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Analysis Of Regions In The Envelope Glycoproteins Of Rous Sarcoma Virus Required For Virion Assembly And Infectivity.

Jianyun Dong
University of Alabama at Birmingham

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Dong, Jianyun, Ph.D.

University of Alabama at Birmingham, 1992

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

ANALYSIS OF REGIONS IN THE ENVELOPE GLYCOPROTEINS OF ROUS SARCOMA VIURS REQUIRED FOR VIRION ASSEMBLY AND INFECTIVITY

by

JIANYUN DONG

A DISSERTATION

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ABSTRACT OF DISSERTATION
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Title Analysis of Regions in the Envelope Glycoproteins of Rous Sarcoma Virus Required for Virion Assembly and Infectivity

The envelope glycoprotein of Rous sarcoma virus (RSV) is translated as a single polyprotein precursor which is then cleaved into two subunits, SU and TM, by cellular enzymes in late Golgi compartment. The amino acid sequence required for the intracellular cleavage of the glycoprotein precursor was investigated by introducing mutations into the region encoding the cleavage recognition site (Arg-Arg-Lys-Arg). In addition to mutants G1 (Arg-Arg-Glu-Arg) and Dr1 (Deletion of all 4 codons) investigated previously, two additional mutants were constructed: Ar1 (Arg-Arg-Arg-Arg) in which the highly conserved lysine is replaced by an arginine and S19 (Ser-Arg-Glu-Arg) in which no dibasic pairs remain. The results of these studies demonstrated that when the cleavage sequence was deleted (Dr1) or modified to contain unpaired basic residues (S19), intracellular cleavage of the glycoprotein precursor was completely blocked. Transport to the cell surface occurred normally with each of the mutant proteins. However, whereas the Dr1 product was resistant to cleavage by exogenous protease, the S19 protein was completely susceptible. This demonstrates that the cellular endopeptidase responsible for cleavage had a stringent requirement for the presence of a pair of basic residues (Arg-Arg or Lys-Arg). Furthermore, it implies that the cleavage enzyme is not trypsin-like, since it is unable to recognize arginine residues that are sensitive to trypsin action. Substitution of the mutated genes into a replication competent avian retrovirus genome showed that cleavage of the glycoprotein precursor was not required for incorporation into virions but was necessary for infectivity. Treatment of turkey cells, transfected with a RSV proviral genome containing the S19 mutation, with low levels of trypsin resulted in the release of infectious virus, demonstrating that exogenous cleavage could generate a biologically active glycoprotein molecule.

Envelope glycoproteins of retroviruses appear to be selectively incorporated into the virion during the budding process, while the cellular membrane proteins are excluded. The protein sequence requirement and the specificity of envelope glycoprotein incorporation into RSV was analyzed using two chimeric HA proteins. In the first, the signal peptide of the RSV envelope glycoprotein was fused in-frame to the entire structural sequence of the HA gene so that an authentic HA protein would be released upon signal peptide cleavage. In the second, the entire transmembrane and cytoplasmic domains of the HA protein were also replaced with those from the RSV *env* gene. When expressed from a SV40 vector in CV-1 cells, both genes yielded functional HA proteins that were transported to the cell surface and retained hemagglutination activity. When either of the chimeric HA genes were co-expressed with the *gag* gene of RSV in a vaccinia-T7 RNA polymerase expression system, the chimeric HA proteins were incorporated into RSV virions. This result indicated that the presence of the transmembrane and cytoplasmic domains form the RSV glycoprotein did not facilitate HA glycoprotein incorporation into virions. When chimeric HA genes were substituted into an RSV proviral vector in the place of the *env* gene, both chimeric genomes yielded virus that could infect avian and human cells with a similar efficiency. These experiments demonstrated that RSV has a limited selectivity for glycoprotein incorporation and that a foreign glycoprotein can confer a broadened host range on the virus. A mechanism is proposed to explain how viral glycoproteins can be incorporated into virions while cellular membrane proteins are excluded.

A novel complementation assay for studying viral assembly and analyzing the incorporation of mutated *env* gene products and foreign glycoproteins into RSV was also described. In this system, assembling virions that were morphologically indistinguishable from those released from wild type virus infected cells were demonstrated.

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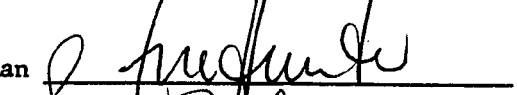
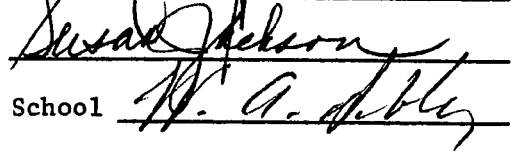
Program Director 

Date Oct 6/1992 Dean of Graduate School 

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LIST OF ABBREVIATIONS

A	the adenine base of a nucleotide (when in nucleic acid sequence) or alanine residue (when in peptide sequence)
A$^-$	a mutated forme of envelope glycoprotein lack of the transmembrane and cytoplasmic domains
Ar1	an env gene mutant carriying a argenine residue substitution for the lysine residue in the cleavage-activation site
ASLV	avian sarcoma leucosis virus
AUG	protein synthesis initiation codonin ribonucleic acid
ATG	protein synthesis initiation codonin deoxyribonucleic acid arg arginine
BHRCAN	a provirus vector of Shmid-Rupin A strain of Rous sarcoma virus
BHRCAN-Hisv	BHRCAN vector containing a hygromycin resistant gene under the control of SV40 virus early promoter
C	the cytidine base of a nucleotide (when in nucleic acid sequence) or cystine residue (when in peptide sequence)
°C	degrees centigrade
cpm	counts per minute
CV-1	African green monkey kidney cells
D.L.S.	dimer linkage site
DNA	deoxyribonucleic acid
Dr1	env gen mutant carrying a deletion of the cleavage-activation site
dr	direct repeated sequence
E	glutamic acid
env	envelope glycoprotein gene
ER	endoplasmic reticulum
G	the guanine base of a nucleotide (when in nucleic acid sequence) or glycine residue (when in peptide sequence)

G1	env gene mutant carrying a glutamic acid for lysine substitution at the cleavage site of the envelope glycoprotein of Rous sarcoma virus
gag	retrovirus nonglycoprotein structural protein genes (group associated antignens of retroviurs)
Glu	glutamic acid residue
gp	glycoprotein
h	hours (s)
HA	hemagglutinin glycoprotein of influenza virus
HIV	human immuno deficiency virus
HTLV	human T-cell leukemia virus
I	isoleucine residue
L	Leucine residue
Lys	Lysine residue
LTR	long terminal repeat
M	molar
mCi	milliCurie (s)
M	methionine
Met	methionine
µCi	microCurie (s)
µg	microgram (s)
µl	microliter (s)
mm	millimeter (s)
mM	millimolar
MoMuLV	Moloney murine leukemia virus
M-PMV	Mason-Pfizer monkey viurs
mRNA	messenger ribonucleic acid
MuLV	murine leukemia virus

NA	neuraminidase
P	proline residue
pol	retroviral RNA-dependent DNA polymerase gene
Pr	protein precursor
Q	glutamine residue
QT6	a chemically induced quail tumer cell line
R	argenine residue or repeat region (when in retroviral genome)
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
rpm	revolutions per minute
RSV	Rous sarcoma virus
S	serine residue
SA	splicing acceptor site
SD	RNA splicing donor site
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophosis
Ser	serine residue
SFV	Semliki forest virus
src	oncogene of Rous sarcoma virus
SRP	signal recognition particle
SV40	simian virus 40
SU	surface protein of the envelope glycoprotein complex
T	thymidine or threonine (when in peptide sequence)
TEF	turkey enbryol fibroblast cells
TM	transmembrane protein of the envelope glycoprotein complex

tRNA	transfer ribonucleic acid
U3	3'-end unique region of a retroviral genome
U5	5'-end unique region of a retroviral genome
V	valine residue
VSV-G	vescular stomatitis virus glycoprotein
Y	tyrocine residue

INTRODUCTION

I. ROUS SARCOMA VIRUS, A MEMBER OF THE RETROVIRUSES

A. History and Classification of Rous Sarcoma Virus

During the last two decades, a great amount of effort has been focused on the basic biology and genetics of retroviruses (or RNA tumor viruses) due to their association with neoplastic diseases. These studies have further intensified since the discovery of human immunodeficiency virus (HIV), the retroviral pathogen that causes acquired immune deficiency syndrome (AIDS) (5, 49, 104, 123). A great amount of information on the molecular biology of retroviruses has been obtained during this period of time. It is anticipated that this knowledge will lead to the development of effective prevention mechanisms and treatments for the diseases caused by retroviruses, as well as to the use of these viruses for other beneficial purposes, such as vectors for genetic therapy.

Although retroviruses can infect an extraordinarily diverse group of animals and can cause a vast array of pathological consequences through a variety of mechanisms, they are unified by the nature of their genomes, their means of entering cells, their biochemical composition, virion structure, and their mechanisms of replication. They all have an RNA genome and an RNA dependent DNA polymerase (reverse transcriptase), which enable them to replicate via a DNA intermediate that is integrated into the host genome. This is the most important feature that distinguishes this family of viruses from DNA and other RNA viruses.

Rous sarcoma virus, an avian retrovirus, was among the first retroviruses to be isolated. It was discovered in 1911 by Peyton Rous in an effort to identify the pathogen that caused tumors in Plymouth Rock chickens (116). Since then this virus has been

used as the prototype retrovirus for studies of biochemical composition, genome structure, mechanisms of replication, and oncogenesis. The name Rous sarcoma virus is now shared by a number of independently isolated chicken viruses which induce sarcomas via similar genetic mechanisms.

B. General Features of a Rous Sarcoma Virus Virion

The schematic diagram of an RSV virion is shown in Figure 1. As with other retroviruses, RSV contains a pair of positive strand RNA molecules of identical genetic content that are encapsidated in a protein shell-the capsid. The genomic RNA is bound by nucleocapsid proteins (NC), which serve to protect the genomic RNA and which are involved in the process of RNA encapsidation. The virus-coded reverse transcriptase is associated with the genomic RNA inside the nucleocapsid. The major building block of the capsid is a 27 kilodalton-protein, the major capsid protein (CA), or p27. While the structure of the CA protein has not been determined, structure predictions have been made based on the amino acid sequence, and it has been speculated that, as with the capsid protein of the picornaviruses, it may form an eight-strand antiparallel ß-barrel with additional loops and extensions that provide structural variations in different viruses (2, 50). This structure serves as building block for the protein shell. Outside the capsid is the matrix protein (MA), the exact function of which is not yet clear, although it is known to be important for the morphogenesis of retroviruses (108, 152). A lipid bilayer derived from the host cell during the viral budding process forms the viral membrane and is the outermost layer of the virion. The envelope glycoprotein complex, seen as knobbed-spike structures in electron microscopy (13, 109), is anchored in the viral membrane. These glycoprotein complexes appear to be oligomeric structures (36), trimers in the case of RSV, and probably tetramers in the case of HIV (119). Each monomer is comprised of two subunits, the transmembrane protein (TM) and a surface protein (SU). In RSV the two subunits remain associated on the cell surface through a disulfide bond (79).

C. Genome Organization of Rous Sarcoma Virus

Like all retroviruses, RSV is a true parasite that relies on the host for its replication. Its genome is relatively small, yet it can replicate itself effectively with a minimal number of viral genes via the host biosynthetic machinery. The virion-associated genetic material is a dimer structure, in which two identical genomic RNAs are held together at one end by a dimer-linkage structure (8, 22, 29, 76). An RSV genomic RNA is schematically diagrammed in Fig. 2. It is 8 to 10 Kb in size and flanked by a terminally redundant sequence (R region), as well as sequences that are unique to each end of the RNA (U_3 and U_5) (23, 121, 130). Near the 5' end of the genomic RNA, there is a primer binding site for initiation of DNA synthesis by a cellular tRNA, and sequences involved in the packaging of genomic RNA into the capsid (106, 134). The viral RNA is post-transcriptionally modified by the host in a way identical to the cellular mRNA molecules. It has a 5'-terminal m^7Gppp capping group (44, 72), a 3' poly(A), and some internal methyl groups (57, 78).

RSV has the basic genes required to be replication competent. These are the *gag*, *pol*, and *env* genes. In addition the virus possesses an oncogene, *src*. The *gag* region encodes the internal structural proteins of the virion. Translation of these sequences results in a high molecular-weight polyprotein precursor that is subsequently cleaved to generate the mature proteins: p19, p10 p27, p12, and p15 (132). The *pol* region encodes the viral RNA-dependent DNA polymerase with its various enzymatic functions. Translation occurs as read through of *gag* and *pol* sequences by a frame shifting mechanism, resulting in a precursor that is subsequently processed into the one or two pol polypeptides (66, 69, 77). The *env* region encodes the envelope glycoproteins found on the surface of the virion envelope (11, 14, 15). The product of the oncogene, *src*, is responsible for the rapid transformation of infected fibroblasts *in vitro* and is not required for viral replication. It is translated from a spliced subgenomic

mRNA into a 60 kilodalton phosphoprotein that is analogous to a cellular protein tyrosine-kinase (24, 39, 40).

D. Life Cycle of a Retrovirus

Although in reality a viral life cycle is a continuous process, for the convenience of description it can be divided into the following stages: (a) the envelope-mediated viral entry into the host, (b) viral genome replication and integration, and (c) protein synthesis, virus assembly, and budding. A simplified life cycle of a retrovirus is diagrammed in Figure 3.

1. Virus entry mediated by envelope glycoprotein protein Infection begins with the absorption of virions to the cell surface of a susceptible host (25, 28, 68). This is mediated by specific binding of viral glycoproteins to receptors on the target cell. The binding event not only attaches the virion to the cell surface but also induces fusion of the viral and cellular membranes, presumably by exposing the sequestered fusion peptide (11, 46, 73, 149). The viral nucleocapsid is then introduced into the cytoplasm of the cell.

2. Viral genome replication Once the viral nucleocapsid is inside the host cells, it probably disassembles to allow the access of cell-derived precursors and DNA synthesis to occur. In a complex process, the virally coded reverse transcriptase which is associated with the genomic RNA then begins to catalyze the synthesis of the first strand of DNA that is complementary to the RNA genome of the virus. It is this unique reverse-transcription activity that gives this family of viruses the name retroviridae. The process of viral genome replication is schematically diagrammed in Figure 4. The DNA synthesis is primed by a cellular tRNA that binds to the primer binding site near the 5'-end of the genomic RNA. DNA polymerization is carried out in the 5' direction of the nascent DNA molecule which results in a short piece of DNA containing the R and U_5 region. This fragment of DNA then "jumps" to the 3' end of the RNA where it binds to the R region through base pairing and serves as a primer for the synthesis of

the complete DNA that is complementary to the genomic RNA (56). During or after the synthesis of the first strand of the DNA, the genomic RNA is digested by an RNaseH activity of the *pol*-gene products, and a second strand of DNA is synthesized to produce a linear double stranded DNA molecule in the cytoplasm. As a result of these jumping mechanisms, the U5 and U3 regions are duplicated which results in two identical long terminal repeats (LTR) flanking the viral genome. This linear DNA is then integrated into the host genome following migration into the nucleus (71, 148). The integration event is catalyzed by viral integrase and is mediated by the two LTRs. Once the viral DNA is integrated, it defines the provirus stage of the viral life cycle in which the viral genome co-exists and replicates with the host genome.

A provirus that has integrated into the host genome is transcribed by the host RNA polymerase II as if it were a cellular gene (66, 69, 77, 87). Transcription of the full-length viral RNA starts at the promoter in the 5'-end LTR and terminates at the polyadenylation site within the 3'-LTR (87, 157). These positive-stranded RNAs then enter one of two pathways. Full length RNAs are directly transported to the cytoplasm, where some of them will be translated into *gag* and *pol* gene products, and others will be packaged into virions. The second class of RNAs are spliced into subgenomic mRNAs within the nucleus before migrating to cytosol where they are translated into envelope glycoproteins and the *src* gene product (60, 131).

3. Viral protein synthesis, virion assembly, and budding In RSV three different species of mRNAs are translated into viral proteins, two of which are produced by an RNA splicing mechanism as diagrammed in Figure 5 (131). This single promoter-RNA splicing mechanism ensures the correct ratio of Gag and Env protein synthesis for virion assembly at the transcription level. The Gag proteins are translated from the full length viral RNA as a single polypeptide precursor of 76 KD, which is further processed by a viral protease (p15) that cleaves the molecule to yield the internal virion proteins late in the maturation pathway (96). The *pol* gene product (reverse

transcriptase) is synthesized as part of a large precursor of 180 KD ($Pr180^{gag}$) that includes the *gag* components in addition to the polymerase moiety. The translation of $Pr180^{gag}$ is the result of suppression of the stop codon at the end of *gag* gene by a minus-one frameshift which occurs 2 to 5 percent of the time (66). As a result, virions contain 20 to 50 fold fewer Gag-Pol molecules than Gag precursors (132). The incorporation of the Gag proteins as precursors ensures an equi-molar ratio of the proteins that form the capsids.

Different sub-types of retrovirus assemble the capsid at different locations in the cell. D-type retroviruses, for example Mason-Pfizer monkey virus (M-PMV), assemble capsids in the cytoplasm. These assembled capsids then migrate to the cell membrane where they incorporate envelope protein. For C-type viruses, such as RSV and HIV, capsid assembly occurs beneath the cell membrane, and capsid assembly, glycoprotein incorporation, and virion budding occur simultaneously (10, 52, 161). The exact pathway for *gag* protein transport is not yet clear, but is presumably through the same pathway as some of the cytosolic proteins. The signals that direct the C-type and D-type Gag proteins to different locations are yet to be determined.

On the other hand, the pathway for envelope glycoprotein biosynthesis and transport is well defined. Envelope proteins are synthesized and transported in the secretory pathway of the host cell. The protein is first translated into a protein precursor of 95 KD (Pr95) from a subgenomic mRNA on membrane bound polyribosomes. During its transport through the Golgi compartments, the precursor is progressively modified by cellular enzymes. It is glycosylated, proteolytically cleaved, and transported to the cell surface where it is incorporated into virions (these aspects of envelope glycoprotein biosynthesis will be discussed in detail in the following sections).

Virions leave the infected cells through a budding process, taking with them a lipid bilayer and the envelope glycoproteins that are embedded in the membrane. After

budding from the cell surface the capsid undergoes a morphological change, and the center of the core becomes highly condensed. Associated with this morphological change is the cleavage of the gag-protein precursor into mature proteins by a viral proteinase (3, 97, 118, 141). Although what triggers this change is not known, it is possible that this post-budding modification enables the capsid to disassemble once it re-enters an infected cell.

II. ENVELOPE GLYCOPROTEINS OF RETROVIRUSES AND RSV

A. Basic features of the envelope glycoprotein

Although the envelope glycoprotein complex of retroviruses is not required for the assembly and budding of enveloped virus particles, it does play a critical role in the virus replication cycle by recognizing and binding to specific receptors and by mediating the fusion of viral and cell membranes (91). Therefore virus particles lacking envelope glycoproteins are non-infectious.

The envelope glycoproteins of RSV and other retroviruses share an overall structural similarity despite their differences in size and amino acid composition. The knobbed-spike structures of the envelope glycoproteins of several retroviruses have been shown to be multimers of SU and TM proteins. The results of sucrose gradient sedimentation analysis suggest that the glycoprotein complex is most likely a trimer in RSV and MuLV, and a tetramer in HIV (70, 75, 119, 138). The basic organization of each monomer is defined by three hydrophobic or apolar regions that are arranged along the envelope molecule, a signal sequence, a fusion peptide, and transmembrane domain (Figure 6).

The signal peptide is a short stretch of hydrophobic amino acids that directs the nascent *env* gene product into the secretory pathway of the infected cell. It is located at the amino-terminus and is removed during translocation by a cellular signal peptidase from the nascent *env* gene product.

The transmembrane domain is a longer hydrophobic region that stops the translocation process, anchors the molecule in the membrane, and causes it to span the lipid bilayer. The molecule is oriented with its N-terminus outside and its C-terminus in the cytoplasm--a type 1 glycoprotein--and is therefore similar to that of the influenza virus hemagglutinin (HA) (53, 105), the vesicular stomatitis virus (VSV) G protein (48, 113), and several membrane spanning cell-encoded glycoproteins, such as the HIV receptor on human T cells, CD4.

The Env protein precursor is cleaved into the two subunits, the SU and TM proteins, in the Golgi compartment by a cellular endopeptidase. The two subunits remain associated through different molecular interactions in different viruses. This cleavage frees the amino-terminus of the fusion peptide, which is postulated to be involved in the process of membrane fusion during virus entry, probably through a hydrophobic interaction with the lipid bilayer. For most retroviruses it is located at the N-terminus of the TM protein; however, in RSV this fusion peptide is preceded by a few polar amino acid residues. The functional organization within the envelope glycoprotein is shared not only by the *env* gene products of other members of the retrovirus family, but also by other enveloped viruses. For example, the HA protein of influenza virus is a similarly-sized glycosylated protein that also possesses the three functional hydrophobic domains mentioned above. The HA precursor itself is cleaved to HA1 and HA2 proteins that are structurally and functionally analogous to those of the SU and TM proteins, respectively. Like its glycoprotein counterpart in RSV, the HA protein is also a trimeric complex. Analyses of potential secondary structure features for the retroviral *env* gene products predict a similar distribution of alpha-helices and beta-sheets within the SU and TM proteins of RSV *env* as is seen in HA1 and HA2 (149), and a predictive modeling of the primary amino acid sequences of several retroviral TM proteins is consistent with their folding in a similar way to the

influenza HA2 protein (47). Definitive structural comparisons will ultimately require X-ray crystallographic information on the retroviral gene products.

B. Functions Of Retroviral Envelope Glycoproteins

1. Glycoprotein Mediated Virus Entry

Once incorporated into the virus particle, the glycoproteins play a critical role in virus entry into a new target cell. The SU protein binds to a specific receptor on the target cell (91), and this binding has been postulated to trigger a conformation change in the glycoprotein to expose the fusion peptide at the N-terminus of the TM protein (149). An interaction of the latter with the cell membrane is postulated to cause fusion between the viral and the cellular membrane and to allow the the viral capsid to enter the target cell. For a majority of retroviruses this fusion event does not require a low pH environment (82, 128) and the mechanisms which trigger fusion peptide activation are not well understood. It is possible that the fusion peptide becomes active after glycoprotein cleavage but is sequestered by protein folding or the formation of the multimer of the glycoprotein complex. The binding of the SU protein to the cellular receptor may be required for inducing the disassembly of the multimer complex or a conformational change to expose the fusion peptide. This is consistent with the results of a recent study, in which expression of the TM protein of HIV(gp41) alone, without the SU protein, results in induction of syncytia formation in a variety of CD4(-) cell cultures--a membrane fusion event that presumably mimicks that of viral infection. In this case the fusion peptide may be exposed due to the absence of the SU protein which is predicated to cover the TM protein in the glycoprotein complex (46, 47). The *env* gene product of MMTV and MuLV, in contrast, appears to resemble the HA of influenza virus. Cells expressing the gp52/gp36 protein of MMTV can be stimulated to undergo cell fusion after a brief exposure to low pH (107), and compounds which increase the pH of endosomes interfere with viral infectivity of MuLV(1). For these murine retroviruses then, it is possible that the conformational change after receptor

binding requires an additional low pH environment, analogous to that seen with the HA trimer (30, 125).

Retroviruses utilize surface molecules as their receptor for attachment and entry. Although receptor binding by the envelope glycoprotein complex is essential for initiation of viral infection, the nature of the receptor for many of the retroviruses is unknown. The receptor molecules have been identified for four groups of viruses, ecotropic murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), HIV and most recently avian leukosis virus (ALV). The receptors for MuLV and GaLV are transporter-like cellular proteins (144), while the CD4 molecule of the T-helper lymphocyte is used by HIV and SIV (117). More recently $ALVR^a$, an LDL receptor-like protein encoded by the TVa locus, has been identified as the receptor for avian leukemia viruses (158).

Even for the best characterized receptors, such as the HIV receptor molecule (CD4), the mechanism of viral and cellular membrane fusion is more complicated then the simple binding of the glycoprotein to the receptor. This is shown by the resistance to HIV infection of murine cell lines that express human CD4 (81). Although it is postulated that a secondary receptor, a fusion receptor, or a difference in the cell membrane composition may be responsible for the phenomenon, no evidence has been found to support any of these hypotheses. Much remains to be understood about the processes involved in the binding/fusion events of the retrovirus replication cycle.

2. Envelope Glycoproteins Determine the Host Range of a Retrovirus

The specific interaction of envelope glycoprotein with receptor molecules on the surface of a target cell determines the host range of a virus (28). It is postulated that a binding site composed of multiple polypeptide chains is responsible for binding to the cellular receptor. Due to the complexity of the tertiary structures of a glycoprotein, the exact sequences that determine a particular host range are not well understood. Sequence analysis of different subgroups of RSV reveals that regions of

conserved sequences are interrupted by clustered variable regions along the envelope glycoprotein molecule (17, 32). Five regions of sequence heterogeneity have been identified: three short stretches (six to twelve amino acids in length) named vr1, vr2, and vr3 and two larger regions of sequence heterogeneity (thirty to fifty amino acids in length) named hr1 and hr2. Sequence similarity is usually greater than 90% among the conserved domains, compared to only about 50% in the variable domains (16). This suggests that differential abilities to infect chicken cells with viruses of different subgroups are determined by these variable regions, while the conserved sequences are important for maintaining the normal structure of the viral glycoprotein. However, even within the variable regions there are conserved amino acids, frequently at positions containing proline, glycine, and cysteine. Since these amino acids residues play significant roles in determining protein structure (i.e. turns and disulfide bonds), it is likely that the SU proteins from the different subgroup viruses have similar overall structures within both the conserved domains and the variable regions. The differences in host range specificity determined by each envelope glycoprotein are probably confined to the variable amino acid sequences that are involved in forming the binding pocket and therefore determine the specificity of interacting with the host cell receptor (32). A schematic diagram of the conserved and variable domain organization of the *env* gene product of RSV is shown in Figure 8.

Evidence supporting the hypothesis that certain variable regions in the envelope glycoprotein determine the host range of different subtypes of a retrovirus were obtained from experiments using recombinant viruses to probe the specific sequence requirements involved in subgroup specificity of the avian sarcoma viruses. Analysis of the specificity of a series of recombinants showed that for all subgroups the major determinants of subgroup specificity lie within the five variable regions (16, 17, 32). A natural recombinant subgroup B virus that contains approximately 200 bases of sequence spanning the hr2 domain of a subgroup E virus has the normal host range of a

subgroup B virus, but is also able to infect turkey cells which are resistant to subgroup B viruses and susceptible to subgroup E viruses (31, 33, 137).

Further evidence supporting the concept that the envelope glycoprotein determines the host range of a retrovirus comes from phenotypic mixing experiments, that is, the host range of a virus can be altered by substituting the envelope glycoproteins with those of a different virus. When VSV infects cells that are chronically infected with RSV, VSV obtains the host range of RSV by incorporating the envelope protein of RSV; similarly, a small number of RSV virions released from cells infected with VSV can infect cells that are normally susceptible to VSV but not to RSV (146, 147). In recent experiments, hybrid virions generated by coexpressing the MuLV Gag proteins with the envelope proteins of HTLV-II, GaLV, amphotropic MuLV and VSV respectively, in the absence of the native envelope glycoprotein of MuLV, demonstrated extended host ranges resembling those of the viruses from which the envelope protein was derived (38, 85, 155). Similarly, in experiments described in this thesis, I have shown that RSV virions which in the absence of RSV *env* gene products, have incorporated the HA protein from influenza virus display a host range characteristic of the influenza virus.

Additional factors also restrict retrovirus replication in certain host cells. For example, subgroup D isolates of the avian sarcoma viruses are able to infect mammalian cells, but the infection does not result in the release of new virus. This type of abortive infection is the result of post-entry restriction by factors other than the envelope glycoprotein. For RSV, abnormal over-splicing of the viral mRNA in murine cells results in the disproportionate expression of the *src* product and only limited expression of the *gag* and *env* gene products (9, 131). Those capsid proteins that are synthesized fail to assemble in mammalian cells. This block is at least partially due to the lack of myristylation of the matrix protein (151). Nevertheless, the ability of the subgroup D-ASV to penetrate mammalian cells is determined by the *env* gene product.

Therefore the main determinant of viral host range is the envelope glycoprotein which specifies the cell type for viral attachment and penetration.

3. Glycoproteins Define the Neutralization Epitopes of a Retrovirus

For an intact virion, the only exposed antigen is the external portion of the envelope glycoprotein. Since the envelope glycoprotein is responsible for initiation of a viral infection, these surface epitopes represent the most important target against which the host can launch an effective neutralizing immune response. Although an infected host will also produce antibodies against the viral core proteins (major capsid and matrix proteins), these antibodies are probably induced by virions that have already been disrupted by the immune system. These antibodies are unlikely to be effective in neutralizing a viral infection because the core proteins are inaccessible to the immune system. Antibodies against the viral core proteins are more effective for diagnostic purposes because the core proteins are more conserved than envelope glycoproteins among different subgroups of retroviruses.

Viral infection may induce different types of host immune responses. Studies with the ASV have shown that the envelope glycoprotein induces two major types of neutralizing responses. One response recognizes determinants on the viral glycoprotein that are common to all viruses within a subgroup. A second type of response is highly specific to the infecting virus with no significant neutralizing activity towards other viruses even within the same subgroup; this response is called type-specific .

The subgroup-specific neutralization response correlates with host range specificity and thus is likely to be induced by the variable sequences that form the region of the glycoprotein which determines the specific host range (140). This is suggested by the study of two subgroup A viruses, where it was shown that both subgroup and type specific epitopes resided within the same domain of the SU protein that spanned the host range determinants (17). The common neutralizing reactions are probably induced by those sequences conserved within a particular subgroup.

It is clear from the preceding sections that the envelope glycoprotein complex plays a critical role during an infection cycle of a retrovirus. Despite the extensive research focused on this area and the large amount of information accumulated during the past decade, prior to initiating the work described in this thesis many aspects of envelope glycoprotein biosynthesis and function remained to be understood. In this dissertation I describe experiments in which I determined the sequence necessary for envelope glycoprotein precursor cleavage, and demonstrated the biological importance of this proteolytic processing step in the biosynthesis of the envelope glycoprotein. In the second part of the thesis I analyzed the requirement for glycoprotein incorporation into the virion and proposed a model for the mechanism by which selective incorporation of envelope glycoprotein into virions occurs. The background and rationale for initiating this research is presented in the following section of the introduction.

C. Biosynthesis of RSV envelope glycoprotein

The genetic organization of replication competent retroviruses, 5'-*gag-pol-env* -3', requires that the envelope gene be translated from a spliced, subgenomic mRNA (Figure 7). In a majority of retroviruses, this moves an initiating methionine codon at the beginning of the *env* open reading frame close to the 5' end of the mRNA. In RSV, however, the splicing event fuses the strong initiation codon from the *gag* open reading frame in-frame with *env* gene sequences. The nascent *env* gene product of this virus is thus a fusion protein of approximately 600 amino acid residues containing the first 6 amino acids from the *gag* gene product.

1. Protein Translocation

The envelope glycoprotein is transported in the secretory pathway, as are all of the cellular membrane and secretory proteins. The initial translation of the *env* mRNA is in the cytoplasm of membrane bound polyribosomes. The first step that introduces the nascent polypeptide into the secretory pathway is mediated by the binding of the

newly translated signal peptide by a component of the cellular transport machinery--the signal recognition particle (SRP). This binding arrests the initial protein synthesis and guides the translation complex to the rough endoplasmic reticulum (RER) through an interaction with a docking protein complex in the RER membrane (143). In the retroviral *env* gene products, the hydrophobic signal peptide is generally located in a longer (30 - 100 amino acids) leader sequence. The function of these sometimes numerous additional amino acids is unknown. However, normal biosynthesis and translocation was observed in experiments in which the HA signal peptide was substituted for the entire RSV *env* leader sequence (98). Similarly, the *env*-derived signal peptides can substitute for the signal peptides of other membrane proteins, such as that of the HA (65). Thus, it seems unlikely that these extra amino acid residues have an additional required function other than the initiation of the translocation process. The length of the hydrophobic domain of the signal sequence appears to be critical for its function, since the deletions of one or two amino acids in this region of the RSV *env* gene product, generating a hydrophobic domain 9 and 10 amino acids long, reduces the efficiency with which it directs molecules across the RER by 50 and 95% respectively (153). Mutations which entirely remove the signal peptide result in the synthesis of a cytoplasmically located product that is rapidly degraded (65)..

During translation of the nascent *env* gene product, the entire signal/leader peptide is removed by a host protease. The site of this cleavage is specified by amino acid residues within the signal peptide itself (101, 142), and a mutational analysis of the RSV *env* gene sequences in this region confirms that amino acid changes carboxyl-terminal to the cleavage site, including the second amino acid of the site itself, have no effect on the efficiency with which cleavage occurred (61).

2. Membrane Anchoring of the Glycoprotein Precursor

The transmembrane domain of RSV is comprised of a stretch of 27 hydrophobic amino acids located near the C-terminus of the TM protein sequences. It

stops translocation of the precursor protein through the RER bilayer and anchors the nascent polypeptide in the membrane. A stop codon introduced into the protein prior to this sequence resulted in secreted forms of the envelope glycoprotein of RSV (99), proving the anchor function for this region.

In an attempt to determine the minimal functional length for the RSV anchor domain, a series of internal deletions were introduced into the transmembrane domain of RSV envelope glycoprotein, reducing its length from 27 amino acids to as few as five (65). Surprisingly, *env* gene products with a hydrophobic domain as short as 16 amino acid residues still arrested translocation and spanned the membrane; however, such proteins were much less stable on the cell surface. Although they were transported to the cell surface, they were not released into the culture medium, thus suggesting they were digested in the endocytosis pathway (65). When the anchor region was reduced to 5 amino acid residues, it could no longer stop translocation; these glycoproteins were secreted outside the cell through the secretory pathway.

Using a novel complementation system established during the course of this thesis work, I have shown that the truncated, membrane-anchored proteins are not incorporated into virions. These data suggest that a proper conformation of the transmembrane domain is required not only for the stability of the glycoprotein in the cell membrane but also for incorporation into virion. Preliminary results from other members of this laboratory have demonstrated that palmitic acid modification of cysteines within the hydrophobic anchor domain is also critical for efficient glycoprotein incorporation into virions (86). It seems likely then that this region plays additional roles to those of stopping translocation and anchoring the molecule in the membrane .

3. The Function of Cytoplasmic Domains

A stretch of hydrophilic amino acid residues is located to the C-terminus of the transmembrane domain. It was thought that a possible role for this cytoplasmic domain

might be to provide a signal for the incorporation of the envelope glycoprotein into virions, since the cytoplasmic residues are ultimately located in the interior of the virion. It was postulated that the cytoplasmic domain of envelope glycoportein might interact with capsid proteins during virion assembly, since it was shown that the TM and MA proteins of RSV could be chemically cross-linked in virions (51). This hypothesis was supported by the conservation of this region among different subgroups of RSV. However, deletion of the cytoplasmic domain, by insertion of a stop codon in place of that for the first residue of the cytoplasmic domain of the RSV TM protein, had no effect on transport of the protein to the membrane, on its incorporation into virions, or on the infectivity of the virus (99). Thus, in the case of Rous sarcoma virus, this region is not apparently required for glycoprotein/capsid recognition. Because this result raised questions about the requirement for specific TM/capsid interactions during the assembly of RSV, I addressed this problem in this thesis. Experimental results presented here show that a chimeric HA protein containing the transmembrane and cytoplasmic domains of the RSV envelope glycoprotein did not have any advantage over a wild-type HA protein for incorporation into RSV virions. Additional experiments not presented here showed that envelope glycoproteins from a variety of different viruses could be efficiently incorporated into RSV virions. These results argue against a specific incorporation signal in the RSV glycoprotein.

Nevertheless, the membrane-spanning domain of the envelope glycoprotein may be involved in some form of interaction with capsid protein. This is suggested by the experiments described in this thesis using mutants with internal deletions in the transmembrane domain and by the cysteine mutants described above. These data suggest that sequences within the hydrophobic anchor region of the TM protein may be necessary for the efficient incorporation of RSV envelope glycoprotein into the virion during assembly.

Additional functions of the cytoplasmic domain are found in MuLV and M-PMV. In these viruses, following assembly the viral-encoded protease cleaves the bulk of the cytoplasmic domain from the TM polypeptide (p15E and p22, respectively) to yield a truncated form of the TM protein (20, 133). This cleavage of the cytoplasmic region appears to be required to activate fusogenic activity of the envelope glycoprotein in M-PMV.

D. Post Translational Modification of the Envelope Glycoprotein

1. Transport of the glycoprotein

Retroviral glycoproteins are transported to the cell surface through the secretory pathway using the same mechanisms as cellular membrane proteins. However, the proposed "sorting signals" that direct the glycoprotein to the cell membrane have not been identified. Unlike the nuclear targeting or mitochondria locating sequence that are identified as specific peptide sequences, the signals for membrane transport are more complex and appear to be at the level of certain tertiary structures of the protein. This is suggested by the fact that disruption of a polypeptide's normal folding can completely prevent its transport from the ER (54, 75), and that sequence analysis of a variety of conditional and non-conditional membrane protein transport mutants did not reveal any consensus sequences that may be involved in the sorting process. The simplest explanation for the phenotypes of those transport defect mutants is that they alter the tertiary structure of the mature protein.

Following the complex series of modifications, the mature Env complex is transported from the trans-Golgi reticulum to the plasma membrane where it can be incorporated into assembling virions. In fibroblastic and lymphoid cells this may be a relatively unregulated process but in epithelial cells, which have apical and basolateral surface membranes with distinct membrane protein compositions, targeting of proteins is specific. All retroviral glycoproteins studied to date are transported exclusively to the basolateral membrane (114, 129). It is possible that since this membrane is also the

site of virus assembly and release, basolateral transport might provide a mechanism by which retroviruses establish a systemic versus a localized infection.

2. Multimerization of the envelope glycoprotein

In a similar way to the influenza virus HA protein, the precursor of the RSV envelope glycoprotein oligomerizes into trimeric structures prior to transport from the ER (36). Since an *env* gene mutant that is transport defective does not form oligomers (36), it is likely that oligomerization is required for intracellular transport. In studies of the influenza HA protein, monoclonal antibodies specific for either the trimeric HA or monomeric HA proteins were isolated. This suggests that oligomerization induces structural changes in the HA protein which result in the immunogenic differences between the two forms of the HA protein. The formation of an oligomeric structure may provide a mechanism for sequestering a potentially active fusion peptide during transport of the glycoprotein through the intracellular compartments. It may also provide a structural rigidity to the Env protein complex and a mechanism to activate the fusion peptide by reordering the oligomer upon binding the cellular receptor. Oligomerization of envelope glycoproteins has also been shown in HIV and MuLV (70, 138). Given the similarity of retroviral *env* genes, it seems likely that other retroviral glycoproteins will form analogous oligomeric structures.

3. Glycosylation

Envelope glycoproteins of retroviruses are glycosylated by the same mechanism as the cellular glycoproteins during the transport into the secretory pathway. In the course of co-translational transfer into the lumen of the rough ER, the newly synthesized envelope protein precursor is first modified by the addition of mannose rich oligosaccharide chains onto the NH_2 groups of asparagine residues that are within a conserved glycosylation sequence (-Asn-X-Ser/Thr-). Since this is a co-translational event (115), the first detectable translation product of the *env* gene is thus

a core--glycosylated precursor. During transport of the polypeptide through the Golgi--complex, a majority of the core oligosaccharides are modified by trimming of mannose residues and addition of N-acetyl-glucosamine, galactose, and fucose residues (74), to yield both complex and hybrid carbohydrate side-chains (55, 64, 74). Residues at certain sites on the glycoprotein appear to remain unmodified in their high-mannose, endoglycosidase-sensitive form in the mature protein (18, 55, 102).

The number and distribution of N-linked glycosylation sites varies widely between different retroviruses, ranging from four in MMTV to 30 potential glycosylation sites in HIV. The positions at which carbohydrate is added also vary significantly among different groups of retroviruses. However, highly conserved carbohydrate residues within a particular retrovirus group can be observed (94, 150). It suggests a specific requirement for carbohydrate at certain positions to maintain a functional structure of the glycoprotein.

The role of carbohydrate side chains in glycoprotein biosynthesis, transport, and stability has been a subject of much interest. Although experiments have failed to demonstrate that oligosaccharide side chains themselves can act as sorting signals to direct the glycoprotein to the cell surface, they may provide the hydrophilicity required to drive or stabilize correct protein folding which in turn may be a prerequisite for protein transport and protein function. Removal of a highly conserved glycosylation site within the gp120 of HIV, for example, resulted in a normally transported but inactive glycoprotein and loss of virus infectivity. However, infectious revertants of the mutant virus did not regain the missing carbohydrate residue but instead contained second site mutations that presumably compensated for structural changes induced by the primary mutation (150). In addition, treatment of virus-producing cells with inhibitors of glycosylation or of enzymes involved in carbohydrate processing can reduce virus infectivity significantly, even under conditions where the modified glycoproteins are efficiently incorporated into virions (12, 59, 92).

It is likely that carbohydrate, through masking of susceptible residues, also enhances the stability of the glycoprotein by providing protection from proteolytic enzymes. In addition this masking may also reduce the immunogenicity of the protein, presumably by preventing immunoreactive cells from interacting with polypeptide epitopes (37).

4. Proteolytic Processing of the Envelope glycoprotein

After transport of the oligomeric Env precursor to the Golgi-complex, it is cleaved by a host-derived protease into the individual SU and TM proteins. This cleavage occurs immediately after a series of basic amino acid residues, Arg-Arg-Lys-Arg, (46, 100) that are highly conserved in different retroviruses. Prior to my entry into the laboratory, a mutational analysis of the cleavage site of the RSV *env* gene product suggested that two host enzymes might exist in the Golgi-complex, one which cleaved preferentially after the Lys-Arg sequence and a second which cleaved less efficiently at other dibasic residues (i.e., Arg-Arg) (100). Nevertheless, when this thesis research was initiated the requirement for precursor cleavage in activating the biological activity of the glycoprotein, in targeting its incorporation into virions, and for initiating virus infection was not known. Previous work in the ortho- and paramyxoviruses had demonstrated the importance of precursor cleavage of both the HA in influenza virus and F protein in parainfluenza viruses for virus infectivity (4, 127). However, other viruses capable of inducing fusion have uncleaved fusion proteins, as with the G protein of VSV (95).

