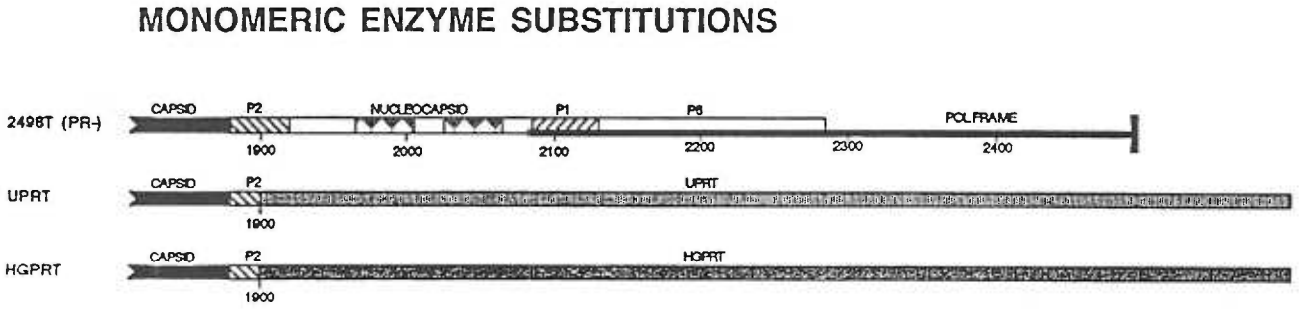
TARK
TAM
ApoTE
PstTARK
PstTAM
PstApo

Fig.2A


MONOMERIC ENZYME SUBSTITUTIONS
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P2
NUCLEOCAPSID
P1
P6
POL FRAME
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HGPRT
1900

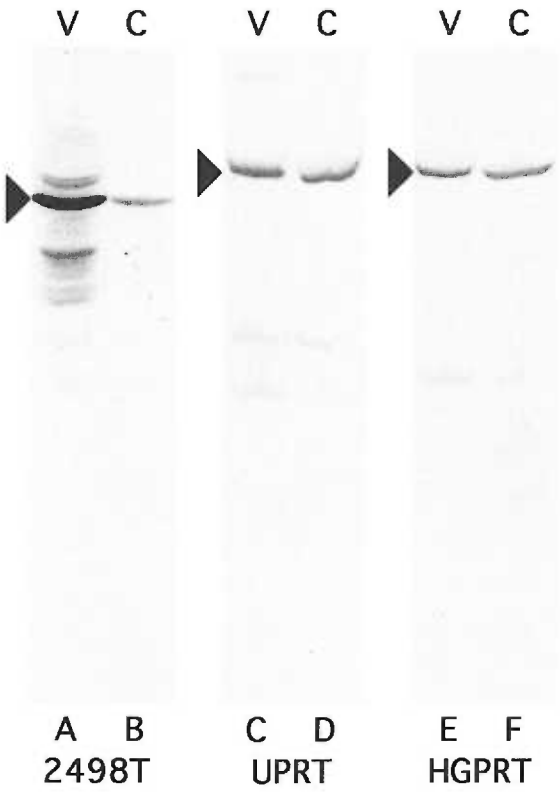

V C V C V C
A B C D E F
2498T UPRT HGPRT

Fig.2C

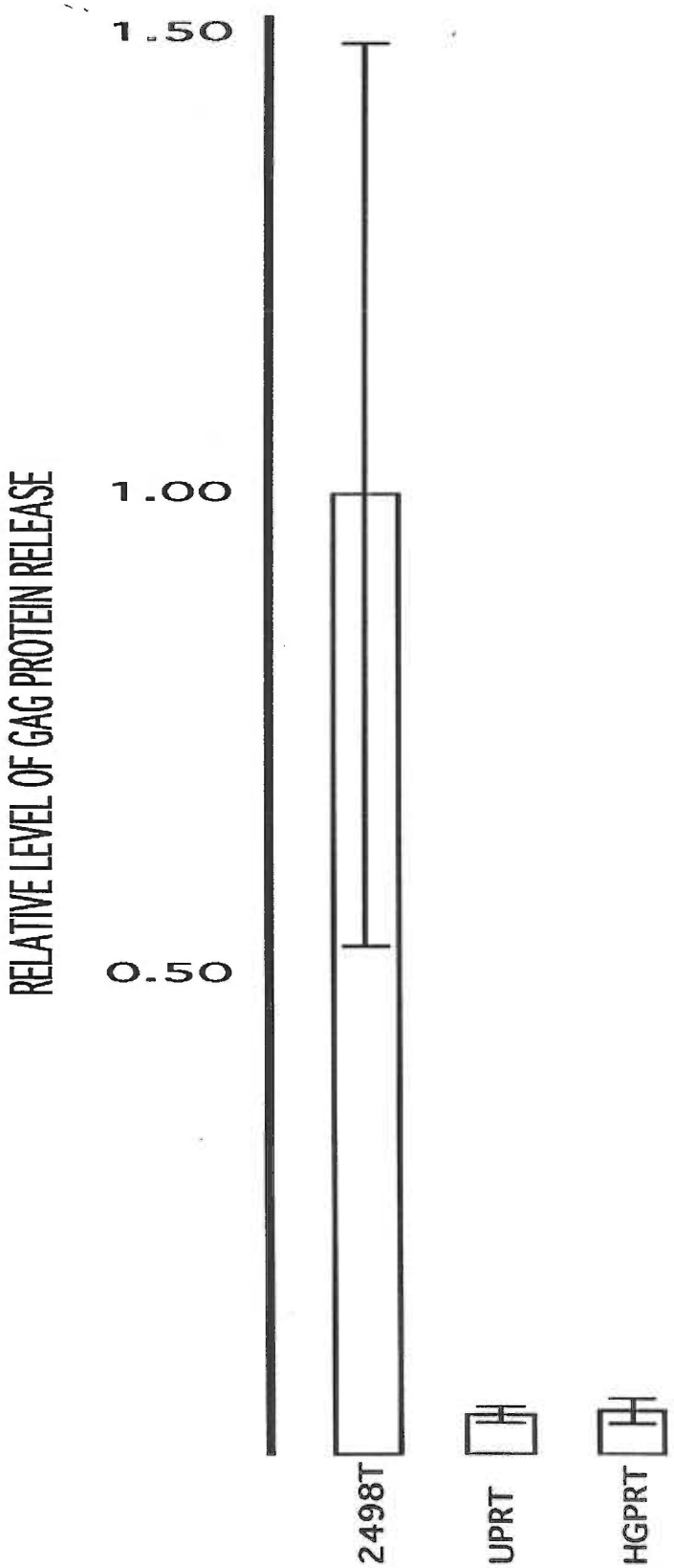
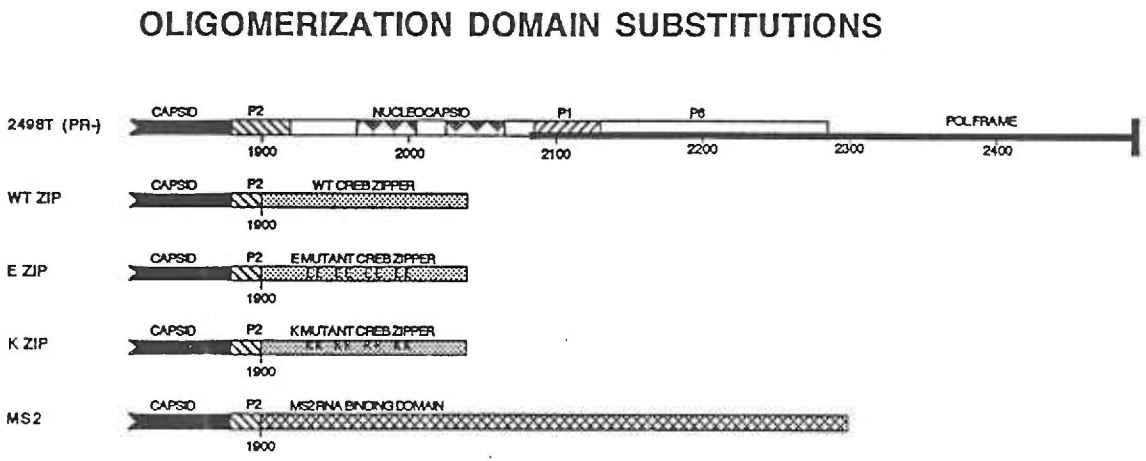

RELATIVE LEVEL OF GAG PROTEIN RELEASE
1.50
1.00
0.50
2498T
UPRT
HGPRT

Fig.3A


OLIGOMERIZATION DOMAIN SUBSTITUTIONS
2498T (PR-)
CAPSID
P2
NUCLEOCAPSID
P1
P6
POL FRAME
1900
2000
2100
2200
2300
2400
WT ZIP
WT CREB ZIPPER
E ZIP
E MUTANT CREB ZIPPER
K ZIP
K MUTANT CREB ZIPPER
MS2
MS2 RNA BINDING DOMAIN

