STUDIES OF THE STRUCTURE AND BIOSYNTHESIS OF THE MEMBRANE GLYCOPROTEINS ENCODED BY THE FRIEND MURINE LEUKEMIA VIRUS

by

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I. INTRODUCTION

The RNA tumor viruses (also called oncornaviruses or retroviruses) form a large and diverse group which occur in different vertebrates and have the capability of inducing tumors. This group of viruses is characterized by having a genome composed of RNA and an enzyme, reverse transcriptase, capable of RNA-dependent DNA polymerization. This unique enzyme[1] generates a double-stranded DNA copy from the viral RNA which then integrates into the host cell genome in an early phase of the viral infection (see below).

The RNA tumor viruses have been identified as causative agents of tumors of connective tissue, hemopoietic and reticulo-endothelial systems in numerous animal species including mice, rats, hamsters, cats, pigs, cows, monkeys, apes and even snakes (1,2). Because the infected hosts contain in their chromosomes an integrated proviral copy of the viral genome (3,4), virus-specific DNA sequence can be detected in these species by molecular hybridization (5-7). As integrated proviral genes, the RNA tumor viruses can be transmitted vertically by inheritance (2,8).

[1]The discovery of the enzyme, reverse transcriptase (36,37), presented enzyme biochemists with an unprecedented capability to generate DNA from RNA. This capability was central to the development of recombinant DNA technology.

Furthermore, the relative homologies of these different proviruses as determined by molecular hybridization parallels the taxonomic phylogeny of the species (5-8). Therefore RNA tumor virologists believe that all vertebrate species, including man, probably contain proviral genomes which are continuously present, are inherited vertically (2,5-8) and which have evolved from ancient RNA tumor virus predecessors.

The significance of studying the RNA tumor viruses is vast, and may contribute to an understanding of cancer in many animals, including man. In addition, the study of these viruses has more recently played an important role in basic research on gene expression and recombinant DNA technology. Therefore it is beyond the scope of this introduction to review more than the details relating to the results and conclusions contained herein. A thorough historical account of the field is available which starts with the first discovery of the Rous sarcoma virus (9) and includes the subsequently isolated viruses up to 1970 (1). Several more recent reviews provide a current perspective on the biology (10,11) and molecular biology (2,12,13) of the RNA tumor viruses.

The RNA tumor virus characterized in this thesis is the replication-competent Friend murine leukemia virus (F-MuLV). The F-MuLV is the helper component of the Friend virus complex which also includes a defective, but potent

spleen focus-forming virus (F-SFFV) that causes rapid splenomegaly and leukemia (14). The Friend virus was first isolated from extracts of enlarged spleens generated by innoculating newborn Swiss mice with an Erlich ascites tumor cell extract (15). The Friend virus has been widely studied because of the leukemia that the virus causes in mice and because the virus-induced tumor cells differentiate through the erythroid cell lineage upon culturing in the presence of dimethyl sulfoxide (16). Thus Friend virus-induced leukemia cells, or Friend cells, also provided a conveniently inducible in vitro model system for analyzing erythroid differentiation (17) and hemoglobin expression (18).

A. Virus Morphology and Life Cycle

As deduced from electron microscopic studies, the leukemia virus particle is a roughly spherical structure about 100 mu in diameter (19), comprising a nucleoid core enclosed in an outer envelope made of a single lipid bilayer. Imbedded in the viral envelope and projecting outward are knobs now known to be the membrane glycoproteins (20). The chemical composition of the MuLV particle is relatively simple and includes RNA (1%), carbohydrate (2%), lipid (20-30%) and protein (60-70%) (2). The major RNA component is located with the nucleoid core as a filamentous ribonucleoprotein structure (2).

The structural arrangement of the components in

the MuLV particle can be described in terms of the biosynthesis of the virus particle. In productively infected cells, the viral mRNA is transcribed from the integrated proviral DNA and translated into protein using the host biosynthetic machinery. The core porteins are synthesized as polyprotein precursors in the cytoplasm (21,22) and assemble with the viral RNA genome at the plasma membrane (23). The structure and function of the individual core proteins in virion assembly will be described below (section I, D2) and their role in this process is a subject of inquiry in this thesis (sections IV and VI). However, during core maturation the assembling core buds from the cell, acquiring its lipid bilayer envelope from the host cell plasma membrane. Furthermore, as discussed below (section I, F1), the viral envelope glycoproteins are syntheized on the rough endoplasmic reticulum by the normal cellular mechanicsm for membrane glycoprotein synthesis. These envelope glycoproteins are then transferred to the plasma membranes and are subsequently acquired as components in the virion envelope when the viron core buds through the cellular plasma membrane.

The infectious phase of the MuLV life cycle begins with a specific binding between the virion envelope glycoprotein and a surface receptor on susceptible host cells. The specificity of this binding defines the host

range of the infecting virus at this level of the infectious process and is described thoroughly in section I, F2. After binding, the virus penetrates through the plasma membrane leaving its envelope in the host's plasma membrane. Once inside the cytoplasm, the core generates a double-stranded DNA intermediate by reverse transcribing the viral RNA genome using the viral enzyme present in the core. The ability of the DNA copy to integrate into the host chromosome is then determined by intracellular host range growth restrictions (described in section I, D2). Once integrated, the viral DNA can reside as a stable, inheritable, potentially expressive proviral DNA.

B. The MuLV Genome Structure and Replication

The MuLV genome is composed of a 70S RNA molecule which upon denaturation yields two single-stranded 35S subunits believed to be identical copies of the viral genome (24-26). The Moloney-MuLV 35S RNA contains 8332 nucleiotides (27) and therefore can potentially encode approximately 2780 amino acids or 305,000 daltons of protein. The MuLV genome encodes three genes called gag, pol and env (28) which are arranged 5'-gag-pol-env-3' on the 35S RNA. This RNA has properties characteristic of eukaryotic mRNAs including a positive messenger sense (29,30), a 5' cap structure (31) and a polyadenylated tail at the 3' end (32).