This problem has been investigated further in the first part of this thesis by construction of additional mutations in the RSV *env* gene. The results of these mutations support the concept of more than a single endoprotease within the Golgi and demonstrate an absolute requirement for glycoprotein precursor cleavage for virus infectivity. Nevertheless, uncleaved precursors could be efficiently incorporated into virions, and in mutants retaining the ability to be cleaved by the exogenous addition of

trypsin, biological activity could be partially restored by addition of the protease. During the performance of these experiments in the RSV system, a study in which multiple mutations were introduced into the HIV-1 cleavage site was published. The study also showed a correlation between inhibition of precursor cleavage and loss of virus infectivity (83).

Cleavage of the precursor is probably required to free the fusion peptide so that it can be exposed after receptor binding. The exact location within the Golgi-complex where cleavage occurs has not been determined; studies with RSV and REV are consistent with cleavage occurring in a late (trans-) compartment of this organelle (136, 154). However, recent studies with HIV-1 have suggested that cleavage of the *env* gene product of this virus might occur prior to carbohydrate modification (55). In some retroviruses, RSV for example, the mature products of cleavage are covalently joined through disulfide linkages in an SU-TM heterodimer (79). In other retroviruses, however, only a fraction of the virion glycoproteins appear to be covalently linked, e.g., MuLV (80, 156), or are held together entirely by non-covalent forces, e.g., HIV, M-PMV (18, 112). The latter arrangement allows the SU protein to be readily shed from the virus (111) .

E. Glycoprotein Incorporation into Virion

During viral assembly, retroviruses specifically incorporate their envelope glycoproteins into virions at the cell plasma membrane, while excluding the bulk of the cellular proteins. The mechanism that specifies incorporation of retroviral glycoproteins into assembling virions is not well understood. It has been suggested that the specificity of this incorporation is due to direct interactions between the viral glycoproteins and the matrix or major capsid protein of the virus, although the nature of this interaction has not been defined. At least in some viruses the cytoplasmic domain of the glycoprotein may be involved in the interaction with the viral nucleocapsid. Using internal image anti-idiotypic antibodies, it was shown that the

nucleocapsid of Semliki Forest virus may contain a specific socket into which the cytoplasmic tail of the E2 glycoprotein fits, in a fashion similar to a receptor-ligand type interaction. Studies in some retroviruses also have shown the importance of the cytoplasmic domain for viral infectivity and glycoprotein incorporation into virions. Truncation of the cytoplasmic domain of MuLV TM glycoprotein resulted in a non-infectious virus, although it was not clear if this defect was due to the failure of glycoprotein incorporation (58). Consistent with the possibility that specific interactions are necessary is the observation that the gp65 glycoprotein of the murine spleen focus-forming virus (SFFV), which has an altered anchor domain and no cytoplasmic domain, is not incorporated into virions (103). In more recent studies of HIV, progressive truncations of the TM protein cytoplasmic domain resulted in a decrease in both viral infectivity and glycoprotein incorporation into virions. When the truncation was greater then 19 amino acid residues, the infectivity was completely blocked and only trace amounts of glycoprotein could be detected by immunoprecipitation in the virions released from a transient expression system (34). A similar study in M-PMV also showed that progressive truncation of the cytoplasmic domain of TM protein reduced the incorporation of the envelope glycoprotein into virions and abolished the infectivity of the virus (20). These results pointed to a requirement for the cytoplasmic domain of the TM proteins for glycoprotein incorporation and viral infectivity in both HIV and M-PMV. These results are supported by the observations that two mutants with single amino acid changes in the MA protein of M-PMV greatly reduced or abolished the amount of envelope protein incorporated into virions even though an abundant amount of envelope glycoprotein was present at the cell surface (108). Moreover, a recent report from Lee and coworkers showed that small deletions within the MA protein of HIV resulted in the release of virions lacking glycoprotein (160).

On the other hand, in the avian retroviruses, a mutant glycoprotein lacking a cytoplasmic domain is still efficiently incorporated into the virion. Since the TM protein and the MA protein of RSV could be chemically cross-linked in virions (51), it seems likely that if such TM-MA interactions are essential, they must occur within the hydrophobic anchor domain of the TM protein (99). Other results also appear to diminish the importance of interactions between the glycoprotein and capsid. Pseudotype viruses were generated when cells chronically infected with different retroviruses were superinfected with VSV (145, 147). In addition, as we have described above, the glycoproteins of VSV, HTLV-I, and the Gibbon ape leukemia virus can be efficiently incorporated into MuLV virions when these glycoproteins are coexpressed with Gag proteins of MuLV (38, 85, 155). Since little conserved sequence can be found in the TM subunits of these different envelope glycoproteins, it seems unlikely that a specific ligand-receptor type interaction with the protein capsid is required for envelope glycoprotein incorporation, at least in retroviruses such as RSV and MuLV.

In the second section of this thesis, I described experiments aimed at probing the requirement for incorporation of glycoproteins into RSV virions. In these studies, I have demonstrated that the influenza virus HA protein can be efficiently incorporated into RSV virions irrespective of whether it retains its native membrane spanning and cytoplasmic domains or those of the RSV gp37 (TM). Both native and chimeric HA proteins can confer infectivity on the RSV virions and an expanded host range for entry that includes mammalian cells. A hypothesis is presented to explain the apparent lack of exclusion of foreign viral glycoproteins in cells where host plasma membrane proteins are excluded.

Assays for virus assembly and release

Both the study of precursor processing and that of HA utilization by RSV highlighted a major drawback of studying the molecular biology of avian retroviruses--the lack of a high level transient expression system for studying virion assembly, such

as the SV40 origin-containing plasmids that can be used for mammalian virus expression in COS cells (27, 108). In the third section of this thesis I describe a novel assembly assay. This assay makes use of a system first described by Fuerst and coworkers in which the bacteriophage T7 polymerase is produced in cells from a vaccinia virus vector and is used to transcribe genes under the control of a T7 promoter from plasmids introduced into the same cell (41, 42). The broad host-range of vaccinia virus has allowed the transcription from independent plasmids, of the Gag and Env precursors of RSV in avian cells. The viral proteins translated from these cytoplasmic mRNAs assemble into virions that are morphologically indistinguishable from native virions. This assay has allowed the analysis of a variety of mutant and foreign glycoproteins for their ability to be incorporated into RSV virions.

Overall, the experiments described in this thesis were aimed at delineating the regions required for the function and incorporation of envelope glycoprotein--the fundamental aspects of the envelope glycoproteins during the replication cycle of retroviruses. Based on the experimental data obtained during my thesis research, I refined our understanding of the requirements and role of precursor cleavage in glycoprotein function, and defined a possible mechanism for the selective incorporation of viral envelope glycoproteins over cellular membrane proteins into virons. A rapid assay for retrovirus assembly is also described in detail. This will provide a powerful tool for analyzing mutants in structural genes with regard to viral assembly.

Figure 1. Schematic Diagram of a Retrovirus.
RSV virions contain two positive-strand RNA genomes (RNA genome) that are located inside the capsid, a spherically-shaped protein shell. The RNA genomes are bound by nucleocapsid proteins (NC). The virus-coded reverse transcriptase (RT) molecules are associated with the genomic RNA inside the nucleocapsid. The capsid is comprised of a 27kD protein, the major capsid protein (CA), or p27. Outside the capsid is the matrix protein (MA). The outermost part of the virion is the viral membrane (Lipid Bilayer), a lipid bilayer derived from the host cell. The envelope glycoprotein complex is seen as a knobbed-spike structure. These glycoprotein complexes are trimeric structures. Each monomer is comprised of two subunits, the transmembrane protein (TM) and a surface protein (SU). The two subunits remain associated on the virus through a disulfide linkage.

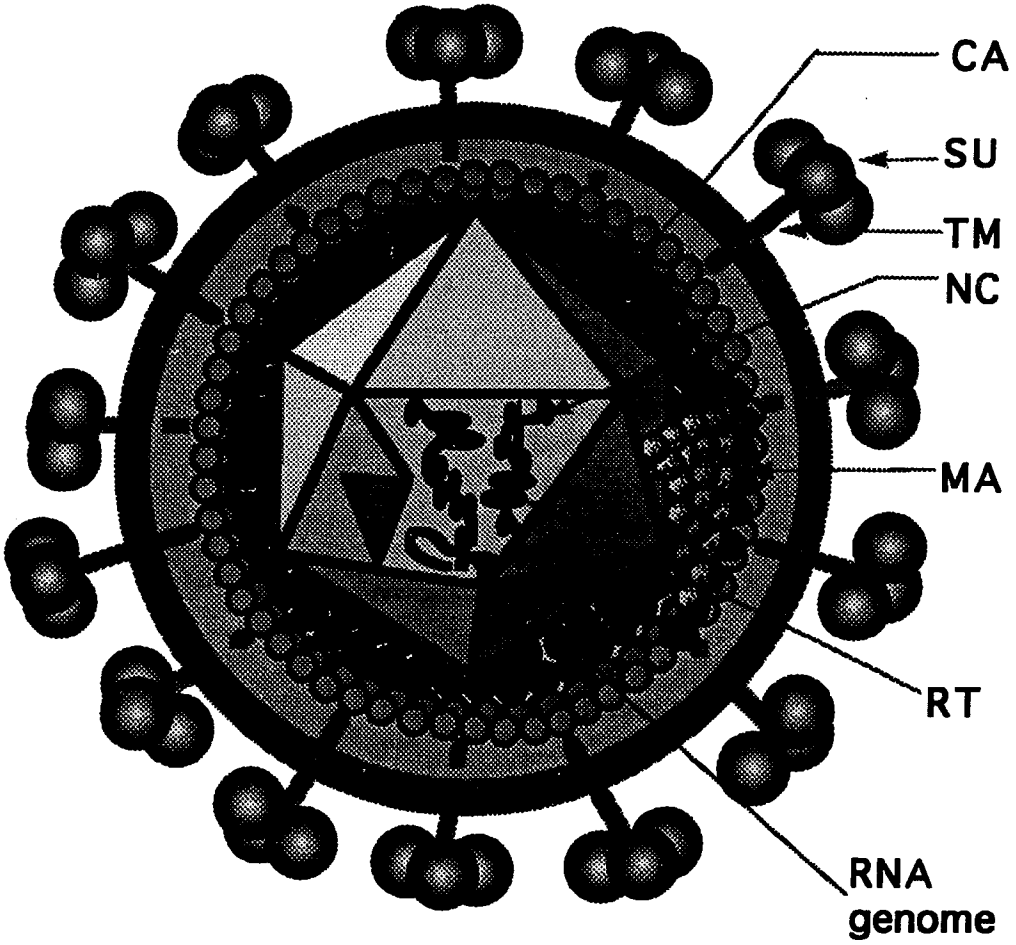

CA
SU
TM
NC
MA
RT
RNA
genome

Figure 2. A schematic diagram of the genomic structure of RSV.

The viral RNA is modified in the same way as a cellular mRNA. It has a 5'-cap (m^7Gppp) and a 3'-polyadenylated terminus(poly A). The structural genes (*gag, pol,* and *env*) and an oncogene (*src*) are flanked by a repeat region at each end (R) and regions unique to each end (U5, U3). The initiator AUG for Gag and Env polyprotein synthesis, the packaging site (Psi), short direct repeats (dr), a dimer linkage site (D.L.S.), splicing donor (SD), and acceptor sites (SA) are labeled in the diagram.

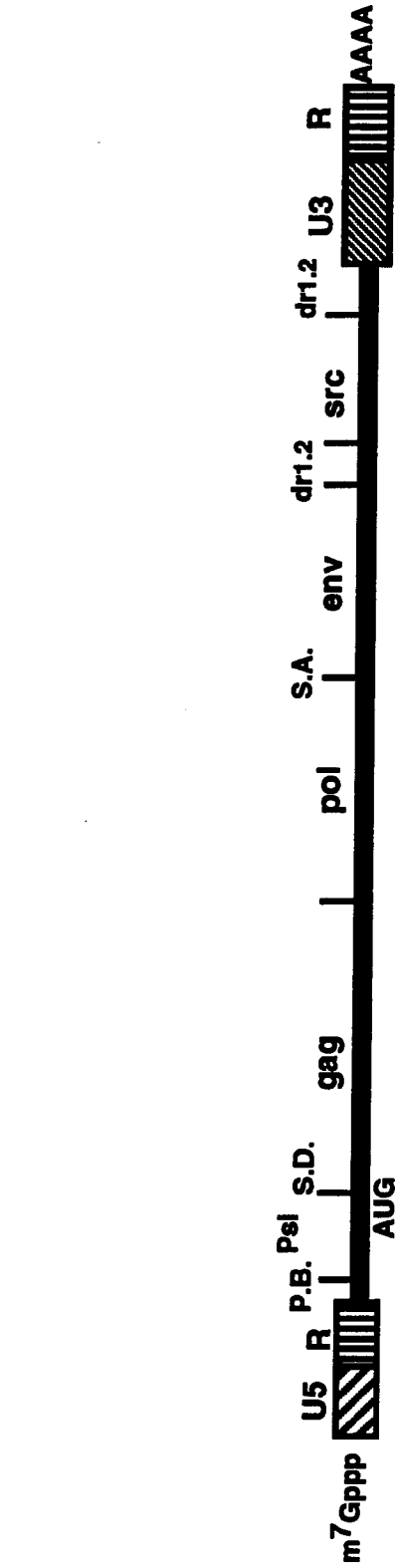

m7Gppp
U5
R
P.B.
Psi
S.D.
AUG
gag
pol
S.A.
env
dr1.2
src
dr1.2
U3
R
AAAA

Figure 3. A simplified diagram of the life cycle of retroviruses.
(a) Infection begins following viral attachment to the host cell and the viral glycoprotein-mediated entry of the capsid into a host cell. (b) After disassembly of the capsid inside the cell, the first strand of DNA is synthesized by the RT. After degradation of the RNA genome the second strand of DNA is synthesized. The linear DNA intermediate then migrates to the nucleus and is integrated into the host genome. (c) After viral RNA transcription, proteins are synthesized and assembled into virions. Infectious viruses then bud off from the cell membrane.

Life cycle of a Retrovirus

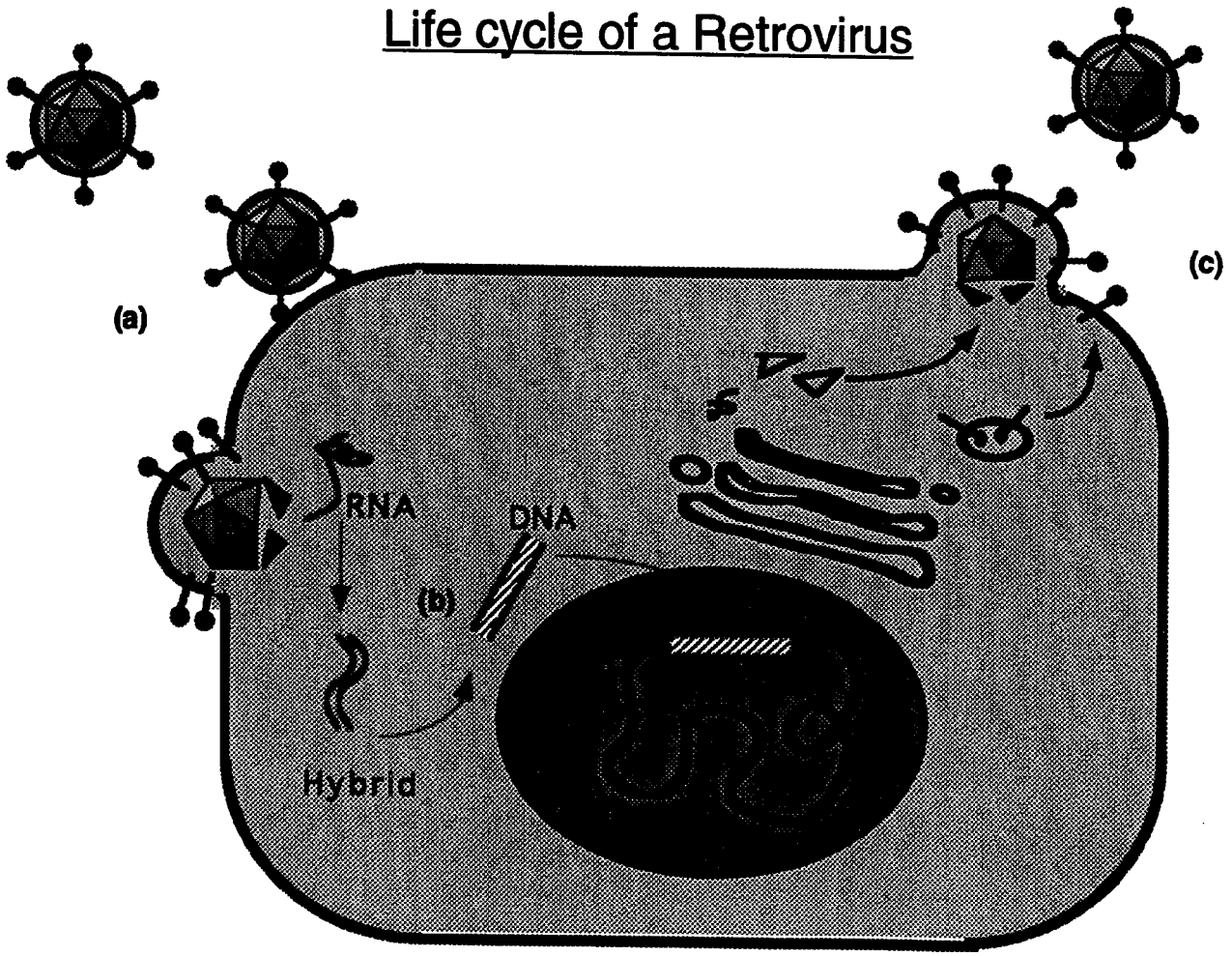
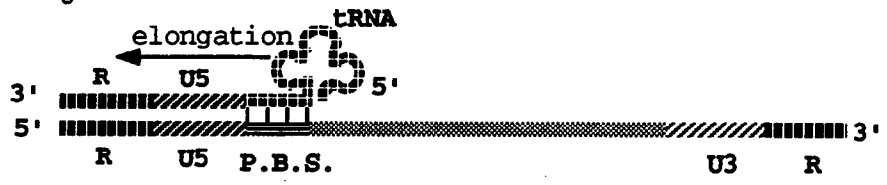
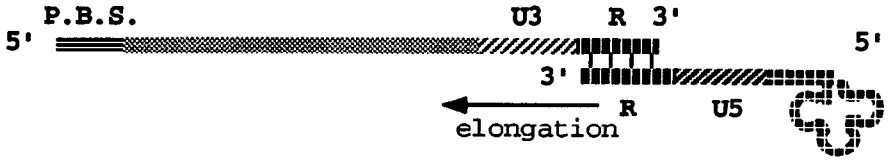


Figure 4. Genome replication of retroviruses by a "jumping mechanism"
Viral genomes are represented by horizontal bars. Gray bars represent RNA and solid black bars represent the newly synthesized DNA. Different regions important for genome replication are represented by bars with distinct patterns and are labeled in the diagram.

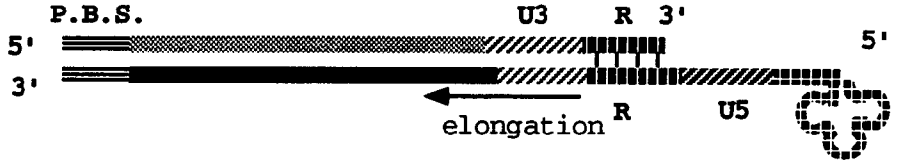
1. tRNA primer binding



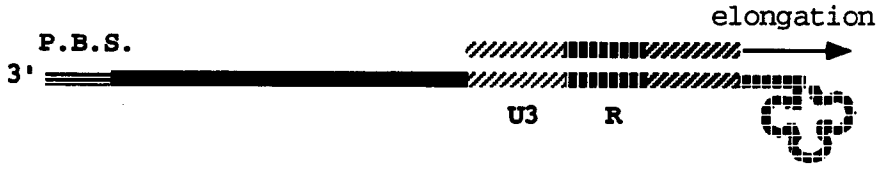
2. first "jump" of the primer



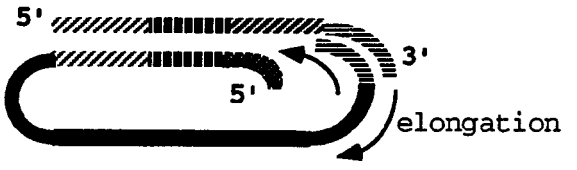
3.synthesis of negative strand DNA



4.second primer "jump"



5. synthesis of the positive strand DNA and formation of LTRs



6. the linear double strand DNA

Figure 5. Genome organization and RNA splicing of RSV
Boxes represent the reading frames of the genes of the virus. Horizontal bars represent the viral RNA genome and spliced subgenomic RNAs. Splicing occurs at the splicing donor (SD) and splicing acceptor (SA) sites.

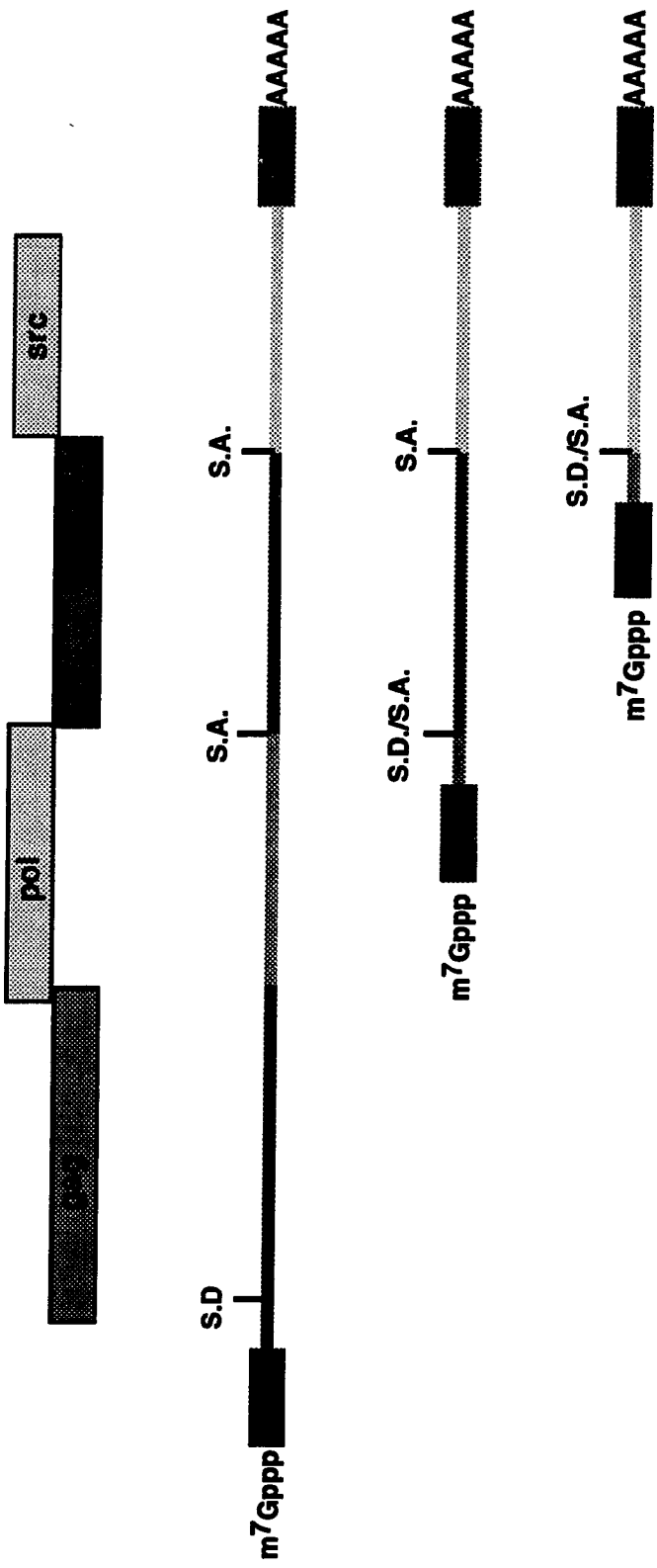

src
pol
m7Gppp
S.D
S.A.
S.A.
AAAAA
m7Gppp
S.D./S.A.
S.A.
AAAAA
m7Gppp
S.D./S.A.
AAAAA

Figure 6. Basic features of envelope glycoprotein molecule of RSV
The horizontal line represents the envelope glycoprotein molecule . The three functionally important hydrophobic regions are represented by boxes and indicated by arrows, respectively. The glycoprotein cleavage-activation site and the signal peptide cleavage site are indicated by bold arrows.

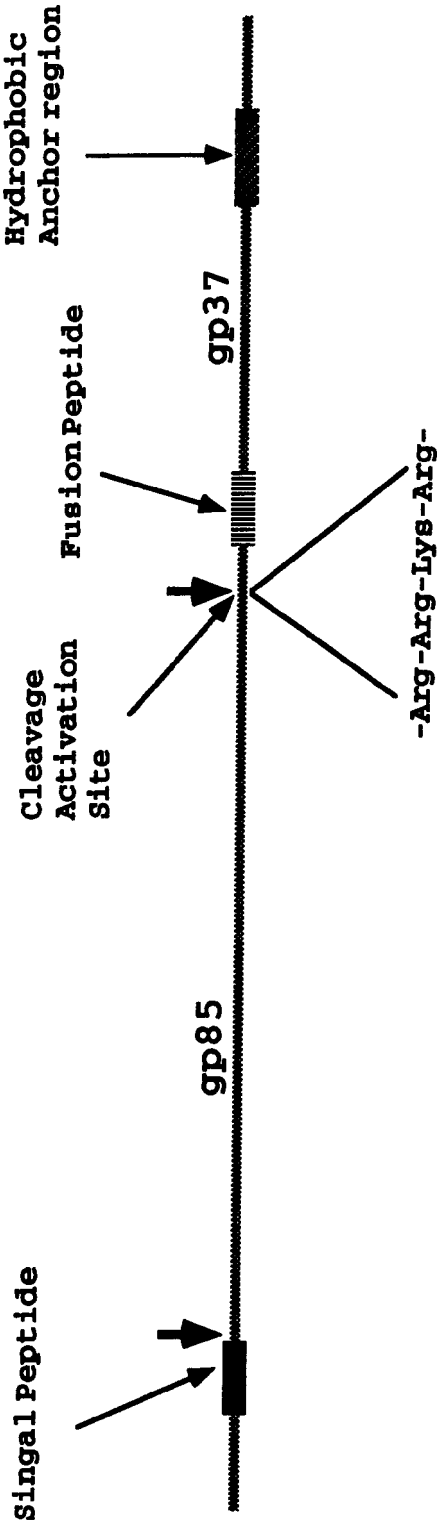

Hydrophobic
Anchor region
gp37
Fusion Peptide
Cleavage
Activation
Site
-Arg-Arg-Lys-Arg-
gp85
Singal Peptide

Figure 7. Structure of the avian sarcoma/leucosis virus env gene product
The horizontal line represents the RSV envelope glycoprotein molecule. An arrow indicates the cleavage site at which the precursor protein is cleaved into gp85 and gp37. Gray boxes indicate the positions of the regions of sequence variability seen in the gp85 domain when the *env* gene sequences from viruses of different subgroups are compared

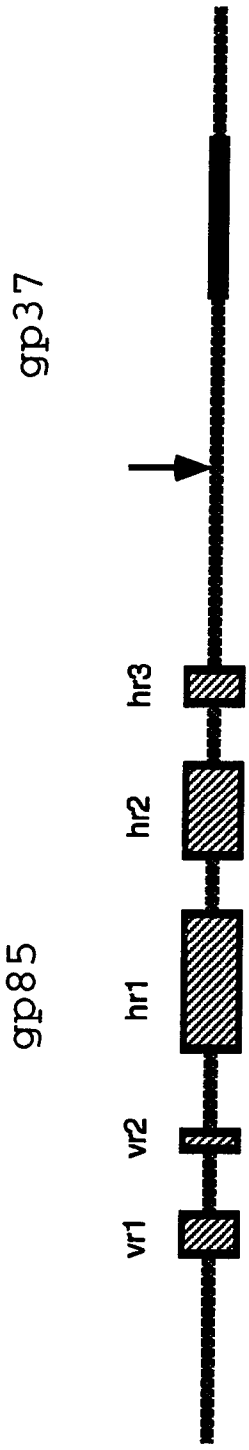
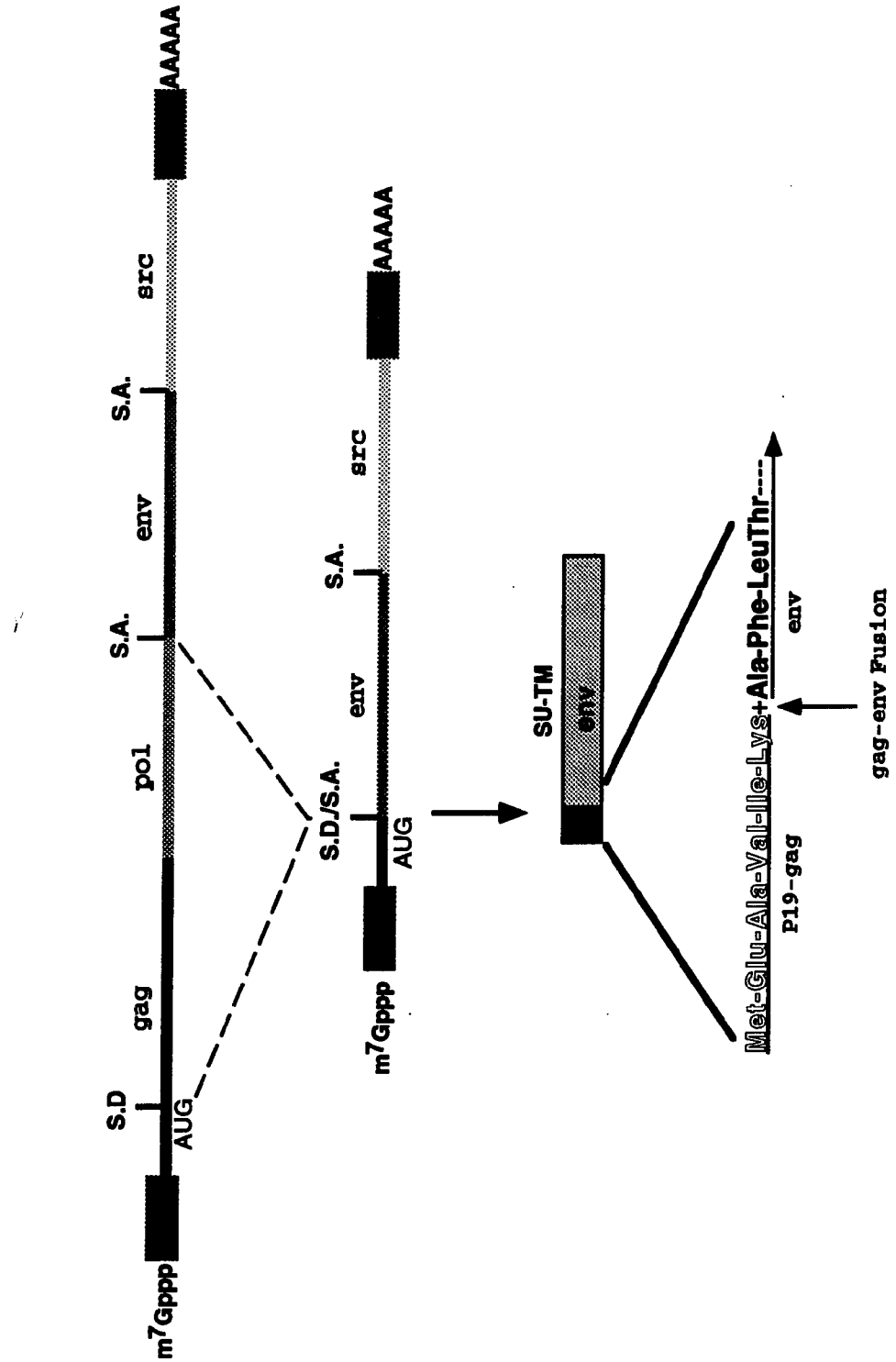

gp37
gp85
hr3
hr2
hr1
vr2
vr1

Figure 8. Schematic diagram of the env mRNA splicing and the translation of the envelope glycoprotein
Genomic and spliced viral RNAs are represented by horizontal bars. The gray box represents the translated envelope glycoprotein. The region translated from the *gag* gene is represented by solid black. The fusion sequence between the p19-gag and gp85-env is shown at the bottom.


m7Gppp
S.D
gag
AUG
pol
S.A.
env
S.A.
src
AAAAA
m7Gppp
S.D./S.A.
AUG
env
S.A.
src
AAAAA
SU-TM
env
Met-Glu-Ala-Val-Ile-Lys+Ala-Phe-LeuThr----
P19-gag
env
gag-env Fusion

MUTATIONS WITHIN THE PROTEOLYTIC CLEAVAGE SITE OF THE ROUS SARCOMA VIRUS GLYCOPROTEIN DEFINE A REQUIREMENT FOR DIBASIC RESIDUES FOR INTRACELLULAR CLEAVAGE.

JIANYUN DONG, JOHN W. DUBAY, LAUTARO G. PEREZ AND ERIC HUNTER*

Department of Microbiology
University of Alabama at Birmingham
Birmingham, AL 35294

* Corresponding author.

ABSTRACT

We have investigated the amino acid sequence requirements for intracellular cleavage of the Rous sarcoma virus glycoprotein precursor by introducing mutations into the region encoding the cleavage recognition site (Arg-Arg-Lys-Arg). In addition to mutants G1 (Arg-Arg-Glu-Arg) and Dr1 (Deletion of all 4 codons) that we reported on previously (43), we constructed two additional mutants, AR1 (Arg-Arg-Arg-Arg), in which the highly conserved lysine is replaced by an arginine and S19 (Ser-Arg-Glu-Arg) in which no dibasic pairs remain. The results of these studies demonstrated that when the cleavage sequence was deleted (Dr1) or modified to contain unpaired basic residues (S19), intracellular cleavage of the glycoprotein precursor was completely blocked. This demonstrated that the cellular endopeptidase responsible for cleavage had a stringent requirement for the presence of a pair of basic residues (Arg-Arg or Lys-Arg). Furthermore, it implies that the cleavage enzyme was not trypsin-like, since it was unable to recognize arginine residues that were sensitive to trypsin action. Substitution of the mutated genes into a replication competent avian retrovirus genome showed that cleavage of the glycoprotein precursor was not required for incorporation into virions but was necessary for infectivity. Treatment of BH-RCAN-S19 transfected turkey cells with low levels of trypsin resulted in the release of infectious virus, demonstrating that exogenous cleavage could generate a biologically active glycoprotein molecule.

INTRODUCTION

The envelope glycoprotein complex of Rous sarcoma virus (RSV), like that of most replication competent retroviruses, is comprised of two polypeptides, an external, glycosylated, hydrophilic polypeptide (gp85 or SU) and a membrane-spanning protein (gp37 or TM), that form a knob or knobbed spike on the surface of the virion (32). Both polypeptides are encoded in the env gene and are synthesized in the form of a polyprotein precursor (Pr95) that is proteolytically cleaved during its transport to the

surface of the cell (reviewed, 22). While these proteins are not required for the assembly of enveloped virus particles, they do play a critical role in the viral replication cycle by recognizing and binding to specific receptors (SU) and by mediating the fusion of viral and cell membranes (TM), virus particles lacking envelope glycoproteins are thus non-infectious (5; 27; 45; 47).

We have shown previously that cleavage of Pr95 to the disulfide-linked gp85 and gp37 occurs after a sequence of four basic amino acids, arginine-arginine-lysine-arginine, which is highly conserved in a variety of retroviruses and resembles the cleavage sequence found in avian and equine influenza viruses and in a variety of peptide hormone precursors (43). The proteolytic cleavage event is mediated by a host cell-encoded enzyme that appears from metabolic labeling studies to be located in a late compartment of the Golgi complex (4, 56, 59).

Initial studies from this laboratory, in which mutations were introduced into the cleavage region, showed that deletion of the codons for all four basic residues (Dr1) resulted in the normal biosynthesis, intracellular transport, oligosaccharide processing, and cell surface expression of an uncleaved glycoprotein precursor; this demonstrated that precursor cleavage was not a prerequisite for intracellular transport (43). In addition, we showed that substitution of a glutamic acid codon for the lysine codon (G1) reduced precursor cleavage by more than 80%, suggesting that this basic amino acid was very important in recognition by the Golgi endopeptidase (43). The biological role of retroviral glycoprotein precursor cleavage was not addressed in these studies but by analogy with the myxoviruses and paramyxoviruses it might be expected that cleavage would be required for glycoprotein function (29, 48, 50). Indeed, subsequent mutagenesis studies with HIV-1 have shown that mutations within the cleavage region of Pr160*env* that interfere with precursor cleavage can block both glycoprotein-induced cell fusion and infectivity of the virus (1, 9, 35). The latter studies, however, have not fully addressed the question of the amino acid sequence

requirements for recognition by the Golgi endopeptidase, nor have they examineed whether cleavage is required for glycoprotein incorporation into virions. This is important since Linial et al. (33) have reported on a non-conditional mutant of RSV (SE521), in which processing of the glycoprotein precursor was blocked. In SE521 infected cells Pr95 remained uncleaved on the cell surface and was not incorporated into virions. Although the nature of the defect in SE521 cells has not been determined, these results raise the possibility that cleavage might be required for glycoprotein incorporation into virus particles.

Using an SV40 late-replacement expression system, we report here on the phenotypic analysis of two additional mutations within the cleavage site of the RSV glycoprotein precursor. The effects of all four mutations (Dr1, G1, Ar1, and S19) on glycoprotein incorporation and virus infectivity in an avian retrovirus expression system are described. The first of the additional mutations, Ar1, substitutes an arginine residue for the highly conserved lysine within the cleavage site and yields an envelope gene that has an essentially wild-type phenotype. In contrast, the second mutant, S19, which contains two substitutions (serine-arginine-glutamic acid-arginine), is not cleaved during its transport to the cell surface even though it remains fully susceptible to cleavage by low levels of exogenously added trypsin. Viral genomes carrying the Dr1 or S19 mutations are non-infectious despite the fact that the uncleaved precursor is efficiently incorporated into virus, thus demonstrating that cleavage is essential for glycoprotein-induced membrane fusion.

MATERIALS AND METHODS

Cells and DNAs

Turkey embryo fibroblasts (TEF) cells were prepared from 10 old day turkey eggs as described previously (21) and cultured in Dulbecco's modified Eagles medium (DMEM) containing 5% Calf serum and 10% tryptose phosphate broth (Growth Medium,(21). The QT6 cells, derived from a methylcholanthrene-induced quail tumor

(37), were grown in growth medium containing 1% heat-inactivated chicken serum. CV-1 cells, used for SV40 expression studies, were grown in DMEM containing 10% fetal calf serum.

Plasmid DNA was propagated in E. coli DH5 cells and was purified on both small and large scale as described previously by Maniatis et al. (34). All DNA used for transfection studies was derived from cesium chloride gradients.

Oligonucleotide Mutagenesis

To modify the cleavage sequence, an EcoRI-XbaI restriction fragment of 717 base pairs, containing the carboxy-terminus of gp85 and the entire coding sequence of gp37, was cloned into M13mp11. Two synthetic oligonucleotides were designed to introduce changes into the cleavage sequence (61). A 23-mer was designed to change the third A (nucleotide 6260) of the codon for arginine to a C, creating a codon for serine(AGC), and the first A (nucleotide 6264) of the codon for lysine(AAA) to a G, resulting in a codon for glutamic acid (GAA). The mutant was designated S19. A 20-mer was used to change the second A (nucleotide 6265) of the codon for lysine (AAA) to a G, generating a codon for arginine (AGA). This mutant was designated Ar1. Mutations were confirmed by dideoxy DNA sequencing (data not shown). According to protein structure predictions (3, 15), these changes do not alter the protein conformation. The *env* gene EcoRI-XbaI fragments carrying the mutations were removed from the replicative form of the M13 phage DNA and exchanged for the wild-type *env* gene in the SV40 *env* expression vector, as described below.

Expression Systems

For the initial characterization of mutant phenotypes, wild-type and mutated *env* genes of the Prague C strain of RSV were expressed in a SV40 late-region replacement vector (pSVenvKX) as described previously (4, 41). In the pSVenvKX slot-in vector, mutated *env* genes were substituted for the wild-type *env* gene through

unique EcoRI and XbaI restriction endonuclease sites which flank a 700bp region encoding the C-terminus of gp85 and the entire gp37.

For infectivity and incorporation studies, RCANBP, a modified proviral vector derived from the Schmidt-Rupin and Bryan high titer strains of RSV was used (20, 44). For the studies described here, the A-subgroup *env* gene was replaced by that of the Pr-C strain of RSV (26) to yield BH-RCAN. Mutant and wild-type genes were transferred from the pSVenvKX vector to RCANBP, using unique KpnI and ClaI sites flanking the *env* gene sequence (4). In some experiments aimed at quantitating trypsin-induced enhancement of infectivity of virions containing the S19 mutant glycoprotein, a second replication competent vector, BH-RCAN-HiSV (Figure 1), was used. In this vector, the RSV *src* gene was replaced by the hygromycin-resistance gene (25) under the transcriptional control of the SV40 early promoter. Transfection of the plasmid into avian cells results in expression of the viral replicative genes and packaging of a viral genome that confers a drug-resistant, selectable phenotype on the cell it infects.

For additional analyses of mutant glycoprotein incorporation into virions, the wild-type RSV-PRC *gag-pol* genes, and wild-type or mutant *env* genes were expressed independently in QT6 cells under the transcriptional control of a T7 promoter. For *gag-pol* expression, a SacI (nucleotide 255) to XbaI (nucleotide 6861) fragment from the pATV8 genomic clone was cloned into the plasmid pSP72 (Promega Biotec, Madison, WI), which contained the T7 promoter region. This yielded pSP72-*gag-pol*. For *env* expression a KpnI (nucleotide 5995) to XbaI (nucleotide 6861) fragment was cloned into pSP72. The resulting plasmids containing wild-type, or mutant G1, S19, and A^- (a secreted form of the RSV *env* ge) (42) *env* sequences, were named pSP72-Wt, pSP72-G1, pSP72-S19, and pSP72-A^- respectively.