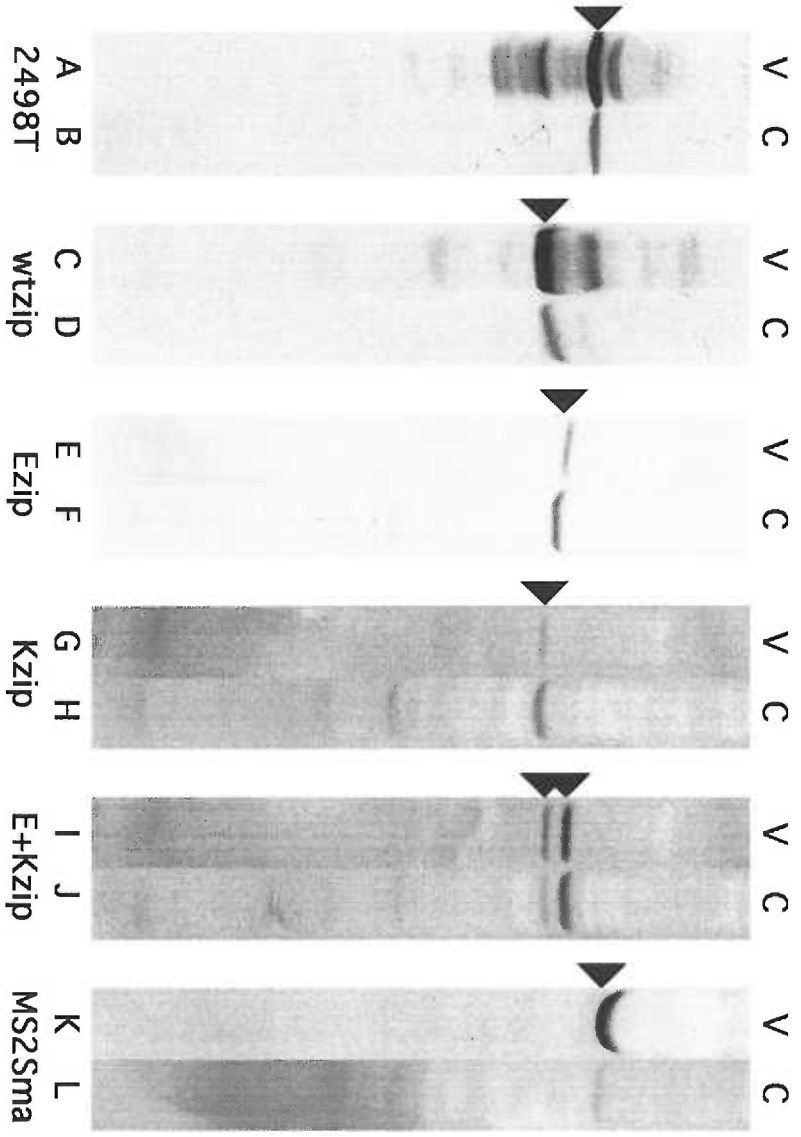
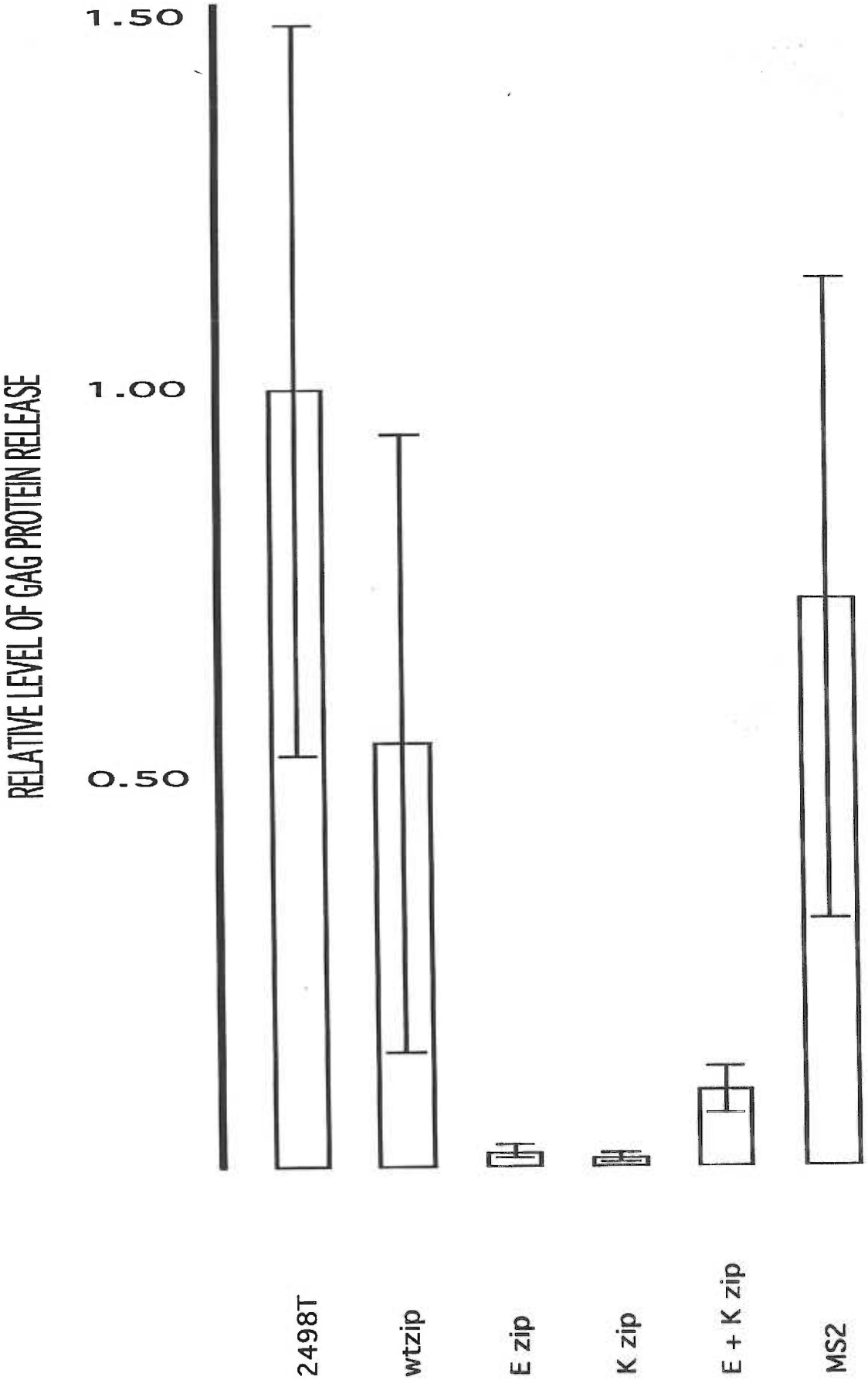
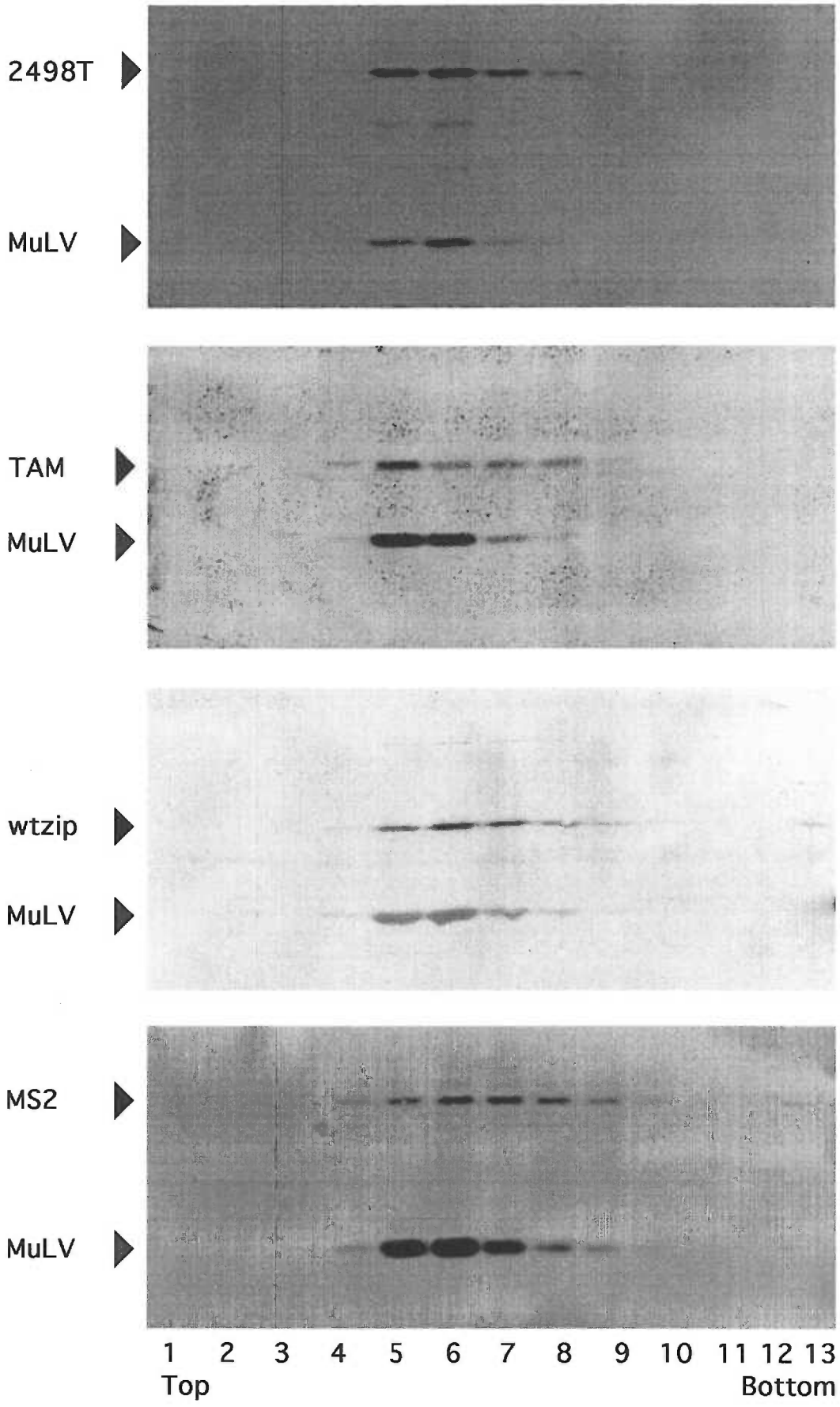

A B
2498T
V C
C D
wtzip
V C
E F
Ezip
V C
G H
Kzip
V C
I J
E+Kzip
V C
K L
MS2Sma
V C

Fig.3C


RELATIVE LEVEL OF GAG PROTEIN RELEASE
1.50
1.00
0.50
2498T
wtzip
E zip
K zip
E + K zip
MS2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MuLV
TAM
MuLV
wtzip
MuLV
MS2
MuLV
1 2 3 4 5 6 7 8 9 10 11 12 13
Top
Bottom

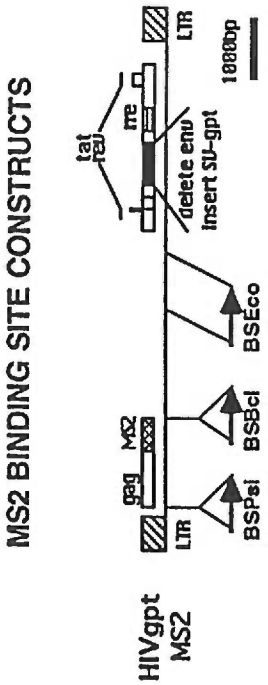
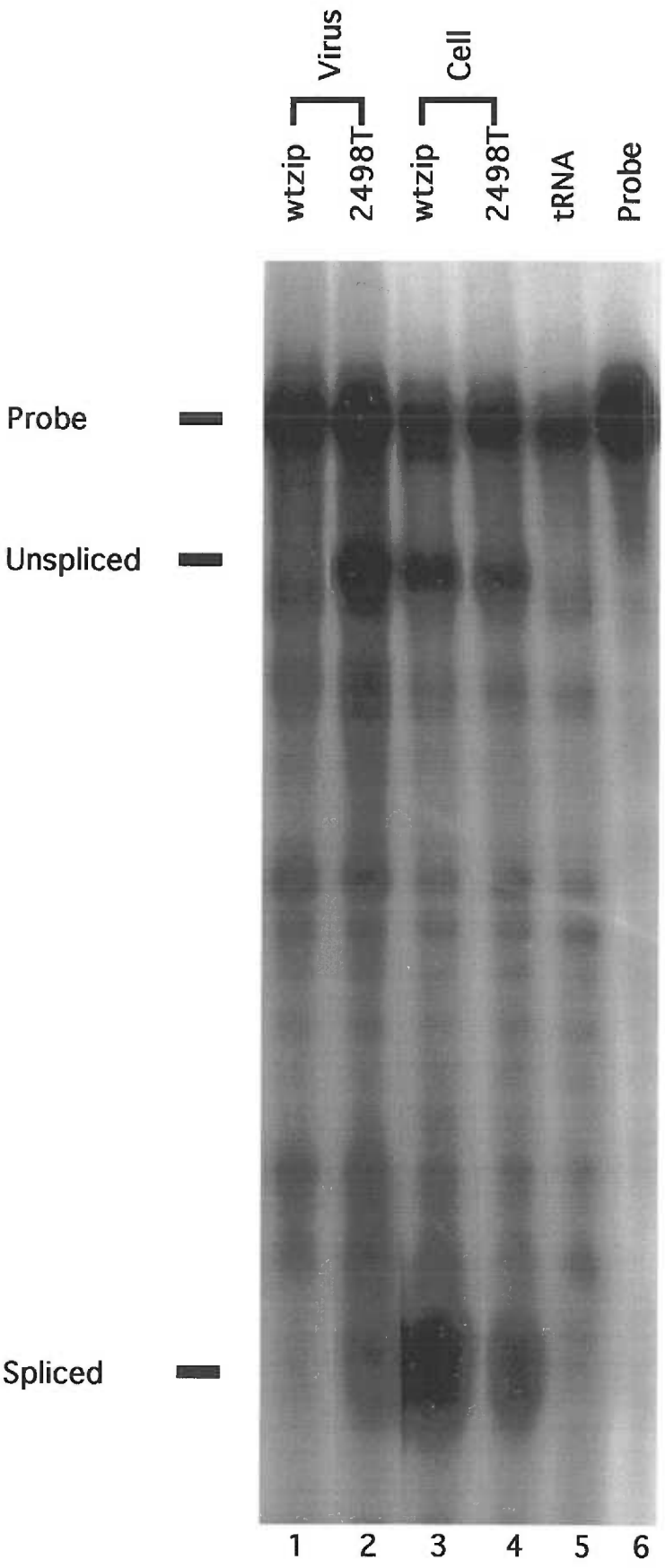

MS2 BINDING SITE CONSTRUCTS
HIVgpt MS2
LTR
gag
MS2
BSPsi
BSBcl
BSEco
tat
rev
nef
delete env
insert SV-gpt
LTR
10000bp