The replication of the viral genome into an integrated proviral DNA copy is an obligatory step in

productive virus infection (33,34). After virion core penetration into the cytoplasm, reverse transcription begins and is catalyzed by the viral enzyme present in the virion core (34-37). During the ensuing RNA-dependent DNA synthesis, the ends of the viral genome, which play a key role in replication and integration (12,38), are duplicated such that the final double-stranded DNA copy contains long terminal repeats (LTR) of approximately 550 nucleotides at each end and therefore is longer than the 35S viral RNA (27). The precise details of structure of the LTRs and the mechanism of replication by which they are generated has been recently reviewed (39). This double-stranded DNA copy of the genome is transported to the nucleus where at least some of the copies are converted into closed circular molecules (40). The DNA copy integrates into the host cell genome in potentially many different sites (33,41) to form a provirus (3,4). However, it is not known if the circular or linear DNA copy is the form which integrates (41). Expression by transcription of the proviral genes is initiated by promoters present in the 5'LTR and terminated by terminators in the 3'LTR (27) to generate either the viral mRNA or the genomic RNAs.

C. General Methodology for Studying Murine Leukemia Virus

MuLVs do not inhibit host protein synthesis during productive infections. As a consequence, the

levels of expressed viral proteins are very small compared to ongoing normal cellular protein synthesis. Studies of MuLV protein synthesis and processing therefore require the use of antisera to selectively identify the viral proteins in the presence of an excess of cellular proteins. Routinely, infected cells are metabolically labeled with radioisotopes, viral proteins are selectively isolated by immunoprecipitation and the immunoprecipitates are then dissolved in sodium dodecyl sulfate (SDS) and analysed by electrophoresis on polyacrylamide slab gels.

A nomenclature for RNA tumor virus-encoded proteins was developed to permit unambiguous communication of data on the structural proteins and intracellular processing intermediates generated by these viruses (42,43). According to this nomenclature, p is for protein, gp is for glycoprotein, Pr is for precursor and gPr is for glycosylated precursor. The number which follows indicates the apparent molecular weight in kilodaltons derived from mobilities on either gel filtration or gel electrophoresis techniques. The superscripts indicate the gene of origin. For example, gPr90$^{\underline{env}}$ indicates a glycosylated precursor protein of apparent Mr 90,000 which is the product of the <u>env</u> gene.

D. <u>gag Gene Products</u>

The <u>gag</u> gene-encodes for two distinct classes of proteins. These are the structural proteins of the virion core which are synthesized and processed in the cytoplasm

(44), and the membrane-associated glycosylated gag polyproteins which are synthesized and processed in the membranous subcellular organelles (45-47).

1. Synthesis of the Virion Core Proteins

The virion structural proteins, p30, p15, p12, and p10, are produced by the processing and cleavage of the precursor polyprotein, $Pr65^{gag}$ (21,22). This $Pr65^{gag}$ precursor is translated in the cytoplasm using the viral 35S RNA as a template (48-51). The cleavage of $Pr65^{gag}$ occurs via a series of intermediate proteolytic events during core assembly and maturation (as decribed below) to form the individual core proteins. A cell-encoded proteolytic factor that is required for $Pr65^{gag}$ cleavage has been detected in virion particles (52). The analysis of the processing intermediates for $Pr65^{gag}$ (53-55) and tryptic peptide mapping of premature termination polypeptides derived from in vitro translation of MuLV genomic RNA (56) originally allowed the ordering of the virion core proteins on the precursor as follows: NH_2-p15-p12-p30-p10-COOH. This order is now clearly established by sequence data for the Moloney-MuLV genome (27).

2. Function of the Virion Core Proteins

p15, characterized as a hydrophobic membrane protein, is located at the amino terminal of intact $Pr65^{gag}$ and therefore is accessible for interaction with plasma membranes during the assembly of virion cores (23). Furthermore, p15 is found at the outermost aspect of

mature virion cores (57) after Pr65^{gag} cleavage. This is consistent with the notion that p15 interacts with the plasma membrane or transmembrane components such as the envelope glycoprotein (23) during the organization of a budding core.

p12, characterized as a type-specific RNA-binding phosphoprotein, exhibits a specificity to bind RNA from homologous viruses but not the RNAs of heterologous viruses (58). Furthermore, sequence homology between MuLV p12 and histone H5, a phosphorylated nuclear protein, suggests that phosphorylation of RNA binding proteins plays a role in viral regulatory functions (55).

p30 is characterized as the major structural protein of the virion core comprising 10-20% of the total virion protein (59). p30 carries the major group-specific antigenic determinants from which the gag gene derived its name (28). p30 has been implicated in an important growth restriction that exists between classes of MuLV and different inbred mouse strains (60). This growth restriction is controlled genetically by a single mouse gene and a determinant present on the p30 molecule. This restriction functions intracellularly at the level of controlling whether incoming virus can functionally integrate into the cell genome (61). Because this thesis does not deal with this portion of the viral life cycle (i.e., proviral DNA integration) and because of its complexity, the interested reader is referred to a recent

review (60).

p10, characterized as a highly basic ribonucleoprotein, is located in the ribonucleoprotein complex associated with viral genomic RNA within the virion core (62). p10's strong non-specific avidity for binding to single-stranded nucleic acid molecules (63) and its location at the carboxy terminus of $Pr65^{gag}$ suggests a role for organizing the viral RNA genome at this time of core assembly and budding (23).

These properties of the virion core proteins combined with their alignment along the $Pr65^{gag}$ molecule has inspired a useful model for the assembly and budding of virion cores (23). In this model, uncleaved $Pr65^{gag}$ organizes at the cytoplasmic face of the host cell plasma membrane because of an interaction of the hydrophobic p15 protein with the plasma membrane and possibly with transmembrane portions of the viral envelope glycoprotein. This alignment facilitates interaction between single-stranded RNA and the p10 located on the opposite (carboxy) terminus of $Pr65^{gag}$, which protrudes away from the membrane and into the cytoplasm. According to this model, the uncleaved $Pr65^{gag}$ organizes an interaction between envelope glycoproteins imbedded in the lipid bilayer and the viral RNA components present in the cytoplasm. After assembly, the core buds from the cell and thereby acquires the lipid bilayer with its incorporated membrane glycoproteins. The maturation by proteolytic cleavage of

the core precursor polyprotein to generate the individual core proteins occurs late in this process, predominantly after assembly and budding has occurred (64-67).