For these experiments, subconfluent QT6 cells were infected with a recombinant vaccinia virus expressing the T7 RNA polymerase at an MOI of 15-20 (11). After one hour the cells were transfected with the different *env* and *gag* gene-

containing plasmids (as described in the text) by the modified polybrene method described below. At 12h post-transfection, the cells were pulse-labeled for 30 min. with 200μCi [^{3}H]leucine and then chased for 6h in complete medium. Released virus particles were pelleted by centrifugation through a 20% sucrose cushion (100,000 rpm for 20min in a TL100 centrifuge, Beckman Instruments) and both virus and cells lysed and immunoprecipitated as described below.

Metabolic labeling, immunoprecipitation, trypsin digestion, and chloroquine inhibition experiments.

Cells expressing viral proteins were starved for leucine in leucine-free DMEM for 1 h and then pulse-labeled at 37°C for 15-20 min in leucine-free labeling medium containing 200μCi of ^{3}H-leucine (Dupont, NEN Research Products). In pulse-chase labeling, labeled cells were chased in complete growth medium for 4-6 h. Immunoprecipitation was carried out as described previously (59). Viral protein was immunoprecipitated with specific antibodies as indicated in the text. Antibodies directed against the RSV glycoproteins were prepared in rabbits using either lentil-lectin purified gp85/gp37 (anti-Env) as described previously (23) or a peptide corresponding to the C-terminal cytoplasmic domain of gp37 (anti-gp37c). These antibodies demonstrated no cross-reaction with the RSV Gag proteins. The antibody to the RSV *gag* gene products was kindly provided by Dr. John Wills. Trypsin treatment and chloroquine inhibition experiments were carried out in a similar manner except that labeled cells were chased in serum-free medium containing 0.5 μg/ml of TPCK-trypsin (Sigma, St. Louis, Mo) or in complete growth medium containing 100μg/ml of chloroquine (4).

Analyses of viral infectivity.

For infectivity studies TEF were transfected with the BH-RCAN constructs using a modified polybrene method (28). Semi-confluent cells were washed twice with serum-free Dulbecco's modified Eagle medium (DMEM), 0.5ml of DMEM containing 30μg of polybrene (Sigma, St Louis. MO) was added, and the cells were incubated at

37°C for 30 minutes. At the end of the incubation, 1μg of DNA was diluted into 0.5 ml DMEM and added to the plate dropwise while the plate was rocked. After addition of the DNA, the plates were incubated at 37°C for 6 h with gentle rocking on a automatic rocker. Cells were then shocked with 30% DMSO in DMEM for 4 minutes and washed three times with DMEM. Fresh growth medium was then added and one of two methods was used to determine infectivity.

A. Modified rapid reverse transcriptase (RT) assay: Medium from transfected TEF cells was collected at three days intervals from day 3 to day 15 post-transfection. A volume of 20μl of the medium from each plate was mixed with 50μl assay buffer (60 mM Tris, pH 8.0, 7mM DTT, 7mM $MgCl_2$, 200mM KCl, 0.2% Triton X-100), 1μl of poly A:oligo dT, and 1μCi of ^{35}S-TTP in a 96-well plate. The reaction was carried out for 1h at 37°C and stopped by the addition of 50μl of 0.5M NaH_2PO_4. Each sample was blotted to an NA45 (Schleicher and Schuel, Keene, NH) membrane with vacuum. After washing 3 times with 0.5M NaH_2PO_4 and air-drying, the incorporated counts were quantitated on a AMBIS radioanalytic imaging system (AMBIS Systems, Inc. San Diego, California).

B. Focus assay. At 6 days post-transfection medium was collected from each plate and diluted 10^{-1}-10^{-5} in DMEM. A volume of 1 ml of each diluted sample was then used to inoculate TEF cells which were about 80% confluent, incubated for 1 h, and then overlaid with agar (21). Foci of transformed cells were counted after 12 days.

Trypsin activation of BHRCAN-HiSV/S19 virions

Duplicate plates of TEF cells were transfected with the BHRCAN-HiSV/S19, BHRCAN-HiSV/Dr1, and wild-type BHRCAN-HiSV genomes. At 24 h post-transfection, cells were washed once with serum free DMEM and cultured in 4 ml of serum-free DMEM with or without 0.5μg/ml of TPCK-trypsin for 24 h. Medium was then collected and mixed with 4 ml of complete growth medium containing 10μg/ml of

polybrene; 2 ml of the mixture was added to each of three 100 mm plates of semi-confluent QT6 cells. Culture medium from cells transfected with the wild type genome was diluted 10^{-1} -10^{-3} before being used to infect QT6 cells in order to obtain a discrete number of colonies on each plate. After 16 h incubation, infected resistant cells were selected with hygromycin-containing medium (350u/ml hygromycin in growth medium) for 10 days. The number of colonies on each plate was counted and the mean determined for each mutant.

Incorporation of viral glycoproteins into virions

For incorporation studies, two expression methods were employed. In the first, turkey embryo fibroblasts (TEF) were transfected with wild-type or mutant BH-RCAN genomes using the methods described previously. Virions were harvested 48-96h post-transfection, pelleted by ultracentrifugation, and virion proteins were identified by immuno-blotting using rabbit anti-Env antibody or anti-capsid antibody, following separation on SDS-PAGE and transfer to nitrocellulose filters (58).

For some mutants which retained the ability to infect cells, the transfected TEC were maintained until equivalent levels of cell transformation were observed (10 days) and then 100mm plates were pulse-labeled with [^{3}H]leucine (0.5mCi in 0.5ml) for 20min. The cells were then lysed immediately (pulse plate) or chased for 6h in complete medium prior to lysis. Virions released into the medium of the chase plate were pelleted through a 25% sucrose cushion by centrifugation for 1h at 50K in an SW55 rotor and the composition of virions was determined by immunoprecipitation with a mixture of anti-Env and anti-gp37c antibodies followed by SDS-PAGE analysis.

In the second approach, we employed the T7 complementation/assembly system described above, in which QT6 cells were cotransfected with *gag-pol* gene and *wt* or the mutant *env* gene containing plasmids.

RESULTS

Expression of the envelope glycoprotein cleavage mutants from an SV40 vector in CV-1 cells

The SV40 late-region replacement vectors carrying the modified envelope genes were cotransfected into CV-1 cells with helper SV40 DNA (dl1055) to obtain infectious recombinant SV40 virus stocks. High-titer viral lysates were diluted 1:50 to infect fresh CV-1 cells. Cells were pulse-labeled with [^{3}H]-leucine and immunoprecipitated using an anti-Env antibody as described in Materials and Methods. The results of a typical pulse-chase labeling experiment are shown in Figure 2. In each of the pulse lanes (labeled P) a single major band of 95kD could be seen. This is the core-glycosylated precursor to the envelope glycoproteins of RSV, gPr95. After a 4h chase period, the intensity of this precursor band decreased and more diffuse bands with MWs of 85,000 (gp85) and 31,000 (gp37) were immunoprecipitated from cells expressing the wild-type glycoprotein (Fig. 2, Wt, lane C). These represent the terminally-glycosylated cleaved products SU and TM, respectively, of the RSV *env* gene.

A similar level of gp85 and gp37 could be seen in chase-lysates of cells expressing the Ar-1 mutant glycoprotein (Fig 2, Ar1, lane C). In this mutant the substitution of arginine for the conserved lysine residue did not significantly reduce proteolytic cleavage of gPr95. In contrast, as we have described previously (43), substitution of glutamic acid for the lysine residue (mutant G1) results in a significant reduction in the amount of gp85 and gp37 precipitated from the chase lysates and a new, more diffuse band of approximately 120kD (gp120*) can be immunoprecipitated. This represents the terminally glycosylated form of uncleaved gPr95.

The substitution of glutamic acid for lysine in mutant G1 significantly reduced cleavage of the precursor, but some residual cleavage to gp85 and gp37 was observed. This residual cleavage was not occurring at a site distal to the tetrapeptide cleavage recognition region since deletion of the four codons for the basic residues in this region

resulted in a complete inhibition of gPr95 cleavage and accumulation of only gp120* (Fig 2, Dr1, Lane C).

To determine if the residual cleavage was occurring after the C-terminal arginine or if it was dependent on the remaining dibasic arg-arg sequence, the S19 mutation, in which the cleavage site was mutated to serine-arginine-glutamic acid-arginine, was expressed in this system. As can be seen from Figure 2 (S19, Lane C), this mutation blocked cleavage completely and no gp85 or gp37 was detectable. As with Dr1 the terminally glycosylated form of gPr95, gp120*, accumulated in the chase period. This result indicates that the enzyme(s) responsible for retroviral glycoprotein cleavage have an absolute requirement for a dibasic sequence.

In previous studies we had shown that, in contrast to cleavage of the *wt* glycoprotein precursor, the residual cleavage observed with the G1 mutant was inhibited by chloroquine (43). We suggested therefore that cleavage of the Arg-Arg-containing G1 cleavage site might be mediated by a different, chloroquine-sensitive enzyme. Since the Ar1 mutant glycoprotein was cleaved as efficiently as that of the *wt* protein, we investigated whether proteolysis of the Lys-deficient cleavage site was also chloroquine-sensitive. The results of a pulse-chase experiment in which 100mM chloroquine was added to one set of the chase plates are presented in Figure 3. As we reported previously, the residual cleavage of the G1 precursor was abrogated by chloroquine treatment, whereas that of the *wt* protein was essentially unaffected. In contrast, a significant inhibition of Ar1 precursor cleavage was observed in the presence of chloroquine. Quantitation by densitometry of the cleavage products observed in the presence and absence of the drug showed that Ar1 cleavage was inhibited by approximately 70%.

The modified processing site of mutant S19 is susceptible to added trypsin

Based on protein structure models, the substitution of a polar amino acid residue, serine, for the first positively charged residue, arginine, within the cleavage

sequence would not be expected to significantly alter the tertiary structure (ß-turn) of the precursor glycoprotein (3; 15). To confirm that the lack of processing was due to a loss of a specific recognition sequence rather than an altered tertiary structure in which the enzyme site is no longer accessible, we have determined the susceptibility of the uncleaved 'gp120* to exogenously added trypsin. Infected cells were pulse-labeled then chased for 4 h in serum free medium containing 0.5 µg/ml of TPCK trypsin at 37°C. As can be seen in Figure 4, the uncleaved S19 precursor gp120* was cleaved completely into gp85 and gp37 by the added protease. A similar result was observed for mutant G1, confirming our previous results with this mutant (43). In contrast, the gp120* precursor of mutant Dr1, which lacked any basic residues in the cleavage region, was unaffected by the same treatment. Similarly the gp85 and gp37 polypeptide products of intracellular cleavage were not cleaved further by the added trypsin. These results indicate that on the mature Env precursor only a single region is susceptible to trypsin cleavage and that in the S19 mutant polypeptide this cleavage site is still accessible to cellular endopeptidase(s).

Cleavage is essential for viral infectivity but not for incorporation of glycoprotein into virion

In order to determine the effects of the cleavage site mutations on the biological activity of the *env* gene products, RSV proviruses carrying the mutated *env* genes were transfected into turkey embryo fibroblasts. The BH-RCAN vector used in these studies contains a replication-competent genome with flanking LTRs, so that following transfection of susceptible cells, viral RNA is transcribed and viral structural components synthesized and assembled even if the viral genome is non-infectious. The infectivity of the released virions was determined by assessing the rate of increase of RT activity in the culture medium at days 3, 6, 9, 12, and 15 post-transfection. Figure 5 shows the results of such an RT assay. RT activity in the supernatants collected from BH-RCAN-G1, BH-RCAN-Ar1 and wild-type transfected cells increased rapidly from days 9-15 after transfection, indicating that the Ar-1 mutation did not reduce the

infectivity of the virus in this *in vitro* assay. While the BH-RCAN-G1 was infectious, the level of RT activity was lower than that of the wild-type and increases at a lower rate. The reduced level of glycoprotein precursor cleavage observed with the G1 mutation was therefore reflected in a reduced level of infectivity. The RT activity of virions released from BH-RCAN-S19 transfected cells remained at the level of background, indicating that in the absence of glycoprotein precursor cleavage virus infectivity was abolished. These RT results were confirmed in an assay for focus-forming virus carried out at 6d post-transfection. At this time the *wt* transfected cell medium contained 3.5 X 10^3 focus forming units (FFU)/ml, while that from AR1 and G1 contained 1.9 and 0.9 X 10^3 FFU/ml respectively. In contrast, no infectious focus-forming virus could be detected in the S19 or Dr1 culture media. The more than one thousand-fold reduction in infectivity for both S19 and Dr1 indicates that envelope glycoprotein precursor cleavage is essential for viral infectivity of RSV.

Since in the above experiments, the level of infectivity appeared to be related to the level of precursor cleavage, we attempted to determine if the uncleaved glycoprotein could be incorporated into virions. In the initial experiments, we utilized the two mutants, Ar1 and G1, which could replicate in turkey cells. Wild-type BH-RCAN, BH-RCAN-Ar1, and BH-RCAN-G1 genomes were transfected into turkey embryo fibroblasts and 10 days post-transfection were pulse-labeled then chased for either 4h or overnight prior to lysis. Virus was pelleted from the overnight chase. Viral proteins were immunoprecipitated from both the 4h chase cell lysate and pelleted virus to compare the amount of cleaved and uncleaved products in the cell and incorporated in virus particles. Fig.6A shows the results of such an experiment. It can be seen that from Ar1 and wild-type infected cells, virions containing only gp85 and gp37 were observed (Figure 6A, Wt Lane V and Ar Lane V). No gp120* was found within the cell or virions. In contrast virus released from BH-RCAN-G1 infected cells contained both uncleaved gp120* and the mature cleaved products (Figure 6A, G1

Lane V). In this case both gp120* and mature cleavage products could be detected in the cell lysates (Figure 6A, G1 Lane C) in a ratio similar to that observed in virions. The above experiment showed that terminally-glycosylated, uncleaved G1 gp120* could be incorporated into virions. However, since retroviral glycoproteins are present in virions as oligomeric structures, it was possible that the cleaved molecules facilitated entry of uncleaved proteins. In order to rule out this possibility, we examined the ability of the S19 and Dr1 glycoproteins to be incorporated into virions. Because these viruses are non-infectious, in preliminary experiments virions were pelleted 48-96h post-transfection and virus-associated glycoproteins were identified by Western Blot analysis. The results of such an experiment are shown in Figure 6B. Low levels of virions were released in these transient experiments, making quantitation difficult, but for both Dr1 and S19 significant amounts of gp120* could be detected in virions (Figure 6B, Lanes D and S respectively). In this experiment the ratio of glycoprotein to capsid proteins was similar for both the mutants and wild-type virus (data not shown).

Since these results suggested that the uncleaved glycoproteins could be incorporated efficiently into virions, we turned to a transient expression system in order to obtain more quantitative results. In these experiments the RSV *gag-pol* genes and the RSV *env* gene were expressed independently under the control of the T7 promoter by a modification of the method of Fuerst and Moss (11). In this complementation system, which will be described in detail elsewhere, QT6 cells were used to co-express the capsid proteins and wild-type or mutant envelope proteins of RSV. The results of immunoprecipitating lysates and fractionated culture medium from cells pulse-labeled for 30min then chased for 6h is presented in Figure 7. In the QT6 cells the gp85 product and gPr95 migrate to the same position in the gel (compare Wt, cell lysate to Wt, viral pellet lanes) and so gp85 is not clearly visible in cell lysates. Uncleaved terminally glycosylated products (gp120*) were only observed in cells expressing the

G1 and S19 mutant *env* genes. After the 5h chase, little Pr76^{gag} precursor was detected in cells but a strong p27 band of cell-associated p27(CA) could be seen in all lanes. An analysis of pelleted virus polypeptides confirmed that uncleaved, terminally-glycosylated gp120* was efficiently incorporated into virus released from both G1- and S19-expressing cells. The ratio of uncleaved to cleaved glycoprotein in G1 virions resembled that observed in virus released from infected turkey cells, thus confirming the validity of the T7 expression system. In contrast, cells expressing a truncated form of the RSV glycoprotein precursor that is secreted from cells showed no glycoprotein associated with virions (Figure 7, viral pellet, A^-) and the glycoprotein was found only in the supernatant following fractionation (Figure 7, supernatant, A^-).

It is clear from these experiments that proteolytic processing of the RSV glycoprotein precursor is not required for its efficient incorporation into virions. Moreover, the uncleaved precursors appear to be incorporated with equal efficiency into virus particles in the presence of normally cleaved glycoprotein.

Infectivity of virions containing the S19-mutant glycoprotein can be activated by exogenous protease.

Because the S19 mutant glycoprotein was efficiently transported to the cell surface, remained sensitive to cleavage by exogenous trypsin, and could be efficiently incorporated into virions, we investigated whether exogenous cleavage of the glycoprotein resulted in a biologically active molecule. For this study, the S19 and Dr1 mutant *env* genes were cloned into a proviral vector, BHRCAN-HiSV, that contained within its genome the gene for hygromycin resistance. The wild-type and mutant genes were transfected into TEF cells and transcription from the input DNA was allowed to occur. From 24 to 36 h post transfection cells were treated with 0.5 μg/ml of trypsin in serum-free medium. Control cells were incubated in parallel in serum-free medium lacking protease. The culture fluids from both treated and untreated cells were harvested at 36h and used to infect fresh QT6 cells. At 16 h post-infection, QT6 cells infected with the drug resistance-carrying virus were selected by addition of

hygromycin. After 10 to 12 days of selection, resistant cell colones were counted. The results of two experiments are summarized in Table 1. As expected from the infectivity data presented above, no virions capable of infecting QT6 cells were released from untreated cells transfected with either the BHRCAN-HiSV-Dr1 or S19 mutant genomes. However, resistant colonies were consistently observed when QT6 cells were infected with the supernatants of trypsin treated cells, transiently-expressing the BHRCAN-HiSV-S19 genome. In contrast no colonies were seen in plates infected with supernatants from trypsin treated, BHRCAN-HiSV-DR1 expressing cells. The latter result was expected since this mutant glycoprotein could not be cleaved by addition of trypsin to expressing cells.

The number of colonies developing in BHRCAN-HiSV-S19 infected cells was approximately 8% of that observed with the wild-type BHRCAN-HiSV virus. Thus, while it is clear that the S19 mutant glycoprotein can be activated by addition of small amounts of trypsin, the level of infectivity is significantly less than that of wild-type virions.

DISCUSSION

In retroviruses, as with many other enveloped viruses, the surface glycoprotein complex initiates virus infection by binding to a receptor on the surface of a target cell (24). A hydrophobic domain (the "fusion peptide") in the membrane spanning component of the complex then induces the viral and cellular lipid membranes to undergo fusion, thus releasing the viral capsid and genetic material into the cytoplasm of the host cell. It is clear from a number of studies that conformational constraints on the glycoprotein complex and the ability to expose the fusion peptide are crucial for functional receptor binding and membrane fusion (24; 31). The glycoprotein complex, on the other hand, must be capable of traversing the secretory pathway of the cell without being non-specifically triggered to induce fusion of internal membranes. One possible way to guard against such an event might be to delay precursor cleavage and

activation of the fusion peptide until a late stage of the transport pathway. The cleavage site of the glycoprotein precursor is not only highly conserved among different retroviruses but also resembles that of many eucaryotic protein precursors (35; 42; 46; 55). Conservation of the cleavage site suggests that a common cellular endopeptidase(s), present in a wide variety of cell lineages, may be responsible for retrovirus envelope protein cleavage.

We reported earlier that a deletion of the four basic amino acid residues within the cleavage site for Pr95 completely blocked its proteolytic processing, and showed that the uncleaved products were resistant to added trypsin to a concentration up to 20 μg/ml. These data proved that the region containing the four basic amino acid residues is the only site in the glycoprotein precursor that is accessible to trypsin-like enzymes (43). We also showed that a substitution of glutamic acid for the highly conserved lysine, a basic amino acid residue at the third position of the cleavage sequence, reduced cleavage by about 90%. We inferred from inhibitor studies (see below) that the residual cleavage observed in this G1 mutant resulted from less efficient cleavage at the residual arginine pair of the arg-arg-glu-arg sequence (43). In this present study, by introducing a second mutation into the G1 mutant (to yield mutant S19), dibasic sequences within the cleavage region were eliminated and we observed no intracellular cleavage of the S19 precursor. Nevertheless this mutated region, containing two unpaired arginine residues remained susceptible to cleavage by exogenously added trypsin, indicating that the changes introduced into the cleavage region had not altered the tertiary structure of the protein to occlude the residual arginines. This result is consistent with the interpretation that a dibasic sequence (arg-arg or lys-arg) is absolutely required for cleavage site recognition by the Golgi-endopeptidase. Furthermore, it implies that the cleavage enzyme is not trypsin-like, since it is unable to recognize arginine residues that are sensitive to trypsin action.

In our previous studies, the residual cleavage seen with the G1 mutant could be inhibited by the addition of 100μM chloroquine even though cleavage of the wild-type precursor was not affected by this compound. We suggested from this result that two enzymes might be present in the Golgi-complex--one choroquine-resistant that had a high preference for lysine-arginine pairs and a second chloroquine-sensitive enzyme that could cleave at arginine-arginine pairs (43). However, we could not rule out the possibility that, in the context of the glutamic acid, cleavage by a single enzyme became choroquine-sensitive. The results obtained with mutant Ar1 demonstrate that the cleavage sequence arg-arg-arg-arg, lacking the highly conserved lysine residue, can be cleaved efficiently in an intracellular compartment to yield biologically functional gp85 and gp37 proteins. However, inhibition studies with choroquine showed that cleavage of the Ar1 precursor was significantly more sensitive to treatment of cells with the drug than was that of the wild-type protein; Ar1 cleavage was inhibited approximately 70% following treatment of cells with 100μM choroquine whereas little effect was observed on cleavage of the wild-type protein. This result is consistent with the hypothesis that two distinct proteinases, capable of cleaving at dibasic sequences, are present within the secretory pathway. One of these would be predicted to favor lysine-argine targets, whereas the other would recognize arginine-arginine pairs. It is interesting to note that when the G1 mutant glycoprotein was expressed in avian cells as much as 50% of the mutant precursor was cleaved to gp 85 and gp37. It is possible that the Golgi-endopeptidase has a broader specificity in these cells or that in avian cells the arginine-arginine pair is more efficiently cleaved by a second enzyme.

In the yeast *Saccharomycetes cerevisiae*, a prohormone processing enzyme encoded by the *KEX2* gene product cleaves the pro-a factor and pro-killer toxin at Lys-Arg and Arg-Arg sites as they transit the secretory pathway (12; 13). This membrane anchored, calcium-dependent, serine proteinase can process mammalian prohormones correctly and displays significant amino acid homology with three recently described

mammalian genes: *fur, pc2* and *pc3* (2; 17; 18; 19; 36; 51; 52; 54; 57). The product of the *fur* gene appears to have the capacity to mediate cleavage at paired basic residues (60), and Hatsuzawa et al. (18) demonstrated that in the mouse this gene is transcribed in a wide variety of tissues. It is thus a possible candidate for an enzyme that mediates retroviral glycoprotein precursor cleavage. Moreover, preliminary experiments in this laboratory have demonstrated the presence of a membrane bound, calcium-dependent protease, within Golgi vesicles of chicken hepatic cells, that specifically cleaves the Pr95 precursor (8). The characteristics of this enzyme resemble those of the KEX2 protein. However, Smeekens et al (53) have suggested that *fur, pc2, and pc3* comprise members of a mammalian proteinase superfamily. It remains to be determined which member(s) might be responsible for retroviral glycoprotein cleavage.

By introducing the mutated *env* genes back into an RSV-derived vector and transfecting avian embryo fibroblasts we were able to address the question of whether proteolytic processing was required for incorporation of the glycoprotein into the virion and for viral infectivity. The hemagglutinin (HA) glycoprotein of the avian and equine influenza viruses is proteolytically cleaved into HA1 and HA2 in a region of basic amino acid residues similar to that of the retroviruses (29, 43). In the human influenza viruses this cleavage occurs at a single basic residue (40). In the influenza viruses it has long been known that while cleavage is absolutely required for infectivity it is not required for assembly into virions, or for receptor binding activity (6, 16, 30, 38, 39, 49, 50). We have demonstrated in this report that in Rous sarcoma virus proteolytic cleavage of the glycoprotein precursor is also a prerequisite for infectivity as evidenced by mutants Dr1 and S19. The infectivity of mutant glycoprotein-containing viruses appears to be related to the amount of cleaved glycoprotein incorporated into the virion, as can be seen from the results of experiments with mutants S19, G1, Ar1, and wild type virus. Recent studies with HIV-1 have also demonstrated that mutations within the glycoprotein precursor cleavage site can reduce the fusogenic potential of

the glycoprotein and infectivity of the virus (1, 9, 35). In the latter experiments, however, the effect of the mutations on virus infectivity was not quantitatively determined.

Although cleavage of the RSV glycoprotein precursor is required for infectivity, it is not a prerequisite for incorporation of the glycoprotein into virions. Moreover, the uncleaved, terminally glycosylated form of the precursor can be incorporated into virions as efficiently as the mature cleavage products. Preliminary results indicated that the ratio of uncleaved precursor to cleaved glycoprotein in pelleted BH-RCAN-G1 viral particles was similar to that present in cells infected by mutant G1 virus. However, since we have shown previously that the RSV glycoprotein is transported intracellularly and incorporated into virions as an oligomeric structure (7), it was possible that the uncleaved glycoprotein was incorporated into virions as a component subunit of an oligomer containing cleaved glycoprotein precursors. This possibility was ruled out by transient expression studies using the BH-RCAN vector and a novel T7-polymerase-based *gag-pol/env* complementation system. In both systems we were able to demonstrate equivalent incorporation of uncleaved, terminally glycosylated envelope glycoproteins (S19, Dr1) and cleaved glycoproteins (Ar1 and Wild-type) into virus particles. Thus the lack of infectivity of the BH-RCAN-S19 and Dr1 virions was not due to an inability to incorporate the uncleaved glycoprotein, but probably reflected the failure to generate an active fusion peptide (14). These results suggest that in the SE521 mutant of PR-RSV-E described previously by Linial et. al. (33) the lack of incorporation of the mutant glycoprotein into virions is not related to the absence of cleavage.

Since the S19 mutant glycoprotein was susceptible to cleavage by trypsin on the surface of expressing cells and could be efficiently incorporated into virions, it was possible for us to determine whether the exogenously cleaved molecule was biologically active. Transfection of TEF with BHRCAN-HiSV-S19 plasmid DNA

resulted in the release of virions that were conditionally infectious; virus infectivity could be activated by mild trypsin treatment. While the infectivity of the released, activated virus appeared to be significantly reduced (to approximately 8%) compared to the wild-type BHRCAN-HiSV, this is likely to be a low estimate. Virions were harvested from 24-36h post-transfection to reduce the possibility of wild-type virus released from transfected cells infecting adjacent cells; however, it is possible that some second rounds of infection did occur. Similarly, while hygromycin selection was initiated at 16 h post infection, some cells infected with wild-type virus could have released virus capable of infecting new cells within this time period.

The exogenous activation of the RSV glycoprotein with trypsin is in contrast to that described for the SV5 fusion protein (40). In the latter system, exogenous cleavage of fusion protein precursors with trypsin did not result in proteins capable of mediating cell-cell fusion. It is possible that in the case of the SV5 F protein, cleavage within an intracellular environment is required for an active protein conformation. Our experiments with the RSV glycoprotein suggest that this is not a prerequisite and that at least a fraction of the molecules cleaved exogenously does achieve an active tertiary structure. The conditional nature of the S19 virus opens up the possibility of defining biochemically the conformational changes that take place upon glycoprotein precursor cleavage in this prototype retrovirus system.

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Figure 1. Schematic representation of BHRCAN-HiSV provirus vector
BHRCAN-HiSV, a replication competent RSV provirus vector, was derived from the modified RCAN vector, RCABP (20, 44). The gene specifying hygromycin resistance (open box) under the control of the SV40 early promoter (arrowed box) was inserted into the BH-RCAN vector (gray box) at the unique Cla 1 site. For the construction of wild-type and mutant genomes with C-subgroup host range, wild-type and mutated *env* genes, flanked by unique Kpn1 and Cla1 sites, were excised from the SV40 expression vector SVenvKX;(4) and used to replace the wild-type subgroup A *env* gene in BHRCAN-HiSV.

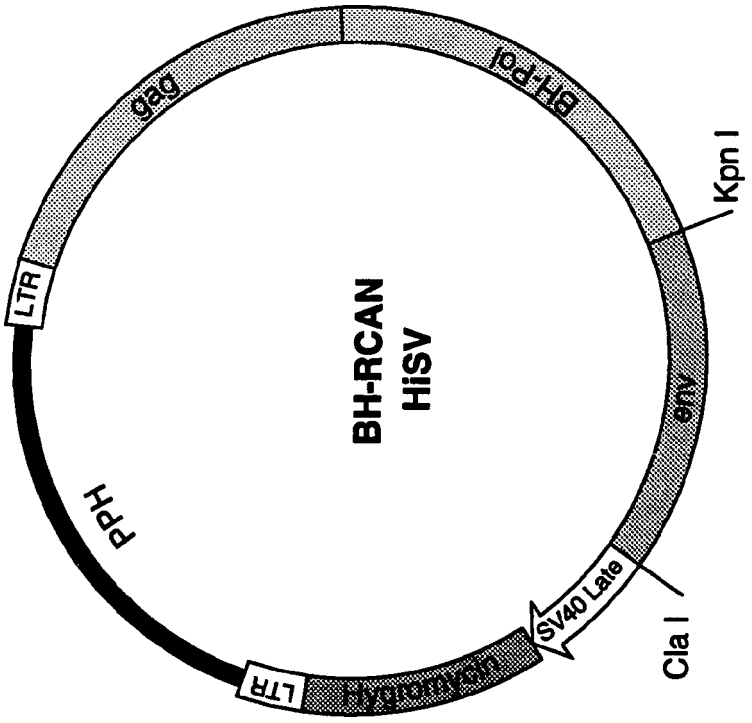
BH-RCAN
HiSV
gag
BH-Pol
Kpn I
env
Cla I
SV40 Late
Hygromycin
LTR
pPH
LTR

Figure 2. Pulse-Chase Analysis of Mutant Glycoprotein Biosynthesis
CV-1 cells were infected with recombinant SV40 virus expressing Wt, Ar1, G1, S19, or Dr1 *env* genes. Three days post-infection, cells were pulse-labeled for 10 minutes (Lane P in each panel) or pulse-labeled and chased with complete DMEM for 4h (Lane C in each panel). Cells were lysed at the appropriate time and immunoprecipitated with a rabbit anti-Env antibody. Precipitated proteins were separated on a 12% polyacrylamide gel and bands were visualized by fluorography. An uncleaved but terminally glycosylated form of Pr95, designated gp120*, was observed in chases of G1, S19, and Dr1.

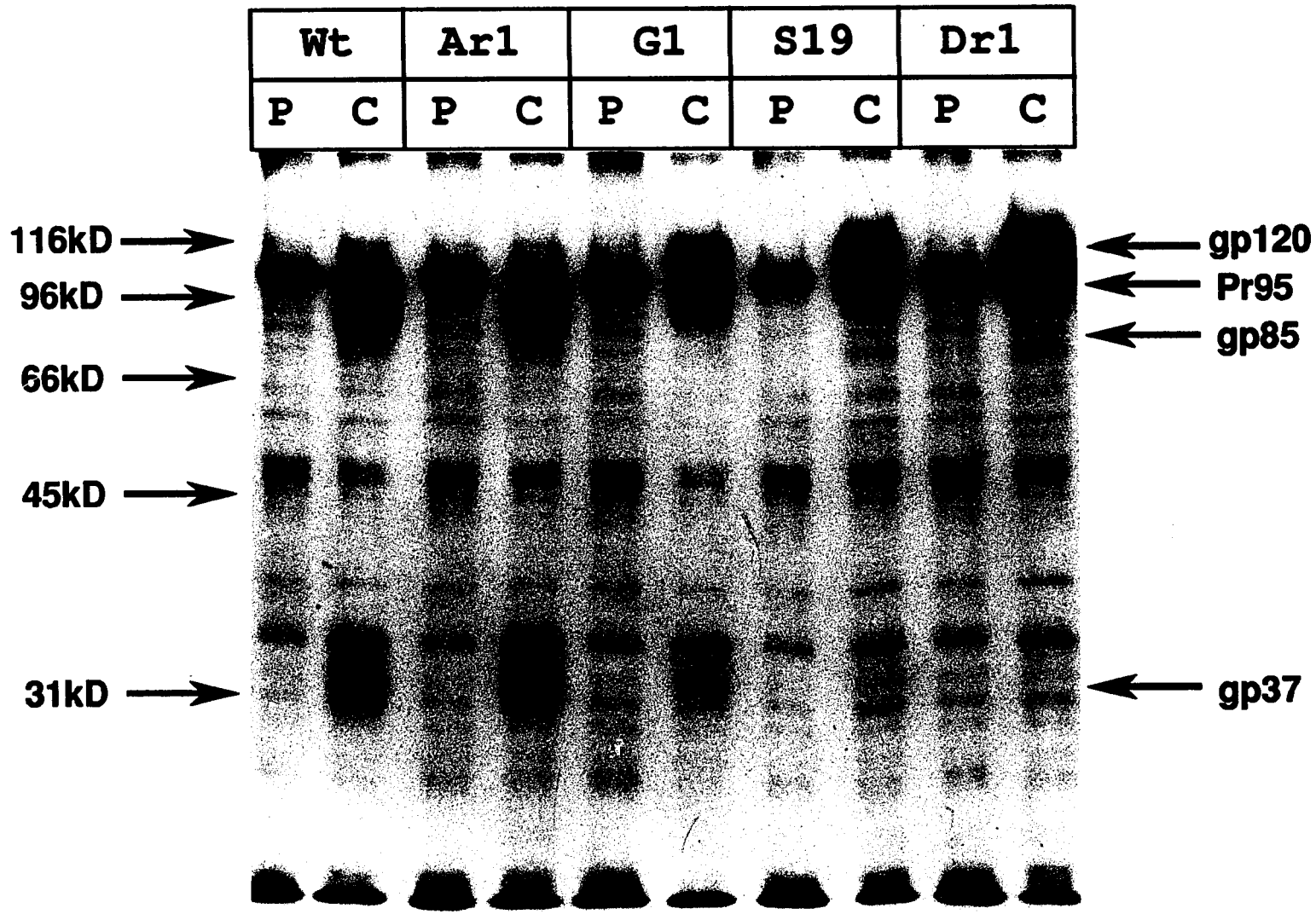
Wt
Ar1
G1
S19
Dr1
P C P C P C P C P C
116kD
96kD
66kD
45kD
31kD
gp120
Pr95
gp85
gp37

Figure 3. Synthesis and processing of mutant Ar1 envelope glycoprotein
CV-1 cells were infected with recombinant SV40 virus containing Wt, Ar1, or G1 *env* genes. At three days post-infection, cells were labeled and processed as described in Figure 2, except that one set was chased in the presence of chloroquine (Lane Ch in each panel). In the presence of chloroquine, both gp85 and gp37 migrate faster due to alterations in the extent of glycosylation (43).

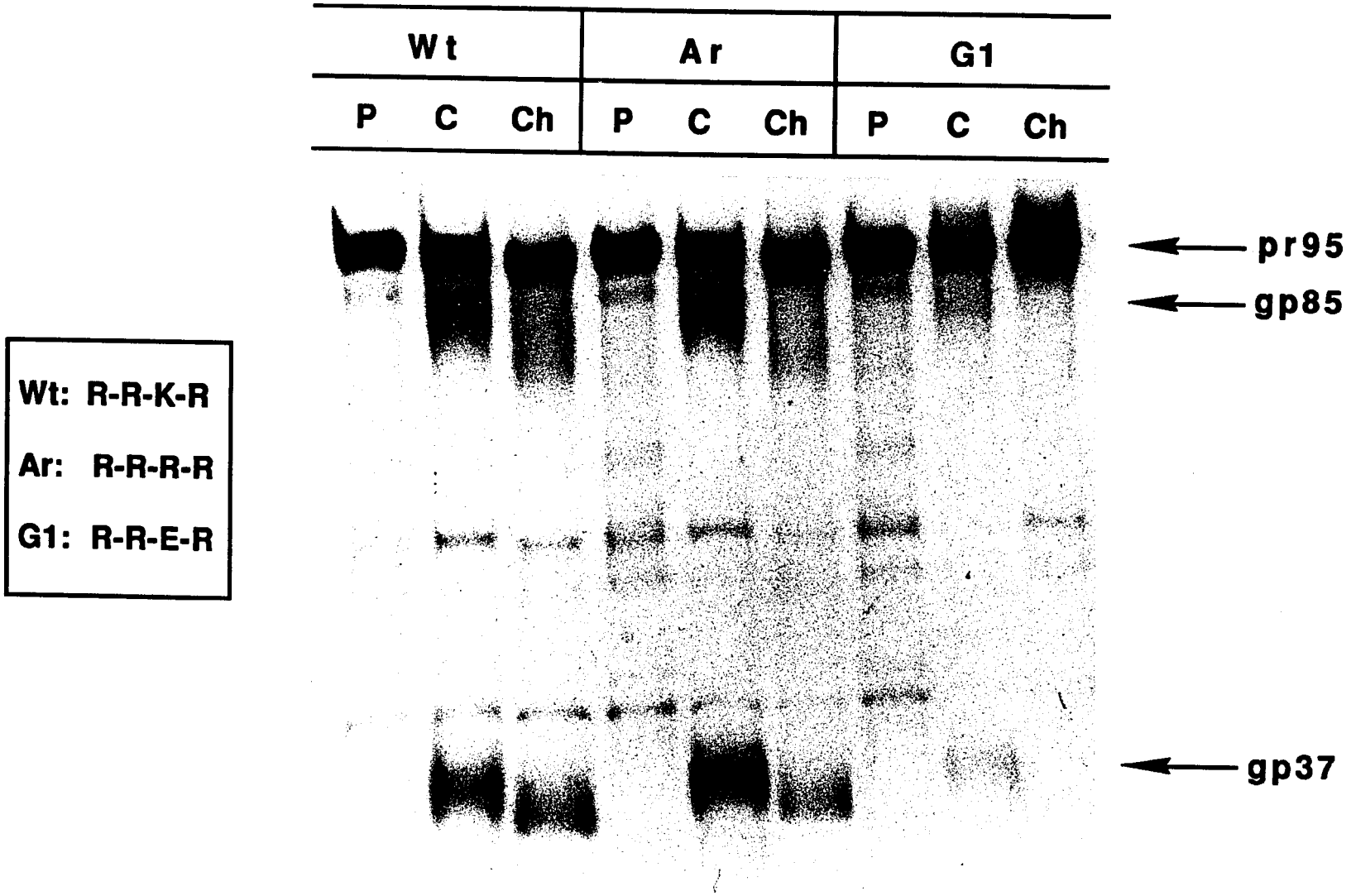
Wt
Ar
G1
P
C
Ch
P
C
Ch
P
C
Ch
pr95
gp85
gp37
Wt: R-R-K-R
Ar: R-R-R-R
G1: R-R-E-R

Figure 4. Terminally glycosylated gp120* of mutant S19 is susceptible to trypsion cleavage.
CV-1 cells were infected with recombinant SV40 virus expressing Wt, G1, S19 and Dr1. Three days after infection, cells were pulse labeled for 10min (P), pulse-labeled and chased for 4h in serum-free DMEM (C), or pulse-labeled and chased for 4h in DMEM containing 0.5 μg/ml of trypsin (T). Viral glycoproteins were precipitated and identified as described in Figure 2.

Wt:	R-R-K-R
G1:	R-R-E-R
S19:	S-R-E-R
Dr:	----Δ-----

P: pulse
C: chase
T: trypsin

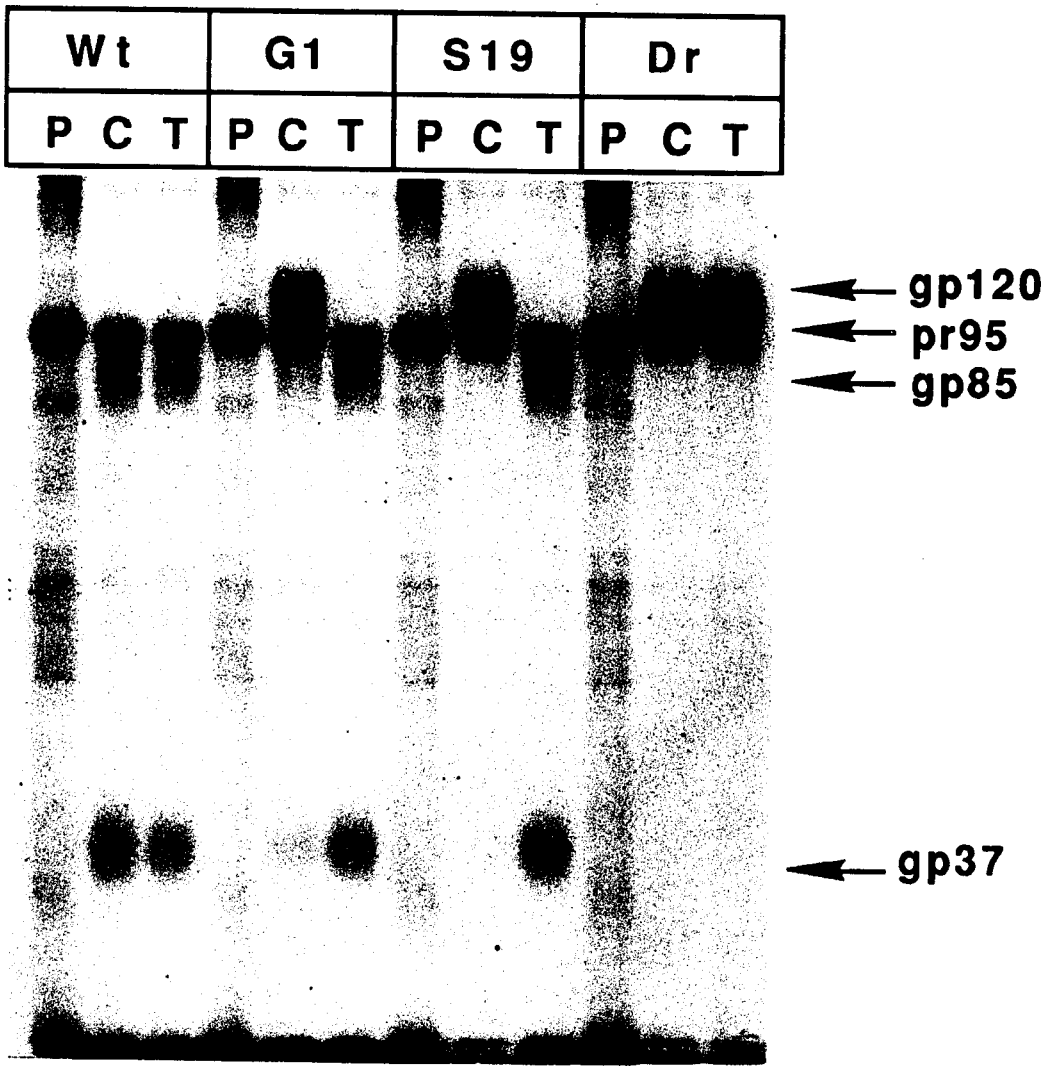

Figure. 5. Infectivity of Envelope Mutant BH-RCAN Genomes
Infectivity of mutant genomes was assessed by measuring the release of reverse transcriptase-containing virions into the culture medium. Wild-type or mutant BHRCAN-HiSV plasmid DNAs were transfected into TEF cells using the polybrene transfection method. At days 3, 6, 9, 12, and 15, medium was collected and assayed for reverse transcriptase activity using a modified RT assay as described in Materials and Methods.