Fig.5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Probe
Probe
Unspliced
Spliced
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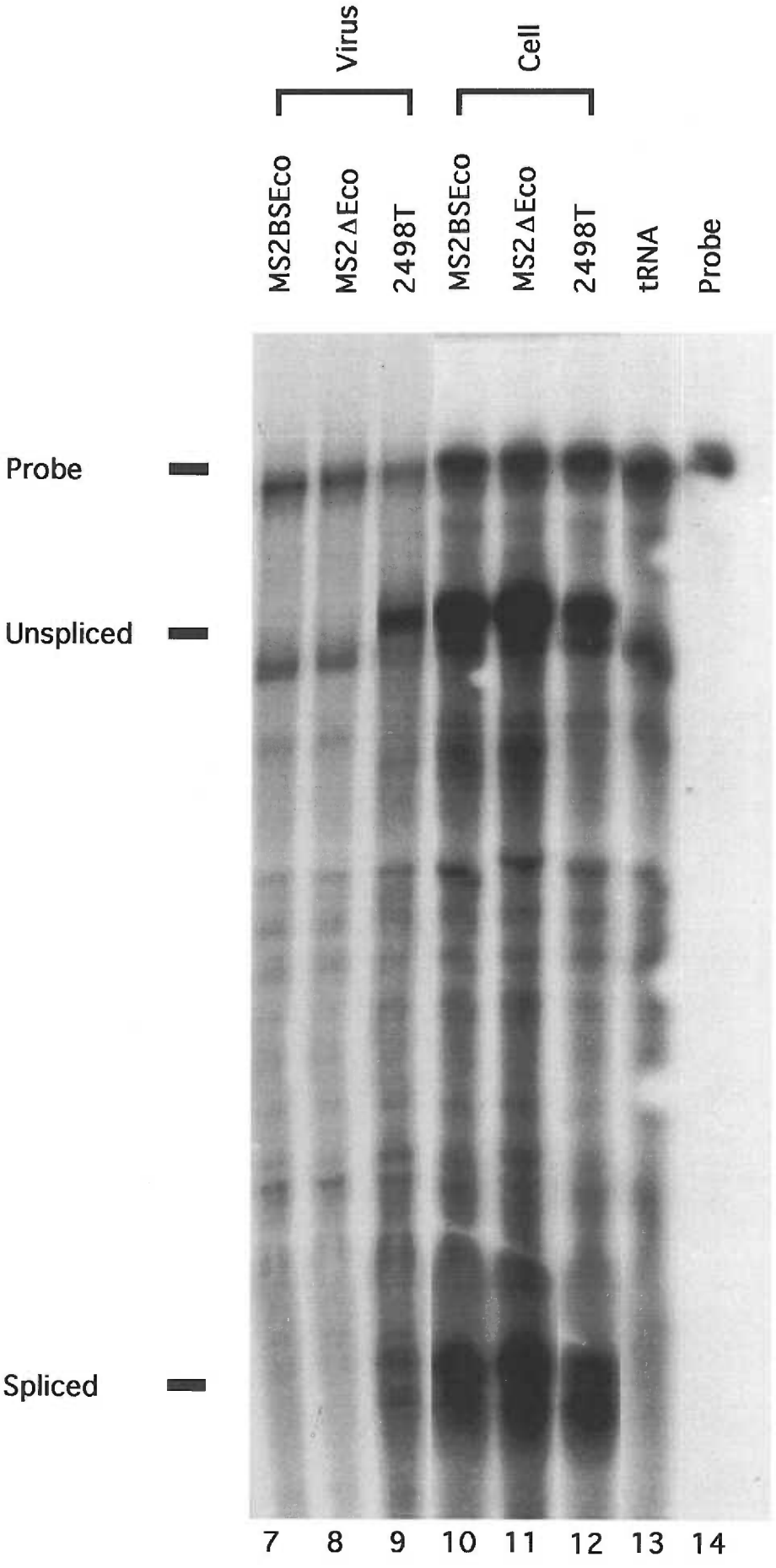
4
5
6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tRNA
Probe
Probe
Unspliced
Spliced
7 8 9 10 11 12 13 14

NUCLEOCAPSID PROTEIN EFFECTS ON THE SPECIFICITY OF RETROVIRUS RNA ENCAPSIDATION

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Running title: Retrovirus RNA encapsidation

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ABSTRACT

We have analyzed the roles of Gag protein nucleocapsid (NC) domains on the packaging or encapsidation of retroviral RNAs into virus particles. We found that mutation of both zinc-finger motifs of the human immunodeficiency virus (HIV) NC domain reduced but did not eliminate encapsidation of the HIV viral RNA. However, the NC mutations also resulted in a 3- to 4-fold reduction in the specificity of RNA encapsidation, as determined by comparison of virus-associated genomic and spliced RNA levels. As a complementary approach, we replaced the NC domain of Moloney murine leukemia virus (M-MuLV) with that of HIV. Chimeric virus particles assembled efficiently, were of wild type M-MuLV density, and crosslinked at NC cysteines. In encapsidation studies, wt M-MuLV precursor Gag (PrGag) proteins packaged M-MuLV transcripts more efficiently than HIV RNAs. In contrast, chimeric PrGag proteins possessing M-MuLV MA, p12, and CA domains, but the HIV-1 NC domain encapsidated HIV transcripts to a greater extent than M-MuLV transcripts. Our results support the notion that retroviral NC domains contribute toward both the efficiency and specificity of viral genomic RNA packaging.

INTRODUCTION

Retrovirus particles contain a genomic complement of two molecules of viral genomic RNA, but a provirus also may give rise to a variety of smaller, singly or multiply spliced subgenomic mRNAs. Although viral spliced RNAs and cellular RNAs may be packaged into virus particles, the packaging of viral genomic RNA is essential for the newly formed virus to be infectious. Thus, the encapsidation of retroviral genomes must be selective enough to overcome the high background of cellular RNAs and subgenomic viral RNAs. Two components are involved in specific encapsidation of viral genomic RNA: the secondary and tertiary structures of genomic viral RNA, and the Gag or Gagpol proteins which form assembling virus particles. The specificity with which retroviral full-length genomic RNAs are encapsidated suggests that specific sequences are required for RNA selection. These cis-acting sequences, called packaging (Psi) or encapsidation signals, have been identified in avian, murine and primate retroviruses (13,14,17,19,23). For Moloney murine leukemia virus (M-MuLV) and HIV, packaging signals are located between splice sites and therefore are not contained in the subgenomic mRNAs. The existence of a specific cis-acting genomic segment required for high efficiency RNA encapsidation suggests that it is a recognition site for a trans-acting factor, presumably the retroviral Gag protein, since gag mutants have been shown to impair RNA encapsidation (1,9,26). Gag proteins initially are synthesized as precursor polyproteins which form assembling particles. During or after budding, PrGag proteins are cleaved by the viral protease (PR) to yield the mature proteins MA (matrix), p12, CA (capsid), and NC (nucleocapsid) for M-MuLV, and MA, CA, NC, and p6 for HIV-1. To date, of these proteins, only mutations in NC have been shown to block encapsidation of

viral RNA into virus particles (1,10,16,26). NC deletions and some NC mutations also impair virus assembly (7), suggesting that NC plays a role in virus assembly in addition to viral RNA packaging.

Although some NC mutations can reduce assembly of RNA into virus particles (1,10), most studies have not addressed how the specificity for encapsidation of the viral genomic RNA is affected. Several in vitro studies have shown that NC binds both specifically and non-specifically to RNA, so it has been difficult to assess whether mutations affect non-specific or specific binding to viral genomic RNA (4,21). One observation which suggests that the NC domain is involved in specific RNA recognition comes from the studies of the mutations of the M-MuLV NC cys-his motif. Several mutations led to the production of noninfectious particles lacking genomic RNA but containing detectable levels of cellular RNA, suggesting an altered binding specificity (9). Similarly, analysis of a chimeric RSV-M-MuLV Gag protein suggested that some specificity is determined by the NC protein (7). Based on the observations above, we decided to examine the specificity of RNA encapsidation into HIV particles. Examination of an HIV-1 NC cys-his motif mutant showed that mutant particles encapsidated less viral RNA than wild type (wt) particles, and that the ratio of encapsidated HIV genomic RNA versus spliced RNA was reduced. Additionally, we analyzed a chimeric M-MuLV PR- Gag protein in which the M-MuLV NC domain was replaced by an HIV-1 NC domain. Our results show that the chimeric protein assembles virus particles, and that the wt M-MuLV PR- Gag protein preferentially encapsidated M-MuLV RNA, while the M-MuLV Gag protein with an HIV NC region preferentially packaged HIV-1 genomic RNA. Our results indicate that at least some of the specificity for encapsidation of HIV RNA is inherent in the NC domain of the Gag protein.

MATERIALS AND METHODS

Recombinant constructs.

BlueHX680-831 (26), the recombinant plasmid used to make antisense riboprobe for detection of HIV RNA, was constructed by using the SacI-SalI fragment from pBluescribe (Stratagene) and an HIV-1 (HXB2) nt 680-831 insert, which was isolated as a SacI-SalI restriction fragment from our HIVgptClaI linker-insertion mutant (26). For detection of Moloney murine leukemia virus (M-MuLV) transcripts, we used the plasmid SPMLV (2), which contains a M-MuLV SacI-BalI fragment corresponding to proviral nt 420-660, inserted into the SacI-HincII fragment of SP64 (Promega). HIV expression constructs were based on HIVgpt (20,25,26), which derives from HXB2 (8). HIVgptA14-15 is a derivative of HIVgpt (25), in which the nucleocapsid (NC) mutations of A14-15 (kindly provided by R. Young) were transfered to the HIVgpt backbone. Thus, HIVgptA14-15 has cysteine to tyrosine mutations at Gag codons 392, 395, 413, and 416, altering both of the NC Cys-His finger motifs.

pXM2453-wt is a protease-deficient (PR-) M-MuLV expression vector, deleted in pol and env genes. The M-MuLV sequence starts from the PstI site at viral nt 563, which was altered to Hind III (19), and ends at the NheI site at nt 7842 of the M-MuLV viral genome. The internal sequence from the BstEII site at viral nt 2453, which was changed to a BamHI site, to ClaI site at viral nt 7674 was deleted and replaced by a BamHI to ClaI fragment derived from a modified pBluescript SK-(Stratagene) polylinker region, in which the pBluescript SK-SmaI site was opened and an all-frame HpaI terminator fragment was inserted. The junction sequences between the BstEII2453 and ClaI 7674 are as follows: 5' CCT AAG GTC ACC GCG GAT CCC CCT **TAA** GTT AAC TTA AGG GCT GCA GGA ATT CGA

TAT CAA GCT TAT CGA T 3', in which the M-MuLV viral nt 2453 (G) and nt 7674 (A) are underlined, and the termination codon is **TAA**. To facilitate cloning, EcoRI adapters were added 5' to the original PstI site at viral nt 563 (now a HindIII site) and 3' of the M-MuLV NheI site at 7842. Thus, the sequence 5' of nt 563 is 5' GAATTCGATATCAAGCTT 3', and the sequence 3' to nt 7842 is 5' GCTAGCAGGATCCCCGGGCGACCTCGAATTC 3', in which the M-MuLV viral nt 7842 (G) is underlined. The above EcoRI fragment was cloned into the EcoRI site of pXM (28), an expression vector which uses the adenovirus major late promoter (MLP) to drive gene expression. The resulting vector, pXM2453-wt, lacks the M-MuLV encapsidation signal (Psi region), and encodes M-MuLV gag, but not pol or env genes. The total length of pXM2453-wt is 7275 bp. Starting from the Hind III site near the SV40 ori at nt 0, upstream of the M-MuLV sequences are the SV40 ori (nt 0-290), the adenovirus MLP and leader (nt 290-960), and the adenovirus intron sequences (nt 960-1070). Downstream are DHFR RNA stabilization sequences (nt 3215-3965), a polyA signal (nt 3965-4815), and the plasmid backbone (nt4815-7275). Starting from the HindIII site near the SV40 ori, approximate map positions for all occurrences of selected enzymes are as follows: ClaI: 3023; HindIII: 0, 960, 1082, 3018; EcoRI: 1070, 3006, 3205; HpaI: 2984, 4075; XbaI: 4565; XhoI: 2079. The vector pXM2453-NCex is the same as pXM2453-wt, except that the nucleocapsid coding region of M-MuLV was substituted by the HIV-1 nucleocapsid coding region. This was done by insertion of an HIV-1 NC PCR fragment, with primer-modified ends, in place of the M-MuLV NC fragment from the BalI site at M-MuLV viral nt 2056 to a BamHI site created at nt 2192. The 5' nucleocapsid junction sequence is 5' GCC ACT GTC GTT AGT GGA **ATG CAG AGA GGC** 3', where HIV nucleotides are in bold text, and M-MuLV nt 2072 (A) and HIV nt 1920 (**A**) are underlined. The 3' junction sequence is 5' TGT **ACT GAG AGA** *GGA TCC CG* T CGG GGA CCA 3`, where HIV sequences again are

bold, non-M-MuLV and non-HIV linker sequences are in italics, and the HIV nt 2075 (**A**) and M-MuLV nt 2192 (T) are underlined.

