ASSEMBLY AND TRANSPORT OF MOLONEY MURINE LEUKEMIA VIRUS

by

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DEDICATION

This thesis is dedicated to Amanda Roberts, my wife and friend.

During our time in Portland, we ran hundreds of miles together on the streets and trails of Portland, got married, and completed graduate school. We arrived in Portland happy and ready to begin. We will leave Portland happy and ready to begin a new stage. Maybe we'll come back to live in Portland one day.

ABSTRACT

Moloney murine leukemia virus (M-MuLV) structural proteins (Gag) assemble particles that encapsidate the retroviral genomic RNA molecules and the viral enzymatic proteins. The mode of transport and the assembly interactions of M-MuLV Gag proteins have not been characterized. In the first manuscript, experiments were designed to identify the mode of Gag protein transport. A previous description of mutant Gag proteins trapped on intracellular membranes, suggested that Gag proteins may be transported to the cell surface associated with vesicles (85). In order to examine this model, virus-producing cells were treated with monensin, a potassium ionophore that inhibits vesicular transport (165). Monensin treatment inhibited the release of Gag by up to 70%. As a control, this treatment inhibited over 90% of the release of Env, which is transported through the Golgi. This suggests that Gag transport is, in part, mediated by a vesicle transport pathway. A second aim of the experiments in manuscript #1 was to identify specific domains of the Gag protein that contribute to Gag protein interactions with either transport machinery or other Gag proteins. A Gag-B-galactosidase fusion protein, composed of M-MuLV MA, p12, and CA fused to B-galactosidase, had been shown by its enzymatic activity to efficiently incorporate into helper virus (85). The fusion gene was used as a template to create 12 mutant fusion genes with insertions of four codons into regions of Gag. The mutant fusion proteins were expressed in the presence of helper virus. Proteins with insertions into the amino-terminal third of the MA protein were trapped on intracellular membranes, suggesting that MA possesses a transport signal. Five of six proteins with insertions in CA were transported to the cell surface, however, they were not efficiently incorporated into particles.

The study of the effects of monensin-treatment on Gag protein transport led to the observation that M-MuLV particles can assemble on intracellular membranes (i.e. non-plasma membranes). In manuscript #2, experiments were designed to characterize the

components packaged within the intracellular particles. A subcellular fractionation protocol was employed to enrich or partially purify intracellular particles. Viral particles and protease activity co-fractioned, indicating that intracellular particles contain pol gene products. To determine whether genomic RNA was encapsidated by intracellular particles, two cell lines were constructed to express an RNA molecule (Inf A) that could be encapsidated by viral proteins. One cell line did not express viral proteins. The other cell line expressed viral proteins that formed particles that could only encapsidate Inf A RNA. Both cell lines were subjected to subcellular fractionation. The fractionation pattern of viral RNA was dependent on the expression of viral proteins, suggesting that intracellular particles contain genomic RNA.

Specific contacts between Gag proteins in retrovirus particles have not been identified. In an attempt to identify domains and specific residues of Gag that interact in particles, we employed a cysteine-specific crosslinking system (manuscript #3). This system, adapted from Pakula and Simon (125), utilized a cysteine-specific crosslinking agent, BMH, that has a small atomic radius, and so only crosslinks proteins that have cysteine residues in very close proximity. Initially, particle-associated Pr65Gag protein was crosslinked into multimers. The cysteines of the nucleocapsid cys-his-motif facilitat intermolecular crosslink formation. The crosslinking cysteines were identified by substitution of Gag cysteine codons with serine codons, singularly or in combination, followed by crosslinker treatment of virus particles composed of cysteine to serine mutatnt Gag proteins. The cys-his-motif forms a zinc finger which packs in close proximity to other zinc-finger motifs in immature particles.

All of the cysteine codons of Pr65Gag were substituted with serine codons, and the cysteine-minus gag gene was used as a template to add-back single cysteine codons into a candidate region for effecting Gag protein interactions. The major homology region (MHR), a region in which amino acid substitutions often disrupt particle assembly (110, 162) , was modeled as an amphipathic alpha helix, and cysteine residues were

substituted on either side of the putative helix. MHR cysteine-mutant proteins in immature particles were treated with crosslinker, and they formed Gag dimers above control crosslinking levels. Interestingly, crosslinking of the MHR-cysteine mutants resulted in the formation of a novel band. The novel band is composed of Gag and a non-viral protein of 140-150 kd that is present in M-MuLV virions produced in 3T3 and Cos7 cells, as well as in HIV virions. A second aim of the experiments in manuscript #3 was to examine the role of nucleocapsid in assembly. Deletion of nucleocapsid significantly reduced Gag protein release, while amino acid substitution of three cysteines in the cys-his-motif did not disrupt particle formation or affect particle density. The nucleocapsid plays a minor, but necessary, role in the Gag assembly interactions. NC cys-his-motifs in immature particles are held in close proximity by regions of CA or non-cys-mtif regions of NC.

INTRODUCTION

I. GENERAL INTRODUCTION

A. Thesis Overview

The work presented in this thesis details aspects of the assembly of Moloney murine leukemia virus, M-MuLV, a retrovirus that causes T-cell lymphoma, or leukemia, in murine species. The thesis contains three manuscripts that address facets of the assembly, transport, and structure of M-MuLV. Before presenting these manuscripts, I will discuss retroviruses in general terms, describing their classification, life cycle, and components. I will also review the literature pertaining to the assembly, transport, and structure of retroviruses.

B. Importance of Studying Retroviruses

Because retroviruses replicate in tissue culture cells and are amenable to genetic manipulation, they have been used to elucidate cellular processes such as transcription (6), translation (130), glycosylation (9), intracellular transport (62), and membrane fusion (63). M-MuLV is among the simplest retroviruses because it encodes only three genes. Therefore, it has been used widely in the study of retroviral processes to model more complex retroviruses, such as HIV.

Acquired immune-deficiency syndrome (AIDS), which is caused by the retrovirus HIV, is a world wide epidemic in the 1990s. Retroviral epidemics such as AIDS necessitate the study of retroviruses in hope of developing strategies for preventative immunization or curative drug treatments. The aim of the work presented here is to contribute to a greater understanding of the processes of retroviral protein transport and assembly.

II. GENERAL DISCUSSION OF RETROVIRUSES

A. Classification of Retroviruses

Retroviruses are RNA viruses that integrate a copy of their genome into a host cell's genome. In other words, a retrovirus is a code of RNA that promotes its own replication, in that a single strand of RNA encodes the directions for a cell to construct, assemble, and release a retrovirus particle that can infect and replicate in another cell. Three of the defining features of retroviruses are a lipid envelope; an RNA genome comprised of two non-covalently associated copies of a single-stranded, positive-sense RNA molecule; and reverse transcription. Reverse transcription is a unique replication process that transforms the genomic RNA into a DNA form that is integrated into a host-cell's genome. The family of retroviruses is classified into three sub-families: oncoviruses, the RNA tumor viruses that cause cancer; spumaviruses, the "foamy" viruses that cause persistent infection in culture but are not associated with disease; and lentiviruses, such as human immunodeficiency virus (HIV), that have slow disease progressions (34).

B. Life Cycle of a Retrovirus

Retroviruses must transmit their genetic information from cell to cell, and the life cycle of a prototype retrovirus, shown in Figure 1, consists of two phases, entry and assembly.

1. Entry Phase

During the entry phase, an extracellular virion usually containing two identical strands of genomic RNA enters a cell (step 1), transforms the RNA genome to a DNA form (the provirus; step 3), and integrates the proviral DNA into a host-cell chromosome (step 4). The integrated provirus encodes the necessary information to assemble and release (steps 5-8) a retroviral particle that encapsidates the genomic information (RNA) and accessory components necessary to perform the entry phase in a new cell. A more detailed

account of the life cycle of a prototype retrovirus follows and serves as a means to introduce both the terminology that will be used and the retroviral components and processes that will be examined further.

Virus particles bind to a target cell by virtue of a specific protein-protein interaction between the viral receptor-binding protein (Env) and a receptor molecule at the cell surface (Figure 1, step 1) (176). Infectivity of cells is determined by the specificity of the receptor-binding protein of the virus for the receptor molecule on the cell's surface. Murine retroviruses are sub-classified by differences in their receptor-binding proteins: Ecotropic retroviruses infect solely murine cells; amphotropic infect most cells; and xenotropic infect non-murine cells (176). In the case of HIV, the cellular receptor is CD4 (40), for ecotropic M-MuLV, the receptor is CAT1, a basic-amino acid transporter (95, 173), and for amphotropic M-MuLV the receptor is Ram-1, a phosphate transporter (94).

After the virus particle has adhered to the cell, it must enter the cell. For M-MuLV, entry appears to be a limiting step of infection and may require a receptor accessory factor (174). HIV receptor binding interactions have been extensively studied, and evidence suggests that for HIV entry is achieved by direct membrane fusion of the retroviral and cellular membranes (109, 159). HIV entry is pH-independent (159), and membrane fusion may be protein-mediated. Fusion of the viral and cellular membranes effectively removes the viral envelope and exposes the core components to cytosolic factors (Figure 1, step 2). At this point, the retroviral components use the plus-stranded genomic RNA molecules as templates for reverse transcription (34).

Reverse transcription (Figure 1, step 3) effects the transition of the genomic information from an RNA form to a double-stranded DNA form. This transition enables retroviruses to integrate their genome permanently into a host's genome. Reverse transcription can begin within the virion (167), and is accomplished by a viral enzyme, reverse transcriptase (RT), that utilizes a tRNA primer. This primer is derived from the host cell where the virus particle was assembled, and it primes transcription of the DNA

minus strand (35). Minus-strand synthesis begins near the 5' terminus of the plus-strande RNA and runs off the RNA template (35). Conveniently, the RNA strand has terminal repeats (see below). The newly-synthesized, minus-strand DNA is repositioned at the repeat near the 3' terminus of the RNA molecule and primes transcription of a full-length minus-stranded DNA molecule (103).

Synthesis of the plus strand of DNA initiates at a site, the poly-purine tract, where RNase H, a domain of RT, cleaves RNA from the RNA-DNA complex (59). The transcript runs off the minus-strand DNA template and is again repositioned and aligned by virtue of the terminal repeat sequences present on both the primer and template (65). This alignment enables the synthesis of the double-stranded DNA provirus to be completed. Reverse transcription products, including double-stranded circular and linear DNAs, are transported into the nucleus (19). However, only the double-stranded, linear DNA copy of the genomic RNA is integrated into a chromosome (shown incorrectly in Figure 1, step 4; 33, 152). Importantly, reverse transcriptase, which is specific to retroviruses, does not discriminate between nucleotides and nucleotide analogs as effeciently as host polymerases It is therefore a target for anti-viral agents such as the nucleoside analog, azidothymidine (AZT) which, when incorporated, terminates DNA elongation (79).

The process of integration (Figure 1, step 4) is facilitated by a viral protein, integrase, which cleaves the host chromosomal DNA in a retrovirus-specific manner (34). M-MuLV integrase cleaves both strands of host DNA and leaves a four nucleotide (nt) overhang of single-stranded DNA on each strand (23), while HIV integrase leaves a five nt overhang (24). Integrase also precesses the proviral DNA, removing two nt from each 3' terminus and leaving 2 unpaired nts (20, 104). The cellular and viral substrates are attached, a strand transfer requiring the integrase protein in vitro (37, 93). After the strands are atteached, they are sealed and filled by cellular factors. In the case of M-MuLV, this creates a duplication of four cellular and two retroviral nts at either end of the integration site (34). The size of the host DNA duplication is retrovirus specific. However, the only

specificity of site selection of integration seems to be into DNase I-hypersensitive sites (147).

2. Assembly Phase

During the assembly phase, retroviral proteins form a particle that encapsidates the retrovirus' genomic RNA and exits the cell by non-lytically budding through the plasma membrane. The retroviral RNA is transcribed by host RNA polymerase II (step 5) (34), and, for the simplest retroviruses, is translated into three polypeptides encoded by the genes gag, pol, and env (step 6, (176)). The gag gene encodes the structural proteins that assemble the housing for both the genomic RNA and the enzymes, encoded by the pol gene, that are necessary for transformation of RNA to DNA and integration of the DNA. Env encodes the receptor-binding protein that spans the viral envelope surrounding the assembled particle (176). A retrovirus particle contains approximately 2,000 Gag polypeptides, 100 Pol polypeptides, a maximum of 2,000 Env proteins, and two viral genomic RNA molecules (119). Therefore, the three types of gene products, along with the genomic RNA, must co-localize and interact in an organized fashion to assemble an infectious retrovirion. The subcellular site of initial interactions between retroviral components varies, and retroviruses are classified by this characteristic as type A, B, C, or D.

Type-C retroviruses, such as M-MuLV, and lentiviruses, such as HIV, are thought to assemble at the plasma membrane, where structural proteins aggregate, position the genomic RNA centrally, and enclose it as more structural proteins aggregate (179). Transport of the type-C structural proteins and the genomic RNA has not been fully characterized. There are several implications of the above model of type-C assembly that could be incorrect. For example, it is possible that the structural proteins form a complete particle and then the genomic RNA is threaded through a pore in the virion, or perhaps the

RNA is transported to the plasma membrane associated with structural proteins that are pre assembled into structural building blocks.

Type B and type D retrovirus particle assembly differs from type C, because it occurs away from the membrane. Again, the genomic RNA could be encapsidated prior to completion of the capsid or threaded through a pore at any time prior to budding. If these particles later associate with the plasma membrane and bud, they are classified as type A particles. Transport of the pre-assembled type B or D particles to the plasma membrane ha not been characterized.

Whether the particle assembles at the plasma membrane or is transported there, the process of budding envelops the particle with a host-derived lipid bilayer containing Env proteins (176). During or after budding, the viral protease cleaves Gag and Gag-Pol polypeptides to their mature components (179). This final step is necessary for retroviral replication (179).

C. Components of a Retrovirus

1. Nucleic Acid

The M-MuLV nucleic acid can be either of two forms depending upon the stage of the life cycle. In a retrovirion, genomic RNA is comprised of two identical strands that are approximately 8 kb each, while in a host-cell chromosome, genomic or proviral DNA coding sequence is slightly longer than the genomic RNA. The provirus contains two long terminal repeats (LTRs) that are divided into three regions based on comparisons with the full-length genomic transcript: U3 is present only at the 3' end of the viral genomic RNAs R is a repeat present at both the 5' and 3' ends of the RNAs; and U5 is present only near the 5' end of the RNAs. Importantly, the presence of R near both ends of the genomic RNA helps facilitate the regeneration, by reverse transcription, of the larger proviral LTR from the smaller RNA LTR (34).

Both LTRs harbor promoter and enhancer elements which are active in the 5' LTR, but, for poorly understood reasons, silenced in the 3' LTR (34). The promoter is regulated by multiple viral enhancer/silencer motifs that affect tissue type expression (28, 44, 94, 96). Transcription initiates in the 5' LTR at the U3 and R junction and terminates after the 3' LTR in host flanking DNA, but ultimately is cleaved and poly-adenylated between the 3' LTR R and U5 regions. Retroviral transcripts can be spliced, or they can remain unspliced. Retrovirus splicing patterns vary. For example, M-MuLV has only one splice donor site, while HIV has a complex RNA splicing pattern that helps provide ordered gene expression.

HIV RNA splicing produces early genes, one of which, rev, encodes a protein that increases the percentage of full-length HIV RNA transcripts in the cytoplasm (54, 150). The full-length or genomic transcript contains a packaging signal that is bound by one of the retroviral structural proteins; an interaction that is necessary for RNA incorporation into virus particles. While there can be several splice acceptor sites in all retroviral genomes, there is only one splice donor site. Its proximity 5' of the packaging region ensures that only full-length, unspliced transcripts (genomic RNAs) contain the packaging signal and are incorporated into virus particles.

Other than genomic RNA, the primary components of a retrovirus are the gag, pol, and env gene products. Some retroviruses, however, also encode accessory proteins. For example, the Rev protein affects RNA transport into the cytoplasm (54); Nef down-regulates cellular CD4 in HIV infected cells (3); and Tat binds to an HIV enhancer element tar, and increases the initiation and elongation of HIV transcripts (100). The following discussion focuses on the genes common to all retroviruses.

2. Genes

All retroviral proviruses encode at least three genes: gag, pol and env, the order of which is conserved. M-MuLV codes for only these three genes, while HIV encodes

several other genes that help orchestrate the expression of gag, pol, and env. The 5' most gene is gag, whose protein product is the major structural protein. The name "gag" derives from the phrase 'group specific antigens', since the protein was identified as being immunologically specific to a group of retroviruses such as the murine leukemia viruses (73). As will be detailed in section III, the Gag protein plays the central role in the assembly of retrovirus particles. Gag protein expression in the absence of all other retroviral genes can produce virus-like particles (75, 101, 154). Gag proteins are translated as precursor polypeptides composed of three to five Gag proteins that are cleaved by a viral protease (179). Gag proteins may direct each retroviral component in their role in the process of viral assembly. As mentioned previously, the viral nucleic acid is encapsidated by Gag as a result of a protein-RNA interaction between Gag and the RNA packaging signal. The pol gene products are also incorporated into particles as a direct consequence of Gag protein.

The pol gene encodes the enzymatic proteins of mammalian retroviruses: protease, reverse transcriptase, RNase H, and integrase. Gag and Pol are synthesized as polypeptides that are cleaved to their mature components by the virally encoded protease. The Pol proteins are dependent on Gag for incorporation into particles, because they are translated only as a Gag-Pol fusion protein. M-MuLV utilizes an amber suppression of the gag termination codon, while HIV utilizes a -1 ribosomal frameshift at the gag termination codon (36, 183). Both mechanisms result in the synthesis of a Gag-Pol fusion protein in approximately 5% of Gag translations. Therefore, both mechanisms control the ratio of Gag to Gag-Pol proteins, which may be very important for assembly of infectious particles. Virus infectivity may require that a minimum number of Gag-Pol fusion proteins incorporate per retrovirion, as protease function requires dimerization (91), and integrase function may require multimerization (55). However, it is also likely that size constraints within a retrovirus particle limit the ratio of Gag to Gag-Pol proteins. Forced Gag-Pol

fusion proteins, expressed in the absence of Gag, do not assemble into virus particles (57, 127).

The env gene encodes the receptor-binding protein, which determines the host-range of the retrovirus. The M-MuLV Env protein is synthesized as a polypeptide, gp80Env, that is transported through the secretory pathway and glycosylated (176). M-MuLV Env is cleaved by a cellular factor to gp70Env and p15Env (176); which remain associated during transport to the plasma membrane (15). As with influenza haemmaglutinin proteins, the Env proteins may function as trimers (53, 63). The Env proteins may incorporate into retroviral particles as a result of an interaction between Gag and Env proteins (105, 184), or, because Env proteins are transported to and expressed at the cell surface, Env may incorporate into particles as the particles bud through the plasma membrane.

III. LITERATURE REVEIW

A. ASSEMBLY - The Role of Gag.

1. Precursor Gag

Now that the components and life cycle of a prototype retrovirus have been described, single facets of the life cycle, shown in Figure 1, can be magnified, examined, and fitted into a more complete understanding of retroviruses. To form a retrovirus particle, approximately 2,000 Gag proteins co-localize, interact in a specific manner, and form a core that is 120 nanometers in diameter (119). There is no information defining any of the initial, specific interactions between Gag proteins. The transport of the M-MuLV structural proteins and the interactions between the structural proteins are the focuses of the work presented in this dissertation.

The major structural protein of M-MuLV (Gag; See Figure 2 for protein sequence.) is synthesized as a 65 kd (Pr65Gag) precursor polypeptide (176), that is co-translationally

myristylated by removal of the initiator methionine and attachment of myristate to the new terminal glycine residue (78). For M-MuLV and HIV, the myristate addition is a requirement for precursor Gag membrane-association which in turn is a requirement for particle assembly (138). Some retroviruses, such as Rous sarcoma virus (RSV), are not myristylated but still associate with membrane due to a membrane-binding protein domain near the amino-terminus of its precursor Gag protein (180). The protein-membrane interaction is necessary for the assembly of RSV (180). Assembly of RSV in mammalian cells requires myristylation (10)

After membrane-association and particle assembly are completed, the Pr65Gag protein is proteolytically cleaved by the virally encoded protease into the four M-MuLV Gag proteins: Matrix (MA) is the amino-terminal Gag protein that associates with membrane; p12, the second protein, is phosphorylated and has no known function; capsid (CA) is the largest of the Gag proteins and forms the capsid; and nucleocapsid (NC) is the carboxy-terminal protein that binds the retrovirus' genomic RNA molecule (11, 176, 179). Because cleavage occurs during or after budding (179), the Gag proteins that initially assemble a particle are solely precursor Gag proteins, and the precursor Gag molecule is the building block in the assembly of retrovirus particles.

Precursor Gag (PrGag) proteins mediate the assembly of retroviral particles. Pr65Gag expressed in the absence of Env, Pol, and RNA still assembles retroviral particles (75, 101, 154), and PrGag appears to interact with each component of the retrovirus during the process of assembly. A useful model for the assembly of M-MuLV-type retroviruses (Figure 3; 14) suggests that the precursor Gag proteins could direct each of the five components in their assembly role: 1) Amino-terminal regions of PrGag molecules associate with the retroviral lipid envelope that surrounds the virus particle; 2) the amino-terminus of PrGag also could be important for establishing interactions with the Env protein, as mutation of this region in HIV PrGag reduced the levels of incorporation of HIV Env (184); 3) PrGag controls the incorporation of the Pol proteins, because, as

described earlier, Pol is synthesized only as a Gag-Pol fusion protein; 4) the genomic RNA is incorporated as a result of a binding interaction with the carboxy-terminal Gag protein, nucleocapsid (11); and 5) PrGag proteins may interact with each other in repeated patterns to form icosahedral particles (119).

2. Matrix (MA)

The matrix protein (MA) is the amino-terminal Gag protein, and it is a membrane-associated protein (179). For M-MuLV and HIV, myristylation helps achieve membrane binding (179), while for RSV, domains of MA are necessary for membrane binding (180). A deletion of the first 50% of the MA protein of RSV Gag disrupts both membrane association and particle assembly (10). This protein was modified by expressing the first ten residues of the src protein, which is a functional myristylation signal, at its amino-terminus. The fusion protein associated with membrane and directed the assembly of RSV (180). Smaller deletions near the amino-terminus of RSV MA that blocked membrane-binding and particle assembly were also overcome by fusing the first ten residues of HIV-1 to their amino termini (10). Together, these experiments demonstrate that membrane association is necessary for particle assembly and can be supplied by functionally equivalent signals. The importance of membrane binding may be to provide an anchor point, so that Gag proteins can align and interact in parallel. In addition, membrane attachment could serve to increase the local concentration of Gag proteins.

A second role of the matrix protein may be to incorporate the Env proteins into the particle. As mentioned previously, mutations in HIV MA reduce the levels of Env protein incorporated into particles (184). Furthermore, in an elegant experiment, HIV Env protein affected HIV particle release in polarized cells. Particles produced in polarized cells in the absence of Env were released from both the apical and basolateral surfaces. However, particles produced in polarized cells that also expressed Env proteins were only released from the basolateral surface (105). This suggests that Env and Gag proteins associate. A

further characterization of the interaction between Env and MA proteins showed that HIV Gag proteins with a deletion of 80% of MA could assemble particles that were pseudotyped with co-expressed Env proteins (172). The HIV MA-deleted Gag and wild type Gag proteins incorporated M-MuLV Env protein at similar levels. Therefore, much of the HIV MA protein is not necessary for incorporation of M-MuLV Env protein. However, gp160Env, the unprocessed Env protein, was preferentially incorporated into particles composed of HIV MA-deleted Gag, while particles composed of wild type Gag protein preferentially incorporated the processed HIV Env proteins. These results may suggest that HIV MA recognizes processed HIV Env with specificity, or they may suggest that the MA-deleted Gag protein is transported aberrantly.

There are contradictory reports as to the importance of the role that matrix plays in the assembly of retroviral particles. One describes a deletion of 80% of HIV MA that only reduces infectivity (172). Another describes a single amino acid substitution in the MA of Mason-Pfizer monkey virus (M-MPV), a type D retrovirus, that changes the assembly characteristics to those of a type C retrovirus (141). That the majority of the matrix protein can be deleted without totally blocking particle assembly suggests that most of the MA is unnecessary for Gag protein interactions. That the assembly site characteristics of a retrovirus can be drastically altered by a single amino acid substitution may suggest a role of MA in the transport of Gag proteins. There is additional evidence that MA is involved in the transport of Gag proteins. A series of M-MuLV Gag fusion proteins with the carboxy-terminus of Gag truncated, leaving just MA or portions of MA, fused to B-galactosidase were expressed in mouse fibroblast cells. These proteins exhibited perinuclear immunofluorescent staining (85). HIV Gag proteins with insertions of four amino acids in the MA protein also exhibit perinuclear subcellular localization (171). The proteins appear to be trapped at the ER or Golgi, which could indicate a role of the MA protein in the transport of Pr65Gag to the plasma membrane.

The matrix protein association with membrane is necessary for particle assembly (179). It apparently also associates with Env protein (184). Matrix may possess a signal for transport of the PrGag molecule to the cell surface (85, 171), and the majority of MA is not essential for Gag protein interactions (172). Matrix also may have a role in a post-entry step of the retroviral life cycle. Single amino acid substitutions in M-MuLV MA (39) and insertions of 4 amino acids in HIV MA (170) can be permissive for particle assembly, but block infectivity. The HIV-1 MA has a nuclear localization signal that may be important in facilitating the transport of the pre-integration complex to the nucleus (21).

3. p12

All retroviral precursor Gag molecules have the MA, CA, and NC proteins in that order. However, many retroviruses also have other Gag proteins on the precursor Gag protein. M-MuLV Pr65Gag contains the p12 protein between MA and CA. The p12 protein is phosphorylated (176), but does not yet have a function ascribed to it. Early reports suggested that p12 binds genomic RNA, and binding is specific to M-MuLV RNA (153). Amino acid substitutions in p12 do not disrupt particle formation, but do block infectivity at a post-entry step (39).

In other retroviruses, there are also uncharacterized Gag proteins between MA and CA: An RSV p10 protein can be deleted without affecting particle assembly; and an M-MPV p12 protein can be deleted without blocking particle assembly (179). While these proteins do not share any homology, it is possible that they serve a similar function in the retroviral life cycle. Since all three proteins can be mutated or deleted without preventing particle assembly, it is also possible that they affect a post-entry stage in retroviral replication.

4. Capsid (CA)

The capsid protein (CA) is the largest of the Gag proteins. Several lines of evidence suggest that regions of CA are important in establishing interaction between precursor Gag proteins and, therefore in the formation of retrovirus particles (85, 170, 177). Support for a role of CA proteins in establishing Gag-Gag contacts comes from the yeast two-hybrid system of examining protein interactions (58). M-MuLV Pr65Gag formed measurable protein interactions with other Pr65Gag molecules, and HIV Pr55Gag formed interactions with other Pr55Gag molecules. However, the two proteins did not form heterologous HIV-M-MuLV interactions (107). The domains of interaction between M-MuLV Pr65Gag proteins have been mapped to the carboxy-terminal half of CA (K. Alin). A chimeric PrGag protein consisting of M-MuLV MA, p12, HIV CA, and M-MuLV NC assembled into virus particles in 3T3 cells, suggesting that assembly domains can be functionally exchanged (43). The apparent difference between the findings of these experiments, chimeric Gag proteins associate while heterologous Gag proteins do not, may be due to the necessity of aligning like-assembly domains.

To examine the role of CA in Gag protein interactions, Jones et al. constructed a series of M-MuLV PrGag proteins which were truncated from the carboxy-terminus into regions in the CA protein and then fused to B-galactosidase. The fusion proteins were expressed in mouse fibroblasts that were producing wild type virus proteins. If the fusion proteins contained regions of CA necessary for Gag-Gag interactions, they were incorporated into assembling retrovirus particles. The efficiency of Gag-B-galactosidase fusion protein incorporation was proportional to the amount of CA protein expressed on the fusion protein (85). The results of this experiment suggest that M-MuLV CA contributes to Pr65Gag protein interactions that facilitate Gag protein incorporation into assembling particles. In a similar experiment, HIV Gag-B-galactosidase truncations were incorporated into assembling wild type particles only when the fusions contained the carboxy-terminal two-thirds of CA (171). An HIV Gag-B-galactosidase fusion protein with an internal deletion of the amino-terminal third of CA was efficiently incorporated into particles (172).

Interestingly, the M-MuLV Gag-B-galactosidase fusion proteins expressed without helper virus were released from cells, but at a lighter density form than wild type virus particles (85). Similarly, an RSV PrGag protein truncated halfway through CA produced virus-like particles of light density (177). One possibility is that Gag proteins that release from cells at a light density form virus-like particles, but do not contain all of the domains necessary for wild type Gag protein packing. In support of this idea, an RSV PrGag protein composed of MA, the carboxy-most 20% of CA, and NC assembled virus-like particles of wild type density (177). Additionally, an HIV PrGag protein with an internal deletion of the amino-terminal third of CA also assembled particles of wild type density (170). These proteins presumably contain the domains necessary for correct Gag protein packing, and at least one of those domains apparently lies in the carboxy-terminal half of CA or in NC.

Within the carboxy-terminal half of all retroviral capsid proteins there is a region of strong protein sequence homology, the major homology region (MHR) (179). This region spans approximately 20 amino acids, and while no function has been attributed to the MHR, amino acid substitutions at conserved residues in this region can disrupt particle assembly (110, 162). Although only three residues of the MHR are invariant, several others are conserved as either aromatic, polar, or hydrophobic. Even conservative substitutions of the invariant or well-conserved residues can prevent particle assembly (110). The effect of MHR amino acid substitutions on particle assembly and its location in the carboxy-terminal half of the CA protein suggest that since the carboxy-terminal half of the CA may contain an assembly domain, the MHR may be an interesting region to examine for a possible role in Gag protein interactions.

The capsid protein apparently contributes to precursor Gag protein interactions, but it may also form capsid-capsid protein interactions after the precursor Gag proteins have been cleaved to their mature components. The M-MuLV mature CA protein exhibits a high degree of self-association (22). Recombinant HIV CA also self-associates in vitro,

forming dimers and a specific multimer that may be composed of 12 CA proteins (52). Proteolytic cleavage of the CA multimer produces two fragments and suggests that the interaction site could be in the carboxy-terminal half of the protein. Identification of the regions of CA-CA interaction could elucidate the mechanism of assembly of immature or mature retrovirus particles.

It is quite likely that the mature capsid protein plays a post-assembly role in the replication of retroviruses. Amino acid substitutions in CA can be permissive for particle formation, but block infectivity (83, 110, 162, 170). Additionally, a small span (N/B) of murine retroviral CA proteins apparently performs a post-entry role in the infection of murine cells (176). The protein sequence at the N/B site determines the retrovirus' capacity to infect NIH 3T3 or BalbC mice. The post-entry infectivity difference could result from the involvement of a cellular protein that differs between the two cell types.

5. Nucleocapsid (NC)

Perhaps the most well understood Gag protein is the nucleocapsid (NC) protein, whose function is to encapsidate the genomic RNA (176). An interaction between the nucleocapsid protein and viral genomic RNA has been characterized in vitro by North-western (11). The nucleocapsid-RNA binding interaction facilitates incorporation of genomic RNA into retroviral particles. The genomic RNA consists of two identical full-length strands of RNA. A packaging signal structure, psi, maps to a region of the RNA that is necessary for incorporation into particles. The two RNA molecules non-covalently dimerize over portions of the genomic sequence, and while interactions between the RNA molecules have not been fully characterized, it is apparent that dimerization alters RNA structure at the packaging signal site (82, 166). Dimerization of both HIV and RSV RNA molecules may be facilitated by the nucleocapsid protein (42, 115), and RNA dimerization may form RNA structures that are not present in RNA monomers. RNA dimers, and not monomers, may be recognized with specificity by the NC protein (11, 82, 166).

Evidence that nucleocapsid is the protein that encapsidates the RNA molecules comes from chimeric Gag proteins composed of M-MuLV Gag protein with an RSV nucleocapsid domain replacing the M-MuLV NC domain. The M-MuLV-RSV chimera formed particles that packaged RSV RNA and did not package M-MuLV RNA. While some protein-RNA contacts may be attributed to protein domains outside NC, apparently the nucleocapsid protein, at least for RSV, can specifically bind and package the genomic RNA of that virus (48).

A nucleocapsid protein motif that is crucial for genomic RNA incorporation is Cys-X_2-Cys-X_4-His-X_4-Cys, termed a Cys-His box. All oncoviruses and lentiviruses contain at least one of these motifs in their NC domain, with M-MuLV NC containing one Cys-His box and HIV NC containing two. Point mutations changing cysteine or histidine residues in the Cys-His box generally disrupt RNA packaging (68). These arrays, deemed zinc-finger motifs, have been shown to coordinate zinc cations and bind nucleic acid (72). Several lines of evidence suggest that the cys-his motif(s) in nucleocapsid function as zinc-fingers and bind RNA. M-MuLV, HIV, HTLV and other retroviruses bind approximately 0.8 moles of zinc per mole of nucleocapsid per zinc-finger motif (12), and the solution structure of an HIV zinc-finger is similar to previously described zinc-finger, nucleic acid binding motifs (118).

A second region of nucleocapsid protein homology is the basic amino acid region that consists of three or more basic residues proximal to each Cys-His box (82). The positively charged nature of these residues make this region a good candidate for associating with negatively charged RNA. This region has been proposed to facilitate NC-genomic RNA binding or to position the tRNA primer onto the RNA primer binding site prior to reverse transcription (41, 82, 136). The basic amino acid region binds RNA in vitro, and mutation of the basic residues reduces infectivity and reverse transcriptase activity, while substitution of the basic residues with different basic residues has no apparent effect on infectivity or reverse transcription (41, 82).

Although the nucleocapsid protein plays a critical and identifiable role in packaging the genomic RNA molecules, NC's role in the assembly of particles is not well understoo The nucleocapsid protein apparently does contribute to PrGag-PrGag protein contacts (85, 171). Fusion proteins comprised of M-MuLV Gag proteins fused to B-galactosidase were expressed with wild type M-MuLV Gag proteins, and the level of B-galactosidase incorporation into M-MuLV particles was assessed to determine the fusion protein's rescu efficiency. NC-deleted fusion proteins incorporated into particles at a 2-3 fold reduced efficiency in comparison to full-length fusion proteins (85). In a similar system using HIV Gag proteins, deletion of HIV NC also reduced Gag incorporation levels (170). NC-deleted RSV Gag proteins assemble particles at reduced efficiency, and the particles are les dense than wild type particles. A small region of HIV nucleocapsid, containing a Cys-His box, restored release efficiency and particle density when added back to the RSV NC-deleted Gag protein (10). These experiments suggest that the NC protein effects protein-protein contact between precursor Gag molecules to a small degree and the cys-his motif may be important for protein interactions and tight packing of Gag proteins within particles However, because residues of the Cys-His box can be mutated without affecting particle assembly, their importance for Gag-Gag interactions is questionable. Conceivably the basic amino acid stretch may have a role in mediating protein contacts.

B. TRANSPORT OF RETROVIRUSES

Expression of the gag gene alone is sufficient for the assembly and release of virus-like particles (75, 101, 154), indicating that the precursor Gag protein possesses the signal for transport and self-association. Type C retroviruses and lentiviruses are thought to assemble at the plasma membrane (179). However, the mode of transport to the plasma membrane is unknown. One model suggests that type C Gag proteins transit to the plasma membrane on the cytoplasmic surface of transport vesicles (85). Evidence for this model i two-fold: (1) M-MuLV Gag proteins truncated into the matrix protein were trapped

intracellularly, possibly in the RER/Golgi (85); and (2) a percentage of murine leukemia virus proteins were shown to associate with cytoskeletal elements (50). Together, these findings suggest that M-MuLV Gag proteins possess a transport signal and are transported to the cell surface on transport vesicles.

In contrast, gag cores of type B and D retroviruses are thought to assemble intracellularly, away from the plasma membrane (179). These Gag proteins are presumably transported to the cell surface as assembled cores that have encapsidated the genomic RNA. Whether the particles passively transit the cytosol or are transported in transport vesicles is not known. Mammalian retroviral Gag proteins, including those of type D retroviruses, are myristylated. Type C and lentivirus Gag proteins require myristylation for both membrane association and particle assembly (138), while type D retroviruses require the myristate only for transport (140). This suggests that transport of Gag proteins could be mediated by a cellular myristate receptor or even the retroviral Env protein.

The env gene product, a glycosylated, transmembrane protein, is transported to the cell surface via the Golgi apparatus (15) and could be incorporated into retrovirus particles as they bud through the plasma membrane. It has been proposed that the Env protein coul serve as an assembly nucleation point (14) through an affinity interaction with the structura proteins. The Env protein could lead Gag proteins through the Golgi to the plasma membrane. As previously described, Env expression affected Gag protein release in polarized cells (105). However, the absence of Env expression does not block particle assembly (154).

Alternatively, Gag proteins could utilize a cellular protein, such as a myristate receptor as an assembly receptor (139). However, the prospect of a specific assembly receptor seems unlikely for the following reasons: 1) some retroviruses can assemble in numerous cell types including murine fibroblast cells, monkey kidney cells, human cervica carcinoma cells, and silkworm ovarian cells mandating that an assembly receptor have a

wide species and tissue distribution (64, 170); and 2) type C and D assembly is similar as evidenced by the elegant demonstration that a single amino acid substitution in the structur protein of a type D retrovirus alters the assembly characteristics to those of a type C retrovirus (141). This suggests that assembly characteristics can be very different for nearl identical viruses in the same cell. This argument is conjectural, and it is certainly possible that either a myristate receptor or the viral Env protein anchors Gag proteins to membranes and supports transport.

If Gag protein transport of type B, C, D, and lentivirus retroviruses is similar, conceivably the difference in the assembly site could be due to the affinity between Gag proteins. For example, type D Gag proteins could have a high affinity for one another an form capsids on intracellular membranes prior to transport, while both type C and lentiviru Gag proteins could have a lower affinity and assemble only after many Gag proteins have been delivered to the plasma membrane. Lastly, it is likely that some cellular factors such as chaperonins help mediate Gag protein interactions with other Gag proteins and cytoskeletal elements.

C. STRUCTURE OF RETROVIRUSES

Retroviral Gag proteins have the capacity to assemble a structure that encloses and protects the genomic nucleic acid and enzymes. The repeated contacts between immature Gag proteins form a particle that is resistant to non-ionic detergent solubilization (161). Amazingly, the proteins are restructured following maturation and form new structures. The immature and mature Gag protein structural differences are evident when compared by electron microscopy (117). Immature particles typically have an electron-dense ring near the envelope, while mature particles are electron dense near the center of the particle (179). The Gag protein components within a mature HIV virion have been localized by immunogold electron microscopy (61), and fit very well with an early model of Gag protein alignment within the virion (14). Matrix is apparently near the envelope, possibly

contacting the Env protein, while CA is centrally localized, apparently surrounding the ribonucleoprotein complex.

The organization of capsid structures has been demonstrated to be icosahedral for many viruses (26, 74, 80, 102, 119, 126). An icosahedron has twenty symmetric faces. However, the number of sub-structures necessary to construct identical facets, the triangulation number, varies depending on the morphology of the virus. The triangulation number, an element of the series 1, 3, 4, 7, 9,12, 13.., is a means to describe the facets of an icosahedron (26).

X-ray crystallographic data to this point shows how the facets of an icosahedron are arranged, but does not necessarily demonstrate contact between residues on neighboring proteins. Structural information about five viruses follows:

(1) The three-dimensional (3-D) structure of poliovirus has been modeled at 2.9-angstrom resolution (80). Although poliovirus is an RNA virus, it differs from retroviruses in that it is much smaller, 310 angstroms in diameter, and is not enveloped. The structure indicates that it is arranged as an icosahedron with 60 copies each of the four capsid-like proteins arranged into 20 facets composed of symmetric thirds; T=3.

(2) The structure of simian virus 40, a DNA virus, has been modeled at a 3.8-angstrom resolution. This virus has 360 surface capsid proteins with 72 pentameric capsomeres arranged in icosahedral T=7 symmetry, which indicates that the shell is not completely close-packed; the shell has "holes" (102).

(3) Another DNA virus, human papillomavirus (HPV-1), was modeled to 2.9-angstrom resolution as a particle composed of 360 surface capsid proteins assembled in 72 pentameric capsomeres in icosahedral T=7 symmetry (74).

(4) A membrane-containing RNA alphavirus, Sindbis virus, has been modeled at low resolution as an icosahedral particle composed of 240 copies of the capsid protein arranged in an icosahedron that surrounds a similar icosahedron composed of nucleocapsid protein (126). Both have T=4 symmetry and have 80 trimeric subunits arranged as 20

tetramers. Each of the 20 faces is essentially an equilateral triangle that is divided into four equivalent domains.