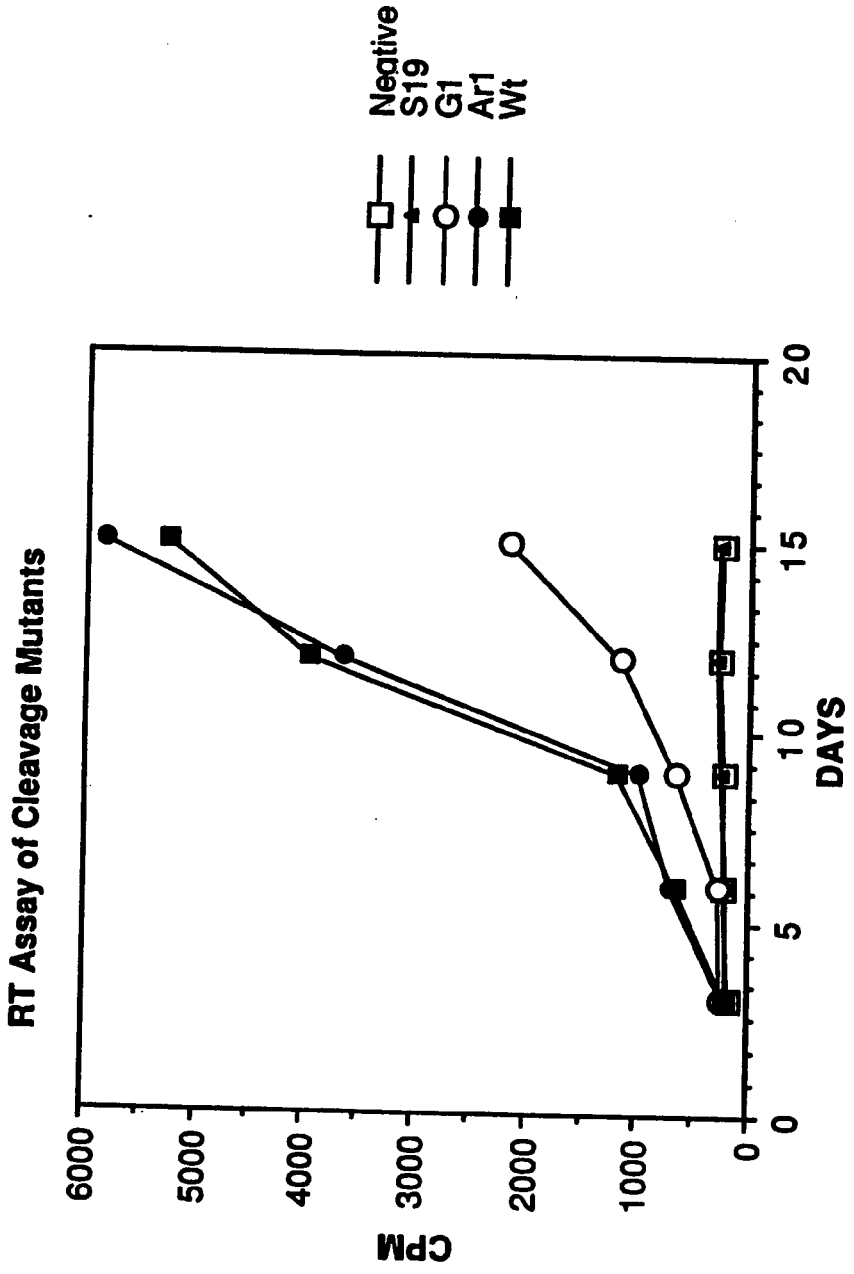
RT Assay of Cleavage Mutants
CPM
DAYS
0
1000
2000
3000
4000
5000
6000
0
5
10
15
20
Negative
S19
G1
Ar1
Wt

Figure 6. Incorporation of Mutant Glycoproteins into BH-RCAN Virions
TEF cells were transfected with BHRCAN containing the Wt, Ar1, or G1 (Panel A) or Wt, S19, and Dr1 (Panel B) *env* mutations as described in Materials and Methods.
Panel A: At 10 days post-transfection, cells were pulse-labeled for 15 min(P) with [^{3}H]-leucine or pulse-labeled and chased for 5 h (C). Medium from pulse-chased cells was collected, cleared of cell debris by low speed centrifuge, and then pelleted by ultra centrifugation (V). Immunoprecipitation of cell lysates and lysed viral pellets with an anti-glycoprotein antibody was as described in Materials and Methods. It can be clearly seen that the uncleaved gp120* protein encoded by the G1 mutant *env* gene is efficiently incorporated into virions (Panel G1, lane V).
Panel B: Virions were harvested 36-96h post-transfection. The pelleted virion proteins were fractionated on a 12% SDS gel, transferred to nitrocellulose, and immunoblotted using an anti-glycoprotein antibody. In both wild-type and mutant virions equivalent amounts of glycoprotein were incorporated.

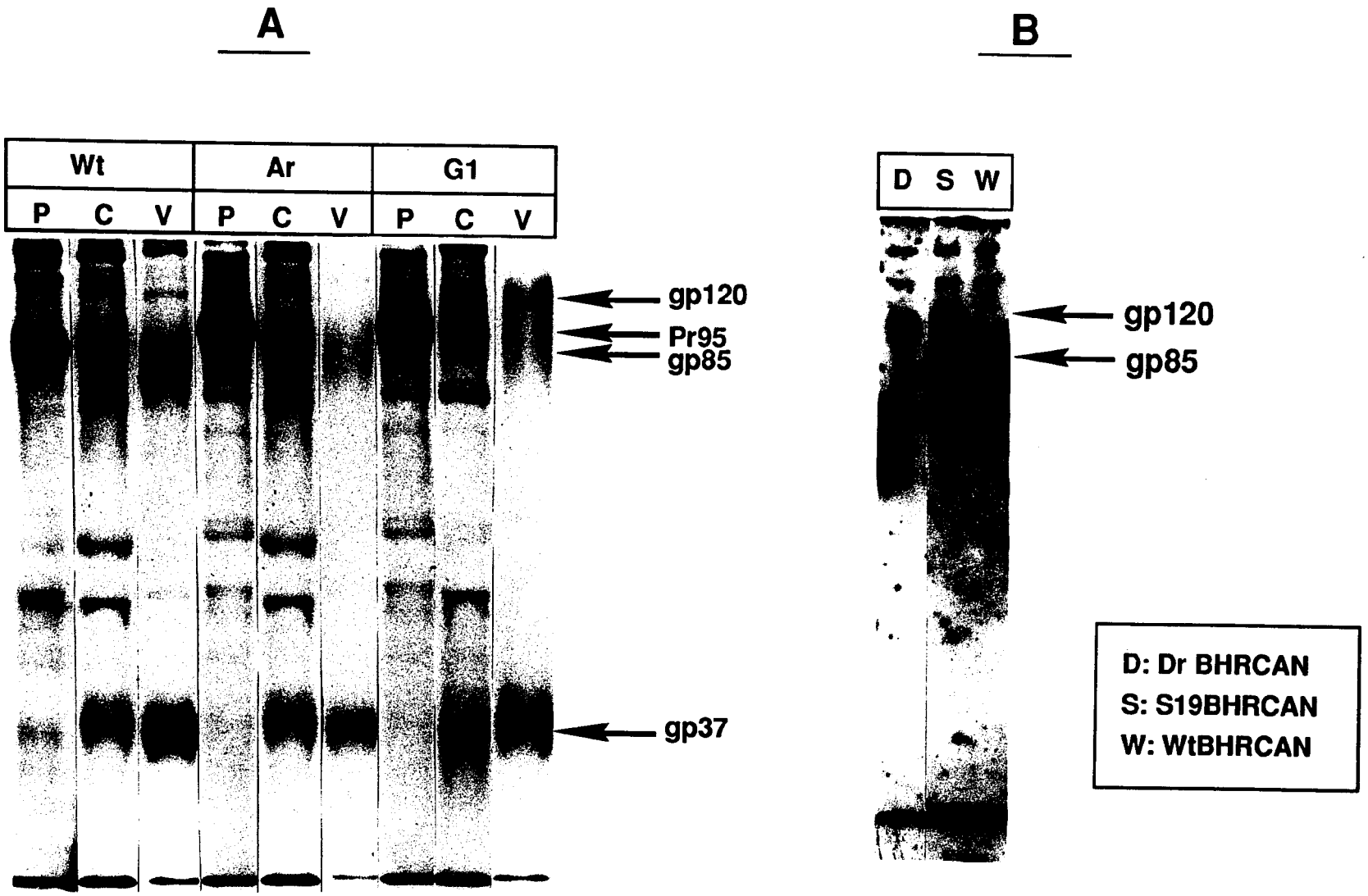
A
Wt
Ar
G1
P C V P C V P C V
gp120
Pr95
gp85
gp37
B
D S W
gp120
gp85
D: Dr BHRCAN
S: S19BHRCAN
W: WtBHRCAN

Figure.7. Analysis of Glycoprotein Incorporation into Virions in a T7 Promoter-Based Expression System
QT6 cells were infected with recombinant vaccinia virus expressing the T7 polymerase gene (10, 11). One hour post infection, the cells were co-transfected with pSP73*gag-pol* and pSP72*env* mutants (pSP72-Wt, pSP72-G1,pSP72-S19, and pSP72-A$^-$) as described in Materials and Methods. A t 14h post-transfection, the cells were labeled for 30min with [^{3}H]-leucine, then chased for 6h in complete DMEM. Viral proteins were immunoprecipitated from lysed cells (cell lysate), pelleted virus (viral pellet), and the supernatant following virus pelleting (supernatant).

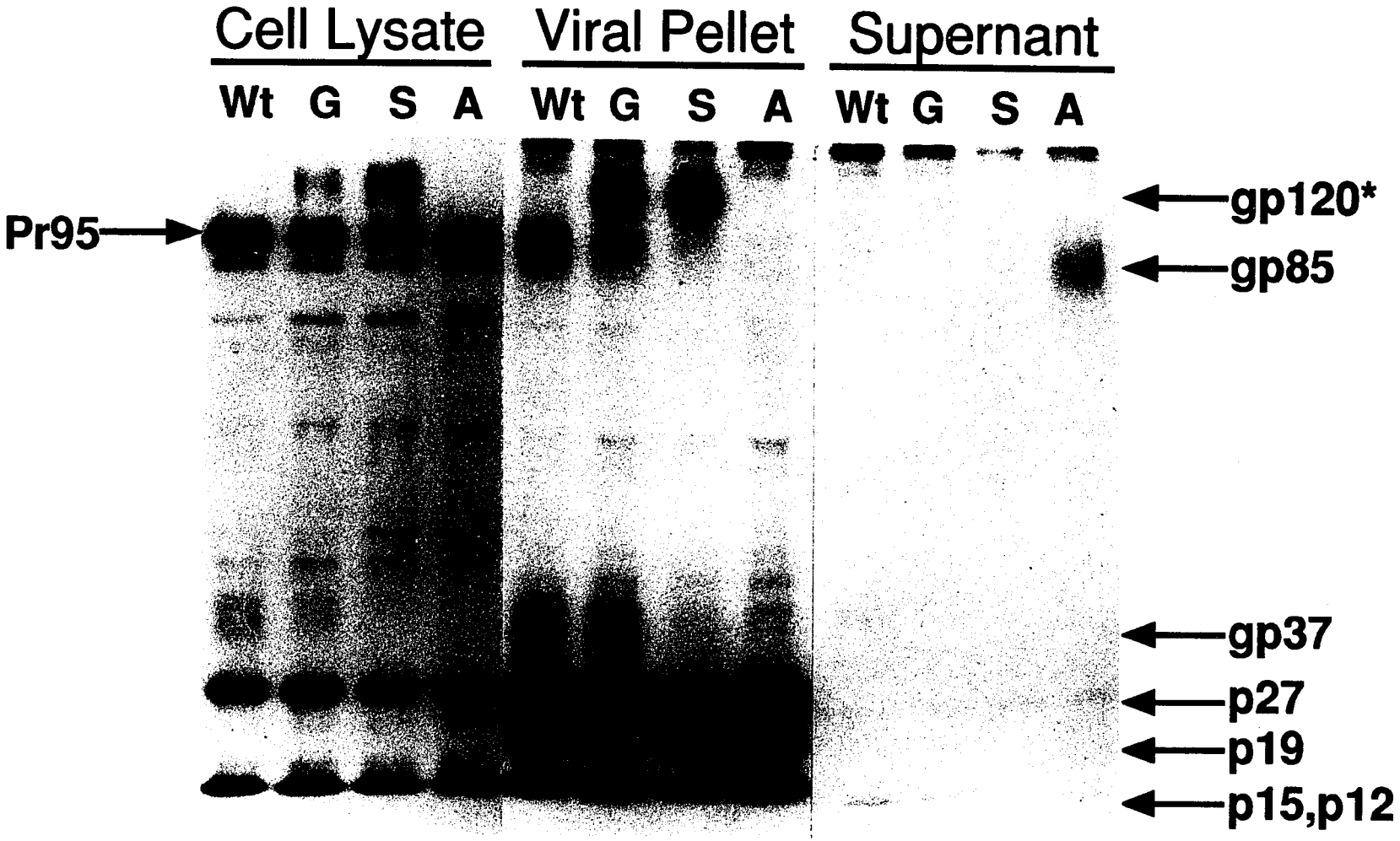
Cell Lysate
Viral Pellet
Supernant
Wt G S A Wt G S A Wt G S A
Pr95
gp120*
gp85
gp37
p27
p19
p15,p12

Table 1. Colony- forming assay of trypsin-treated BN-RCAN-HiSV virions

Virus Type/Treatment	Titer of Virus (Colony-Forming Units/ml)	
	Experiment 1	Experiment 2
Wild-type	7×10^1	2.4×10^2
S19/Trypsin	6×10^0	1.8×10^1
Dr1/Trypsin	0	0
S19/Mock	0	0
Dr1/Mock	0	0

Table 1 Turkey embryo fibroblasts (TEF) were transfected with the specified proviral DNA-containing (BH-RCAN-HiSV) plasmids. At 24h post-transfection cells were incubated in serum-free DMEM with or without 0.5μg TPCK-trypsin; 24 hours later virus was harvested from the culture supernatants. QT6 cells were incubated with serial dilutions of the virus and at 16h were subjected to hygromycin selection. Colonies of surviving cells were counted after 10 days. The results of two representative experiments are presented.

REFERENCES

1. **Bosch, V. and M. Pawlita.** 1990. Mutational analysis of the human immunodeficiency virus type 1 *env* gene product proteolytic cleavage site. J. Virol. **64**:2337-2344.

2. **Bresnahan, P. A., R. Leduc, L. Thomas, J. Thorner, H. L. Gibson, A. J. Brake, P. J. Barr, and G. Thomas.** 1990. Human fur gene encodes a yeast KEX2-like endoprotease that cleaves pro-beta-NGF in vivo. J. Cell Biol **111**:2851-2859.

3. **Chou, P. Y. and G. D. Fasman.** 1978. Prediction of the secondary structure of proteins from their amino acid sequence. Adv. Enzymol. **47**:45-148.

4. **Davis, G. L. and E. Hunter.** 1987. A charged amino acid substitution within the transmembrane anchor of the Rous Sarcoma virus envelope glycoprotein affects surface expression but not intracellular transport. J. Cell Biol. **105**:1191-1203.

5. **de Giuli, C., S. Kawai, S. Dales, and H. Hanafusa.** 1975. Absence of surface projections of some noninfectious forms of RSV. Virology **66**:253-60.

6. **Deshpande, K. L., V. A. Fried, M. Ando, and R. G. Webster.** 1987. Glycosylation affects cleavage of an H5N2 influenza virus hemagglutinin and regulates virulence. Proc Natl Acad Sci U S A **84**:36-40.

7. **Einfeld, D. and E. Hunter.** 1988. Oligomeric structure of a prototype retrovirus glycoprotein. Proc Natl Acad Sci U S A **85**:8688-8692.

8. **Einfeld, D. and E. Hunter.** 1991. Specific *in vitro* cleavage of retroviral glycoprotein precursors by a Golgi-derived endopeptidase. Manuscript in preparation.

9. **Freed, E. O., D. J. Myers, and R. Risser.** 1989. Mutational analysis of the cleavage sequence of the human immunodeficiency virus type 1 envelope glycoprotein precursor gp160. J. Virol. **63**:4670-4675.

10. **Fuerst, T., P. L. Earl, and B. Moss.** 1987. Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. Mol. Cell. Biol. 7:2538-2544.

11. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eucaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. U S A **83**:8122-8126.

12. **Fuller, R. S., A. Brake, and J. Thorner.** 1989. Yeast prohormone processing enzyme (KEX2 gene product) is a Ca2+-dependent serine protease. Proc Natl Acad Sci U S A **86**:1434-8.

13. **Fuller, R. S., A. J. Brake, and J. Thorner.** 1989. Intracellular targeting and structural conservation of a prohormone-processing endoprotease. Science **246**:482-6.

14. **Gallaher, W. R.** 1987. Detection of a fusion peptide sequence in the transmembrane protein of human immunodeficiency virus. Cell **50**:327-328.

15. **Garnier, J., D. J. Osguthorpe, and B. Robson.** 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J Mol Biol **120**:97-120.

16. **Garten, W., A. Stieneke, E. Shaw, P. Wikstrom, and H. D. Klenk.** 1989. Inhibition of proteolytic activation of influenza virus hemagglutinin by specific peptidyl chloroalkyl ketones. Virology **172**:25-31.

17. **Germain, D., L. Zollinger, C. Racine, F. Gossard, D. Dignard, D. Y. Thomas, P. Crine, and G. Boileau.** 1990. The yeast KEX-2-processing endoprotease is active in the Golgi apparatus of transfected NIH 3T3 fibroblasts. Mol Endocrinol **4**:1572-9.

18. **Hatsuzawa, K., M. Hosaka, T. Nakagawa, M. Nagase, A. Shoda, K. Murakami, and K. Nakayama.** 1990. Structure and expression of mouse furin, a yeast Kex2-related protease. Lack of processing of coexpressed prorenin in GH4C1 cells. J Biol Chem **265**:22075-8.

19. **Hosaka, M., M. Nagahama, W. S. Kim, T. Watanabe, K. Hatsuzawa, J. Ikemizu, K. Murakami, and K. Nakayama.** 1991. Arg-X-Lys/Arg-Arg motif as a signal for precursor cleavage catalyzed by furin within the constitutive secretory pathway. J Biol Chem **266**:12127-30.

20. **Hughes, S. and E. Kosik.** 1984. Mutagenesis of the region between *env* and *src* of the SR-A strain of Rous scarcoma virus for the purpose of constructing helper-independent vectors. Virology **136**:89-99.

21. **Hunter, E.** 1979. Biological techniques for avian sarcoma viruses. Methods Enzymol **58**:379-93.

22. **Hunter, E.** 1988. Membrane Insertion and Transport of Viral Glycoproteins: A Mutational Analysis. In: Protein Transfer and Membrane Biogenesis. Das R.C. and P.W. Robbins, eds. Academic Press, Inc., San Diego, CA.

23. **Hunter, E., E. Hill, M. Hardwick, A. Bhown, D. E. Schwartz, and R. Tizard.** 1983. Complete sequence of the Rous sarcoma virus env gene: identification of structural and functional regions of its product. J. Virol **46**:920-936.

24. **Hunter, E. and R. Swanstrom.** 1990. Retrovirus Envelope Glycoproteins. Curr Topics Immunol. Microbiol. **157**:188-251.

25. **Kaster, K. R., S. G. Burgett, R. N. Rao, and T. D. Ingolia.** 1983. Analysis of a bacterial hygromycin B resistance gene by transcriptional and translational fusions and DNA sequencing. Nucleic Acids Res. **11**:6895-6911.

26. **Katz, R. A., C. A. Omer, J. H. Weis, S. A. Mitsialis, A. J. Faras, and R. V. Guntaka.** 1982. Restriction endonuclease and nucleotide sequence analyses of molecularly cloned unintegrated avian tumor virus DNA: structure of large terminal repeats in circle junctions. J Virol **42**:346-51.

27. **Kawai, S. and H. Hanafusa.** 1973. Isolation of defective mutant of avian sarcoma virus. Proc Natl Acad Sci U S A **70**:3493-7.

28. **Kawai, S. and M. Nishizawa.** 1984. New Procedure for DNA transfection with Polycation and Dimethyl Sulfoxide. Mol. Cell. Biol. **4**:1172-1174.

29. **Kawaoka, Y. and R. G. Webster.** 1988. Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells. Proc Natl Acad Sci U S A **85**:324-8.

30. **Klenk, H. D., W. Garten, and R. Rott.** 1984. Inhibition of proteolytic cleavage of the hemagglutinin of influenza virus by the calcium-specific ionophore A23187. Embo J **3**:2911-5.

31. **Kowalski, M., J. Potz, L. Basiripour, T. Dorfman, W. G. Goh, E. Terwilliger, A. Dayton, C. Rosen, W. Haseltine, and J. Sodroski.** 1987. Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. Science **237**:1351-1355.

32. **Leamnson, R. N. and M. S. Halpern.** 1976. Subunit structure of the glycoprotein complex of avian tumor virus. J. Virol. **18**:956-968.

33. **Linial, M., J. Fenno, W. N. Burnette, and L. Rohrschneider.** 1980. Synthesis and processing of viral glycoproteins in two nonconditional mutants of Rous sarcoma virus. J Virol **36**:280-90.

34. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. In Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

35. **McCune, J. M., L. B. Rabin, M. B. Feinberg, M. Lieberman, J. C. Kossek, G. R. Reyes, and I. L. Weissman.** 1988. Endoproteolytic cleavage of gp160 is required for the activiation of human immunodeficiency virus. Cell **53**:55-67.

36. **Misumi, Y., M. Sohda, and Y. Ikehara.** 1990. Sequence of the cDNA encoding rat furin, a possible propeptide-processing endoprotease. Nucleic Acids Res **18**:6719.

37. **Moscovici, C., M. G. Moscovici, H. Jimenez, M. M. Lai, M. J. Hayman, and P. K. Vogt.** 1977. Continuous tissue culture cell lines derived from chemically induced tumors of Japanese quail. Cell **11**:95-103.

38. **Ohuchi, M., M. Orlich, R. Ohuchi, B. E. Simpson, W. Garten, H. D. Klenk, and R. Rott.** 1989. Mutations at the cleavage site of the hemagglutinin alter the pathogenicity of influenza virus A/chick/Penn/83 (H5N2). Virology **168**:274-80.

39. **Ohuchi, R., M. Ohuchi, W. Garten, and H. D. Klenk.** 1991. Human influenza virus hemagglutinin with high sensitivity to proteolytic activation. J Virol **65**:3530-7.

40. **Paterson, R. G., M. A. Shaughnessy, and R. A. Lamb.** 1989. Analysis of the relationship between cleavability of a paramyxovirus fusion protein and length of the connecting peptide. J Virol **63**:1293-301.

41. **Perez, L., J. W. Wills, and E. Hunter.** 1987. Expression of the Rous sarcoma virus env gene from a simian virus 40 late-region replacement vector: effects of upstream initiation codons. J. Virol. **61**:1276-1281.

42. **Perez, L. G., G. L. Davis, and E. Hunter.** 1987. Mutants of the Rous sarcoma virus envelope glycoprotein that lack the transmembrane anchor and/or cytoplasmic domains: analysis of intracellular transport and assembly into virions. J. Virol. **61**:2981-2988.

43. **Perez, L. G. and E. Hunter.** 1987. Mutations within the proteolytic cleavage site of the Rous Sarcoma virus glycoprotein that block processing to gp85 and gp37. J. Virol. **61**:1609-1614.

44. **Petropoulos, C. J. and S. H. Hughes.** 1991. Replication-Competent retrovirus vectors for the transfer and expression of gene cassettes in avian cells. J. Virol **65**:3728-3737.

45. **Rhee, S. S., H. X. Hui, and E. Hunter.** 1990. Preassembled capsids of type D retroviruses contain a signal sufficient for targeting specifically to the plasma membrane. J Virol **64**:3844-52.

46. **Rholam, M., P. Nicolas, and P. Cohen.** 1986. Precursors for peptide hormones share common secondary structures forming features at the proteolytic processing sites. FEBS Lett. **207**:1-6.

47. **Scheele, C. M. and H. Hanafusa.** 1971. Proteins of helper-dependent RSV. Virology **45**:401-10.

48. **Scheid, A. and P. W. Choppin.** 1974. Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis and infectivity by

proteolytic cleavage of an inactive precursor protein of Sendai virus. Virology **57**:475-490.

49. **Schlesinger, R. W., G. L. Bradshaw, F. Barbone, M. Reinacher, R. Rott, and P. Husak.** 1989. Role of hemagglutinin cleavage and expression of M1 protein in replication of A/WS/33, A/PR/8/34, and WSN influenza viruses in mouse brain. J Virol **63**:1695-703.

50. **Scholtissek, C., R. T. Schwarz, W. Keil, and H. D. Klenk.** 1984. A mutant of fowl plague virus (influenza A) with an altered glycosylation pattern in its hemagglutinin. Virology **136**:1-9.

51. **Seidah, N. G., L. Gaspar, P. Mion, M. Marcinkiewicz, M. Mbikay, and M. Chretien.** 1990. cDNA sequence of two distinct pituitary proteins homologous to Kex2 and furin gene products: tissue-specific mRNAs encoding candidates for pro-hormone processing proteinases. DNA Cell Biol. **9**:415-24.

52. **Seidah, N. G., M. Marcinkiewicz, S. Benjannet, L. Gaspar, G. Beaubien, M. G. Mattei, C. Lazure, M. Mbikay, and M. Chretien.** 1991. Cloning and primary sequence of a mouse candidate prohormone convertase PC1 homologous to PC2, Furin, and Kex2: distinct chromosomal localization and messenger RNA distribution in brain and pituitary compared to PC2. Mol Endocrinol **5**:111-22.

53. **Smeekens, S. P., A. S. Avruch, J. LaMendola, S. J. Chan, and D. F. Steiner.** 1991. Identification of a cDNA encoding a second putative prohormone convertase related to PC2 in AtT20 cells and islets of Langerhans. Proc Natl Acad Sci U S A **88**:340-4.

54. **Smeekens, S. P. and D. F. Steiner.** 1990. Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease Kex2. J Biol Chem **265**:2997-3000.

55. **Stoller, T. J. and D. Shields.** 1989. The role of paired basic amino acids in mediating proteolytic cleavage of prosomatostatin. J. Biol. Chem. **264**:6922-6928.

56. **Tsai, W. P. and S. Oroszlan.** 1988. Novel glycosylation pathways of retroviral envelope proteins identified with avian reticuloendotheliosis virus. J Virol **62**:3167-74.

57. **van de Ven, W., J. Voorberg, R. Fontijn, H. Pannekoek, A. M. van den Ouweland, H. L. van Duijnhoven, A. J. Roebroek, and R. J. Siezen.** 1990. Furin is a subtilisin-like proprotein processing enzyme in higher eukaryotes. Mol Biol Rep **14**:265-75.

58. **Weldon, R. J., C. R. Erdie, M. G. Oliver, and J. W. Wills.** 1990. Incorporation of chimeric gag protein into retroviral particles. J Virol **64**:4169-79.

59. **Wills, J. W., R. V. Srinivas, and E. Hunter.** 1984. Mutations of the Rous sarcoma virus env gene that affect the transport and subcellular location of the glycoprotein products. J. Cell Biol. **99**:2011-2023.

60. **Wise, R. J., P. J. Barr, P. A. Wong, M. C. Kiefer, A. J. Brake, and R. J. Kaufman.** 1990. Expression of a human proprotein processing enzyme: correct cleavage of the von Willebrand factor precursor at a paired basic amino acid site. Proc Natl Acad Sci U S A **87**:9378-82.

61. **Zoller, M. J. and M. Smith.** 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. **100**:468-500.

CHIMERIC AVIAN RETROVIRUS CONTAINING THE INFLUENZA VIRUS HEMAGGLUTININ GENE HAS AN EXPANDED HOST RANGE

JIANYUN DONG[1], MICHAEL G. ROTH[2] AND ERIC HUNTER[1,*]

[1]Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294 and [2]Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75235

* Corresponding author.

ABSTRACT

We have investigated what protein sequences are necessary for glycoprotein incorporation into Rous sarcoma virus (RSV) virions by utilizing the hemagglutinin (HA) protein of influenza virus. Two chimeric HA genes were constructed. In the first the coding sequence for the signal peptide of the RSV *env* gene product was fused in-frame to the entire HA structural gene, and in the second the hydrophobic anchor and cytoplasmic domain sequences of the HA gene were also replaced with those from the RSV *env* gene. Both chimeric genes, expressed from an SV40 expression vector in CV-1 cells, yielded functional hemagglutinin proteins that were transported to the cell surface and were able to bind to erythrocytes. When the genes were expressed in combination with the RSV *gag-pol* gene region in QT6 cells using a Vaccinia-T7 expression/complementation system, virions were assembled that efficiently incorporated either chimeric protein. This result indicated that the presence of the RSV *env* membrane-anchor and cytoplasmic sequences did not facilitate HA glycoprotein incorporation into virions. The presence of the RSV *env* signal sequence allowed the chimeric HA genes to be substituted into the RSV-derived BHRCAN-HiSV viral genome in place of the RSV *env* gene. Both chimeric genomes yielded infectious virus that could infect human and avian cells with high efficiency. These experiments demonstrate that a foreign glycoprotein, efficiently incorporated into virions lacking a native glycoprotein, can confer a broadened host range on the virus. Moreover, because the HA of influenza virus requires the acidic pH of the endosome to be activated, these results imply that foreign proteins can modify the normal route of entry of this avian retrovirus.

INTRODUCTION

The envelope glycoprotein complex of Rous sarcoma virus (RSV), like that of most replication competent retroviruses, is comprised of two polypeptides, an external glycosylated, hydrophilic polypeptide (gp85 or SU) and a membrane-spanning protein

(gp37 or TM), which form a knob or knobbed spike on the surface of the virion (25). This glycoprotein complex appears to be specifically incorporated into virions while the bulk of the host cell membrane proteins are excluded from the assembling particle. Electron micrographs of virions released from cells infected with an *env*-deleted RSV provirus show a lack of surface projections on the 'bald' particles, and protein analyses did not demonstrate any significant incorporation of cell membrane components (6). The fact that the TM protein (gp37) of RSV could be chemically cross-linked to the MA protein (pp19) in virions supported the concept that an interaction between the glycoprotein and capsid directed the incorporation of glycoproteins into virus (14). Moreover, mutations within both the MA protein and the TM protein of the primate retrovirus (Mason-Pfizer monkey virus or M-PMV) can reduce the efficiency of glycoprotein incorporation into virions (3, 33), and mutations within the MA protein of this virus can interfere with a viral protease-mediated cleavage of the TM cytoplasmic domain (4).

In the case of RSV, a truncation of the gp37 that removed 22 amino acids from the C-terminus of the cytoplasmic domain did not block glycoprotein incorporation (10, 31). In addition, pseudotype RSV virions, resistant to neutralizing antibody and with an extended host-range, were generated when cells chronically infected with RSV were superinfected with vesicular stomatitis virus (VSV) (35), suggesting that foreign glycoproteins could be incorporated into RSV virions. Young et al (42) extended this observation when they demonstrated that the human lymphocyte membrane protein CD4 could be incorporated efficiently into RSV virions when it was expressed in avian cells producing virus. Moreover, in the murine retroviruses, recent experiments have demonstrated that the host range of an MuLV vector could be extended by co-expressing either the VSV-G protein or Gibbon ape leukemia virus envelope protein with the Gag-Pol proteins of MuLV (11, 28, 41).

In an effort to determine whether specific sequences were required for the efficient incorporation of biologically active glycoproteins into RSV virions, we constructed two chimeric influenza virus HA genes. Both of these contained the coding sequence for the RSV Env signal peptide, which contained the splice acceptor sequences for *env* expression, fused in-frame to the HA structural gene. In one chimeric gene, *sHAa*, the membrane-anchor and cytoplasmic domain of HA were replaced with the analogous regions from the RSV *env* gene. Expression of these biologically functional genes together with the *gag-pol* genes of RSV showed that they could be incorporated into virions with the same efficiency and could confer an extended host-range for infection.

MATERIALS AND METHODS

Cells and DNAs

Turkey embryo fibroblasts (TEF) cells were prepared from 10 day old turkey eggs as described previously (20) and cultured in Dulbecco's modified Eagles medium (DMEM) containing 5% calf serum and 10% tryptose phosphate broth (Growth Medium; 20). The QT6 cells, derived from a methylcholanthrene-induced quail tumor (29), were grown in growth medium containing 1% heat-inactivated chicken serum. CV-1 cells, used for SV40 expression studies, were grown in DMEM containing 10% fetal calf serum.

Two genomic plasmid clones of RSV were used in these studies. For infectivity studies a modification of a vector (pRCAN (17, 32)) based on the Schmidt-Rupin, subgroup A (SR-A) strain of RSV was used in which the *pol* gene sequences from the Bryan High Titer strain were substituted to yield pRCANBP (19, 32). This plasmid vector was modified further by substituting the *src* gene of RSV with a gene for hygromycin resistance under the control of the simian virus 40 early promoter, as descibed below. For construction of chimeric envelope glycoprotein genes, *env* gene sequences from the pATV8 molecular clone (22) of the Prague subgroup C (Pr-C)

strain of RSV and HA sequences from a plasmid clone, pJHB23 (15), of the HA gene of influenza virus A/Jap/305/57 were used.

Plasmid DNA was propagated in E. coli DH5 cells and was purified on both small and large scale as described previously by Maniatis et al. (26). All DNA used for transfection studies was derived from cesium chloride gradients.

Construction of chimeric HA proteins

We constructed two chimeric RSV *env*-influenza HA genes, sHA and sHAa. In the sHA construction, the mature HA coding sequence was fused to the RSV signal peptide sequence, so that a full length HA protein was formed following signal peptide cleavage (Figure 1A). In the single stranded phage M13 mp18, a KpnI-XhoI fragment encoding the long signal peptide sequence of *env* and the six amino-terminal amino acids of gp85 was ligated in frame with a HindIII-SalI fragment of the HA gene from pJHB23, a plasmid containing an HA gene which lacked the initiation codon and signal sequence coding regions (16). An oligonucleotide, 36 nucleotides in length, was then used to fuse the coding region for the *env* signal peptide with that for the amino-terminus of the HA1 protein by oligonucleotide mutagenesis in a similar manner to that described previously (30). The Kpn-SalI fragment was then cloned into the HA-expressing SV40 late replacement vector, pSVEHA3 (15), using unique KpnI and SalI sites within the vector in order to replace the natural signal sequence coding region of HA. A KpnI to BamHI fragment encompassing the entire chimeric gene was then subcloned into pSVenvKB (5) to generate pSVsHA (Figure 2A).

In sHAa, the C-terminal-anchor region of HA was replaced by the RSV *env* transmembrane and cytoplasmic domains. Site-directed mutagenesis was used to create the fusion between the ectodomain of the HA protein (HA nucleotide 1618) and the C-terminus of the TM domain of the RSV envelope glycoprotein (*env* sequences at nucleotide 6716). Thus, as shown in Figure 1B, in this chimeric protein the last amino acid of the HA ectodomain was Gln and the first amino acid of the Env transmembrane

domain was Gly (24). For the studies described here, the SalI-BamHI fragment of this chimeric gene was cloned into pSVsHA to generate pSVsHAa.

For later studies, a ClaI site was inserted into both pSVsHA and pSVsHAa at the 3' end of the HA gene by digesting with BamHI, blunt-ending with the Klenow enzyme, and ligating in a ClaI linker. This allowed the entire chimeric gene to be excised via KpnI-ClaI digestion.

Expression Systems

For the preliminary characterization of the biosynthesis and transport of chimeric proteins, both sHa and sHAa genes were expressed in CV-1 cells from the SV40 late-region replacement vector pSVsHA (Figure 2A) and pSVsHAa (not shown) essentially as described previously (30, 39). In this system the bacterial plasmid sequences were removed from pSVsHA (or pSVsHAa) by digesting with KpnI and the SV40/HA-containing sequences were religated to yield a recombinant SV40 genome in which the late region sequences were substituted by glycoprotein sequences. Co-transfection of CV-1 cells with this DNA and a helper SV40 genome (dl1055, deleted in the early region) allowed complementation and a high titer stock of infectious, recombinant SV40 virions to be established. This virus stock was diluted 1:20 prior to infection and pulse-chase analyses of the expressed chimeric proteins were carried out at 72h post-infection (39).

For initial infectivity studies of RSV genomes encoding a chimeric glycoprotein, we used BH-RCAN/HA, a modified proviral vector derived from the Schmidt-Rupin, and Bryan High titer strains of Rous sarcoma virus (19, 32), in which the A-subgroup *env* gene was replaced by the chimeric genes using unique KpnI and ClaI sites flanking the *env*/HA gene sequences (9) in a similar manner to that described below. The coding region of the *pol* gene of RSV overlapped the signal peptide coding region of *env* (Figure 1C) and the splice acceptor site for *env* gene expression was located 108 nucleotides upstream of the hydrophobic signal sequence itself. The

chimera constructions took advantage of the unique KpnI site just upstream of these important sequences and so their insertion did not interfere with the expression of either the *pol* or *env*/HA gene sequences. In more quantitative analyses, we utilized BH-RCAN.HiSV in which the RSV *src* gene was replaced by the hygromycin-resistance gene (21) under the transcriptional control of the SV40 early promoter (Figure 2B) (9). Transfection of the pBH-RCAN.HiSV plasmids into avian cells resulted in expression of the viral replicative genes and packaging of a viral genome that conferred a drug-resistant, selectable phenotype on the cell it infected.

For additional analyses of chimeric glycoprotein incorporation into virions, wild-type RSV-PrC *gag-pol* genes, and wild-type RSV *env* or chimeric HA genes were expressed independently in QT6 cells under the transcriptional control of a T7 promoter essentially as described previously (9). In these experiments, subconfluent QT6 cells were infected with a recombinant vaccinia virus expressing the T7 RNA polymerase at an MOI of 15-20 (12, 13). After one hour the cells were transfected with the different *env* or *env*/HA and *gag-pol* gene-containing plasmids by the modified polybrene method described previously (9). These plasmids were constructed so that the WT or chimeric *env* genes were under the transcriptional control of a phage T7 RNA polymerase promoter. Each gene was excised from the SV40-based vector using KpnI and ClaI and cloned into the same sites in pSP73 to yield pSP.*env*, pSP.*s*HA and pSP*s*HA*a* respectively. The RSV *gag*-expressing plasmid pSP.*gag-pol* has been described previously (9). At 12h post-transfection, the cells were pulse-labeled for 30 min with 200μCi [^{3}H]leucine and then chased for 16h in complete medium. Released virus particles were pelleted by centrifugation through a 15% sucrose cushion (100,000 rpm for 20min in a TL100 centrifuge, Beckman Instruments) and both virus and cells were lysed and immunoprecipitated as described below.

Metabolic labeling and immunoprecipitation

Cells expressing viral proteins were starved for leucine in leucine-free DMEM for 1 h and then pulse-labeled at 37°C for 15-20 min in leucine-free labeling medium containing 200μCi of ^{3}H-leucine (Dupont, NEN Research Products). In pulse-chase labeling, labeled cells were chased in complete growth medium for 2-6 h. Immunoprecipitation was carried out as described previously (40). Viral protein was immunoprecipitated with specific antibodies as indicated in the text. Antibodies directed against the RSV glycoproteins were prepared as described previously (9). Antibody to the RSV *gag* gene products was provided by Dr. John Wills and to the HA of the A/Jap/305/57 strain of influenza virus by Dr. Mary-Jane Gething.

Analysis of HA/Erythrocyte binding

To evaluate the functional surface expression of the chimeric HA genes, an erythrocyte binding assay was employed. Fresh human or guinea pig blood was washed four times with 0.9% saline solution in a low-speed centrifuge (J6B, Beckman), resuspended in serum-free DMEM medium (Gibco BRL, Gaithersburg,MD), and stored at 4°C until use. CV-1 cells infected with recombinant SV40 virus expressing the chimeric HA genes or TEF cells transfected with BHRCAN-HA were washed twice with serum-free DMEM, and 3 ml of serum-free DMEM was added to each plate. Five drops of washed red blood cells (3%) were then added, and distributed evenly by gently rocking the plates. The plates were incubated at 4°C for 20 minutes and then gently washed 4 to 6 times with serum-free medium to remove unbound red cells. Photographs were taken on an Olympus inverted microscope.

Analyses of viral infectivity

In initial experiments TEF cells were transfected with the BHRCAN-sHA genome and spread of virus infection was monitored by red blood cell binding assays as described above. For more quantitative analyses of specific virus infectivity, duplicate plates of TEF cells were transfected with the BHRCAN-HiSV/*env*

(containing the wild-type RSV PR-C *env* gene), BHRCAN-HiSV/sHA and BHRCAN-HiSV/sHAa genomes. At 24 h post-transfection, cells were washed once with serum free DMEM and cultured in 4 ml of serum-free DMEM with or without 0.6μg/ml of TPCK-trypsin for 24 h. In some experiments 0.025U/ml of neuraminidase (*vibrio cholera,* Sigma) was also included to determine the effect of this enzyme on virus infectivity. Medium was then collected and mixed with 4 ml of complete growth medium containing 10μg/ml of polybrene, and 2 ml of the mixture was added to each of three 100 mm plates of semi-confluent QT6 or HeLa cells. After 16 h incubation, infected resistant cells were selected with hygromycin-containing medium (250u/ml hygromycin in growth medium) for 10 days. The number of colonies on each plate was counted and the mean determined for each construct. All experiments utilizing these recombinant viruses were performed under BSL2/P2 laboratory conditions.