Because pXM-based constructs do not possess either M-MuLV or HIV encapsidation signals, it is necessary to supply packageable HIV and M-MuLV transcripts in trans. For this purpose, we have employed the vectors HIVGBG831 and B2BAG. HIVGBG831 has the same structure as HIVgpt, except that the ClaI831 to SalI5786 fragment was replaced by the E. coli B-galactosidase (B-gal) gene, in frame so that HIVGBG831 expresses 15 codons of HIV Gag fused to the ninth codon of B-gal (27). HIVGBG831 has HIV long terminal repeats (LTR), a intact Psi region, and the HIV intron structure, so it produces both spliced and unspliced RNA sequences which can be detected by the HIV probe made from Blue HX680-831. B2BAG is a proviral expression vector, where the Moloney murine leukemia virus (M-MuLV) long terminal repeat (LTR) promoter drives expression of B-gal, and an internal SV40 early promoter with replication origin function (SV40 ori) drives expression of a selectable neomycin gene (5). It also has the pBR322 bacterial origin of replication (pBR ori) and a M-MuLV Psi region, but no splice donors or acceptors.

Cells, viruses, and protein analysis.

Cells were grown and transfected as described previously (11,25-27). Detection of Gag proteins in cell lysates and virus particles was by immunoblotting (25-27): M-MuLV Gag proteins were detected with a mouse anti-p30 monoclonal antibody (12) as the primary antibody and an alkaline phosphatase-conjugated goat anti-mouse antibody (at 1: 1,500) as the

secondary antibody, while HIV Gag proteins were detected with an anti-HIV CA monoclonal (Hy183), obtained from Dr. Bruce Chesebro through the AIDS Research and Reference Reagent Program. For bis-maleimido hexane (BMH; Pierce) crosslinking of viral Gag proteins, BMH was prepared in dimethylsufoxide (DMSO) as a 100 mM solution, and crosslinking followed established protocol (12). For sucrose density gradients, 60 ml supernatants from transfected Cos7 cells were first centrifuged at 4 deg C for 10 min at 1,000 x g to remove cells and debris, after which the cell-free supernatants were centrifuged through 4 ml 20% sucrose cushions in TSE at 4 deg C for 2 hr at 83,000 x g (25,000 rpm on an SW28 rotor). The viral pellets were resuspended in 0.5 ml TSE, and applied to sucrose gradients consisting of 1.1ml steps of 20, 30, 40. and 50% sucrose in TSE that had been prepared at least 60 min in advance and placed at 4 deg C to permit mixing. Gradients were centrifuged at 300,000 x g (50,000 rpm on an SW50.1 rotor) overnight at 4 deg C, and 0.4 ml fractions were collected from top to bottom. Fractions then were mixed, and aliquotted for measurement of both sucrose densities, and Gag protein levels, which were quantitated from immunoblot bands using a Bio-Rad model#620 densitometer (25).

RNAse protections.

For RNAse protection assays, viral pellets were prepared from 30 ml supernatants of transfected Cos7 cells in the same way as described for the sucrose density gradients. Viral pellets were resuspended in 600 ul of RNAse-free TE-SDS (50 mM Tris (pH7.4), 5 mM EDTA, 0.1% SDS), and 0.1 ml aliquots were taken for protein analysis as described above. For viral RNA preparation, 30ug of yeast tRNA was added to each of the remaining 0.5 ml viral suspensions, and

the mixtures were extracted once with phenol-chloroform, once with chloroform, twice with phenol-chloroform, once with chloroform, and then precipitated with ethanol. Pellets were dried and resuspended in 0.1ml of 10 mM Tris (pH 7.4), 0.1 mM EDTA. Cellular RNA was isolated from transfected cells as described previously (26), and cellular RNA concentrations were quantitated by measuring the 260 nm absorbance using Beckman DU-64 Spectrophotometer. Riboprobe transcriptions used linearized template plasmids and followed standard procedures (26). After synthesis, phenol-chloroform extraction, and ethanol precipitation, probe pellets were resuspended in sequence loading dye, heated at 85 deg C for 5 min and electrophoresed on a small 5% denaturing polacrylamide sequencing gel to purify and isolate the probes. Probes excised from gels were eluted by two 30 min incubations at 50 deg C in 250 ul of 1 M ammonium acetate (pH 7.4), 0.1% SDS, and 1 mM EDTA, and pooled elutions were combined with 20 ug of yeast tRNA, phenol-chloroform extracted twice, and ethanol precipitated. Dried probe pellets were resuspended in 30 ul of 10 mM Tris (pH7.4), 0.1 mM EDTA, and stored frozen at -80 deg C for less than 10 days.

For hybridizations with riboprobes, 10% of the viral RNA samples, or 40 ug of cellular RNAs were mixed with 10 ug of yeast tRNA, ethanol precipitated, dried and resuspended for use. Hybridizations, RNAse digestions, electrophoresis, and detection have been described previously (26). The HIV probe prepared from BlueHX680-831 is 183 bases in length including 5' and 3' non-HIV sequences derived from the pBluescribe vector. Spliced and unspliced genomic HIV RNAs are expected to yield protected fragments of 63-64 bases and 150 bases, respectively. The probe made from SPMLV is 274 bases long, including 5' and 3' non-M-MuLV vector sequences. Hybridization of the SP6 transcript to the genomic unspliced M-MuLV RNA should yield a protected

fragment of 214 bases after RNAse digestion. The protected bands were measured densitometrically. For the RNAse and DNAse control experiments, equal amounts of viral or cellular RNA were incubated at 0 ug/ml, 30 ug/ml, 100 ug/ml and 300 ug/ml RNAse or DNAse (Boehringer) at 37 deg C for 1 h. As controls, RNAse and DNAse solutions of 100ug/ml concentration also were mock-incubated at 37 deg C for 1 h without adding any RNA. Incubated samples then were mixed with 10 ug of yeast tRNA, phenol-chloroform extracted three times, chloroform extracted twice, and ethanol precipitated. Hybridizations and RNAse digestions were carried out as described above, while aliquots of mock treated samples were processed for electrophoresis directly after hybridization incubations without RNAse post-treatment.

RESULTS

RNA encapsidation into wild type and nucleocapsid mutant HIV particles.

Previously, we (26) and others (1) observed reduced incorporation of HIV-1 viral RNA into the NC mutant HIVgptA14-15, which substitutes tyrosines for the first two cysteines in each of the NC cys-his boxes. However, in repeated experiments we observed that the incorporation of viral RNA into the mutant particles was not blocked completely. Because previous experiments suggested that some NC mutations might affect specificity of viral RNA incorporation (1, 26), we examined more closely the specificity of viral RNA encapsidation of the NC cys-his motif mutant. To do so, Cos7 cells were transfected with wt HIVgpt or NC mutant HIVgptA14-15 constructs, and cell and virus RNAs were collected. For RNA analysis, we used a 183 base antisense RNA HIV probe which crosses the major splice donor site of HIV-1. Not surprisingly, protection of the HIV probe (Figure 1, Panel A, lane B) was not observed with yeast tRNA (lane A) or RNA preparations for mock-transfected Cos7 cells (lane E) or mock media samples (lane H). However, as observed previously, cellular wt or A14-15 mutant HIVgpt genomic transcripts protected a fragment of 150 b while cellular HIV spliced RNAs protected fragments of 63-64 b (lane C). As shown in lane F, wt virus preferentially encapsidated genomic versus spliced viral RNA (compare lanes F and C), while HIVgptA14-15 virions packaged genomic and spliced RNAs at roughly equal levels (lane G). Because the amount of Gag protein in virus samples was slightly higher for HIVgptA14-15 than HIVgpt (Figure 1, panel B), it appears that HIVgptA14-15 reduced both the specificity of viral RNA packaging (as determined by the

reduced ratio of genomic RNA versus spliced RNA; 23) and the amount of viral RNA encapsidated (as determined by viral RNA to Gag protein ratio).

In order to quantitate our results, viral Gag protein levels and cellular and viral RNA levels were quantitated densitometrically to obtain the results shown in Table 1. As shown, the HIVgpt viral genomic RNA signal was higher than that of HIVgptA14-15 (second column), and the ratio of the viral genomic RNA signal to viral Gag protein levels were higher for HIVgpt than for HIVgptA14-15 (right-most column). However, the difference between wt and mutant genomic RNA to Gag ratios was 3- to 4-fold, suggesting that an intact NC cys-his motif is not absolutely essential for incorporation of RNA into virus particles. In terms of specificity, the viral genomic RNA to spliced RNA ratio of HIVgpt was 9-fold higher than that of HIVgptA14-15 done in parallel (column 3). Since the levels of genomic (G) and spliced (S) RNAs in virus samples were determined at the same time in the same samples, and virus versus cell G/S RNA ratios were 10-fold higher for wt than for mutant virus (column 4), the observed specificity differences in virus particles appeared independent of potential sample differences or major differences in cellular RNA levels.

Although these results showed the cys-his mutant (HIVgptA14-15) affected levels and specificity of encapsidation, the effect was not as high as one might have predicted, based on the observation that wt HIV is over 1,000-fold more infectious than such NC mutants (10,25,26). Since this could have been due to some unusual feature of the Cos7-SV40 expression system, we repeated our experiments in Hela cells, and obtained the similar results (data not shown). A separate concern was that the apparent viral genomic RNA signals in our cell or virus samples could have been due to contamination of

RNA samples with transfected DNA. To control for this possibility, viral and cellular RNA samples were pretreated with either DNAse or RNAse before phenol-chloroform extraction, ethanol precipitation and hybridizations. As shown in Figure 2, DNAse treatment of viral (lanes L-N) or cellular (lanes D-F) RNA samples did not affect signal detections relative to mock treated controls (lanes G, O). In contrast, RNAse pretreatment (lanes H-J and P-R) eliminated RNAse protection signals relative to mocks (lanes K, S). Since sample pretreatment with either DNAse (lane B) or RNAse (lane C) did not cause probe degradation, these results indicate that the protected fragments are due to signals from viral and cellular RNAs, and not transfected DNA.

RNA encapsidation into chimeric virus particles.

Because the HIV NC mutant, HIVgptA14-15, appeared to lose some degree of viral genomic RNA packaging specificity, we attempted the complementary experiment, to see if increased encapsidation specificity could be transfered to another virus with the HIV NC domain. To do so, we chose to make a chimera of the M-MuLV Gag MA, p12 and CA domains with the HIV-1 NC domain, which would allow us to test NC effects on encapsidation. The parental construct, pXM2453-wt (see Figure 3), expresses the M-MuLV Gag protein from the adenovirus major late promoter, and deletes almost all of the pol gene, to eliminate potential difficultes of Gag and Gagpol interactions. The construct also lacked the M-MuLV Psi signal, so it would be possible to provide HIV or M-MuLV packagable RNAs separately. To substitute the HIV NC domain for the M-MuLV NC domain in pXM2453-wt, the HIV-1 NC coding region (excluding the 3 carboxy-terminal codons) was inserted in place of the central 40 codons of M-MuLV NC. The resulting chimeric construct, pXM2453-NCex, has M-MuLV MA,

p12, CA and first 6 residues of M-MuLV NC, 52 of the 55 codons of HIV-1 NC, a linker containing 3 foreign residues (GSD), and the last 14 codons of M-MuLV NC (see Figure 3).