(5) Retroviruses, being relatively large and membrane-associated, are not amenable to x-ray crystallography. However, the organization of HIV-1 has been modeled by negatively staining virus particles, digitizing an image, and transforming the image by fourier transform to establish unit cell averages (119). The structural information reported is that linear rods of Pr55Gag, 85 angstroms in length, are arranged in pentameric or hexameric capsomeres that form an icosahedron of T=63 with 632 "holes". Each of the 20 facets contains essentially 10.5 rings of six Gag monomers arranged so that they form a subunit that is identical to another facet of 10.5 hexameric rings. This model suggests that the rod-like (immature) protein subunits form rings that closely pack, surrounding 632 pores, and forming a nearly spherical shape. The morphology of a mature virion has not been analyzed in this fashion.

IV. THESIS RATIONALE

The research presented here pertains to the assembly, transport, and structure of Moloney murine leukemia virus (M-MuLV). Examination of retroviral protein transport may elucidate an uncharacterized aspect of retrovirus replication. The entry and transcription steps of retroviral replication have been targets of anti-viral agents, and perhaps the transport and assembly replication steps may, in time, provide effective anti-viral targets.

In the first manuscript, experiments were designed to identify the mode of Gag protein transport. A previous description of mutant Gag proteins trapped in the Golgi suggested that Gag proteins transport to the cell surface associated with Golgi-derived vesicles (85). In order to examine this model, virus-producing cells were treated with

monensin, an inhibitor of vesicular transport (89). A second aim of the experiments in manuscript #1 was to identify specific domains of the Gag protein that contributed to Gag protein interactions with either transport machinery or other Gag proteins. A Gag-B-galactosidase fusion protein, composed of M-MuLV MA, p12, and CA fused to B-galactosidase, had been shown by its enzymatic activity to efficiently incorporate into helper virus (85). The fusion protein was used as a template to create 12 separate fusion proteins with insertions of four amino acids into regions of Gag. The mutant fusion proteins were expressed in the presence of helper virus. Comparison with the parental fusion protein, in regard to transport and efficiency of incorporation into helper virus, elucidates the function of the mutated domains.

The study of the effects of monensin-treatment on Gag protein transport led to the observation that M-MuLV particles can assemble on intracellular membranes (i.e. non-plasma membranes). In manuscript #2, experiments were designed to characterize the components packaged within the intracellular particles. A subcellular fractionation protocc was employed to enrich or partially purify intracellular particles, and the fractions were analyzed for enrichment of both particles and retroviral components, such as protease. In order to determine whether genomic RNA was encapsidated by intracellular particles, two cell lines were constructed to express an RNA molecule (Inf A) that could be encapsidated by viral proteins. One cell line did not express viral proteins. The other cell line expresse viral proteins that formed particles that could only encapsidate Inf A RNA. Both cell lines were subjected to subcellular fractionation to determine if the Inf A RNA fractionation pattern was dependent on virus protein expression.

Specific contacts between Gag proteins in retrovirus particles have not been identified. In an attempt to identify domains and specific residues of Gag that interact in particles, we employed a cysteine-specific crosslinking system (manuscript #3). This system, adapted from Pakula and Simon (125), utilized a cysteine-specific crosslinking agent, BMH, that has a small atomic radius, and so only crosslinks proteins that have

cysteine residues in very close proximity. Initially, particle-associated Pr65Gag protein was crosslinked into multimers. In order to identify the cysteine residues involved in crosslink formation, cysteine residues were substituted with serine, singularly or in combination, and the mutant proteins were crosslinker-treated.

All of the cysteines of Pr65Gag were substituted with serine, and the cysteine-minus Gag protein was used as a template to add-back single cysteine residues into a candidate region for effecting Gag protein interactions. The major homology region (MHR), a region in which amino acid substitutions often disrupt particle assembly (110, 162), was modeled as an amphipathic alpha helix, and cysteine residues were substituted on either side of the putative helix. The MHR cysteine-mutant proteins were treated with crosslinker in order to determine if the region forms self-associations or interacts with cellular factors. The cysteine-specific crosslinking system is especially useful for structura analysis of retroviruses, because domains or residues of interaction can be identified in either the immature or mature particle. A second aim of the experiments in manuscript #3 was to examine the role of nucleocapsid in assembly. Proteins with amino acid substitutions in NC or a full deletion of NC were assayed for particle assembly .

Figure 1. **The life cycle of a prototype wild type retrovirus.** The cycle of retroviral replication is shown in eight steps that are as follows: 1, adsorption and entry of the retroviral particle into a cell is mediated by an interaction between the viral receptor-binding protein (Env) and a cellular recptor; 2, uncoating of the protein shell from the retroviral genomic RNA molecules; 3, reverse transcription of the genomic RNA molecule produces a double-stranded DNA copy of the retroviral genome, the provirus, which enter the nucleus of the cell; 4, integration of the linear provirus into the host-cells' genome; 5, transcription of retroviral messenger RNA (mRNA) is accomodated by cellular machinery; 7, assembly of the retroviral proteins which encapsidate genomic RNA and form a particle; 8, non-lytic budding through the host-cell plasma membrane generates a retroviral lipid bilayer. The envelope glycoprotein (Env), internal structural proteins (Gag), and reverse transcriptase are shown.

THE LIFE CYCLE OF A PROTOTYPE WILD TYPE RETROVIRUS

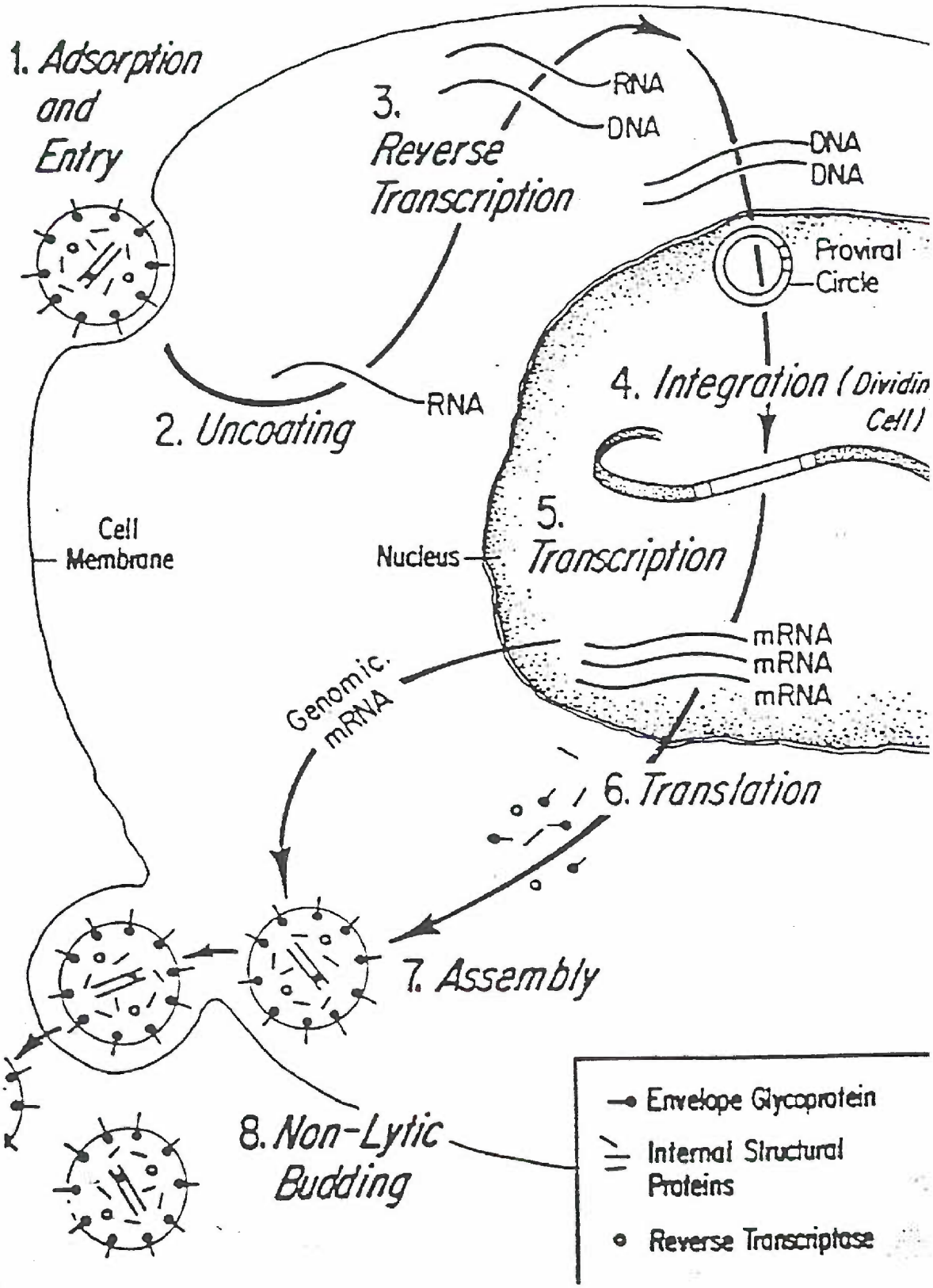
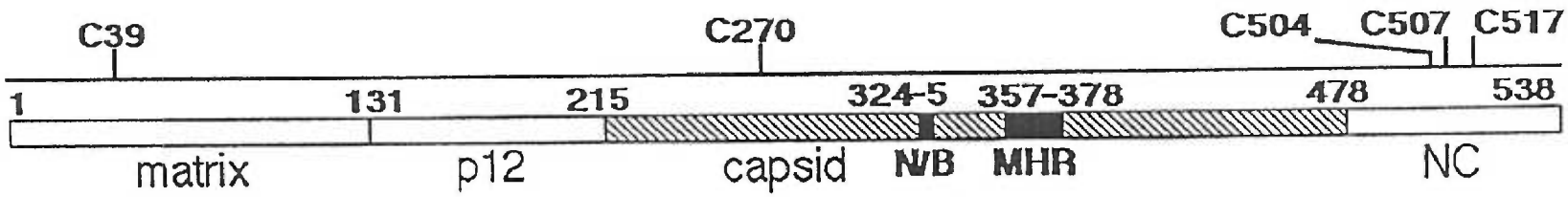


Figure 2. **Map and codon sequence for the Moloney murine leukemia virus (M-MuLV) gag gene.**

Top panel: The M-MuLV Pr65Gag protein consists of four domains, matrix (MA), p12, capsid (CA;stippled lines), and the nucleocapsid, that are cleaved to maturation by the viral protease (PR) during or after budding. As shown, the gag gene encodes a precursor polypeptide of 538 codons, but the amino-terminal methionine is cleaved and replaced with an N-terminal myristate during translation. The boundaries between the four gag domains are shown, as are the residues which define the N/B tropism determinant (176), the major homology region (MHR), and the five gag cysteine codons.

Bottom panel: The deduced codon sequence of the M-MuLV gag gene is shown in single letter code with domain boundaries indicated, the MHR underlined, and the cysteine codons in lower case. (From E. Barklis)

M-MuLV GAG



MOLONEY MURINE LEUKEMIA VIRUS GAG GENE CODONS

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MGQTVTTPLS LTLGHWKDVE RIAHNQSVDV KKRRWVTFcS AEWPTFNVGW PRDGTFNRDL    60
ITQVKIKVFS PGPHGHPDQV PYIVTWEALA FDPPPWVKPF VHPKPPPPLP PSAPSLPLEP   120
PRSTPPRSSL Y<<MA//p12>>PALTPSLGA KPKPQVLSDS GGPLIDLLTE DPPPYRDPRP   170
PPSDRDGNGG EATPAGEAPD PSPMASRLRG RREPPVADST TSQAF<<p12//CA>>PLRAG   220
GNGQLQYWPF SSSDLYNWKN NNPSFSEDPG KLTALIESVL ITHQPTWDDc QQLLGTLLTG   280
EEKQRVLLEA RKAVRGDDGR PTQLPNEVDA AFPLERPDWD YTTQAGRNHL VHYRQLLLAG   340
LQNAGRSPTN LAKVKGITQG PNESPSAFLE RLKEAYRRYT PYDPEDPGQE TNVSMSFIWQ   400
SAPDIGRKLE RLEDLKNKTL GDLVREAEKI FNKRETPEER EERIRRETEE KEERRRTEDE   460
QKEKERDRRR HREMSKLL<<CA///NC>>AT VVSGQKQDRQ GGERRRSQLD RDQcAYcKEK   510
GHWAKDcPKK PRGPRGPRPQ TSLLTLDD                                      538
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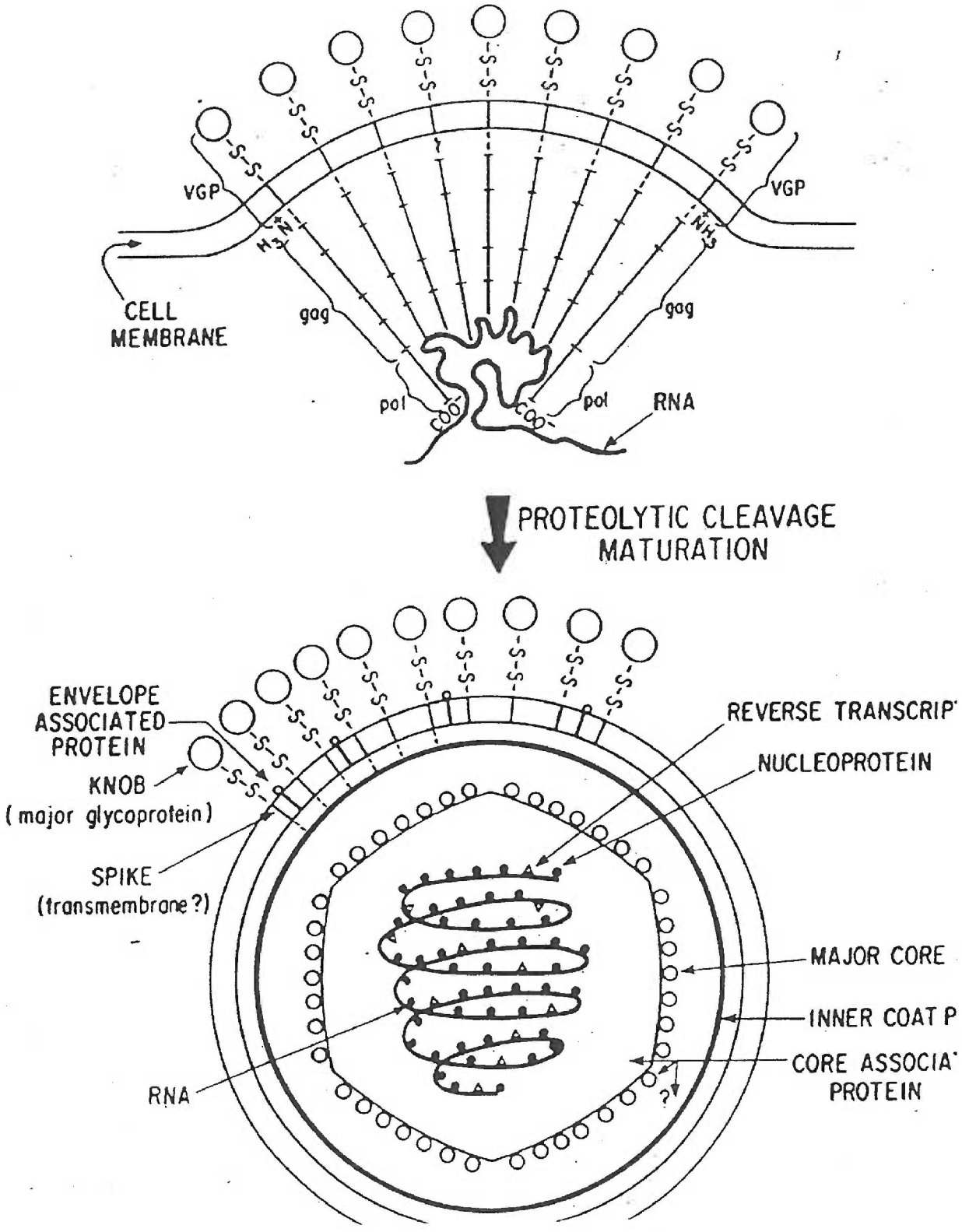
Figure 3. **A model for the assembly of a retroviral particle.**

Top panel: The formation of an immature retrovirus particle is shown. The model suggests that precursor Gag proteins interact with each of the retroviral components. The viral glycoprotein (VGP; Env) is a transmembrane protein shown spanning the cellular membrane which will be the retroviral lipid bilayer upon budding. The cytoplasmic doma of Env may associate with Gag proteins. Gag may also associate with the cellular membrane and force a curvature as shown. Pol enters retroviral particles only as a Gag-P fusion protein. The carboxy-terminus of Gag protein binds the genomic RNA. The alignment of the Gag proteins suggests that Gag forms homotypic interactions.

Bottom panel: During or after budding, a viral protease cleaves Gag and Gag-Pol proteins into their mature components. This cleavage results in the internal rearrangement of Gag proteins as suggested in the diagram. The mature Gag proteins are shown in three discrete layers, the inner coat, the core, and the centrally located nucleoprotein complex. Adapted from Bolognesi et al. (14).


VGP
VGP
CELL MEMBRANE
gag
gag
pol
pol
RNA
PROTEOLYTIC CLEAVAGE
MATURATION
ENVELOPE ASSOCIATED PROTEIN
KNOB
(major glycoprotein)
SPIKE
(transmembrane?)
REVERSE TRANSCRIP
NUCLEOPROTEIN
MAJOR CORE
INNER COAT P
CORE ASSOCIA
PROTEIN
RNA

MANUSCRIPT #1

TRANSPORT AND ASSEMBLY OF GAG PROTEINS INTO MOLONEY MURINE LEUKEMIA VIRUS

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Running Title: Transport and Assembly of Gag Proteins

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ABSTRACT

We have studied the process of Moloney murine leukemia virus (M-MuLV) assembly by characterization of core (gag) protein mutants and analysis of wild type (wt) gag proteins produced by cells in the presence of the ionophore monensin. Our genetic studies involved examination of linker insertion mutants of a gag-B-galactosidase (gag-B-gal) fusion protein, GBG 2051, which is incorporated into virus particles when expressed in the presence of wt viral proteins. Analysis indicated that the amino-terminal two thirds of the gag matrix domain is essential for targeting of proteins to the plasma membrane: mutant proteins localized to the cytoplasm or were trapped on intracellular membranes. Mutations through most of the coding region of the gag capsid domain generated proteins which were released from cells in membrane vesicles, but not in virions. In contrast, linker insertions into gag p12 or carboxy-terminal portions of the matrix or capsid coding regions did not affect assembly of fusion proteins into virus particles. Monensin, which blocks vesicular transport, inhibited gag protein intracellular transport and release from cells. Our results suggest that a significant proportion of M-MuLV myristylated gag proteins travel via vesicles to the cell surface. Specific matrix protein polypeptide regions and myristic acid

modification are both necessary for appropriate gag protein transport, while capsid protein interactions appear to mediate the final phase of virion formation.

INTRODUCTION

The process of retrovirus assembly requires that several viral constitutents must localize to the plasma membrane of an infected cell and assemble into a budding virus particle. Incorporated into virions are the viral genomic RNAs; core (gag) and envelope (env) proteins; and viral enzymatic functions, including pol gene products such as reverse transcriptase. Central to this process is the retroviral gag polyprotein. With Moloney murine leukemia virus (M-MuLV), the gag polyprotein is synthesized as a myristylated precursor Pr65gag, which is cleaved during assembly to form the mature M-MuLV gag proteins p15 (matrix, MA), p12, p30 (capsid, CA), and p10 (nucleocapsid, NC) (2,4,15,29,35,42). Evidence suggests that synthesis of the M-MuLV gag proteins is necessary and sufficient for assembly of (non-infectious) virus particles (14,23,33,40), and that M-MuLV enzymes initially are incorporated into virions as a gag-pol fusion protein, Pr180gag-pol (41).

One clear requirement for virus assembly is a myristic acid addition at the amino-terminal glycine of the matrix domain of Pr65gag (15,31). This modification appears necessary for a membrane association event which is a prerequisite for virion formation (31). While myristylation is essential for gag protein membrane association, the mechanism by which myristylated Pr65gag travels to the plasma membrane is unclear. It is possible that myristylated Pr65gag transits freely through the cytoplasm to assembly sites at the inner face of the plasma membrane. Alternatively, gag and gag-pol proteins may associate with intracellular membranes shortly after synthesis, and travel to the cell surface via vesicular transport (17). Also unresolved are details concerning gag protein assembly resulting in the formation of a budded virus particle.

To investigate the process of M-MuLV virion formation, we previously expressed gag-B galactosidase (gag-B-gal, GBG) fusion proteins in Psi2 cells (17), which express wild typ M-MuLV proteins (26). During these studies, we identified a construct (GBG 2051; see Figure 1B) encoding a fusion protein which was incorporated efficiently into virus particles, and also released from cells in a low density non-viral form. While GBG 2051, containing intact gag matrix, p12, and capsid coding regions, was assembled into virions, fusion proteins with deletions in capsid, or capsid plus p12, were released from cells only in the non-viral form. Also excluded from virions were gag-B-gal fusion proteins containing only myristylated fragments of the gag matrix domain. These proteins were trapped at intracellular membranes, reminiscent of the intracellular trapping of mutant integral plasma membrane proteins (12,22,32).

With the goal of clarifying the processes of M-MuLV gag protein transport and assembly we have extended our analysis of gag and gag-B-gal fusion proteins. In particular, we have characterized a myristyl-minus version of the GBG 2051 fusion protein, as well as eleven linker insertion mutants of the GBG 2051 fusion protein.We also have studied the effect of monensin, an inhibitor of vesicular traffic (5,16,19,34), on the transport and release of gag, gag-pol, and gag-B-gal proteins from cells. Our results indicate that interactions involving the amino-terminal two thirds of the gag capsid domain are essential for incorporation of proteins into virus particles, whereas mutations in p12, and the carboxy-terminal portions of the matrix and capsid domains do not impair assembly into virions. As expected, mutations in the amino-terminal regions of the matrix protein affect the intracellular transport of fusion proteins and may result in the loss of membrane association, or in trapping of proteins at intracellular membranes. These results, in conjunction with our observation that monensin inhibits transport and release of gag, gag-pol, and gag-B-gal proteins, suggest that a large proportion of myristylated Pr65gag travels via vesicular transport to assembly sites at the plasma membrane.

MATERIALS AND METHODS

Cell culture. Psi2 cells (26) and PA317 cells (27) were grown as described previously (3). Psi2 cell populations expressing BAG (7) or GBG constructs were generated by transfection of PA317 cells, infection of Psi2 cells with the PA317 supernatant collected 24 h after transfection, and selection of Psi2 cells with G418 as described previously (17).

Recombinant plasmids. Freak plasmids derive from pFreak, a plasmid generated by circularizing the neomycin gene-containing XhoI cassette of MP10 (3). In pFreak derivatives, the original EcoRI site of pFreak was altered to make the following sequences: BamHI-Freak, GAATTCGGATCCGAATTC; ClaI-Freak, GAATTCATCGATGAATTC; EcoRV-Freak, GAATTCGATATCGAATTC (see Figure 1).All recombinant retroviral constructs derive from the B-gal expression vector, BAG (7), which was generously provided by Dr. Connie Cepko. Construction and sequencing methodologies followed standard protocols (25). The construct GBG 2051, described by Jones et al. (17), encodes a M-MuLV gag-B-galactosidase fusion protein which includes the M-MuLV gag matrix, p12, and capsid domains fused to the ninth residue of B-galactosidase. When expressed in Psi2 cells, this protein is efficiently incorporated into virions (17). GBG myr- is identical to GBG 2051 except a point mutation preventing myristylation, kindly provided by Dr. Alan Rein (31), has been inserted into the GBG 2051 backbone. All GBG 12in constructs are identical to the GBG 2051 parent construct except for 12 bp linker insertions in their gag coding regions. Linker insertions were created as described in Figure 1. Briefly, EcoRI minus, ampicillin resistance conferring target plasmids containing the 5' portion of the M-MuLV proviral genome up to viral nt. 2051 (34) were randomly linearized and ligated to compatible ends of the plasmids EcoRV-Freak, ClaI-Freak, or BamHI-Freak. Recombinants were selected in bacteria with ampicillin plus kanamycin. After sequencing

of insertion sites, 12 bp insertions in target plasmids were generated by deletion of the large Freak-derived EcoRI fragment from recombinants. Target plasmids with linker insertions were used to replace wild type GBG 2051 gag coding regions, yielding GBG 12in plasmids. Eleven linker insertion mutation constructs (GBG 12in 676, 747, 919, 1074, 1084, 1436, 1560, 1672, 1752, 1862, and 1908; see Figure 1B) were produced in this fashion. Sequences in mutated regions are shown in Figure 1C.

Analytical Procedures. Enzyme assays, cell fractionation, virus pelleting, sucrose density gradients, immunoblotting, and immunofluorescence protocols were performed as described previously (10,13,17,24,28).

Antibodies and golgi localization. Mouse anti-B-galactosidase antibody was from Promega and was used at a 1:3000 dilution for immunofluorescence studies. A goat anti-feline leukemia virus gp71 antibody , used at a 1:1,500 dilution was employed for immunodetection of M-MuLV envelope proteins. Tissue culture supernatants of rat hybridoma cells R187 and R548 (8) were used at 1:2 dilutions for immunoblotting or immunofluorescence detection of M-MuLV p30gag and p12gag proteins, respectively. These cells were the kind gift of Dr. Bruce Chesebro. For immunoblotting studies, alkaline phosphatase-anti-immunoglobulin G conjugates (Promega or Boeringer Mannheim) were used at 1:7,500 dilutions. For immunofluorescence, rhodamine-conjugated goat anti-mouse antibody (TAGO) was used at a 1:100 dilution. Golgi complex localization by FITC-conjugated wheat germ agglutinin (U.S. Biochemicals) followed the protocol of Rose and Bergmann (32), but excluded the preincubation with unconjugated wheat germ agglutinin.

Monensin treatment. Monensin was from Sigma and was stored as a 10 mM stock in ethanol at -20 degrees C. For immunofluorescence studies, 10 or 25 micromolar monensin was added to cells on coverslips for 4 h prior to fixation. For enzyme or protein analysis,

confluent or nearly confluent 10 cm plates of cells were pretreated for 1 or 3 h at 37 degree C with 10 ml of DMEM/Calf (Dulbecco modified Eagle's medium plus penicillin, streptomycin, and 10% heat-treated calf serum) plus 10 or 25 micromolar monensin. Following pretreatment, cells were washed with DMEM/Calf and reincubated for 5 or 6 h at 37 degrees C with DMEM/Calf plus monensin, after which cells and media supernatants were collected for analysis.

RESULTS

Assembly of gag-B-gal proteins into virus particles.

In our previous experiments (17), we established a system for studying the assembly of gag-B-galactosidase (gag-B-gal) fusion proteins into M-MuLV virions. In those studies, fusion proteins were expressed in Psi2 cells, which produce wild type (wt) M-MuLV proteins, and the ability of fusion proteins to become incorporated into virus particles was analyzed. Our negative control construct, BAG, expressed a free B-gal protein which was cytoplasmic and not incorporated into virions. In contrast, the fusion protein expressed by the GBG 2051 construct was readily incorporated into virions. As shown in Figure 1B, th GBG 2051 protein is expressed in Psi2 cells from the M-MuLV long terminal repeat (LTR promoter and contains M-MuLV matrix, p12, and capsid domains fused to B-gal: incorporation of this protein into virions indicated that the M-MuLV nucleocapsid domain i not required for this process.

For fine mapping of M-MuLV gag domains involved in assembly, we constructed 12 mutant versions of the GBG 2051 construct (Figure 1). Twelve base pair linker insertions at M-MuLV viral positions 676, 747, 919, 1074, 1084, 1436, 1560, 1672, 1752, 1862, and 1908 were generated as described in the Materials and Methods and Figure 1A. These mutations were introduced into the GBG 2051 construct, yielding the constructs GBG 12in676, 747, and 919, with mutations in the matrix domain; GBG 12in1074 and 1084 with mutations in the p12 domain; and GBG 12in1436, 1560, 1672, 1752, 1862, and 1908, with mutations in the capsid domain (see Figure 1B). As shown in Figure 1C, all linker insertions are expected to encode mutant proteins with 4 amino acid insertions, plus an additional amino acid mutation in the case of GBG 12in1672. In addition to linker insertion mutations, we transfered the previously created (31) glycine to alanine mutation a

the second codon of M-MuLV gag to the GBG 2051 backround. Since this mutation prevents myristylation, membrane association, and assembly of the wt gag polyprotein (31), we have designated our construct GBG myr- (Figure 1B,C).

The GBG 2051, BAG, GBG myr- and all eleven GBG 12in constructs were expressed in Psi2 cells after introduction via the transfection-infection protocol of Jones et al. (17), which introduces proviral constructs into recipient cells by precise retrovirus integration rather than nonhomologous recombination, as seen in direct transfections. Our initial characterization of proteins involved quantitation of total B-gal activity in matched samples of medium released from cells (supernatant) and in cell lysates: previous experiments showed that normalization for total cellular protein and/or total reverse transcriptase activity in the supernatant did not alter relative ratios (17). As shown in Table 1, the free B-gal produced from the BAG construct is not released from cells, as demonstrated by a supernatant to cell ratio of 0.016. In contrast, the fusion protein produced by the GBG 2051 construct gave a ratio of 0.482, indicating that it is released from cells at a high rate. These results were comparable to our previous studies, which gave ratios of 0.017 and 0.567 for BAG and GBG 2051, respectively (17).

Analysis of our twelve mutant constructs indicated that only the three most amino-terminal mutations (GBG myr-, GBG 12in676, and GBG 12in747) prohibited release of B-gal activity from cells. All other linker insertion mutants (GBG 12in 919 and longer) expressed proteins that were released from cells at levels comparable to the GBG 2051 parental construct and at least 10 times higher than free B-gal produced by the BAG construct. In general, these results corroborate our previous results (17), indicating that determinants necessary for fusion protein release from cells are contained within the M-MuLV matrix domain. One minor disparity is that the GBG 12in919 gag-B-gal protein was released from cells (supernatant/cell ratio equals 0.596), while our previous studies (17)

indicated that a gag-B-gal protein fused at nt. 917 (construct GBG 917) was not released from cells (ratio=0.005). This discrepancy could be caused by insertion of non-disruptive amino acids in the GBG 12in919 construct, or by disruption of neighboring matrix protein domains when B-gal is fused directly to the carboxy-terminal region of matrix in the GBG 917 construct.

While our previous work indicated that supernatant and cellular B-gal activity levels reflected protein levels (17), release of B-gal activity from cells does not necessarily indicate that fusion proteins are incorporated into virions. Indeed, the GBG 2051 protein is released from cells in two forms: one form is a virus-associated high density form, and the other form is associated with a low density membrane vesicle form (17). Thus, in order to determine whether supernatant gag-B-gal proteins were in virions, it was necessary to fractionate supernatant material on sucrose density gradients. Figure 2 illustrates the results of our density fractionations on all GBG 12in proteins that are released from cells. As shown, our marker for virus particles, reverse transcriptase activity, fractionated to the bottom of our gradients, at a density of 1.15-1.16 g/ml. Fusion constructs GBG 12in919, 1074, 1084, and 1908 produce gag-B-gal proteins that fractionated similarly to the GBG 2051 protein: 30-80% of the released B-gal activity is virion associated, while the remainder was in the light density fraction. However, constructs with mutations in the amino-terminal portions of the capsid region (GBG 12in 1436-1862) express proteins that, for the most part, were excluded from virus particles. These results suggest that interactions involving the amino-terminal two thirds of the capsid domains are required for protein assembly into virions.

Subcellular localization of gag-B-gal proteins.

Why are matrix mutant fusion proteins not released from cells? To answer this question, we examined the subcellular localization of proteins produced by BAG, GBG 2051, and a matrix mutant proteins. Our method was to measure B-gal activities in crude membrane (P2) and cytosol (S2) fractions (10) of cell lysates (see Table 2). Not surprisingly, virtuall all of the free B-gal produced by the BAG construct fractionated to the cytosol, while over two thirds of the gag-B-gal from the GBG 2051 construct was membrane associated. On the basis of specific activities, the GBG 2051 protein was enriched in membranes 70-fold relative to free B-gal. The GBG 12in919 protein, which is efficiently released from cells and incorporated into virions, also fractionated to cellular membranes. However, the GBG myr-, 12in676, and 747 proteins had reduced levels of membrane association. The GBG 12in747 protein, which has an insert at the forty-second residue of the myristylated matrix domain, was reduced two-fold in its membrane affinity relative to the wt GBG 2051 protein, but still partitioned over half of its B-gal activity to the P2 fraction. GBG myr- and 12in676 proteins were largely cytosolic, although their levels of membrane association were slightly higher (2 to 4-fold) than that of free B-gal.

Since the above fractionation experiments implied altered subcellular localization patterns for proteins with mutations in the gag matrix domain, we performed immunofluorescence studies to visualize these patterns. Figure 3 shows examples of our B-gal and gag-B-gal immunofluorescence localization studies. As illustrated, free B-gal was present throughout cells expressing the BAG construct, although the protein was reduced somewhat in cell nuclei (Figure 3A). In contrast, the GBG 12in919 protein stained a heterogenous perinuclear area as well as a microfilamentous region at the cell periphery (Figure 3D): this pattern is similar to that seen with the GBG 2051 protein (17; see also Figure 5C). Due to low expression levels, we were unable to perform immunofluorescence studies on the GBG 12in676 protein. However, our results with the GBG myr- mutant protein (Figure 3B), as anticipated (31), gave a cytoplasmic staining pattern similar to free B-gal, with

little, if any, nuclear staining. Interestingly, the GBG 12in747 protein yielded a perinuclear staining pattern (Figure 3C) that suggested the protein was trapped on intracellular membranes (12,22,32). This pattern was different than the staining pattern we obtained when staining the golgi apparatus with FITC-conjugated wheat germ agglutinin: while the golgi appeared as a flattened disc of fluorescence on one side of the cell nucleus, the GBG 12in747 protein formed a diffuse rosette around the entire nucleus. Experiments concerning the interpretation of the GBG 12in747 staining pattern are described below.

Inhibition of gag and gag-B-gal transport by monensin

Although we did not assess the myristylation status of GBG myr- or 12in proteins in these studies, our previous work (17), and that of others (6,18,20,31) would indicate that the GBG 12in proteins should be myristylated, and that the glycine to alanine mutation of GBG myr- should prevent myristylation. Likewise, our previous work would suggest that variant glycosylated gag-B-gal proteins (1,11,30,39), if present, should represent a minor fraction of the gag-B-gal proteins in our study (17). Thus, we feel that the GBG 12in747 protein is trapped at intracellular membranes not because it is modified inappropriately, but as a direct consequence of its linker insertion mutation.

The aberrent localization of GBG 12in747 (and GBG 648, 730, and 917 proteins in our previous study; 17) suggests two possibilities. First, the localization may reflect an unnatural missorting of a mutant protein. Alternatively, the GBG 12in747 protein may be blocked at a stage in the natural routing of viral gag proteins via vesicular transport to the cell surface. Assuming this latter hypothesis, it should be possible to inhibit transport of gag proteins by inhibition of intracellular vesicular transport. To test this prediction, we decided to examine the effect of monensin on virus assembly. Since monensin is an ionophore for monovalent cations and blocks vesicular transport of newly synthesized

membrane proteins at the golgi (38), we felt it might disrupt the process of M-MuLV particle assembly.

Initially, we decided to assess the effects of monensin on the release of reverse transcriptase and B-gal activity from Psi2 cells expressing the gag-B-gal GBG 2051 protein. Since Psi2 cells express wt M-MuLV proteins and the GBG 2051 protein is efficiently incorporated into M-MuLV virions, we felt monensin might affect release of these activities similarly. Control experiments indicated that treatment of cells with 25 micromolar monensin for up to 9 h had no apparent effect on cell morphology or total cell protein levels (data not shown). However, in both subconfluent (Table 3, top experiment) or confluent (Table 3, middle experiment) cells, release of reverse transcriptase activity from treated cells was reduced approximately nine-fold. Not surprisingly, monensin treatment also diminished gag-B-gal release from cells, although the level of reduction was only 2 to 7-fold.

We have not established whether concentrations of less than 25 micromolar monensin will inhibit M-MuLV reverse transcriptase release, but our results indicate that 10 micromolar monensin is adequate for inhibition of gag-B-gal release (Table 3, bottom experiment). Immunofluorescence experiments also indicate that 10 micromolar monensin inhibits intracellular transport of gag-B-gal proteins (see below). The timecourse of the monensin effect on gag-B-gal release was studied by collection of supernatants and refeeding of cells at 2 h timepoints following initial monensin application. Relative to mock treated cells, 2 h 25 micromolar monensin treatment reduced B-gal release only 1.4-fold. However, at 4, 6, and 8 h timepoints B-gal release, relative to mocks, was reduced 3.9, 4.6, and 3.8-fold, respectively. These results suggest that a steady state inhibition of release is achieved by 4 h of monensin treatment.

Although our initial results indicated that release of reverse transcriptase activity from M-MuLV producing cells was inhibited by monensin, these experiments did not show conclusively that monensin acts by transport blockage of the reverse transcriptase precursor, Pr180gag-pol. Since we did not control for intracellular reverse transcriptase activity directly, it is possible that intracellular Pr180gag-pol levels were reduced by monensin treatment, while levels of our control protein, gag-B-gal, were unperturbed. Alternatively, monensin could have inhibited the proteolytic processing of Pr180gag-pol tc the enzymatically active form of reverse transcriptase. To circumvent these objections, we decided to measure the direct effects of monensin on the levels of gag in cells and virions.

For direct measurement of gag levels, Psi2 cells expressing only wt M-MuLV proteins were collected after mock or monensin treatment. Matched media supernatants from experimental cells were centrifuged at 4 degrees C for 30 min at 274,000 x g to pellet virus particles. Proteins in cell lysates and virus pellets were subjected to polyacrylamide gel electrophoresis, and electroblotted onto a nitrocellulose membrane. Afterwards, p12gag, capsid (p30), and envelope related viral proteins were visualized by successive rounds of immunodetection. Our results (Figure 4) on three separate sets of mock and monensin treated cells showed that cellular Pr65gag levels were not affected by monensin treatment (compare lanes g,h; i,j; and k,l). However, clear differences in virus-associated gag protei levels were detected. Specifically, supernatants from monensin treated cells had reduced amounts of p30gag and p12gag relative to mocks (Figure 4; lanes a,b; c,d; e,f): we estimat these differences to be 3 to 5-fold. In all monensin supernatants, Pr65gag was present at higher levels than in mocks, probably reflecting a monensin effect on precursor processing (see below). Nevertheless, as Pr65gag was a minor constituent in viral samples relative to p30 and p12, our data suggests that monensin treatment inhibits gag protein release from cells.

Our positive control for sensitivity to monensin was the M-MuLV envelope protein, an integral membrane protein whose transport should be inhibited by monensin (5,41). As shown, while total cellular levels of envelope proteins (Pr80env plus gp70) were relatively unaffected by monensin treatment (Figure 4, lanes g-l), monensin supernatants (Figure 4, lanes a-f) were reduced 5 to 10-fold in envelope protein levels relative to mocks. These results suggest that, while inhibited by monensin, gag release from cells is somewhat less sensitive to treatment than envelope protein release. Another point is that Pr80env processing, like that of Pr65gag, was inhibited by treatment. Monensin treated cells and supernatants possessed higher ratios of Pr80env to mature gp70 than did mocks, supporting the observation that processing is transport dependent (5,42). Of additional interest are minor 28 and 25 kd gag related proteins of unknown origin (denoted by black dots) which were reduced in abundance in treated cells (25 kd protein) or supernatants (28 kd protein). The significance of these minor species, and the p12gag related 27 kd protein present in all viral supernatants (possibly an unprocessed matrix-p12 polypeptide) is presently unclear.

The results described above indicate that monensin inhibits release of reverse transcriptase activity, gag-B-gal activity, and wt gag proteins from cells. While some of these effects may be attributable to blocked precursor processing, reduced capsid and p12gag levels in treated supernatants (Figure 4) implied that direct effects on release were involved. Since it was conceivable that drug treatment might block either transport to the plasma membrane, or assembly and release of virions at the plasma membrane, we examined the subcellular distributions of gag and GBG 2051 gag-B-gal proteins after monensin or mock treatment. As shown (Figure 5A) wt gag protein (presumably Pr65gag) in untreated Psi2 cells detected with an anti-p12gag antibody stained a heterogeneous pattern extending to peripheral cellular projections, with slightly enhanced fluorescence around the nucleus. The GBG 2051 gag-B-gal protein demonstrated a roughly similar

pattern (Figure 5C), with more noticeable staining of the cell periphery and perinuclear region. Cells treated with 25 (Figure 5B,D) or 10 (Figure 5E) micromolar monensin showed different patterns. Both gag (Figure 5B) and gag-B-gal (Figure 5D,E) proteins localized to a perinuclear region in a distinct punctate pattern: peripheral regions remained unstained.