Numerous studies with core precursor synthesis and of processing mutants have suggested a critical role for $Pr65^{gag}$ in core assembly and budding (44,68,69) consistent with the model above. However, the role of either the envelope glycoprotein or the viral genomic RNA in virion assembly and budding is not well supported. Mutants which lack the envelope glycoproteins on their plasma membranes were found to release virion cores (70-73). Furthermore, virus particles lacking virus-specific 70S RNA are released from cells in the presence of actinomycin D (74-76). There remains the possibility that functionally equivalent cell-encoded proteins and non-viral RNA can fulfill the essential roles of interaction with $Pr65^{gag}$ for virion core assembly and release.

Studies presented in section IV and VI of this thesis further investigate the requirements for virion core assembly and release. In these experiments, a role for the glycosylated polyproteins (discussed below in section I, D3) in core release is also not supported.

3. The gag Gene-Encoded Membrane Glycoproteins

In addition to the structural proteins of the virion core, the gag gene also encodes glycosylated proteins (46,47) detectable on the surface of MuLV-infected cells (45,77-80).

The synthesis of glycosylated gag gene-encoded molecules occurs via the normal cellular pathway for membrane glycoprotein biosynthesis (described in section I, G). In this pathway, the primary gag gene translation product is a protein of approximately 75,000 daltons ($Pr75^{gag}$). $Pr75^{gag}$ is translated directly into the lumen of the rough endoplasmic reticulum and glycosylated by the "en bloc" addition of preassembled high-mannose oligosaccharides to yield a glycosylated precursor molecule, $gPr80^{gag}$ (47). Because of this nascent insertion into the rough endoplasmic reticulum, $Pr75^{gag}$ is only detectable when labeled in the presence of inhibitors of glycosylation. The presence of two N-linked high mannose oligosaccharides has been determined in $gPr80^{gag}$, one located on the p30 portion of the polyprotein and the other near the amino-terminal (81). In addition, MuLV nucleotide sequence data shows the presence of four possible glycosylation sites (Asn-X-Thr/Ser) (27). $gPr80^{gag}$ then migrates to the Golgi apparatus where it is processed by additional oligosaccharide modifications to yield $gp93^{gag}$ (46,47,79). The latter glycoprotein is then transported to the cell surface (45,77,79,80).

The polypeptide sequence of $Pr75^{gag}$ is nearly identical to that of $Pr65^{gag}$, the precursor to the structural core proteins, except that $Pr75^{gag}$ contains in addition to p15, p12, p30 and p10 (45,77), an additional

polypeptide of 7,000 daltons (82) located at the amino terminus (81). This amino terminal polypeptide would presumably function as a signal sequence (83) to facilitate entry into the rough endoplasmic reticulum.

Several lines of evidence suggest that $Pr65^{\underline{gag}}$ and $Pr75^{\underline{gag}}$ are synthesized independently rather than with a precursor-product relationship. As mentioned above, $Pr65^{\underline{gag}}$ is synthesized and processed to form the virion core proteins whereas $Pr75^{\underline{gag}}$ is processed to become a cell membrane glycoprotein (46,47). Also, cell-free translation studies (47) indicate that $Pr65^{\underline{gag}}$ and $Pr75^{\underline{gag}}$ are translated from separate initiation sites on the mRNA. Finally, the complete nucleic acid sequence of Moloney-MuLV genomic RNA reveals the presence of several initiation codons, one immediately preceding the $Pr65^{\underline{gag}}$ reading frame and several further towards the 5' end of the 35S RNA (27). From this sequence, a spliced mRNA could be proposed which would have the necessary additional information to generate a signal sequence polypeptide (27). On the basis that eukaryotic mRNAs usually contain only a single initiation site with a solitary exception (84), it has been proposed that $Pr65^{\underline{gag}}$ and $Pr75^{\underline{gag}}$ are produced from two different 35S mRNAs (47).

Although $gp93^{\underline{gag}}$ has been identified as a cell surface glycoprotein on MuLV-infected cells, the function of $gp93^{\underline{gag}}$ is almost completely unknown. $gp93^{\underline{gag}}$

does interact with the host immune system (85) and has been shown to carry the antigenic determinants for the Gross cell surface antigen (86). Therefore gp93^{gag} is probably important in the host response to virus-induced cancers. Furthermore, gp93^{gag} is not found in released virions (47,70,78), suggesting that it does not play a role in infectivity or in proviral integration (73).

Studies which are presented in this thesis address the role of gp93^{gag} as a surface component in the budding and release of virions. As is shown in section IV, mutants lacking gp93^{gag} bud infectious virus efficiently (73). Therefore gp93^{gag} does not appear to play a role in virus assembly, release or in exogenous infection.

E. pol Gene Products

1. Biosynthesis of Reverse Transcriptase

The pol gene encodes the viral enzyme reverse transcriptase, which is an RNA-dependent DNA polymerase (35-37). The enzyme is derived from a large precursor polyprotein, Pr180$^{gag\text{-}pol}$, which contains both gag and pol antigens (29,50,87). This precursor is first incorporated into budding virions (88) and is then proteolytically cleaved to form the mature enzyme, a single polypeptide of 70,000 daltons (28,35).