Since NC has been implicated in retrovirus assembly, one concern about our chimeric construct pXM2453-NCex, was that it might not produce virus particles, or that the particles produced might not be like wt viruses. The first analysis of whether our chimeric protein could produce virus particles was to measure whether the chimeric Gag proteins were released from transfected cells. To do so, plates of Cos7 cells were transfected with pXM2453-wt or pXM2453-NCex, and Gag proteins in cell lysates and virus particles were examined by immunoblotting (Figure 4). Comparison of the ratios of cell versus virus Gag protein levels for pXM2453-wt (Figure 4; lanes A, B) and pXM2453-NCex (lanes D, E) showed that chimeric Gag proteins were released at levels equal to that of the wt M-MuLV PrGag proteins. To test whether chimeric Gag proteins formed virus particles that were structurally similar to wt viruses, we next tested the densities of the chimeric and wt viruses. Supernatants from Cos7 cells transfected with pXM2453-wt or pXM2453-NCex were collected, pelleted, resuspended, mixed with internal control wt HIV virus and fractionated by 10-50% sucrose density gradient centrifugation. Aliquots of fractions were collected to measure sucrose densities, and levels of both M-MuLV and HIV Gag proteins. As shown in the top panel of Figure 5, virus particles formed by wt M-MuLV PrGag proteins expressed by pXM2453-wt had a density of 1.15 g/ml, while the internal control (HIV; lighter line) had a slightly lower density of 1.14 g/ml. This result is consistent with the slightly different densities observed for different retroviruses by others (3). As shown in the bottom panel of Figure 5, virus particles assembled from the chimeric

Gag protein expressed by pXM2453-NCex had the same density as wt M-MuLV, again slightly higher than that of HIV (lighter line). To further validate the structural similarity between the chimeric virus and wt M-MuLV, we tested the abilities of PrGag proteins in chimeric and wt particles to be crosslinked by bis-maleimidohexane (BMH). Previous studies showed that PrGag nucleocapsid cysteine residues in intact M-MuLV and HIV particles can be crosslinked with either iodine or BMH to yield Gag protein dimers (12), demonstrating that NC domains in immature viruses are in close proximity. To test chimeric and wt M-MuLV viruses, pelleted particles were mock-treated or BMH-crosslinked, after which Gag proteins were separated by SDS-PAGE, and detected by immunoblotting. As expected, PrGag proteins in wt virions crosslinked via cysteines to form dimers when BMH-treated (Figure 6, lane B), while dimer formation was not observed for the parallel mock-treated sample (lane A). We also observed that PrGag proteins in chimeric particles crosslinked via Gag cysteines (compare Figure 6, lanes D and C). These experiments suggest that the chimeric virus particles produced from pXM2453-NCex are similar to wt M-MuLV viruses, at least at this level of biochemical analysis.

With our assembly-competent chimeric virus, it was possible to test whether encapsidation specificity could be transfered with the HIV NC domain. Since the transcripts from pXM2453-wt and pXM2453-NCex do not possess the Psi signals, they can not be packaged into the virus particles produced by these constructs. However, this feature has allowed us to provide potentially packageable transcripts from other constructs in trans, and evaluate encapsidation efficiencies. The packagable M-MuLV vector was B2BAG (3; Figure 3) which expresses a viral genomic transcript retaining the M-MuLV encapsidation signal and encoding B-galactosidase (B-gal). For HIV, we

employed HIVGBG831 (6; Figure 3), which encodes an HIV Gag-B-galactosidase fusion protein that has only 15 residues of HIV Gag, produces both virus genomic and spliced transcripts, and retains the putative HIV encapsidation signal on its genomic RNA. To examine encapsidation, Cos7 cells were transfected with pXM-2453wt or pXM2453-NCex plus either B2BAG or HIVGBG831. At 72 h post-transfection, viral protein samples, and cellular and viral RNA samples were collected and processed for analysis. As shown in Figure 7 (panel A) when pXM2453-wt and pXM2453-NCex were cotransfected with B2BAG, the detected cellular levels of the B2BAG message were approximately equal (Figure 7, Panel A, lanes C and D). In contrast, viruses produced from pXM2453-wt showed higher B2BAG RNA levels than did viruses made from pXM2453-NCex (compare Figure 7, Panel A, lanes A and B). Since the amount of PrGag protein in the pXM2453-wt virus sample was equal or less than that of pXM2453-NCex (compare Figure 7, Panel C, lanes A and B), these results suggest that the specificity of wt M-MuLV PrGag is greater than that of the chimeric PrGag protein for the M-MuLV B2BAG transcript. The reverse was true when pXM2453-wt and pXM2453-NCex were cotransfected with HIVGBG831. While HIVGBG831 genomic and spliced RNAs were detected at approximately equal levels in cell samples (Figure 7, Panel B, lanes C and D), when viral RNAs were detected with the HIV probe prepared from BlueHX680-831, we found that the viruses produced from pXM2453-wt packaged spliced RNA, but very little HIV genomic RNA (Figure 7, Panel B, lane A). In contrast, the viruses produced from pXM2453-NCex showed more particle-associated HIV genomic RNA (Figure 7, Panel B, lane B), and the ratio of genomic to spliced HIV RNA in these particles was higher than that of the viruses produced from pXM2453-wt. Again, because the levels of Gag proteins in the viral samples were approximately equal (Figure 7, Panel C, lanes C and D), these data suggest

that retrovirus nucleocapsid domains provide some specificity toward the recognition of viral transcripts.

To quantitate our results, RNA and Gag protein levels were quantitated densitometrically to obtain the data shown in Table 2. As shown, the raw signal of viral genomic RNA was higher for B2BAG with pXM2453-wt, and higher for HIVGBG831 with pXM4253-NCex (left column). Similar results were obtained when genomic RNA levels were normalized for total Gag protein levels (right column). Also, the virus to cell B2BAG RNA ratio of pXM2453-wt was higher than that of pXM2453-NCex, and the reverse was true when the virus to cell ratio of the genomic HIVGBG831 transcript was considered (column 2). Furthermore, the specificity of HIVGBG831 RNA packaging, which could be followed by measuring ratios of genomic versus spliced RNAs in single hybridizations reactions, showed that particles produced from pXM2453-NCex encapsidated genomic HIV RNA with a specificity ratio (genomic versus spliced) 4-fold higher than the viruses produced from pXM2453-wt (column 3). Taken together, these experiments show that the HIV nucleocapsid domain contributes toward the specificity of RNA encapsidation, and that this contribution can act independently of the natural PrGag context, and pol or env gene products.

DISCUSSION

Retroviral genomic RNA is packaged preferentially into the virus particles over a high background of cellular RNAs and spliced viral messages. This appears to be achieved by an interaction between the Psi packaging (encapsidation) signal on the viral genomic RNA and the viral Gag proteins, but the precise nature of the interaction is unknown. The results of our study confirm that mutations in the cys-his motif of HIV NC reduce the total viral genomic RNA packaged into the particles, as we and others had observed before (1,9,26). However, the zinc-finger motif mutations in the construct HIVgptA14-15 did not eliminate encapsidation of viral RNA completely, and comparison of genomic versus spliced RNA levels in mutant NC particles produced showed that the specificity of viral genomic RNA encapsidation was reduced (Figure 1, Table 1). These results imply that the HIV nucleocapsid domain is not just a non-specific RNA-binding moeity, but also provides at least some specificity to RNA encapsidation, in agreement with several previous studies (4,6,7,22).

One implication of our result with the NC cys-his motif mutant was that encapsidation specificity might be transfered to another virus with the HIV nucleocapsid domain. Thus, we exchanged the M-MuLV NC domain for HIV NC, making a chimera of M-MuLV Gag MA, p12, and CA domains with the HIV NC domain. Although we previously observed that M-MuLV NC deletion mutants do not assemble virus particles (12), the chimeric PrGag protein produced from pXM2453-NCex directed the assembly of particles (Figures 4-6). In encapsidation studies, we found that, in an M-MuLV context, M-MuLV NC contributed to some specificity for M-MuLV transcripts, and HIV NC

contributed to specificity for HIV transcripts (Figure 7, Table 2). These results are in agreement with those of Dupraz and Spahr (7), who showed that chimeric Gag proteins containing the Rous sarcoma virus (RSV) NC domain in place of M-MuLV NC showed an increased specificity to packageable RSV transcripts. Taken together, these results suggest that retroviral nucleocapsid domains contribute toward the specificity of encapsidation. Furthermore, this level of specificity is not dependent on pol gene products, since pol is deleted in pXM2453-wt and pXM2453-NCex. However, in our experiments with HIV NC mutants and exchanges (Figures 1, 7; Tables 1, 2), the maximum encapsidation differences observed were only about 10-fold. Consequently, it seems likely that other regions in Gag or Gagpol may add to encapsidation specificity. For instance, NC may interact with another Gag or Gagpol domain to allow more efficient recognition of the genomic packaging signal and drive viral genomic RNA encapsidation specificity. Such possibilities currently are under investigation.

ACKNOWLEDGEMENTS

We are grateful to Chin-tien Wang, Mark Hansen, Marylene Mougel, Lori Farrell, Jenny Stegeman-Olsen, and Jason McDermott for assistance and advice throughout the course of this work. The anti-M-MuLV-CA monoclonal antibody was a gift from Dr. Bruce Chesebro, who also made the anti-HIV CA Hy183 hybridoma cell line that was obtained from the AIDS Research and Reference Program, Division of AIDS, NIAID, NIH. This work was supported by a grant from the National Institutes of Health (NCI Grant 5R01 CA 47088-06).

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TABLE AND FIGURE LEGENDS

Table 1. RNA encapsidation in wild type and nucleocapsid mutant HIV particles.

Cellular and viral RNAs were detected and Gag protein levels were monitored as described in Figure 1. RNAse protection bands and Gag immunoblot bands from Figure 1 were densitometrically quantitated using a Bio-Rad Model 620 scanning densitometer. Results are shown with columns for viral genomic (G) RNA levels, and ratios of viral to cellular G RNA levels; viral G to spliced (S) RNA levels; viral to cellular G/S ratios; and viral G RNA to Gag protein ratios. Note that in five separate transfections, HIVgpt viral G/S ratios were 2.7- to 9.4-fold higher than HIVgptA14-15 ratios. The values listed are in arbitrary densitometer units.

Table 2. HIV nucleocapsid effects on RNA encapsidation into M-MuLV particles.

Protein and RNA levels were quantitated from immunoblots and RNAse protection gels by densitometry and expressed as arbitrary densitometer units. In the table, virus-associated B2BAG or HIVGBG831 genomic (G) RNA levels are shown in the second column from the left, while the right-most four columns show normalized values. In the third column from the left, the virus to cell genomic RNA ratio is given; while in the fourth column, virus genomic (G) to virus spliced (S) RNA ratios are shown. The fifth column gives values for the double ratio (viral G/S)/(cell G/S), while the right most lane provides a comparison of virion genomic RNA and Gag protein levels. Note that G/S and double ratios are not provided for B2BAG cotransfections since B2BAG does not encode a spliced message.

Figure 1. Encapsidation of HIV RNA into wild type and nucleocapsid mutant particles.

Panel A. Cellular (lanes C-E) and viral (lanes F-H) RNAs were collected from Cos7 cells which were either mock transfected (lanes E, H), or transfected with DNAs encoding wt HIVgpt (lanes C, F) or nucleocapsid mutant HIVgptA14-15 (lanes D, G). Viral (10% total sample) or cellular (40 ug) RNAs were detected by RNAse protection using the BlueHX680-831 probe as described in the Materials and Methods. The probe itself (lane B) is 183 nt, and yields little background with the control yeast tRNA sample (lane A). Since the antisense probe crosses the HIV-1 splice donor, viral genomic transcripts protect a probe fragment of 150 b, while spliced HIV transcripts protect 63-64 b fragments (indicated). **Panel B.** For quantitation of Gag protein levels in virus samples from which RNAs were obtained, one third of the HIVgpt and HIVgptA14-15 virus samples was taken prior to RNA extraction and electrophoresed and immunoblotted with a mouse anti-CA monoclonal primary antibody for HIV Gag protein detection. Lanes A and B are immunoblots of proteins from HIVgpt and HIVgptA14-15 transfections, respectively. The positions of the HIV Gag proteins Pr55, p41, and p24 (CA) are indicated on the left. Note that although minor differences in proteolytic processing were observed, the average ratios of Pr55:p41:CA for HIVgpt were 20:24:56 (29 transfections) and 17:28:55 for HIVgptA14-15 (11 transfections).