The localization patterns of gag related proteins in monensin treated cells were similar to those seen for intracellularly trapped mutant gag-B-gal proteins (17; see Figure 3C). Because monensin appears to block vesicular transport of membrane proteins at a post-RER stage (38), we do not feel this is a RER pattern, although this assumption has yet to be tested. Based on immunofluorescence analysis with FITC-conjugated wheat germ agglutinin (32), these do not appear to be golgi staining patterns (data not shown): it is feasible that the monensin-induced staining patterns represent post-golgi or RER-golgi-independent vesicles. In any case, our observations suggest that a significant proportion of M-MuLV gag proteins travel to the cell surface via vesicular transport. That these are myristylated gag proteins and not the variant glycosylated gag (1,11,30,39) is strongly supported by the fact that the glycosylated gag initiation codon (30) and leader region is deleted in the Psi2 construct (26).

DISCUSSION

We have studied the process of M-MuLV gag protein assembly into M-MuLV particles using both mutants and the ionophore, monensin. Our method has been to assess virion incorporation of gag proteins, or mutants of the GBG 2051 gag-B-gal fusion protein, which is ordinarily targeted into virions (17). In support of previous experiments (17,31), our current studies indicate that mutations in the amino-terminal portion of the gag matrix domain prohibit protein release from cells (Table 1; GBG myr-, GBG 12in676 and 747). These proteins had reduced affinities to cellular membranes (Table 2), and were either cytoplasmically localized, or trapped on intracellular membranes (Figure 3). In this regard, we feel the discrepancy between the GBG 12in747 staining pattern (Figure 3) and its slightly reduced retention in the P2 membrane fraction (Table 2) may be due to dissociation from membranes during fractionation.

Our experiments with monensin support the notion that mutant proteins such as GBG 12in747 were trapped on intracellular membranes that are on the normal gag transport route to the cell surface. Monensin, which blocks vesicular transport of newly synthesized transmembrane proteins at the golgi (5,16,19,38), clearly reduced release of reverse transcriptase and gag-B-gal activities from treated cells (Table 3). Almost certainly, part of this activity release inhibition was due to impaired precursor processing. However, direct assay of cell and supernatant gag levels in mock and monensin treated cells showed a reduction of released gag p12 and p30 proteins from treated cells (Figure 4). This result, and the observation that monensin treatment resulted in an accumulation of wt gag and GBG 2051 gag-B-gal proteins to a perinuclear region (Figure 5), suggest that monensin inhibits the intracellular transport of M-MuLV gag proteins. Because the glycosylated gag

initiation codon and amino terminus is deleted in the Psi2 M-MuLV provirus (26,30), we feel that myristylated Pr65gag is the molecule affected by monensin treatment.

We do not know where newly synthesized and myristylated gag proteins first become membrane associated. No obvious RER or golgi staining pattern was apparent with either the GBG 12in747 protein (Figure 3) or with gag proteins in monensin treated cells (Figure 5). Until detailed subcellular fractionation studies prove otherwise, we consider it possible that myristylated gag proteins associate with a variety of intracellular membranes prior to cell surface routing. We also consider it feasible that some Pr65gag travels freely through the cytoplasm to the plasma membrane. This possibility, suggested by the differential effec of monensin on gag and envelope protein release from cells (Figure 4), may explain the inability of monensin to completely block retrovirus assembly (5), and relate to the partial effect of human immunodeficiency virus (HIV) vpu mutants on HIV budding (21,36,37).

Regardless of the gag pathway to the plasma membrane, our mutant studies demonstrate the importance of gag capsid determinants in M-MuLV particle formation. It is clear that linker insertions into the capsid domain of GBG 2051 did not inhibit fusion protein release from cells (Table 1). However, five of six capsid linker insertion mutants (GBG 12in 1436, 1560, 1672, 1752, and 1862) blocked fusion protein assembly into virions, permitting release only in the low density, presumably plasma membrane vesicle derived fraction (Figure 2). The one capsid mutation which does not affect fusion protein assembly into virions is GBG 12in1908, a RIRI insertion preceeding the highly charged carboxy-terminus of the capsid domain (Figure 1C). This may be either a neutral mutation for M-MuLV, or may affect some other aspect of the viral life cycle: further experimentation with replication competent constructs may distinguish these alternatives.

In addition to the GBG 12in1908 mutant, three other mutants, GBG 12in919, 1074, and 1084 also did not eliminate gag-B-gal assembly into virions. As noted for GBG 12in1908, these mutations may also be neutral for M-MuLV. Alternatively, these mutations may influence viral functions other than assembly. Indeed, Crawford and Goff (9) have found that deletions of as many as 144 nt at the M-MuLV matrix-p12 border (M-MuLV nt. 944-1088) do not impair virus assembly. However, such mutations resulted in the formation of mutant virions which were blocked at an early step of infection, possibly at the uncoating stage (9). It will be of interest to determine whether our linker insertions demonstrate similar phenotypes when transfered to M-MuLV proviral constructs.

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Table 1. B-gal released from cells.

Psi2 cells expressing the indicated recombinant constructs were split 1:20 or 1:40 from confluence into 10 cm tissue culture plates and grown for 48 to 72 h. Supernatant medium (10 ml) was collected, filtered through a 0.45 micron pore size filter, pelleted at 4 degrees C for 2 to 4 h at 274,000 x g (SW41 rotor at 40,000 rpm), and suspended in PBS before enzyme assay. Cells were washed twice with PBS, scraped from plates in 1 ml PBS, pelleted in a microcentrifuge, and resuspended in PBS. Enzyme assays on aliquots of cell and supernatant samples were performed as described previously (17). From these aliquots, total cell and supernatant B-gal activities were calculated. Results are expressed as the ratio of total enzyme activity in the supernatant sample versus the cell sample, with standard deviations as indicated when possible. BAG values derived from 5 independent trials; GBG 2051 and GBG 12in747, 919, 1436, 1752, and 1862 values are from 3 trials each; GBG 12in676 was from two experiments; all others derived from one experiment.

Table 1. B-gal released from cells.

CONSTRUCT	SUPER/CELL RATIO (total B-gal supernatant/ total B-gal activity in cells)
BAG	0.016 +/- 0.009
GBG myr-	0.011
GBG 12in676	0.020 +/- 0.023
GBG 12in747	0.049 +/- 0.020
GBG 12in919	0.596 +/- 0.415
GBG 12in1074	1.49
GBG 12in1084	0.671
GBG 12in1436	0.632 +/- 0.245
GBG 12in1560	0.356
GBG 12in1672	0.327
GBG 12in1752	0.202 +/- 0.067
GBG 12in1862	0.437 +/- 0.182
GBG 12in1908	0.570
GBG 2051	0.482 +/- 0.226

Table 2. Membrane association of proteins.

Membrane association of proteins was monitored by crude cell fractionation (10) into a crude membrane fraction, P2, and a cytosolic fraction, S2. Proteins were expressed from the indicated constructs in Psi2 cells. Fractions were assayed for B-gal activity (28) and protein (24). In the center column, ratios of total membrane (P2) versus cytosol (S2) activities are shown, along with standard deviations when possible. In the right hand column, ratios of specific enzyme activities are given. GBG 2051 values derive from 4 independent trials; BAG values are from 3 trials; GBG myr-, 12in676, and 12in747 results are from 2 experiments each; GBG 12in919 is from one experiment.

Table 2. Membrane Association of Proteins

Construct (in Psi2 cells unless indicated)	Total B-gal ratio P2 B-gal / S2 B-gal	Membrane Association (B-gal P2/Protein P2) / (B-gal S2/Protein S2)
BAG	0.025 +/- 0.010	0.359 +/- 0.227
GBG myr-	0.143 +/- 0.087	1.49 +/- 1.44
GBG 12in676	0.092 +/- 0.031	0.734 +/- 0.379
GBG 12in747	1.40 +/- 1.05	8.36 +/- 4.75
GBG 12in919	2.35	29.8
GBG 2051	2.76 +/- 1.84	25.7 +/- 17.3

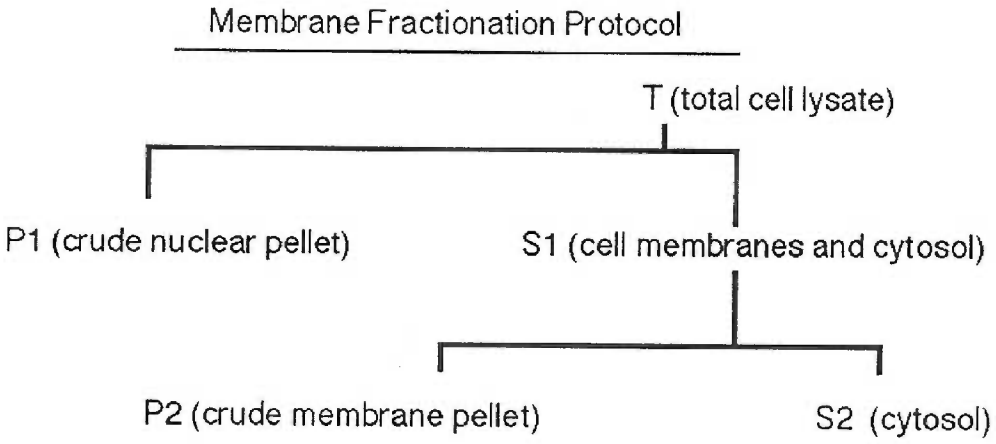

Table 3. Enzyme activity released from virus producing cells treated with monensin.

50% confluent (top experiment) or confluent (bottom two experiments) 10 cm plates of Psi2 cells expressing wt M-MuLV proteins as well as the GBG 2051 construct were mock treated or treated with 10 or 25 micromolar monensin as indicated. Treatments consisted of a 1 h (bottom experiment) or 3 h (top two experiments) pretreatment with the indicated amount of monensin, followed by a 5 h (bottom experiment) or 6 h (top two experiments) incubation with monensin. Following treatments, supernatant medium was collected, pelleted and suspended as described in Table 1 prior to enzyme assays. Cells were collecte for B-galactosidase assays as described in Table 1. Total units of supernatant reverse transcriptase activities were determined as described in the Materials and Methods, using dilutions of purified AMV reverse transcriptase as standards. Total units of supernatant anc cellular. B-galactosidase activities were determined as described in Table.1. Total enzyme activities in treated supernatants were normalized by multiplication to a normalization factor (total cellular B-gal activity in treated/ mock treated cells) to account for monensin effects on myristylated fusion proteins in cells. Ratios of normalized supernatant enzyme activities from treated versus mock treated cells provides a rough estimate of the effect of monensin treatments on enzyme activity release.

TABLE 3. Enzyme activity released from virus producing cells treated with monensin

	----------REVERSE TRANSCRIPTASE ACTIVITY----------			----------B-GALACTOSIDASE ACTIVITY----------			
treatment	supernatant	normalized	ratio to mock	supernatant	cells	normalized	ratio to moc
mock	36.8	36.8	1.0	37.1	312.4	37.1	1.0
25 mcM	3.9	4.1	9.0	5.7	299.8	5.9	6.3
mock	346.0	346.0	1.0	61.8	558.6	61.8	1.0
25 mcM	22.7	37.1	9.3	14.8	340.9	24.3	2.5
mock	-----	-----	-----	27.6	193.8	27.6	1.0
10 mcM	-----	-----	-----	10.3	220.4	9.1	3.0
25 mcM	-----	-----	-----	9.9	209.0	9.2	3.0

Figure 1. M-MuLV linker insertion mutations.

A. Linker insertion mutations were generated initially in gag gene containing, EcoRI minus, ampicillin resistance conferring plasmids. Target plasmids linearized with EcoRV, ClaI, or BamHI compatible restriction enzymes were ligated to EcoRV, ClaI or BamHI Freak plasmids. The Freak plasmids contain the designated unique restriction sites bracketed by EcoRI sites, and possess the bacterial neomycin phosphotransferase gene. Freak/target plasmid recombinants were selected with kanamycin plus ampicillin, and junction regions were sequenced using Freak specific sequencing primers. Following analysis of junction sites, deletion of the Freak EcoRI fragments from Freak/target intermediates yields target plasmids with 12 bp inserts of ATCGAATTCGAT for EcoRV-Freak, GATGAATTCATC for ClaI-Freak, and TCCGAATTCGGA for BamHI-Freak.

B. Linker insertion mutations in gag coding regions were transferred into the GBG 2051 construct for analysis. The GBG 2051 construct, previously described (17), derives from the retroviral B-galactosidase expression vector BAG (7), and expresses a protein which contains M-MuLV gag matrix (MA), p12, and capsid (CA) coding regions fused to B-galactosidase. The GBG 2051 gag-B-galactosidase (GBG) fusion protein is incorporated efficiently into virions when expressed along with M-MuLV wild type proteins. As shown six CA linker insertions (GBG 12in1436, 12in1560, 12in1672, 12in1752, 12in1862, and 12in1908), two p12 linker insertions (GBG 12in1074, and 12in1084) and three MA linker insertions (GBG 12in676, 12in747, 12in919) were generated. In addition, a myristylation minus mutation (kindly provided by Dr. A. Rein; 31) was transferred into the GBG 2051 backbone, yielding the construct GBG myr-.

C. Viral RNA sequences and encoded protein sequences in mutated M-MuLV gag coding regions are shown. In the case of GBG myr-, the underlined mutated nucleotide (nt.) corresponds to M-MuLV nt. 625 (34), converting the second gag codon from glycine to alanine and preventing myristylation (31). In the GBG 12in linker insertion mutants, the underlined nucleotide corresponds to the viral nt. designated by the construct name.

Inserted and mutated amino acid residues are indicated in bold lettering.

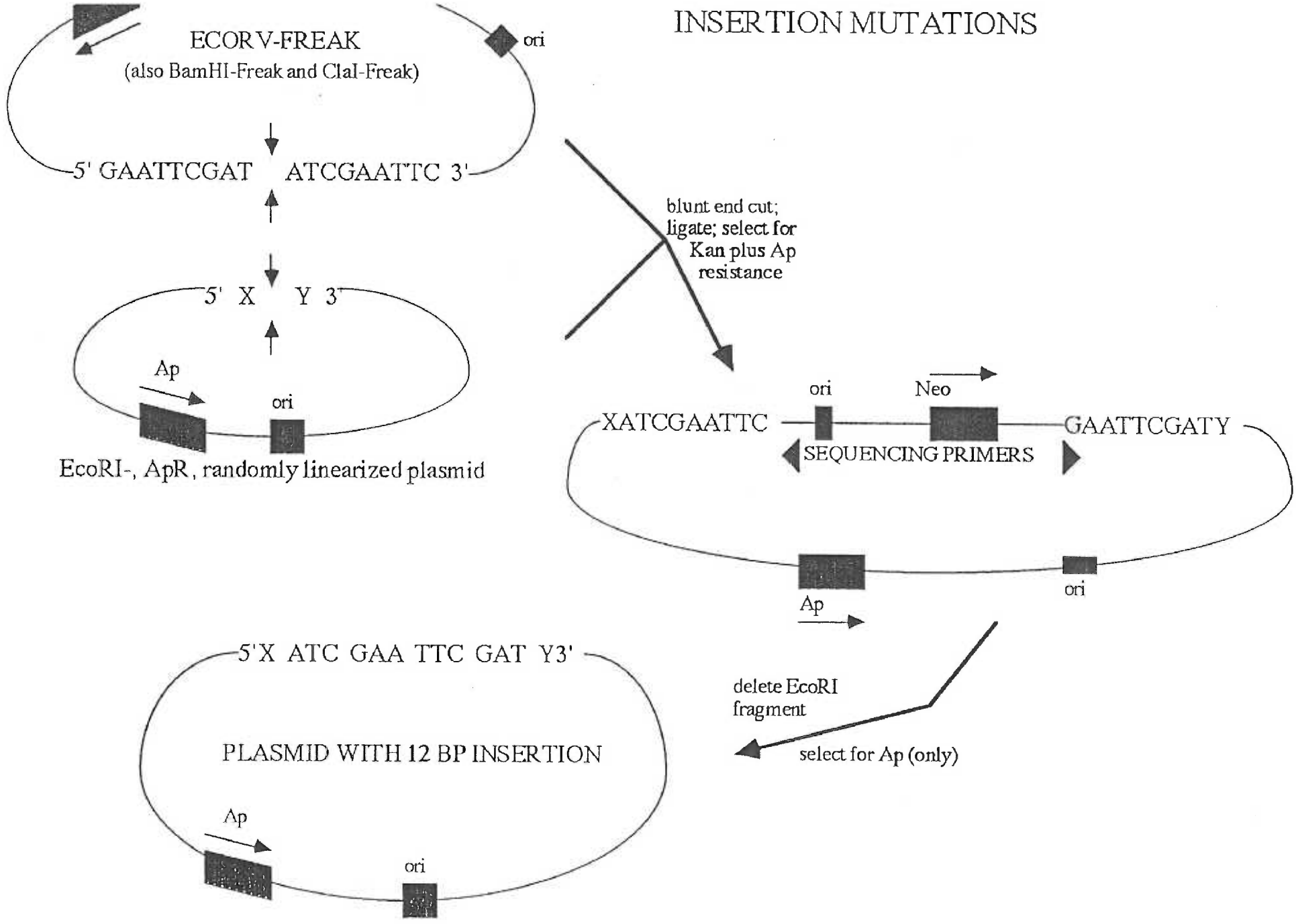

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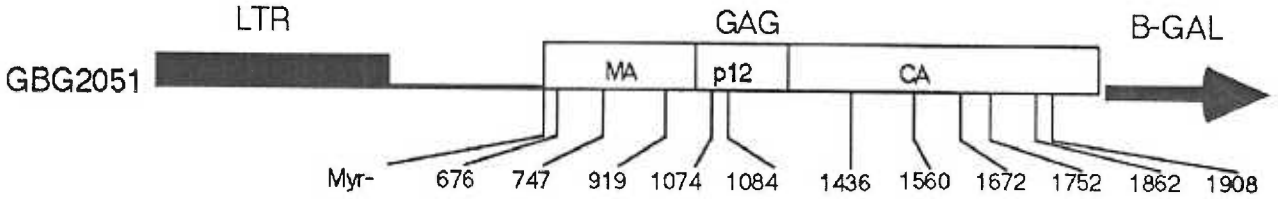
Figure 1A

MOLONEY MURINE LEUKEMIA VIRUS GAG MUTANTS



GAG MUTATIONS

GBG myr-	AUG GCC CAG ACU GUU ACC ACU CCC UUA AGU
	M **G>A** Q T V T T P L S
GBG 12in676	AAA GAU GUC GAU GAA UUC AUC GAG CGG AUC
	K D V **D E F I** E R I
GBG 12in747	GCA GAA UGG AUC GAA UUC GAU CCA ACC UUU
	A E W **I E F D** P T F
GBG 12in919	CCC UUU GUA UCG AAU UCG AUA CAC CCU AAG
	P F V **S N S I** H P K
GBG 12in1074	AGU GGG GGG AUC GAA UUC GAU CCG CUC AUC
	S G G **I E F D** P L I
GBG 12in1084	CUC AUC GAU GAA UUC AUC GAC CUA CUU ACA
	L I D **E F I D** L L T
GBG 12in1436	UGU CAG CAG AUC GAA UUC GAU CUG UUG GGG
	C Q Q **I E F D** L L G
GBG 12in 1560	UUU CCC CUC GAU GAA UUC AUC GAG CGC CCA
	F P L **D E F I** E R P
GBG 12in1672	ACU AAU UUG GAU CGA AUU CGA UCC AAG GUA
	T N L **D R I R A>S** K V
GBG 12in1752	UAU CGC AGG UAU CGA AUU CGA UAC ACU CCU
	Y R R **Y R I R** Y T P
GBG 12in1862	GAA GAU UUA UCG AAU UCG AUA AAA AAC AAG
	E D L **S N S I** K N K
GBG 12in1908	GAA AAG AUC CGA AUU CGG AUC UUU AAU AAA
	E K I **R I R I** F N K

Figure 2. Sucrose density gradient fractionation of supernatant material.

Supernatant material from cells expressing the indicated constructs was fractionated by sucrose density gradient centrifugation (20-50% gradients) as described in the Materials and Methods. Fractions of 0.5 ml were collected from the bottom of the gradients, and fraction densities, B-gal activities, and reverse transcriptase activities were determined. In each gradient, the bottom of the gradient is at the left, and B-gal activities (units per fraction on each left vertical axis) are depicted by open boxes, while reverse transcriptase activities (cpm per 10 microliters on each right vertical axis) are depicted by black diamonds. In all cases where densities were determined (A,C,D,F,G,H,I), reverse transcriptase peaks were at densities of 1.15-1.16 g/ml and light density B-gal peaks were at 1.11-1.13 g/ml.

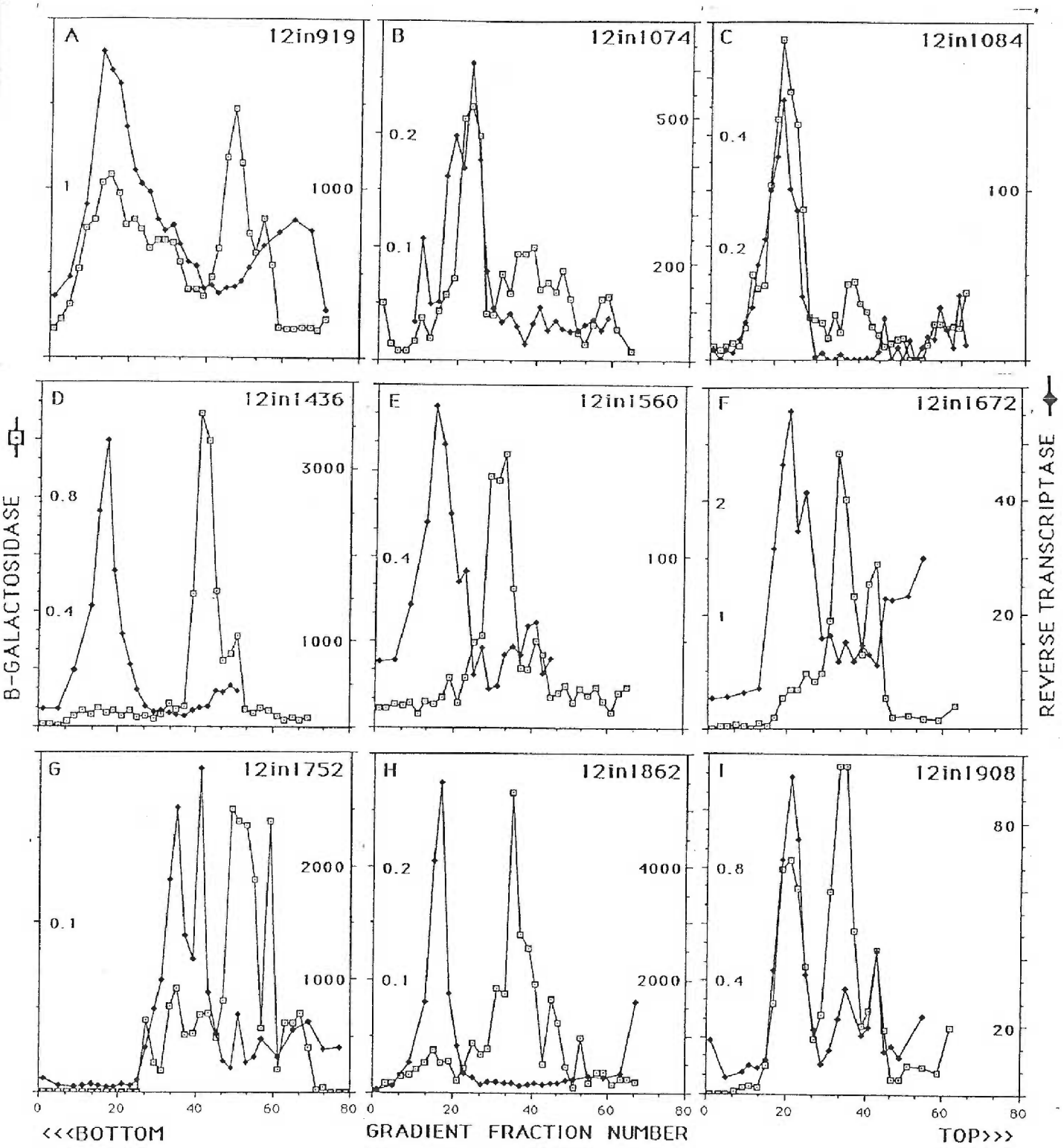


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Figure 3. Indirect immunofluorescence detection of B-gal and gag-B-gal fusion proteins in cells.

Cells grown on cover slips were fixed, permeabilized, and subjected to indirect immunofluorescence analysis, utilizing mouse antiB-gal antibody as the primary reagent, followed by rhodamine-conjugated goat antimouse antibody (see Materials and Methods). The white bar indicates 20 microns. All photographs are of proteins expressed in Psi2 cells. (a) BAG; (b) GBG myr-; (c) GBG 12in747; (d) GBG 12in919.

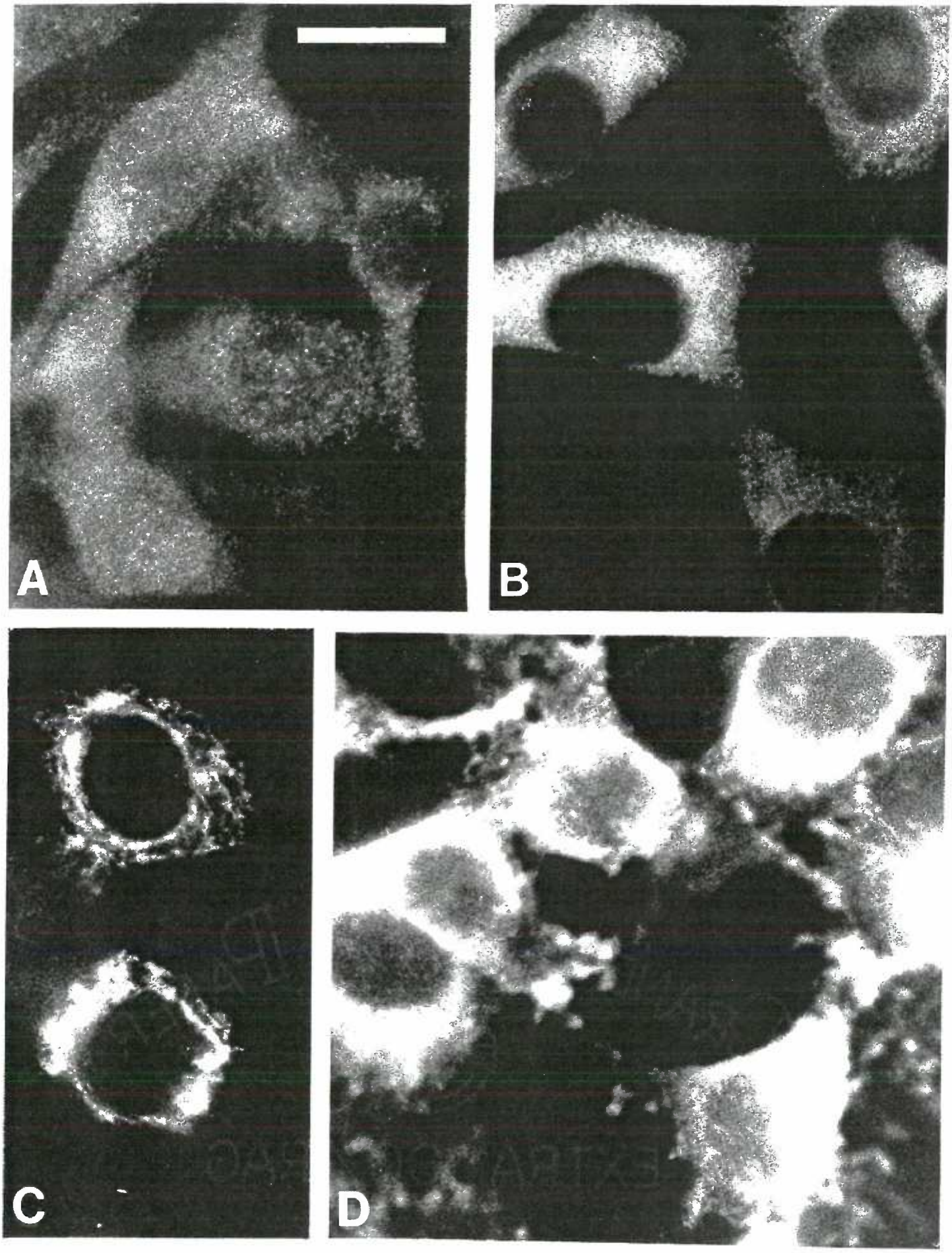

A
B
C
D

Figure 4. Effect of monensin on release of gag and envelope proteins from cells. Matched 10 cm plates of Psi2 cells at 50% (lanes a,b,g,h), 75% (lanes c,d,i,j) and 100% confluence (lanes e,f,k,l) were mock treated (lanes a,c,e,g,i,k) or pretreated with 25 micromolar monensin for 3 h, washed, then incubated 6 h with monensin (lanes b,d,f,h,j,l). Following treatments, cell lysates were collected as described in the Materials and Methods. Virus particles in supernatants were collected by filtration through a 0.45 micron pore size filter, followed by centrifugation at 4 degrees C for 30 min at 274,000 x (SW41 rotor at 40,000 rpm) through a 2 ml cushion of 20% sucrose in PBS. Supernatant samples (lanes a-f; corresponding to 50% of the total sample) and cell samples (lanes g-l; corresponding to 10% of the total cell sample) were subjected to SDS-polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose filter. M-MuLV proteins were detected immunologically with anti-viral protein antibodies, followed by secondary alkaline phosphatase-conjugated antibodies and detection of alkaline phosphatase activity. Three successive rounds of protein detection were employed using differing primary antibodies in the following order: mouse anti-p12gag monoclonal, mouse anti-p30gag monoclonal, and goat antiFeLV gp71 polyclonal. Viral Pr80env, gp70env, Pr65gag, p30gag (capsid), and p12gag proteins are as indicated. A 27 kd protein detected in supernatant samples with the anti-p12gag antibody is also indicated. Black dots additionally show minor 28 and 25 kd gag related proteins of unknown origin which are reduced in abundance in treated cells (25 kd protein) or supernatants (28 kd protein). Supernatant and cell samples on filters were processed separately for immunodetection.

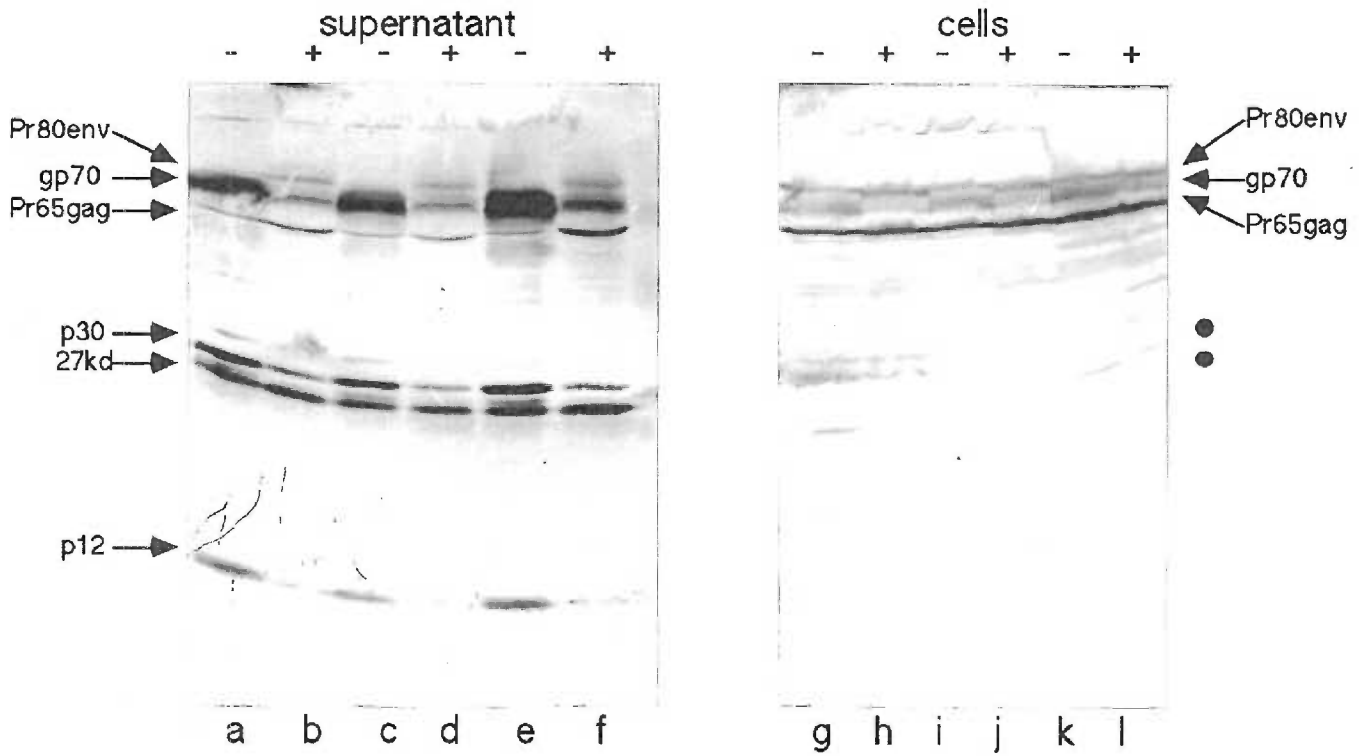
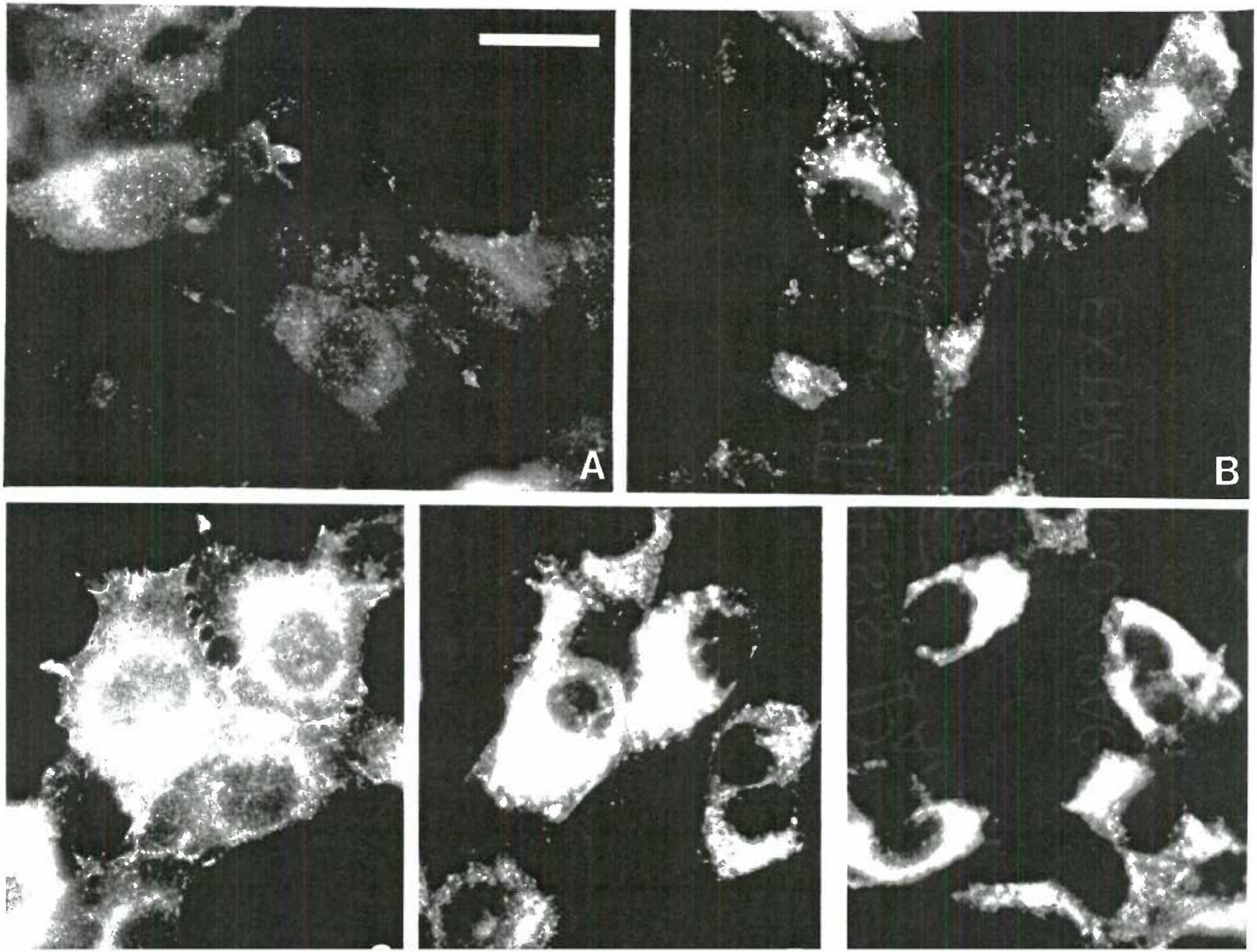

supernatant
cells
- + - + - +
- + - + - +
Pr80env
gp70
Pr65gag
p30
27kd
p12
Pr80env
gp70
Pr65gag
a b c d e f
g h i j k l

Figure 5. Immunofluorescence detection of gag and gag-B-gal fusion proteins in monensi treated cells.

Psi2 cells (A,B) or Psi2 cells expressing the gag-B-gal fusion protein from the GBG 2051 construct (C,D,E) were grown on cover slips and mock treated (A,C) or treated 4 h with 1 micromolar (E) or 25 micromolar (B,D) monensin. Following treatment, cells were fixed, permeabilized, and subjected to indirect immunofluorescence analysis as described in the Materials and Methods. In panels A and B, gag proteins were detected using mouse anti-p12 gag monoclonal antibody and in panels C-E, gag-B-gal fusion proteins were detected using mouse anti-B-gal antibody. In each case, visualization of localized proteins was accomplished using a secondary, rhodamine-conjugated goat anti-mouse antibody. The white bar indicates 20 microns.


A
B

MANUSCRIPT #2

ASSEMBLY AND COMPOSITION OF INTRACELLULAR PARTICLES FORMED BY MOLONEY MURINE LEUKEMIA VIRUS

Mark Hansen, Laura Jelinek, Russell S. Jones, Jenny Stegeman-Olsen and Eric Barklis*

Running title: Assembly of M-MuLV Intracellular particles.

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ASSEMBLY AND COMPOSITION OF INTRACELLULAR PARTICLES FORMED BY MOLONEY MURINE LEUKEMIA VIRUS

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ABSTRACT

Assembly of type C retroviruses such as Moloney murine leukemia virus (M-MuLV) ordinarily occurs at the plasma membranes of infected cells, and absolutely requires the particle core precursor protein, Pr65*gag*. Previously we have shown that Pr65*gag* is membrane associated, and that at least a portion of intracellular Pr65*gag* protein appears to be routed to the plasma membrane by a vesicular transport pathway. Here we show that intracellular particle formation can occur in M-MuLV-infected cells. M-MuLV immature particles were observed by electron microscopy (EM) budding into and within rough endoplasmic reticulum (RER), Golgi and vacuolar compartments. Biochemical fractionation studies indicated that intracellular Pr65*gag* was present in non-ionic detergent-resistant complexes of greater than 150 S. Additionally, viral RNA and pol functions appeared to be associated with intracellular particles, as were *gag*-B-galactosidase fusion proteins which have the capacity to be incorporated into virions. Immature intracellular particles in post-nuclear lysates could be proteolytically processed in vitro to mature forms, while extracellular immature M-MuLV particles remained immature as long as 10 h during incubations. The occurence of M-MuLV-derived intracellular particles demonstrates that Pr65*gag* can associate with intracellular membranes, and indicates that if a plasma membrane Pr65*gag* receptor exists, it also can be found in other membrane compartments.

These results support the hypothesis that intracellular particles may serve as a virus reservior during in vivo infections.

INTRODUCTION

In order to assemble an infectious retrovirus particle, several components must colocalize and assemble in an ordered manner. The major viral structural protein (*gag*) is central to the assembly process (40). The *gag* protein is co-translationally myristylated at its amino terminus (17), and acylation of the matrix domain is required for membrane association and assembly (30). There appear to be five to ten thousand precursor *gag* molecules in each virus particle (40), which oligomerize to form the viral core. In the case of Moloney murine leukemia virus (M-MuLV) and other retroviruses, expression of the *gag* protein alone produces non-infectious virus particles (15, 21, 37). The M-MuLV *gag* protein is synthesized as a 65 kilodalton precursor polypeptide, (Pr65*gag*), which is cleaved proteolytically into the four mature *gag* proteins: matrix (MA), p12, capsid (CA), and nucleocapsid (NC). The protease responsible for this cleavage is encoded by the virus, and the processing of Pr65*gag* is required for infectivity: M-MuLV protease (PR) mutants assemble to produce non-infectious virus particles that bud in an apparently norma fashion, and contain strictly Pr65*gag* (19).

In addition to *gag*, three other components normally are assembled into infectious M-MuLV virions, and each may be directed by the Pr65*gag* protein. The matrix domain of *gag* has been postulated to associate with the transmembrane envelope (*env*) protein, which travels to the plasma membrane by vesicular transport (2, 40). The M-MuLV envelope protein complex is synthesized as a precursor (Pr80*env*) which is cleaved to form a receptor binding (RB) domain (gp70) associated with a transmembrane (TM) anchor domain (p15E). Assembly of infectious virions also mandates inclusion of the retroviral genome, consisting of two strands of RNA which may be bound by the carboxy-terminal *gag* nucleocapsid domain (14). The other components are the products of the *pol* gene

and include protease (PR), reverse transcriptase (RT), and integrase (IN) activities. The *pol* proteins depend on *gag* protein domains during assembly as *pol*, and *pol* proteins appear to be incorporated into virions initially as *gag-pol* fusion proteins.