Incorporation of viral glycoproteins into virions

For incorporation studies, we employed the vaccinia T7 complementation/assembly system described above, in which QT6 cells were cotransfected with *gag-pol* gene and *wt env* or chimeric HA-gene containing plasmids.

RESULTS

Expression of chimeric HA proteins in CV-1 cells

To determine if functional proteins were expressed from the chimeric genes, the sHA and sHAa constructs were cloned into an SV40 late replacement vector and co-transfected with a helper virus into CV-1 cells. An infectious recombinant SV40 viral stock was collected at Day 4. The virus was then used to infect semi-confluent monolayers of CV-1 cells. Three days post infection the expression of HA proteins was examined in a pulse-chase labelling experiment. As shown in Figure 3, equivalent levels of sHA and sHAa precursors were synthesized in CV-1 cells during a 10 min [^{3}H]-leucine pulse-label (Lane P). After a 2hr chase (Lane C2), the precursor HAs migrated with a reduced mobility consistent with normal transport through the

secretory pathway and conversion of the core glycosylated oligosaccharides to their terminally glycosylated form. After a 4 h (Lane C4) or 6h (Lane C6) chase, we did not observe any significant reduction in the amount of cell-associated HA proteins. Moreover, when the 4h chase was carried out in the presence of 1.0 μg/ml of TPCK/trypsin, both sHA and sHAa were completely cleaved to HA1 and HA2 (Lanes Tr). These results indicated that both chimeric HA proteins were efficiently transported to the cell surface and retained a conformation that allowed normal proteolytic processing.

In order to determine whether the chimeric proteins retained biological activity an erythrocyte binding experiment was performed. The results are shown in Fig. 4. Panels A and B show the red cell binding activities of sHA and sHAa, respectively. In each case a monolayer of erythrocytes was tightly bound to cells infected with the 'HA' expressing virus. These results indicated that both chimeric HAs were not only efficiently expressed on the cell surface but also retained the biological activity of binding to the sialic acid on erythrocytes.

The sHA and sHAa glycoproteins can be incorporated into RSV virions

The incorporation of the HA proteins into RSV virions was determined by a complementation/assembly experiment using a vaccinia-T7 expression system in which it had been demonstrated that morphologically normal viral particles could be assembled and released (9). Following infection with a recombinant vaccinia virus carrying the T7 polymerase gene, QT6 cells were co-transfected with plasmids carrying the RSV *gag-pol* and either the RSV *env* gene (pSP.*env*) or the chimeric HA genes (pSP.*sHA* or pSP.*sHAa*). Control cells were transfected with each of the plasmids individually. The cells were labelled, virus was pelleted and viral proteins immunoprecipitated as described in Materials and Methods. Figure 5 shows the results of a typical experiment in which cell lysates and viral pellets were immunoprecipitated with a mixture of antibodies to the RSV *gag* and *env* gene products and/or influenza

virus A/Jap/307/57 as described in the figure legend. In cells transfected with the glycoprotein-expressing plasmids alone, no glycoprotein was found in the viral pellet (Fig. 5, Viral Pellet, lanes env, sHA, and sHAa), despite the presence of glycoprotein in the cell lysates following the 16h chase (Fig. 5, Cell Lysate, lanes env, sHA, and sHAa). Expression of the *gag* gene alone resulted in the release of pelletable, glycoprotein-free virus particles containing the Gag cleavage products p27, p19, and p15/p12 (Fig 5., Viral Pellet, gag lane). Some residual uncleaved $Pr76^{gag}$ as well as cell-associated p27 can be seen in the cell lysates (Fig 5., Cell Lysate, gag lane). As we have observed previously (9), co-expression of RSV *gag* and *env* genes in the QT6 cells resulted in release of particles containing amounts of gp85(SU) and gp37(TM) similar to that seen in cells infected with RSV (Fig 5., Viral Pellet, g/env lane). Cells co-expressing *gag* and sHA or sHAa genes released virions containing similar levels of the chimeric proteins (Fig.5, Viral Pellet, lanes g/sHA and g/sHAa). This result indicated that both proteins could be incorporated into virus particles with similar efficiency. While pulse-chase experiments showed that the level of synthesis of the RSV *env* gene product was similar to that of the chimeric proteins, at the end of the 16h chase a significant fraction of the Pr95 and gp85/gp37 cleavage products had turned over. This is consistent with studies of RSV where less than 5% of the glycoprotein products made in a pulse-label are incorporated into virus (2). In contrast the HA proteins were stable and large amounts of cell-associated protein could be observed at the time of immunoprecipitation. However, as can be seen in Figure 5, even after a 16h chase a significant amount of core glycosylated HA (HA*) remains in the cell suggesting that in QT6 cells the HA protein is transported and processed to the terminally glycosylated (HA_0) less efficiently than in CV-1 cells.

In influenza virus, neuraminidase, an enzyme capable of cleaving sialic acid from oligosaccharide chains, is also incorporated into the viral envelope. It has been suggested that this enzyme may be required for releasing virions from cell surfaces by

removing sialic acid residues to which HA proteins might bind (1, 18). Although we did not observe significant differences in the levels of virions released from HA-expressing and *env*-expressing cells (Fig 5. Viral Pellet, lane g/env versus g/sHA or g/sHAa), we determined the effect of adding exogenous NA in this system. In an experiment similar to that described above, ^{3}H-leucine-labeled, transfected QT6 cells were chased in the presence of NA. The results of such an experiment are presented in Figure 6. Addition of NA did result in a small increase in the number of virions released from cells , as shown by the increased levels of capsid proteins in the viral pellet (Fig 6., Viral Pellet, lanes g/sHA+NA vs g/sHA and g/sHAa vs g/sHAa+HA). Quantitation of the p19 bands by densitometry showed an average increase of approximately 50%. However, in the case of both sHA- and sHAa-expressing cells, the amount of incorporated HA in released virions had increased approximately 70% relative to Gag, suggesting that neuraminidase treatment facilitated incorporation of the HA proteins into virions.

The chimeric HA glycoproteins can confer infectivity on the RSV virions

In order to determine whether the chimeric HA proteins could confer infectivity on RSV virions, the *env* gene in an RSV-derived proviral vector, BH-RCAN, was replaced with the chimeric HA genes. Since this construction lacks an RSV envelope glycoprotein, it allowed us to determine if the HA proteins could mediate entry in the absence of the *env* gene product. In preliminary experiments we assayed for spread of HA-containing virus in the culture by using the ability of cells expressing HA to bind erythrocytes. After transfection of BH-RCAN/HA into TEF, cells were trypsinized every three or four days to keep the cells constantly growing and erythrocyte-binding assays were done during a period of 12 to 15 passages. Red cell binding activity was observed to propagate slowly through the cell population (Figure 4, panels D and E). Under higher magnification, the agglutinated red cells could be seen as clusters on the cell surface in contrast to the tight monolayers of red cells seen in the HA expressing

CV-1 cells (Figure 4, panels A and B). Moreover following an overnight incubation, the red cells were released as cross-linked rafts of cells rather than as individual erythrocytes, consistent with the red cells being linked to each other by released virions bearing the HA proteins.

In order to quantitate the infectivity of HA-containing RSV virions and to determine whether the sHAa protein (with the gp37 membrane-spanning and cytoplasmic domains) yielded a more infectious virus, a hygromycin-resistance-gene cassette was inserted into the viral genome (Figure 2, BH-RCAN.HiSV/sHA). Virus released from cells transfected with this genome would thus carry the hygromycin resistance marker into the newly infected cells.

The titer of infectious particles released from transfected turkey embryo cells was determined by counting the number of hygromycin resistant colonies arising following infection and hygromycin treatment. Infectivity assays were initially performed in QT6 cells, an avian cell line susceptible to RSV infection, to compare the infectivity of the Env-, sHA-, and sHAa-containing virions. We then determined if these virions demonstrated a host range that extended to mammalian cells by infecting Hela cells, a human cell line which was not susceptible to wild-type RSV Pr-C infection. The results of these experiments, summarized in Table 1, and demonstrate that the trypsin-treated, HA-containing virions released from TEF cells in the absence of neuraminidase had a titer approximately 1/3 that of the Env-containing virus. Treatment of cells expressing the HA chimeric genes with neuraminidase, however, resulted in an increase in the titer of the released virus to levels similar to that of the Env-containing virus. This is consistent with the biochemical studies described earlier. It was of interest to note that both the sHA- and sHAa-containing virus had similar titers, indicating that the presence of the C-terminal sequences from gp37 did not potentiate the infectivity of the virions. While the levels of virions released from TEF cells at the time points used for these infectivity assays (~102 CFU/ml) preclude an

accurate quantitation of reverse transcriptase activity or virion proteins, the reproducibility of the results in several experiments with different preparations of plasmid DNAs suggest that these results reflect the specific infectivities of the virions.

In the absence of trypsin-treatment (required for cleavage-activation of HA0 to HA1 and HA2) cells infected with the sHA- and sHAa-containing virions yielded no colonies (data not shown), confirming the requirement for this cleavage in the infection process. In addition, cells infected with a BH-RCAN.HiSV vector containing an uncleavable mutant Env precursor, Dr1, which we have previously shown could be incorporated into virions but could not confer infectivity on virus (9), yielded no background hygromycin-resistant colonies.

When the culture supernatants tested on QT6 cells were used to infect HeLa cells, we observed that the HA-containing virions were as infectious for these mammalian cells as they were for the QT6 cells (Table 1). Thus the incorporation of the HA proteins into RSV resulted in an expanded host-range for the virus.

DISCUSSION

The mechanisms by which retroviral glycoproteins are selected for incorporation into the nascent virion during its budding from the plasma membrane have not been well defined. In this study we demonstrated that the influenza virus HA, a non-retroviral glycoprotein, can be incorporated into RSV virions. Using the T7 complementation/assembly system described previously, we were able to show that similar amounts of HA and Env were incorporated into the virus particles produced. Moreover, the amount of HA incorporated could be increased significantly if the cells producing virions were treated with exogenous neuraminidase (NA). In these experiments the incorporation of glycoprotein into particles required that cells be co-transfected with both the *gag-pol*- and glycoprotein-expressing plasmids. It was not possible, therefore, to determine whether the NA treatment resulted in the release of additional virions that were attached to cell-surface sialic acid or whether it freed HA

molecules from plasma membrane proteins that prevented incorporation into nascent virions. Although both mechanisms may have contributed to our results, the latter possibility was supported by the observation that, proportionally, there was a significantly greater increase in the amount of virion-associated HA relative to the increase in the level of virions released. This raises the possibility that NA may play an additional role in influenza virus assembly by freeing the HA molecules for incorporation. The system described here may therefore be useful for additional studies on the role of NA in the influenza virus life cycle.

In these studies, we constructed two functional chimeric genes, *sHA* and *sHAa*, which contained the coding sequences for the RSV Env signal peptide, overlapping reverse transcriptase sequences, and the splice acceptor site required for processing of the *env*-specific subgenomic mRNA. These chimeric genes allowed us to replace, within an infectious provirus, the complete RSV *env* gene sequences (encoding gp85 and gp37) with those of the influenza virus HA. These HA encoding proviruses were shown to express HA at high levels, as evidenced by red cell binding to infected cells. Moreover, virions produced during transient transfection of TEF with the chimeric proviruses were as infectious on QT6 cells as the parental Env-containing virus. This result was somewhat surprising since it has been well established that HA-induced fusion requires an acid pH mediated conformational change that exposes the fusion peptide (7, 8, 38). This conformational change normally occurs in the acidic environment of the endosome, in which fusion of the influenza virus and endosomal membrane takes place. In contrast, entry of RSV into cells does not require an acidic pH (27) and it is generally accepted that this virus enters by fusing its membrane with the plasma membrane of the target cell (37). Because acidic activation of HA is a prerequisite for its function, we can conclude that efficient entry of RSV can also occur via the endosome and that by substituting the Env glycoproteins with HA we have redirected the route of entry into the cell for this virus.

Earlier studies of vesicular stomatitis virus (VSV)/RSV pseudotyping indicated that a small percentage (0.2-1%) of the RSV released from cells coinfected with VSV contained the VSV G protein, and that these pseudotypes could infect chicken cells normally resistant to the subgroup of RSV used (36). However, in these studies VSV was much less efficient (10^{-4}) than the RAV-1 (Env donating) helper virus in complementing the *env*-deficient RSV(-). The question of whether a specific interaction occurs between the assembling capsid and the cytoplasmic domain of the retroviral glycoprotein in order to facilitate Env incorporation and exclude cell-encoded plasma membrane proteins, has not been answered to-date. The observation that the gp37 of RSV could be chemically cross-linked to the matrix protein (pp19) supported the concept that a close physical association existed between the two (14). However, a mutant of RSV, in which a premature termination codon resulted in the truncation of the C-terminal 22 amino acids of gp37, efficiently assembled gp85/gp37 into virions and retained infectivity, suggesting that this region was not involved in glycoprotein incorporation (31). The results presented here support the concept that incorporation of glycoproteins into RSV does not require a specific interaction between the capsid and the cytoplasmic domain, since both the sHA and sHAa glycoproteins were incorporated with equal efficiency into RSV particles, and since both types of HA-containing virions showed equivalent infectivity. Nevertheless, it is not possible to rule out a specific interaction between the native Env protein and capsid that facilitates but is not required for assembly and infectivity.

Other C-type viruses also appear to exhibit a similar promiscuity with regard to glycoprotein incorporation. Recently it was demonstrated that co-expression of the VSV G or Gibbon ape leukemia virus *env* gene with the MuLV *gag-pol* genes in a murine cell resulted in assembly of viruses that incorporated the foreign protein and exhibited an expanded host range characteristic of the glycoprotein incorporated (11, 28, 41). Similarly, Landau et al and Spector et al have shown that HIV virions can

incorporate glycoproteins of both HTLV-I and amphotropic MuLV to generate infectious virus with an expanded host-range (23, 34). Thus it is difficult to reconcile these results with a specific requirement for strong capsid-glycoprotein interactions. How then might viral proteins be preferentially incorporated into virions? It is possible that virus assembly occurs in areas of the plasma membrane not associated with underlying cytoskeleton or cellular cortex (indeed capsid precursor association with the membrane may displace such structures). Mobile viral (and cellular) membrane proteins might freely diffuse into this region and be incorporated into virions in amounts that would reflect both their concentration in the plasma membrane and any affinity for viral capsid proteins they might have. Thus, the glycoprotein composition of the RSV virion might normally be determined by the efficiency with which the gp85/gp37 oligomer competes for available space in the membrane above the capsid proteins according to two separate factors: (1) a weak interaction with capsid and (2) its relative abundance as a mobile suface protein. We have shown in our experiments in which treatment of cells with neuraminidase increased the ratio of chimeric envelope proteins to Gag proteins in virions that the amount of envelope proteins in RSV virions can vary. Thus if the interaction between gp85/gp37 and capsid proteins is weak or the number of such interactions is limited, space for additional gp85/gp37 or other mobile proteins that do not interact with the underlying capsid might be available. The observation that human CD4, expressed in chicken cells, could be efficiently incorporated into RSV virions is consistent with this possibility.

Virions containing either sHA or sHAa in their membrane were equally infectious on both human and avian cells, as assayed by their ability to introduce a genome expressing the hygromycin-resistance marker into these cells. Functional incorporation of a non-retroviral envelope glycoprotein into RSV can thus provide a mechanism to target the virus into host cells that are normally not susceptible to RSV infection. We do not know, at the present time, the limitations for the functional

incorporation of glycoproteins into RSV; however, the relaxed specificity of incorporation for RSV may be particularly useful in the development of retroviral vectors that can target a therapeutic gene into a specific tissue. We are currently investigating the potential for RSV in this context.

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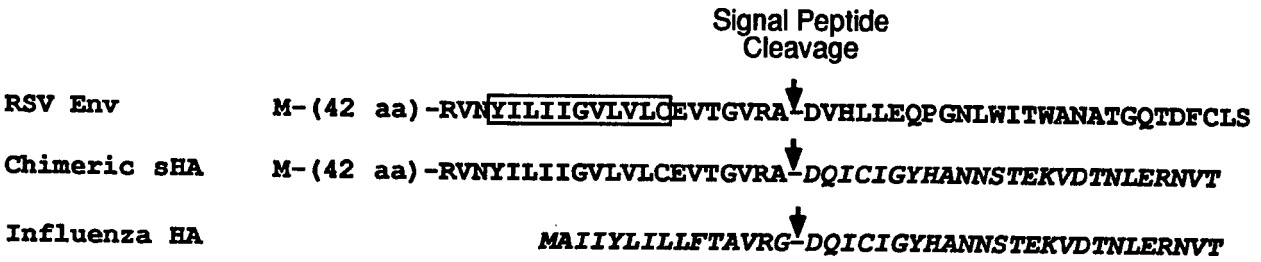
Figure 1 (A and B). Schematic diagram of the chimeric *sHA* (A) and *sHAa* (B) constructions.

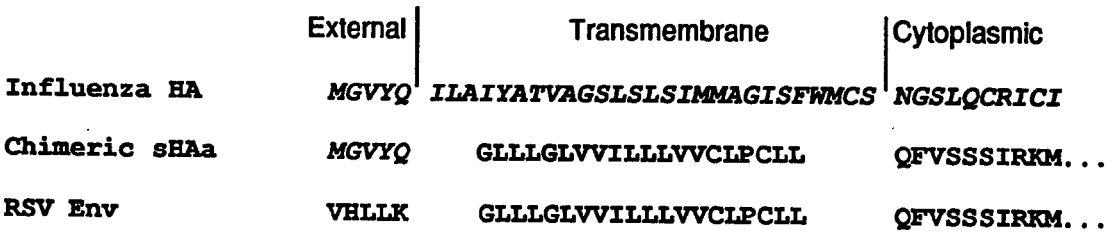
In (A) influenza virus HA sequences are shown in italics. The 11 amino acid hydrophobic core of the long RSV *env*-encoded signal peptide is boxed and the site of signal peptidase cleavage is denoted by an arrow. Signal sequences and structural protein sequences are separated by a hyphen. In (B) the location of external, transmembrane and cytoplasmic domain boundaries are as defined by Roth and coworkers (24).

C) Schematic representation of the KpnI-ClaI fragment containing the sHA chimeric gene that was used for introduction into the BH-RCAN.HiSV vector. The location of the splice acceptor site used for *env* expression and the overlapping *pol* and *env* genes are depicted.

A.


Signal Peptide Cleavage
RSV Env
M-(42 aa)-RVNYILIIGVLVLCEVTGVRA-DVHLLEQPGNLWITWANATGQTDFCLS
Chimeric sHA
M-(42 aa)-RVNYILIIGVLVLCEVTGVRA-DQICIGYHANNSTEKVDTNLERNVT
Influenza HA
MAIIYLILLFTAVRG-DQICIGYHANNSTEKVDTNLERNVT

B.


External
Transmembrane
Cytoplasmic
Influenza HA
MGVYQ
ILAIYATVAGSLSLSIMMAGISFWMCS
NGSLQCRICI
Chimeric sHAa
MGVYQ
GLLLGLVVILLLVVCLPCLL
QFVSSSIRKM...
RSV Env
VHLLK
GLLLGLVVILLLVVCLPCLL
QFVSSSIRKM...

C.

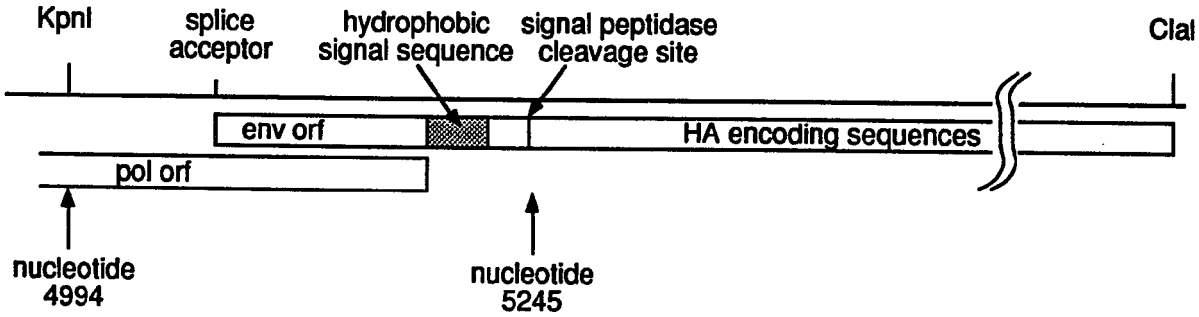

KpnI
splice acceptor
hydrophobic signal sequence
signal peptidase cleavage site
ClaI
env orf
HA encoding sequences
pol orf
nucleotide 4994
nucleotide 5245

Figure 2. Schematic diagram of SV40 expression vector, and RSV provirus vector.

(A) Schematic diagram of the SV40 expression vector, pSVsHA, in which, after digestion with KpnI and religation, the chimeric sHA gene is under the transcriptional control of the SV40 late promoter.

(B) Schematic diagram of the RSV proviral vector, BH-RCAN.HiSV/sHA, in which the *env* gene of RSV was replaced by the chimeric sHA gene using the unique, flanking KpnI and ClaI restriction endonuclease sites.

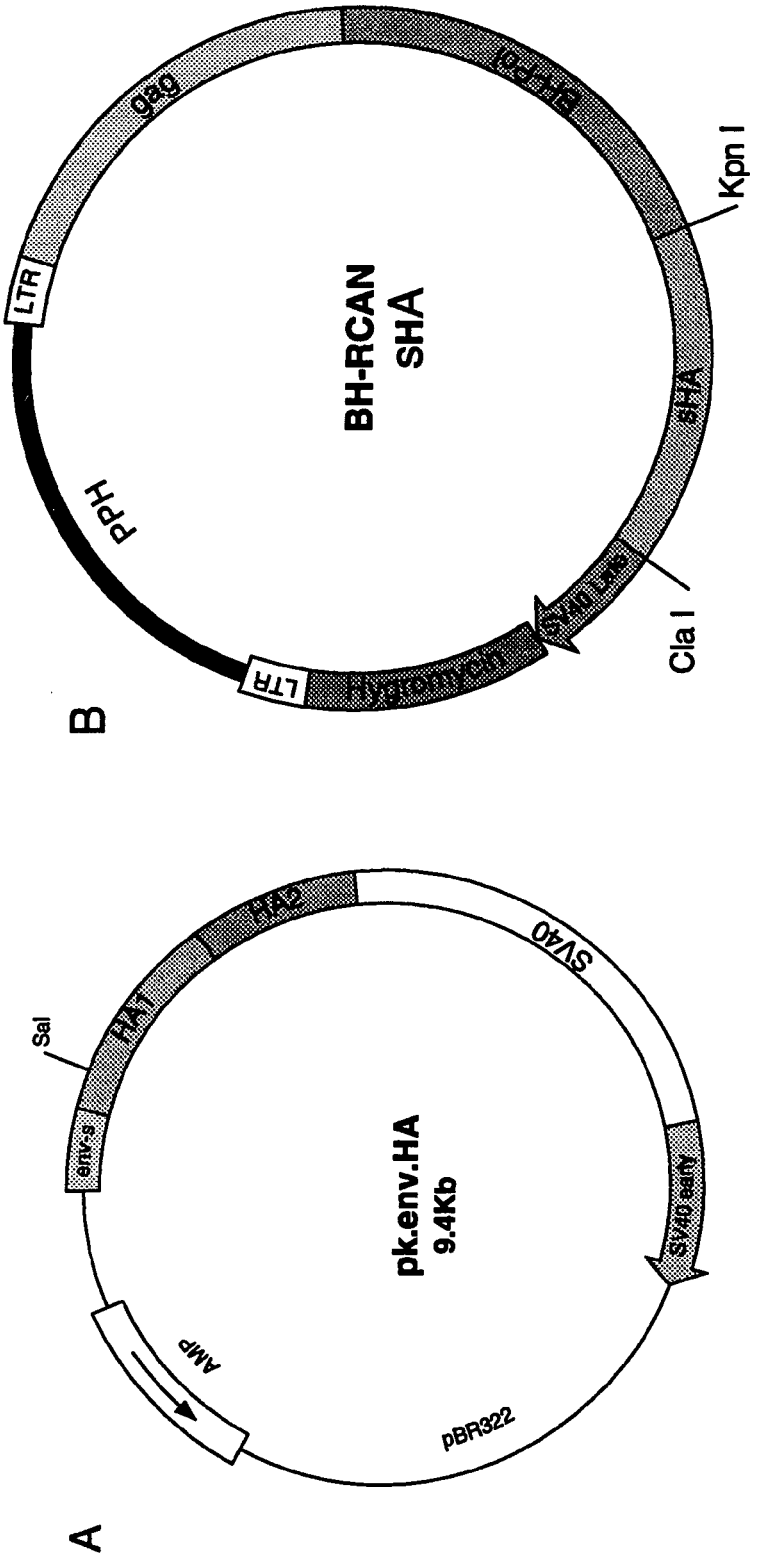

B
BH-RCAN
SHA
gag
EF-P.D
Kpn I
δHA
SV40 pA
Cla I
LTR
Hygromycin
LTR
PPH
A
pk.env.HA
9.4Kb
HA2
SV40
HA1
Sal
env
SV40 early
AMP
pBR322

Figure 3. Pulse-chase labeling and immunoprecipitation of chimeric HA proteins in CV-1 cells infected with recombinant SV40 viruses.

Lane P shows the precipitated precursor chimeric HA proteins (HA_0) after a 10 minute pulse-labeling with ^3H-leucine. In lanes C2-C6, pulse-labelled plates were chased for 2, 4, and 6h in complete medium as marked on top of each lane. Lane Tr, HA proteins were chased for 4h in the presence of 1μg/ml of trypsin yielding the cleavage products HA1 and HA2.

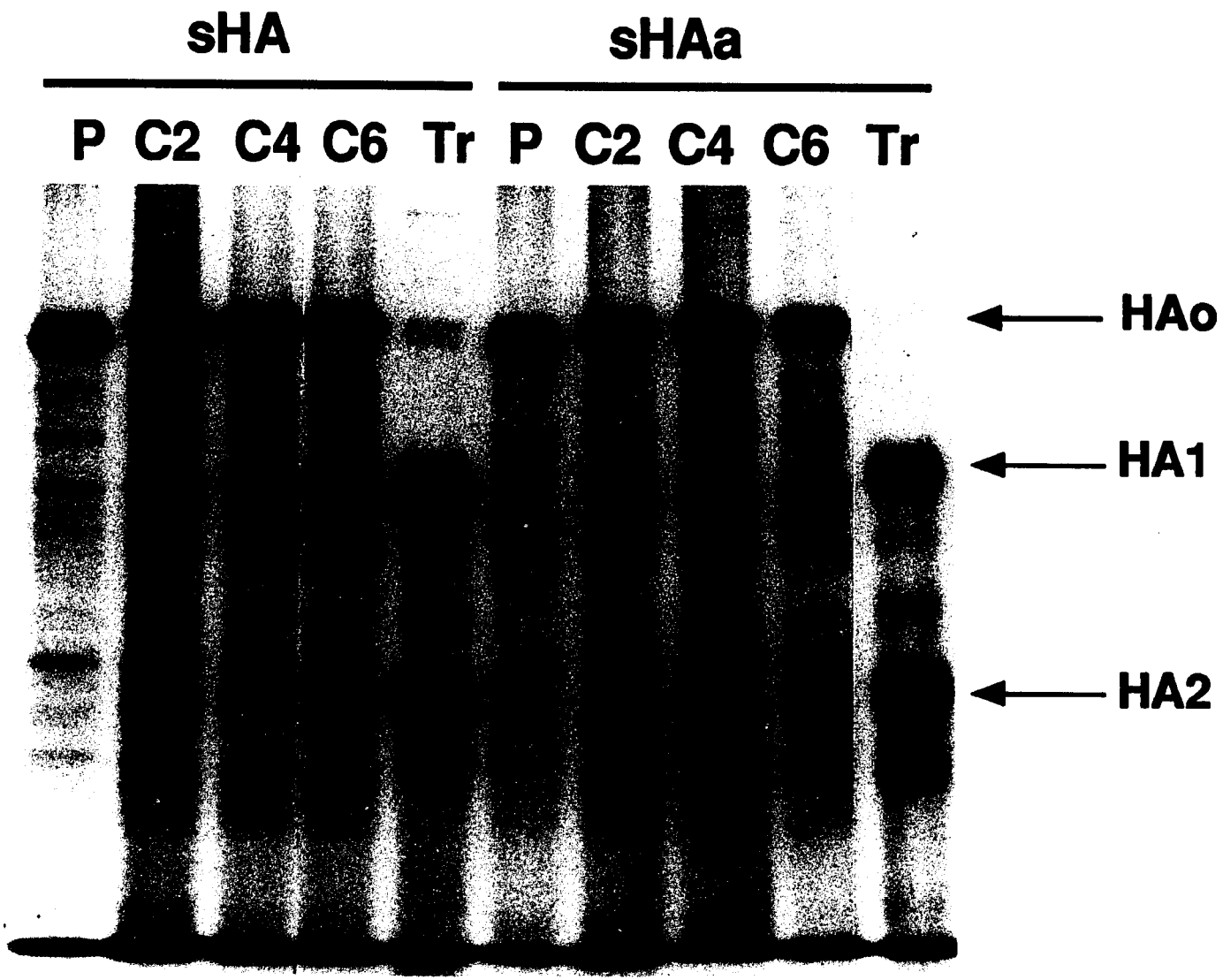

sHA
sHAa
P C2 C4 C6 Tr P C2 C4 C6 Tr
HAo
HA1
HA2

Figure 4. Red cell binding assay of CV-1 and turkey embryo fibroblast cells expressing chimeric HA genes.

Panels A and B CV-1 cells were infected with recombinant SV40 virus, expressing chimeric *sHA* and *sHAa*, respectively. In both cases, a monolayer of red blood cells can be observed tightly bound to the surface of cells expressing the HA proteins. Panel (C) CV-1 cells were infected with recombinant SV40 virus pSVenvKX, expressing the RSV *env* gene as a negative control. Panel (D), TEF cells infected with BHRCAN-sHA virus, showing clusters of cross-linked red cells. Panel (E), uninfected control TEF cells passaged in parallel.

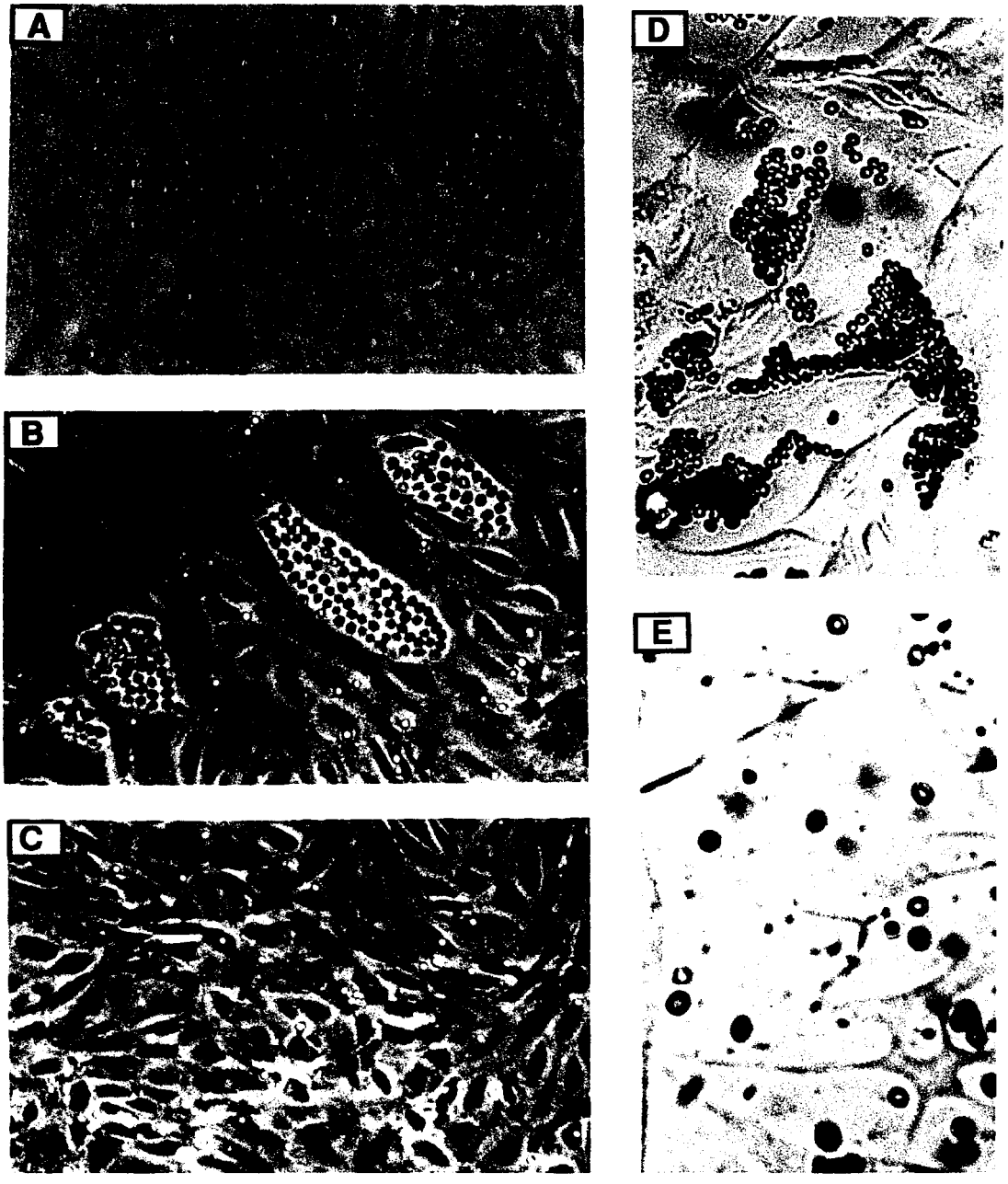

A
B
C
D
E

Figure 5. Assembly of chimeric HA and RSV Env proteins into virions.

QT6 cells, expressing *gag* and different glycoprotein genes, were labeled with 200 μCi of ^{3}H-leucine for 30min and chased for 16h. (Left Panel--viral pellet) Virus particles released into the medium were centrifuged at 100,000g through a 15% sucrose cushion prior to solubilization in lysis buffer and immunoprecipitation with specific antibodies against RSV Gag proteins (all lanes), the RSV Env glycoproteins (lanes env and g/env) and influenza virus (lanes sHA, sHAa, g/sHA and g/sHAa).

(Right Panel--cell lysate) Viral proteins remaining in the cells at the end of 16h-chase were immunoprecipitated from lysed cells using the same panel of antibodies. The core-glycosylated and terminally glycosylated HA proteins are denoted by HA* and HA_0 respectively.

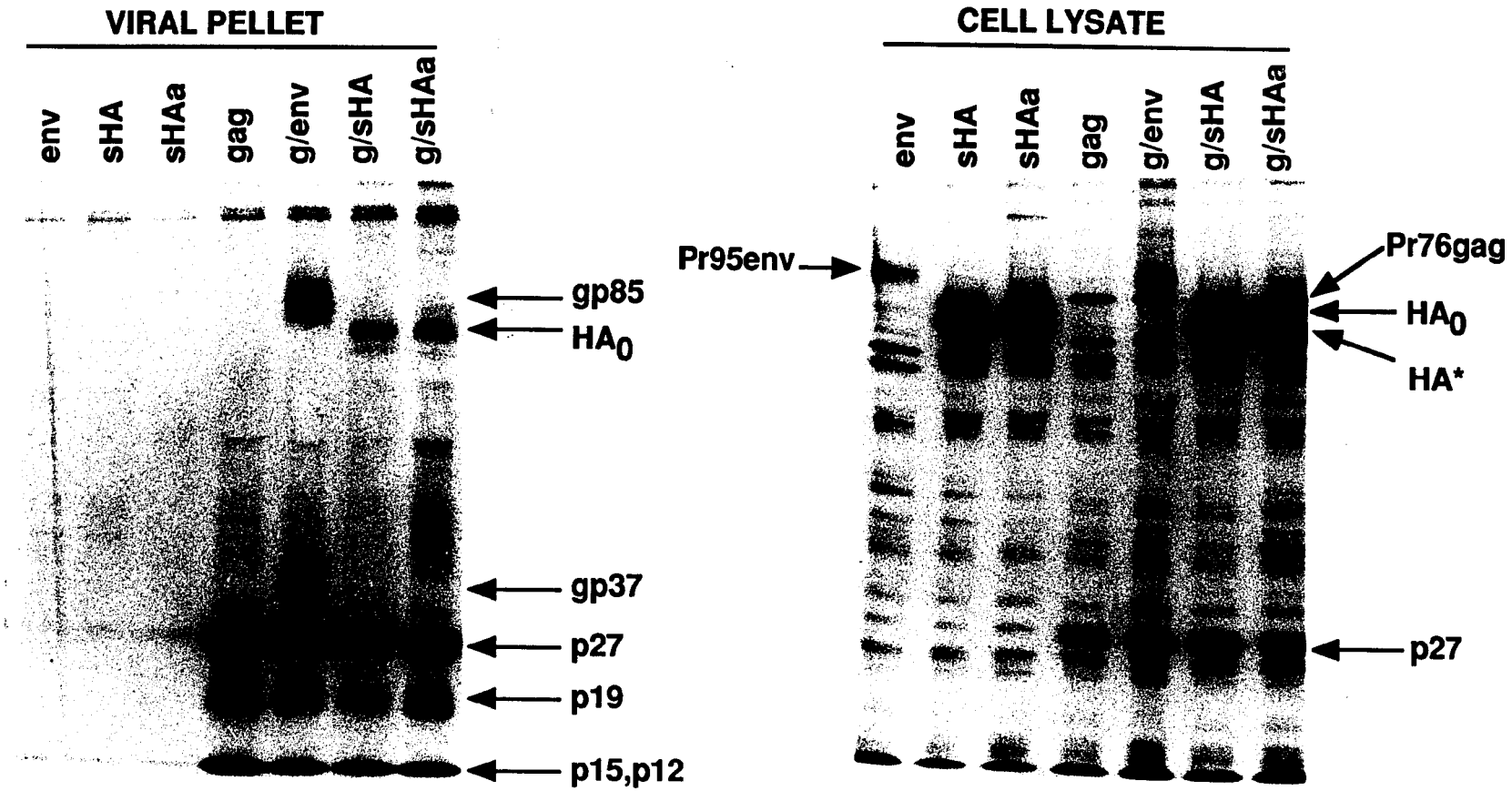

VIRAL PELLET
env
sHA
sHAa
gag
g/env
g/sHA
g/sHAa
gp85
HA0
gp37
p27
p19
p15,p12
CELL LYSATE
env
sHA
sHAa
gag
g/env
g/sHA
g/sHAa
Pr95env
Pr76gag
HA0
HA*
p27

Figure 6. Effect of neuraminidase on hemagglutinin incorporation into virions.

QT6 cells were labelled with 200μCi of ^{3}H-leucine for 30 and chased for 6 hours in the presence or absence of 0.1U of neuraminidase as indicated on top of each lane. Virus and cell associated proteins were immunoprecipitated as described in Figure 5. The incorporation of hemagglutinin was quantitatively determined on a lightly exposed film using a densitometer to determine band intensity. Glycoprotein levels were then normalized to the density of p27 and p19 bands.

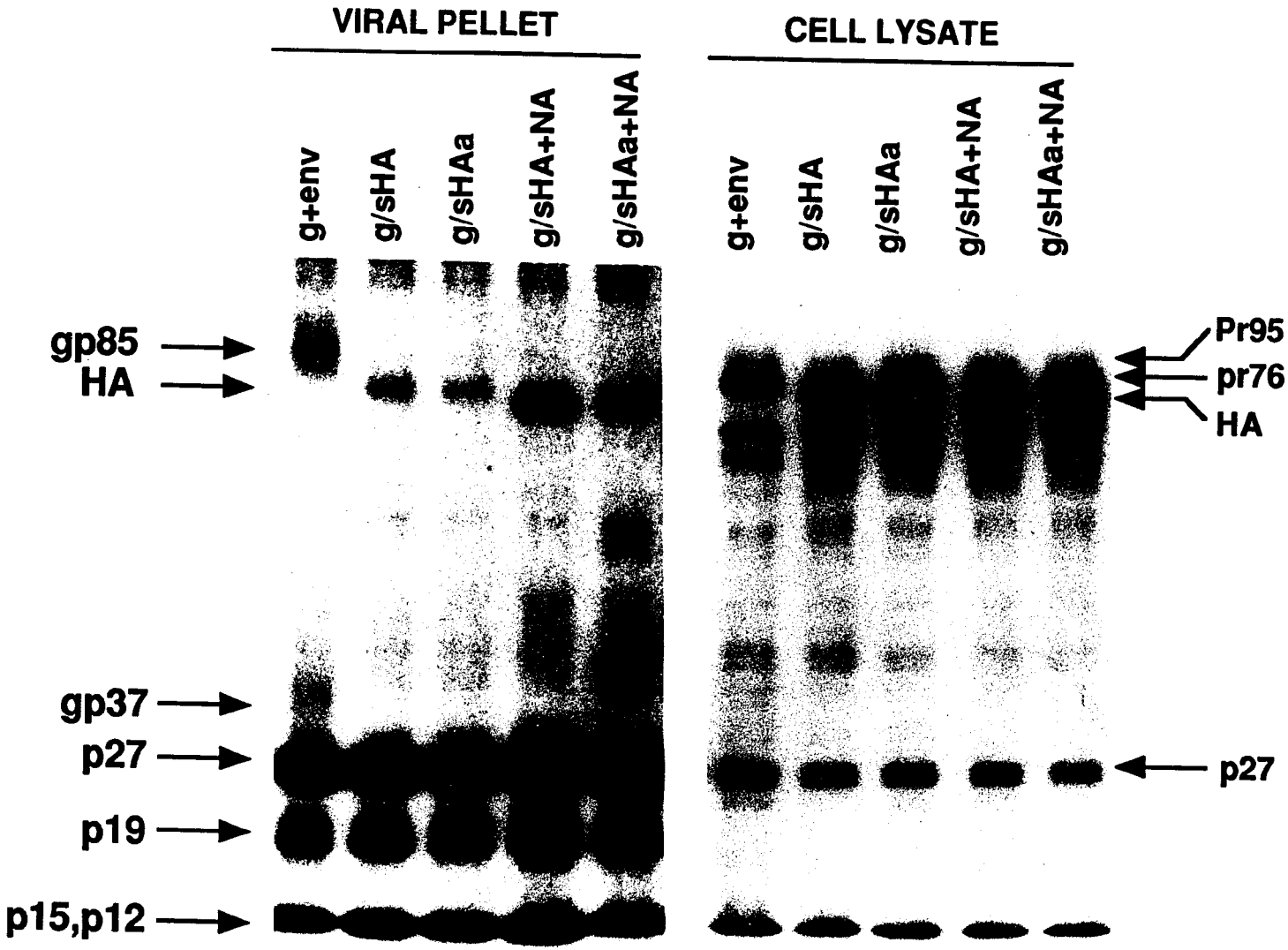

VIRAL PELLET
CELL LYSATE
g+env
g/sHA
g/sHAa
g/sHA+NA
g/sHAa+NA
g+env
g/sHA
g/sHAa
g/sHA+NA
g/sHAa+NA
gp85
HA
gp37
p27
p19
p15,p12
Pr95
pr76
HA
p27

Table 1. Infectivity and host-range analysis of hybrid RSV virions containing chimeric HA proteins

	Number of Colonies/plate[a]			
	No neuraminidase		Neuraminidase[b]	
Virus[c]	QT6	HeLa	QT6	HeLa
BH-RCAN.HiSV	111	0	ND	ND
BH-RCAN.HiSV/sHA	38	28	93	74
BH-RCAN.HiSV/sHAa	29	31	105	93
BN-RCAN.HiSV/Dr1	0	0	0	0
MOCK	0	0	0	0

[a] After 10 days of hygromycin selection, cell colonies were stained and counted. The average number of colonies from three plates is shown.