Figure 2. RNAse protection controls.

To demonstrate that probe protection was from sample RNA rather than transfected plasmid DNAs, equal amounts of HIV viral RNA (lanes L-S) and equal amounts of HIVgpt-transfected Cos7 cellular RNA (lanes D-K) were incubated 1 h at 37 deg C in 300 ug/ml, 100 ug/ml, 30 ug/ml and 0 ug/ml of RNAse (lanes H-K and lanes P-S) or DNAse (lanes L-O and lanes D-G). RNAse and DNAse solutions also were incubated at 37 deg C without adding any RNA (lanes C and B). After incubations, samples were mixed with 10 ug of yeast tRNA, phenol-chloroform extracted three times, chloroform extracted twice, and ethanol precipitated. Hybridizations and RNAse digestion were carried out as described in Materials and Methods. For lanes C and B, RNAse digestions was not performed after hybridization , but aliquots of the hybridization mixtures were loaded directly onto the gel instead. The positions of the probe (lane A), and unspliced and spliced HIV RNAs are shown on the left. Note that these results were obtained in three separate experiments, and that input DNA came from pooled stocks of cell and virus DNA, so that cell to virus RNA levels are not meaningful here.

Figure 3. Recombinant viral constructs.

pXM2453-wt is an expression vector, which uses the adenovirus major late promoter (black oval) to drive the expression of the Moloney murine leukemia virus (M-MuLV) gag gene. The encapsidation signal and pol and env genes of the M-MuLV sequences were deleted, and the M-MuLV coding region terminates at the protease region (grey box). Other features of this construct are the adenovirus leader and intron (int), 3' non-coding DHFR sequences (striped box) and the poly A signal. SV40 ori and ColEI ori permit plasmid replication in both Cos7 cells and bacteria. Shown in the figure is the 4815 bp M-MuLV gag gene expression unit, and not the pUC 12 plasmid backbone. The EcoRI sites are at nt 1070 and 3205, and occurences of other selected restriction endonuclease sites are given in the Materials and Methods. pXM2453-NCex is similar to pXM2453-wt, except that the M-MuLV nucleocapsid coding region was substituted by the HIV-1 nucleocapsid coding region as described in the Materials and Methods. Consequently, pXM2453-NCex encodes a gag fusion gene, retaining M-MuLV gag matrix, p12, and capsid domains fused to a C-terminal HIV NC domain. Because neither pXM2453-wt or pXM2453-NCex retains a packaging signals, we have used B2BAG and HIVGBG831 in encapsidation studies. B2BAG is a retroviral expression vector and is shown in its proviral form. The M-MuLV long terminal repeat (LTR) promoter drives expression of the E. coli B-galactosidase gene and an internal SV40 early promoter, which also retains replication origin capsbilities (SV ori), drives the expression of the selectable neomycin gene. The M-MuLV encapsidation signal (black diamond) is intact but splice sgnals have been removed. HIVGBG831, shown in its proviral form, is similar to B2BAG in that it expresses both B-gal and a second gene (gpt) from a internal SV40 promoter. The HIVGBG831 B-gal gene is driven by the HIV-1 LTR promoter, and retains 15 amino-terminal codons from

the HIV gag matrix domain. HIVGBG831 also retains the HIV-1 leader and encapsidation signal region (white diamond), and expresses a genomic transcript, as well as spliced massages which lack the encapsidation signal.

Figure 4. Release of M-MuLV and nucleocapsid exchange Gag proteins from transfected cells.

Twenty micrograms each of pXM2453-wt or pXM2453-NCex were transfected onto separate plates of Cos7 cells. Three days post-transfection, supernatants and cells were collected and prepared for SDS-PAGE as described in Materials and Methods. Supernatant samples (lane B, pXM2453-wt; lane E, pXM2453-NCex) and cell samples (lane A, pXM2453-wt; lane D, pXM2453-NCex) were electrophoresed and electroboltted onto a nitrocellulose filter. M-MuLV Gag proteins were detected by immunoblotting with a mouse anti-M-MuLV CA monoclonal antibody as the primary antibody and a secondary alkaline phosphatase-conjugated goat anti-mouse antibody at a 1:1,500 dilution. Size standards are in lanes C and F, molecular weights are indicated on the right, and the M-MuLV Pr Gag proteins are indicated by arrowheads.

Figure 5. Density gradient fractionation of wild type and nucleocapsid exchange viruses.

Six plates of Cos7 cells were transfected with either pXM2453-wt or pXM2453-NCex. Three days post-transfection, media supernatants were collected and viral pellets were prepared as described in Materials and Methods. The pellets were resuspended in 0.5 ml TSE mixed with an internal control HIV (HIVgpt) virus stock, and fractionated on 10-50% sucrose density gradients. Fractions, shown on the X-axis, were collected from top to bottom, and aliquots were used to measure sucrose densities, and HIV and M-MuLV Gag protein levels. As shown, the thick black line indicates gradient densities in g/ml as indicated on the right hand Y-axis. Internal control HIV Gag protein levels, shown as percentages of the peak fraction value for a gradient, are indicated by thin black lines; and 2453-wt (top panel) or 2453-NCex protein levels (bottom panel) are indicated by intermediate black lines.

Figure 6. Crosslinking of Gag proteins in viruses.

Virus pellets prepared from supernatants of Cos7 cells transfected with pXM2453-wt (lanes A, B) or pXM2453-NCex (lanes C, D) were resuspended in 200 ul of TSE and split into equivalent 100 ul fractions that were mock treated (lanes A, C) or crosslinked at cysteines with bis-maleimido hexane (BMH; lanes B, D). After treatment, samples were electrophresed and immunoblotted for detection of Gag proteins as described in Materials and Methods. Molecular weight marker sizes are shown on the left, while PrGag monomers and dimers are indicated on the right.

Figure 7. RNA incorporation into M-MuLV and mucleocapsid exchange viruses.

The plasmids pXM2453-wt or pXM2453-NCex were cotransfected with either B2BAG or HIVGBG831 onto Cos7 cells, using six plates of cells for each cotransfection. Viruses were pelleted from media supernatants, and an aliquot was used for Gag protein detection. In parallel, virus RNAs were prepared, and RNAse protection assays were performed as described in Material and Methods. **Panel A.** The SPMLV probe was used as described in the Materials and Methods to detect B2BAG RNA in virus (lanes A, B) and cell (lanes C, D) samples from cells transfected with pXM2453-wt plus B2BAG (lanes A, C) or pXM2453-NCex plus B2BAG (lanes B, D). The probe band (lane F) is indicated, as is the fragment protected by B2BAG genomic message. In lane E, yeast tRNA was used as a negative control. **Panel B.** The BlueHX680-831 probe was used as described in Figure 1 to detect HIVGBG831 genomic and spliced RNAs in virus (lanes A, B) and cell (lanes B, D) samples of cells transfected with pXM2453-wt plus HIVGBG831 (lanes A, C) or pXM2453-NCex plus HIVGBG831 (lanes B, D). Lane F contained probe alone (size indicated on left), and lane E derived from a negative control yeast tRNA sample. The HIVGBG831 genomic and spliced RNA protection fragments are as indicated. **Panel C.** Aliquots of the virus samples used for RNAse protections were subjected to SDS-PAGE and immunoblot detection of Gag proteins for control quantitation purposes. Proteins correspond to the cotransfection from panels A and B as follows: lane A, pXM2453-wt plus B2BAG; lane B, pXM2453-NCex plus B2BAG; lane C, pXM2453-wt plus HIVGBG831; lane D, pXM2453-NCex plus HIVGBG831. Protein standard marker sizes are shown on the right, and PrGag bands are indicated on the left.

Table 1. RNA Encapsidation in wild type and nucleocapsid mutant HIV particles.

Virus	viral G RNA (densitometer units)	viral G RNA / cell G RNA	viral G RNA / viral S RNA	viral G/S RNA / cell G/S RNA	viral G RNA / viral Gag
wild type	1750	0.54	5.81	7.85	0.57
A14-15	694	0.17	0.62	0.76	0.15

Table 2. HIV nucleocapsid effects on RNA encapsidation into Moloney murine leukemia virus particles.

Virus	viral G RNA (densitometer units)	viral G RNA / cell G RNA	viral G RNA / viral S RNA	viral G/S RNA / cell G/S RNA	viral G RNA / viral Gag
2450T+B2BAG	1337	2.45	----	----	0.34
NCex+B2BAG	312	0.55	----	----	0.06
2450T+HIVGBGCla	85	0.15	0.14	0.20	0.03
NCex+HIVGBGCla	265	0.60	0.54	0.44	0.06