Assembly of type C retroviruses, such as M-MuLV, is thought to occur at the plasma membrane, following the cytoplasmic transport of *gag* precursor monomers (2). Electron micrographs have shown assembling and budding virus particles at the plasma membrane (36, 40, 41), and assembly of temperature-sensitive (ts) mutants can occur at the plasma membrane within minutes of shifting to the permissive temperature (41). This process is distinct from assembly of D type retroviruses, where virus cores are formed intra-cytoplasmically and are delivered to the plasma membrane (32-34). However, in either case, preferential assembly at or association with the plasma membrane appears essential, and could be mediated either by an inhibition of assembly at internal membranes, or specific binding to a plasma membrane receptor. Precedent for the latter mechanism has been provided by characterization of a membrane receptor specific for the myristylated *src* protein (31).

During our investigations on M-MuLV *gag*-B-galactosidase (*gag* -B-gal) fusion proteins, we observed fusion proteins that were released from cells in both viral and non-viral forms, but were surprised to observe fusion proteins which localized to intracellular membranes (18), reminiscent of mutant secretory proteins trapped in the vesicular transport pathway (12, 35). That some percentage of *gag* proteins might be transported to the cell surface via vesicles was suggested by monensin inhibition of M-MuLV particle assembly (16). Consistent with these results were electron microscope (EM) observations of intracisternal particles formed during occasional avian, murine and human retrovirus

infections (40). In our efforts to corroborate previous ultrastructural studies, we examined several cell types expressing M-MuLV and have observed virus particles within intracellular membrane compartments. Biochemical analyses support our EM studies, showing that intracellular particles are not endogenous intracisternal type A particles (IAPs), and indicating that a proportion of the intracellular M-MuLV gag precursor protein is present in a detergent resistant particle form. These intracellular MuLV particles appear to contain both viral RNA and *pol* gene products. Our observations have specific implications with regard to retrovirus infections and *gag* protein transport and assembly mechanisms.

MATERIALS AND METHODS

Cell culture. NIH 3T3, Psi2 (23) and PA317 (25) cells were grown as described previously (1). 3T3 and Psi2 cells expressing recombinant retrovirus constructs were obtained by infection and selection (18). The TR291F cell line, expressing a M-MuLV protease mutant, was the kind gift of Alan Rein (19). NIH 3T3 cells expressing infectious M-MuLV were derived by chronic infection with M-MuLV derived from pMOV9 or Sup1 (29).

Viral constructs. BAG, a retroviral vector which expresses neomycin phosphotransferase and B-galactosidase proteins, was the kind gift of Connie Cepko (6). GBG1560 and GBG2051 are retroviral vectors which express *gag*-B-galactosidase (GBG) fusion proteins which are poorly (GBG1560) or efficiently (GBG2051) incorporated into retrovirus particles (18). MP10 is a neomycin transducing retrovirus described previously (1).

Immunofluorescence. Indirect immunofluorescence of M-MuLV *gag* protein was performed by standard methods (18). The primary antibody was a tissue culture supernatant of rat hybridoma cells R161 (kindly provided by Bruce Chesebro; 7) used at a 1 to 3 dilution for detection of M-MuLV matrix p15*gag* protein. The secondary antibody was rhodamine conjugated anti-mouse IgG antibody (TAGO) used at a 1:100 dilution. After the final DMEM/Calf (Dulbecco's Modified Eagles Media supplemented with 10% calf sera, penicillin, streptomycin, and Hepes buffer solution(Gibco BRL)) washes, coverslips were washed three times for 5 minutes each in phosphate buffered saline (PBS) and mounted in 50% glycerol in PBS. Cells were viewed with a standard rhodamine filter.

Electron Microscopy. Confluent 10 cm plates of cells were prepared for analysis by decanting the supernatant and fixing the cells for 5 min at 25 degrees C in a fixative of 1.5% glutaraldehyde plus 1.5% paraformaldehyde in 100 mM sodium cacodylate buffer, pH 7.4. The fixative was decanted and fresh fixative was added and the cells were maintained at 4 degrees C for 1 hr. Cells then were scraped off the petri dishes with a rubber policeman and pelleted. The fixative was removed and cells were gently resuspended in 0.1 M Na cacodylate buffer, pH 7.4, for 25 min at 25 degrees C, after which cells were pelleted and then resuspended in 6% bovine serum albumin (BSA) in 0.1 M Na cacodylate buffer, pH 7.4, for 5 min. Cells were pelleted and all but 4 mm of overlaying buffer was removed. Fixative was carefully layered above the BSA/buffer solution and the sample was left overnight at 4 degrees C. This crosslinks the BSA and gelatinizes the sample so that it is removed from the centrifuge tube as a solid block that is sectioned into 2 mm cubes for processing. The tissue was washed in 0.1 M Na cacodylate buffer pH 7.4 for 25 min at 2 degrees C, and then post-fixed in 2% OsO_4 for 90 min at 25 degrees C. The samples wer dehydrated through graded ethanols, infiltrated with toluene, and infiltrated and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined on a Philips EM 301 transmission electron microscope (Phillips Electronic Instruments, Inc.). In order to tabulate intracellular particle localization, 22 Psi2 and 22 M-MuLV-infected 3T3 cells were extensively examined for virus particle content. Cells were chosen solely by the criterion that they lay fully within one grid sector. Intracellular particles were distinguished as to whether they were within vacuoles, vesicles, RER or golgi. In each compartment, the number of budding virions was enumerated.

Subcellular fractionation. Typically, three 10 cm plates of confluent cells were washed twice, pelleted in PBS, and resuspended in 1 ml of dounce buffer (20 mM Hepes pH 7.4, 100mM KCl, 85 mM sucrose, 100 micromolar EGTA). The cells were lysed by douncing 200 times in a 2 ml Wheaton dounce using the type A (tight) pestle. Whole cell

lysates were spun for 5 min at 5,000 x g (8,500 rpm on an Eppendorf microfuge) at 4 degrees C. Post-nuclear supernatant (PNS) was collected and either treated or not treated with Triton X-100 to a final concentration of 0.5%. Treated or untreated PNS was spun at 201,000 x g (55,000 rpm on a Beckman TLS-55 rotor) for 15 min at 4 degrees C. Second supernatants (S2) and second pellets (P2) were resuspended with either electrophoresis sample buffer or TSE (10 mM Tris HCl pH 7.4, 100 mM NaCl, 1mM EDTA) depending upon the experiment. In experiments testing the effects of salt on fractionation patterns, 50(mM NaCl was substituted for Triton X-100, giving a final NaCl concentration of 600 mM in these samples. Alternatively, Triton X-100 P2 pellets were resuspended in dounce buffer, resuspended by douncing an additional 200 times, mock treated or treated with 500 mM NaCl as above, and recentrifuged to obtain third supernatants (S3) and pellets (P3) for analysis as above. For proteolysis time course studies of Pr65*gag*, Psi2 PNS prepared as described above but also containing protease inhibitors (0.1 mM phenylmethylsulfonylfluoride {PMSF}, 5 micrograms/ml leupeptin, 2.5micrograms/ml pepstatin A, 2 micrograms/ml benzamidine), or media supernatants from Psi2 cells, were incubated at 37 degrees C for the times indicated prior to electrophoresis. As a control for intracellular proteolysis experiments, PNS from the M-MuLV protease deficient cell line TR291F (19) was employed but, in this case, protease inhibitors were omitted.

Concentration of virus particles. Supernatants collected from cells grown to confluency on 15 cm plates were layered on sucrose step gradients consisting of 3.5 ml 20% sucrose and 2.5 ml 60% sucrose in TSE. Samples were centrifuged at 83,000 x g (25,000 rpm using an SW28 rotor) for 60 min at 4 degrees C. The 20/60% interface from (tubes collected in a total volume of 6 ml was diluted by addition of 5 ml TSE and layered onto a 2 ml 60% sucrose cushion (in TSE) and centrifuged at 151,000 x g (SW41 at 35,00(rpm) at 4 degrees C for 75 min. The final interface was collected in 1 ml and frozen at -80 degrees C in aliquots.

Enzymatic assays. Protein quantification was done by the method of Bradford (4) using Bio-Rad reagent and the microassay procedure. B-gal assays were performed by the method of Norton and Coffin (26). Galactose transferase assays were a modified version of Bretz et al. (5), while cytochrome oxidase assays were performed by the method of Omura et al. (27).

Immunoblotting. Samples for immunoblot analysis were prepared in elctrophoresis sample buffer (without DTT) and applied to 7.5% or 10% Laemmli SDS-PAGE protein gel (20) and run at 35 mA. Proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) on a Hoefer electroblotter at 4 degrees C and 0.15 A overnight, after which membranes were blocked at 25 degrees C in 3% gelatin (Bio-Rad) in TBST (20 mM Tris hydrochloride pH 7.6, 150 mM NaCl, 0.05% Tween 20 (Bio-Rad)) prior to antibody binding steps. Primary antibody treatments for 30 min at 25 degrees in 1% gelatin in TBST were with a goat anti-FeLV gp71 antibody (1:1,500 dilution); tissue culture supernatants of rat hybridoma cells R548 and R187 (from B. Chesebro) used at 1:4 dilutions for immunodetection of p12*gag* and p30*gag* proteins; or a rabbit anti-human placental alkaline phosphatase (PLAP) antibody (DAKO), at a 1:1000 dilution (22). After three ten min TBST washes, immunodetection was performed with a 1:100 dilution of alkaline phosphatase anti-immunoglobulin G conjugates (Promega anti-mouse; Boehringer Manheim Biochemicals anti-goat and rabbit). After the secondary antibody binding for 30 min at 25 degrees C in 1% gelatin in TBST, the membranes were washed three times in TBST for ten min each, and then incubated in 20 ml of a color reaction solution of 0.33 mg Nitro Blue Tetrazolium, 0.17 mg 5-bromo-4-chloro-3 indolyl phosphate, 100 mM Tris hydrochloride pH 9.5, 100 mM NaCl, and 5 mM MgCl2 until bands appeared.

RNase Protection. Total cellular RNA was isolated by collecting cells in 1 ml per 10 cm plate of 6M guanidium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% Sarkosyl and 100 mM B-mercaptoethanol and pelleting through a 2 ml 6.2 M cesium chloride plus 100 mM EDTA pH 7 per 3 ml lysate for 18 hours at 115,000 x g (35,000 rpm on an SW 50.1 rotor; 8). Pellets were washed with 70% ethanol, dried, and resuspended in 10 mM Tris pH 7.4 and 1 mM EDTA. Membrane associated RNA was isolated from cell lysates (PNS which were untreated or treated with 0.5% Triton X-100 . Samples were centrifuged at 201,000 x g for 15 min at 4 degrees C. The pellets were rinsed in 10 mM Tris 7.4, 100 mM NaCl, 1mM EDTA, suspended in 200 microliters 10 mM Tris pH 7.4, 25 mM EDTA 50 mM NaCl containing 10 micrograms yeast tRNA and extracted twice with phenol/chloroform 1:1, twice with chloroform, and ethanol precipitated. For RNase protection assays, 10 microgram samples of tRNA, membrane associated RNA or total cellular RNA were precipitated and dried just prior to annealing reactions.

Riboprobe transcription templates were prepared in the following manner. A 250 bp Rsa I fragment of ribosomal protein L3 (rpL3; 28) was inserted into the Sma I site of bluescript -SK. This construct was linearized with Bam HI and transcribed from the T7 promoter resulting in a run-off transcript approxiamately 250 nt long. A neomycin (neo) probe was constructed by cloning a 542 bp Pst I - Hind 3 fragment from the neo gene into the bluescript -KS cloning vector. This plasmid was linearized at the Bgl 2 site and transcribed from the T7 promoter resulting in a run-off transcript approximately 260 nt lon

Probe transcription reactions contained 1 microgram of linearized plasmid, 100 microcuries ^{32}P-rGTP (specific activity of 600 Ci/mMol), 500 micromolar of each of the other ribonucleotides, 40 mM Tris pH 7.9, 10 mM NaCl, 6 mM MgCl2, 2 mM spermidin and 1 mM DTT and RNasIn (40u; Promega). Reaction incubations were for 1 hr at 37 degrees C and were terminated by phenol/chloroform extraction after the addition of 10

micrograms tRNA, 120u RNasIn (Promega), and Na Acetate pH 7.6 to a final concentration of 300 mM. Ethanol precipitated probes then were purified away from free nucleotides and partial synthesis products by denaturing DNA acrylamide gel electrophoresis. After elution from the gel by a 50 degree C incubation with 1 M ammonium acetate pH 7.4, 0.1% SDS, and 1 mM EDTA, probes were extracted twice, precipitated and suspended in 20 microliters water.

Annealing reactions combined 1-5 x 10^5 cpm of probe with the precipitated RNA samples in 80% formamide, 400 mM NaCl and 40 mM Pipes pH 6.4. These were denatured at 85 degrees C for 5 min and incubated at 37 degrees C overnight. Unbound probe was eliminated by incubation at 30 degrees C for 30 min in 350 uL of 300 mM NaC 10 mM Tris pH 7.5, 5 mM EDTA, 15 ug RNase A, and 1.4 ug RNase T1. This reaction was terminated by incubation at 37 degrees C for 15 min with 50 ug Proteinase K (Boehringer Manheim Biochemicals) and 0.1% SDS. The protected probe was extracted after addition of 20 ug tRNA and ethanol precipitated. The pellet was rinsed, dried and suspended in 5 uL sequence loading dye. Samples were denatured and run on a 6% acrylamide sequencing gel that was exposed with a screen for 1-7 days.

RESULTS

Morphological characteristics of intracellular particles.

To study the mechanism of M-MuLV Pr65*gag* transport and assembly, we initially used electron microscopy (EM) to visualize the effects of monensin treatment on virus producing and non-virus producing cells. Surprisingly, virus producing cells showed intracellular particles, even in the absence of monensin (Figure 1). Since these observations were unexpected for type C retrovirus assembly, we verified them by analysis of Psi2 cells, M-MuLV-infected 3T3 cells and Psi2 cells expressing a retroviral vector. To exclude the possibility that intracellular particles represented endogenous type A particles, we analyzed two lines of NIH 3T3 fibroblasts in precisely the same manner and did not observe any virus particles in any of the fields from all the grids examined. In contrast, particles from virus producing cells (Figure 1) were roughly 120 nm in diameter and appeared to be immature, based on their electron density at the particle rim, and the lack of a centrally located dense core. Many of the virions appeared within the RER, (Figures 1A, 1B) but there also were those that appeared to be within vesicles. Interestingly, some particles appeared to be assembling into intracellular membranes, (note the uppermost arrow in Figure 1A). As an attempt to quantify our EM observations, we tabulated the numbers of virus particles at the plasma membrane or within intracellular compartments. Table 1 shows that 70 and 90% of the particles in 3T3-infected and Psi2 virus producer cell were intracellular: this is a maximum estimate, since released particles which do not remain cell associated are not counted. There was a significant difference between the two cell lines analyzed with respect to the location of intracellular virions. Particles were localized predominantly to RER/golgi/vesicle compartments of Psi2 cells, while the majority of particles in M-MuLV-infected 3T3 cells were located within vacuoles. The reason for this

difference is unclear, but could be caused by a general impairment of vesicular transport function in Psi2 cells (M. Yatvin, pers. comm.).

To extend our observations, we also examined Psi2 cells by immunofluorescence light microscopy using an antibody against the amino terminal (p15) matrix domain of Pr65*gag* (Figure 2). While this anti-p15 antibody (7) gave no signal on uninfected 3T3 cells (Figure 2F), panels 2B and D show that the cells exhibited fluorescence at the periphery of the cell and along cell projections, possibly indicative of assembly and release of viral particles. Additionally, cells showed intense punctate fluorescence (Figure 2B, D surrounding the nuclei in the vicinity of the RER, consistent with the presence of intracellular particles within RER and/or golgi compartments. We estimate the smallest resolvable spots of fluorescence to be 100-500 nm in diameter, and the smallest of these may represent individual virus particles. The distinct punctate pattern demonstrated by the p15*gag* antigen supports the notion that a significant proportion of intracellular gag protein is not in monomeric form (see below).

Detergent resistance of intracellular particles.

Having observed intracellular virus particles, we wanted to characterize their status of assembly and determine which components were within the particles. Since *gag* protein of type D intracellular particles are not solubilized by 0.5% Triton X-100 (32), we reasoned that we might be able to separate fully formed type C cores from monomeric Pr65*gag* protein by subcellular fractionation in the presence of Triton X-100. Such an approach could serve as biochemical support of our EM observations if Pr65*gag* were enriched in post-nuclear Triton-resistant pellets. Figure 3 shows subcellular fractionation results with three cell lines and the effects of treatment of post-nuclear supernatants (PNS) with Triton X-100 prior to separating crude membranes (P2) from cytosol (S2). Not surprisingly,

3T3 cell lysates showed little or no reactivity to an anti-p12*gag* (7) antibody (Figure 3, lanes E-H). In contrast, Psi2 packaging cells (lanes A-D, M-T) and 3T3 cells chronically infected with M-MuLV (lanes I-L) showed three immunoreactive bands: Pr65*gag*, p12*ga* and p27, which may be a Pr65*gag* cleavage intermediate consisting of the M-MuLV matrix (p15) and p12 domains (16). In both Psi2 and M-MuLV-infected cells, unprocessed Pr65*gag* appeared to be present in greater quantities than p12*gag*, but in infected 3T3 cells, the p27 *gag* product appeared at roughly the same levels as Pr65*gag*, whereas Psi2 Pr65*ga* levels were greater than those of the putative intermediate: we have not determined the reason for this difference. Nevertheless, a consistent observation was that Pr65*gag* was membrane associated in the absense of detergent (compare lanes A versus B and I versus J and that addition of 0.5% Triton X-100 did not affect the Pr65*gag* fractionation pattern (compare lanes C versus D and K versus L). One possible explanation for this fractionation pattern is that intracellular Pr65gag may be bound in a non-ionic detergent-resistant form to cytoskeletal elements (10,11). To test this hypothesis, Psi2 cell PNS was treated with 600 mM NaCl, which reportedly eliminates cytoskeleton binding (10,11). However, while such treatment solubilized p27, it had a much smaller effect on the Pr65gag pattern (Figure 3, lanes O,P). Even when Psi2 PNS was treated sequentially with Triton X-100 and NaCl, a considerable amount of membrane-associated Pr65gag (approximately 25-50%) remained in the pellet fraction (Figure 3, lanes S,T). These results are consistent with the interpretation that at least a quarter of the intracellular Pr65*gag* protein tends to be present in a Triton X-100 resistant multimeric form. Furthermore, the appearance of a Pr180*gag-pol* band in Triton X-100 PNS pellets (evident when larger amounts of material were used; Figure 3, lanes R, T) suggests that *pol* gene products also are present in intracellular aggregates, an interpretation which is supported below.

To examine further the effects of the Triton X-100 treatment on particles and demonstrate to what extent membrane proteins were solubilized, additional studies were

performed. Figure 4 shows the effect of Triton treatment on the distribution patterns of several proteins in virus-producing cells expressing human placental alkaline phosphatase (PLAP) which were subjected to subcellular fractionation protocols. As in Figure 3, Pr65*gag* pelleted even in the presence of Triton X-100 (Figure 4, lane D versus C). However human placental alkaline phosphatase (PLAP), which localizes to the plasma membrane, and gp70*env*, the mature proteolytic product of Pr80*env*, were both predominantly in the pellet of untreated fractionation (lane B), while the addition of Triton X-100 shifted these proteins toward the supernatant fraction (lane C). Pr80*env*, which is the RER and early golgi M-MuLV *env* protein form (40), was solubilized considerably by the detergent treatment (compare lanes C and A). This experiment shows that the detergen treatment had a much greater effect on membrane proteins than on Pr65*gag*.

To confirm the effects of Triton X-100 treatment on cellular and viral proteins, we subjected two additional cell lines to subcellular fractionation with and without detergent treatment, and assayed the contents of each fraction. The two cell lines expressed different enzymatically active *gag*-B-galactosidase (GBG) fusion proteins, both of which are transported to the plasma membrane and released from cells. However, only 5-20% of the GBG1560 fusion protein is incorporated into virions, while 50-80% of the GBG2051 protein enters virus particles (18). Table 2 shows the results of our fractionations with Psi packaging cells expressing the 1560 or 2051 proteins. In agreement with our results from Figure 4, the RER NADPH cytochrome c reductase and golgi galactosyl transferase activities were solubilized 2-3 fold by detergent treatments (compare activity percentages in treated versus untreated pellets in each experiment). Also, the GBG1560 protein which assembles poorly into particles, is Triton-solubilized (Table 2, part A). In contrast, the GBG2051 protein is relatively resistant to solubilization (Table 2, part B). From Table 2 and the previous two figures, it appears that up to half of the M-MuLV intracellular Pr65*gag*

and GBG2051 proteins present were in Triton-resistant forms. These results are consisten with our EM observations.

Characterization of intracellular particles.

To examine whether viral RNA was associated with intracellular particles, we designed an experiment to ascertain whether viral RNA is enriched in pellet fractions of Triton treated PNS material. Our approach was to determine whether packageable RNA expressed from proviral retroviral vector constructs was enriched in Triton-resistant pellets of virus producing (Psi2) versus non-virus producing (3T3) post-nuclear supernatants. Tc do so, we used matched Psi2 and 3T3 cells expressing the neomycin (neo) gene from proviral constructs, and assayed the subcellular distributions of viral neo transcripts and, as a control, cellularly derived ribosomal protein L3 (rpL3; 28) transcripts by RNase protectio assays (see Materials and Methods).

Figure 5A shows the results of an RNase protection assay using the rpL3 probe to analyze total RNA, mock-treated PNS pellet (P2) RNA and Triton treated PNS pellet (Triton-P2) RNA of the Psi2 and 3T3 neo-expressing (Psi2-neo and 3T3-neo) cells. Lane shows the probe alone without RNase treatment, lane H contained total cellular RNA from a positive control cell line, and lane G is a negative (tRNA) control. As shown in lanes C and D where total cellular RNA was employed, the rpL3 transcript was expressed to similar levels in Psi2-neo and 3T3-neo cell lines. The P2 subcellular fractions, (lanes A, B) also contained similar quantities of rpL3 transcript. Similarly, when the PNS was treated with 0.5% Triton X-100 prior to centrifugation, the Triton-P2 fractions from Psi2-neo (lane E) and 3T3-neo (lane F) cells contained roughly equivalent levels of rpL3 RNA. Note that while the rpL3 RNA is present in P2 (lanes A, B) and to a lesser extent, Triton-P2 (lanes E, F) of Psi2-neo and 3T3-neo cells, these results can not be used as an absolute indicator of

the subcellular localization patterns of the rpL3 transcript: this is because we found it difficult to assess transcript levels in nuclease-rich nuclear pellet and supernatant (S2) fractions. However, since the subcellular localization of rpL3 RNA was unaffected by virus production, it served as a control for cellular RNA levels within given P2 and Triton-P2 fractions.

Our results with packageable neo RNA in 3T3 and Psi2 cells are shown in Figure 5B, where lane designations and fractions are identical to those in Figure 5A: lane I contained the neo probe alone without RNase treatment, lane H was the positive control, and lane G was the tRNA negative control. Total neo RNA levels in Psi2-neo and 3T3-neo cells are shown in lanes C and D, respectively. As shown, the levels of neo RNA were similar, but the 3T3 cells, lane D, expressed slightly more than the infected Psi2 cell, lane C. The P2 fractions (lanes A and B) both contained neo transcript, with the Psi2 cells having slightly higher levels: this suggested that the presence of virus particles might enrich packageable RNA to the P2. Such an interpretation was supported by results on RNA from Triton-P2 fractions, where Psi2 derived cells contained significantly more neo RNA in the Triton-P2 than their 3T3-neo counterparts. This difference indicates that neo transcript fractionation was dependent on the expression of the Psi2 genome and implies that viral RNA is associated with intracellular particles.

As shown in Figure 3 (lanes R,T), the gag-pol protein precursor Pr180*gagpol* appeared enriched in the Triton X-100 P2 fraction, suggesting that *gagpol* proteins become associated with intracellular *gag* protein complexes. However, as an independent attempt to determine whether *pol* gene products were incorporated into intracellular particles, we initially attempted reverse transcriptase assays, but were hampered by high background activity levels in subcellular compartments. However, we next examined the potential presence of the *pol* gene protease (PR) product in intracellular particles. For these

experiments, Psi2 PNS, containing Pr65*gag* exclusively in a Triton resistant form (Figure 3,4), was isolated and incubated at 37 degrees C for 0, 30, 60, or 120 min, to monitor protease-mediated cleavage of Pr65*gag*. As illustrated in Figure 6 (lanes A-D), during such a time course, Pr65*gag* decreased with a concommitant increase in p30*gag* levels. In contrast, Pr65gag in the PNS from the viral protease-deficient cell line TR291F did not become converted to p30 during incubations (Figure 6, panel B). These results suggested that Psi2 intracellular Pr65*gag* in its Triton resistant form is accessible to the viral protease Thus, it appeared that intracellular particles contain both viral RNA (Figure 5) and at least one *pol* gene product.

Detergent treatment of extracellular M-MuLV particles.

Because we assumed that, like type D intracellular particles (32), immature type C particles were resistant to dissociation by non-ionic detergents, we wished to verify this notion with extracellular M-MuLV particles. To do so, we isolated extracellular virus particles, incubated them in the absence or presence of detergent and separated supernatant from pellet material after centrifugation at 150,000 x g (65,000 rpm using a Beckman TLA-100 rotor) for 15 min at 4 degrees C. Figure 7 shows the results of such an experiment after electrophoresis and immunoblotting of supernatants and pellets to detect *gag* proteins. In untreated samples, both processed p30*gag* and the small amount of Pr65*gag* were confined to the pellet (lanes B versus A). In contrast, in the presence of Triton X-100 (lanes C, D, G, H) or NP-40 (lanes E, F) Pr65*gag* still pelleted (lanes D, F, H) while p30*gag* was solubilized (lanes C, E, G). Similar results were obtained with virus from M-MuLV-infected 3T3 cells and from M-MuLV-protease mutant expressing TR291F cells (19). In all experiments, Pr65*gag* pelleted in the presence of non-ionic detergent, while p30*gag* was solubilized. This implied that processed p30*gag* and Pr65*gag*

were not present in the same virus particles, or that conversion from a completely immature to completely mature particle must be very rapid after the initial cleavages occur. To distinguish these possibilities, we examined early collections of virus particles from Psi2 cells. At our earliest time points (less than 2 hours), the major portion of virus particle-associated *gag* antigen in media supernatants was present in the mature form. Significantly, particle-associated media Pr65*gag* did not become converted to the mature form over a 10 h, 37 degrees C incubation (data not shown). These results suggest that if a particle is released in the immature form, it remains so. This result contrasts with those from intracellular particles (Figure 6, lanes A-D), a difference that is discussed below.

DISCUSSION

By electron microscopy (EM) of virus producing cells, we observed M-MuLV virions assembling on intracellular membranes within infected cells. EM analysis of M-MuLV-infected and virus particle packaging Psi2 cells, but not of several different 3T3 lines, showed that particles can assemble on and bud through intracellular membranes. The results in Table 1 showed that although both Psi2 and M-MuLV infected 3T3 cells contained intracellular particles, the 3T3 infected line has a very high percentage of vacuole-associated virus rather than RER/ Golgi/ vesicle particles. While we have no data which clarifies this difference, it is possible that a slight impairment of vesicular transport processes in either of the cell lines might account for this variability. Our immunofluorescence microscopy results also suggested that M-MuLV *gag* proteins may assemble intracellularly into higher order structures. This interpretation was suggested by the punctate quality of p15 staining, rather than the haze of fluorescence expected for non-aggregated cytoplasmic antigens. We believe that these intracellular particles represent assembling structures rather than infecting virions for several reasons. Firstly, infection of cells by ecotropic retroviruses should be inhibited by receptor interference (40). Secondly, intracellular particles appeared to be immature (Figure 1), which is not consistent with infection by mature extracellular virions. Finally, several of the particles depicted in the micrographs (Figure 1) appeared to be assembling onto intracellular membranes.

Biochemical studies support the notion that M-MuLV particles were formed within Psi2 and M-MuLV infected cells. Most of the intracellular *gag* protein in these cells consisted of the precursor Pr65*gag* and, using a modification of the protocol for isolation of type D particles, we found that at least 25% of the intracellular Pr65*gag* was present in a non-ionic detergent resistant form (Figures 3,4). Similarly, a *gag*-B-galactosidase fusion protein (GBG2051) which assembles into extracellular virions (18) also remained in a

pelletable form after Triton X-100 treatment, while a control B-galactosidase fusion protein (GBG1560) and membrane marker proteins became more solubilized (Figure 4, Table 2). Interestingly, both intracellular and extracellular mature M-MuLV *gag* proteins also became solubilized on non-ionic detergent treatment (Figures 3, 7). These results with M-MuLV are in concordance with observations on mature and immature avian type C (38) and human immunodeficiency virus (Wang and Barklis, unpublished observations) particle sensitivities. Additionally, the fractionation of Pr65*gag* to Triton-pellets and p30*gag* to Triton-supernatants, coupled with the observation that particle-associated media Pr65*gag* does not become processed in a 10 h incubation (data not shown) suggests that extracellular immature particles may be protease defective (Figure 7). This result is in contrast with our observations of intracellular particles, which appear to possess PR activity (Figure 6). Such a difference may result from the absence of *pol* gene products in extracellular immature particles, which would imply that ordinarily, a very small number of *gag-pol* proteins enter virions. Alternatively, the disparity in extracellular versus intracellular particle processing of Pr65gag might be due to an as yet undetermined structure or cofactor difference between the two immature forms.

We do not know the amount of Pr65*gag* oligomerization necessary to confer resistance to Triton solubilization. However, based on the clearing rates during one centrifugation step, we estimate that the predominant intracellular form of Triton-resistant Pr65*gag* is in a complex, or complexes, of greater than 165 S. Furthermore, based on our electron micrographs (Figure 1) we assume that the intracellular particles are lipidated, although we have no direct evidence showing this. Also unclear for methodological reasons is whether the M-MuLV *env* protein is associated with intracellular particles. However, both viral RNA (Figure 5) and protease (Figure 6) appear to be associated with intracellular Pr65*gag*.

We do not claim that intracellular assembly of M-MuLV particles is the natural rout of virus assembly, yet our observations shed some light on the process. In particular, models of C type retrovirus assembly often involve a critical role for the plasma membrane possibly by virtue of a specific membrane binding interaction. Our results necessitate a qualification of such a model. In this regard, we note that intracellular particles have been observed with other type C retrovirus-infected cells (3), and that HIV particle assembly can occur in murine and insect cells (Wang and Barklis, unpublished observations; 13). Thus there is no absolute species or subcellular membrane specificity to the process: assembly of membranes conceivably could be nucleated by a specific receptor, but such a receptor is not absolutely membrane nor species specific.

What determines the incidence of intracellular versus cell surface assembly of C type viruses? By our simplest model, the determining factor may be the rate of *gag* transport. We have proposed that a high proportion of cotranslationally myristylated *gag* proteins bind to the cytoplasmic faces of intracellular membranes and travel in association with vesicles to the cell surface (16, 18). Presumably, concentration of Pr65 in two dimensions at the plasma membrane achieves a high enough accumulation of monomers for virion formation. If this is the case, then perturbation of *gag* transport should result in its increased accumulation on intracellular membranes and the formation of intracellular particles. Possibly with HIV, a protein such as *vpu* (39) may be required to counteract this effect under certain circumstances.

A functional role, if any, for internally formed particles in virus infections is unclear. At one extreme, it could be a viable and frequently used pathway for the elaboration of infectious virions. However, retrovirus assembly undeniably can occur at the plasma membrane, as evidenced in numerous EM studies (36, 38, 41). Additionally virion assembly by an internal route would require *env* protein association at the RER, as well as

transport of the enveloped particle through the lumenal compartments of the RER and Golg to the cell surface. Nevertheless, we do not consider intracellular particles to represent a complete dead end, since our studies suggest a small percentage of them may be infectious (data not shown). As suggested for HIV infections (24), intracellular particles could constitute a reservoir of virus during the progression of disease.

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Table 1. Electron Microscopic localization of virus particles.

Psi2 cells expressing the BAG retroviral vector, M-MuLV infected 3T3 cells, and 3T3 cel were prepared for electron microscopic (EM) analysis as described in Materials and Methods. For tabulation of virus particles, sectioned cells were chosen solely by the criterion that they lay fully within one grid sector. No virus particles were observed in an of the fields of 3T3 cells examined. For both Psi2 and M-MuLV-infected 3T3 cell lines, cells were completely examined for virus particle content. Total extracellular and intracellular particles were counted, and intracellular particles were distinguished as to whether they were within vacuoles or the rough endoplasmic reticulum (RER), golgi, or vesicles. In each compartment, the number of budding particles was determined, as shown. Note that EM photography (Figure 1) and tabulation of intracellular particles occured on separate occasions.

Table 1. ELECTRON MICROSCOPIC LOCALIZATION OF VIRUS PARTICLES

	Psi2 cells	3T3 infected
cells counted	22	22
total virions counted	166	287
extracellular virions	15	81
intracellular virions	146	206
in vacuoles	5	167
in RER	141	39
budding virions	16	3
at plasma membrane	10	2
intracellular	6	1
% intracellular virions	91	72

Hansen et al.
Table 1.

Table 2. Fractionation of untreated and Triton X-100 treated cell lysates. Pellets or supernatants from untreated or Triton treated PNS of Psi2 cells (2×10^7 per experiment) expressing *gag*-B-galactosidase (GBG) fusion proteins were obtained as described in the Materials and Methods. As described previously (18), the GBG1560 protein is released from cells in a low density fraction but is not efficiently incorporated into virions, while the GBG2051 protein is incorporated efficiently into virions. Pellet and supernatant fractions were assayed for total protein (in micrograms), as well as for cpm of galactosyl transferase activity (a golgi marker), and NADPH cytochrome c reductase activity (an RER marker; 1 unit is (change in Abs.) x 1000/ min), and units of B-galactosidase activity. Values shown reflect the total calculated amount in each sample.

Table 2. FRACTIONATION OF UNTREATED AND TRITON X 100 TREATED CELL LYSATES.

A. Psi2 cells expressing M-MuLV gag-B-gal fusion protein GBG1560

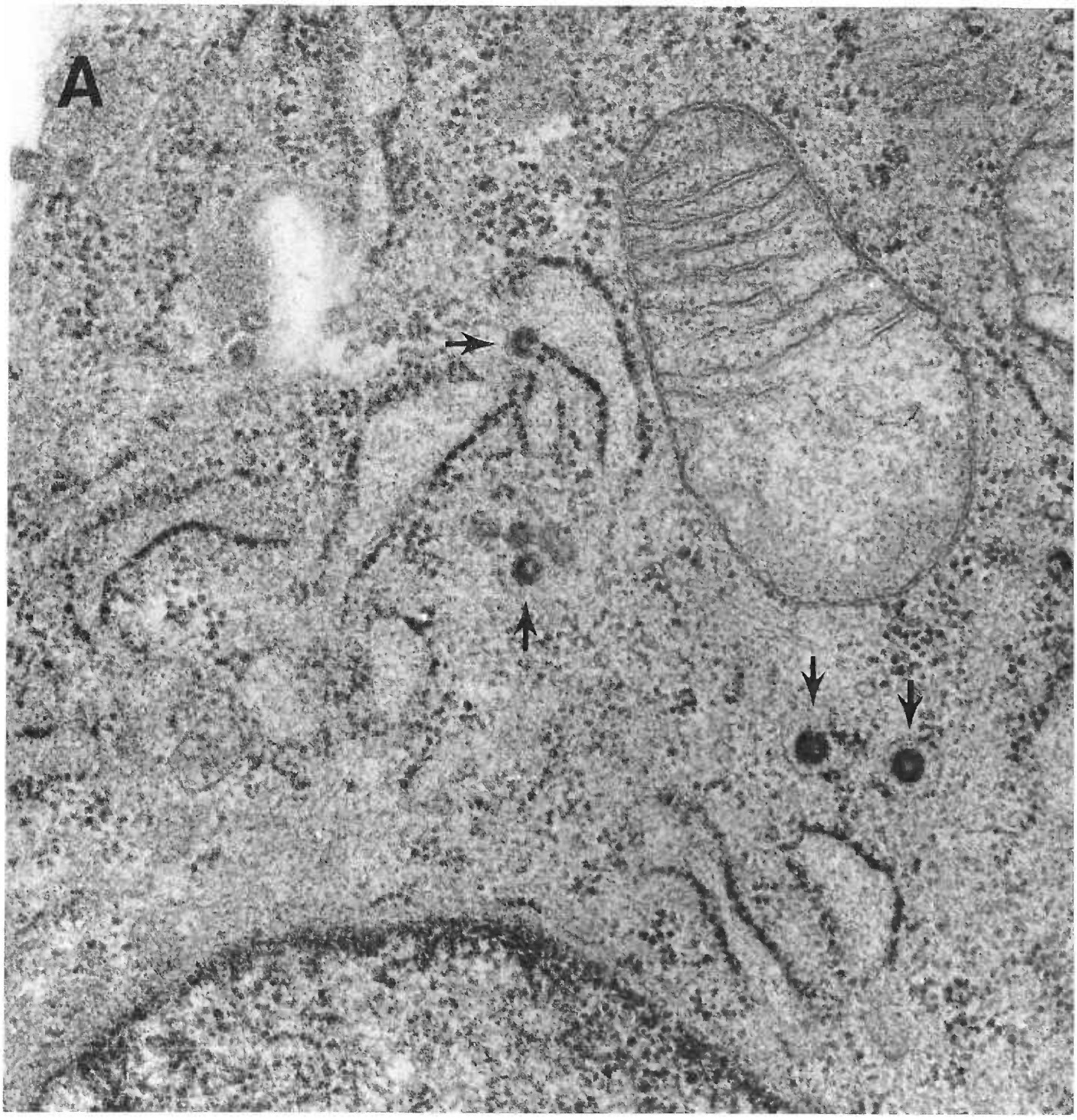
MARKER	UNTREATED CELLS			TREATED CELLS		
	pellet	super	% in pellet	pellet	super	% in pellet
total protein (ug)	270	700	28%	260	750	26%
gal transferase	2209	770	74%	1104	3806	22%
NADPH cyt. c red.	388	204	66%	138	548	20%
B-galactosidase	21.2	2.7	89%	6.2	18.6	25%

B. Psi2 cells expressing M-MuLV gag-B-gal fusion protein GBG2051

MARKER	UNTREATED CELLS			TREATED CELLS		
	pellet	super	% in pellet	pellet	super	% in pellet
total protein (ug)	270	650	29%	240	900	21%
NADPH cyt. c red.	224	142	61%	120	344	26%
B-galactosidase	12.0	5.6	68%	9.1	8.1	53%

Hansen et al., Table 2.

Figure 1. Electron microscopy showing intracellular M-MuLV particles. A. The micrograph shows a Psi2 cell with intracellular, assembled M-MuLV cores. Arrows denote particles which are assembling or within RER, Golgi, or vesicle compartments B. Large numbers of intracellular particles are shown within a Psi2 retrovirus packaging cell which expresses a neomycin vector. In each case the scale bar represents 1000 nm.