Because so few reverse transcriptase molecules are incorporated into virions compared to the virion core precursor (88), the controlling mechanism for

synthesis of reverse transcriptase is an intriguing one. In vitro translation of viral genomic RNA predominantly synthesizes the virion core precursor, $Pr65^{gag}$, and the cell surface glycoprotein precursor, $Pr75^{gag}$ (29). However the addition of suppressor tRNA to this cell-free system will increase the levels of $Pr180^{gag\text{-}pol}$ and decrease the levels of $Pr65^{gag}$ and $Pr75^{gag}$. This result suggests a model in which the level of $Pr180^{gag}$ synthesis is controlled by the inefficient read-through of a UAG (amber) termination codon located between gag and pol coding sequences on the 35S RNA (88). Moloney-MuLV sequence data has established that gag and pol are in the same reading frame and are separated by a single amber codon (27). A second model suggests that the terminator codon for the gag polyprotein is removed from the message by an RNA processing event (89) analogous to the processing of yeast tRNA (90).

2. Function of Reverse Transcriptase

The viral polymerase catalyses the synthesis of double-stranded DNA using the viral genomic 35S RNA as template. This reverse transcription is described in more detail in section I, B. In addition, this enzyme has a ribonuclease H activity which degrades the RNA present in a RNA-DNA hybrid (91,91).

F. env Gene Products

1. Synthesis of the Envelope Proteins

The env gene encodes the virion envelope

glycoproteins, gp70 and p15(E)[1] (28).

The synthesis of these envelope glycoproteins occurs from a subgenomic 22S mRNA (51,93) which derives from the genomic 35S RNA by splicing of small amounts of the 5' end to large portions of the 3' end of the viral genome (94). It is presumed that this RNA splicing occurs as is described in other eukaryotic systems (95). Surprisingly, as a result of this splicing, a small portion of the 3' terminus of the pol gene is contained in the env mRNA sequence (27).

The primary translation product of the 22S mRNA is the glycosylated protein, gPr90^{env}, precursor to the virion envelope proteins, gp70 and p15(E) (22,51,96-99). gPr90^{env} is known to enter the normal pathway for membrane glycoprotein processing during nascent translation (described in section I, G) because the earliest detectable product in vitro and in vivo contains the simple oligosaccharides (22,96,99) characteristic of membrane glycoproteins (100,101) and because it is synthesized on membrane bound ribosomes (93). Furthermore, MuLV genomic sequence data (27) supports the idea the gPr90^{env} enters the rough endoplasmic reticulum (RER) for glycosylation. The deduced nucleotide sequence

[1]The suffix (E) indicates a protein of the viral envelope (42) and is used here to distinguish this protein from the core protein, p15.

indicates the presence of an appropriate amino terminal hydrophobic signal polypeptide proposed to direct nascent polypeptides into the RER (83). Also, seven Asn-X-Ser/Thr sites for the lipid-linked oligosaccharide addition (102,103) are present on the gp70 coding portion of the env gene, of which six or seven are known to be glycosylated (104).

The processing of gPr90^{env} to form gp70 and p15(E) occurs during its migration from the RER via the Golgi apparatus to the plasma membrane (97). In the course of this migration, oligosaccharide structures are enzymatically modified from the simple to complex type (22,99). Furthermore, disulfide bridges are formed between the gp70 and p15(E) portions of the precursor (97,98,105,106) probably before it is cleaved to yield gp70 and p15(E). The final event in this processing sequence is the placement of gp70, the major envelope glycoprotein, and p15(E), its membrane anchor (27,107), onto the plasma membrane (45,77,96,108,109).

Because of the heterogeneous and membranous nature of the membrane glycoprotein processing organelles (110,111), it has been very difficult biochemically to determine the precise location and sequence of events whereby gPr90^{env} is processed as described above. Kinetic labeling experiments demonstrate that cleavage of the precursor occurs shortly before the surface expression of the cleaved products (22,97,112). However, the processing

of gPr90^{env} must occur before departure from the Golgi apparatus where complex sugar processing is known to occur (113-115), because gp70 contains the complex type terminal sugars fucose (22,97,99) and sialic acids (116). These results suggest that transport from the Golgi apparatus to the plasma membrane is very rapid after these processing events occur. Attempts to differentiate the precursor cleavage event from the oligosaccharide modifications which convert simple carbohydrate structures to complex were unsuccessful suggesting that these two events are very closely linked (112).

Work presented in section V of this thesis supports the hypothesis that until cleavage and complex sugar processing of gPr90^{env} is completed, the precursor is stored intracellularly, presumably in the RER. After transport to the Golgi apparatus where these processing events are completed, transfer to the surface is very rapid. In support of this hypothesis, there are envelope precursor processing mutants which do not cleave gPr90^{env} and also do not transport this molecule to the cell surface (72,73).

2. Biology of gp70

As discussed below, the envelope glycoproteins of the MuLV play a key role in the infectivity and host range of the virus (117). Furthermore, as a surface protein, gp70 molecules also have important implications in processes such as

lymphocyte differentiation (118-121), host immune response to viral antigens (85) and in leukemogenesis (122,123).

gp70 is the major component on the virus particle and comprises approximately 10% of the total virion protein (124). The envelope glycoprotein appears as "spikes" when viewed in the electron microscope (20). In the virus particle, at least a portion of gp70 is found disulfide bridged to p15(E) (57,105).

As the viral envelope glycoprotein, gp70 plays a pivotal role in the infectivity of the virus particle. A binding interaction between gp70 and cell surface receptors is required for successful virus infection (125). An investigation of this binding using [^{125}I]-labeled gp70 to probe for cell surface receptors revealed that MuLV gp70 binds to uninfected murine cells and not to other mammalian cell types (117). In fact, a restriction in the host range of the infecting MuLV is defined by this binding interaction between the virion gp70 and the host's cell surface receptor.

For example, ecotropic MuLV, which (by definition) can only infect mouse cells, possess a gp70 which binds specifically to murine receptors. Amphotropic MuLV, which infect both non-mouse and mouse cells, possess a gp70 which binds to both types of receptors. Finally, xenotropic MuLV, which only infect non-mouse cells, possess a gp70 which only binds to those cells which it can infect.