[b] At 24h post-transfection, the cells were washed and 4 ml of serum-free medium containing 0.025u neuraminidase and 0.6μg trypsin/ml was added to the cells. Virus was harvested 16h post-transfection. In the no neuraminidase plates, cells were treated with trypsin only.

[c] Plasmid DNAs encoding the BH-RCAN.HiSV genome containing the wild-type Pr-C *env* gene (BH-RCAN.HiSV), the *sHA* or *sHAa* genes (BH-RCAN.HiSV/sHA or BH-RCAN.HiSV/sHAa) or the cleavage defective *env* gene (BH-RCAN.HiSV/Dr1) were transfected into TEF cells. The cells were treated as described in [b] and virus harvested after 48h was used to infect QT6 or HeLa cells as described in Materials and Methods.

REFERENCES

1. **Basak, S., M. Tomana, and R. W. Compans.** 1985. Sialic acid is incorporated into influenza hemagglutinin glycoproteins in the absence of viral neuraminidase. Virus Res. **2**:61-8.

2. **Bosch, J. V., R. T. Schwarz, A. Ziemiecki, and R. R. Friis.** 1982. Oligosaccharide modifications and the site of processing of gPr92env, the precursor for the viral glycoproteins of Rous sarcoma virus. Virology **119**:122-32.

3. **Brody, B. A., S. S. Rhee, and E. Hunter.** 1992. Post assembly cleavage of a retroviral cytoplasmic domain removes a necessary incorporation signal and activates fusion activity. Submitted.

4. **Brody, B. A., S. S. Rhee, M. A. Sommerfelt, and E. Hunter.** 1992. Viral protease-mediated cleavage of the transmembrane glycoprotein of Mason-Pfizer monkey virus can be suppressed by mutations within the matrix protein. Proc. Natl. Acad. Sci. U.S.A. **89**:3443-3447.

5. **Davis, G. L. and E. Hunter.** 1987. A charged amino acid substitution within the transmembrane anchor of the Rous Sarcoma virus envelope glycoprotein affects surface expression but not intracellular transport. J. Cell Biol. **105**:1191-1203.

6. **de Giuli, C., S. Kawai, S. Dales, and H. Hanafusa.** 1975. Absence of surface projections of some noninfectious forms of RSV. Virology **66**:253-60.

7. **Doms, R. W. and A. Helenius.** 1986. Quaternary structure of influenza virus hemagglutinin after acid treatment. J. Virol. **60**:833-9.

8. **Doms, R. W., A. Helenius, and J. White.** 1985. Membrane fusion activity of the influenza virus hemagglutinin. The low pH-induced conformational change. J. Biol. Chem. **260**:2973-81.

9. **Dong, J. Y., D. J. W. , L. G. Perez, and E. Hunter.** 1992. Mutations within the proteolytic cleavage site of the Rous sarcoma virus glycoprotein define a requirement for dibasic residues for intracellular cleavage. J. Virol. **66**:865-74.

10. **Einfeld, D. and E. Hunter.** 1992. Unpublished.

11. **Emi, N., T. Friedmann, and J. K. Yee.** 1991. Pseudotype formation of murine leukemia virus with the G protein of vesicular stomatitis virus. J. Virol. **65**:1202-7.

12. **Fuerst, T., P. L. Earl, and B. Moss.** 1987. Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. Mol. Cell. Biol. **7**:2538-2544.

13. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eucaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. **83**:8122-8126.

14. **Gebhardt, A., J. V. Bosch, A. Ziemiecki, and R. R. Friis.** 1984. Rous sarcoma virus p19 and gp35 can be chemically cross linked to high molecular weight complexes: An insight into viral association. J. Mol. Biol. **174**:297-317.

15. **Gething, M. J. and J. Sambrook.** 1981. Cell-surface expression of influenza haemagglutinin from a cloned DNA copy of the RNA gene. Nature **293**:620-5.

16. **Gething, M. J. and J. Sambrook.** 1982. Construction of influenza haemagglutinin genes that code for intracellular and secreted forms of the protein. Nature **300**:598-603.

17. **Greenhous, J. J., C. J. Petropoulos, L. B. Crittenden, and S. H. Hughes.** 1988. Helper-independent retrovirus vectors with Rous-associated virus type O long terminal repeats. J. Virol. **62**:4909-4812.

18. **Griffin, J. A., S. Basak, and R. W. Compans.** 1983. Effects of hexose starvation and the role of sialic acid in influenza virus release. Virology **125**:324-34.

19. **Hughes, S. and E. Kosik.** 1984. Mutagenesis of the region between *env* and *src* of the SR-A strain of Rous sarcoma virus for the purpose of constructing helper-independent vectors. Virology **136**:89-99.

20. **Hunter, E.** 1979. Biological techniques for avian sarcoma viruses. Methods Enzymol **58**:379-93.

21. **Kaster, K. R., S. G. Burgett, R. N. Rao, and T. D. Ingolia.** 1983. Analysis of a Bacterial hygromycin B resistance gene by transcriptional and translational fusions and DNA sequencing. Nucleic Acids Res. **11**:6895-6911.

22. **Katz, R. A., C. A. Omer, J. H. Weis, S. A. Mitsialis, A. J. Faras, and R. V. Guntaka.** 1982. Restriction endonuclease and nucleotide sequence analyses of molecularly cloned unintegrated avian tumor virus DNA: structure of large terminal repeats in circle junctions. J Virol **42**:346-51.

23. **Landau, N. R., K. A. Page, and D. R. Littman.** 1991. Pseudotyping with human T-cell leukemia virus type I broadens the human immunodeficiency virus host range. J. Virol. **65**:162-9.

24. **Lazarovits, J., S. P. Shia, N. Ktistakis, M. S. Lee, C. Bird, and M. G. Roth.** 1990. The effects of foreign transmembrane domains on the biosynthesis of the influenza virus hemagglutinin. J. Biol. Chem. **265**:4760-7.

25. **Leamnson, R. N. and M. S. Halpern.** 1976. Subunit structure of the glycoprotein complex of avian tumor virus. J. Virol. **18**:956-968.

26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. (eds.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.,

27. **McClure, M. O., M. A. Sommerfelt, M. Marsh, and R. A. Weiss.** 1990. The pH independence of mammalian retrovirus infection. J. Gen. Virol. **71**:

28. **Miller, A. D., J. V. Garcia, N. von Suhr, C. M. Lynch, C. Wilson, and M. V. Eiden.** 1991. Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. J. Virol. **65**:2220-4.

29. **Moscovici, C., M. G. Moscovici, H. Jimenez, M. M. Lai, M. J. Hayman, and P. K. Vogt.** 1977. Continuous tissue culture cell lines derived from chemically induced tumors of Japanese quail. Cell **11**:95-103.

30. **Perez, L., J. W. Wills, and E. Hunter.** 1987. Expression of the Rous sarcoma virus env gene from a simian virus 40 late-region replacement vector: effects of upstream initiation codons. J. Virol. **61**:1276-1281.

31. **Perez, L. G., G. L. Davis, and E. Hunter.** 1987. Mutants of the Rous sarcoma virus envelope glycoprotein that lack the transmembrane anchor and/or cytoplasmic domains: analysis of intracellular transport and assembly into virions. J. Virol. **61**:2981-2988.

32. **Petropoulos, C. J. and S. H. Hughes.** 1991. Replication-Competent Retrovirus Vectors for the Transfer and Expression of Gene Cassettes in Avian Cells. J. Virol **65**:3728-3737.

33. **Rhee, S. S. and E. Hunter.** 1990. A single amino acid substitution within the matrix protein of a type D retrovirus converts is morphogenesis to that of a type C retrovirus. Cell 77-86.

34. **Spector, D. H., E. Wade, D. A. Wright, V. Koval, C. Clark, D. Jaquish, and S. A. Spector.** 1990. Human immunodeficiency virus pseudotypes with expanded cellular and species tropism. J. Virol. **64**:2298-308.

35. **Weiss, R. A., D. Boettiger, and H. M. Murphy.** 1977. Pseudotypes of avian sarcoma viruses with the envelope properties of vesicular stomatitis virus. Virology **76**:808-25.

36. **Weiss, R. A. and A. L. Wong.** 1977. Phenotypic mixing between avian and mammalian RNA tumor viruses: I. Envelope pseudotypes of Rous sarcoma virus. Virology **76**:826-34.

37. **White, J., M. Kielian, and A. Helenius.** 1983. Membrane fusion proteins of enveloped animal viruses. Q. Rev. Biophys. **16**:151-195.

38. **White, J., K. Matlin, and A. Helenius.** 1981. Cell fusion by Semliki Forest, influenza, and vesicular stomatitis viruses. J Cell Biol **89**:674-9.

39. **Wills, J. W., J. M. Hardwick, K. Shaw, and E. Hunter.** 1983. Alterations in the transport and processing of Rous sarcoma virus envelope glycoproteins mutated in the signal and anchor regions. J. Cell Biochem. **23**:81-94.

40. **Wills, J. W., R. V. Srinivas, and E. Hunter.** 1984. Mutations of the Rous sarcoma virus env gene that affect the transport and subcellular location of the glycoprotein products. J. Cell Biol. **99**:2011-2023.

41. **Wilson, C., M. S. Reitz, H. Okayama, and M. V. Eiden.** 1989. Formation of infectious hybrid virions with gibbon ape leukemia virus and human T-cell leukemia virus retroviral envelope glycoproteins and the gag and pol proteins of Moloney murine leukemia virus. J. Virol. **63**:2374-8.

42. **Young, J. A., P. Bates, K. Willert, and H. E. Varmus.** 1990. Efficient incorporation of human CD4 protein into avian leukosis virus particles. Science **250**:1421-3.

A RAPID ASSAY SYSTEM FOR THE ANALYSIS OF RETROVIRAL ASSEMBLY

JIANYUN DONG AND ERIC HUNTER*

Department of Microbiology
University of Alabama at Birmingham
Birmingaham, AL 35294

* Author to whom reprint requests should be made.

ABSTRACT

The nature of protein-protein interactions during retroviral assembly is not well understood, and mutational analyses of the potential signals involved in the viral assembly process has been difficult, particularly with the avian retroviruses due to the level of viral proteins expressed in the clonal cell lines containing defective viral genomes. We describe here a complementation system in which the retroviral *gag/pol* and *env* gene products were expressed independently from different plasmids under the control of the bacteriophage T7 promoter, in avian cells. Coexpression of the T7 polymerase from a vaccinia virus vector resulted in a high level of biosynthesis of retroviral structural proteins and efficient assembly of virus particles. Electron microscopy and protein composition analyses demonstrated that these virions were indistinguishable from those produced from RSV-infected cells. The applicability of this system to other retroviral systems is described.

INTRODUCTION

Retroviruses have a protein capsid which contains the viral genetic material and reverse transcriptase. Outside the capsid is the lipid bilayer derived from the host cell membrane in which envelope glycoproteins are embedded. During the infection cycle, these envelope glycoproteins initialize infection by recognizing and binding specific receptors on the surface of a host cell, and induce fusion of the viral and cell membranes. After genomic replication and integration into the cell chromosome viral RNAs encoding structural proteins are produced and nascent virions are assembled. Newly synthesized viral capsids specifically incorporate viral glycoprotein from the cell plasma membrane during viral budding while, for the most part, excluding the cellular proteins. This retroviral assembly process is an important aspect of the basic molecular biology of retroviruses.

The nature of protein-protein interactions during retrovirus assembly is not well understood, and molecular genetic analyses of functional regions within the *gag* and

env gene products are only begining to provide information in this regard. For the primate virus Mason-Pfizer monkey virus (M-PMV) and human immunodefficiency virus (HIV), transient expression in COS cells of viral genomes cloned into plasmids containing an SV40 origin of replication has allowed rapid biochemical analyses of mutations(2, 13, 21). Studies of the prototypical retrovirus, Rous sarcoma virus, have been hampered by the virus' inability to assemble in COS cells (1, 18, 23). While modifications of the RSV gag gene, which result in a myristylated Gag polyprotein have allowed the studies of capsid assembly in COS cells (24), this system does not appear to be suitable for studying glycoprotein incorporation (R. Craven--personal communication). The RSV system also suffers from the fact that there are few permanent avian cell lines in which genomes containing non-conditional lethal mutations can be established. Moreover, when such quail tumor cells (QT6 or QT35) are transfected with a replication-defective RSV genome they express only low levels of viral protein (Stewart et al, 1990; Dong, Enfield, Miller--unpublished results).

Because we were interested in studying both RSV capsid assembly and the incorporation of native as well as foreign glycoproteins into RSV, we established a complementation system in avian cells in which gene expression was under the control of the bacterophage T7 promoter. The functional expression of bacteriophage T7 polymerase in eukaryotic cells via recombinant vaccinia virus provided one of the most versatile systems for high level expression of biologically functional proteins (7, 9, 10). This system takes advantage of the high efficiency and specificity of phage T7 polymerase and provides the eukaryotic machinery for protein transport and modifications.

In this complementation approach, the *gag-pol* and *env* genes are expressed from independent plasmids that are transfected into avian cells immediately after infection with vaccinia virus expressing the T7 polymerase. The large amount of the viral protein made in the cell makes it possible to analyze biochemically the virus that

is assembled and was not dependent on the infectious nature of the retrovirus. Thus the defects within non-infectious mutants of RSV can be dissected. Moreover, we show that this approach is applicable to the transient expression analysis of the assembly of other retroviruses.

METHODS AND MATERIALS

Cells and DNA.

QT6 cells, derived from a methylcholanthrene-induced quail tumor (16), were grown in growth medium containing 1% heat-inactivated chicken serum and 5% fetal calf serum. HeLa and Hep2 cells were grown in DMEM containing 10% fetal calf serum.

Expression vectors pSP72 or pSP73 were purchased from Promega (Promega Biotech, Madison, WI). For RSV *gag-pol* expression, a SacI (nucleotide 255) to XbaI (nucleotide 6861) fragment from the pATV8R genomic clone of the Prague C strain of RSV was cloned into the plasmid pSP72 which contains the T7 promoter region. This yielded pSP73.gag-pol. For *env* expression a KpnI (nucleotide 5995) to XbaI (nucleotide 6861) fragment was cloned into the respective sites in pSP72. Plasmids containing wild-type, or mutant (S19, T24 and A^-) *env* sequences, were named pSP72.env, pSP72.S19, pSP72.T24 and pSP72.A^- respectively. The MPMV *env* gene was cloned from an *env*-expressing plasmid TML (2) into pSP73 using flanking BamHI-EcoRI sites to yield pSP73.MPenv. The MPMV *gag-pro-pol* genes were excised from a plasmid containing a complete infectious M-PMV genome, pSRHM15 (19), using NarI and BglII, and cloned into the pSP73 vector using compatible restriction sites (ClaI and BamHI). This plasmid was named pSP73.MPgag-pol.

Plasmid DNA was propagated in E. Coli DH5 cells and purified on both small and large scales as described previously by Maniatis et al. (15). All DNA used for transfection studies was derived from cesium chloride gradients.

Vaccinia Virus.

Recombinant vaccinia virus, VT7, carrying the bacteriophage T7 polymerase gene under the control of the 7.5K promoter, was a gift from Dr. B. Moss. This virus was propagated in Hep2 cells as follows: Hep2 cells were infected with the recombinant vaccinia virus at an MOI of 0.1 and incubated at 37°C in a 5% CO_2 incubator for 3 days or until over 90% of the cells began to show CPE. Cells were then scraped off the plate and suspended in 4 ml of DMEM for each 100 mm plate. The cell suspension was frozen and thawed three times and sonicated for 2 min. before being subjected to centrifugation at 15,000 rpm for 15 min. The supernatant was aliquoted and stored at -100°C until use. The viral titer was determined by serial dilution as described previously (10).

Complementation system for studies of retrovirus assembly

The assembly complementation approach was based on the expression system described by Feurst et al. (7, 9, 10). For the initial expression of *gag-pol* and *env* genes of RSV, subconfluent QT6 cells were first infected with the VT7 recombinant vaccinia virus at an MOI of 20. Two hours post infection cells were co-transfected with a mixture of 5μg each of pSP73.gag-pol and pSP72.env DNAs, using the Lipofectin™ transfection method with slight modifications (7, 8). At five hours post-transfection, cells in 60mm dishes were were starved in leucine-free DMEM (Gibco life technologies,Inc., Grand Island, N.Y.) for 1 h and then pulse-labeled at 37°C for 30-60 min in 1ml leucine-free labelling medium containing 200μCi of ^{3}H-leucine (Dupont, NEN Research Products). In order to allow incorporation of the labelled polypeptides into virus, labelled cells were chased in complete growth medium for 5-8 h. After that time virus released into medium was collected by ultracentrifugation at 80,000 rpm for 15 minutes in a table-top ultracentrifuge (TLA100 rotor, Beckman) after being cleared of cell debris by low speed centrifugation. Viral pellets were then lysed in lysis buffer B for immunoprecipitation (12). Following removal of the virus-containing

supernatent, cells were lysed in lysis buffer A (12), cleared of undisrupted nuclei, and immunoprecipitated as described previously (12) with the antibodies delineated below.

After optimization of the complementation procedure (see results for details) infection with vaccinia virus and transfection with DNA was done at 8 and 5 hours prior to cell lysis, respectively.

For electron microscopy, cells co-transfected with pSP73.gag-pol and pSP72.env were scraped off the plate and pelleted at 14 hr post infection with recombinant vaccinia virus. Following fixation in glutaraldehyde (1%), the tannic acid staining method (4, 14) was used to enhance the ability to visualize glycoprotein on the surface of virions. Photographs were taken following thin-section electron microscopy.

Antibodies.

A rabbit antibody prepared against the Prague C subgroup of RSV was a gift from Dr. John Wills. This antibody reacted strongly with *gag*-gene products of the virus and therefore was used for the precipitation of major capsid proteins (24). Antibodies directed against the RSV glycoproteins were prepared in rabbits using either lentil-lectin purified gp85/gp37 (anti-Env) as described previously (12) or a peptide corresponding to the C-terminal cytoplasmic domain of gp37 (anti-gp37c). These anti-glycoprotein antibodies demonstrated no cross-reaction with the RSV Gag proteins. The MPMV *gag*- and *env*-gene products were immunoprecipitated with a goat anti-MPMV antibody obtained through the Division of Research Resources, NCI, as described previously(2, 19, 20).

RESULTS

Expression of *gag-pol* and *env* genes from the phage-T7 promoter results in assembly of viral particles.

To determine if Gag-Pol and Env proteins made from to separate constructs under the control of a T7-promoter could be properly assembled into viral particles, SP73.gag-pol and SP72.env were transfected separately or cotransfected into QT6 cells that were infected with the recombinant vaccinia virus, VT7. After pulse labeling with

^{3}H-leucine and chasing for 6h in complete medium, virus particles were collected by ultra-centrifugation and immunoprecipitated with antibody as described in Materials and Methods. The results are shown in Figure 1A. When the *env* gene was expressed alone, envelope glycoprotein was found associated with cell lysate (Figure 1A, lane env, cell lysate) but was not precipitated from the viral pellet (Figure 1A, lane env, viral pellet). When the *gag-pol* gene was expressed in the absence of the *env* gene, the gag-gene products including the major capsid protein, p27, were detected in the pelletable fraction as well as in the cell lysate. This result indicated that envelope glycoproteins expressed alone were not released into the culture medium associated with a pelletable fraction and that particles lacking the envelope glycoprotein could be released in the medium in the absence of *env* gene (Figure 1A, lane gag, viral pellet and cell lysate). This is consistent with previous studies of *env*-deficient mutants of RSV (5). In contrast, when *gag-pol* and *env* genes were co-expressed in the cells, the envelope glycoproteins as well as capsid proteins were found in the pelletable fraction (Figure 1A, lane e/g; viral pellet). This indicated that glycoproteins were incorporated into the assembled virions.

Moreover, a mutant lacking the entire transmembrane and cytoplasmic domains (17) was not associated with pelletable viral particles (Fig. 1B viral pellet, lane A), even though the amount of mutant envelope glycoprotein synthesized was similar to that of wild type (Figure 1B lanes Wt and A; Cell lysate). As shown previously, the mutant glycoprotein was found secreted into the medium in a soluble, non-pelletable form (Figure 1B, A; supernant). In addition, a three amino acid deletion with the membrane spanning domain of *env* (T24), which resulted in the synthesis of non-infectious virus ((11) Davis et al, in preparation), completely blocked incorporation of the env-gene products into virons (Figure 1A, lane T24/g,viral pellet). Thus, we would argue that the incorporation of envelope glycoprotein in this complementation system is specific for membrane proteins that are competent for incorporation.

Electron microscopic analysis of released virions.

Thin-section electron microscopy was used to determine whether the particles released possessed a typical morphology of RSV virions. Figure 2 shows thin sections of QT6 cells transfected with pSP73.gag-pol and pSP72.env, or pSP73.gag-pol alone. Only 'bald' particles were released from the cells transfected with the *gag-pol* construct alone, as indicated by the smooth surface of each particle (Figure 3A, indicated by arrow). In contrast, when *gag-pol* and *env* genes were transfected into the QT6 cells, glycoprotein incorporation into particles could be clearly visualized as projections on the surface of the viral particles (Figure 3B, indicated by arrow). In certain sections, a regular, apparently symmetrical, pattern of glycoproteins was observed on the surface of a virion (Figure 2B, open arrow). Figure 2C shows two morphologically normal virions in the process of budding; the incorporated glycoproteins are clearly visible (indicated by the arrow).

A cluster of virions indistinguishable from those released from RSV-infected cells are shown in Fig. 3D. This mature morphology is consistent with the presence of a normal complement of cleaved capsid proteins observed in gels of released virions. A recombinant vaccinia virus can be seen in this panel.

Optimization of the time of vaccinia infection and DNA transfection.

Many of the protocols for the vaccina-T7 expression system have been optimized for mammalian cells and utilize a long-period post-vaccinia infection (often 24 hr) prior to analyzing the protein under study (7, 9, 10). For studies on virus assembly that utilize metabolic labeling techniques, the optimal time point for labelling would be the point at which the rate of protein synthesis was highest. Moreover, we observed that at late times of vaccinia virus infection (>12h), retroviral glycoprotein transport and processing can be significantly inhibited (Brody and Hunter, unpublished). Therefore, we carried out experiments to investigate the optimal time for

infection and transfection within a time-frame that allowed native processing of foreign glycoproteins.

In order to optimize the time of infection of cells with vaccinia virus, duplicate 35 mm-plates were infected with the recombinant VT7 at a multiplicity of infection (MOI) of 20, at 13,11,9,7,5, and 3h prior to metabolic labelling. All of the plates were transfected with 5μg of SP72.env plasmid DNA 12h prior to pulse labeling. After a 15 minute label, cells were lysed and the RSV envelope precursor protein was immunoprecipitated as described in Materials and Methods. The results are shown the in the left-panel (Infection Time Course) of Figure 3, . Under the conditions of this experiment, the highest rate of protein synthesis appeared to occur approximately 7 hours post-infection.

The transfection time course experiment was performed in a similar manner to the infection study. Duplicate plates were transfected at 12,10,8,6,4, and 2 h prior to pulse labeling. All of the plate were infected at the same time at 11h prior to labeling. The results are shown in the right-panel of Figure 3. The highest rate of protein synthesis occurred when transfection preceded the pulse-label by 4h. Therefore we conclude that for QT6 cells, the optimal time point for metabolic labelling of the cells is at 7h post infection and 4h after transfection.

Optimization of the ratio of *gag* and *env* gene expression vectors

During the replication of avian retroviruses the ratio of *gag* and *env* gene expression is controlled by the extent of RNA splicing. In the complementation system this ratio is directly determined by the ratio of the pSP73.gag-pol and the pSP72.env plasmids used for transfection. To optimize the efficiency of viral assembly, different ratios of the *gag* and *env* gene-containing constructs were co-transfected into QT6 cells. After an 8h chase, viral particles were collected and analyzed by immunoprecipitation (Figure 4). As might be expected, if there is a limit to the amount of glycoprotein that can be incorporated into a virion, a saturation phenomenon was

observed in this experiment. At low ratios of envelope expression vector (1:4 to 1:1; *env:gag*) the amount of envelope glycoprotein precipitated from the pelleted virions increased with the level of envelope glycoprotein expression. The highest amount of envelope glycoprotein incorporation was observed at a 1:1-2:1 ratio of *env* and *gag* constructs. An additional increase in env-gene expression, that can be seen in the cell lysates (Figure 4, cell lysate, lane 8.0), did not yield a significantly higher level of glycoprotein incorporation into the virus.

Incorporation of functional and non-functional glycoproteins

One of the most useful applications of this complementation system is to study the biochemical defects of non-infectious mutants of avian retroviruses. Due to the high specificity and efficiency of the T7 RNA polymerase activity, a large amount of mRNA can be made; this results in a high level expression of the retroviral proteins independent of retroviral infectivity. To examine this capability we compared the incorporation of the wild-type RSV glycoprotein with a non-infectious mutant, S19,(6). This mutant contained a double mutation in the glycoprotein precursor cleavage site that completely blocks the proteolytic cleavage of the envelope protein precursor, and thus blocked infectivity (6). The result of such an experiment is shown in Fig.4. The amount of viral glycoprotein incorporated was comparable for both the mutant and the wild-type *env* expressing systems (Figure 5, lanes S19 and Wt). Therefore, the efficiency of assembly and the incorporation of glycoprotein for replication defective mutants can be compared. This complementation system thus provides a convenient way to biochemically dissect the defects of a non infectious mutant.

Application of the system to mammalian viruses and host range studies

The broad host range of vaccinia virus makes it possible to use this complementation method for other retroviruses, including those of mammalian origin, and to utilize the system to explore host cell restrictions on virus assembly and release.

In a preliminary test of this possibility, we investigated whether expression of the *gag-pol* and *env* genes of the primate type-D retrovirus, M-PMV, under the control of the T7 promoter would result in assembly and release of capsids, and whether this could occur in avian cells. As shown in Figure 5 (lane MPMV), efficient release of M-PMV virions containing a normal complement of envelope glycoproteins was observed. More importantly, both Gag precusor maturational cleavage and the viral protease-mediated processing of gp22 to gp20 (3, 22) were observed in the virions released from avian cells.

DISCUSSION

Although avian sarcoma viruses have been used as models for the study of retroviral biology and molecular biology, it has been difficult to study biochemically their assembly and glycoprotein incorporation using replication defective mutants. This is mainly due to two reasons; lack of available cell lines for establishing stable clonal cells expressing at high levels defective avian retroviruses, and the low and variable level of transient gene expression following transfection of avian cells. Although some chemically induced quail cell lines have been used for propagation of avian sarcoma viruses, these cells have failed to support stable expression of viral genes at levels that make extensive biochemical analyses feasible (7, 9, 10).

In this study wedemonstrated an alternative approach for the study of retroviral assembly using a T7-*gag-pol/env* complementation system. In this system, a high level of gene expression made it straightforward to detect viral protein synthesis, transport, and assembly into virions. More importantly, the level of virus protein expression was independent of the infectivity of the retroviral genome. Thus it would be possible to compare in a quantitative fashion the assembly of capsid protein mutants and the incorporation efficiencies of different glycoprotein mutants. Experiments with the T24, Anchor(-), and S19 mutants suggest that it will be possible to use this system to distinguish between mutants defective in viral glycoprotein incorporation and those in

which the function of the glycoprotein itself is impaired. Moreover, the lack of incorporation of the T24 mutant protein into virions, despite equivalent levels of cell-associated protein to that in wild-type *env* expressing cells, suggests that there must be some mechanism to preclude incorporation of this modified protein into virions.

In the complementation-expression system presented here, we demonstrated that morphologically normal virions bud from cells expressing the structural polypeptides of RSV, and that the components of these virions undergo the normal process of precursor protein cleavage and virion maturation. The assembled mature virions appeared to have the same protein content as virions released from virus-infected cells and in some instances the glycoprotein spikes of the budding and released virions appeared to be arranged in a regular fashion. Because the *gag* and *env* genes of the virus were expressed independently, this system provides a useful approach to investigate the requirements for glycoprotein incorporation into the avian retrovirus particle. Initial studies in this regard suggested that the hemagglutinin glycoprotein of influenza virus could be efficiently incorporated into RSV virions when the HA gene was substituted for the RSV *env* gene in this system (Dong, Roth, and Hunter, submitted). The basis for this in the light of the T24 result remains to be determined.

The broad host range of vaccinia virus makes it possible to study retrovirus assembly and release in a variety of cells that are susceptible to vaccinia infection, including a wide variety of mammalian and avian cells. We have shown in this paper that, following expression of *gag-pol* and *env* genes of the primate retrovirus M-PMV in avian cells, virus particles are produced which contain a normal complement of mature virus proteins. Moreover, the virions appear to have assembled the viral glycoprotein in a native conformation since processing of the C-terminal cytoplasmic domain by the viral protease was observed. Previous studies showed this to be a specific process that could be disrupted by mutations within the matrix (MA-p10)

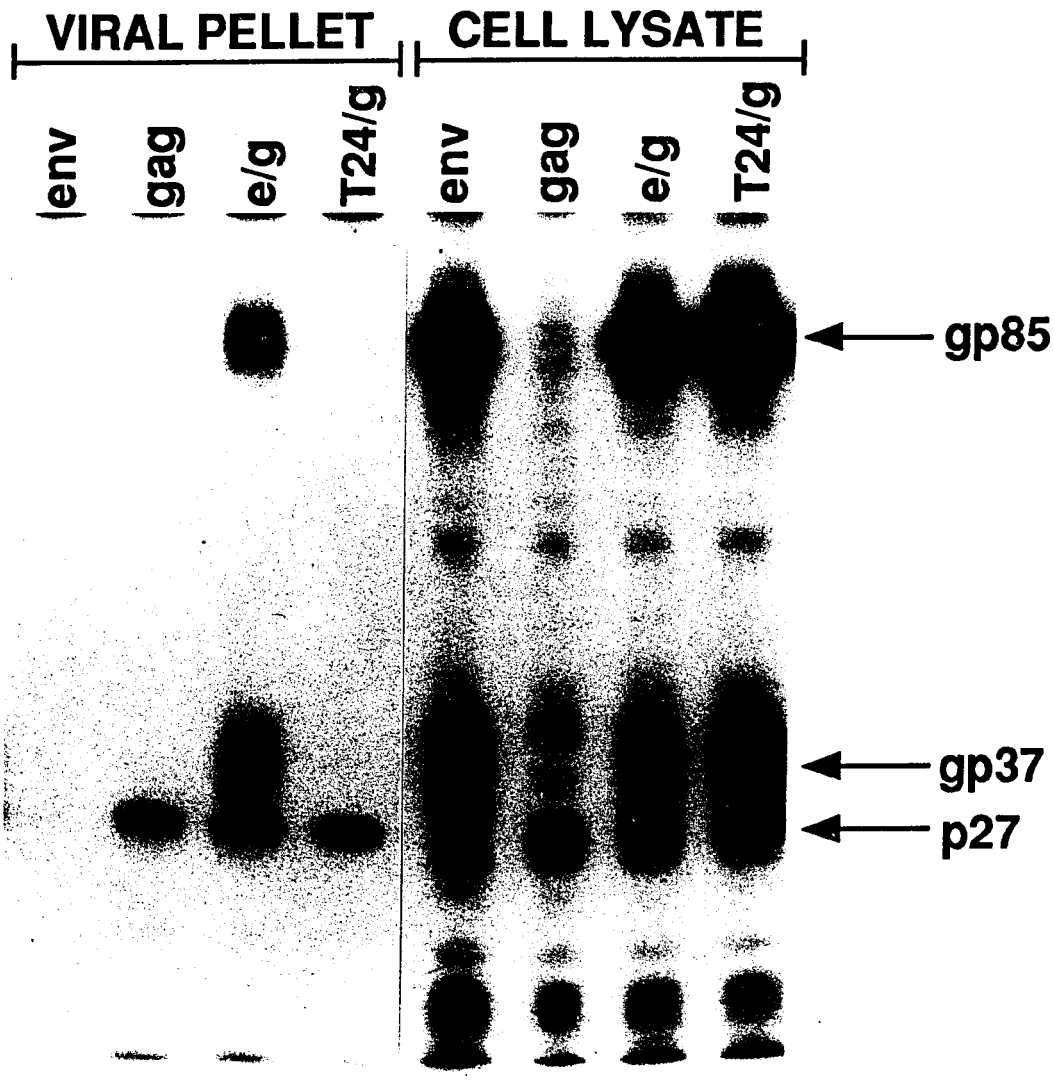
protein of the virus (19, 20),. RSV, in contrast, appears to be defective in assembly in mammalian cells (1, 23). Wills and colleagues have shown using an SV40-based expression system that at least part of this defect can be overcome by modifying the *gag* gene so that the Gag precusor is myristylated. It will be of interest to express the myristylated and non-myristylated *gag* gene products using the vaccinia expression system that we have described here in a variety of mamalian cells to compare the relative ability of these proteins inorder to assemble in different intracellular environments.

Figure 1. Immunoprecipitation analysis of virus assembly in cells expressing *gag-pol* and *env* genes.

Viral proteins were immunoprecipitated from virus pellets (left panel) and cell lysate (right panel) of QT6 cells which were infected with VT7, and transfected with plasmids containing the viral genes, as described in Materials and Methods.

A. Cells were transfected either with pSP72.env or pSP73.gag-pol alone, or co-transfected with pSP73.gag-pol/pSP72.env, pSP73.gag-pol/pSP72.T24, labeled as env, gag, e/g and T24/g, respectively, on top of each lane. Arrows indicate the positions of different viral proteins, on the autoradiograph of a SDS 10% PAGE gel.

B. Cells were transfected with both pSP73.gag-pol and pSP72.env (Wt) or pSP73.gag-pol and pSP72A$^-$ (A).


VIRAL PELLET
CELL LYSATE
env
gag
e/g
T24/g
env
gag
e/g
T24/g
gp85
gp37
p27

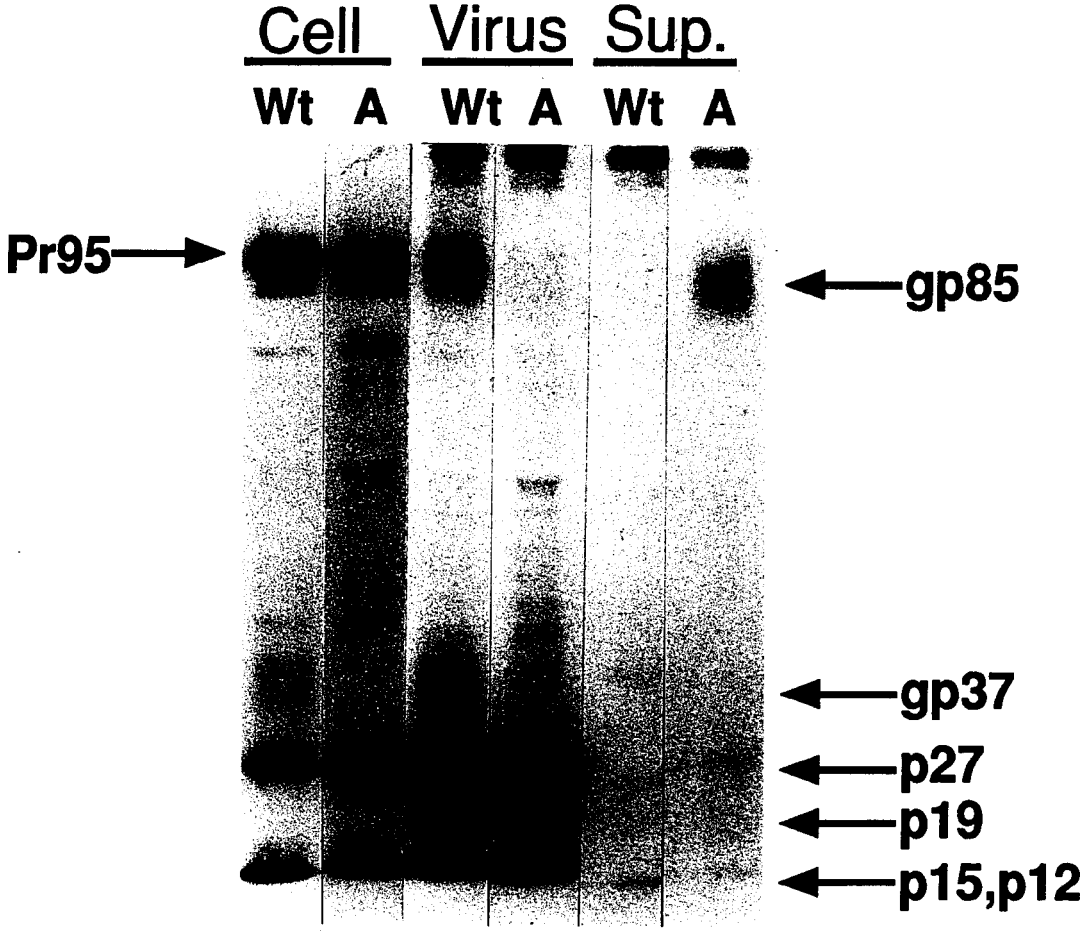

Cell
Virus
Sup.
Wt
A
Wt
A
Wt
A
Pr95
gp85
gp37
p27
p19
p15,p12

Figure 2. Electron microscopy of virus particles assembled in expressing QT6 cells.
Thin section electronmicrographs were taken of QT6 cells that were infected with recombinant vaccinia virus and transfected with pSP73.gag-pol alone or cotransfected with both pSP72.env and pSP73.gag-pol. In panel A, the arrow indicates the smooth surface of "bald" viral particles released from cells transfected with pSP73.gag-pol alone. In panel B, the black arrow indicates the "glycoprotein spikes" on the surface of virions released from cells cotransfected with both pSP73.gag-pol and pSP72.env. The open arrow points to a section in which regular patterns of incorporated envelope glycoproteins can be distinguished. Panel C shows budding virions that are morphologically indistinguishable from wild type virus. An arrow points at the glycoproteins on the viral surface. Panel D is a field of lower magnification showing the recombinant vaccinia virus (denoted by black arrow) and mature retroviral particles (denoted by open arrow) released from a single cell.

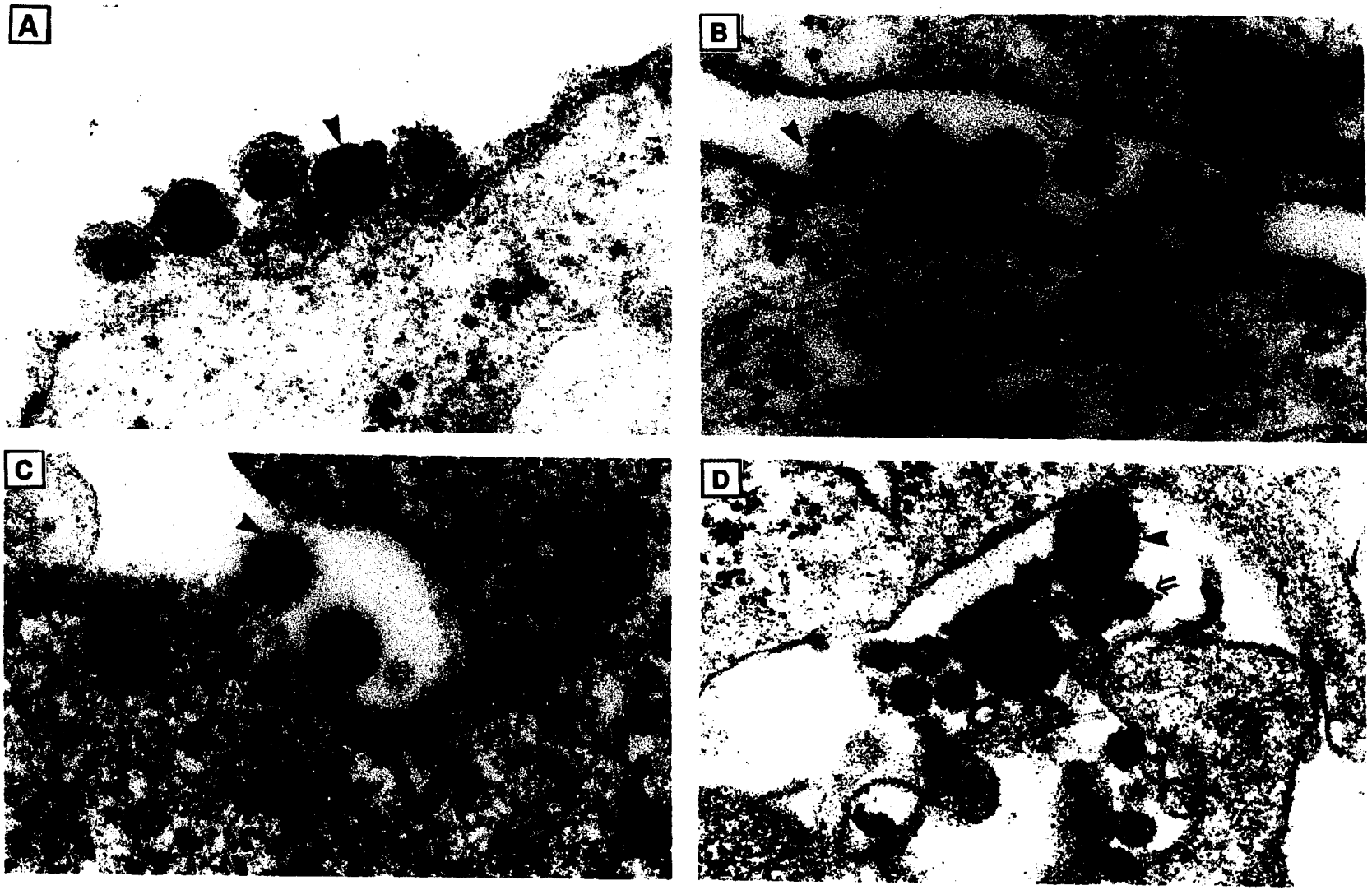

A
B
C
D

Figure 3. Optimization of recombinant vaccina virus infection and plasmid transfection

For the transfection study (right panel), QT6 cells were infected at 11h prior to cell lysis and transfected with pSP72.env at different time points prior to cell lysis as indicated by the numbers on top of each lane on the left half of the panel. For the infection study (left panel), QT6 cells were transfected at 12h before cell lysis and infected at the times indicated by the number on top of each lane in the right half of the panel. In each case labeling and immunoprecipitation were carried out as described in Materials and Methods.