A

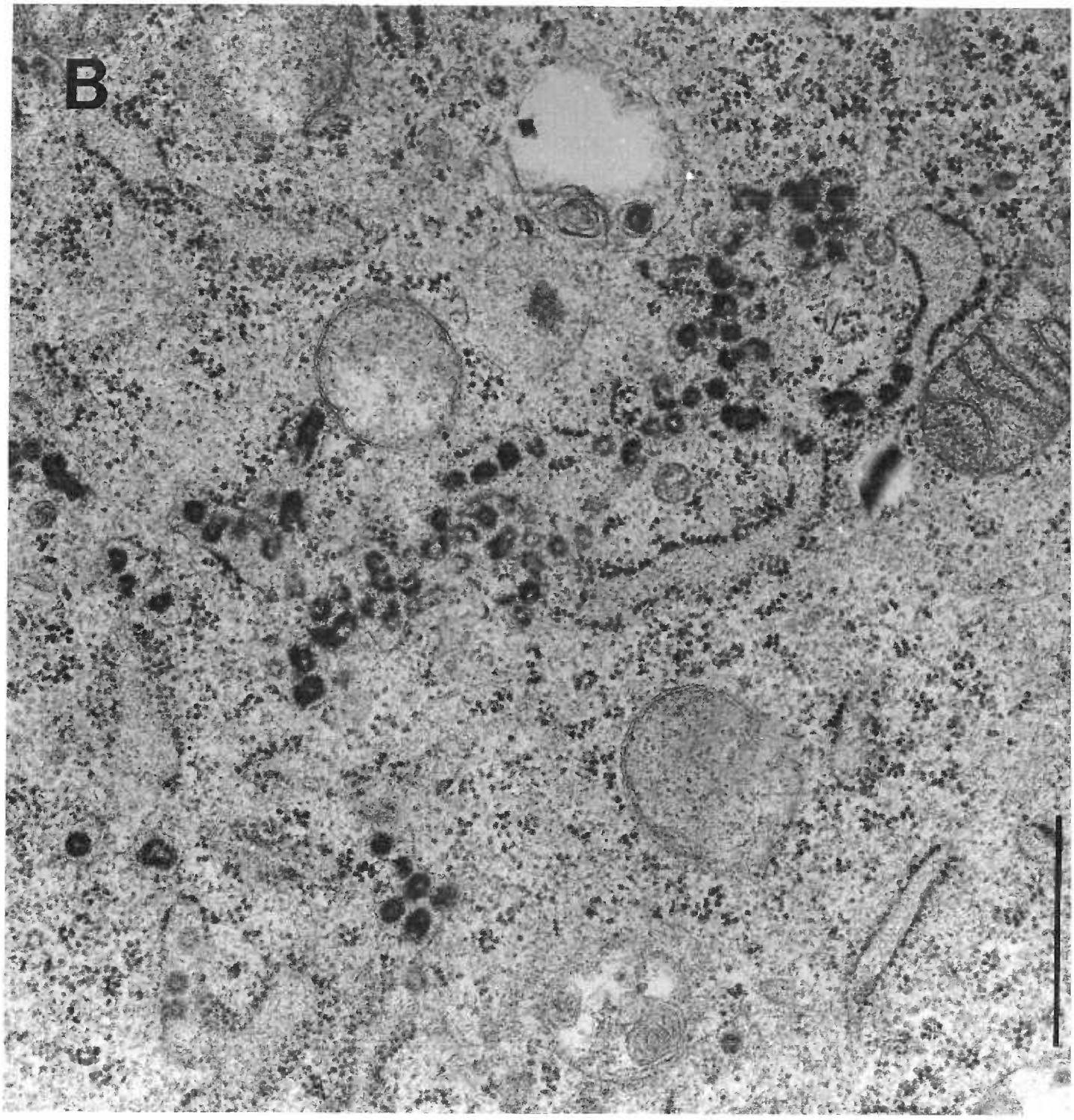
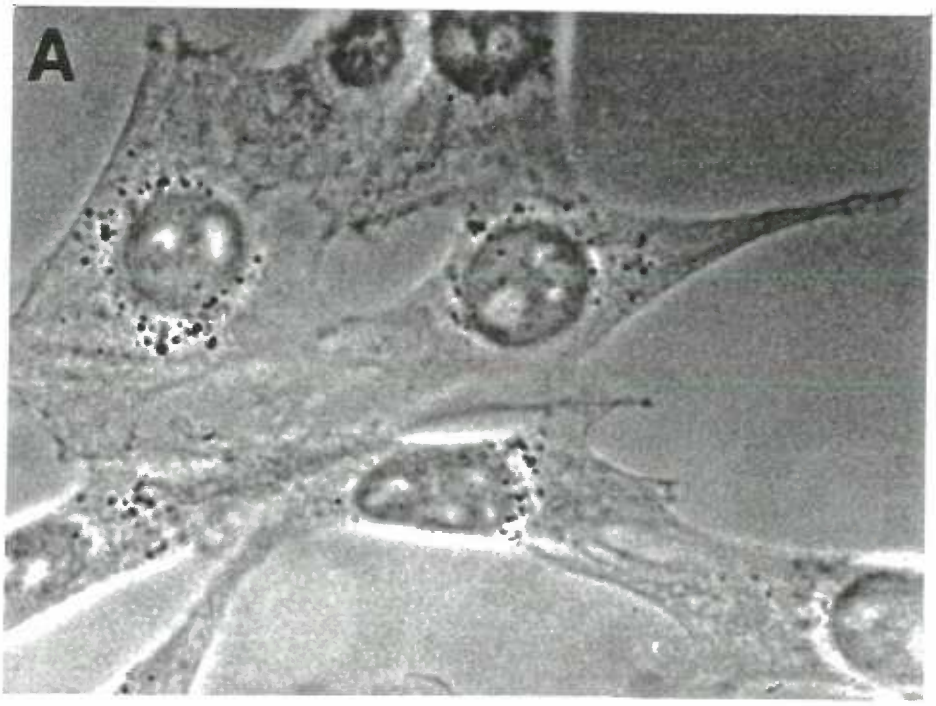
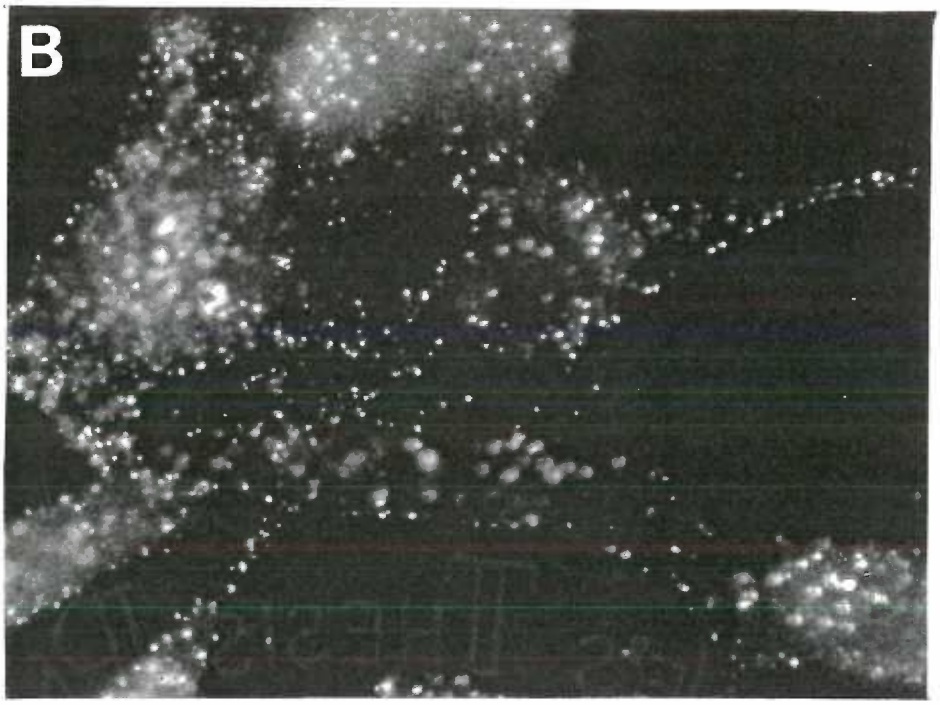

B

Figure 2. Immunofluorescent localization of M-MuLV *gag* proteins in Psi cells. Matched phase contrast (A, C, E) and anti-p15*gag* immunofluorescence (B, D, E) micrographs of Psi2 cells expressing the BAG retroviral vector (A-D) or negative control 3T3 fibroblast cells (E, F) are shown. For the negative control panel F, photography exposure times and conditions were identical to those in panels B and D. As illustrated, the M-MuLV p15*gag* antigen shows plasma membrane as well as perinuclear staining. The size bar in Panel D represents 20 microns, while we estimate the smallest resolvable immunofluorescent spots to be 100-500 nm.


A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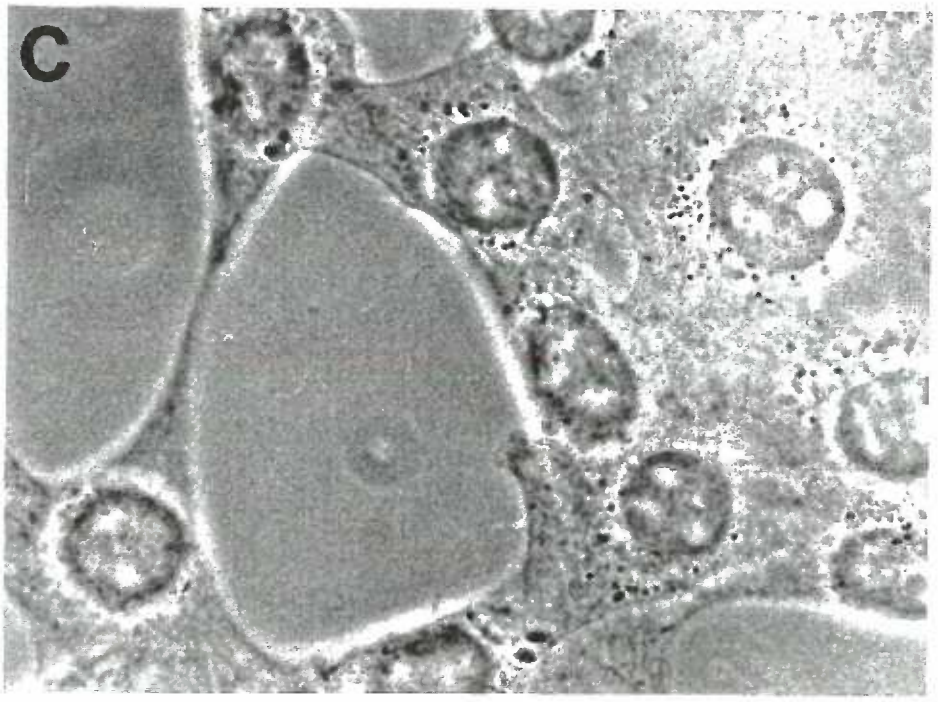
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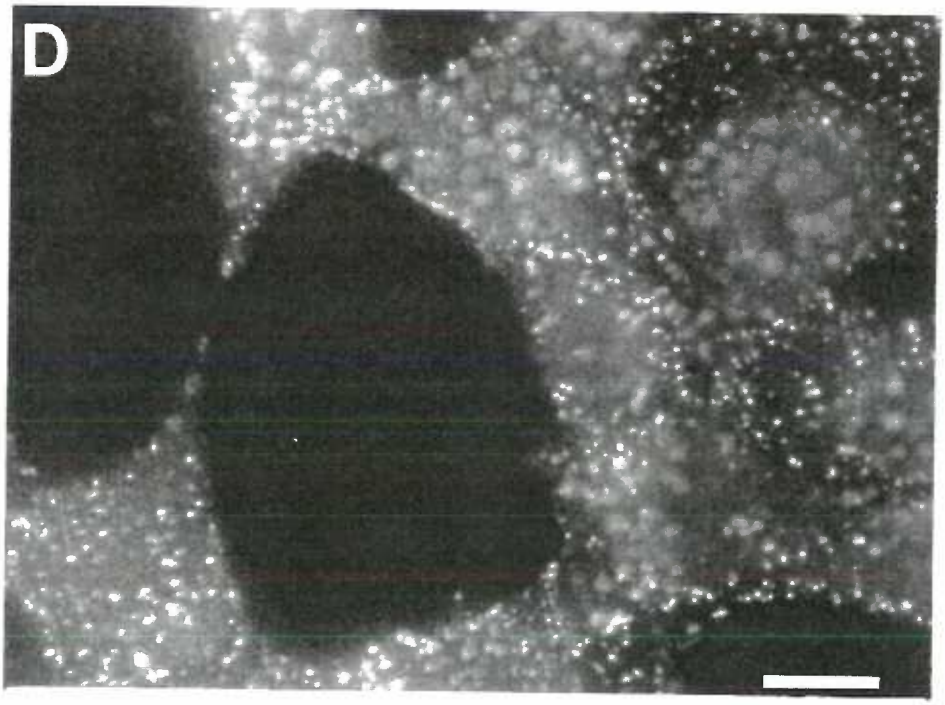
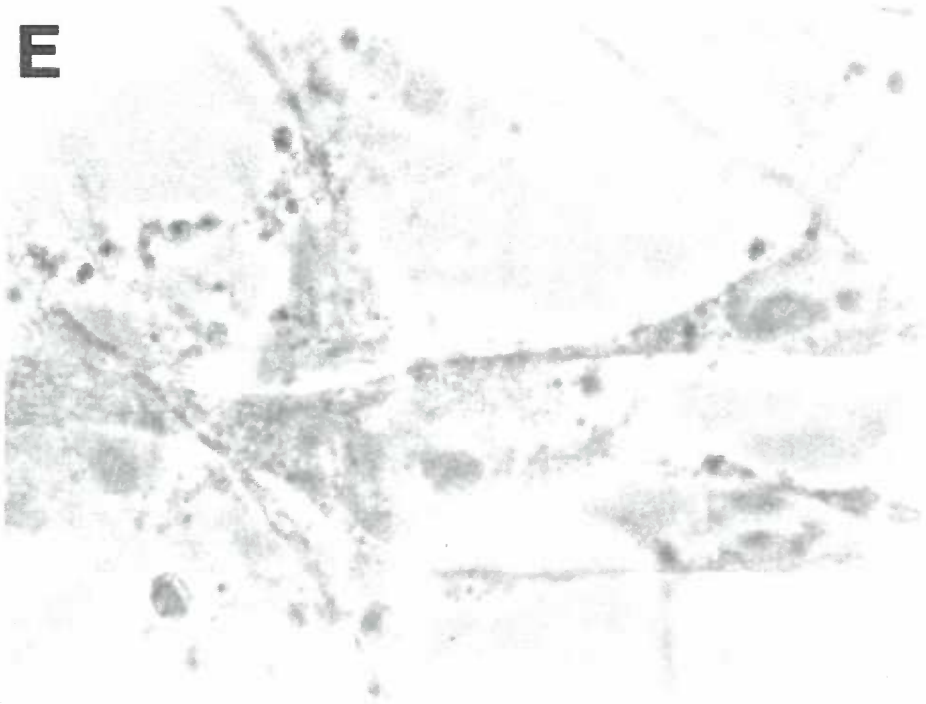

D


E

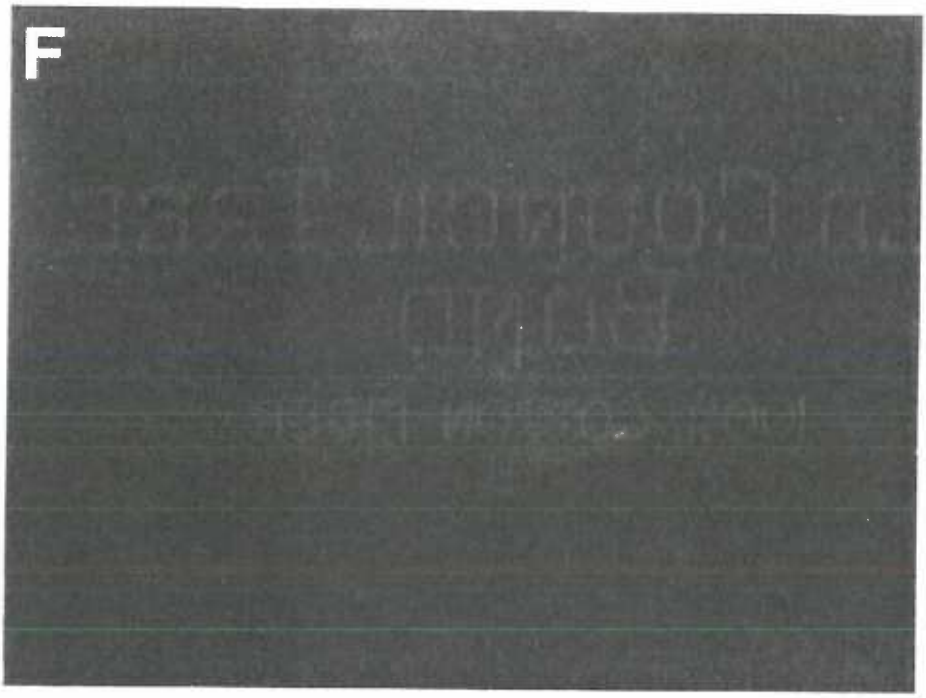
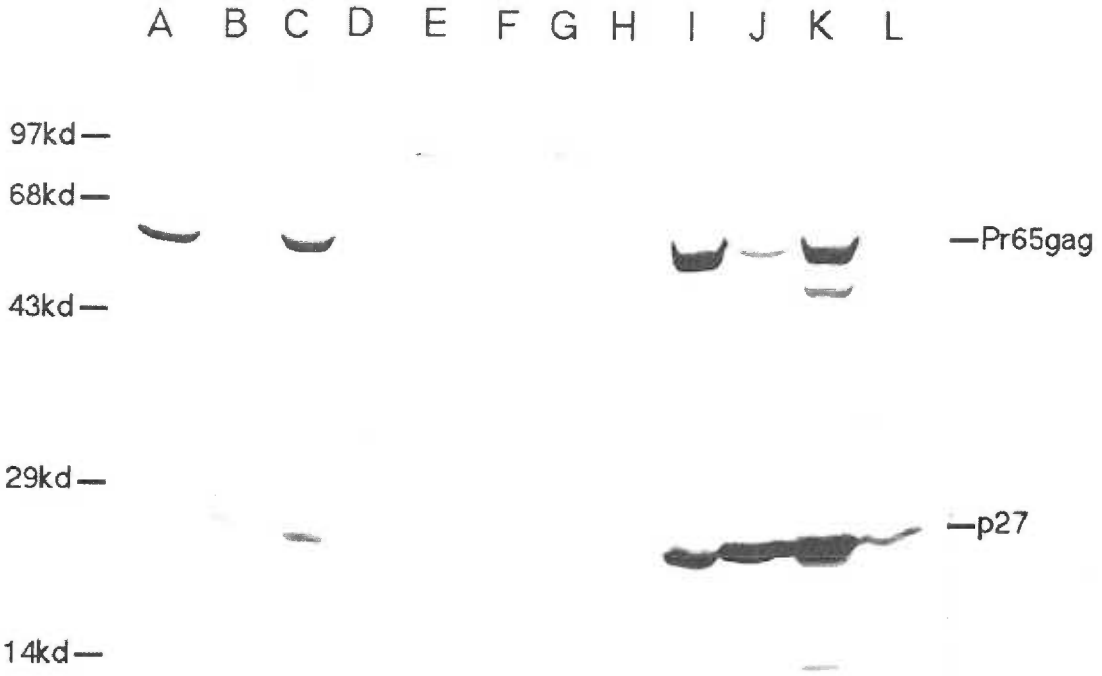
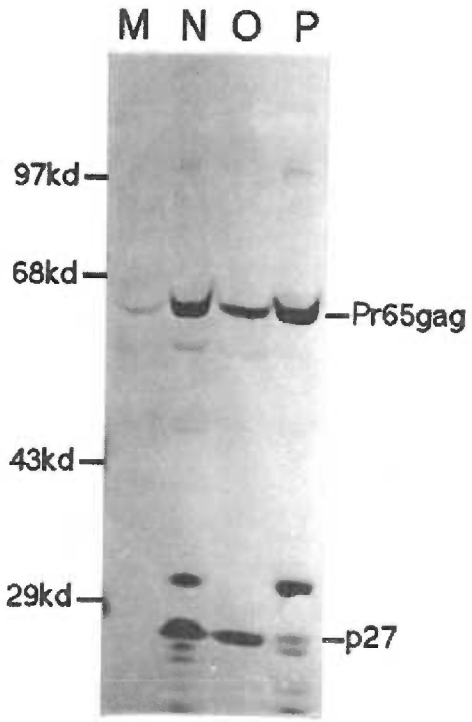

F

Figure 3. Effect of Triton treatment on intracellular *gag* fractionation. Post-nuclear supernatant (PNS) fractions of Psi2 (lanes A-D and M-T), 3T3 (lanes E-H), and M-MuLV-infected 3T3 (lanes I-L) cells were electrophoresed and immunoblotted for detection of Pr65 and p12*gag* proteins. Supernatants and pellets from PNS fractions were treated with and without 0.5% Triton X-100 or NaCl (600 mM final concentration) and centrifuged for 15 min at 201,000 x g. Lanes were pellets (A,C,E,G,I,K,N,P) and supernatants (B,D,F,H,J,L,M,O) from untreated (C,D,G,H,K,L,M,N), NaCl (O,P) or Triton X-100 treated (A,B,F,G,I,J) PNS samples. In the last four lanes (Q-T), Triton X-100 pellets were resuspended by douncing, mock treated (Q,R) or treated with NaCl (S,T) and fractionated again into supernatant (Q,S) and pellet (R,T) fractions. In all experiments, supernatant and pellet material represented one tenth and one third of the total respective supernatant and pellet samples. Pr65*gag*, p12*gag*, Pr180*gagpol* and a proteolytic intermediate, p27, are indicated.


A B C D E F G H I J K L
97kd
68kd
43kd
29kd
14kd
Pr65gag
p27


M N O P
97kd
68kd
43kd
29kd
Pr65gag
p27

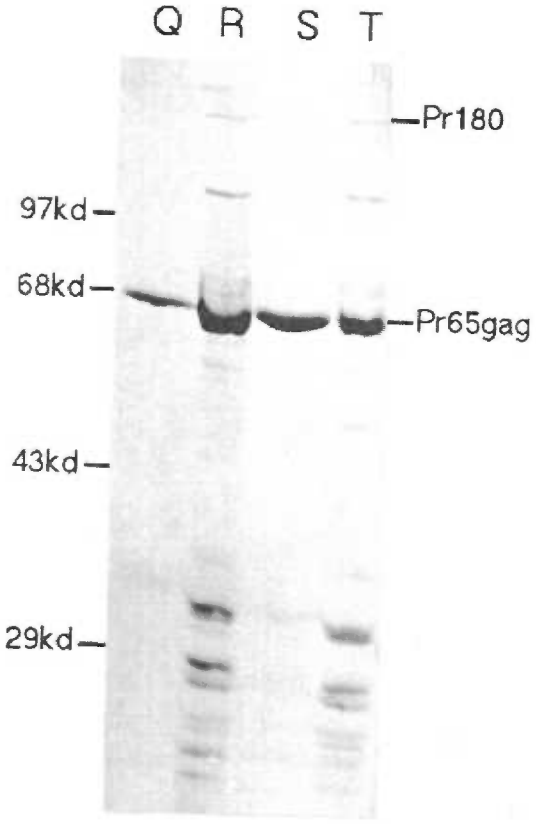

Q R S T
Pr180
97kd
68kd
43kd
29kd
Pr65gag

Figure 4. Resistance of Pr65*gag* to Triton X-100 solubilization.

Psi2 virus producing cells expressing human placental alkaline phosphatase (PLAP; 22) were harvested and dounce homogenized as described in Materials and Methods. Post-nuclear supernatants (PNS) were treated with Triton X-100 or untreated. The samples we fractionated into soluble and insoluble fractions by centrifugation at 201,000 x g for 15 m at 4 degrees C. Matching supernatants (10% of total sample) and pellets (25% of total sample) were subjected to SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose filter. Pr65*gag*, PLAP, and M-MuLV Pr80*env* and gp70*env* were detected by sequential antibody binding and color reaction steps. Protein bands are as indicated. Lanes A and B are untreated supernatant and pellet samples while lanes C and D correspon to Triton X-100 treated supernatant and pellet samples. Note that only part of the gel is magnified to distinguish each of the proteins.

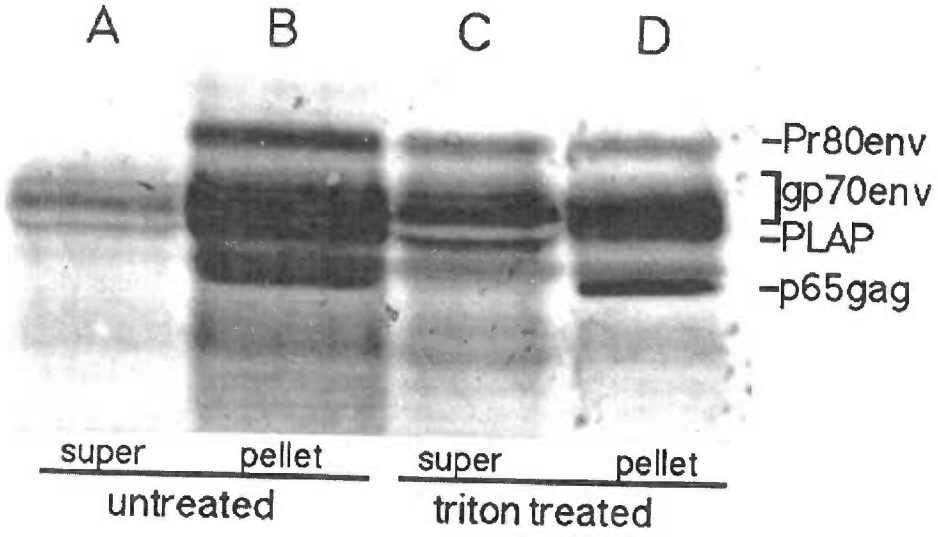
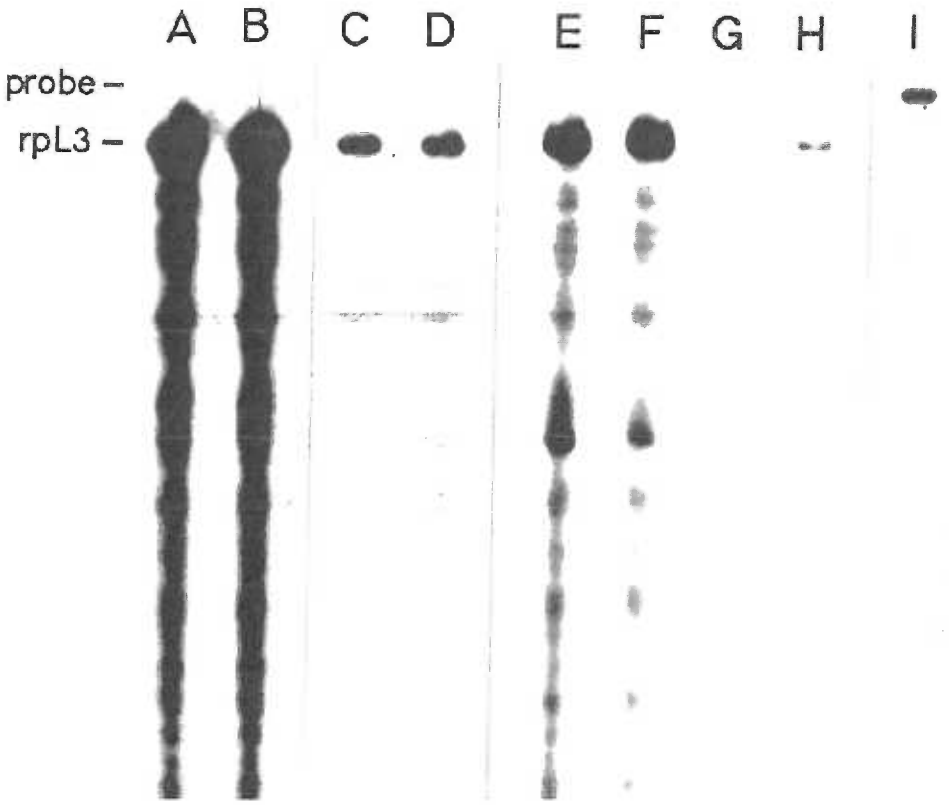

A
B
C
D
-Pr80env
gp70env
-PLAP
-p65gag
super
pellet
super
pellet
untreated
triton treated

Figure 5. Viral RNA content of untreated and Triton-treated pellet fractions. A. Ribosomal protein L3 (rpL3) labeled anti-sense RNA was prepared and used to detect rpL3 transcript by RNase protection in various samples as described in the Materials and Methods. Psi2 virus-producing cells expressing a packageable neomycin-transducing retroviral vector (lanes A, C, E) and 3T3 cells expressing the neo vector (lanes B, D, F) were analyzed for the presence of the cellular ribosomal protein L3 (rpL3) transcript in post-nuclear supernatant (PNS) pellet RNA (lanes A, B), total cellular RNA (lanes C, D), and Triton X-100 treated PNS pellet RNA (lanes E, F).
Lanes G, H, I contain negative control (tRNA), positive control, and undigested probe sam respectively. Note that rpL3 RNA levels in pellet fractions are used to normalize for the no specific cellular RNA content of total cellular and pellet fractions. **B.** Neomycin (neo) label sense RNA was used to detect neo transcript. Lane designations and procedures are identi those in Figure 5A. Note that film processor roller marks are evident, and that only a porti gel is magnified to show the largest protected bands.


A B C D E F G H I
probe –
rpL3 –

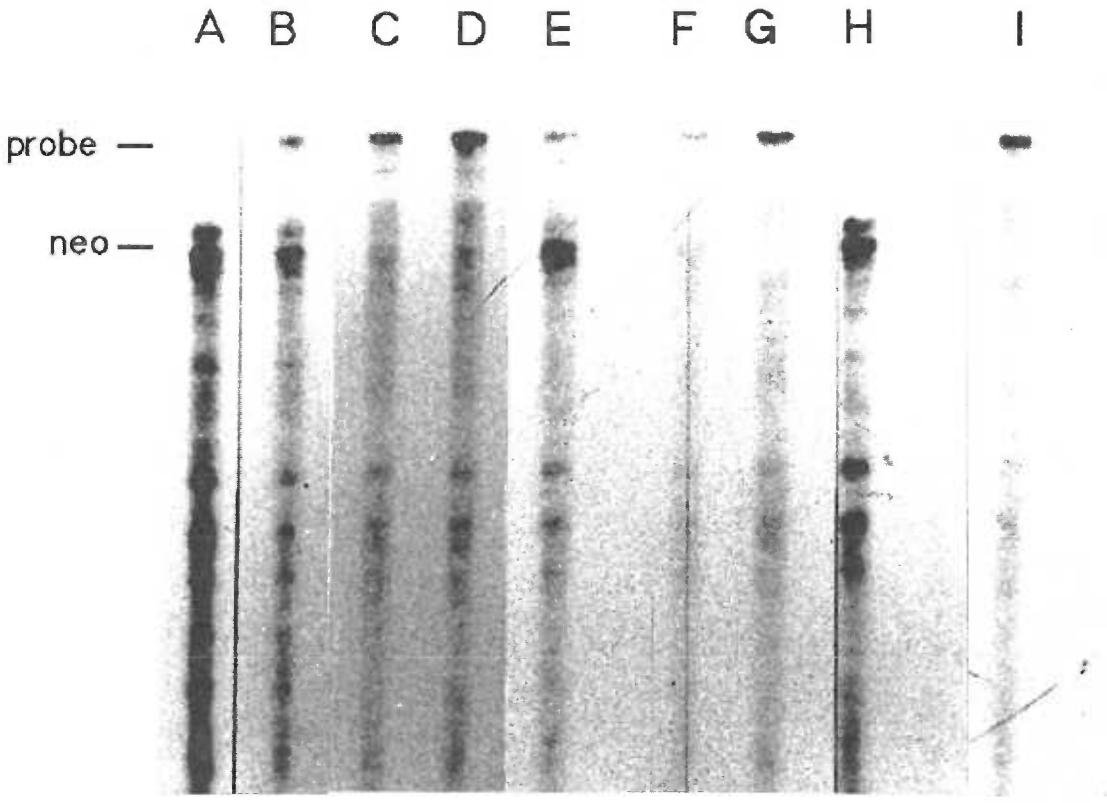

A B C D E F G H I
probe —
neo —

Figure 6. Proteolysis of Pr65*gag* in Psi2 post-nuclear supernatants.

Psi2 cell (lanes A-D) or M-MuLV protease deficient TR291F cell (lanes E-H) post-nuclear supernatants were incubated at 37 degrees C in time course studies of Pr65*gag* proteolysis Psi2 PNS (A-D), lysates contained a cocktail of protease inhibitors including 0.1 mM phenylmethylsulfonylfluoride (PMSF), 5 micrograms/ml leupeptin, 2.5micrograms/ml pep 2 micrograms/ml benzamidine; for TR291F PNS incubations (lanes E-H), inhibitors were Incubations were stopped at various time points by the addition of electrophoresis sample b and boiling for 10 min. Samples were subjected to SDS-PAGE and electroblotted onto a nitrocellulose filter. M-MuLV p30 and Pr65*gag* proteins were detected immunologically a described in Figure 4. Pr65*gag* and p30*gag* are as indicated. Note that the low levels of p TR291F cells probably reflect the fact that this mutant virus gradually reverts over time in n fibroblasts. Incubation times were A, 0 minutes; B, 30 minutes; C, 60 minutes; D, 120 m E, 0 minutes; F, 30 minutes; G, 90 minutes; H, 270 minutes.

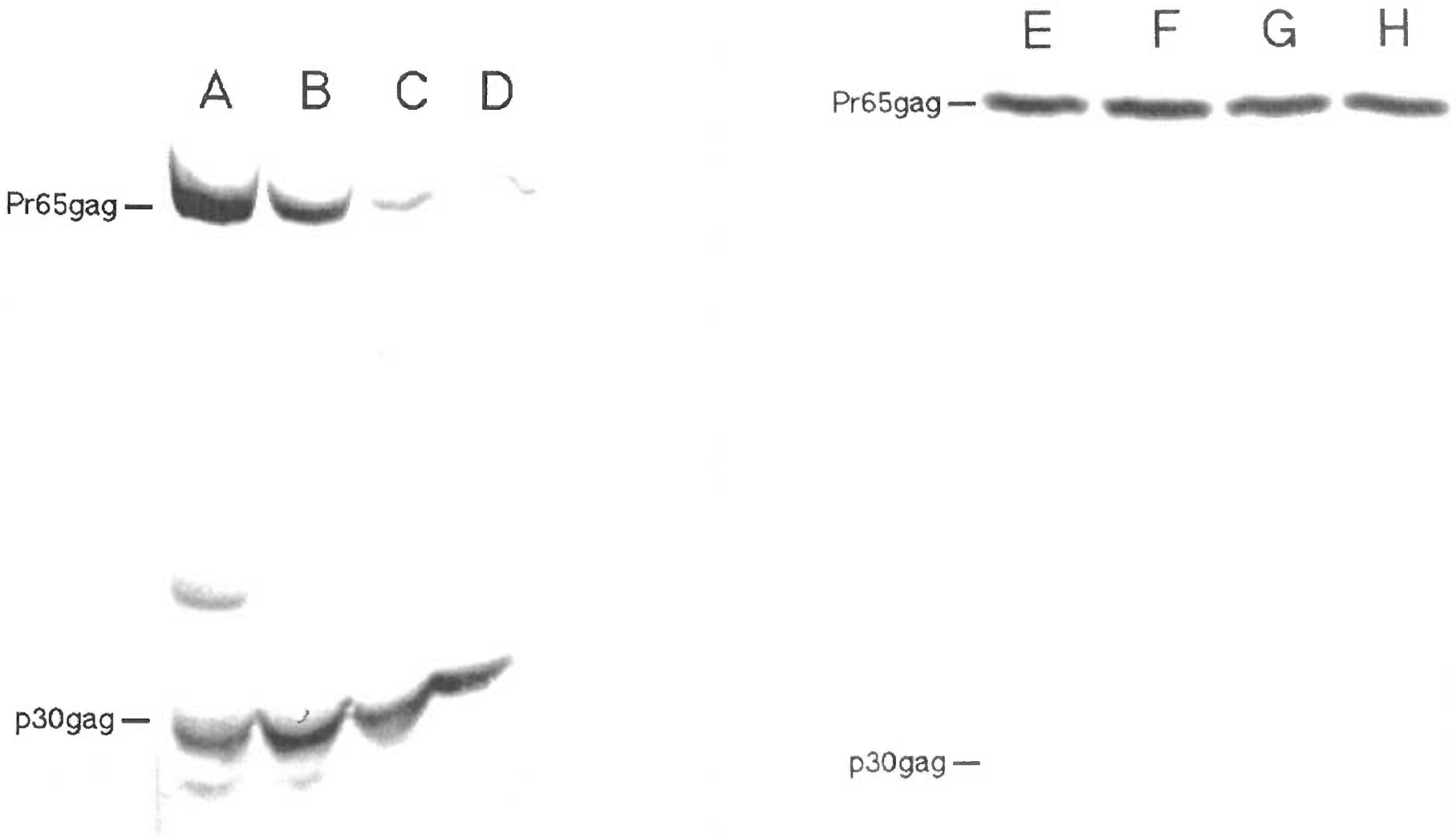
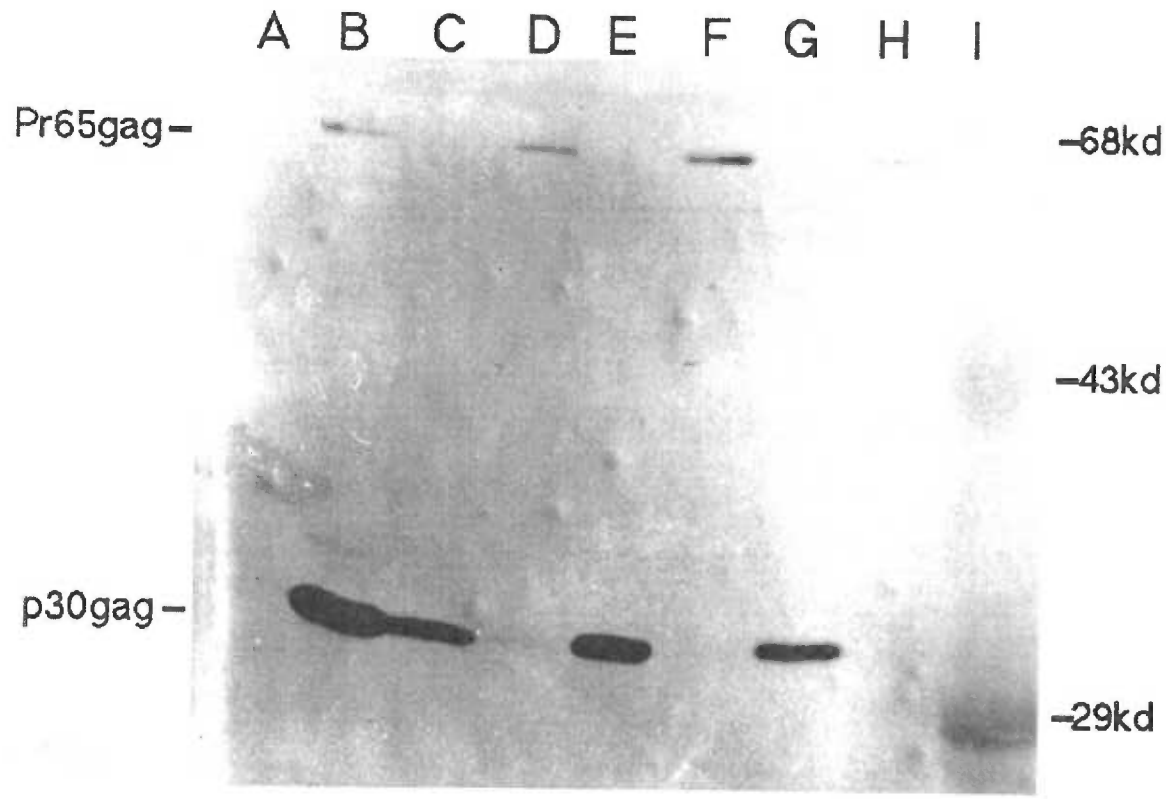

A
B
C
D
Pr65gag
p30gag
E
F
G
H
Pr65gag
p30gag

Figure 7. Detergent sensitivity of extracellular virions.

Psi2 extracellular virions, enriched by purification, were treated with detergents and centrifuged to examine the difference in protein interactions in immature versus mature virions. Matching supernatants (16% of the total sample; lanes A, C, E, G) and pellets (50% of the total sample; lanes B, D, F, H) were obtained by centrifugation at 150,000 x g (65,000 rpm on a TLA-100) for 15 min at 4 degrees C after mock treatment (lanes A, B); 0.1% Triton X-100 treatment (lanes C, D); 0.1% NP-40 treatment (lanes E, F); or 0.1% Triton X-100 plus 100 mM DTT treatment. *Gag* proteins in samples were detected with a anti-p30 antibody after electrophoresis and electroblotting. Marker protein sizes, indicated on the right, are from marker proteins in lane I.


A B C D E F G H I
Pr65gag
p30gag
68kd
43kd
29kd

MANUSCRIPT #3

STRUCTURAL INTERACTIONS BETWEEN RETROVIRAL GAG PROTEINS EXAMINED BY CYSTEINE CROSSLINKING

Mark S. T. Hansen and Eric Barklis*

Running title: Retroviral Gag protein structural interactions.

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STRUCTURAL INTERACTIONS BETWEEN RETROVIRAL GAG PROTEINS EXAMINED BY CYSTEINE CROSSLINKING

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ABSTRACT

We have examined structural interactions between Gag proteins within M-MuLV particles by making use of the cysteine-specific crosslinking agents iodine and bis-maleimido hexane (BMH). Virion associated wild-type (wt) M-MuLV Pr65Gag proteins ir immature particles were intermolecularly crosslinked at cysteines to form Pr65Gag oligomers, from dimers to pentamers, or hexamers. Following a systematic approach of cysteine to serine mutagenesis, we have shown that crosslinking of Pr65Gag occured at cysteines of the nucleocapsid (NC) cys-his motif, suggesting that the cys-his motifs within virus particles are packed in close proximity. The M-MuLV Pr65Gag protein did not crosslink to HIV Pr55Gag protein when the two molecules were co-expressed, indicating that either they did not co-assemble or that heterologous Gag proteins were not in close enough proximity to be crosslinked. Using an assembly-competent, protease-minus, cysteine-minus Pr65Gag protein as a template, novel cysteine residues were generated in the M-MuLV capsid domain major homology region (MHR). Crosslinking of proteins containing MHR cysteines showed above background levels of Gag-Gag dimers, but also identified a novel cellular factor, present in virions, that crosslinked to MHR residues. Although NC cysteine mutation was compatible with M-MuLV particle assembly, deletions of the NC domain were not tolerated. These results suggest that the cys-his motif is held ir close proximity within immature M-MuLV particles by interactions between CA domains and/or non-cys-his motif domains of the NC.