The ability of a MuLV-infected cell to resist superinfection by exogenous MuLV is also governed by gp70 (117,126,127). Mouse cells infected by ecotropic MuLV express gp70 on their plasma membranes (see above). These infected cells are no longer susceptible to superinfection by ecotropic MuLV, but are susceptible to infection by xeno- or amphotropic MuLV (2). This resistance to infection, called interference, occurs because the gp70 that is expressed on the surface of infected cells occupies the receptor and therefore blocks superinfecting virus from attachment (117). Because an ecotropic gp70 does not bind the receptor for xeno- or amphotropic MuLVs, ecotropic MuLV infections do not interfere with superinfection by these other tropic classes of MuLV.

An awareness of this central role which the gp70-receptor binding interaction plays in susceptibility to infection and interference has understandably resulted in studies to characterize the binding properties (117,128-133) and identity (134-137) of the receptor molecule. From the binding studies, the number of ecotropic MuLV gp70 binding receptor molecules was found to vary from $1x10^4$/cell for BALB/c thymus cells to $5x10^5$/cell for NIH/3T3 fibroblasts. However, the identity of this receptor has not been unambiguously defined since putative purified receptor preparations have varied widely in content. The mouse H-2 histocompatibility antigens, which were found to be the receptor for Semliki Forest virus

(138), were also found to associate with the MuLV glycoproteins (139,140). However, this interaction was not shown to occur in normal receptor assay conditions (135) suggesting that mouse histocompatibility antigens are not the receptor for ecotropic MuLV. The ecotropic MuLV receptor has been mapped on chromosome 5 of the mouse (141).

As discussed above, gp70 is a critical envelope component of the virus particle which defines the host range of the infecting virus. In addition, gp70 functions on the surface of cells which are expressing MuLV proviral genes in ways which are less well characterized (45,77,80,96,109,119,120).

For example, in virus biosynthesis it has been suggested that gp70 plays some essential role in the budding of virus cores from host cell plasma membranes (23,142). Specifically, interactions between the p15 moiety of the core precursor, $Pr65^{gag}$, and transmembrane portions of the envelope glycoproteins have been proposed (see section I, D2). These interactions would i) bring viral envelope proteins into the vicinity of a virus budding event and ii) direct $Pr65^{gag}$ to a specific membrane-associated site at which to organize for core assembly. However, numerous MuLV mutants which lack cell surface gp70 have been reported which do bud virion cores (68,71-73,143). Although not demonstrated, it seems plausible that normal cellular proteins may fulfill the

essential suggested roles which the viral envelope components provide. In this regard, murine cells contain in their genome a family of gp70-related genes (see below) whose products could be candidates for fulfilling the proposed requirements. However, studies presented in section IV of this thesis support the hypothesis that MuLV-encoded membrane components are not required for efficient budding of MuLV core particles.

It has been observed that mice express a polymorphic group of gp70-related products on their cell surfaces (109,119,120). gp70-related proteins are in fact encoded by a family of endogenous viral genes, members of which are expressed in different tissues and at different but specific stages of differentiation. Therefore they are implicated as normal mouse differentiation antigens. One of these expressed gene products is the mouse differentiation antigen G_{IX} (108), and has been extensively characterized (104,144).

Whether cells are productively infected by MuLV or are only expressing gp70-related antigens, the presence of gp70 on the surface of cells makes it a candidate for an interaction with the host's immune system. In fact, the MuLV env and gag gene-encoded surface molecules play a role in the immune response (59,85).

gp70 has also been implicated in playing a role in leukemogenesis because changes in gp70 structure

are believed to alter the leukemogenicity of the virus expressing the gp70 molecule (122,123,145). Replication-competent MuLVs (such as the one studied in this thesis) are capable of inducing lymphatic leukemia, but only after a lengthy 6 month latent period following innoculation into adult mice (145). In contrast, highly leukemogenic MuLVs such as the murine mink cell focus-inducing virus (MCF) (146) or the Friend spleen focus-forming virus (F-SFFV) (14) induce rapidly progressing fatal leukemias upon innoculation. Analyses of the env gene or env gene products of these viruses reveals in both cases sequences present which are homologous to both ecotropic and xenotropic gp70 molecules (122,123). These results suggest that the env genes of the highly leukemogenic MCF and F-SFFV viruses were produced by recombination in the env gene between viruses with distinct host range and interference properties. The similar acquisition of a recombinant env gene in different isolated viruses strongly suggests that gp70 is involved in the disease process. As a consequence, it has been proposed that recombinant env genes or their encoded glycoproteins might play a causal role in leukemogenesis (122,145).

G. Membrane Glycoprotein Biosynthesis in General

Plasma membrane and secretory proteins are believed to follow a similar pathway from their synthesis in the rough endoplasmic reticulum (RER) via

the Golgi apparatus to the cell surface (110,147,148). The transit between organelles may be facilitated by transfer or shuttle vesicles which in some cases contain a clathrin coat on their cytoplasmic surfaces (149,223). During this process, the protein being transferred may be covalently altered by glycosylation (101,148,150), oligosaccharide modifications (115,151,152), acylation (153,154), sulfation (155) or proteolysis (83,98,156). Although many plasma membrane or secretory proteins are specifically affected by such covalent modifications, it is believed that these modifications are not prerequisite for the successful transfer of all proteins (157-161). Alternatively, transfer of some proteins from the Golgi apparatus may require specific modification such as the addition of 6-phosphomannosyl residues or attached oligosaccharides (162-164). Sections IV and V present data which demonstrates that transfer of MuLV envelope glycoprotein precursor is impeded because of processing blockades.

The biosynthesis, glycosylation and processing of membrane associated glycoproteins occurs on membrane bound polyribosomes (83,100) of the RER. In vitro membrane protein synthesis systems have been developed which allow nascently synthesized polypeptides to be sequestered and glycosylated in membranous vesicles (165,166). Thus studies on the biosynthesis of vesicular stomatitis virus glycoprotein "G" have

elucidated the timing of translocation and glycosylation into these vesicles. As an example, insertion into the microsomes begins within 6 minutes of the start of translation. Glycosylation, which occurs on two acceptor sites by "en bloc" transfer (see below), begins at 10 and 20 minutes after the start of initiation. Furthermore, the synthesis of G protein, which has an apparent Mr 63,000, is complete in 40 minutes.