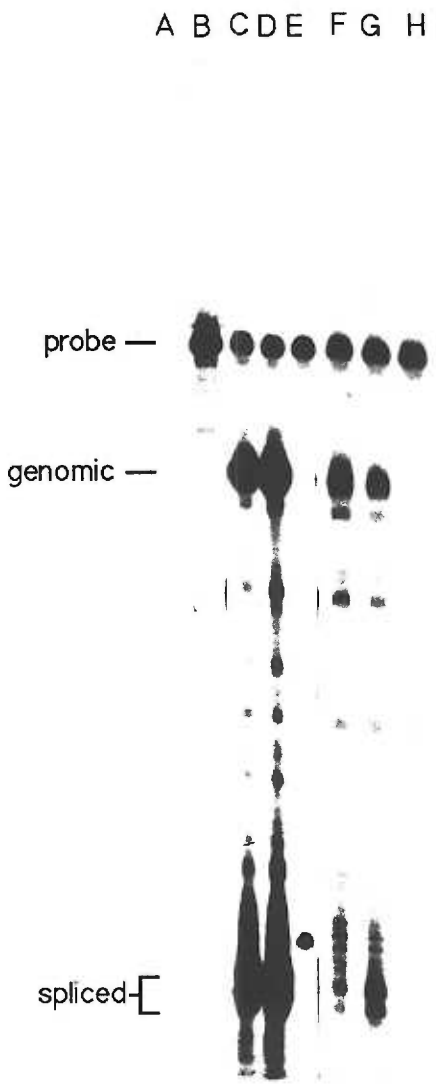


Fig.1A

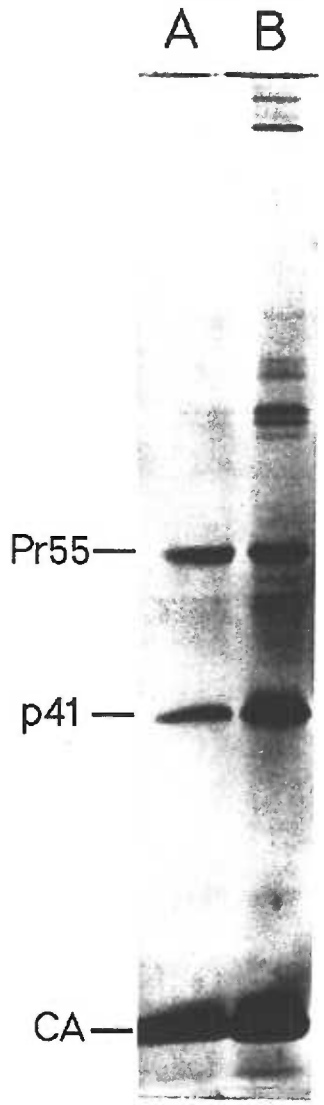
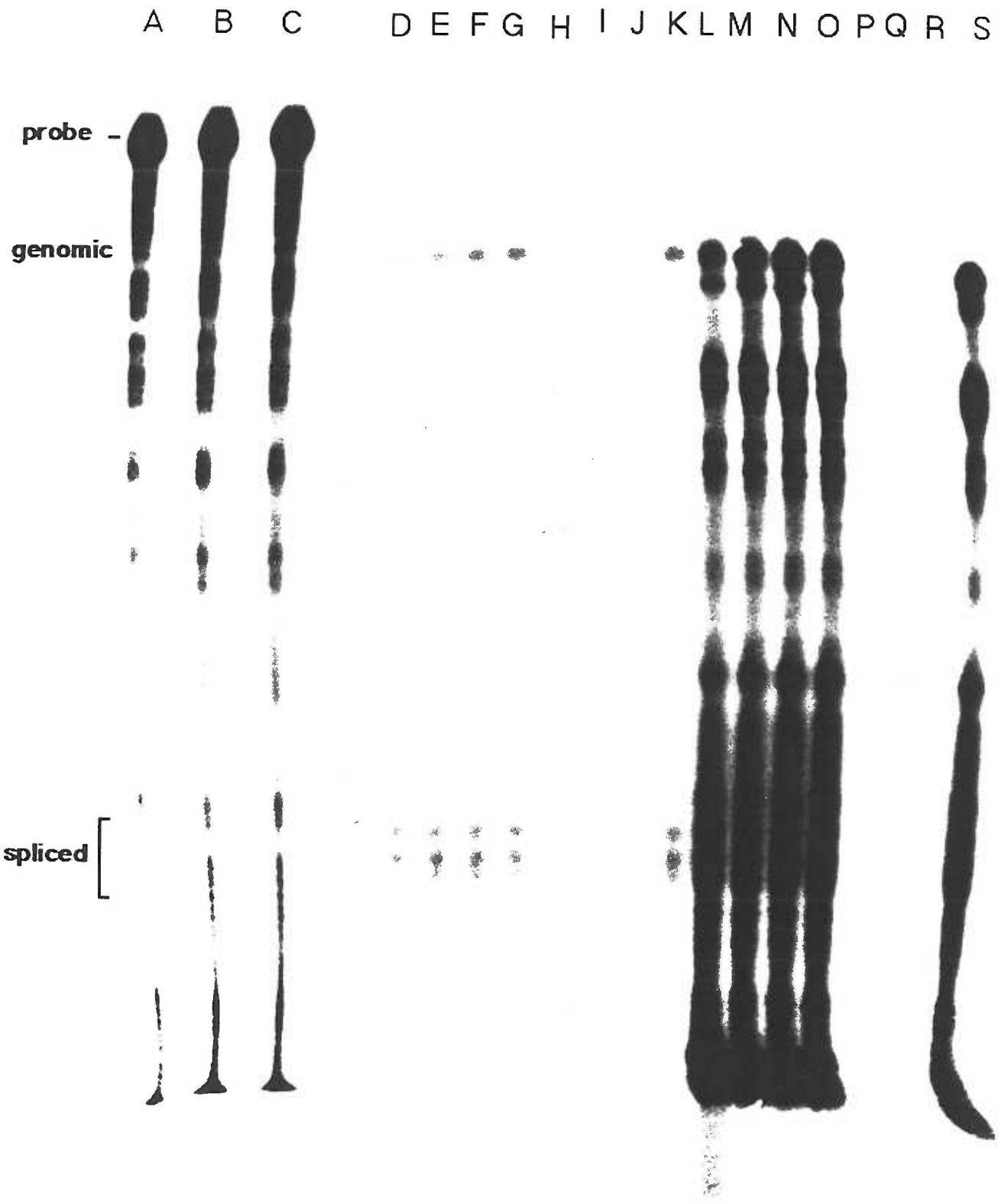
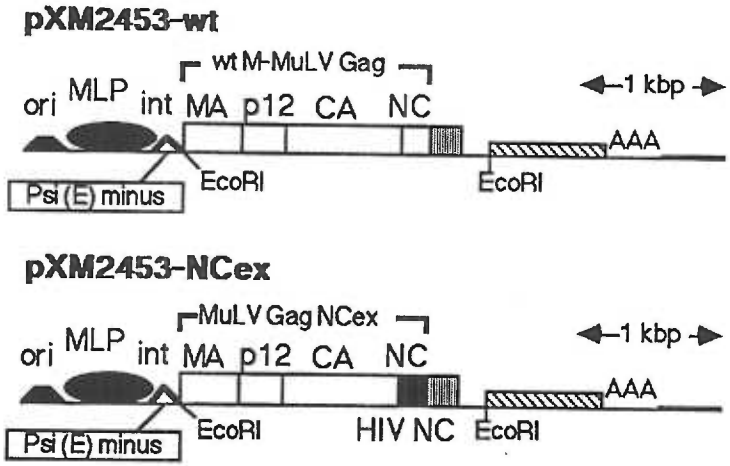
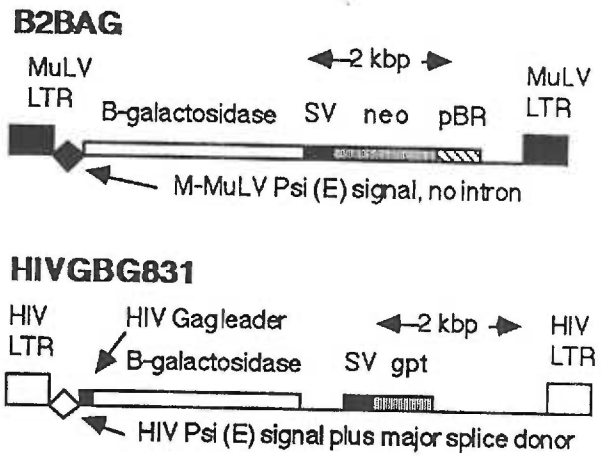


Fig.1B

Fig.2

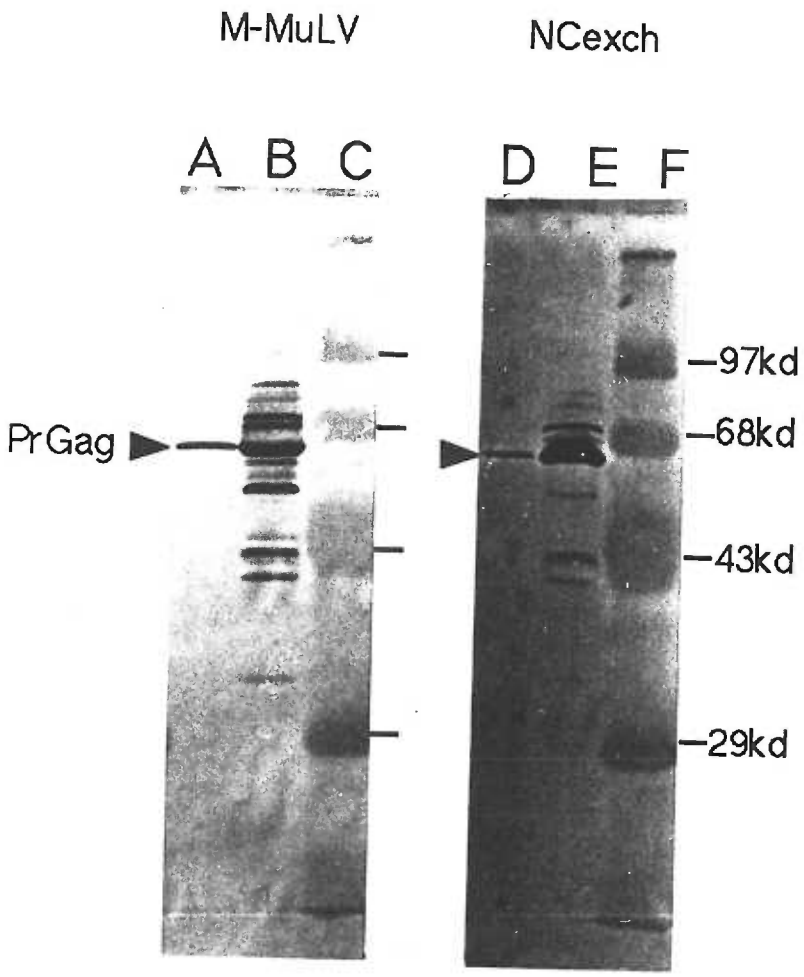




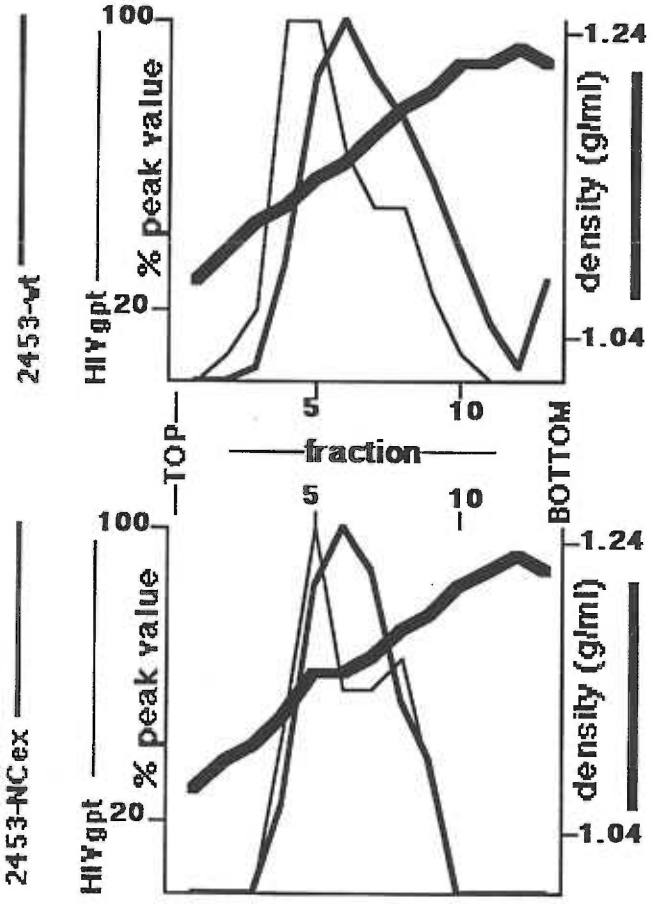


Zhang and Barklis
Figure 3

Fig.4



DENSITY GRADIENT FRACTIONATION OF WT AND NC EXCHANGE VIRUSES



Zhang and Barklis
Figure 5

Fig.6

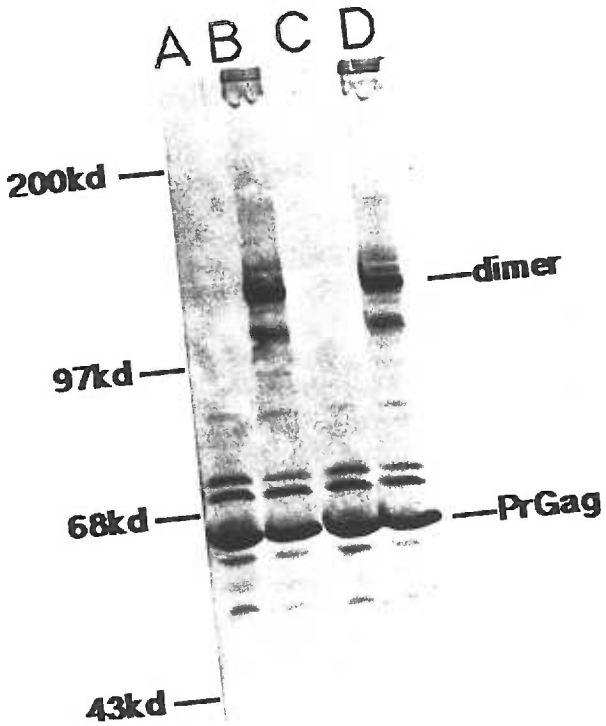
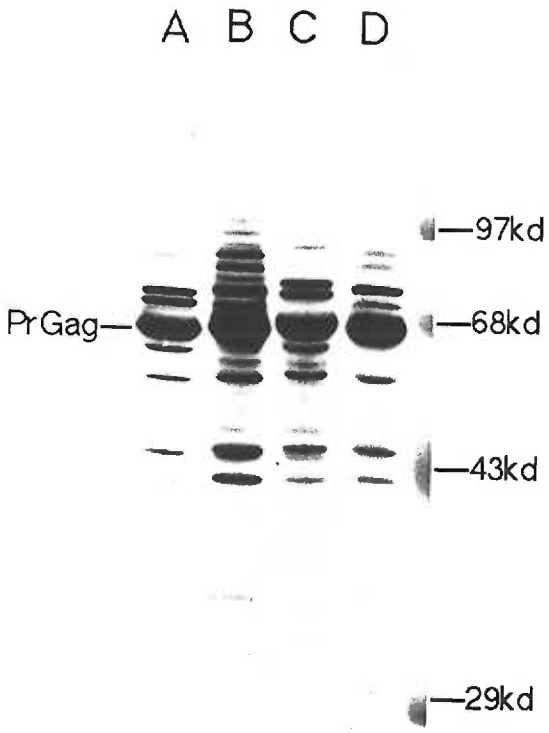


Fig.7C



Discussion and Conclusions

The major target cells for human immunodeficiency virus (HIV) infection are the CD4+ cells that are pivotal to the development of humoral and cell mediated immunity. It is generally considered that virally mediated destruction of these cells is a major contributor to immune breakdown (215).Gradual but accelerating decline in immune competence leads to overwhelming infection with one or more other microorganisms which exploit the immunocompromised state, culminating in the debilitating and generally fatal disease, AIDS (acquired immune deficiency syndrome). Many efforts have been made to study the HIV life cycle. However, the molecular basis of HIV virus assembly and genomic RNA encapsidation processes are still not clear.