INTRODUCTION

Successful completion of the retrovirus life cycle requires that viral components in an infected cell co-localize and assemble particles that bud through the plasma membrane, mature properly, enter a target cell, and integrate their genomes into the genome of the host. During assembly of Moloney murine leukemia virus (M-MuLV), core (Gag) proteins form a protein capsid including the viral genomic RNA, and enzymatic (Pol) proteins, which are incorporated into virus particles only as a Gag-Pol fusion protein that results from the suppression of termination of approximately 5% of Gag translations (48). Expression of the Gag protein is necessary and sufficient for the formation of enveloped, virus-like particles (13, 22, 36, 43), and assembly of type C retroviruses such as M-MuLV usually occurs at the plasma membrane (44), but also can occur on intracellular membranes (14), which may or may not be a productive route of assembly.

The Gag protein of M-MuLV is synthesized as a myristylated precursor polypeptide, Pr65Gag (16), which is processed by the viral protease (PR) during or after budding, into four mature Gag proteins: matrix (MA), p12, capsid (CA), and nucleocapsid (NC) (2, 5, 29, 38, 46). The MA protein is myristylated (34), associates with membranes, and may contact the carboxy-terminal region of the viral transmembrane (Env) protein (44, 49). The M-MuLV p12 protein has no avian or primate retroviral homologs, and is dispensable for virus particle assembly (7). In contrast, the Gag capsid domain has been shown to facilitate interactions between Pr65Gag proteins and appears to be essential for particle assembly (15, 17, 18, 41, 45). Within the CA protein sequence lies the determinant of murine C-type NIH/BalbC (N/B) cell tropism (44), as well as the major homology region (MHR), a stretch of 20-30 residues which represents the only highly

conserved sequence among retroviral CA proteins (24, 40, 45). At the C-terminus of CA is the NC protein, which specifically binds viral genomic RNA and is required for RNA encapsidation (4, 12): point mutations in the nucleocapsid cys-his finger eliminate this function (11).

Although general functions have been attributed to the mature M-MuLV Gag protein products, little is known of specific protein-protein contacts in immature or mature virions. Some genetic analyses have implicated CA as a major region of intermolecular contacts (17 18, 42), and Gag-Gag binding in a yeast two-hybrid system has been attributed to the CA domain (23). The pioneering use of crosslinking agents to examine virus structure (10, 32 33) demonstrated that chemical treatment of avian retroviruses produced homotypic dimers of all mature Gag proteins, but did not yield any heterotypic dimers (33). Similar results were shown for murine C-type retroviruses, where matrix and nucleocapsid domains also crosslinked in Pr65Gag (32). A limitation of these experiments is that the crosslinkers employed were amine-reactive and had multiple reaction target sites, making it difficult to identify specific regions of protein-protein contact. To extend previous results, we have adapted the method of Pakula and Simon (31) for specific crosslinking at cysteine residues, in which proteins or protein complexes are treated with membrane-permeable cysteine-specific crosslinking agents, such as molecular iodine or bis-maleimido hexane (BMH), and cysteines on neighboring proteins can form a covalent bond, creating an intermolecular crosslink. Using these reagents, we have found that neither mature CA nor NC showed significant homo-dimerization, but that Pr65Gag in immature particles crosslinked via NC cysteines to form oligomers up to pentamers or hexamers. Pr65Gag crosslinking was resistant to non-ionic but sensitive to ionic detergent treatments and Pr65Gag did not crosslink with co-expressed HIV Pr55Gag. Cysteines created in the MHR of the M-MuLV capsid domain showed increased levels of Pr65Gag intermolecular crosslinking and also

crosslinked to a non-viral protein with a molecular weight of 140-160 kd. Interestingly, while cysteine-minus Gag proteins formed virus-like particles of wild-type (wt) density, implying that the NC cys-his motif is unnecessary for assembly, deletion of the NC domain greatly reduced the assembly efficiency and stability of particles.

MATERIALS AND METHODS

Recombinant Constructs. The M-MuLV stable expression vectors, 2051T dl. NC and 2189T, are nucleocapsid (NC) truncation mutants derived from previously described Gag-B-galactosidase fusion proteins (18) by replacing the B-galactosidase moiety with a termination signal. 3229T C504S/C507S (numbers indicate Gag cysteine codons mutated to serine) has the same plasmid backbone and was derived from C26S/C29S (numbers indicate NC codons; the mutations are identical), the kind gift of Dr. Alan Rein, and has been described (11). The sequences at gag termination junctions, in which the underlined nt marks the M-MuLV nt position (37) designated in the construct name and TAA is the termination codon, are as follows: 2051T dl. NC 5' CTA TTG GCG GAT CCC CCT TAA GTT AAC TTA AGG GCT GCA GGA ATT 3'; 2189T 5' GGA CCT CGG CGG GAT CCC CCT TAA GTT AAC TTA AGG GCT GCA GGA ATT 3'; and 3229T C504S/C507S 5' CGG ATC CCC CTT AAG TTA ACT TAA GGG CTG CAG GAA TTC 3'. M-MuLV transient expression vectors were derived from pXM (47) that contains an SV40 origin of replication and the adenovirus major late promoter. M-MuLV was modified at the Psi2 position 563 Hind III site (26) by addition of 5' EcoRI and EcoRV sites (GAATTCGATATCAAGCTT) and used to create pXMGPE, which expresses gag, pol, and env genes and spans M-MuLV nt 566 to 7846; pXM2453 is protease-deficient (PR-), deleted in pol and env genes, and encodes M-MuLV nt 566 to 2453, where a termination signal was placed downstream of nt 2453. Junction sequences are as follows: pXM2453 5' GGT AAG GTC ACC GCG GAT CCC CCT TAA GTT AAC TTA AGG G CTG CAG GAA TTC 3', in which the M-MuLV nt indicated in the name is underlined and the termination codon is TAA; pXMGPE 5' TTTGGCAAGCTAGA 3', in which M-MuLV nt 7840 from 3' untranslated sequence is underlined. The parental vectors, pXMGPE and pXM2453, were used as templates to introduce cysteine to serine point mutations at each of the M-MuLV cysteine codons. All five cysteine to serine point

mutations, including two from the previously described 3229T C504S/C507S (11), then were cloned singularly or in combination into the parental vectors (In particular note that all five mutations were cloned into two vectors to yield pXMGPE cysteine-minus (C-) and pXM2453 cysteine-minus). Using the C- constructs as templates, we then introduced novel cysteine codons into the M-MuLV major homology region. The sequences of mutant pXM constructs, in which the altered M-MuLV codon designated in the construct name appears in bold and point mutations, conservative and non-conservative, are underlined, are as follows: C39S, 5' TGG GTT ACG TTC TCC TCT GCA 3'; C270S, 5' TGG GAC GAT AGT CAA CAG 3'; C517S, 5' AAA GAT TCT CCC AAG AAG CCT CGA 3'; L369C, 5' TCT CCC AGC GCC TTC TGT GAG AGA C 3'; E370C, 5' TCT CCC TCG GCC TTC CTA TGT CGA C 3'; and, E370C/K373C, 5' TTC CTA TGT CGA CTT TGC GAA GCC TAT CGC ACG TAC ACT CCA TAT 3'. Two nucleocapsid truncation mutants, pXM2051 dl. NC and pXM2189, were derived from their stable expression counterparts, described above, and have the same junction/termination sequences. pXM2453 dl. p12 has a deletion of p12 coding sequence from nt 1035 to nt 1265 and the junction sequence, in which nt 1034 and nt 1266 are underlined and the first codon of CA is in bold, is 5' CT CTA GGC GAT ACC GTC GAT CCC CTC CGC 3'. The vector HIVgpt Bcl was used to express HIV-1 Pr55Gag and HIV-1 Pr160GagPol and has been described previously (30). All mutants were constructed by standard cloning techniques (1, 25, 27), and verified by dideoxy sequencing (35).

Cells and Viruses. NIH 3T3, Psi2 (26), and PA317 (28), cells were grown as described previously (18). The TR291F cell line, expressing a M-MuLV protease-deficient (PR-) mutant, was the kind gift of Dr. Alan Rein (20). Stable cell lines expressing retroviral constructs were established in 3T3 cells by the transfection-infection protocol of Jones et al. (18). Cos7 cells were grown and transfected with transient expression vectors as described previously (42). Briefly, confluent 10-cm-diameter dishes of Cos7

cells were split 1:5 the day prior to transfection. Fifteen micrograms of plasmid DNA per dish was mixed with 1 ml Hepes-buffered saline (HBS [pH 7.05]; 21 mM N-2-hydroxy-ethylpiperazine-N-2'-ethanesulfonic acid [HEPES; CalBiochem], 137 mM NaCl, 5 mM KCl, 0.7 mM sodium phosphate, and 5 mM dextrose), and 40 ul of 2M CaCl2 was added while vortexing. The DNA solution was incubated at room temperature for 45 min, added dropwise to the monolayer, and then incubated at room temperature for 20 min. After addition of 10 ml of Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma), 500 U/ml penicillin and streptomycin, 10 mM HEPES buffer solution (GIBCO BRL), and 25 ul of 50 mM chloraquine, plates were incubated for 4-6 hr at 37 deg C. Following incubations, cells were washed with 5 ml DMEM, incubated in 3 ml of 15% glycerol in HBS (pH 7.05) for 3 min at 37 deg C, washed twice, and fed with 10 ml DMEM with FCS and 50 ug/ml gentamycin (GIBCO BRL). Supernatants and cells were collected at 72 hr, and either filtered through a 0.45 micron filter (Gelman) or spun at 560 x g (2,000 rpm on an MSE centrifuge) for 5 min to clear cells.

Protein preparation. Cell free supernatants from transfected Cos7 cells were centrifuged through a 4 ml sucrose cushion consisting of 20% sucrose in TSE (10 mM Tris hydrochloride (pH 7.4), 100 mM NaCl, 1 mM EDTA) at 83,000 x g (25,000 rpm on an SW28 rotor) for 2 hr at 4 deg C. Pellets were suspended in TSE or electrophoresis sample buffer (12.5 mM Tris hydrochloride [pH 6.8], 2% SDS, 20% glycerol, and 0.25% bromphenol blue) with 5% B-mercaptoethanol (B-me), depending on the experiment. In experiments comparing cellular and extracellular Gag proteins, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS; 9.5 mM sodium-potassium phosphate [pH 7.4], 137 mM NaCl, 2.7 mM KCl), collected in 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, 0.02% sodium azide), vortexed 1 min, and

microcentrifuged (13,700 x g, 4 deg C, 10 min) to remove insoluble material. Solubilized cellular material was mixed with an equal volume of 2x electrophoresis sample buffer with 5% B-mercaptoethanol (B-me), unless otherwise indicated. In most experiments, supernatant and cell samples, 50% and 10% of the total samples respectively, were prepared for electrophoresis and immunoblot analysis.

Sucrose Gradients. Extracellular virions were pelleted through a 4 ml sucrose cushion of 20% sucrose in TSE at 83,000 x g for 2 hr at 4 deg C. The pellets were resuspended in 0.5 ml TSE, and applied to sucrose gradients consisting of 1.1 ml 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 400 ul fractions were collected from top to bottom. Fractions were mixed, and aliquotted for measurement of sucrose densities, and Gag protein levels by densitometric quantitation of immunoblot bands (Bio-Rad model #620). Gag protein levels were normalized to the most dense band from each gradient.

Detergent treatments. In crosslinking experiments utilizing detergents, 10% Triton X-100 (Bio-Rad) was added to a final concentration of 0.5%, and 10% SDS was added to a final concentration of 0.1%. Samples were vortexed gently and incubated at room temperature 5 min prior to further treatment.

Subcellular fractionation. Typically, three 10-cm-diameter plates of confluent cells were washed twice, pelleted in PBS, and resuspended in 1 ml of Dounce buffer (20 mM HEPES [pH 7.4], 100 mM KCl, 85 mM Sucrose, 100 uM EGTA). The cells were lysed by Dounce homogenization 200 times in a 2 ml Wheaton Dounce homogenizer, using the type A (tight) pestle. Whole cell lysates were spun for 5 min at 5,000 x g (8,500 rpm on an Eppendorf microfuge) at 4 deg C. Postnuclear supernatant (PNS) was collected and

either treated or not treated with Triton X-100 to a final concentration of 0.5%. Treated or untreated PNS was spun at 201,000 x g (55,000 rpm on a Beckman TLS-55 rotor) for 15 min at 4 deg C. Second supernatants (S2) and second pellets (P2) were resuspended in electrophoresis sample buffer with B-me, or untreated second pellets were suspended in 200 ul TSE for intracellular particle crosslinking experiments.

Immunoblotting. Samples for immunoblot analysis were prepared in electrophoresis sample buffer (without dithiothreitol (DTT), and where indicated, without B-me) and applied to Laemmli sodium dodecyl sulfate (SDS)-polyacrylamide gels (21). After electrophoresis, proteins were electroblotted to nitrocellulose membranes (0.45 micron pore size; Schleicher and Schuell). For immunoblotting, non-specific binding sites were blocked in TBST (20mM Tris hydrochloride [pH 7.6], 150 mM NaCl, 0.05% Tween 20 [Bio-Rad]) with 3% gelatin (Bio-Rad) for 30 min at 25 deg C on a shaker platform. Blocking solution was replaced with a primary antibody solution of 1% gelatin in TBST for 30 min and then washed 3 times for 5 min with TBST. After washes, filters were incubated in a solution of alkaline phosphatase-conjugated secondary antibody in TBST with 1% gelatin for 30 min, and then washed 3 times for 5 min with TBST. After the final washes, filters were incubated in 20 ml color reaction solution of 0.33 mg Nitro Blue Tetrazolium, 0.17 mg of 5-bromo-4-chloro-3-indolyl phosphate, 100 mM Tris hydrochloride (pH 9.5), 100 mM NaCl, and 5 mM MgCl2 until bands appeared. Primary antibodies were as follows: rat monoclonal antibody hybridoma cell supernatants anti-M-MuLV p12Gag or anti-M-MuLV p30Gag (6, 14) used at a 1:10 dilutions; mouse anti-HIV p24Gag hybridoma #183 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Hybridoma 183, Clone H12-5C from Dr. Bruce Chesebro) supernatant used at a 1:10 dilution; purified mouse monoclonal anti-vinculin used at a 1:1,500 dilution, a gift from P. Stenberg; polyclonal goat anti-M-MuLV p30Gag (NCI) used at a 1:4000 dilution; and rabbit anti-M-MuLV Gag-interacting protein,

the kind gift of K. Alin and S. Goff, used at a 1:4000 dilution. Secondary alkaline-phosphatase conjugated antibodies were goat anti-mouse IgG (Promega) used at a 1:20,000 dilution, rabbit anti-goat IgG (Boehringer Mannheim) used at a 1:10,000 dilution, and goat anti-rabbit IgG (Boehringer Mannheim) used at a 1:10,000 dilution.

Crosslinking techniques. For bis-maleimido hexane (BMH; Pierce) treatments, virus particles or proteins were prepared as described above. BMH was prepared in dimethyl sulfoxide (DMSO) as a 100 mM solution. Samples were prepared in 200 ul of TSE and halved into equivalent 100 ul fractions that were mock treated (1 ul DMSO) or BMH treated (1 ul 100 mM BMH in DMSO). Reaction mixes were vortexed gently, incubated for 60 min at room temperature, and terminated by the addition of electrophoresis sample buffer with 5% B-me and boiled for 5 min. Iodine treatments were performed in a fume hood following the protocol of Pakula and Simon (31). Briefly, 500 mg of iodine crystals (Aldrich) were placed in a flask of 100 ml water that was parafilmed twice and vortexed 5 min. The saturated solution turned orange in color and was used immediately by mixing 25 ul with 25 ul of sample in parallel with distilled water mock treatments. Reactions progressed for 1 min, and were quenched by pipetting 50 ul of 80 mM iodoacetic acid (Aldrich) directly into the reaction, and quickly adding 100 ul of electrophoresis sample buffer without DTT or B-me. Iodoacetic acid was prepared 10 min prior to use by dissolving 185 mg in 10 ml of water, adjusting the pH to 6.8 with 5 M and 100 mM NaOH solutions, and bringing the volume to 12.33 ml with water. For some control samples, B-me (10 ul) was added directly into sample tubes, and all samples were immediately heated at 90 deg C for 4 min prior to electrophoresis. Note that excessive boiling of iodine-treated samples appeared to cause oxidative protein degradation.

RESULTS

Cysteine crosslinking of the M-MuLV Pr65Gag protein.

To identify specific regions of contact between Pr65Gag molecules in immature virus particles, we followed the experimental design of Pakula and Simon (31) which is illustrated in Figure 1A. Briefly, unique cysteines on adjacent molecules will not crosslink with membrane-permeable, cysteine-specific crosslinking agents such as molecular iodine (crosslink distance approximately 5 angstroms) or bis-maleimido hexane (BMH; crosslink distance approximately 15 angstroms) if the cysteine residues are far apart. However, adjacent molecules with cysteine residues in close proximity should crosslink, and oligomers can be detected after gel electrophoresis by immunoblotting. The M-MuLV Gag protein is particularly amenable to this type of analyses, since it possesses only 5 cysteine residues (Figure 1B), three of which occur in the cys-his box of the nucleocapsid (NC) domain. As an initial test of this strategy, we subjected Pr65Gag proteins in immature, protease-minus (PR-) virions to iodine-catalyzed cysteine crosslinking. As shown in Figure 2A (lane C), iodine treatment of PR- particles yielded a novel Gag antibody-reactive band at 130 kd, the predicted size of a Pr65Gag dimer. This band was not present in mock samples (lanes A, B), or after reduction with B-mercaptoethanol (lane D), suggesting that iodine-catalyzed cystine formation between Pr65Gag proteins was responsible for the 130 kd protein in lane C. Similar results were observed when BMH was employed as a crosslinker. As shown in Figure 2B, BMH treatment yielded a ladder of putative oligomers from dimers to pentamers (lane B) with a relative reduction of putative trimers. The mobility of each oligomer, as calculated in semi-log plots, was slightly less than predicted, presumably because crosslinking reduced the Stokes' radius permitting the molecules to traverse the acrylamide gel matrix faster than predicted. Interestingly, the

reduction in putative trimer band intensity is reminiscent of results reporting the *in vitro* oligomerization of purified HIV capsid protein (8).

The protein at 130 kd in Figure 2 is anti-p30 Gag immunoreactive and contains at least one Pr65Gag moiety. However, the mobility shift of monomeric Pr65Gag could be due to a crosslink formed with an unidentified protein of approximately 65 kd such as bovine serum albumin. In order to prove that crosslinked molecules were composed of Pr65Gag dimers, we deleted a portion of the p12gag coding sequence to create a smaller Gag protein: the p12 domain contains no cysteines and similar deletions have been shown to be assembly-competent (7). To examine the composition of Gag-reactive crosslinked molecules, protease-minus (PR-) wt, and dl. p12 proteins in virus particles were subjected to BMH crosslinking (Figure 3). As expected, extracellular wt virions showed Gag-reactive bands at 65 and 130 kd (lane A). Crosslinking of extracellular virions composed of dl. p12 PR- proteins produced bands at 57 and 115 kd (lane D), consistent with the notion that the dl. p12 protein assembles and crosslinks as dimers. When virions composed of homogenous wt protein and virions composed of homogenous dl. p12 protein were mixed and treated with BMH, bands at 115 and 130 kd were observed (lane B), but the absence of an intermediate crosslinking product suggests that crosslinking of free protein in supernatant did not occur, and that proteins in separate particles did not crosslink. However, when mixed virus particles were produced by co-expression of wt and dl. p12 proteins, BMH treatment yielded homogenous dimers as well as a novel intermediate band at 122 kd. The appearance of the 122 kd band is indicative of wt-dl. p12 heterodimers and shows that cysteine crosslinking produces Gag-Gag dimers. Larger oligomers, tetramers and pentamers, also appeared to be composed of the Gag protein as seen in the same experimental system (data not shown).

Based on the above results, our assumption was that crosslinking of Gag proteins takes place primarily between Gag proteins within an individual particle. As an independent test of this, we BMH-treated particles that had been incubated in our standard buffer; in buffer supplemented with 0.5% Triton X-100, which should not disrupt immature particles (14, 39); and in buffer plus 0.1% SDS, which dissociates particles (14, 39). As shown in Figure 4, dimer formation occured after BMH treatment under standard conditions (lane B) or with Triton (lane C), but the dimer band was reduced to near control levels on SDS treatment (lane D). Taken together, these results suggest that oligomers are formed only by particle-associated Gag proteins.

We used cysteine crosslinking to examine whether M-MuLV Gag and HIV Gag proteins could co-assemble into particles and crosslink. To do so, M-MuLV and HIV PrGag molecules were co-expressed in Cos7 cells, and extracellular virions were collected and treated with BMH (Figure 5). Duplicate control and experimental samples were electrophoresed and immunoblotted for detection of HIV Pr55Gag and M-MuLV Pr65Gag proteins. In lanes A and B, detection of HIV Gag shows that HIV Gag proteins were released from cells (lane A), and that HIV Pr55Gag dimers were formed (lane B). Similarly, lanes C and D show M-MuLV Gag protein release (lane D) and BMH dimers (lane C). However, neither of the treated lanes (lanes B, C) showed a mixed dimer band at a predicted size of 120 kd. The absence of a heterodimer indicates that either the HIV and M-MuLV PrGag proteins did not co-assemble, or that they co-assembled but did not crosslink. That M-MuLV Pr65Gag and HIV Pr55Gag do not bind to each other in the yeast two-hybrid system (23) supports the interpretation that these heterologous PrGag proteins do not co-assemble.

While all our results strongly indicated that Pr65Gag proteins were crosslinked via cysteines in immature particles, treatment of mature M-MuLV particles showed no evidence of cysteine-specific crosslinking of mature capsid or nucleocapsid proteins; although previous studies indicated that mature NC domains could be crosslinked at other residues (32). Since p12 contains no cysteines (Figure 1B), one plausible explanation for our results might be that matrix domains mediate cysteine crosslinking of both immature Pr65Gag and mature MA proteins. Alternatively, structural differences of immature versus mature particles could account for differences in crosslinking results. Since we have not found suitable anti-M-MuLV matrix antibodies for our purposes, we do not know if mature M-MuLV MA proteins crosslink via the cysteine at Gag codon 39. However, the observance of oligomers larger than dimers mandates that more than one cysteine residue per Pr65Gag molecule is a constituent of a crosslink. In order to identify residues directly involved in Pr65Gag cysteine crosslinking, we removed gag cysteine codons, singularly or in combination, changing them to serine residues via point mutations. As shown in Figure 6A, our Pr65Gag variant with the MA and CA cysteines mutated to serines (C39S/C270S) was crosslinked above background oxidation levels by iodine treatment (compare lane C versus lanes A, B). The covalent bond was reduced with B-mercaptoethanol (lane B), indicating that the iodine treatment was responsible for cystine bond formation. Dimer formation, by a protein lacking the MA and CA cysteines, indicates that the remaining cysteines, in the NC domain, were responsible for Pr65Gag cysteine-crosslinking. To support this assessment, Pr65Gag variants with single, double, and triple cysteine to serine NC point mutations were BMH-treated (Figure 6B). The wt (lane A), single (C504S, lane B), and double mutations (C504S/C507S, lane C), all crosslinked to form dimers, while the triple point mutation (C504S/C507S/C517S, lane D) did not crosslink detectably. The intensity of the dimers decreased as the number of NC cysteines decreased, and larger oligomers were present only when there were at least two cysteines in the NC domain.

Thus we conclude that most of the cysteine-specific crosslinking of Pr65Gag occured between cysteine residues in the NC domain, and an intact cys-his motif does not appear to be required, as its structure presumably is disrupted in the single and double point mutants.

Cysteine crosslinking of the Pr65Gag capsid domain.

Through our studies, we found that it was possible to eliminate all cysteine codons of Pr65Gag without apparent impairment of assembly (Figure 6, and see below). Consequently, the M-MuLV Gag protein appeared suitable for analysis of intermolecular associations by crosslinking of mutant Gag proteins possessing unique cysteines at novel locations. To do so, we constructed a PR- cysteine minus (C-) Pr65Gag mutant that assembled particles, and introduced cysteine residues into the major homology region (MHR). We examined this region because it maintains a high degree of homology throughout the retroviridae family, as shown in Figure 7A. Retroviral MHRs have been shown to be sensitive to mutagenesis at conserved residues (24, 40), but the region has not been characterized structurally. Based on its putative potential to form an amphipathic alpha helix, we modeled the MHR using the FRODO software (19; Figure 7B). For mutagenesis, we selected MHR positions with some degree of evolutionary variability on either the putative hydrophobic helical face (L369C), or the modeled hydrophilic face (E370C, E370C/K373C; see Figure 7A, B).

Having created Gag proteins with cysteine residues in the MHR domain, we wished to determine if these residues could facilitate the crosslinking of Pr65Gag molecules. Figure 8 shows the results of such an experiment after electrophoresis and immunoblotting to detect Gag proteins of untreated or BMH-treated PR- virions. As

shown (lanes C, D), positive control wt virions crosslinked to form Pr65Gag dimers (lane D) above background levels, while cysteine minus (C-) Gag proteins did not crosslink to detectable levels when treated with BMH (lane F). As illustrated in lanes A and B, particles composed of C-/270C Gag proteins that contain a single cysteine, the natural CA cysteine at position 270, crosslinked above background levels (lane B versus lane F), but not nearly to the extent of wt Pr65Gag (lane B versus lane D). Proteins with cysteines introduced at the MHR, L369C (lanes G, H) and E370C (lanes I, J) both dimerized above negative control levels (lanes H and J versus lane F), and somewhat above 270C levels (lanes H and J versus lane B). Interestingly, proteins with two cysteine residues in the MHR domain, E370C/K373C, did not produce intermolecular crosslinks (Figure 8, lane L). The structure of these non-crosslinking proteins may be slightly different from E370C proteins, or the two cysteine residues preferentially could form an intramolecular crosslink: we have not delineated between these two possibilities. However, in addition to Gag dimers, a novel band was detected when proteins with cysteine residues in the MHR were chemically crosslinked (Figure 8, lanes H, J, L; designated Pr65 + X). The novel band was evident with L369C proteins, pronounced with E370C, and the major crosslink product with E370C/K373C proteins. This band was not seen with 270C proteins (lane B), but may correspond to a wt crosslink band (lane D). Evidence suggests that the Pr65+X band is the product of a crosslink formed between the Pr65Gag mutant protein and a non-viral protein, since viral particles were generated by transfection of plasmids lacking env and pol sequences. Although the novel band, approximately 215 kd, is of similar size to Pr65Gag trimers (approximately 195 kd), the presence of only one cysteine residue for L369C or E370C proteins should preclude the formation of Pr65Gag trimers. Thus, the 'X' factor appears to be a cellular 140-160 kd protein, present in virions, with a cysteine in close proximity to the MHR. A similar crosslink product has been seen after crosslinking of HIV Gag proteins possessing introduced MHR cysteines (Farrell and Barklis, unpublished observations), and when particles were derived from different cells (data not shown).

The role of NC in virus assembly.

The formation of Pr65Gag oligomers by cysteine specific crosslinking of residues in the cys-his motif (Figures 2, 6), suggests a close association of these motifs, corroborating previous results (32). The arrangement of cys-his motifs may facilitate a functional interaction, although several observations have seemed to point against such a role in immature particle structure. Pr65Gag cys-his motif point mutants that presumably disrupt the structure of the motif crosslinked at remaining cys-his motif cysteines, and mature NC domains apparently did not cysteine crosslink. We also found no apparent interaction in the yeast two-hybrid system (9) when M-MuLV NC domains were expressed on two complementary constructs (data not shown). Furthermore, we previously found that a fusion protein comprised of B-galactosidase fused at the carboxy-terminus of the CA domain, replacing the NC domain (GBG2051; 18), incorporated into assembling wt particles and, in the absence of wt proteins, the fusion protein assembled to form low density particles.

Because of the seemingly contradictory NC results, further analysis was necessary to explore the effect of NC on assembly and structure. To test potential effects on viral densities, mature wt virus particles, PR- but otherwise wt virus particles, and protease-minus cysteine-minus (PR- C-) particles were subjected to sucrose density gradient fractionation. As shown in Figure 9, all three particles sedimented at 1.14-1.16 g/ml, the expected density of M-MuLV in sucrose (44). The correct density of PR-C- particles supports the proposal that NC cysteines are held in close proximity by elements outside of the cys-his motif and that NC cysteines are not required for close virus core packing. We further examined the role of NC in particle formation by expressing truncation mutants, pXM2051 dl. NC, a complete deletion of the nucleocapsid domain, and pXM2189, a

carboxy-terminal deletion of the final 6 NC residues. To obtain a rough measure of wt and mutant particle assembly, the amounts of Gag proteins in matching cellular and particle-associated supernatant samples were compared (Figure 10). As shown, protease-minus, but otherwise wt Pr65Gag proteins, were efficiently released from cells (compare lane D versus C). Similarly, pXM2189 (lanes J, K), and single (C504S; lanes L, M), double (C504S/C507S; lanes E, F), and triple (C504S/C507S/C517S; lanes G, H) NC cysteine point mutant proteins were efficiently released, although there appeared to be a slight reduction in the case of the triple point mutant (Figure 10, lane H versus G). However, we found that release of the complete NC-deleted protein (pXM2051 dl. NC) to be reduced drastically in transient expression assays (Figure 10, lane O versus N), as well as stable expression assays (data not shown). These data suggest that some non-cys-his motif component of the NC domain is needed for efficient assembly, or that the exposed carboxy-terminus of dl. NC is detrimental to Gag protein transport or assembly.

The implication from Figure 10 is that the presence of NC affects virus assembly, structure, and/or release. However, examination of virus structure with extracellular virions composed of dl. NC proteins was not possible due to a lack of sufficient extracellular protein for either gradients or stability analysis. As an alternative, we examined intracellular proteins, characterizing their subcellular fractionation patterns in the presence or absence of Triton X-100, which helps dissociate Gag protein complexes (14). To do so, postnuclear cell supernatants (PNS) were either untreated or treated with Triton X-100 and centrifuged to generate second supernatant (S2) and pellet (P2) fractions. As expected, all Gag proteins associated with intracellular membranes in the absence of detergent (Figure 11A, lanes F, J, N, R versus E, I, M, Q). However, wt, C504S/C507S and 2189T intracellular proteins remained largely associated with the pellet fraction after Triton-treatment (Figure 11A, lanes H, L, and P versus G, K, and O), while the NC-

deleted intracellular Gag protein (2051T dl. NC) was solubilized almost completely by Triton (lane S versus T), suggesting that dl. NC proteins did not form stable Gag-Gag contacts. As a further test, P2 fractions from cells expressing wt and 2051T dl. NC proteins were treated with iodine (Figure 11B) to examine the crosslinking profile of intracellular particles: wt Gag proteins were crosslinked to form B-me-sensitive dimers (lanes A-D), and not surprisingly, dl. NC proteins did not form dimers (lanes E-H). From these data we conclude that M-MuLV CA and non-cys-his motif regions of NC are necessary for particle assembly, and interactions between these regions hold zinc-finger motifs in close enough proximity to crosslink.

DISCUSSION

The results of this study demonstrate that particle-associated M-MuLV Pr65Gag monomers can be crosslinked via cysteines into oligomers, from dimers to at least pentamers or hexamers, upon exposure to cysteine-specific crosslinkers (Figures 2, 3, 4, 6). Assignment of crosslink residues to NC cysteines agrees with previous reports, which identified NC domain crosslinks in Pr65Gag, but did not identify specific bridging residues (32, 33). In our crosslinking studies, the relative reduction in trimer formation, in comparison with tetramers (Figure 2B, 4), was reminiscent of results reporting HIV CA *in vitro* oligomerization (8). The lack of observed M-MuLV Pr65Gag trimers could be due to an unknown crosslinking preference, or could indicate that trimers are an unlikely subunit structure in the formation of immature M-MuLV particles. Since the minimum dimension of BMH is approximately 5 angstroms and the nucleocapsid region is presumably within the immature virus shell, the BMH-mediated crosslinking of NC domains suggests one or more of the following: immature particles form cores which are not completed prior to budding (maintaining a complete lipid bilayer but an incomplete protein shell); immature cores are not close-packed shells of protein, but rather a cage of protein with lattice fibers at least 5 angstroms apart; or, our virus preparations have a high percentage of broken particles. We currently are trying to distinguish between these possibilities. Interestingly, M-MuLV Pr65Gag proteins did not crosslink to HIV Pr55Gag proteins when the molecules were co-expressed (Figure 5), suggesting that either the two PrGag proteins did not co-assemble or that they co-assembled but did not crosslink via cysteines. We favor the former interpretation of these results, since M-MuLV and HIV Gag proteins do not appear to interact in the yeast two-hybrid system (23).

We interpret the cysteine crosslinking results to indicate that nucleocapsid cysteines in particle-associated Pr65Gag proteins are held in close proximity by domains in CA and non-cys-his motif regions in NC. Proteins possessing NC cysteine mutations retained thei ability to crosslink (Figure 6B, lanes B, C), implying that residues of these motifs are maintained in close proximity to one another, but their structure is not required for crosslinking. What interactions keep cys-his motifs in close proximity? In the yeast 2-hybrid system, Gag-Gag interactions were localized to CA (23), an important region for particle assembly (17, 18, 41). Pr65Gag protein with a deleted NC domain (dl. NC) was assembly-impaired (Figure 10, lanes N, O) and Triton-sensitive (Figure 11). Thus it appears that both CA and a sub-region of NC are important to the M-MuLV core structure. These results are consistent with those of Bennet et al. (3) which implicate the HIV NC domain as necessary for tight packing of particle-associated Gag proteins.

To extend our observations on wt Pr65Gag proteins, we used cysteine mutagenesis to create novel cysteines in the major homology region (MHR; 24, 40) of a cysteine-minus template (Figure 8). The natural CA cysteine residue, 270C, contributed little to crosslinking of adjacent Pr65Gag proteins, while cysteine residues created in the M-MuLV MHR crosslinked at slightly above control levels. We also observed a reduction of dimer formation when two cysteines were created in the MHR which could have resulted as a consequence of preferential intramolecular crosslink. In interpreting these results, it is important to note that mutant protein structures may deviate from that of wild type (wt) and that crosslinkers may not have had complete access to cysteine residues. However, with protease-competent variants of our MHR mutants we observed no obvious processing defects (data not shown), arguing against global conformational disparities. Also, an indication that the MHR cysteines were accessible to reagents was the formation of a novel crosslink band (Figure 11; designated Pr65 + X). The unknown moiety appeared to be 140-160 kd, and while this band may correspond to a band present when wt proteins are

crosslinked, it appeared to be pronounced with hydrophilic MHR substitutions. A similar or identical factor has been seen when parallel HIV MHR cysteine substitutions were chemically crosslinked (Farrell, McDermott, and Barklis, unpublished results). Evidence suggests that the X factor is non-viral. The expression constructs used in these experiments were env- pol-, and although the mobility of the novel band is similar to the expected mobility of Gag trimers, the factor was present after crosslinking of proteins that contained single cysteine residues, which should preclude Pr65Gag oligomers larger than dimers. At this point evidence suggests that the X factor is not a membrane-anchored protein, as crosslinking was stable in 0.5% Triton (data not shown), and additional analysis suggests that the protein is neither vinculin, nor one of the recently identified Gag-interacting proteins (GIP; identified by K. Alin and S. Goff). We currently are examining the identity and significance of the X factor, as well as the role of the amino-terminal region of NC in particle assembly, transport, and/or release.

ACKNOWLEDGEMENTS

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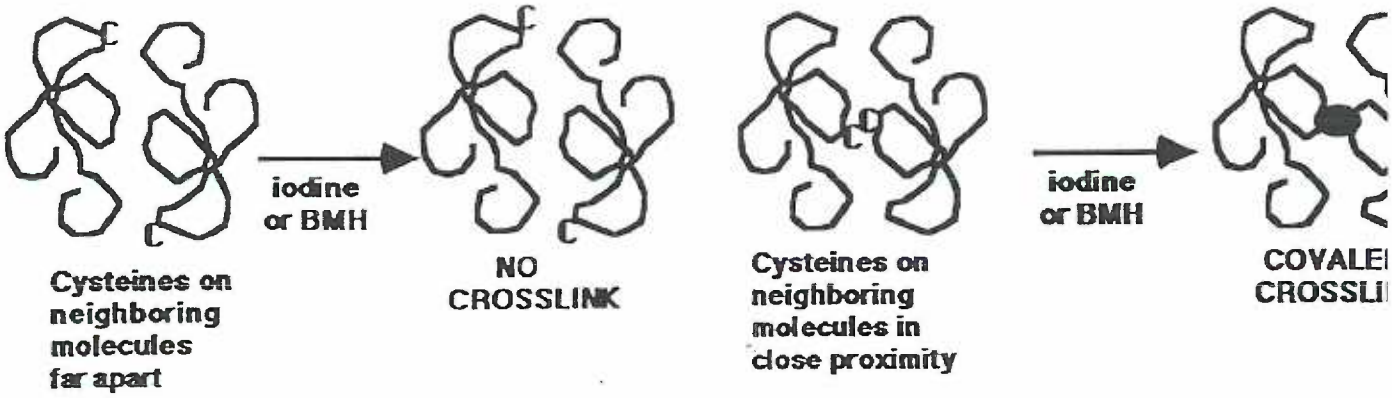
Figure 1. Cysteine crosslinking and location of cysteine residues in Pr65Gag. **A.** A schematic illustration of the consequences of molecular iodine or bis-maleimido hexane (BMH) cysteine-specific crosslinking is shown. Distant cysteine residues from neighboring molecules do not crosslink covalently when exposed to membrane-permeable iodine or BMH, but neighboring cysteine residues in close proximity can form a covalent intermolecular crosslink. The iodine crosslink is a reversible oxidative formation of cystines, while BMH produces an irreversible, maleimide-bridged crosslink. The maximum crosslink distance between cysteine residues is approximately 5 and 15 angstroms for iodine and BMH, respectively. **B.** The Moloney murine leukemia virus (M-MuLV) Pr65Gag protein consists of four domains, matrix, p12, capsid (stippled lines), and nucleocapsid, that are cleaved to maturation by viral protease (PR) during the budding process. The precursor polypeptide is 538 codons in length, and the juncture codons are indicated. Pr65Gag contains five cysteine residues that are indicated on the top bar with codon numbers. Within the capsid domain, the NIH 3T3/BalbC tropism determination site is localized to codons 324-325, and the major homology region (MHR) spans residues 357-378. The nucleocapsid domain contains a cys-his zinc-finger motif with 3 cysteines, C504, C507, and C517.