The nascent glycosylation of glycoproteins occurs enzymatically (102) by the "en bloc" transfer (167) of a preassembled high-mannose "core" oligosaccharide with the structure, $Glc_3Man_9GlcNAc_2$ (168). The attachment of the N-acetylglucosamine of the core oligosaccharide occurs by an N-glycoidic linkage to the asparagine side group on the amino acid tripeptide glycosylation site NH_2-Asn-X-Thr/Ser-COOH, where X is a vairable amino acid (102,103). This initial glycosylation can be specifically blocked with the antibiotic, tunicamycin (150).

The insertion of nascent polypeptides into the RER has been proposed to be guided by a "signal sequence" present on the amino terminus (83). These signal sequences are approximatley 20-30 amino acids long and include a continuously hydrophobic central portion which presumably facilitates insertion and translocation through the membranes of the RER. After translocation, this signal sequence may be

proteolytically cleaved (169).

The majority of covalent modifications described for glycoproteins after their intital glycosylation are belived to occur in some aspect of the Golgi apparatus including the addition of complex type sugars (111,114,115,152). However, prior to departure from the endoplasmic reticulum, terminal glucose residues are quickly removed for the simple core structure (111,168). Processing of glycoproteins then proceeds with their transfer to the cis aspect of the Golgi apparatus (81,111) where fatty acids are added (153,154) and the degradation of outer mannose residues occurs (111). Complex sugar processing occurs in the trans aspect of the Golgi apparatus including terminal additions of galactose, fucose, N-acetylglucosamine or sialic acids (101), sulfation (155) and selective cleavage of propeptides from secretary proteins (111).

An important question in this field concerns the mechanism for the selection and transfer of newly synthesized proteins between organelles during their processing. Generally, it is believed that transfer occurs in a linear manner as a cohort of simultaneously made proteins which passes through different organelles or processing stages during definite subsequent time intervals (165), perhaps carried along by lipid flow (110,147). However, Rothman (111) has recently proposed that the process may be more complex and that the cis

aspect of the Golgi apparatus may fractionate proteins by a reflux type mechanism. Kinetic studies have also suggested that certain secretory proteins are transported intracellularly more slowly than others and that the slowly transported proteins may reach the cell surface throughout a prolonged time span (170). However, previous investigators have indicated that different plasma membrane proteins are transported intracellularly with similar kinetics and that they reach the cell surface approximately 30-45 minutes after synthesis (148,158,165). Studies presented in section V address this issue and support the hypothesis that processing is a highly selective process and that transport between organelles involves interaction with specific carrier molecules that bind to different proteins with different affinities.

H. Thesis Objectives and Experimental Rationale

The predominant objective of the work presented in this dissertation is to analyze the synthesis, processing and functions of the membrane associated glycoproteins encoded for by the murine leukemia virus (MuLV). These membrane glycoproteins are gp70 and $gp93^{gag}$, and are encoded by the distinctive MuLV genes, *env* and *gag*, respectively. As a virion structural protein, gp70 plays an important role in the infectivity and host range of the virus. However, the functions of gp70 and $gp93^{gag}$ as surface proteins on infected cells,

gp70 are poorly understood. As discussed in the introduction, both of these membrane glycoproteins interact with the host immune system, and are therefore probably important in the host response to virus-induced cancers. gp70 is also implicated in lymphocyte differentiation and in leukemogenesis. For these reasons, methods to probe for their synthesis and placement onto the cell surface and to investigate their functions as cell surface components were pursued.

The approaches utilized to achieve this objective involve the genetic technique of isolating mutants with defects in the synthesis and surface placement of the membrane glycoproteins. To do this, infected cell mutants which lack virus-encoded antigens on their surface membranes were immunoselected (171-173). In addition, simple virus cloning, which results in a high rate of formation of spontaneous viral mutations (68), was used to isolate viral mutants. The processing of the membrane glycoproteins in the mutant and normal infected cells was then characterized. This involved labeling the viral proteins metabolically with radioisotopes, isolating these proteins by specific immunoprecipitation and analysing the immunoprecipitates by polyacrylamide gel electrophoresis. Furthermore, because a primary focus is on the cell surface disposition of these membrane glycoproteins, numerous methods were employed which specifically detect cell

surface components. These include lactoperoxidase-catalysed iodonation of cell surface proteins, measuring relative sensitivities to antibody-directed complement-dependent cytolysis, detection of cell surface antigens by an antibody-dependent erythrocyte rosette assay and the specific immunoprecipitation of cell surface proteins after they are endogenously labeled with isotopes and processed to the cell surface. The biological role which these glycoproteins play in virus biogenesis (i.e., requirements for budding of virus particles) is also investigated using standard assays for virion particle release from cells, for infectivity and for virion particle protein content and morphology.

Several properties of the MuLV system provide unique advantages to the studies described in this thesis. The glycoproteins encoded by MuLV are ideal for genetic analysis because the glycoproteins can often be purified in substantial amounts, specific antisera can be easily prepared and virus genes are relatively easy to isolate, manipulate and analyse. Furthermore, because the virus-encoded glycoproteins are not essential for cellular viability, infected cell mutants would be expected to survive. Also, the presence of two membrane glycoproteins encoded by two distinct genes in one virus offer a unique opportunity to characterize membrane glycoprotein processing in general, as shown in

section V. In this regard, the persistent and non-lytic aspect of the MuLV infection may present important advantages as a model system over the widely studied 'G' protein of vesicular stomatitis virus (148,165) in which virus infection shuts down synthesis of normal host proteins.