Time Course of Infection and Transfection

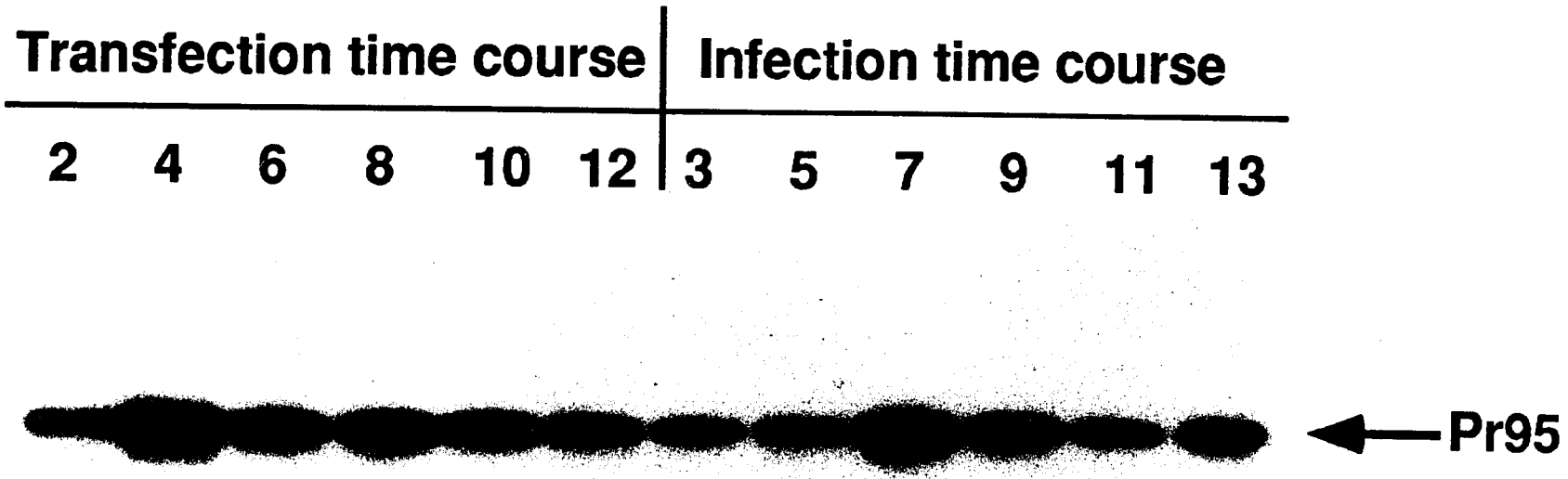


Figure 4 Optimization of the ratio of *gag* and *env* gene expression

QT6 cells were cotransfected with 2μg of pSP73.gag-pol and 0.5, 1, 2, 4, and 8μg of pSP72.env respectively as indicated by the number on top of each lane. Products of the gag gene were precipitated with anti-PrC antibody, and envelope proteins were precipitated with excess amount of antibody against the C-terminus of gp37 (see Material and Methods for details).

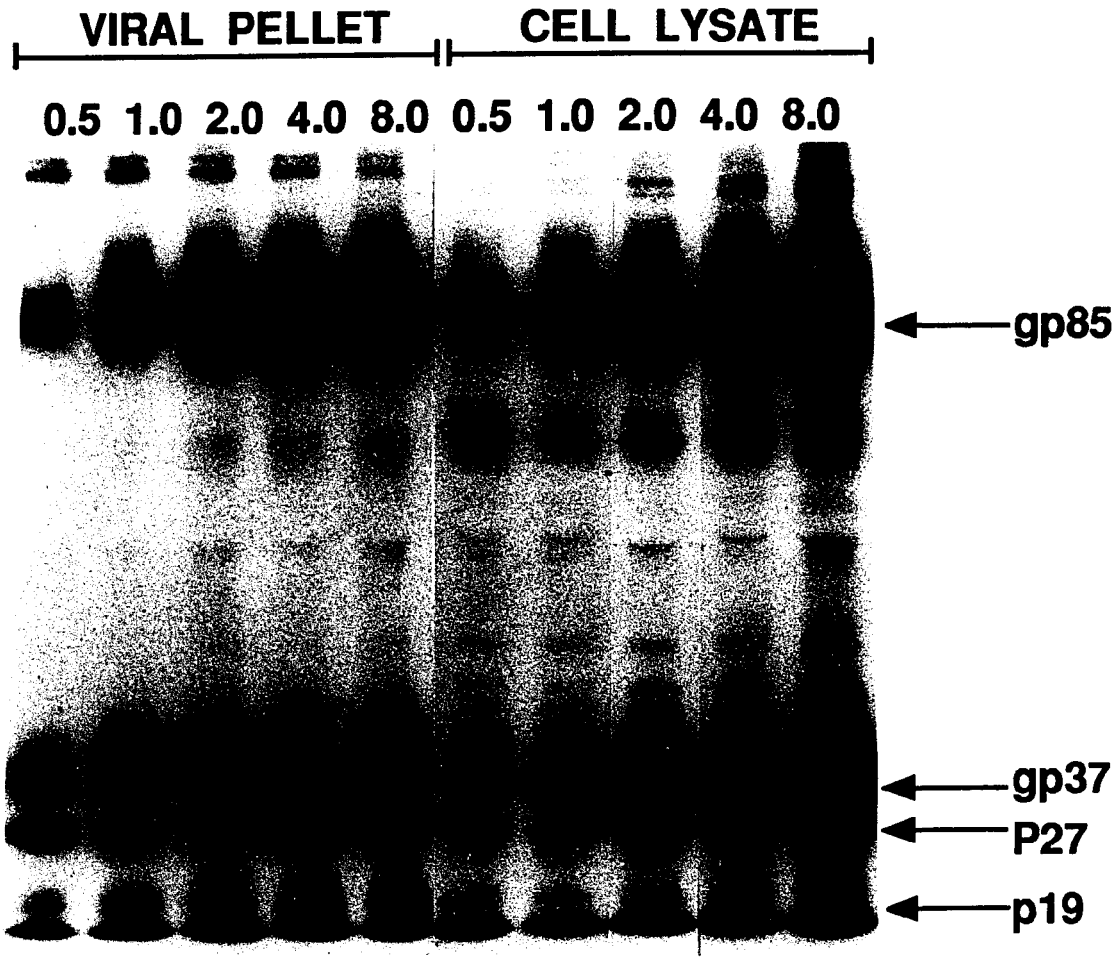
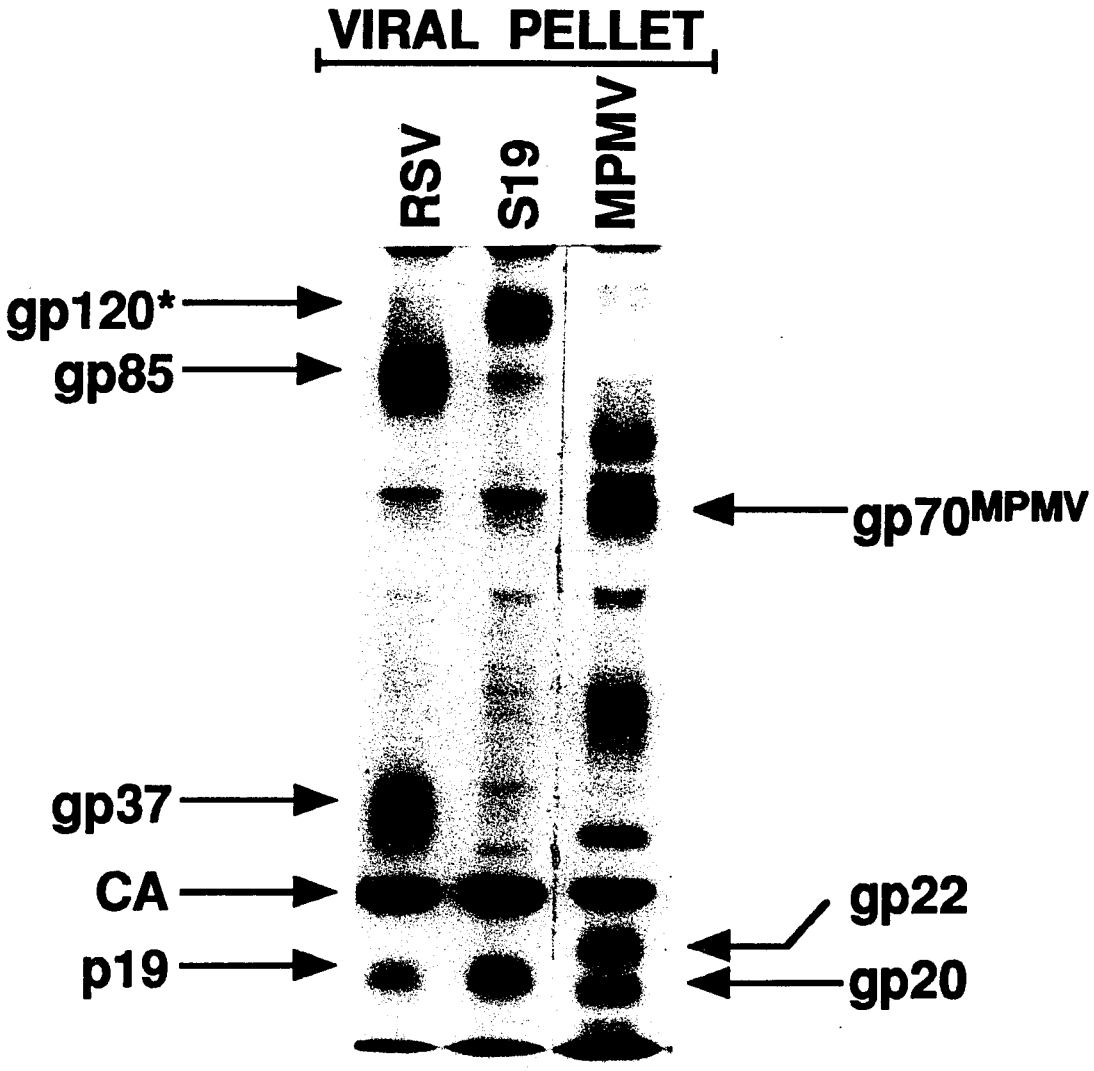

VIRAL PELLET
CELL LYSATE
0.5 1.0 2.0 4.0 8.0 0.5 1.0 2.0 4.0 8.0
gp85
gp37
P27
p19

Figure 5. Assembly assays of defective avian glycoproteins and mammalian viruses.
Viral proteins were immunoprecipitated from virus released from QT6 cells cotransfected with pSP73.gag-pol and pSP72.env or SP72.S19, a cleavage defective RSV *env* gene, as described in Materials and Methods. Cells cotransfected with the *gag-pol* and *env* genes of MPMV in vectors pSP73.MPgag-pol and pSP73.MPenv, respectively, were analysed in a similar manner. The figure shows a fluorograph of the ^{3}H-leucine labelled viral proteins immunoprecipitated from viral pellets and separated on an SDS polyacrylamide gel (10%).


VIRAL PELLET
RSV
S19
MPMV
gp120*
gp85
gp70MPMV
gp37
CA
p19
gp22
gp20

REFERENCES

1. **Altaner, C., and H. M. Temin.** 1970. Carcinogenesis by RNA sarcoma viruses. XII. A quantitative study of infection of rat cells in vitro by avian sarcoma viruses. J. virol. **12**:431-439.

2. **Brody, B. A. and E. Hunter.** 1992. Mutations within the env gene of Mason-Pfizer monkey virus: effects on protein transport and SU-TM association. J Virol **66**:3466-75.

3. **Brody, B. A., S. S. Rhee, M. A. Sommerfelt, and E. Hunter.** 1992. A viral protease-mediated cleavage of the transmembrane glycoprotein of Mason-Pfizer monkey virus can be suppressed by mutations within the matrix protein. Proc Natl Acad Sci U S A **89**:3443-7.

4. **Burton, P. R. and R. H. Himes.** 1978. Electron microscope studies of pH effects on assembly of tubulin free of associated proteins: Delineation of substructure by tannic acid staining. J Cell Biol **77**:120-33.

5. **de Giuli, C., S. Kawai, S. Dales, and H. Hanafusa.** 1975. Absence of surface projections of some noninfectious forms of RSV. Virology **66**:253-60.

6. **Dong, J. Y., D. J. W. , L. G. Perez, and E. Hunter.** 1992. Mutations within the proteolytic cleavage site of the Rous sarcoma virus glycoprotein define a requirement for dibasic residues for intracellular cleavage. J. Virol. **66**:865-74.

7. **Elroy, S. O., and B.Moss.** 1990. Cytoplasmic expression system based on constitutive synthesis of bacteriophage T7 RNA polymerase in mammalian cells. Proc Natl Acad Sci U S A. **87**:6743-7.

8. **Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen.** 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci U S A **84**:7413-7.

9. **Fuerst, T., P. L. Earl, and B. Moss.** 1987. Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. Mol. Cell. Biol. **7**:2538-2544.

10. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eucaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. **83**:8122-8126.

11. **Hunter, E.** 1988. Membrane insertion and transport of viral glycoproteins: A mutational analysis. In: Protein transfer and organelle biogenesis. (eds.) R. C. Das and P. W. Robbins Academic Press, pp. 109-158.

12. **Hunter, E., E. Hill, M. Hardwick, A. Bhown, D. E. Schwartz, and R. Tizard.** 1983. Complete sequence of the Rous sarcoma virus env gene: identification of structural and functional regions of its product. J Virol **46**:920-36.

13. **Ivanoff, L. A., J. W. Dubay, J. F. Morris, S. J. Roberts, L. Gutshall, E. J. Sternberg, E. Hunter, T. J. Matthews, and S. R. J. Petteway.** 1992. V3 loop region of the HIV-1 gp120 envelope protein is essential for virus infectivity. Virology **187**:423-32.

14. **Kelly, D. E. and F. L. Shienvold.** 1976. The desmosome: fine structural studies with freeze-fracture replication and tannic acid staining of sectioned epidermis. Cell Tissue Res **172**:309-23.

15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. (eds.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

16. **Moscovici, C., M. G. Moscovici, H. Jimenez, M. M. Lai, M. J. Hayman, and P. K. Vogt.** 1977. Continuous tissue culture cell lines derived from chemically induced tumors of Japaness quail. Cell **11**:95-103.

17. **Perez, L. G., G. L. Davis, and E. Hunter.** 1987. Mutants of the Rous sarcoma virus envelope glycoprotein that lack the transmembrane anchor and cytoplasmic domains: analysis of intracellular transport and assembly into virions. J Virol **61**:2981-8.

18. **Quintrell, N., S. H. Hughes, H. E. Varmus, and J. M. Bishop.** 1980. Structure of viral DNA and RNA in mammalian cells infected with avian sarcoma virus. J. Mol. Biol. **143**:363-393.

19. **Rhee, S. S. and E. Hunter.** 1990. A single amino acid substitution within the matrix protein of a type D retrovirus converts its morphogenesis to that of a type C retrovirus. Cell **63**:77-86.

20. **Rhee, S. S. and E. Hunter.** 1991. Amino acid substitutions within the matrix protein of type D retroviruses affect assembly, transport, and membrane association of a capsid. Embo J **10**:535-46.

21. **Smith, A. J., M. I. Cho, M. L. Hammarskjold, and D. Rekosh.** 1990. Human immunodeficiency virus type 1 Pr55gag and Pr160gag-pol expressed from a simian virus 40 late replacement vector are efficiently processed and assembled into viruslike particles. J Virol **64**:2743-50.

22. **Sommerfelt, M. A., S. R. Petteway, G. B. Dreyer, and E. Hunter.** 1992. Effect of retroviral proteinase inhibitors on Mason-Pfizer monkey virus maturation and transmemebrane glycoprotein cleavage. J. Virol. **66**:4220-427.

23. **Vogt, V. M. R. Eisenman, and H. Diggelmann.** 1975. Generation of avian myeloblastosis virus structural proteins by proteolytic cleavage of a precursor polypeptide. J. Mol. Biol. **96**:471-493.

24. **Wills, J. W., R. C. Craven, and J. A. Achacoso.** 1989. Creation and expression of myristylated forms of Rous sarcoma virus gag protein in mammalian cells. J Virol. 1989Oct. **63**:4331-43.

SUMMARY AND DISCUSSION

The main goal of this dissertation was to study biologically important regions involved in the processing, incorporation, and function of the envelope glycoprotein of an avian retrovirus using Rous sarcoma virus as a model.

Rous sarcoma virus is one the earliest discovered and best defined retroviruses. It possesses the basic genes required for replication competence (*gag*, *pol*, and *env*) and a typical life cycle that is shared by other members of the retroviral family. Indeed the study of Rous sarcoma virus and its related avian leukosis/sarcoma viruses has been a rich source of knowledge of the basic molecular biology of retroviruses. Since these viruses adopt the biological machinery of the host cell for the biosynthesis, processing, and transport of their gene products during replication, the study of retroviruses also provides insight into the protein chemistry and biology of the host cells.

The envelope glycoproteins of retroviruses play a critical role in the life cycle of a retrovirus by determining the host cell and initiating the infection process. Like the envelope glycoproteins of all the other retroviruses, the RSV *env* gene products are synthesized on membrane bound ribosomes and translocated into the cell's secretory pathway, then transported and modified in the same way as cellular membrane proteins.

I. STRUCTURAL REQUIREMENTS FOR RSV GLYCOPROTEIN FUNCTION:

A. Non-trypsin-like enzymes are responsible for envelope glycoprotein cleavage, and a dibasic amino acid residue is absolutely required for cleavage.

All known retroviruses synthesize their envelope glycoprotein as a glycosylated precursor protein which is then cleaved in a late Golgi compartment by cellular enzymes into two mature subunits, the SU protein and the TM protein. The fact that

this strategy of glycoprotein synthesis is adopted by all retroviruses suggests that it is biologically important. Although it is economical for a virus that has a limited genome size to synthesize two functionally and structurally related proteins from a single gene, when these studies were initiated little was known about the significance of the cleavage, the sequence at which the cleavage event occurred, or the cellular enzymes responsible for this proteolytic processing. The cleavage site in the RSV glycoprotein precursor was first identified by sequence comparison with that of different retroviruses (100). A stretch of basic amino acid residues with the sequence -Arg-X-Lys-Arg- was found to be highly conserved at the SU-TM junction of several retroviruses. Direct evidence that this was the target for the cellular enzyme came from mutational studies. Deletion of the four basic amino acid residues, mutant Dr1, resulted in a complete lack of proteolytic processing of the envelope precursor. Substitution of the conserved lysine residue at the third position with a glutamic acid residue, mutant G1, reduced the cleavage by approximately 80%, indicating the requirement for a basic amino acid residue at the third position of the cleavage site (100). Furthermore, the residual cleavage of the G1 mutant was inhibited by chloroquine while cleavage of the wild type was not significantly affected. This raised the possibility of the existence of a second enzyme that was sensitive to chloroquine inhibition. To further characterize the cleavage sequence and the cellular enzymes involved, additional mutations were introduced into the cleavage site of the RSV envelope glycoprotein. Since some hormone convertases (a group of cellular enzymes that have been localized in the late Golgi) are chloroquine-sensitive and cleave at dibasic sequences, it seemed possible that the enzyme responsible for the residual cleavage of mutant G1 was a hormone convertase-related enzyme (6, 43, 89, 135). Indeed the requirement of the dibasic sequence by the second enzyme was indicated by two lines of evidence, First, envelope glycoprotein cleavage was completely blocked in the double mutant, S19, in which the arginine at the first position was changed to a serine in addition to the lysine

to glutamic substitution of G1 (-Ser-Arg-Glu-Arg-). Since this mutant contained two unpaired arginine residues that could still be cleaved by exogenously added trypsin, it not only indicated that the mutated cleavage site was still accessible to proteolytic enzymes (blockage is due to sequence alteration, not a conformational change of the glycoprotein) but also proved that the enzyme responsible for intracellular cleavage was not a trypsin-like enzyme. Second, when the lysine residue at the third position was replaced by another basic amino acid residue (arginine, mutant Ar1), the percentage of the cleavage carried out by the second enzyme increased. This was shown by the inhibition of a majority of the cleavage of Ar1 in the presence of chloroquine. In contrast, a similar amount of cleavage of wild-type precursor was observed in the presence and absence of the inhibitor. This altered ratio of cleavage by the two enzymes, one responsible for the normal cleavage and chloroquine resistant and another recognizing a dibasic sequence and chloroquine sensitive, can be explained as a combination of two factors. The lysine at the third position is highly preferred by the major enzyme responsible for cleavage; changing it to an arginine reduced the efficiency of cleavage by this enzyme and left the majority of the envelope glycoprotein precursor to be cleaved by a second enzyme that was sensitive to chloroquine inhibition. The fact that Ar1 is cleaved much more efficiently than the G1 mutant is probably due to the fact that Ar1 (-Arg-Arg-Arg-Arg-) contains multiple dibasic sequences while G1 (-Arg-Arg-Glu-Arg-) contains only one dibasic sequence that can be recognized by the less efficient and chloroquine sensitive enzyme. Based on these data, we conclude that the cleavage sequence in the envelope glycoprotein is recognized by at least two enzymes. The first prefers a lysine at the third position and is highly efficient and chloroquine resistant, while the second recognizes dibasic sequences and is chloroquine sensitive. These enzymes are non-trypsin like enzymes.

Given the degree of conservation of the cleavage sequences in the envelope protein precursor of all retroviruses known to date, it is likely that all retroviruses

utilize an enzyme or enzymes common to the cells they infect for their glycoprotein cleavage. Although the ultimate identification of the enzyme(s) will rely on their isolation, mutational analyses do provide important information on the properties and characteristics of the enzyme. This information is in turn essential for the isolation of the cellular enzymes. Indeed partial purification of the enzyme(s) has been achieved by identifying the cell fractions that have the highest activity for cleaving a synthetic peptide containing the cleavage site of the envelope glycoprotein precursor (35). This enzymatic activity is strongly inhibited by Pepstatin, but weakly inhibited by soybean trypsin inhibitor. This *in vitro* evidence supports the concept that the enzyme(s) is not a trypsin-like enzyme.

Based on the profile of the enzyme, one of the candidates is a group of prohormone-processing enzymes (prohormone convertase) analogous to the KEX2 gene products that were first discovered in the yeast *Saccharomyces cerevisiae.* The KEX2 gene products cleave Arg-Arg or Lys-Arg dibasic sequences, which are also found in retroviral envelope glycoproteins, in prohormones such as pro-alpha factor or pro-killer toxin (43, 88). When expressed in mammalian cells these gene products demonstrate an identical specificity to some of the mammalian hormone convertases and can correctly process prohormones into the mature products (19, 135). In more recent studies, several genes in mammalian cells (PC2, PC3 and *fur*) that encode mammalian counterparts of the KEX2 products have been identified. The products of these genes share significant structural similarity with the catalytic domain of KEX2 and can accurately cleave prohormone in mammalian cells (6, 88, 126, 135, 139). These membrane-bound, calcium-dependent enzymes appear to be widely distributed, at least in mammalian cells (63). Given the similarities of the cleavage sites of prohormones and the viral glycoprotein and the parasitic nature of the viruses, it is likely that retroviruses utilize a member of this group of hormone convertases for their envelope glycoprotein processing.

B. Envelope glycoprotein cleavage is a prerequisite for viral infectivity but not for incorporation of the glycoprotein.

The conserved cleavage sequence and the commonly adopted precursor-cleavage strategy retroviruses employ pointed to an important role for this process in the replication cycle of these viruses. The function of the cleavage was analyzed in this thesis by replacing the wild type *env* gene with the cleavage site mutant genes in a provirus vector of RSV (BH-RCAN). The infectivity of these mutants was analyzed by reverse transcriptase activity and focus assays. We were able to demonstrate that the mutants which have a non-cleavable *env* product were not infectious (Dr1 and S19) while mutant G1 was partially infectious. Ar1 was as infectious as the wild type, although the cleavage of Ar1 is carried out by the chloroquine-sensitive enzyme. The infectivities of these different mutants corresponded closely to the amount of the cleaved glycoprotein found in cells expressing the mutant. This indicates that cleavage of envelope glycoprotein is not only a prerequisite for infectivity but is also the restricting factor for the replication of the mutants.

Although proteolytic cleavage of the envelope glycoprotein is required for its function, the basis for this is not known. Since the N-terminus of the TM protein is generated by the proteolytic cleavage, it is likely that the cleaving event is required to free up the fusion peptide (see Introduction), and triggers a conformational change in the envelope glycoprotein to sequester the activated fusion sequence. During infection of a new host cell, the fusion peptide is then exposed through the dissociation of the multimer complex or through a conformational change within the glycoprotein that is induced by the binding of the envelope glycoprotein complex to a cellular receptor on a target cell. The fact that mutant S19 glycoproteins can be activated on the cell surface by exogenously added trypsin supports the notion that this cleavage/fusion peptide region is flexible even after the maturation of the protein. This flexibility is likely to be required to expose the fusion peptide following a conformational change in the SU protein. The interaction of the fusion peptide with the cell membrane catalyzes the

fusion of the viral and cellular membranes (46, 149) and the introduction of the viral genetic material into the host cells. A hypothesized model for the conformation change in the glycoprotein and activation of the fusion peptide is represented in Figure 9.

C. Proteolytic processing of the *env* gene precursor is not required for glycoprotein incorporation into virions.

Glycoprotein cleavage was shown to be a prerequisite for viral infectivity, but the nature of the defect was not known. There could be two possible explanations for the lack of infectivity of mutant S19 and the partial infectivity of G1 mutant: either the uncleaved glycoproteins could not be incorporated into virus or they were incorporated but not functional. This was determined initially by protein composition analysis of the released mutant virions by metabolically labelling the protein . Since G1 is partially infectious, it allowed the mutant to propagate slowly until most of the cells were infected. Virions collected from such cultures contained both cleaved and uncleaved glycoproteins in a similar ratio to that in the cells. This indicated that uncleaved glycoproteins could be incorporated as efficiently as the cleaved envelope proteins. Using a vaccinia-T7 RNA polymerase expression system, it was shown that uncleaved glycoprotein could be incorporated into virions in the absence of the cleaved product. This eliminated the possibility that the uncleaved glycoprotein found in G1 virions might have been passively incorporated into virions as part of the multimer complex.

Since the glycoprotein of mutant S19 could be cleaved by exogenously added trypsin, we further questioned if virions bearing the mutated envelope glycoprotein could be activated by trypsin treatment. Using a hygromycin resistance gene as a marker, we were able to show that about 10% of the infectivity was restored by trypsin treatment in mutant S19, but not in Dr1 in which the cleavage site was completely deleted. The low percentage of trypsin-treated S19 mutant was probably due to the low specificity of the enzyme that may induce damage to the glycoprotein outside the cleavage site.

It is interesting that about 20% of the cleaved glycoprotein of G1 mutant conferred infectivity to the G1 virions at a lower efficiency. This indicates that a small percentage of functional glycoproteins in the virion is enough to initiate a viral infection. This is similar to the situation of HIV in which the SU protein (gp120) can easily dissociate from the TM protein due to the lack of covalent interactions between the two subunits (62, 84, 90, 120). The biological significance of this phenomenon is not yet clear. It is possible that in addition to the regulatory mechanism of viral replication, this may also play a role in the long latency and slow progression of the HIV infection similar to that of G1 mutant. The second possibility is that the loosely associated SU protein serves as a "lizard tail" for the virus to escape from a neutralizing response. When the SU protein is bound by a neutralizing antibody, it will dissociate from the TM protein and leave the virus free of antibody. Even viruses with only a limited number of functional glycoproteins are able to infect a new host cell. This may be one of the reasons that the neutralizing response is not effective in eliminating the infection in an AIDS patient, despite the high levels of neutralizing antibodies found in their serum (7, 21, 110, 122, 124).

The results of the infectivity analyses are consistent with the hypothesis that the proteolytic cleavage of the envelope glycoprotein precursor frees up the fusion peptide and induces a conformation change to sequester the activated fusion peptide, so that it will not induce fusion of internal membranes of the cell during transport. Studies in HIV also show the cleavage of gp160 is required for syncitium formation, a generally accepted assay for envelope glycoprotein mediated fusion. The conformation change is probably subtle or localized, so that the overall structure of the glycoprotein is not affected. This is suggested by the incorporation of the uncleaved glycoprotein and the restored infectivity of S19 by trypsin treatment.

II. REQUIREMENTS FOR ENVELOPE GLYCOPROTEIN INCORPORATION

The envelope glycoproteins of retroviruses are incorporated into their virions at the plasma membrane of the host cells. For type-C retroviruses, such as RSV, virion assembly and glycoprotein incorporation occur simultaneously during the viral budding process, while for type-D viruses, virions are preassembled in the cytosol and then are transported to the cell membrane to incorporate the glycoprotein. Regardless of the differences in the morphology of these viruses, glycoprotein incorporation is a specific process, since the bulk of cellular membrane proteins is excluded from the budding virions. It has been postulated that a specific interaction between the cytoplasmic domain of the glycoprotein and the viral capsid is responsible for this specificity. This is supported by early studies in Semliki Forest virus where a receptor-ligand type interaction between the cytoplasmic domains of the glycoprotein and the capsid protein might be required for glycoprotein incorporation and by the chemical cross-linking of gp37(TM) and the MA protein of RSV (51). Recent studies also have shown that truncation of the cytoplasmic domain of M-PMV envelope glycoprotein diminished the glycoprotein incorporation and resulted in virions that were non-infectious. This result indicated that the cytoplasmic domain of the M-PMV envelope glycoprotein is important for incorporation (20). In contrast, the infectivity of a "tail-less" mutant of RSV, in which the cytoplasmic tail of gp37 was deleted by introducing a premature stop codon, suggested that this domain is not essential for envelope glycoprotein incorporation in RSV (99).

D/S only (2 single returns)

A. RSV has a relaxed specificity for envelope incorporation.

In this dissertation, the incorporation of two chimeric HA-*env* proteins was compared to determine the degree of specificity of envelope glycoprotein incorporation in RSV. In the first chimeric protein, the signal peptide sequence was fused to the entire structural sequence of influenza HA protein, so that cleavage of the signal

sequence released an authentic HA protein. In the second chimeric protein, in addition to the signal peptide fusion, the transmembrane and cytoplasmic domains of the HA protein were replaced by their counterparts in the RSV envelope glycoprotein. When expressed in an SV40-COS-1 system, both chimeric HAs were processed normally and retained hemagglutination activity. The incorporation of the two proteins into RSV was analyzed in a vaccinia-phage T7 expression system. When either of the HA proteins was co-expressed with the RSV *gag* gene products, it was incorporated efficiently into RSV virions. Somewhat to our surprise, the incorporation efficiency of the two proteins was comparable. This indicated that the syngenic transmembrane and cytoplasmic domains of the RSV did not enhance the incorporation of the HA protein. RSV thus appears to have a relaxed specificity for envelope glycoprotein incorporation, consistent with the observation that a cytoplasmic-domain deleted mutant is infectious.

B. Foreign glycoproteins can confer infectivity to a hybrid RSV virion.

The function of the chimeric HAs was analyzed by replacing the *env* gene with the gene that coded for either of the chimeric HA proteins in a RSV proviral vector that contained a hygromycin resistance gene as a marker. Infectivity of the hybrid virions bearing either of chimeric HAs was determined by their ability to transfer hygromycin resistance into infected cells. The results of such experiments show that both proteins conferred infectivity to the hybrid virions with similar efficiency. Moreover, these virions had a host range that extended into mammalian cells. This indicates that the cytoplasmic domain and anchor regions of the RSV glycoprotein do not provide any subtle advantage to the virions for incorporation and infectivity. Moreover, it demonstrates that a non-retroviral glycoprotein, the influenza HA protein, can be functional in an RSV virion in the absence of any RSV glycoprotein. Similar results have recently been reported for MuLV. When the VSV-G protein, or the envelope glycoproteins of HTLV-II or GaLV, were co-expressed with an MuLV vector that had the *env* gene deleted, the pseudotyped viruses demonstrated an extended host range

(38, 85, 155). Since there are no consensus sequences discernible in the cytoplasmic domains of these different viruses, it is difficult to reconcile these results with the concept that specific interactions between the envelope glycoprotein and the protein capsid are required for the incorporation of envelope glycoproteins into virions.

Although it can not be ruled out that minor interactions may occur during envelope glycoprotein incorporation, it is unlikely that any essential protein-protein interaction is responsible for the specific selection of viral glycoproteins over cellular membrane during the budding process. One possibility for the specificity of envelope glycoprotein incorporation is that normal cellular membrane proteins are excluded from the virion due to their association with the cellular protein network. In contrast any truly mobile membrane proteins that lack interactions with the cellular matrix proteins could be incorporated into virions. As hypothetically diagrammed in Figure 10, the formation of viral capsids would be expected to push away the cytoskeleton and the cellular membrane proteins that are associated with it, leaving an area in the membrane devoid of protein where mobile viral glycoproteins could move in and thus be concentrated. This scenario is supported by the experimental result that human CD4 molecule expressed in avian cells can be incorporated into RSV virions. In these cells the human protein may not have an interaction with underlying cellular proteins that is strong enough to prevent the protein from being incorporated into virions (159). Another line of evidence that supports this hypothesis is our observation that HA protein is incorporated into RSV virions much less efficiently in the absence of neuraminidase, an enzyme that cleaves off the sialic acid residues from polysaccharides. This suggests that binding to the sialic acid residues on the cell surface by the HA can prevent the incorporation of the membrane protein that normally would be efficiently incorporated into virions. Although this inhibition of incorporation was incomplete, it does suggest that interaction with other membrane proteins on the cell surface can inhibit the incorporation of a membrane protein into

virions. Since only the extracellular regions of HA can bind to the sialic acid residues on the cell surface, the anchor region of the HA protein remains mobile within the membrane where incorporation initiates. It is possible that interactions between the cytoplasmic domain with the cell matrix that restrict the movement of the anchor region, as in the case of most cellular membrane proteins, may be more efficient in preventing the cellular membrane proteins from being incorporated into virions.

C. Viral glycoproteins of diverse origins can be incorporated into RSV.

To further investigate the specificity of envelope incorporation of RSV and the possible mechanism for the selective envelope glycoprotein incorporation, a series of envelope glycoproteins with significantly different cytoplasmic domains were tested for their incorporation into RSV. These included the envelope glycoproteins of VSV (G-protein), a non-retroviral glycoprotein with shorter cytoplasmic domain; MuLV, a type-C retrovirus with a similarly-sized cytoplasmic domain; M-PMV, a type-D primate virus in which a specific incorporation signal may be required for it incorporation; HIV, which has a long cytoplasmic domain of 150 amino acid residues that is predicted to contain additional interaction with the cell membrane; and NA, a type-II membrane glycoprotein which has its N-terminus inside the cytoplasm. The incorporation of these glycoproteins was analyzed using the vaccinia phage-T7 complementation system. Protein composition analyses of virions collected from cells in which the RSV *gag* gene products and each of the different glycoprotein genes were co-expressed showed that all of the glycoproteins, except that of M-PMV, could be incorporated into RSV virions (data not shown). These results further support the hypothesis that mobile membrane proteins that lack interactions with the cellular protein network can be incorporated into virions, with the exception of the M-PMV envelope glycoprotein.

Although the exact reason for the failure to incorporate the M-PMV envelope glycoprotein into RSV is not clear, it is possible that additional factors beside the free

mobility may influence incorporation of membrane glycoproteins into virions. Indeed the results of mutational analysis of the transmembrane domain of the RSV envelope glycoprotein have shown that certain protein conformation may be required for the membrane protein to be incorporation competent. The incorporation is greatly reduced when the two conserved cysteine residues (164 and 167) within the transmembrane domain of RSV envelope glycoprotein are converted to glycine residues (86). It also renders the glycoprotein incorporation incompetent when two amino acid residues within the transmembrane domain, including one of the conserved cysteine and the adjacent proline, are deleted (26). The fact that these mutated envelope glycoproteins are transported normally to the cell membrane and are stable enough that would allow their incorporation into virions indicates that the block is at the level of incorporation.

Given the results discussed above, it can be concluded that the nature of envelope glycoprotein incorporation of retroviruses is complicated. It is difficult to reconcile that a single mechanism is responsible for the specific incorporation of a virus envelope glycoprotein during viral assembly. It is likely that at least two criteria have to be met for the incorporation of a membrane protein into assembling virions: (1) a truly mobile membrane protein that lacks interactions with cellular protein network, (2) a protein conformation that is competent for incorporation. Other factors may also play a role in determining the incorporation of the membrane protein, such as the concentration of the protein in the cell membrane. Although it is not clear what kind of protein conformation is required for incorporation, it is unlikely to be a linear peptide sequence, since there are no homologous sequences among the envelope glycoproteins which can be incorporated into RSV virions. Even in some of the viruses in which the cytoplasmic region is required for incorporation, other envelope glycoproteins with non-homologous cytoplasmic domains can be incorporated. For example, in HIV progressive truncations of the cytoplasmic domain of gp41 greatly reduced or abolished the efficiency of the glycoprotein incorporation and the infectivity

of the virus (34, 45), even though the envelope glycoprotein of SIV can efficiently complement an *env* minus genome of HIV for infectivity (67). Since there are no significantly homologous sequences in the cytoplasmic regions of these two envelope glycoproteins, it is unlikely the decreased efficiency of envelope glycoprotein incorporation is due to the removal of a specific incorporating signal in the cytoplasmic domain by the truncation. It is possible that the truncation of the cytoplasmic domain affected the overall structure of the envelope glycoprotein which is important for incorporation. This is consistent with the observations that truncation of the cytoplasmic domain can result in the increased fusogenicity (a function of the external region of the envelope glycoprotein complex) of the envelope glycoproteins of several viruses, including HIV, SIV, and M-PMV (20, 93). At least in retroviruses, it is unlikely that a receptor-ligand type interaction is required for envelope glycoprotein incorporation, as was proposed in Semliki Forest virus; rather, a general tertiary conformation that is acceptable for the lipid-protein combined environment of the virion is necessary. Although the importance of the transmembrane domain has only been demonstrated for the envelope glycoprotein of RSV, it is unlikely that the cytoplasmic domain is the only region that affects envelope glycoprotein incorporation, even for those viruses in which the cytoplasmic domain is required for envelope glycoprotein incorporation. This is because a lipid membrane must be incorporated into virions independent of the glycoprotein, thus the incorporation of the envelope glycoprotein must be accepted by the lipid-protein combined environment in the assembling virion. Any glycoprotein that has a conformation that is unfavorable in such an environment or is unstable in the membrane is unlikely to be incorporated.

Little is known about the conformation of the anchoring region (the transmembrane and cytoplasmic domains) of an envelope glycoprotein other than the predicated structure based on the protein sequence. We can only postulate that the required "shape" of the cytoplasmic domain may be different in each virus. Some may

be more general, or more tolerable so that modifications of the cytoplasmic domain may not affect the envelope glycoprotein incorporation into virus. In this group of tolerant viruses, different envelope proteins, including certain cellular proteins that resemble the necessary "shape", such as CD4, may be incorporated into such virions. Other viruses may be more specific, so that modifications of the cytoplasmic domain renders the envelope protein unacceptable for incorporation. In such a case only a few envelope glycoproteins that closely resemble the "shape" can be incorporated. It would be interesting to see if the incorporation-incompetent mutants such as truncated cytoplasmic domain mutants of HIV or M-PMV can be incorporated into a more tolerant virus, such as RSV, and if they can confer infectivity to the pseudotype virions. This will provide information on the nature of the defect caused by the truncation.

The relaxed specificity for envelope glycoprotein incorporation may be a useful feature of RSV. Although it is not known what limitations it may have, it may be possible to specifically target viruses into different host cells by replacing the envelope glycoproteins of the virion. It may provide a convenient way to deliver a gene of interest into targeted tissue with vectors established for the purpose of *in vivo* study of a particular gene function or for genetic therapy. Incorporation of the envelope protein of a pathogenic virus, such as HIV, into a benign retrovirus, may provide an alternative way to produce a replication competent vaccine that can be easily propagated in cell culture and would be safer to use.

The functions of retroviral envelope glycoprotein are complicated. While a great amount of information has been accumulated through individual studies, much remains a mystery. It can be confidently predicted that with a better understanding of this unique group of viruses, the Retroviridae, we will not only be able to eliminate the diseases caused by these pathogens but we will also obtain a better understanding of the molecular biology, genetics, and biochemistry of other living organisms, and thus benefit mankind.

Figure 9. Hypothesized representation of the conformational changes induced by glycoprotein precursor cleavage and receptor binding.
The light gray area represents the lipid bilayer. The cartoon shows the hypothetical conformational changes in the envelope glycoprotein induced by proteolytic cleavage, oligomerization, and receptor binding.