CYSTEINE-SPECIFIC CROSSLINKING



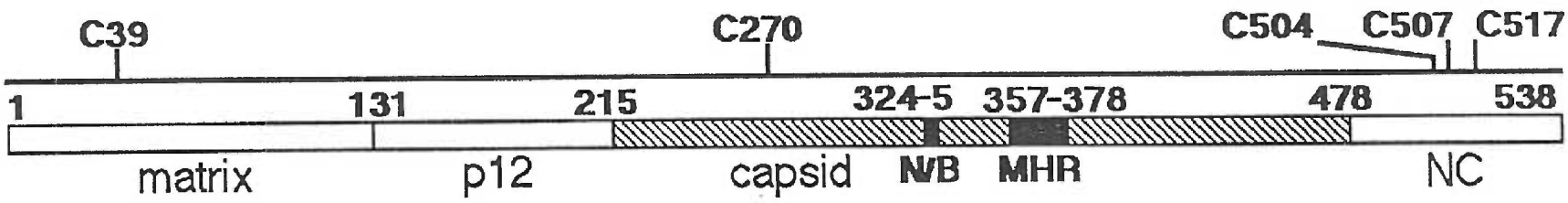
Cysteines on neighboring molecules far apart

NO CROSSLINK

Cysteines on neighboring molecules in close proximity

COVALE
CROSSLI

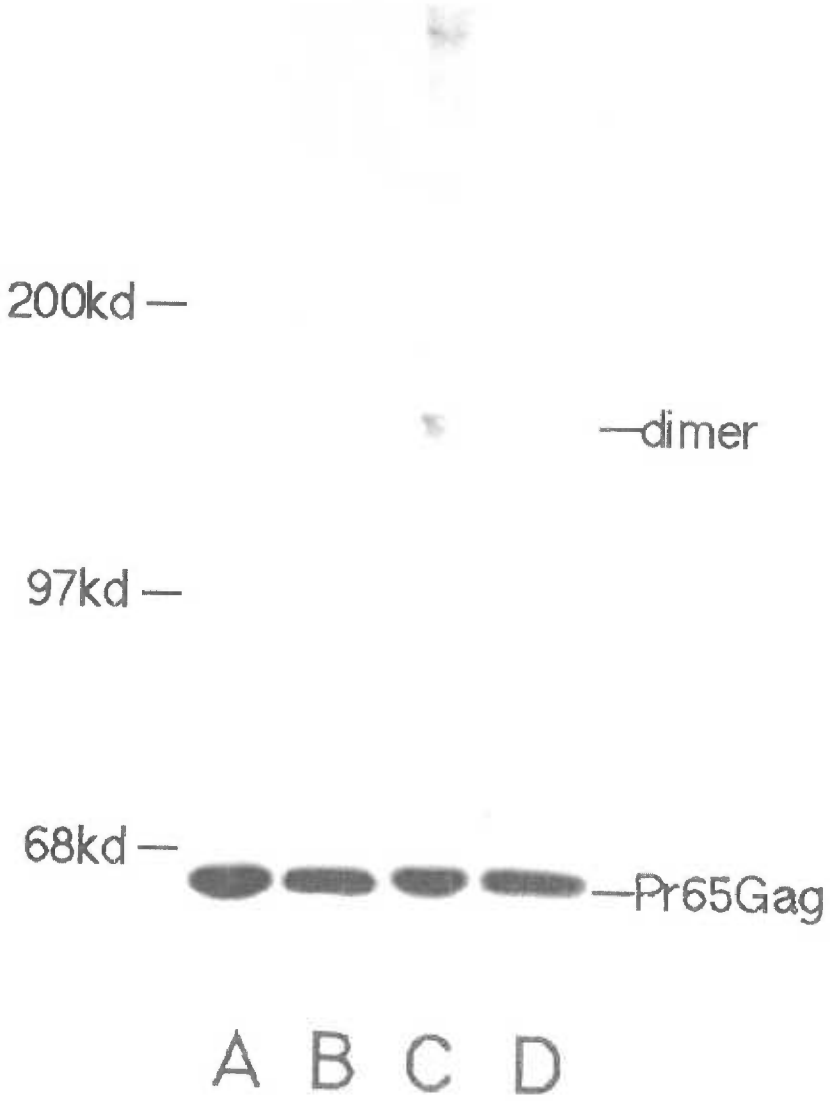
Hansen and Barklis
Figure 1a

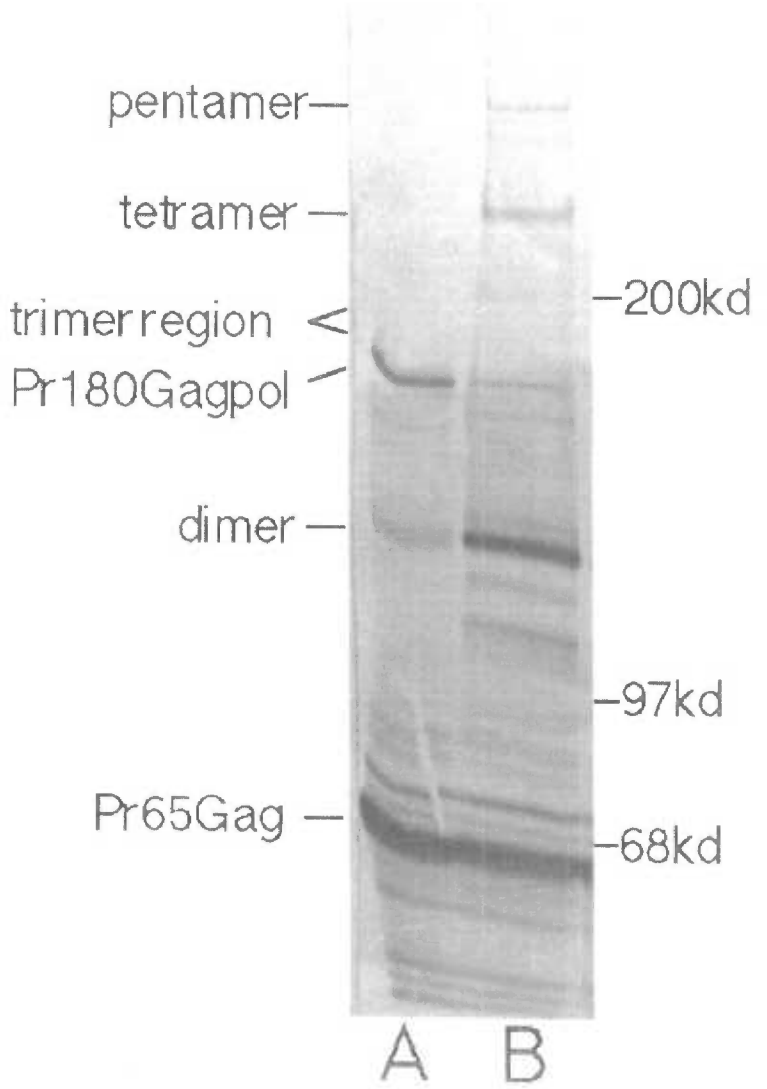


Hansen and Barklis
Figure 1b

Figure 2. M-MuLV Gag protein crosslinking.

M-MuLV protease-deficient (PR-) TR291F (20) extracellular virions were treated with cysteine-specific crosslinking agents, molecular iodine or bis-maleimido hexane (BMH), to examine the effect of crosslinking on M-MuLV Gag proteins. **A.** Parallel 50 ul samples of TR291F virions in TSE were mock-treated (lanes A, B) or iodine-treated (lanes C, D). Reactions were terminated after 1 min by addition of iodoacetic acid and 2x sample buffer. B-me, to 5% was added to two samples (lanes B, D). Samples were immediately boiled, subjected to SDS-PAGE on a 7.5% gel, elctroblotted, and Gag proteins were detected by the sequential addition of anti-p30 antibody, goat anti-mouse alkaline phosphatase-conjugated antibody, and color detection solution. Lanes are A, untreated; B, B-me-treated; C, Iodine-treated; D, Iodine and B-me-treated. Size standard proteins, Pr65Gag, and dimers are indicated. **B.** Parallel samples of TR291F virions were mock-treated (lane A), or BMH-treated (lane B) at 25 deg C for 1 hr. Reactions were terminated by the addition of electrophoresis sample buffer with 5% B-me, and samples were subjected to SDS-PAGE on a 7.5% gel, transferred to a nitrocellulose filter, and M-MuLV Gag proteins were detected with an anti-p30Gag antibody. Marker protein sizes, Pr65Gag, Pr180GagPol, and oligomers are indicated.

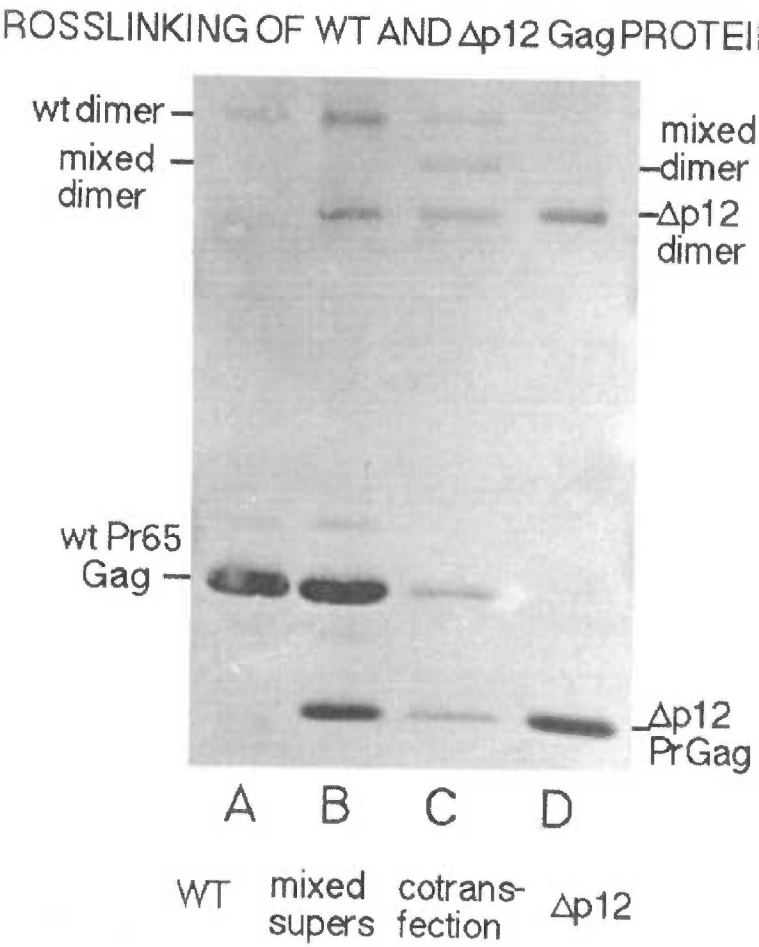




Hansen and Barklis
Figure 2A

Figure 3. Composition of crosslinked species.

Homogenous and heterogenous virus particles composed of protease-minus (PR-) wild type (wt) and/or dl. p12 M-MuLV Gag proteins were treated with BMH to examine the protein composition of crosslinked species. Virus particle preparation and BMH crosslinking reactions were as described in the Materials and Methods. Proteins were subjected to SDS-PAGE on a 6% gel, electroblotted, and M-MuLV Gag proteins were detected by immunoblotting. Lanes derive from BMH-crosslinking of the following PR- virus samples: A, pXM2453 wild type (wt) virus; B, mixed supernatants of wt and pXM 2453 dl. p12 virus; C, co-transfected wt and pXM2453 dl. p12 virus; D, pXM2453 dl. p12 virus alone. Marker protein sizes, wt and dl. p12 monomers and dimers, and mixed dimers are indicated. Note that dimers did not appear with mock treated samples (data not shown).



Hansen and Barklis
Figure 3

Figure 4. Detergent effects on Gag protein crosslinking.

M-MuLV PR-deficient virus particles from Cos7 cell supernatants were mock treated (lane A) or BMH-crosslinked in the absence (lane B) or presence of either 0.5% Triton X-100 (lane C), or 0.1% SDS (lane D). Samples were processed and proteins were detected as in Figure 2B. Pr65Gag and dimers are as indicated.

DETERGENT SENSITIVITY
OF Pr65 Gag CROSSLINKING

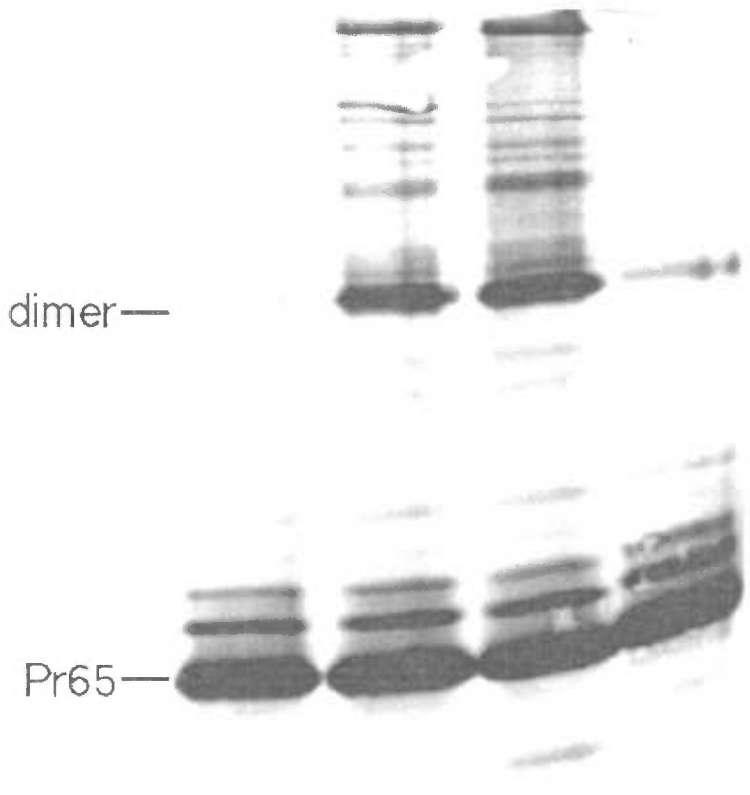
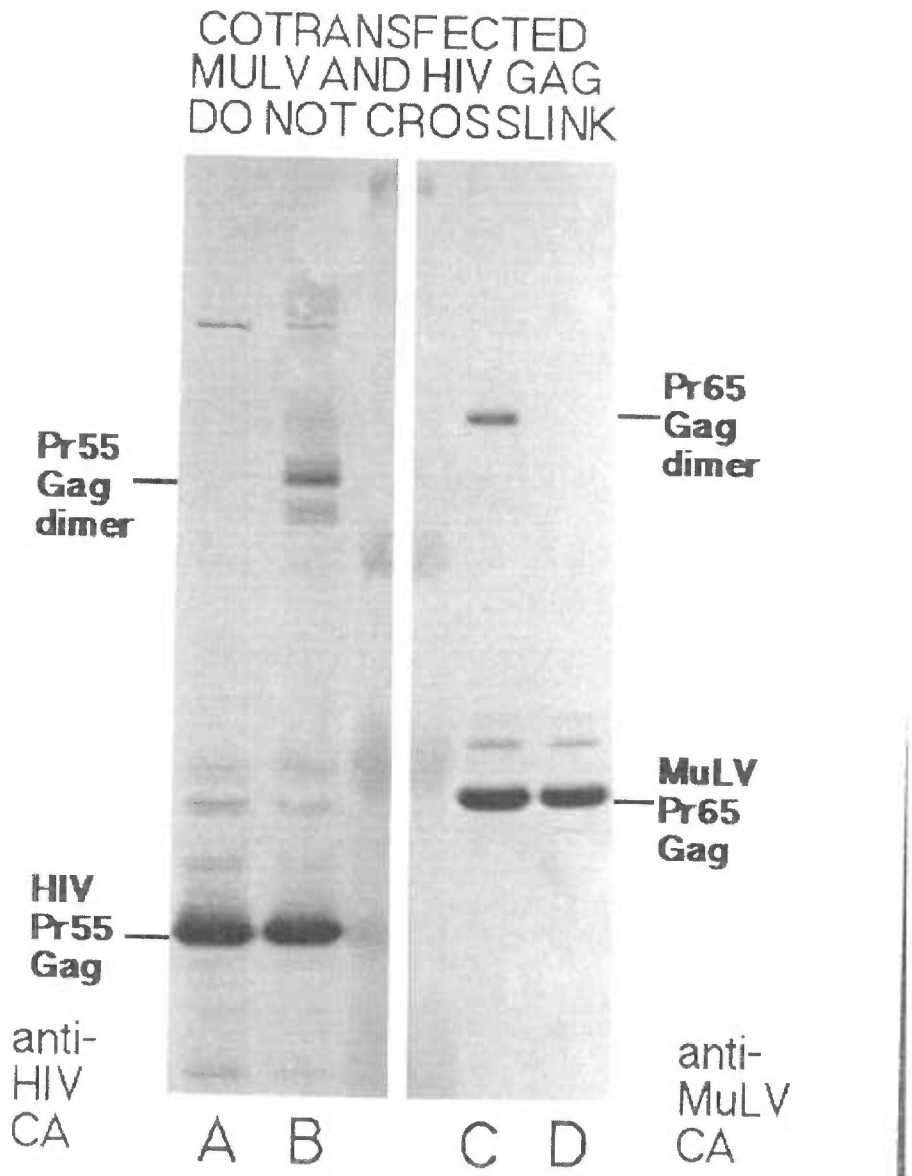


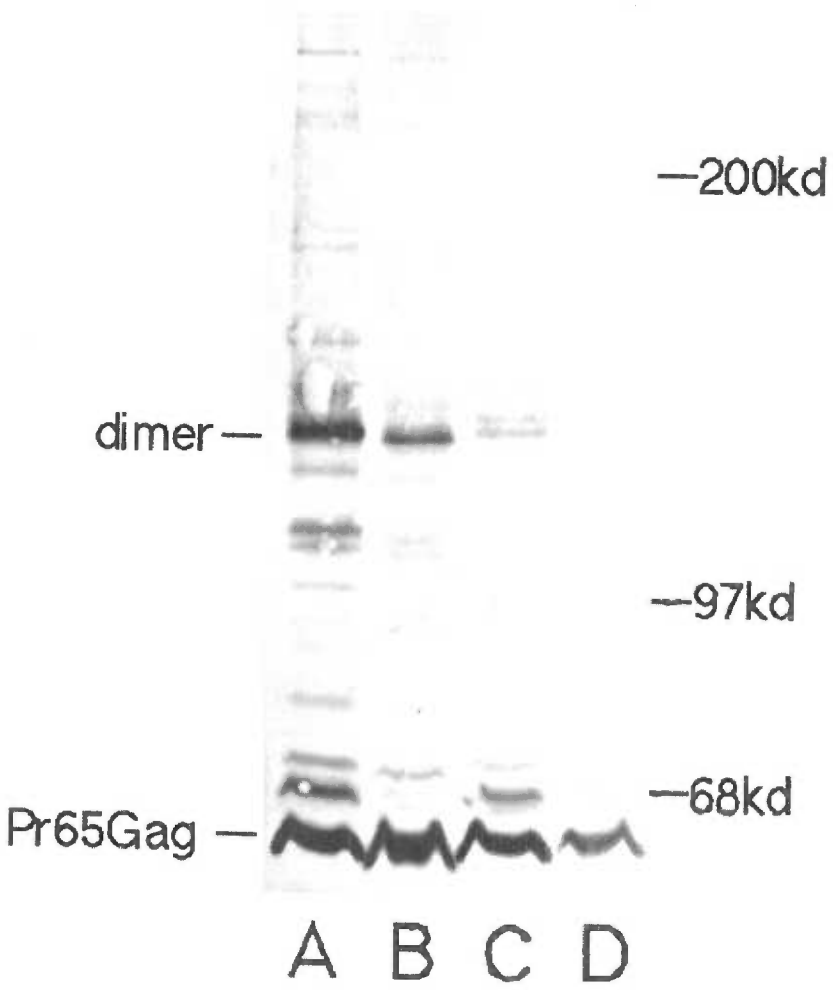
Figure 5. M-MuLV and HIV Gag proteins do not crosslink.

Cos7 cells were co-transfected with 5 ug of pXM2453 plasmid DNA encoding wild type (wt) PR- M-MuLV Pr65Gag protein and 10 ug of HIVgpt Bcl plasmid DNA encoding wt PR- HIV-1 Pr55Gag protein. Supernatants were collected, pooled, and prepared as in Figure 3. Supernatant pellets were suspended in 200 ul TSE, halved, and mock treated (lanes A, D) or BMH-treated (lanes B, C). Reactions were terminated and duplicate samples were electrophoresed, electroblotted, and immunoblotted for detection of HIV Ga proteins (lanes A, B) or M-MuLV Gag proteins (lanes C, D). HIV Pr55Gag and M-MuLV Pr65Gag monomers and dimers are indicated: no mixed dimers were observed. A size standard between lanes B and C was used to align the left and right hand panels.

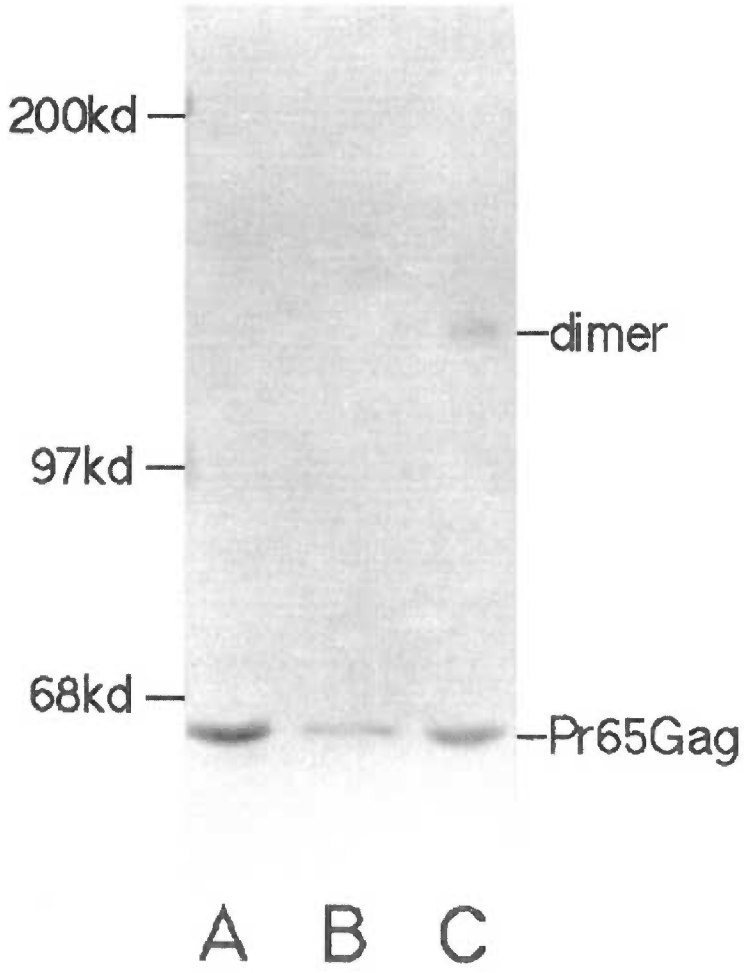


Hansen and Barklis
Figure 5

Figure 6. Gag proteins crosslink at cysteines in the nucleocapsid domain. The five cysteine residues of Pr65Gag were changed to serine residues by point mutation either singularly or in combination to determine the site of cysteine crosslinking. **A.** Cos7 cells were transfected with pXM2453 C39S/C270S plasmid DNA encoding the M-MuLV gag gene containing cysteine to serine point mutations to the cysteines in the matrix (MA) and capsid (CA) domains but retaining NC cysteines. Supernatants were collected, pooled and processed as in Figure 3. The supernatant pellet was suspended in 150 ul, divided intc three equivalent samples of 50 ul, and mock treated (lane A) or treated with molecular iodine (lanes B, C) and subjected to reducing (lane B) or non-reducing (lanes A, C) conditions. Electrophoresed and electroblotted proteins were immunoblotted, and Pr65Gag and dimers are as shown. **B.** Supernatants from Cos7 cells transfected with protease-minus M-MuLV Pr65Gag expression constructs encoding pXM2453 wt (lane A), and nucleocapsid single (lane B, C517S), double (lane C, C504S/C507S), and triple (lane D, C504S/C507S/C517S) cysteine to serine mutants were collected and BMH-crosslinked as in Figure 2B. Samples were electrophoresed, electroblotted and immunoblotted with an anti-p30 capsid antibody as described in the Materials and Methods. Pr65Gag monomer and dimer bands are as indicated.



Hansen and Barklis
Figure 6B

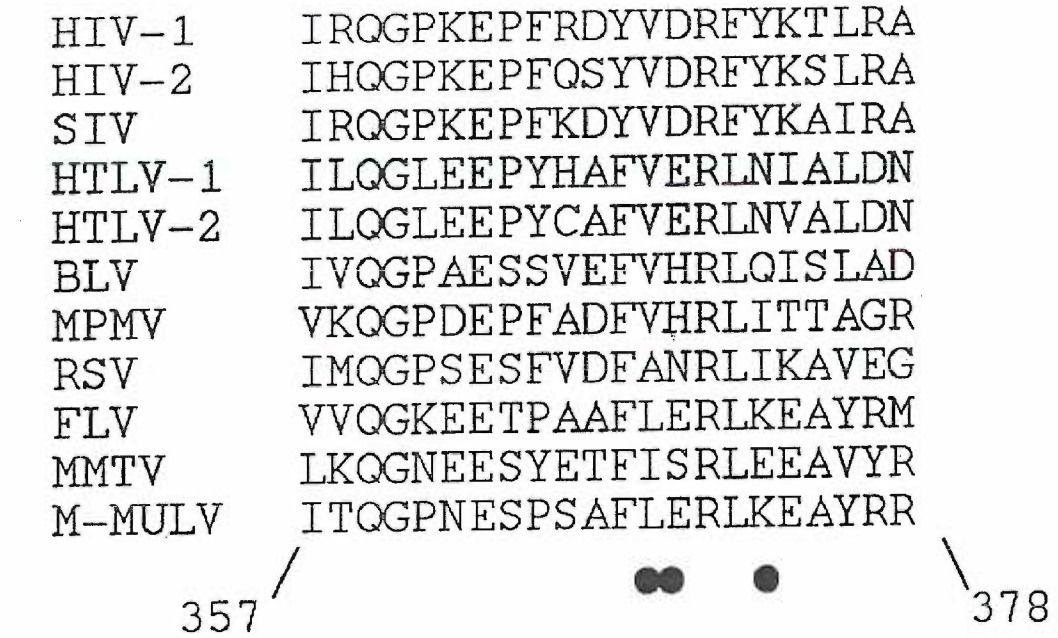


Hansen and Barklis
Figure 6A

Figure 7. Molecular modeling of the major homology region.

A. The major homology region (MHR; 24, 40) of several retroviridae family members ha: been aligned to demonstrate the residue conservation. Virus sequences are as follows: HIV-1 and HIV-2, human immunodeficiency virus types 1 and 2; SIV, simian immunodeficiency virus; HTLV-1 and HTLV-2, human T-cell leukemia virus types 1 and 2; BLV, bovine leukemia virus; MPMV, Mason-Pfizer monkey virus; RSV, Rous sarcoma virus; FLV, feline leukemia virus; MMTV, mouse mammary tumor virus; M-MuLV, Moloney murine leukemia virus. The M-MuLV Pr65Gag residue numbers from 357-378 are indicated at the bottom, and sites of point mutations creating one or two novel cysteine residues (L369C, E370C, and E370C/K373C) are indicated by filled circles. **B.** The M-MuLV MHR was modeled as an alpha helix from residues 366 (top) to 378 (bottom) using an Evans and Sutherland work station with the FRODO software package (19). The modeled helix is amphipathic, with a polar face on the right of the figure and a hydrophobic face to the left. The mutated leucine (L369C), glutamate (E370C), and lysine (K373C) residues are indicated by white arrowheads.

RETROVIRAL CAPSID MAJOR HOMOLOGY REGIONS



Hansen and Bar
Figure 7a

Hansen and Barklis
Figure 7B

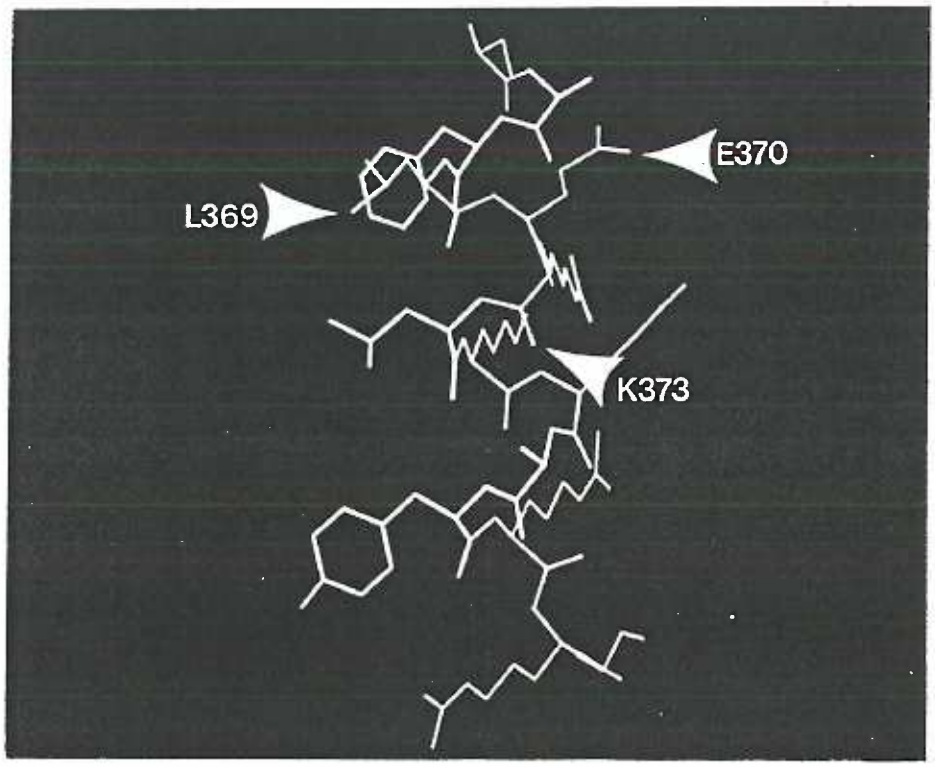


Figure 8. Crosslinking M-MuLV Gag proteins with capsid mutations. COS7 cells were transiently transfected with the designated pXM2453-derived PR-Pr65Gag expression plasmids and, after 72 hr, supernatants were collected and prepared for crosslinking treatment as described in Figure 3. Samples were halved and mock treated (lanes A, C, E, G, I, K) or BMH-treated (lanes B, D, F, H, J, L), and processed for Gag protein detection as described in the Materials and Methods. Plasmids used in transfections were as follows: lanes C-D, wt; lanes E-F, all cysteines to serines (C-); lanes A-B, C- plus 270C; lanes G-H, C- plus L369C; lanes I-J, C- plus E370C; lanes K-L, C- plus E370C/K373C. Size markers are as indicated, as are Pr65Gag monomers (black arrowhead), dimers (grey arrowhead), and crosslinks to a cellular 140-160 Kd protein (Pr65+X; white arrowhead).

Crosslinking of M-MuLV PR- Capsid Mutants

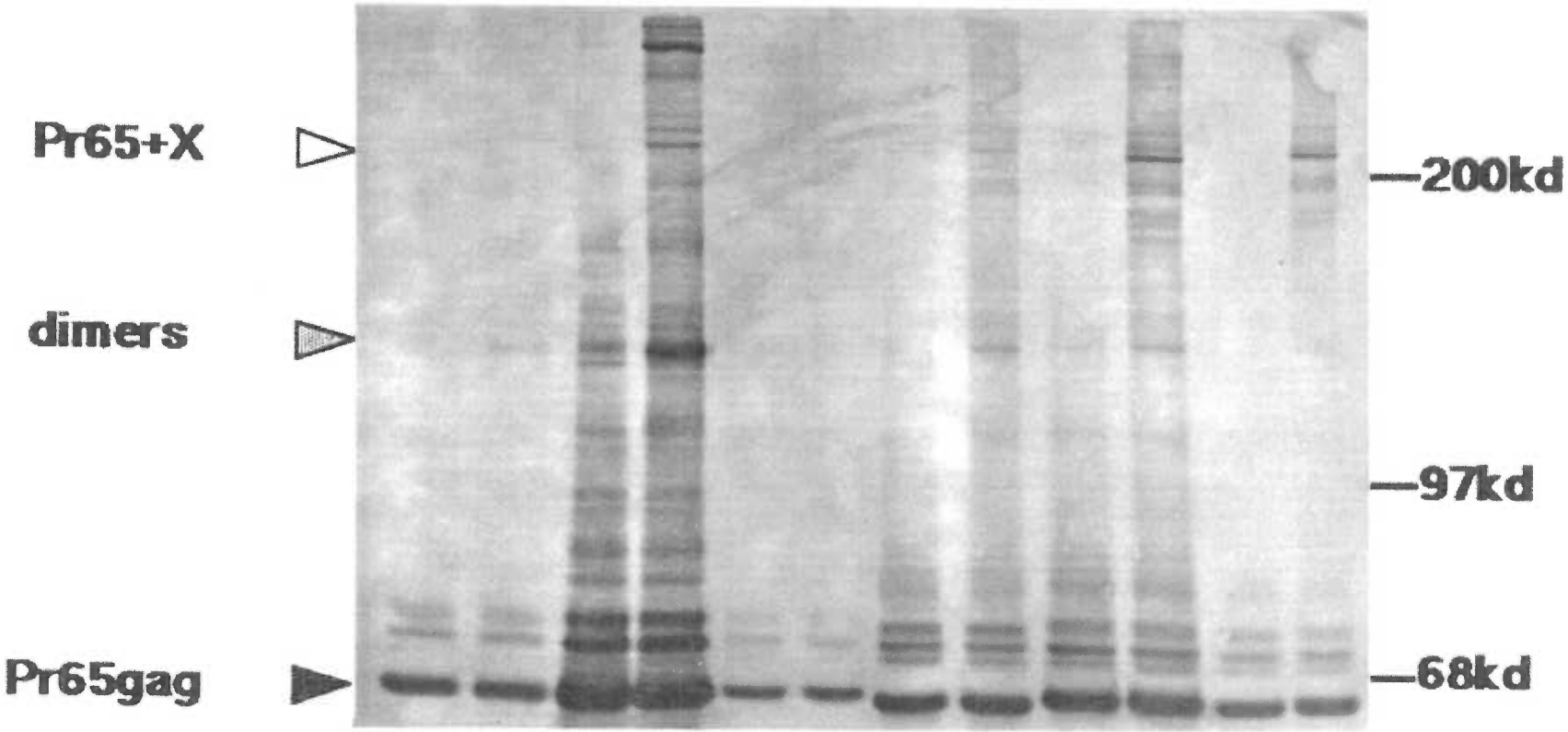


Figure 9. Sucrose density gradient fractionation of M-MuLV particles. Wild type (wt; thick black line), protease-minus (PR-; thin black line), and PR- cysteine-minus (PR- C-; dotted line) extracellular particles were fractionated by sucrose density centrifugation on a 20-50% gradient as described in the Materials and Methods. Fractions were collected and aliquots were assayed for density, and for total Gag protein content by immunoblot detection. Sucrose density (g/ml) is plotted on the x-axis, while Gag protein levels are plotted as a percentage of the most dense band from each immunoblot on the y-axis.

Hansen and Barklis
Figure 9

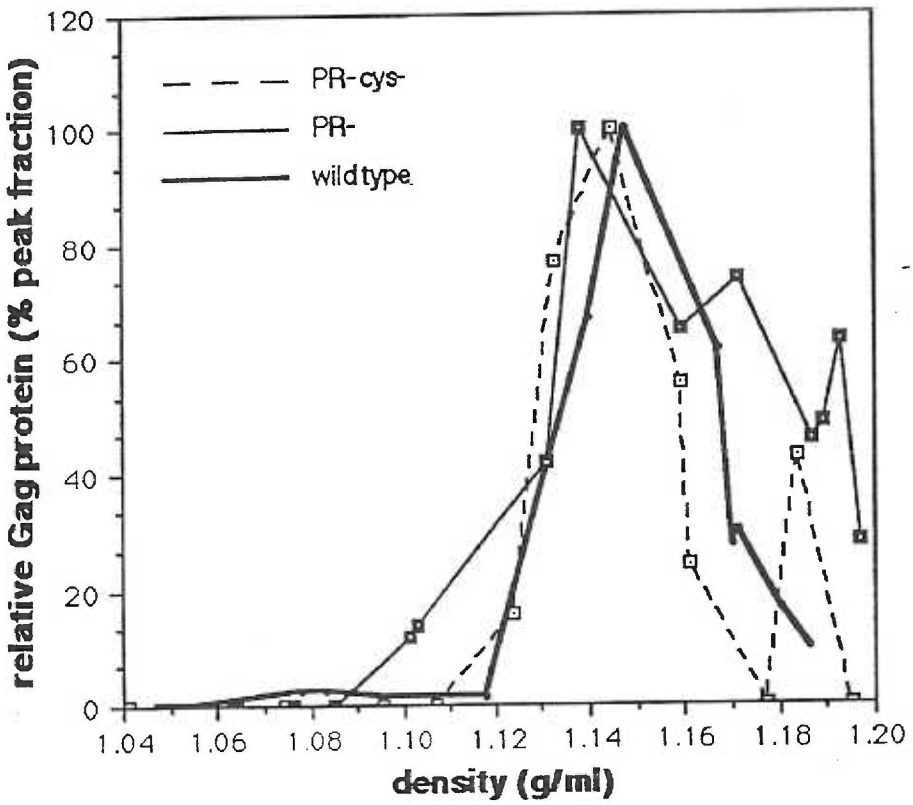
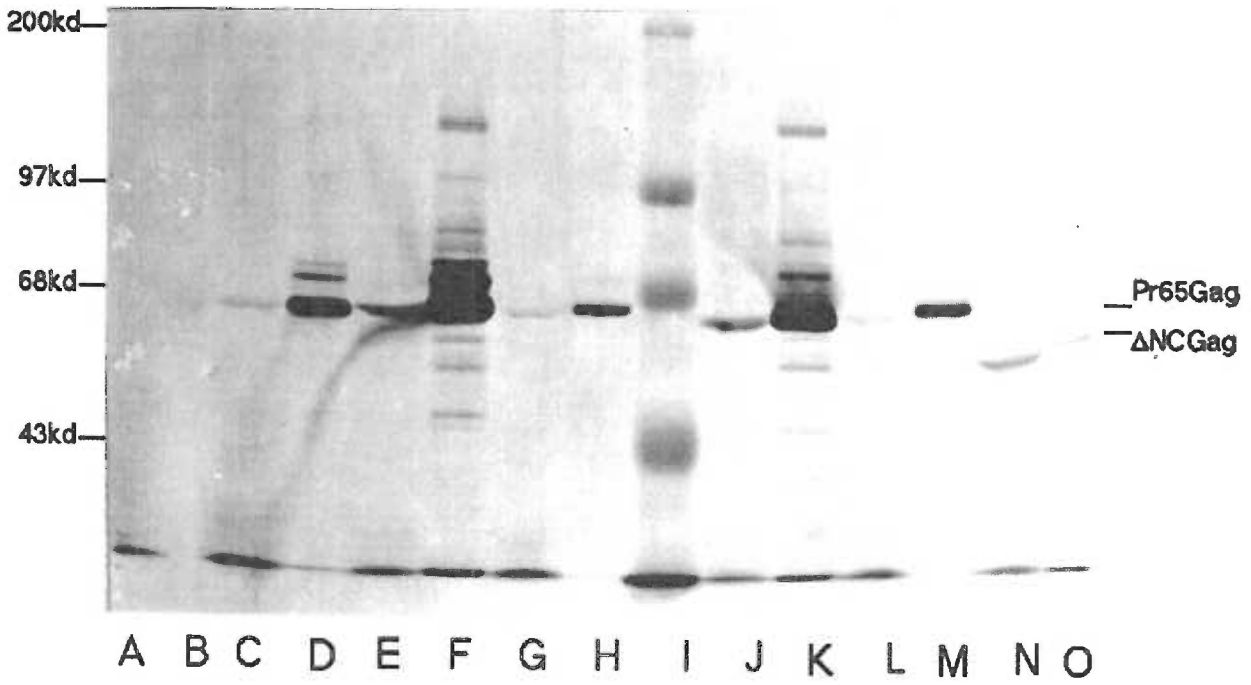


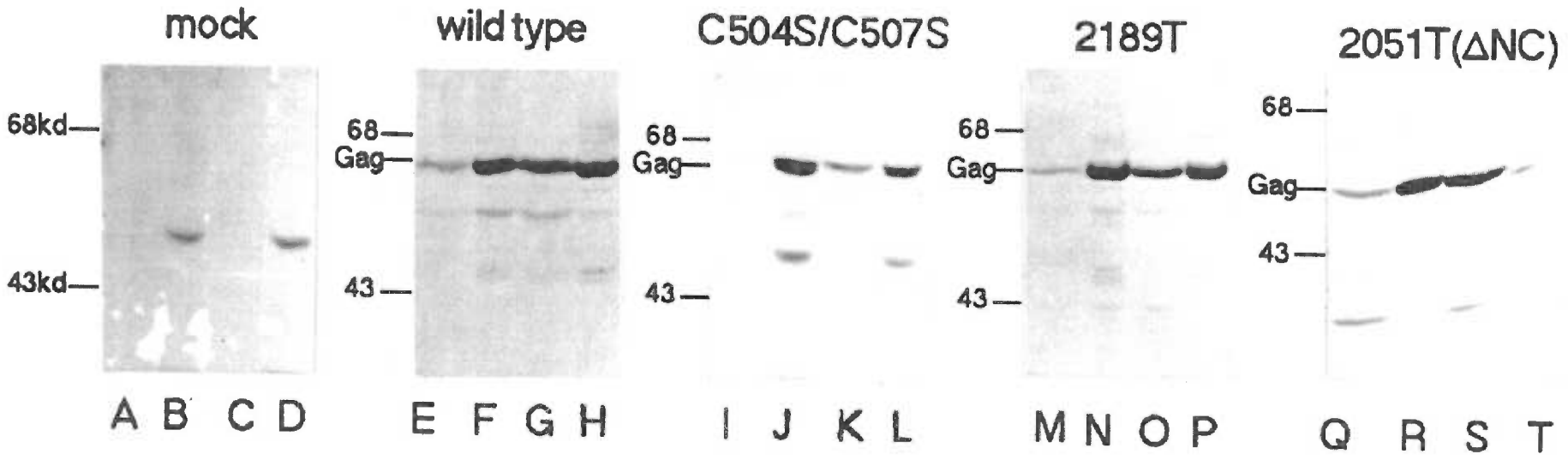
Figure 10. Expression and release of M-MuLV nucleocapsid domain mutant proteins. Cos7 cells were transfected with the designated PR- pXM2453, pXM2189, or pXM2051 derived expression plasmid and, at 72 hrs, cell lysates and supernatants were collected and processed as described in the Materials and Methods. Matching supernatant (lanes B, D, F, H, K, M, and O; corresponding to 50% of the total sample) and cell samples (lanes A, C, E, G, J, L, and N; corresponding to 10% of the total sample) were fractionated by SDS-PAGE on a 10% gel, transferred to a nitrocellulose filter, and Gag proteins were detected with an anti-p30 antibody as described in Figure 3. Samples derive from transfections as follows: lanes A-B, mock; lanes C-D, pXM2453 wt; lanes E-F, pXM2453 C504S/C507S; lanes G-H pXM2453 C504S/C507S/C517S; lanes J-K, pXM2189; lanes L-M, pXM2453 C517S; lanes N-O, pXM 2051 dl. NC. Molecular weight markers were run in lane I, and are indicated at the left, and wt and dl. NC Pr65Gag proteins are indicated on the right.