I. Organization of the Thesis

The results presented in this thesis are organized into five chapters (sections III-VII). These chapters correspond to different categories of results obtained in pursuit of the objectives stated in the preceding section (I, H). Section III describes a purification protocol for obtaining the MuLV envelope glycoprotein, gp70, from F-MuLV-infected Eveline suspension culture cells. Section IV presents the characterization of several cellular mutants with defects in the processing of MuLV-encoded membrane glycoproteins. Section V presents an analysis of the kinetically distinct processing of two different MuLV-encoded membrane glycoproteins from their initial synthesis to their placement on the surface of the cellular plasma membrane. Section VI presents the characterization of two viral mutants with defects in the synthesis of the gag gene-encoded membrane glycoproteins. Section VII describes the application of an immunological cell-surface rosetting technique to a focus assay for titering infectious MuLV. Because of

the distinctive nature of these sections, the results are discussed independently at the end of each section and a general summary is presented at the end (section VIII).

II. MATERIALS AND METHODS

A. Materials.

The following materials for cell culture were obtained from Grand Island Biological Company (GIBCO): cell culture media, fetal calf and calf serum, antibiotics, rabbit complement, trypsin-EDTA solutions and phosphate buffered saline (PBS). The normal goat antiserum (NGS) and the antisera made against Rauscher MuLV gp70, p30, and p12 have been previously characterized (46). The monospecific antiserum to Friend MuLV gp70 was provided by J. Collins (Duke University, Durham, N.C.) and has been previously characterized (172). The source of other materials used in these studies are indicated in the text of this chapter.

B. Cell and Virus Culture.

A subline of Eveline cells (Eveline II cells) were kindly provided by D. Bolognesi (Duke Univeristy Medical Center, Durham, N.C.). These cells, which were originally derived by infection of STU mouse cells with a Friend virus[1] (174), produce large amounts of F-MuLV and negligible amounts of spleen focus-forming virus (F-SFFV) (46). The culturing of Eveline cells has been previously described (116).

[1]Friend virus is a complex of MuLV (F-MuLV) and the replication defective spleen focus-forming virus (F-SFFV).

The Friend virus-induced erythroleukemia cell line (F4-6) was obtained from W. Ostertag. Originally derived from the spleen of a DBA/2 mouse infected with the Friend virus complex (175), the F4-6 cell line has been grown in this laboratory for several years and is therefore designated as the subline F4-6/K (176). The F4-6/K cell line has been shown to produce both MuLV and SFFV in a ratio of 1:2 (177).

The Rauscher murine leukemia virus-infected cell line, R-61, was generated by M. Ruta while working in D. Kabat's laboratory. This variant arose spontaneously (68) by cloning the virus released from the Rauscher erythroleukemia cell line RVTCT 187GG into NIH 3T3 cells as previously described (178).

The xenotropic BALB virus-2 (BC117, Electro-Nucleonics Laboratories, Inc., Bethesda, Md.) was obtained as highly purified frozen virus stock (40 X 10^6 pfu/ml), and was propagated in CC164 mink lung fibroblasts.

The F12 NRK cell line was generated by infecting NRK cells with a MuLV clone (F12) derived by cloning F4-6/K virus in SC-1 cells as previously described (177). The F12 NRK cell line is the wild-type parental cell line from which the immunoselected cell lines were generated (section II, I). These variant cell lines include H-4 (obtained by selection using anti-gp70 antiserum), p30-2 and p30-5 (obtained by selection using anti-p30 antiserum), and M-13 (obtained by selection using anti-p12

antiserum after mutagenesis of F12 NRK as described below in this section).

The NIH 3T3 fibroblasts were provided by S. A. Aaronson (Laboratory of Tumor Virus Genetics, National Cancer Institute, Bethesda, Md.). The CCL64 mink lung fibroblasts were obtained from P.J. Fischinger (Laboratory of Tumor Virus Genetics, National Cancer Institute, Bethesda, Md.). The SC-1 cells were provided by contract E-73-2001-NO 1 within the Special Virus-Cancer Program, National Institute of Health, Public Health Service, through the courtesy of Jack Weaver (Cell Culture Laboratory, University of California School of Public Health, Oakland, Calif.). Normal rat kidney (NRK) and Balb/c 3T3 fibroblasts were obtained from D. Troxler (Laboratory of Tumor Virus Genetics, National Cancer Institute, Bethesda, Md.).

The NRK, NIH 3T3, BALB/c 3T3 and CLL64 cell lines were all maintained as monolayer cultures in Dulbecco-modified Eagle medium supplemented with 10% complement-inactivated fetal calf serum or calf serum, 3.69 g $NaHCO_3$ per liter and antibiotics [penicillin (100 units/ml), streptomycin (100 ug/ml) and gentamycin (Schering, 100 ug/ml)].

The SC-1 cells were maintained as monolayer cultures in McCoy's Modified 5A medium supplemented with 10% complement-inactivated fetal calf serum and the antibiotics mentioned above for NRK cells.

The S^+L^- cells were kindly provided by P. J. Fischinger (National Institute of Health). These cells were maintained as monolayer cultures with McCoy's Modified 5A medium supplemented with 10% fetal calf serum (not heat-inactivated) and Antibiotic-Antimycotic solution (GIBCO).

Where indicated, cell monlayers are mutagenized by incubating at 37°C for 9 h with culture medium supplemented with 1.0 ug/ml N-methyl-N'-nitro-N-nitrosoguanidine (NTG, Sigma). The media containing NTG were then replaced with fresh medium and the cells were grown to confluency and utilized for immunoselection as described (section II, I).

All of the above mentioned fibroblast monolayer cultures, whether they are MuLV-infected or uninfected were maintained and routinely passaged twice weekly as described previously (179).

C. Virus Methodology.

1. Assays for Virus and Viral Components.

The titre of MuLV was measured using a variation of the S^+L^- assay of Bassin et al. (180).