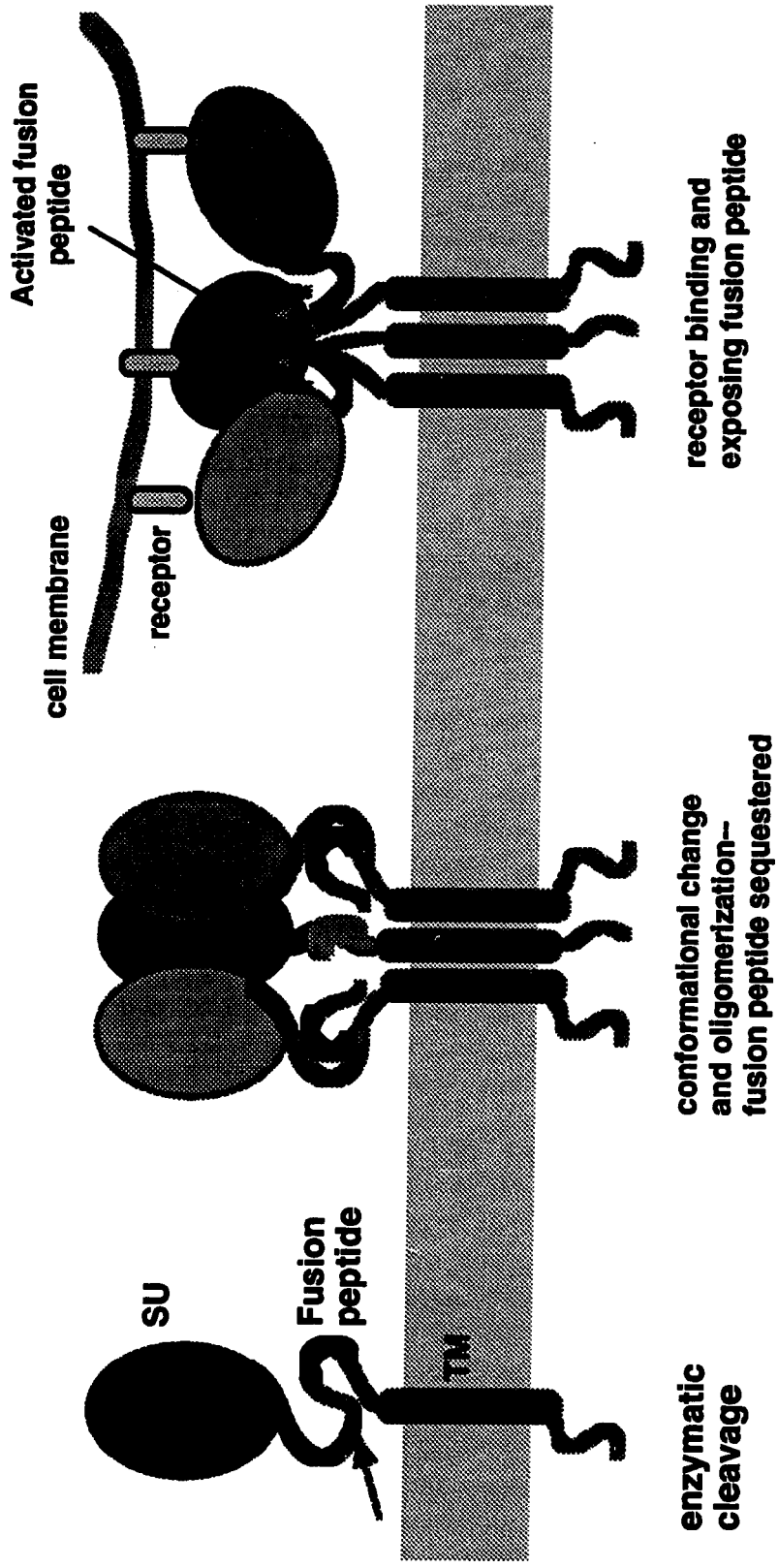
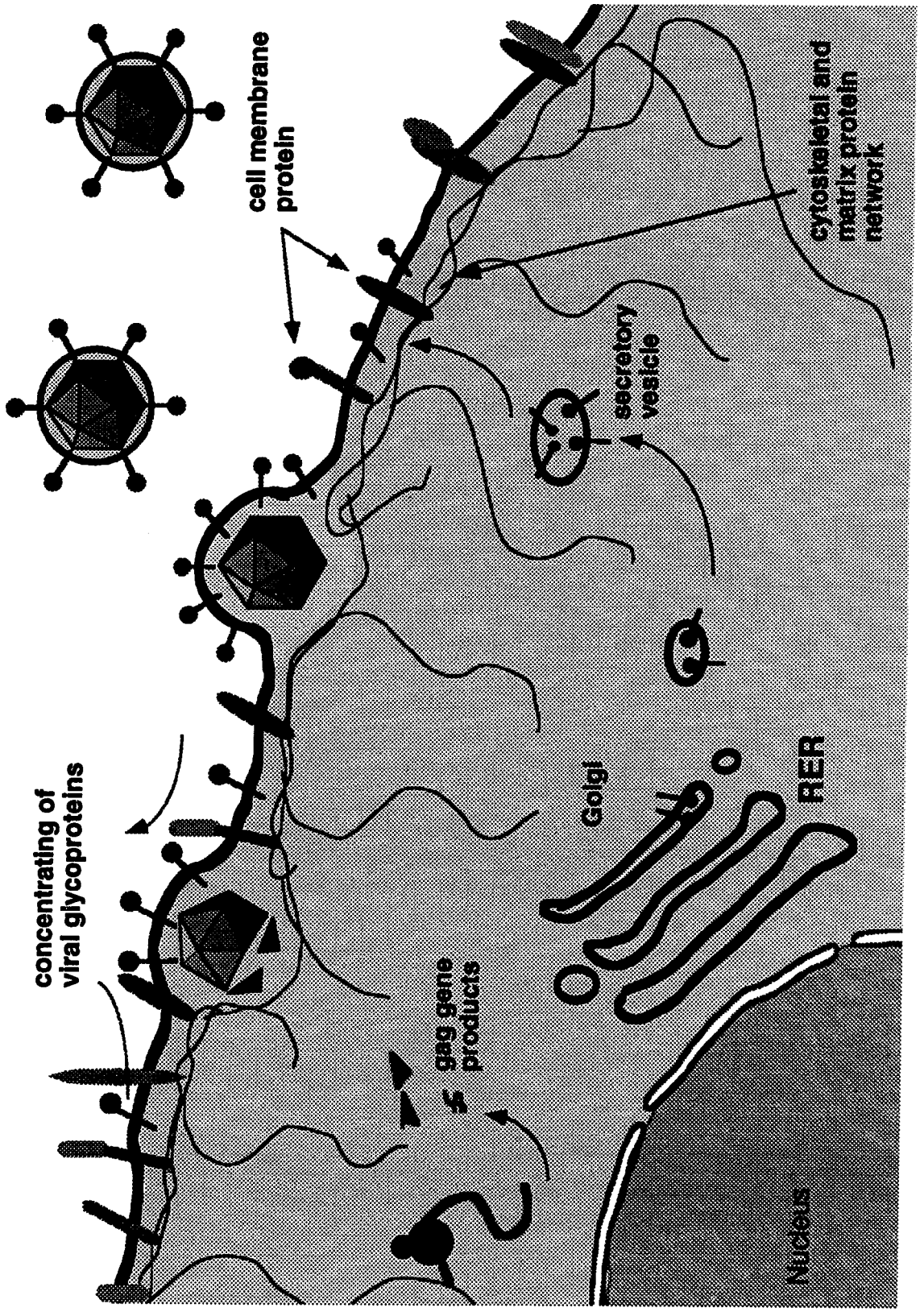

SU
Fusion peptide
TM
enzymatic cleavage
conformational change and oligomerization--fusion peptide sequestered
cell membrane
receptor
Activated fusion peptide
receptor binding and exposing fusion peptide

Figure 10. A hypothesized selection mechanism for viral glycoprotein incorporation during viral budding process. During virus assembly, the formation of the protein capsid pushes away the cytoskeletal and matrix proteins along with the cellular membrane proteins that are associated with the protein network. Mobile viral glycoproteins move into the space left by the cellular proteins in the membrane and form patches. During viral budding only viral glycoproteins are incorporated while the cellular membrane proteins are excluded due to the interactions with the cellular protein network.


concentrating of viral glycoproteins
cell membrane protein
gag gene products
Golgi
secretory vesicle
cytoskeletal and matrix protein network
RER
Nucleus

GENERAL REFERENCES

1. **Anderson, K. B. and B. A. Nexo.** 1983. Entry of murine retrovirus into mouse fibroblasts. **125**:85-98.

2. **Argos, P.** 1989. A possible homology between immunodeficiency virus p24 core protein and picornaviral VP2 coat protein: prediction of HIV p24 antigenic sites. Embo J **8**:779-85.

3. **Ashorn, P., T. J. McQuade, S. Thaisrivongs, A. G. Tomasselli, W. G. Tarpley, and B. Moss.** 1990. An inhibitor of the protease blocks maturation of human and simian immunodeficiency viruses and spread of infection. Proc Natl Acad Sci U S A **87**:7472-6.

4. **Barbey, M. C., T. N. Oeltmann, K. M. Edwards, and P. F. Wright.** 1987. Role of respiratory tract proteases in infectivity of influenza A virus. J Infect Dis **155**:667-72.

5. **Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Brun-Vezinet, C. Rouzioux, W. Rozenbaum, and L. Montagnier.** 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science **220**:868-871.

6. **Bathurst, I. C., S. O. Brennan, R. W. Carrell, L. S. Cousens, A. J. Brake, and P. J. Barr.** 1987. Yeast KEX2 protease has the properties of a human proalbumin converting enzyme. Science **235**:348-50.

7. **Beilke, M. A., H. Minagawa, G. Stone, M. M. Leon, and C. J. J. Gibbs.** 1991. Neutralizing antibody responses in patients with AIDS with neurologic complications. J Lab Clin Med **118**:585-8.

8. **Bender, W., Y.-H. Chien, S. Chattopadhyay, P. K. Vogt, M. B. Gardner, and N. Davison.** 1978. High-molecular-weight RNAs of AKR, NZB, and wild mouse viruses and avian reticuloendotheliosis virus all have similar dimer structures. J Virol **25**:888-96.

9. **Berberich, S. L., M. Macias, L. Zhang, L. P. Turek, and C. M. Stoltzfus.** 1990. Comparison of Rous sarcoma virus RNA processing in chicken and mouse fibroblasts: evidence for double-spliced RNA in nonpermissive mouse cells `published erratum appears in J Virol 1990 Dec;64(12):6360. J Virol **64**:4313-20.

10. **Berhard, W.** 1960. The detection and study of tumor viruses with the electron microscope. Cancer Res **20**:712-727.

11. **Bernhard, W., T. A. Bonar, D. Beard, and J. W. Beard.** 1958. Ultrastructure of viruses of myeloblastosis and erythroblastosis isolated from plasma of leukemic chickens. Proc. Soc. Exp. Biol. Med. **97**:48-52.

12. **Blough, H. A., R. Pauwels, C. E. De Clercq, J. Cogniaux, G. S. Sprecher, and L. Thiry.** 1986. Glycosylation inhibitors block the expression of LAV/HTLV-III (HIV) glycoproteins. Biochem Biophys Res Commun **141**:33-8.

13. **Bolognesi, D. P., H. Bauer, H. Gelderblom, and H. Gudrun,** 1972. Polypeptides of avian RNA tumer virus. IV. Components of the viral envelope. Virology **47**:551-566.

14. **Bolognesi, D. P., H. Gelderblom, H. Bauser, E. Moling, and H. Gudrum.** 1972. Polypeptides of avian RNA tumor viruses. V. Analysis of the virus core. Virology **47**:567-578.

15. **Bolognesi, D. P., R. C. Montelaro, H. Frank, and W. Schafer.** 1978. Assembly of Type C onocornaviruses: A model. Science **199**:183-186.

16. **Bova, C. A., J. C. Olsen, and R. Swanstrom.** 1986. *Env* genes of avian retroviruses: nucleotide sequence and molecular recombinants define host range determinants. Virology **152**:343-54.

17. **Bova, C. A., J. C. Olsen, and R. Swanstrom.** 1988. The avian retrovirus env gene family: molecular analysis of host range and antigenic variants. J Virol **62**:75-83.

18. **Bradac, J. and E. Hunter.** 1986. Polypeptides of Mason-Pfizer Monkey virus. II. Synthesis and processing of the env gene products. Virology **150**:491-502.

19. **Brennan, S. O., R. J. Peach, and I. C. Bathurst.** 1990. Specificity of yeast KEX2 protease for variant human proalbumins is identical to the in vivo specificity of the hepatic proalbumin convertase. J Biol Chem **265**:21494-7.

20. **Brody, B. A., S. S. Rhee, and E. Hunter.** 1992. Post assembly cleavage of a retroviral glycoprotein cytoplasmic domain removes a necessary incorporation signal and activates fusion activity. submitted to Cell

21. **Casareale, D., S. Dewhurst, J. Sonnabend, F. Sinangil, D. T. Purtilo, and D. J. Volsky.** 1984. Prevalence of AIDS-associated retrovirus and antibodies among male homosexuals at risk for AIDS in Greenwich Village. Aids Res **1**:407-21.

22. **Chien, Y. H., R. P. Junghans, and N. Davison.** 1980. Electron microscopic analysis of the structure of RNA tumor virus nucleic acids. In Molecular Biology of RNA Tumer Viruses (ed. J.R. Stephenson) 395-446.

23. **Coffin, J. M., T. C. Hageman, A. M. Maxam, and W. A. Haseltine.** 1978. Nucleotide sequence relationships between the genomes of an endogenous and an exogenous avian tumor virus. J Virol **28**:972-91.

24. **Collett, M. S., E. Erikson, A. F. Purchio, J. S. Brugge, and R. L. Erikson.** 1979. Evidence that the avian sarcoma virus transforming gene product is a cyclic AMP-independent protein kinase. Proc Natl Acad Sci U S A **76**:6260-4.

25. **Dalgleish, A. G., P. C. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature **312**:763-767.

26. **Davis, G., K. S. Shaw, J. Dong, and E. Hunter**. 1992. Mutational analysis of the membrane anchor domain of the RSV TM protein. In preparation.

27. **de Chasseval, R. and J. P. de Villartay**. 1992. High level transient gene expression in human lymphoid cells by SV40 large T antigen boost. Nucleic Acids Res **20**:245-50.

28. **DeLarco, J. and G. J. Todaro**. 1976. Membrane receptors for murine leukemia viruses: characterization using the prurified viral envelope glycoprotein, gp71. Cell **8**:365-371.

29. **Delius, H., H. Westphal, and A. N.** 1973. Length measurements of RNA synthesized *in vitro* by *Escherichia coli* RNA polymerase. J. Mol. Biol. **74**:677-687.

30. **Doms, R. W. and A. Helenius**. 1986. Quaternary structure of influenza virus hemagglutinin after acid treatment. J Virol **60**:833-9.

31. **Dorner, A. J. and J. M. Coffin**. 1986. Determinants for receptor interaction and cell killing on the avian retrovirus glycoprotein gp85. Cell **45**:365-74.

32. **Dorner, A. J., J. P. Stoye, and J. M. Coffin**. 1985. Molecular basis of host range variation in avian retroviruses. J Virol **53**:32-9.

33. **Dorner, A. J., J. P. Stoye, and J. M. Coffin**. 1988. The avian retrovirus env gene family: molecular analysis of host range and antigenic variants. J Virol **62**:75-83.

34. **Dubay, J. W., S. J. Roberts, B. H. Hahn, and E. Hunter**. 1992. Truncation of the HIV-1 Transmembrane Glycoprotein Cytoplasmic Domain Blocks Virus Infectivity. Submitted

35. **Einfeld, D., L. Heck, and E. Hunter**. 1992. In preparation.

36. **Einfeld, D. and E. Hunter**. 1988. Oligomeric structure of a prototype retrovirus glycoprotein. Proc Natl Acad Sci U S A **85**:8688-8692.

37. **Elder, J. H., J. S. McGee, and S. Alexander**. 1986. Carbohydrate side chains of Rauscher leukemia virus envelope glycoproteins are not required to elicit a neutralizing antibody response. J Virol **57**:340-2.

38. **Emi, N., T. Friedmann, and J. K. Yee**. 1991. Pseudotype formation of murine leukemia virus with the G protein of vesicular stomatitis virus. J. Virol. **65**:1202-7.

39. **Erikson, R. L., J. S. Brugge, E. Erikson, and M. S. Collett**. 1980. Avian sarcoma virus-transforming protein, pp60src shows protein kinase activity specific for tyrosine. Nature **285**:167-9.

40. **Erikson, R. L., M. S. Collett, E. Erikson, and A. F. Purchio**. 1979. Studies on the structure and function of the avian sarcoma virus transforming gene product. Hamatol Bluttransfus **23**:261-9.

41. **Fuerst, T., P. L. Earl, and B. Moss**. 1987. Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. Mol. Cell. Biol. **7**:2538-2544.

42. **Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss.** 1986. Eucaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. **83**:8122-8126.

43. **Fuller, R. S., A. Brake, and J. Thorner.** 1989. Yeast prohormone processing enzyme (KEX2 gene product) is a Ca2+-dependent serine protease. Proc Natl Acad Sci U S A **86**:1434-8.

44. **Furuichi, Y., A. J. Shatkin, E. Stavnezer, and J. M. Bishop.** 1975. Blocked, methylated 5'-turminus of the avian sarcoma virus RNA. Nature **257**:618-620.

45. **Gabuzda, D. H., A. Lever, E. Terwilliger, and J. Sodroski.** 1992. Effects of deletions in the cytoplasmic domain on biological functions of human immunodeficiency virus type 1 envelope glycoproteins. J Virol. 1992 Jun **66**:3306-15.

46. **Gallaher, W. R.** 1987. Detection of a fusion peptide sequence in the transmembrane protein of human immunodeficiency virus. Cell **50**:327-328.

47. **Gallaher, W. R., J. M. Ball, R. F. Garry, M. C. Griffin, and R. C. Montelaro.** 1989. A general model for the transmembrane proteins of HIV and other retroviruses. AIDS res and Hum retroviruses **5**:431-440.

48. **Gallione, C. J. and J. K. Rose.** 1983. Nucleotide sequence of a cDNA clone encoding the entire glycoprotein from the New Jersey serotype of vesicular stomatitis virus. J. Virol. **46**:162-169.

49. **Gallo, R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Oleske, B. Safai, G. White, P. Foster, and P. D. Markham.** 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science **224**:500-503.

50. **Garry, R. F.** 1990. Extensive antigenic mimicry by retrovirus capsid proteins `letter. Aids Res Hum Retroviruses **6**:1361-2.

51. **Gebhardt, A., J. V. Bosch, A. Ziemiecki, and R. R. Friis.** 1984. Rous sarcoma virus p19 and gp35 can be chemically crosslinked to high molecular weight complexes: An insight into viral association. J. Mol. Biol. **174**:297-317.

52. **Gelderblom, H. R., E. H. Hausmann, M. Ozel, G. Pauli, and M. A. Koch.** 1987. Fine structure of human immunodeficiency virus (HIV) and immunolocalization of structural proteins. Virology **156**:171-6.

53. **Gething, M.-J., J. Bye, J. Skehel, and M. Waterfield.** 1980. Cloning and DNA sequence of double-stranded copies of haemagglutinin genes from H2 and H3 strains elucidates antigenic shift and drift in human influenza virus. Nature **287**:301-306.

54. **Gething, M.-J., K. McCammon, and J. Sambrook.** 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. Cell **46**:939-950.

55. **Geyer, H., C. Holschbach, G. Hunsmann, and J. Schneider.** 1988. Carbohydrates of human immunodeficiency virus. Structures of oligosaccharides linked to the envelope glycoprotein 120. J Biol Chem **263**:11760-7.

56. **Gilboa, E., S. W. Mitra, S. Goff, and D. Baltimore.** 1979. A detailed model of reverse transcription and tests of crucial aspects. Cell **18**:93-100.

57. **Gillespie, D., S. Marshall, and R. C. Gallo.** 1972. RNA of RNA tumer viruses contains poly A. Nat. New Biol. **236**:227-231.

58. **Granowitz, C., J. Colicelli, and S. P. Goff.** 1991. Analysis of mutations in the envelope gene of Moloney murine leukemia virus: separation of infectivity from superinfection resistance. Virology **183**:545-54.

59. **Gruters, R. A., J. J. Neefjes, M. Tersmette, R. E. de Goede, A. Tulp, H. G. Huisman, F. Miedema, and H. L. Ploegh.** 1987. Interference with HIV-induced syncytium formation and viral infectivity by inhibitors of trimming glucosidase. Nature **330**:74-7.

60. **Hackett, P. B., H. E. Varmus, and J. M. Bishop.** 1981. The genesis of Rous sarcoma virus messenger RNAs. Virology **112**:714-28.

61. **Hardwick, J. M., K. E. S. Shaw, J. W. Wills, and E. Hunter.** 1986. Amino-terminal deletion mutants of the Rous sarcoma virus glycoprotein do not block signal peptide cleavage but can block intracellular transport. J. Cell Biol. **103**:829-838.

62. **Hart, T. K., R. Kirsh, H. Ellens, R. W. Sweet, D. M. Lambert, S. R. J. Petteway, J. Leary, and P. J. Bugelski.** 1991. Binding of soluble CD4 proteins to human immunodeficiency virus type 1 and infected cells induces release of envelope glycoprotein gp120. Proc Natl Acad Sci U S A **88**:2189-93.

63. **Hatsuzawa, K., M. Hosaka, T. Nakagawa, M. Nagase, A. Shoda, K. Murakami, and K. Nakayama.** 1990. Structure and expression of mouse furin, a yeast Kex2-related protease. Lack of processing of coexpressed prorenin in GH4C1 cells. J Biol Chem **265**:22075-8.

64. **Hunt, L. A., S. E. Wright, J. R. Etchinson, and D. F. Summers.** 1979. Oligosaccharide chains of avian RNA tumor virus glycoproteins contain heterogeneous oligomannosyl cores. J. Virol. **29**:336-343.

65. **Hunter, E.** 1988. Membrane insertion and transport of viral glycoproteins: A mutational analysis. In: Protein transfer and organelle biogenesis. (eds.) R. C. Das, and P. W. Robbins Academic Press, pp. 109-158.

66. **Jacks, T. and H. E. Varmus.** 1985. Expression of the Rous sarcoma virus pol gene by ribosomal frameshifting. Science **230**:1237-42.

67. **Johnston, P.** 1992. Personal Communication.

68. **Kabat, D.** 1989. Cell surface receptors for ecotropic murine retroviruses: mobile membrane proteins that mediate binding and slow endocytosis of the viral envelope glycoprotein. Virology **171**:467-74.

69. **Kacian, D. L., K. F. Watson, A. Burny, and S. Spiegelman.** 1971. Purification of the DNA plymerase of avian myeloblastosis virus. Biochim. Biophys. Acta **246**:365-383.

70. **Kamps, C. A., Y. C. Lin, and P. K. Wong.** 1991. Oligomerization and transport of the envelope protein of Moloney murine leukemia virus-TB and of ts1, a neurovirulent temperature-sensitive mutant of MoMuLV-TB. Virology **184**:687-94.

71. **Katzman, M., R. A. Katz, A. M. Skalka, and J. Leis.** 1989. The avian retroviral integration protein cleaves the terminal sequences of linear viral DNA at the in vivo sites of integration. J Virol **63**:5319-27.

72. **Keith, J. and H. Fraenkel-Conrat.** 1975. Identification of the 5' end of Rous sarcoma virus RNA. Proc. Natl. Acad. Sci. **72**:3347-3350.

73. **Kielian, M. and S. Jungerwirth.** 1990. Mechanisms of enveloped virus entry into cells. Mol Biol Med **7**:17-31.

74. **Kornfeld, R. and S. Kornfeld.** 1985. Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. **54**:631-664.

75. **Kreis, T. E. and H. F. Lodish.** 1986. Oligomerization is essential for transport of Vesicular stomatitis virus glycoprotein to the cell surface. **46**:929-937.

76. **Kung, H.-J., J. M. Bailey, N. Davidson, P. K. Vogt, M. O. Nicolson, and R. M. McAllister.** 1975. Electron microscope studies of tumor virus RNA. Cold Spring Harbor Symp. Quant. Bilol. **39**:827-834.

77. **Lai, M. H. and I. M. Verma.** 1978. Reverse transcriptase of RNA tumor viruses. V. In vitro proteolysis of reverse transcriptase from avian myeloblastosis virus and isolation of a polypeptide manifesting only RNase H activity. J Virol **25**:652-63.

78. **Lai, M. M. C. and P. H. Duesberg.** 1972. Adenylic acid-rich sequence in RNAs of Rous sarcoma virus and Rauscher mouse leukaemia virus. Nature **235**:383-386.

79. **Leamnson, R. N. and M. S. Halpern.** 1976. Subunit structure of the glycoprotein complex of avian tumor virus. J. Virol. **18**:956-968.

80. **Leamnson, R. N., M. H. M. Shander, and M. D. Halpern.** 1977. A structural protein complex in Moloney leukemia virus. **76**:437-439.

81. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47**:333-48.

82. **Maddon, P. J., J. S. McDougal, P. R. Clapham, A. G. Dalgleish, S. Jamal, R. A. Weiss, and R. Axel.** 1988. HIV infection does not require endocytosis of its receptor, CD4. Cell **54**:865-74.

83. **McCune, J. M., L. B. Rabin, M. B. Feinberg, M. Lieberman, J. C. Kosek, G. R. Reyes, and I. L. Weissman.** 1988. Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus. Cell **53**:55-67.

84. **McKeating, J. A., A. McKnight, and J. P. Moore.** 1991. Differential loss of envelope glycoprotein gp120 from virions of human immunodeficiency virus type 1 isolates: effects on infectivity and neutralization. J Virol **65**:852-60.

85. **Miller, A. D., J. V. Garcia, N. von Suhr, C. M. Lynch, C. Wilson, and M. V. Eiden.** 1991. Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. J. Virol. **65**:2220-4.

86. **Miller, D., S. Rhee, C. Roberts, and E. Hunter.** 1992. Palmitylation of the TM protein of Rous sarcoma virus is necessary for glycoprotein incorporation and virus infectivity. In preparation.

87. **Mitsialis, S. A., J. L. Manley, and R. V. Guntaka.** 1983. Localization of active promoters for eucaryotic RNA polymerase II in the long terminal repeat of avian sarcoma virus DNA. Mol Cell Biol **3**:811-8.

88. **Mizuno, K., T. Nakamura, T. Ohshima, S. Tanaka, and H. Matsuo.** 1988. Yeast KEX2 genes encodes an endopeptidase homologous to subtilisin-like serine proteases. Biochem Biophys Res Commun **156**:246-54.

89. **Mizuno, K., T. Nakamura, T. Ohshima, S. Tanaka, and H. Matsuo.** 1989. Characterization of KEX2-encoded endopeptidase from yeast Saccharomyces cerevisiae. Biochem Biophys Res Commun **159**:305-11.

90. **Modrow, S., B. H. Hahn, G. M. Shaw, R. C. Gallo, S. F. Wong, and H. Wolf.** 1987. Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions. J Virol **61**:570-8.

91. **Moldow, C. F., F. H. J. Reynolds, J. Lake, K. Lundberg, and J. R. Stephenson.** 1979. Avian sarcoma virus envelope glycoprotein (gp85) specifically binds chick embryo fibroblasts. Virology **97**:448-53.

92. **Montefiori, D. C., W. E. J. Robinson, and W. M. Mitchell.** 1988. Role of protein N-glycosylation in pathogenesis of human immunodeficiency virus type 1. Proc Natl Acad Sci U S A **85**:9248-52.

93. **Mulligan, M. J., G. V. Yamshchikov, G. D. J. Ritter, F. Gao, M. J. Jin, C. D. Nail, C. P. Spies, B. H. Hahn, and R. W. Compans.** 1992. Cytoplasmic domain truncation enhances fusion activity by the exterior glycoprotein complex of human immunodeficiency virus type 2 in selected cell types. J Virol **66**:3971-5.

94. **Patarca, R. and W. A. Haseltine.** 1984. Similarities among retrovirus proteins. **312**:496.

95. **Paternostre, M. T., R. J. Lowy, and R. Blumenthal.** 1989. pH-dependent fusion of reconstituted vesicular stomatitis virus envelopes with Vero cells. Measurement by dequenching of fluorescence. Febs Lett **243**:251-8.

96. **Pawson, T., G. S. Martin, and A. E. Smith.** 1976. Cell-free translation of virion RNA from nondefective and transformation-defective Rous sarcoma viruses. J Virol **19**:950-67.

97. **Peng, C., B. K. Ho, T. W. Chang, and N. T. Chang.** 1989. Role of human immunodeficiency virus type 1-specific protease in core protein maturation and viral infectivity. J Virol **63**:2550-6.

98. **Perez, L., J. W. Wills, and E. Hunter.** 1987. Expression of the Rous sarcoma virus env gene from a simian virus 40 late-region replacement vector: effects of upstream initiation codons. J. Virol. **61**:1276-1281.

99. **Perez, L. G., G. L. Davis, and E. Hunter.** 1987. Mutants of the Rous sarcoma virus envelope glycoprotein that lack the transmembrane anchor and/or cytoplasmic domains: analysis of intracellular transport and assembly into virions. J. Virol. **61**:2981-2988.

100. **Perez, L. G. and E. Hunter.** 1987. Mutations within the proteolytic cleavage site of the Rous Sarcoma virus glycoprotein that block processing to gp85 and gp37. J. Virol. **61**:1609-1614.

101. **Perlman, D. and H. O. Halvorson.** 1983. A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. J. Mol. Biol. **167**:391-409.

102. **Pinter, A. and W. J. Honnen.** 1984. Characterization of structural and immunological properties of specific domains of Friend ecotropic and dual-tropic murine leukemia virus gp70s. J Virol **49**:452-8.

103. **Pinter, A. and W. J. Honnen.** 1985. The mature form of the Friend SFFV envelope protein, gp65, is efficiently secreted from cells. **143**:646.

104. **Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo.** 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science **224**:497-500.

105. **Porter, A. G., C. Barber, N. H. Carey, R. A. Hallewell, G. Threlfall, and J. S. Emtage.** 1979. Complete nucleotide sequence of an influenza virus haemagglutinin gene from cloned DNA. Nature **282**:471-477.

106. **Pugatsch, T. and D. W. Stacey.** 1983. Identification of a sequence likely to be required for avian retroviral packaging. Virology **128**:505-11.

107. **Redmond, S., G. Peters, and C. Dickson.** 1984. Mouse mammary tumor virus can mediate cell fusion at reduced pH. **133**:393-402.

108. **Rhee, S. S. and E. Hunter.** 1990. A single amino acid substitution within the matrix protein of a type D retrovirus converts is morphogenesis to that of a type C retrovirus. Cell 77-86.

109. **Rifkin, D. B., and Compans, R. W.** 1971. Identification of the spike proteins of Rous sarcoma virus. Virology 485-489.

110. **Robert, G. M., J. M. Oleske, E. M. Connor, L. G. Epstein, A. B. Minnefor, and R. C. Gallo.** 1987. Relationship between HTLV-III neutralizing antibody and clinical status of pediatric acquired immunodeficiency syndrome (AIDS) and AIDS-related complex cases. Pediatr Res **21**:547-50.

111. **Robey, W. G., L. O. Arthur, T. J. Matthews, A. Langlois, T. D. Copeland, N. W. Lerche, S. Oroszlan, D. P. Bolognesi, R. V. Gilden, and P. J. Fischinger.** 1986. Prospect for prevention of human immunodeficiency virus infection: purified

120-kDa envelope glycoprotein induces neutralizing antibody. Proc Natl Acad Sci Usa **83**:7023-7.

112. **Robey, W. G., P. L. Nara, C. M. Poore, M. Popovic, M. F. McLane, F. Barin, M. Essex, and P. J. Fischinger.** 1987. Rapid assessment of relationships among HIV isolates by oligopeptide analyses of external envelope glycoproteins. Aids Res Hum Retroviruses **3**:401-8.

113. **Rose, J. K. and J. E. Bergmann.** 1983. Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein. Cell **34**:513-24.

114. **Roth, M. G., R. V. Srinivas, and R. W. Compans.** 1983. Basolateral maturation of retroviruses in polarized epithelial cells. J. Virol. **45**:1065-1073.

115. **Rothman, J. E., F. N. Katz, and H. F. Lodish.** 1978. Glycosylation of a membrane protein is restricted to the growing polypeptide chain but is not necessary for insertion as a transmembrane protein. **15**:1447-1454.

116. **Rous, P. a. J. B. M.** 1911. Experiments with a transmissible sarcoma of the fowl. J. Exp. Med. **13**:397-411.

117. **Sattentau, Q. J., P. R. Clapham, R. A. Weiss, P. C. Beverley, L. Montagnier, M. F. Alhalabi, J. C. Gluckmann, and D. Klatzmann.** 1988. The human and simian immunodeficiency viruses HIV-1, HIV-2 and SIV interact with similar epitopes on their cellular receptor,the CD4 molecule. Aids **2**:101-5.

118. **Schatzl, H., H. R. Gelderblom, H. Nitschko, and d. H. K. von.** 1991. Analysis of non-infectious HIV particles produced in presence of HIV proteinase inhibitor. Arch Virol **120**:71-81.

119. **Schawaller, M., G. E. Smith, J. J. Skehel, and D. C. Wiley.** 1989. Studies with crosslinking reagents on the oligomeric structure of the env glycoprotein of HIV. Virology **172**:367-9.

120. **Schneider, J., O. Kaaden, T. D. Copeland, S. Oroszlan, and G. Hunsmann.** 1986. Shedding and interspecies type sero-reactivity of the envelope glycopolypeptide gp120 of the human immunodeficiency virus. J Gen Virol

121. **Schwartz, D. E., P. C. Zamecnik, and H. L. Weith.** 1981. Secondary structural features in the 70S RNAs of Moloney murine leukemia and Rous sarcoma viruses as observed by electron microscopy. J Virol **37**:411-19.

122. **Sei, Y., P. H. Tsang, J. P. Roboz, P. S. Sarin, J. I. Wallace, and J. G. Bekesi.** 1988. Neutralizing antibodies as a prognostic indicator in the progression of acquired immune deficiency syndrome (AIDS)-related disorders: a double-blind study. J Clin Immunol **8**:464-72.

123. **Shaw, G. M., B. H. Hahn, S. K. Arya, J. E. Groopman, R. C. Gallo, and F. Wong-Staal.** 1984. Molecular characterization of human T-cell leukemia (lymphotropic) virus Type III in the Acquired Immune Deficiency Syndrome. **226**:1165-1171.

124. **Sirianni, M. C., P. Rossi, B. Scarpati, G. Ragona, R. Seminara, G. Bonomo, and F. Aiuti.** 1985. Immunological and virological investigation in patients with

lymphoadenopathy syndrome and in a population at risk for acquired immunodeficiency syndrome (AIDS), with particular focus on the detection of antibodies to human T-lymphotropic retroviruses (HTLV III). J Clin Immunol **5**:261-8.

125. **Skehel, J. J., P. M. Bayley, E. B. Brown, S. R. Martin, M. D. Waterfield, J. M. White, I. A. Wilson, and D. C. Wiley.** 1982. Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. Proc. Natl. Acad. Sci. U.S.A. **79**:968-972.

126. **Smeekens, S. P. and D. F. Steiner.** 1990. Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease Kex2. J Biol Chem **265**:2997-3000.

127. **Someya, A., N. Tanaka, and A. Okuyama.** 1990. Inhibition of influenza virus A/WSN replication by a trypsin inhibitor, 6-amidino-2-naphthyl p-guanidinobenzoate. Biochem Biophys Res Commun **169**:148-52.

128. **Stein, B. S., S. D. Gowda, J. D. Lifson, R. C. Penhallow, K. G. Bensch, and E. G. Engleman.** 1987. pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. Cell **49**:659-668.

129. **Stephens, E. B., R. W. Compans, P. Earl, and B. Moss.** 1986. Surface expression of viral glycoproteins is polarized in epithelial cells infected with recombinant vaccinia viral vectors. EMBO J **5**:237.

130. **Stoll, E., M. A. Billeter, A. Palmenberg, and C. Weissmann.** 1977. Rous sarcoma virus genome is terminally redundant: the 3' sequence. Proc Natl Acad Sci U S A **74**:994-8.

131. **Stoltzfus, C. M.** 1988. Synthesis and processing of avian sarcoma retrovirus RNA. Adv Virus Res **35**:1-38.

132. **Stromberg, K., N. E. Hurley, N. L. Davis, R. R. Rueckert, and E. Fleissner,.** 1974. Comparison of polypeptides in virion and core component by dodecyl dulfate-polyacrylamide gel electrophoresis. J. Virol. **13**:513-528.

133. **Sutcliffe, J. G., T. M. Shinnick, I. M. Verma, and R. A. Lerner.** 1980. Nucleotide sequence of Moloney leukemia virus: 3' end reveals details of replication, analogy to bacterial transposons, and an unexpected gene. Proc. Natl. Acad. Sci. USA **77**:3302-3306.

134. **Taylor, J.** 1977. An analysis of the role of t-RNA species as primers for the transcription into DNA of RNA tumor virus genomes. Biochim. Biophys. Acta **473**:57-71.

135. **Thomas, L., R. Leduc, B. A. Thorne, S. P. Smeekens, D. F. Steiner, and G. Thomas.** 1991. Kex2-like endoproteases PC2 and PC3 accurately cleave a model prohormone in mammalian cells: evidence for a common core of neuroendocrine processing enzymes. Proc Natl Acad Sci U S A **88**:5297-301.

136. **Tsai, W. P. and S. Oroszlan.** 1988. Novel glycosylation pathways of retroviral envelope proteins identified with avian reticuloendotheliosis virus. J Virol **62**:3167-74.

137. **Tsichlis, P. N., K. F. Conklin, and J. M. Coffin.** 1980. Mutant and recombinant avian retroviruses with extended host range. Proc Natl Acad Sci U S A **77**:536-40.

138. **Tucker, S. P., R. V. Srinivas, and R. W. Compans.** 1991. Molecular domains involved in oligomerization of the Friend murine leukemia virus envelope glycoprotein. Virology **185**:710-20.

139. **van den Ouweland, A. M., H. L. van Duijnhoven, G. D. Keizer, L. C. Dorssers, and W. J. van de Ven.** 1990. Structural homology between the human fur gene product and the subtilisin-like protease encoded by yeast KEX2 `published erratum appears in Nucleic Acids Res 1990 Mar 11;18(5):1332. Nucleic Acids Res **18**:

140. **Vogt, P. K.** 1977. Genetics of RNA tumor viruses. In: Fraenkel-Conrat H, Wagner R (eds) Comprehensive Virology, Vol9, Plenu, New York, pp341-455.

141. **Vogt, V. M., A. Wight, and R. Eisenman.** 1979. In vitro cleavage of avian retrovirus gag proteins by viral protease p15. Virology **98**:154-67.

142. **von Heijne, G.** 1983. Patterns of amino acids near signal-sequence cleavage sites. Eur. J. Biochem. **133**:17-21.

143. **Walter, P. and V. R. Lingappa.** 1986. Mechanism of protein translocation across the endoplasmic reticulum membrane. Annu Rev Cell Biol **2**:499-516.

144. **Wang, H., M. P. Kavanaugh, R. A. North, and D. Kabat.** 1991. Cell Surface Receptor for Ecotropic Murine Retroviruses is a Basic Amino Acid Transporter. Nature **352**:729-731.

145. **Weiss, R. A., D. Boettiger, and H. M. Murphy.** 1977. Pseudotypes of avian sarcoma viruses with the envelope properties of vesicular stomatitis virus. Virology **76**:808-25.

146. **Weiss, R. A., P. Clapham, K. Nagy, and H. Hoshino.** 1985. Envelope properties of human T-cell leukemia viruses. **115**:235-245.

147. **Weiss, R. A. and A. L. Wong.** 1977. Phenotypic mixing between avian and mammalian RNA tumor viruses: I. Envelope pseudotypes of Rous sarcoma virus. Virology **76**:826-34.

148. **Whitcomb, J. M. and S. H. Hughes.** 1991. The sequence of human immunodeficiency virus type 2 circle junction suggests that integration protein cleaves the ends of linear DNA asymmetrically. J Virol **65**:3906-10.

149. **White, J., M. Kielian, and A. Helenius.** 1983. Membrane fusion proteins of enveloped animal viruses. Q. Rev. Biophys. **16**:151-195.

150. **Willey, R. L., D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin.** 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity.J Virol **62**:139-47.

151. **Wills, J. W., R. C. Craven, and J. A. Achacoso.** 1989. Creation and expression of myristylated forms of Rous sarcoma virus gag protein in mammalian cells. J Virol. 1989Oct. **63**:4331-43.

152. **Wills, J. W., R. C. Craven, R. A. J. Weldon, T. D. Nelle, and C. R. Erdie.** 1991. Suppression of retroviral MA deletions by the amino-terminal membrane-binding domain of p60src. J Virol **65**:3804-12.

153. **Wills, J. W., J. M. Hardwick, K. Shaw, and E. Hunter.** 1983. Alterations in the transport and processing of Rous sarcoma virus envelope glycoproteins mutated in the signal and anchor regions. J. Cell Biochem. **23**:81-94.

154. **Wills, J. W., R. V. Srinivas, and E. Hunter.** 1984. Mutations of the Rous sarcoma virus env gene that affect the transport and subcellular location of the glycoprotein products. J. Cell Biol. **99**:2011-2023.

155. **Wilson, C., M. S. Reitz, H. Okayama, and M. V. Eiden.** 1989. Formation of infectious hybrid virions with gibbon ape leukemia virus and human T-cell leukemia virus retroviral envelope glycoproteins and the gag and pol proteins of Moloney murine leukemia virus. J. Virol. **63**:2374-8.

156. **Witte, O. N., A. Tsukamoto-Adey, and I. L. Weissman.** 1977. Cellular maturation of oncornavirus glycoprotiens: topological arrangement of precursor and product forms in cellular membranes. **76**:539-553.

157. **Yamamoto, T. B. de Crombrugghe, and I. Pastan.** 1980. Iden ification of a functional promoter in the long terminal repeat of Rous sarcoma virus. Cell **22**:787-97.

158. **Young, J., P. Bates, and H. Varmus.** 1992. Personal Communication

159. **Young, J. A., P. Bates, K. Willert, and H. E. Varmus.** 1990. Efficient incorporation of human CD4 protein into avian leukosis virus particles. Science **250**:1421-3.

160. **Yu, X., X. Yuan, Z. Matsuda, T.-H. Lee, and M. Essex.** 1992. The matrix protein of human immunodeficiency virus type 1 is required for incorporation of viral envelope protein into mature virions. J. Virol **66**:4966-4971.

161. **Zhdanov, V. M., V. D. Soloviev, and T. A. Vektemirov.** 1973. Isolation of oncoviruses form continuous human cell cultures. Intervirology **1**:19-26.

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Dissertation Committee:

_____, Chairman

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Director of Graduate Program Susan Jackson

Dean, UAB Graduate School W. R. Sibley

Date _____

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