HIV Gag proteins have been shown to be necessary and sufficient for directing virus particle assembly. It is initially synthesized as an 55 kD polyprotein precursor, which assembles into viral cores at the plasma membrane. After virus budding through the cell membrane, it is cleaved into matrix protein (MA), capsid protein (CA), p2 peptide, nucleocapsid protein, p1 peptide, and p6 protein. While Gag-Gag protein interactions which affect HIV assembly occur in the capsid (CA) domain of Pr55Gag, the nucleocapsid (NC) domain and the C-terminal portion of retroviral Gag proteins appear to possess a region involved in increasing the efficiency of virus particle assembly, this region has been referred to as an assembly domain (AD) (18), although its exact contribution to virus assembly is not clear (35, 66, 91, 100, 122, 161). We have evaluated the effects of p2, NC, p1 and p6 domains of HIV-1 on virus assembly.

We found that the PR- construct, 2498T, seemed to release particles more efficiently than wt HIVgpt in our experimental system. This result differs

somewhat from results with avian (265) and murine (56, 144) retroviruses in which protease activity does not appear to affect release levels, and contrasts with one report on HIV, in which PR- particles were released less efficiently than PR+ particles (137). It is unclear how the PR+ phenotype might impair Gag protein transport or establishment of interprotein contacts required for assembly and budding, although a number of studies have shown that perturbation of Gag protein to PR activity ratios can alter virus release efficiencies (126, 137, 200, 219, 220, 230). Because of the differences observed with wtHIVgpt and 2498T, we compared mutant particle release efficiencies with either wt HIVgpt or 2498T, depending on the PR phenotype of the mutant construct. In performing such comparisons, we observed that mutants without p6 were released at reasonable efficiencies, which is consistent with the notion that the effects of p6 on virus release may be affected by PR or cell-type specific (126, 137, 200, 260). However, constructs with mutations in p2 and the amino-terminal portion of NC were released much less well than wt, supporting the notion that this region defines an assembly domain which appears to function after Gag protein delivery to the plasma membrane (35, 161).

Although our study indicates that NC is essential to HIV virus assembly, the mechanisms based on which NC influences this process needs to be investigated. Some formal possibilities of how NC deletions affect virus assembly could be conceived as follows: Deletion of NC might unmask a C-terminal p2 or CA sequence that is toxic to assembly. Or, NC might form a stable monomeric conformation which maintains more amino-terminal sequences in an assembly competent conformation. It is also possible that formation of interprotein contacts by NC is essential to assembly. Or maybe NC is required

for assembly because it mediates RNA encapsidation and RNA is essential to virus particle assembly.

Our results support the hypothesis that NC is an active assembly domain (18), which can be substituted by heterologous domains which form interprotein contacts. We found that duplication of p2 and CA at the C-terminus of Gag did not inhibit virus assembly, suggesting that p2 and CA sequences are not toxic to virus assembly. Replacement of NC by monomeric proteins UPRT and HGXPRT did not facilitate virus-like particle release, which suggests that NC does not act passively, by forming a stable structure that restricts p2 or CA in a assembly -competent conformation. MS2 coat protein and wt zipper domain from human CREB both can form interprotein contacts, and replacing NC with either of them increases release levels, relative to the NC deletion proteins. Additionally, replacing NC with mutant E or K zipper which are incapable of forming homodimers did not facilitate assembly of virus-like particles. In contrast, in cotransfection, which permits the formation of heterodimers, release levels are increased. Substitution of NC region with the unrelated *B. subtilis* RNA binding protein MTRB also permitted efficient release of virus-like particles. This result is reminiscent of the abilities of some M-MuLV Gag fusion proteins to direct particle assembly (107, 133). Both of these proteins or domains are known to be able to form interprotein contacts. All these data suggest that formation of interprotein contacts by NC might be essential for normal virus assembly process

Retroviral genomic RNA is packaged preferentially into the virus particles over a high background of cellular RNAs and spliced viral messages. This appears to be achieved by an interaction between the Psi packaging (encapsidation) signal on the viral genomic RNA and the viral Gag protein sequences, but the precise nature of the interaction is unknown. HIV NC has

two Cys-His motifs, reminiscent of the zinc finger motifs of many DNA binding proteins. It has been shown that mutations affecting retrovirus NC Cys-His motifs can reduce levels of genomic RNAs packaged into retrovirus particles (4, 9, 45, 70, 97, 132, 202), although the extent to which NC contributes to encapsidation specificity has not been elucidated completely (19, 20, 49, 134, 179, 241, 294). The results of our study confirm that mutations in the Cys-His motif of HIV NC reduce the total viral genomic RNA packaged into the particles, as we and others had observed before (4, 34, 114). However, the zinc-finger motif mutations in the construct HIVgptA14-15 did not eliminate encapsidation of viral RNA completely, and comparison of genomic versus spliced RNA levels in mutant NC particles produced showed that the specificity of viral genomic RNA encapsidation was reduced. These results imply that the HIV nucleocapsid domain is not just a non-specific RNA-binding moiety, but also provides at least some specificity to RNA encapsidation, in agreement with several previous studies (14, 19, 20, 105). As a complementary approach, we exchanged the M-MuLV NC domain for HIV NC, making a chimera of M-MuLV Gag MA, p12, and CA domains with the HIV NC domain. Although it was observed that M-MuLV NC deletion mutants do not assemble virus particles (49), the chimeric PrGag protein produced from pXM2453-NCex directed the assembly of apparently wt virus. In encapsidation studies, we found that, in an M-MuLV context, M-MuLV NC conferred specificity for M-MuLV transcripts, and HIV NC conferred specificity for HIV transcripts.

These results are in agreement with those of Dupraz and Spahr (19), who showed that chimeric Gag proteins containing the Rous sarcoma virus (RSV) NC domain in place of M-MuLV NC showed an increased specificity to packageable RSV transcripts. Taken together, these results suggest that retroviral nucleocapsid domains contribute toward the specificity of

encapsidation. However, in our experiments with HIV NC mutants and exchanges, the maximum encapsidation differences observed were only about 10-fold. Consequently, it is possible that other regions in Gag or Gag-pol may add to encapsidation specificity. For instance, NC may interact with another Gag or Gag-pol domain to allow more efficient recognition of the genomic packaging signal and drive viral genomic RNA encapsidation specifically.

In accord with these observations (134, 147), major deletions, truncations, or replacements of the NC domain of HIV-1 *gag* were found to eliminate the encapsidation of HIV genomic or spliced RNAs into virus particles. Although theoretically possible, we do not believe these results are due to a defect of mutant construct cis-active encapsidation (Psi) signals, since the mutations occurred away from the known Psi signals (14, 108, 114, 195, 241). While major NC mutations apparently eliminated encapsidation, other mutants showed more subtle effects. Linker insertion between the two NC zinc fingers in the mutant ApaI, reduced the specificity of encapsidation, while the total amount of RNA packaged was normal. This observation supports the notion that NC contributes to the specificity of RNA encapsidation (19, 294). Perhaps more surprisingly, mutants of p1 or p6 maintained wt levels of encapsidated RNA, but the specificity of RNA encapsidation was reduced. This strongly suggests that PrGag determines the packaging specificity, and implicates the entire C-terminal region of HIV-1 Gag in the process.

As mentioned above, substitution of NC region with the unrelated *B. subtilis* RNA binding protein MTRB permitted efficient release of virus-like particles. Although the MTRB domain was fused to HIV-1 *gag* because it potentially acts as an RNA binding protein (214), no evidence of specific or non-specific RNA incorporation into ApoMTRB particles was observed in our experiments using either HIV RNA or MTRB target RNA (data not shown).

Similar situation happened to our other assembly competent NC substitution mutants. The wt zipper domain is not known to bind RNA. The same is true when it is used to replace the NC region of Gag: no viral genomic or spliced RNA appear to be encapsidated into the wtzip virus particles . Although the bacteriophage MS2 coat protein can bind to its own RNA genome at a specific hairpin structure (11, 166, 210, 222, 223, 276), it did not appear to bind spliced or unspliced HIV-1 viral RNA sequences. Thus, it appears that the NC assembly function can be replaced by a protein that does not encapsidate detectable levels of RNA in our system. Although we have not exhaustively tested assembly of all cellular RNAs into virus particles, if spliced RNA encapsidation is indicative of non-specific RNA incorporation, we expect little RNA in these particles, although this assumption has yet to be proven. While our evidence suggests that fusion proteins of Gag with *B. subtilis* RNA binding protein MTRB, MS2 coat protein and wt zipper domain do not need to bind RNA for assembly purposes, a number of studies suggest that loss of NC RNA binding function correlates with decreased efficiency of assembly (65). It is possible that NC interprotein contacts might be mediated indirectly by RNA; or RNA might be required for assembly in the context of NC. Even in the presence of its binding sequence in the HIV-1 genome, MS2 protein fused to Gag in place of NC did not appear to bring viral RNA into virus particles (see Fig. 5b, and Table 1). It could be that the MS2 fused to Gag is non-functional for RNA binding, its RNA binding site may not be folded appropriately, or may be masked by other proteins, or, it is also possible that the MS2 protein domain and it RNA binding site were never at the same locality, which might suggest that encapsidation is not just a simple binding function.

Conclusions of the thesis:

(1) The p6 domain did not appear to affect virus release efficiency, but p6 deletions and truncations reduced the specificity of genomic HIV-1 RNA encapsidation.

(2) Mutations in the nucleocapsid region reduced particle release, especially when the p2 interdomain peptide or the amino-terminal portions of the NC region were mutated.

(3) NC mutations reduced both the specificity and efficiency of HIV-1 RNA encapsidation.

(4) For virus-like particle assembly and release, NC can be replaced by a protein that does not appear to encapsulate RNA.

(5) The p2 and the C-terminus of CA are not necessarily toxic to assembly.

(6) For assembly, NC does not act passively by just providing a stable folded monomeric structure to restrict p2 or CA in an assembly-competent conformation.

(7) Formation of interprotein contacts by NC are essential for normal assembly process.

(8) Chimeric Moloney murine leukemia virus (M-MuLV) particles with M-MuLV NC replaced by HIV NC assembled efficiently, were of wild type M-MuLV density, and crosslinked at NC cysteines.

(9) Wild type M-MuLV precursor Gag (PrGag) proteins packaged M-MuLV transcripts more efficiently than HIV RNAs. In contrast, chimeric PrGag proteins possessing M-MuLV MA, p12, and CA domains, but the HIV-1 NC domain encapsidated HIV transcripts to a greater extent than M-MuLV transcripts.

Medical relevance

Some of our HIV-1 nucleocapsid (NC) mutants are defective in virus particle assembly, and our data suggest that formation of interprotein contacts by NC are essential for normal assembly process. These findings could provide insights for developing anti-AIDS treatments. Chemical compounds could be screened for specific interaction with HIV-1 NC region and tested for interference of virus particle assembly. Additionally, our data suggest that NC mutations reduce both the specificity and efficiency of HIV-1 RNA encapsidation, which could be another potential target for blocking HIV replication.

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