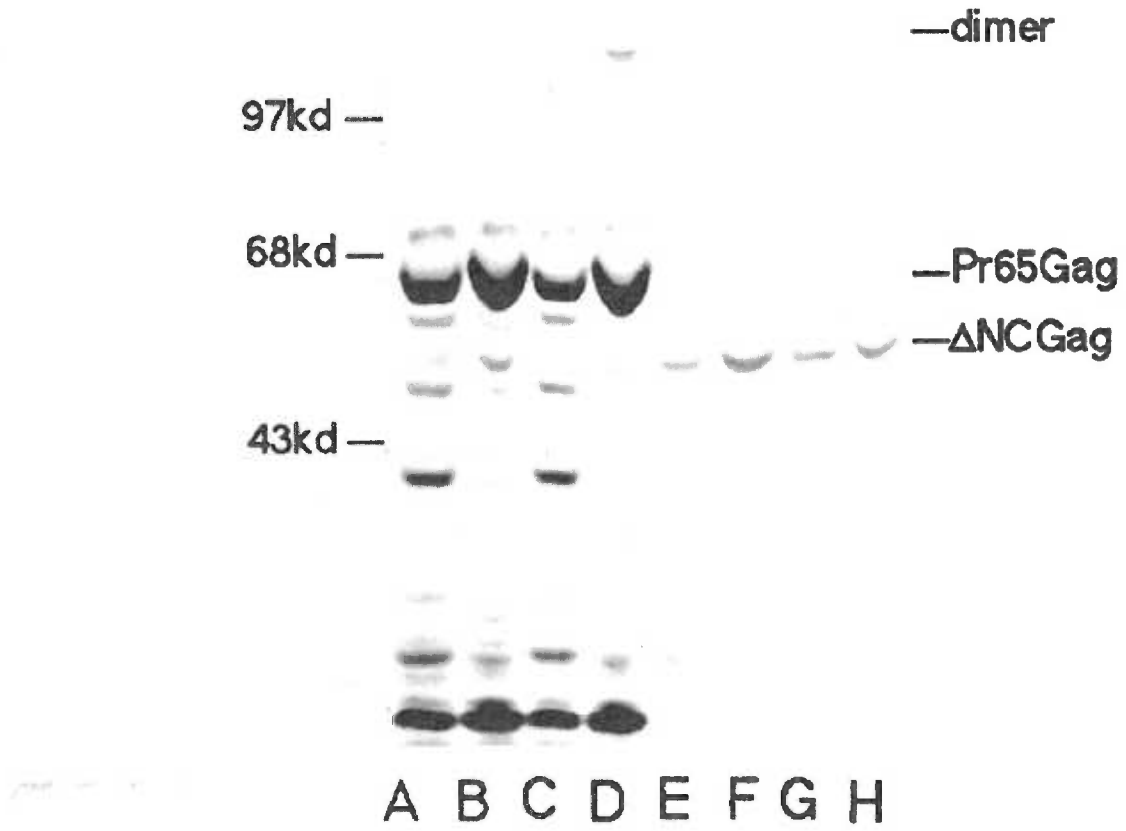
Hansen and Barklis
Figure 10

Figure 11. Intracellular Gag protein fractionation and crosslinking.

A. Stable cell lines expressing the designated protease-minus proteins were established as described in the Materials and Methods. NIH3T3 mock cells (lanes A-D), 3T3 cells expressing wt (lanes E-H), C504S/C507S (lanes I-L), 2189T (lanes M-P), and 2051T (lanes Q-T) proteins were washed in ice-cold PBS, scraped in 1 ml PBS, and pelleted at 630 x g. Cellular pellets were dounced, and centrifuged at 5,000 x *g* at 4 deg C for 5 min to obtain a post-nuclear supernatant (PNS) fraction. PNS fractions were either untreated (lanes A-B, E-F, I-J, M-N, Q-R) or treated with 0.5% Tx-100 (lanes C-D, G-H, K-L, O-P, S-T) for 5 min at 25 deg C prior to centrifugation at 201,000 x g. PNS second supernatants (S2, corresponding to 10% of the total sample, lanes A, C, E, G, I, K, M, O Q, S) and second pellets (P2, corresponding to 33% of the total sample, lanes B, D, F, H J, L, N, P, R, T) were collected, subjected to SDS-PAGE on a 10% gel, transferred to a nitrocellulose filter, and Gag proteins were detected with an anti-p12 antibody. Molecular weight markers and Gag proteins are indicated. B. Intracellular wt (lanes A-D) and NC-deleted 2051T (lanes E-H) Gag proteins were iodine-treated to detect Gag-Gag dimers. Second pellets (P2) from 3T3 cells stably expressing viral constructs were prepared as described in Figure 11A, resuspended in 100 ul PBS, separated into 4 equivalent 25 ul samples and treated with 25 ul water (lanes A, B, E, F), or 25 ul iodine solution for 1 min (lanes C, D, G, H). Reactions were quenched by the addition of 50 ul of 80 mM iodoacetic acid (pH 6.8) and 100 ul of sample buffer with (lanes A, C, E, G) or without (lanes B, D, F, H) B-mercaptoethanol. Samples were immediately heated at 90 deg C for 4 min, electrophoresed, electroblotted, and immunoblotted with an anti-p12 antibody. Molecular weight markers, dl. NC and Pr65Gag proteins, and dimers are indicated.



Hansen and Barklis
Figure 11A


—dimer
97kd —
68kd —
—Pr65Gag
—ΔNCGag
43kd —
A B C D E F G H

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DISCUSSION AND CONCLUSIONS

I. SUMMARY

The three manuscripts that I have presented address aspects of the assembly, transport, and structure of Moloney murine leukemia virus (M-MuLV). The widely accepted model for the assembly of type C retroviruses is that Gag proteins aggregate at the plasma membrane, forming a particle that buds through the plasma membrane (176). Numerous electron microscopy studies have demonstrated that type C retrovirus and lentivirus assembly can take place at the plasma membrane (92, 117, 122). However, the mode of Gag protein transport to the cell surface has not been characterized, although the assumption has been that the proteins traverse the cytosol on their own (176).

Three key observations by Jones et al. (85) pertain to the mechanism of transport and assembly of M-MuLV (Figure 1): M-MuLV Gag proteins, tagged by fusing a B-galactosidase moiety to their carboxy-termini and expressed in the presence of helper virus exhibited two-part immunofluorescence staining, a punctate perinuclear staining and a diffuse cytosolic staining; mutant M-MuLV fusion proteins that were truncated in the matri domain and fused to B-galactosidase exhibited solely the perinuclear staining pattern and were trapped apparently at the endoplasmic reticulum (ER) and/or Golgi apparatus; and truncations that contained the capsid protein exhibited wild type staining patterns and were incorporated into assembling helper virus. These observations suggested a model for both the transport of Gag proteins to the cell surface and their incorporation into retroviral particles. In this model (Figure 1), myristylated Gag proteins associate with intracellular membranes soon after translation and are transported to the cell surface on the cytoplasmic face of vesicles. By this model, the matrix domain contains a signal or region that is necessary for the transport of the protein, and at the cell surface, Gag proteins aggregate and form Gag-Gag interactions that are mediated by the capsid domain.

In a direct examination of the mode of Gag protein transport, virus-producing cells were treated with monensin, a carboxylic ionophore that blocks vesicular transport (165). Monensin treatment reduced Gag protein release by approximately 70%. As a control, in the same cells, monensin treatment reduced by 90% release of the Env protein, which is known to be transported through the Golgi apparatus (176). These results suggest that some, but not all, of Gag proteins appear to be transported to the cell surface by vesicles. At the same time, in an effort to identify transport signals on the MA protein, spans of four codons were inserted into the gag coding region of a Gag-B-galactosidase fusion protein which previously was shown to transport to the cell surface and efficiently incorporate into particles (GBG2051). Two of the three matrix-insertion mutants, 12in 676 and 12in 747, were trapped in a manner similar to the MA truncation mutants. These proteins both contained insertions in the amino terminal third of the matrix protein, while a third mutant protein, which contained an insertion in the carboxy-terminal third of MA, was transported and incorporated into virus particles as efficiently as a wild type protein. These studies supported the hypothesis that Gag protein transport to the plasma membrane involves intracellular membranes, and that Gag proteins contain a transport signal in the amino-terminal third of the MA region.

Four codon inserts into the capsid coding region of Gag-B-galactosidase fusion proteins did not affect protein transport to the plasma membrane. However, five of six capsid-insertion mutant proteins did not incorporate efficiently into assembling virus particles, implying that the capsid domain forms contacts with other Gag proteins, and that capsid-mediated interactions are necessary for particle formation. The specific sites of insertions could be important for either protein-protein contact or effecting the folding of other portions of the Gag protein that are important for protein interactions.

In an effort to characterize the effects of monensin treatment, treated and control virus-producing cells were examined by electron microscopy (EM). Surprisingly, the untreated control cells contained assembled particles, distant from the plasma membrane,

apparently assembling on intracellular membranes. Since repeated EM observations have suggested that type C particle assembly occurs at the plasma membrane (92, 117), this observation in untreated cells was unexpected. The identity of the particles as M-MuLV particles was confirmed by stably infecting naive 3T3 cells and repeating the EM observations. While some particles were formed at the plasma membrane in classic type C fashion, the majority formed on intracellular membranes. The intracellular particles were shown to contain both genomic RNA and viral enzymatic proteins. However, none of our experiments characterized the intracellular as infectious. It is possible that assembly of intracellular particles is a dead-end process and that these particles are not released from cells. However, it also is possible that particles which assemble on intracellular membranes obtain Env proteins from these membranes, are delivered to the cell surface, and are released from cells as infectious particles. We have not been able to distinguish these possibilities. Taken together, manuscripts #1 and #2 suggest that M-MuLV Gag proteins may be transported to the cell surface by two modes. One mode utilizes a vesicular transport pathway in which a matrix-transport signal is important, while a second mode does not utilize vesicles. M-MuLV particles can be formed "intracellularly". However, fates of intracellular particles and their relations to other types of assembly modes are undetermined.

In order to identify structural interactions in retroviral particles, we adapted the cysteine-specific crosslinking protocol of Pakula and Simon. Two cysteine-specific crosslinking agents, molecular iodine and bis-maleimido hexane (BMH), which have atomic radii of approximately 5 and 17 angstroms respectively, were utilized to demonstrate that the particle-associated Gag protein cysteines of the NC domains are packed in close proximity, and could be crosslinked to form Pr65Gag oligomers, from dimers to at least pentamers. For the following reasons, we propose that the NC zinc-finger-motif cysteines are held in close proximity by domains in CA or a non-cys-his motif region of NC: Immature Gag proteins crosslink at the NC cysteine residues, while mature NC proteins do

not crosslink at the cysteines; two of the three cys-his motif cysteines can be substituted with serine, presumably disrupting the zinc finger, and yet the mutant proteins with a singl NC cysteine residue crosslink at that residue; and finally, the capsid domain has been shown to be important in forming Gag-Gag protein interactions.

We used this cysteine-specific crosslinking system to examine structural interactions involving a region in capsid. A cysteine-minus M-MuLV gag gene was create by combining all five cysteine to serine codon mutations. The cysteine-minus gene was used as a template to generate novel cysteine codons in the major homology region (MHR) which has been implicated as an important region in particle assembly (110, 162). Mutant proteins containing these singular, novel cysteines assembled into particles of wild type density. Particles composed of mutant Gag proteins that possessed MHR-cysteines were treated with a cysteine-specific crosslinking agent in order to examine a possible homotypic interaction region between MHR domains of neighboring proteins. The mutant particle-associated Gag proteins formed dimer bands slightly above background control levels, suggesting that the MHR conceivably could be an assembly nucleation domain. The mutants also crosslinked to a putative cellular factor that is 140-160 kd and is present in both 3T3 and Cos7 cells as well as HIV virions. The identity of this factor has yet to be determined.

II. A MODEL OF TRANSPORT AND ASSEMBLY

Commonalities of retrovirus assembly are that the Gag proteins are synthesized, transited through the cytoplasm to the cell surface, and utilized to construct a particle via Gag-Gag affinity interactions. However, the mode of transit to the plasma membrane is unknown. Retroviruses utilize a variety of modes of protein transport and particle assembly, and the order of the steps involving transport and particle construction instead

could be particle construction and then transport. For convenience, I will present a model for the transport and assembly of M-MuLV that incorporates many of the findings in this dissertation, and this model addresses the possibility that the various modes of assembly li on a continuum.

The representation of Gag protein transport and assembly that I propose is that Gag proteins transit to the plasma membrane on the cytoplasmic face of transport vesicles. Fusion of transport vesicles to the plasma membrane would facilitate delivery of Gag proteins to the plasma membrane where they could interact with other Gag proteins in orde to assemble a particle. However, Gag proteins that have high affinity for other Gag proteins could aggregate and form assembly sub-units, or even particles, before departure of the transport vesicle. Overexpression of Gag proteins also could lead to assembly interactions on intracellular membranes, transport, and delivery of a formed particle to the plasma membrane. A receptor could mediate Gag binding to a specific membrane type that is involved in vesicular transport, or a gag receptor could nucleate assembly at the plasma membrane.

I will discuss why this model is relevant to various types of retrovirus assembly and present experimental evidence that supports some of the claims. Caveats will also be discussed. To summarize this model, Gag protein transport to the cell surface could be mediated by transport vesicles, and the site of assembly dependent on the rates of Gag protein synthesis, Gag protein interactions, and transport of Gag-bearing vesicles. Assembly at the plasma membrane occurs when transport is fairly fast and Gag synthesis or interaction slow, while assembly on intracellular membranes occurs when transport is slow and/or Gag synthesis or interaction high.

Type C Gag proteins are thought to transit to the plasma membrane as monomers, interact with other Gag proteins, and assemble particles (176). Electron micrographs depic partially assembled particles on the cytoplasmic face of the plasma membrane (92, 117). Mammalian type C Gag proteins are myristylated, a protein modification that is essential fc

membrane-association (179). Similarly, Gag proteins of avian C-type retroviruses, such a RSV, are membrane associated (10). However, these Gag proteins are not myristylated, and achieve membrane binding via a Gag protein interaction with lipid (10). For all C-type Gag proteins, membrane association is required for assembly (179). The function of membrane association may be two-fold: first, to restrict Gag movement to two dimensions, thereby increasing local Gag protein concentration; and second, to anchor Gag proteins in a way that aligns interaction domains of neighboring proteins. It is not clear when the initial interactions between Gag and membrane take place or which membranes are involved. However, monensin treatment of M-MuLV-producing cells reduced Gag protein release by as much as 70%, suggesting that intracellular membranes were required for particle release. The majority of M-MuLV Gag proteins may associate with intracellular membranes, transit as monomers or low number oligomers through the cytoplasm on the face of transport vesicles, and assemble particles at the plasma membrane. This is compatible with the previous assumptions for type C virus assembly.

In contrast to C-type assembly, type D retroviruses, Mason-Pfizer monkey virus (M-PMV), are thought to assemble particles in the cytoplasm that transit to the cell surface, where they associate with the plasma membrane which contains Env protein (141). Budding through the plasma membrane achieves both envelopment of the particle with a lipid bilayer and incorporation of the Env proteins (141). It is possible that D-type Gag proteins have a higher affinity for one another than do C-type Gag proteins. A higher affinity could explain how type D Gag proteins form particles intracellularly. Although type D Gag proteins are normally myristylated, type D Gag proteins that are myr- can assemble particles intracellularly (140). D-type Gag proteins assemble in a myristylation independent manner, while type C assembly is myristylation dependent. This observation could support the notion that D-type Gag proteins have a higher affinity for one another than do C-type Gag proteins. The mode of transport of the particles is not known. A point that is important in this discussion is whether or not type D Gag proteins are membrane-

associated. Although this has not been biochemically documented, in some electron micrographs intracellular type D particles appear to be enveloped (29).

In an early examination of type D transport, cells producing the type D M-PMV were treated with monensin (29). This treatment did not block transport or release of Gag proteins, but did alter the assembly characteristics (29). Indicative of C-type assembly, partially formed particles were seen at the plasma membrane apparently still in the process of assembly. The concentration of monensin used in the type D study was five logs less than the concentration used in our studies. In fact, this concentration did not block transport of Env proteins to the cell surface, so the secretory pathway was still functional. It is possible that, like M-MuLV, M-PMV utilizes vesicular transport to transit Gag proteins, but in the form of a particle, to the plasma membrane. A consideration in this hypothesis is that intracellular, membrane-associated particles could bud through intracellular membranes. This event could trap the particle in the lumen of an organelle. Perhaps D-type particles are not trapped intracellularly because they remain on the cytoplasmic face of a transport vesicle or they enter the lumen of a transport vesicle.

A second demonstration of the relationship between type C and D assembly characteristics is that a single amino acid substitution in the M-PMV MA protein caused type C-like assembly (141). One explanation is that the high affinity of type D Gag proteins was reduced to that of type C Gag proteins, while a second explanation is that the substitution affected an interaction between Gag protein and the transport machinery. The apparent relation between type C and type D Gag protein assembly suggests that their mod of transport may also be similar. Both may use vesicular traffic to some degree to deliver Gag proteins to the surface, and the status of proteins delivered, either monomeric or oligomeric, could depend on the Gag protein affinity. By this hypothesis, D-type particles and Env protein could be delivered simultaneously to the plasma membrane, where particles that bud acquire their lipid bilayer.

The lentivirus, HIV, is thought to assemble at the plasma membrane (176). However, there are several reports of intracellular assembly (56, 122). HIV Pr55Gag is myristylated, and myr- Pr55Gag does not assemble particles in either HeLa or Cos7 cells (170, 171). However, when expressed in insect cells using a recombinant baculovirus vector, the myr- protein does assemble intracellular particles that are not released (81). Particle formation could result from overexpresssion of the Gag protein which overcomes a typically insufficient affinity of the interaction domains to cause cytoplasmic particle formation in mammalian cells.

A consequence of the model is that Gag proteins that are not membrane-associated could interact with membrane-associated Gag proteins and be incorporated into viral particles by virtue of an affinity interaction. Along these lines, HIV Gag-B-galactosidase (GBG) fusion proteins that were truncated in NC (HIVGBG 2289) or in MA (HIVGBG 831) were incorporated into particles when co-expressed with wild type HIV Gag (171). However, in the same system myr- HIVGBG831 was incorporated inefficiently into particles, while myr- HIVGBG 2289 was incorporated at low levels. The phenotypic difference between these two proteins may be due to the presence of interacting domains of the CA in HIVGBG, and therefore a higher affinity for other Gag proteins. This suggests that interactions between Gag proteins can to a small degree overcome the lack of membrane binding. Although the transport mode of HIV Gag has not been analyzed by monensin treatment in detail, HIV and M-MuLV assembly appears to be fairly similar.

Binding of retroviral Gag proteins to cellular membranes could be specified by a Gag receptor. In terms of the aforementioned model, a Gag receptor could direct Gag to a membrane that forms vesicles which target to the plasma membrane. Alternatively a Gag receptor could serve as an assembly nucleation point on intracellular membranes or at the plasma membrane. Importantly, a Gag receptor is not a requirement in this model. However, at least two molecules have been proposed to serve as Gag receptors, a myristat receptor and the viral Env protein. Evidence in support of a myristate receptor is that type

D Gag proteins that are myr- do form particles, but those particles do not release from cells This suggests that myristylation is somehow important for transport, and possibly that membrane interactions are required for transport (140). A counter-argument to the proposal that myristylation is required for type D transport is that fully 50% of the expressed mutant proteins were initiated at an in-frame initiation codon near the carboxy-terminus of MA that apparently was not used in wild type control proteins. Therefore, half of the mutant proteins were essentially MA deletions, and since MA has been implicated as containing a transport signal, this could account for the transport deficit. A second argument against the necessity of a cellular myristate receptor is that identical gag genes expressed in numerous cell types, from different species, can result in assembly of virus particles on both intracellular and plasma membranes (64, 122, 170). Therefore, the putative cellular myristate receptor is not species, tissue, or membrane specific.

The Env protein also has been hypothesized to serve as a Gag receptor. Env transits to the plasma membrane via the secretory pathway. Interactions between Gag and Env could determine Gag transport on membranes. A strong argument against the utilization of Env as an assembly receptor is that Gag proteins assemble and release in the absence of Env in many systems (176). Env transport mutants, expressed in the presence of Gag, also do not appear to affect Gag particle formation (143). However, in polarized cells, Gag protein expressed in the absence of Env protein forms particles that are released from both the basolateral and apical surfaces, while gag expressed in the presence of Env forms particles that are released preferentially from the basolateral surface (105). This experiment suggests that, alone Gag follows a default transport pathway to either surface, but when Env is present, Gag associates with Env and is transported to the basolateral surface by virtue of Env trafficking signals. Thus Env may influence Gag protein transpor even though it does not play a pivotal role.

To summarize the description of Gag transport to the plasma membrane, monensin treatment altered the assembly of type D Gag proteins (143) and blocked up to 70% of type

C M-MuLV Gag protein release, suggesting that a portion of Gag transport is mediated by a vesicular transport pathway. For this portion of Gag transport, monomeric Gag could b delivered to the plasma membrane by vesicles where Gag proteins aggregate, form specific interactions, and assemble a particle. This is compatible with previous assumptions for type C virus assembly. However, if Gag affinities or concentrations were increased, aggregation could occur at intracellular membranes and capsomeres or whole particles could be transported. This may be the mode of type D particle assembly.

The observation that M-MuLV forms intracellular particles could be a result of high expression of type C Gag or could be due to a difference in a facet of the transport machinery in these cells. Through biochemical characterization, the intracellular particles have been shown to contain genomic RNA and enzymatic proteins. Particles could accrue Env protein by budding through Env-containing Golgi or plasma membrane. The intracellular particles may be transported to the cell surface, or they may be a dead-end assembly route. No experiments delineate between these possibilities.

A caveat that must be stated clearly is that classical type C assembly does appear to take place in all of the systems that I have used. At least 30% of Gag protein release in monensin-treated cells was attributed to some mode other than a monensin-sensitive vesicular pathway, and in all of our electron microscopy studies, at least some particles were observed assembling at the plasma membrane. It should be noted that for type C and lentivirus retroviruses, intracellular particle formation has not been demonstrated to be a viable route for release of infectious particles, and it is possible that it is a dead-end route o assembly. One interesting point is that intracellular particles formed in fusiogenic cells would not need to release, but would need to be cleaved to maturation, in order to be infectious.

III. STRUCTURE

Regardless of the site of retrovirus assembly, Gag proteins must interact to form contacts that are repeated in constructing a virus particle. The structure of an immature M-MuLV virus particle is likely to have icosahedral symmetry. The structure of icosahedra are defined by their axes of two-fold, three-fold, and five-fold symmetry. The ratio of enclosed volume to number of identical subunits employed is maximized in structures with icosahedral symmetry (26). At least nine types of viruses have been crystallized and modeled at below 3.5 angstroms resolution to have icosahedral symmetry (30). Immature HIV-1 particle structure has also been modeled as icosahedral (119). In order to form an icosahedron, each subunit must make at least three contacts that are repeated to build the structure (26). The capsid domain has been shown to be critical in forming Gag-Gag interactions, and it is likely that at least one of the repeated interaction domains lies within capsid (77, 85, 171). In the yeast two-hybrid system, at least two domains of M-MuLV CA facilitate Gag-Gag interactions (K. Alin pers. comm.).

The HIV capsid domain has been modeled as an eight-stranded wedge (5), and the sequence of M-MuLV CA also suggests it could fit the eight-stranded wedge (Argos) model (E. Barklis, pers. comm.). In this model, eight beta-strands form a wedge, and one vertex of the wedge lies at a face of three-fold symmetry (See Figure 2; vertex face is w1, w2), if this modeled subunit were used to construct a spheroid. The Argos model structure is conserved in at least nine icosahedral viruses (30). Th model can be used to map and evaluate M-MuLV mutations in capsid and their role in assembly. Interestingly, of the six mutant M-MuLV capsid proteins with insertions of four amino acids, described in manuscript #1, four map near the vertex face of the model and a fifth maps relativley near the vertex face (See Figure 2). None of these five mutant proteins were incorporated into particles when co-expressed with helper virus. The sixth capsid mutant, which maps distant from the vertex face, was incorporated into particles as efficiently as wild type proteins. This suggests that the vertex face may be a region of capsid-capsid interaction.

While these results, when applied to the Argos model, are exciting and attractive, it is important to consider that it is only a model. A different model of capsid structure predicts that capsid composition is at least 50% alpha-helical and only 12% beta-stranded (22). Furthermore, one beta-strand in the wedge model has been modeled as an alpha helix based on NMR data (D. Peyton, pers. comm.). This region is of particular interest, because it is the major homology region (MHR).

The major homology region of retroviral capsid proteins represents the only region of significant protein homology in CA proteins. The stretch of 20 amino acids contains several positions where residues are invariant or highly conserved, and can be modeled as an amphipathic alpha helix for both M-MuLV and HIV. Amino acid substitutions of MHR residues can block particle assembly (110, 162) In a direct examination of the possibility that the MHR forms homotypic interactions in virus particles, MHR residues on either face of the putative amphipathic helix were substituted with cysteine. These MHR cysteine substitutions were generated on a cysteine-minus (cys-) template, so that the MHR cysteines were novel in the mutant Gag protein. The rationale in generating novel cysteine residues is that cysteine-specific crosslinking agents with small atomic radii can be utilized to crosslink cysteine residues that are in close proximity on neighboring proteins. Crosslinker treatment of MHR cysteine-containing Gag proteins produced Gag protein dimers slightly above background crosslinking levels, suggesting that some of the MHR cysteines were packed in close apposition in immature particles. However, when the MHR cysteine mutants were expressed in a protease-competent background, CA-CA dimerization was not observed. The difference in MHR-cysteine-mediated crosslinking between immature and mature Gag proteins suggests either that the crosslinker was not accessible to the mature proteins, or that there is a structural rearrangement following proteolysis, which is consistent with electron microscopy observations (117).

Cysteine-specific crosslinking was used to identify structural interactions within the nucleocapsid domain. The M-MuLV nucleocapsid contains three cysteine residues that are

part of a cys-his box. Retroviruses in general possess a cys-his-motif that functions as a zinc finger in binding the genomic RNA (179). When immature M-MuLV particles were treated with a cysteine-specific crosslinker, Pr65Gag molecules were crosslinked to form oligomers from dimers to at least pentamers. The cysteine residues involved in crosslink bond formation were identified as the cysteines in the cys-his-motif by substitution of all the Gag cysteines singularly or in combination to serine. In order to form the observed Pr65Gag tetramers, at least two residues must form intermolecular bonds. Therefore, at least two of the NC cysteines are packed in close proximity in immature particles. One of the NC cysteine residues that can be crosslinked is C39. Substitution of both of the other two NC cysteines with serine still resulted in dimer formation. Taken together, the observations that cys-his-motifs are tightly packed in particles, and can be crosslinked at a single cysteine when the motif is presumably disrupted, suggest that the region is held in close proximity by either regions of CA or non-cys-his-motif regions of NC.

Mature NC proteins in particles do not crosslink, indicating that there is a structural rearrangement of NC interactions, and possibly that it is capsid that tethers NC motifs in immature Gag. However, the NC protein apparently does contribute to the assembly of particles as NC-deletions do not efficiently assemble particles. HIV and M-MuLV Gag fusion proteins lacking NC were less efficiently incorporated into particles than wild type proteins (85, 171). Nevertheless, the wild type structure of the cys-his-motif is not essential for particle assembly, as amino acid substitutions in this region, including all NC cysteines to serine, do not disrupt particle formation (46, 47, 68, 69). These results support the notion that non-cys-his-motif regions in NC are important for assembly.

A candidate region in NC is the basic amino acid stretch of retroviral NC proteins that is proximal to the cys-his-motif(s). An RSV Gag protein truncated in CA assembled particles of light density. However, assembly efficiency and particle density were restored by fusing a region of NC containing the basic amino acid stretch and a single cys-his-motif to its carboxy-terminus. Amino acid substitutions of all of the basic residues in the HIV

basic amino acid stretch did not block particle assembly, suggesting that the basic residues of this region are not critical for particle assembly.

Summarizing nucleocapsid protein structural information, NC protein appears to play at least a minor role in particle assembly and protein packing. Disruption of the M-MuLV or HIV cys-his-motif does not affect particle assembly or density, yet the presence of little more than a single cys-his-motif can apparently provide a protein interaction domain. One explanation is that more than one region of NC, including the cys-his-motif, can provide a protein interaction that contributes to particle assembly and tight packing of Gag proteins.

IV. LAST WORDS

A final point about chemical crosslinking is that cysteine-specific crosslinking is a useful method for indentifying protein-protein interaction domains. In this case, a single mutation can be evaluated in the context of both immature and mature Gag proteins. Chemical crosslinking agents previously have been used to analyze protein interactions within retrovirus particles (60, 132). In an avian retrovirus, mature Gag proteins were crosslinked to form homotypic dimers of each Gag protin. No heterotypic dimers were formed (132). These observations suggest that mature Gag proteins align in homogenous strata which is consistent with electron microscopy studies (61). However, these studies utilized an amine-reactive crosslinker (DMS), and the multiple reaction target sites precluded the identification of specific residue contacts.

Cysteine-specific crosslinking can be used to identify specific residue contacts between Gag proteins or between Gag and cellular proteins. Interestingly, cysteine-specific crosslinking of particles composed of Gag proteins with an MHR cysteine produced a novel product at approximately 215 kd. Since the mutant Gag proteins were expressed in an Env and Pol minus system, and they contained a single cysteine residue,

which should preclude trimer formation, it is likely that the novel product consists of Gag crosslinked to a non-viral protein that is 140-150 kd. This factor is present in 3T3 and Cos7 cells, as well as in HIV particles. The identity of this factor and its importance in the assembly of particles remains a mystery.

The aim of the work presented in this dissertation is to contribute to the understanding of retrovirus protein transport and assembly. The findings of this research include:

(1) The transport of M-MuLV Gag proteins sensitive to monensin, and therefore, maybe mediated, in part, by transport vesicles.

(2) The M-MuLV MA protein appears to possess a signal that is necessary for the transpo of Pr65Gag to the cell surface.

(3) Regions of the M-MuLV CA protein are important for capsid-capsid interactions that facilitate incorporation of Pr65Gag into assembling particles.

(4) M-MuLV in Psi2 and 3T3 cells forms intracellular particles that may or may not be infectious.

(5) M-MuLV intracellular particles contain genomic RNA and the pol-encoded enzymatic proteins.

(6) The cysteine residues of the M-MuLV cys-his-motif can be crosslinked by cysteine-specific crosslinkers, and are packed in close proximity in immature particles.

(7) The zinc finger motifs are held in close proximity by regions in CA and/or non-cys-hi motif regions in NC.

(8) Cysteines substituted into the major homolgy region (MHR) of M-MuLV mediate chemical crosslinking of immature Gag proteins to levels above background.

(9) A non-viral protein of 140-150 kd is incorporated into M-MuLV particles and interact with Pr65Gag at the MHR.

Figure 1. **Model of Gag protein assembly into particles.**

According to this model, the M-MuLV Pr65Gag protein is co-translationally myristylated and associates with the cytoplasmic face of intracellular membranes. Myristylated Pr65Ga (indicated by the M and wavy line) travels to the plasma membrane by vesicular transport where it may be released in low-density vesicles (I) or into virus particles (II), depending on capsid protein interactions. Viral RNA (R) is assembled into virions by virtue of association with the carboxy-terminus of Pr65Gag and/or Pr180Gag-Pol. (Taken from Jones et al. (85).)

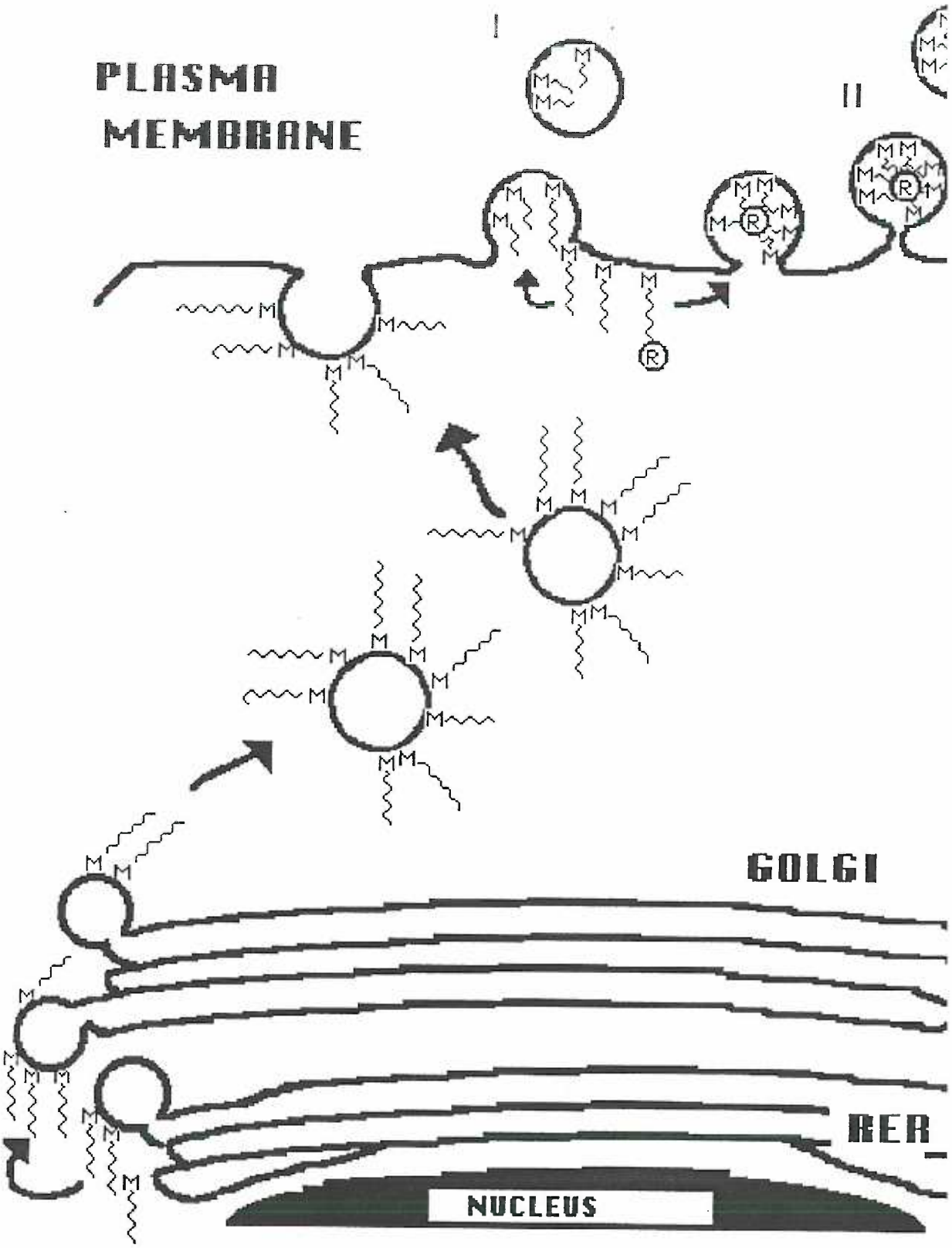
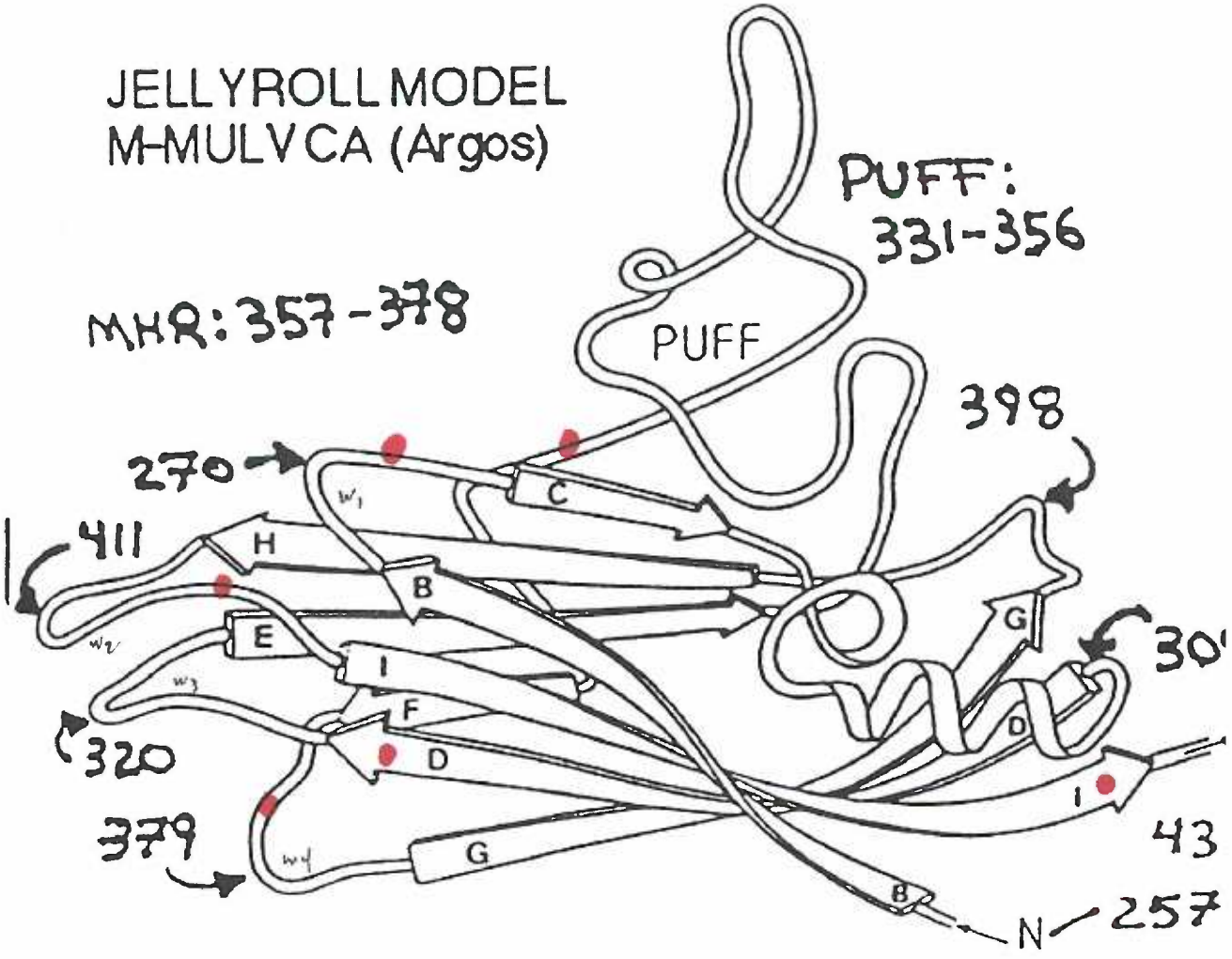

PLASMA
MEMBRANE
I
II
GOLGI
RER
NUCLEUS

Figure 2. **Eight-stranded wedge model of the capsid (CA) domain of the M-MuLV Gag protein.** The eight-stranded wedge model, adapted from Argos (5), predicts that CA will form a B-barrel, and is based on comparison with other viral capsid proteins. Using the codon assignments from Figure 2 of the Introduction, CA extends from 216-478. As modeled here, the major homology region (MHR) and puff region cover residues 357-378 and 331-356, respecively. The eight B-strands comprise codons as follows: B, 257-268; C, 272-277; D, 304-317; E, 323-330; F, 368-375; G, 383-397; H, 400-408; I, 414-431. As modeled by Argos (5), the upper and lower parts of the figure are located on the exterior and interior of capsid shells, respectively. If an SV 40 VP-1 like conformation is assumed (102), left and right hand sides are outside and inside, respectively. For six capsid protein mutants, the approximate sites of the four codon insertions within CA are indicated with a red dot. The construct names followed by the CA codon numbers at insertion site are as follows: 12in1436, 273; 12in1560, 314; 12in1672, 351; 12in1752, 378; 12in1862, 415; 12in1908, 430. (Adapted from E. Barklis)


JELLYROLL MODEL
M-MULV CA (Argos)
PUFF:
331-356
MHR: 357-378
PUFF
398
270
411
320
379
30
43
257
N
C
H
B
E
I
F
D
G
w1
w2
w3
w4

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