A second method to assay for infectious MuLV titre was developed in this laboratory (section VII) and is called a rosette focus assay. To conduct the rosette focus assay, two special reagents are required in addition to a source of virus, susceptible fibroblasts and an appropriate antiserum. (A) Sheep erythrocyctes are

coupled to Staphylococcus aureus protein A (see below, section II, H2) using a modification of Goding's $CrCl_3$ method (181). (B) A hemoglobin stain is prepared (182) by mixing o-dianisine or benzidine (100 mg per 70 mls ethanol), 0.15 M sodium acetate (pH 4.7), distilled water and 30% hydrogen peroxide in a ratio of 20:5:7:1 just prior to use. The methodology for the rosette focus assay is as follows. Briefly, $1x10^5$ cells are plated in a $25cm^2$ tissue culture flask containing 5 mls of the growth medium appropriate for the cell type and containing 10% heat-inactivated fetal calf serum and antibiotics. After 24 hrs of incubation in 5% CO_2 at 37°C, the medium is removed and replaced with 1 ml of the same medium but also containing 25 ug of DEAE-dextran (Sigma Chemical Co.) per ml for 30 min. at 37°C. After decanting the dextran, 1.0 ml of virus sample (in various dilutions) is added, and the flasks are incubated for 30 min. Five milliliters of fresh medium are then added and the flasks are incubated at 37°C until the monolayers reach confluency (e.g., 5 days for the cell types used in this study). Rosette analysis is conducted on the confluent monolayers as described below (Section II, H2). Following rosetting, the monolayers are washed twice with prewarmed PBS and are immediately fixed and stained by flooding the flask with staining solution "B". After 10 min the stain is removed and the flasks are rinsed with distilled water and air dried.

A final method for assaying viral components is the assay for reverse transcriptase activity described in section II, K. This method specifically detects the release of virion cores into culture medium which contain reverse transcriptase.

2. Harvesting and Purification of Virus.

Eveline virus was harvested for gp70 purification from the medium of logarithmically growing Eveline cell cultures as described (46). The cells were removed from the medium by centrifugation at 3000 rpm for 10 min in a Sorvall GSA rotor at 4°C, and the supernate was further clarified by centriguation at 10,000 rpm for 10 min. Virus was then collected by high-speed pelleting in a Beckman SW 27 rotor for 90 min at 25,000 rpm or in a Beckman T 30 rotor for 60 min at 27,000 rpm. Virus pellets obtained were either used at this stage of preparation or resuspended in a small volume of TSE (0.1 M NaCl, 0.01 M Tris-HCl [pH 7.4], 1 mM EDTA) and layered onto linear gradients of 15-60% sucrose in TSE for isopycnic banding. After centrifugation of sucrose gradients in a Beckman SW 27 rotor for 3 hours at 25,000 rpm, the band at 1.15-1.17 g/cm^3 was collected, diluted in TSE and re-pelleted in a Beckman SW 27 rotor for 90 min at 25,000 rpm. This preparation of virus is referred to as "sucrose-banded virus" in contrast to "pelleted virus" in which sucrose-gradient banding is not conducted. Sucrose-gradient banding of virus will remove contaminants present

in the media (e.g., serum albumin) but will also produce osmotic shock in which gp70 will be partially released from virions (124,183). Conversely, "pelleted virus" contains higher yields of gp70 but also contains more contaminants (section III).

The harvesting of virus from the culture medium of fibroblast monolayers grown to 50-75% confluency was conducted as described above for Eveline virus.

When virus samples were harvested for later use in assays for titre (e.g. S^+L^- assay or other plaque assays) or for infecting cells, the media over logarithmically growing cells was collected, clarified by low speed centriguation in a Beckman SS34 rotor for 10 min at 10,000 rpm and frozen in sterile 2 ml aliquots at -70°C until needed.

3. Virus Infections.

Cells to be infected exogenously were plated at 5×10^4 cells per 25 cm^2 culture flask 24 hours prior to infection. The infecting virus samples were adjusted to 8 ug polybrene (Aldrich) per ml. 1.0 ml of this infecting virus sample was overlaid onto receipient cells and incubated for 1 hour at 37°C. Afterwards, 4 mls of fresh medium containing polybrene (8 ug/ml) was added to the cells and they were grown to confluency before subsequent transfers.

4. Radioactive Labeling of Viral Proteins.

Viral proteins were metabolically labeled

with L-[^{35}S]-methionine or D-[^{3}H]-glucosamine (New England Nuclear). Amino acid labeling was performed by incubating virus-infected cells in methionine-free medium (MEM) containing 10% dialysed fetal calf serum supplemented with L-[^{35}S]-methionine at concentrations of 50-100 uCi per ml using 1.0 ml per 25 cm^{2} culture flask for monolayers or at concentrations of 1-1.5 mCi per 10^{8} cells per 75 mls for suspension cultures. Before labeling, the cells are first washed with PBS and then preincubated for 15 min at 37°C in methionine-free medium. D-[^{3}H]-glucosamine labeling was performed simply by adding it to complete medium and incubating the cells 4-18 hours at concentrations of 100 uCi per ml per 25cm^{2} culture flask for monolayers or at 1 mCi per 2 x 10^{6} cells per 25 mls for suspension cultures. Labeling for a pulse and chase period was conducted by incubating the cells as indicated above for a "pulse" interval with L-[^{35}S]-methionine, removing the medium containing the isotope at the indicated time, washing the cells once with PBS and adding fresh complete medium to the cells for a "chase" interval.

For labeling cells in conditions of glycoylation inhibition, cultures were initially exposed to 0.6 ug of tunicamycin (Calbiochem) per ml of complete medium for three hours. The cultures were then radioactively labeled as usual but including tunicamycin in both the pre-pulse and pulse incubations. During the subsequent immunoprecipitations, the cell lysates were

preadsorbed with normal goat serum twice to remove non-specific immunoprecipitates.

D. Purification of the Murine Leukemia Virus Envelope Gylcoprotein, gp70.

1. Solubilization of Virion Components.

Eveline virus which had been harvested from Eveline cells by either sucrose-gradient banding or by simply pelleting (as described in section II, C2) was solubilized in Triton X-100 containing buffers to disperse all the virion components including gp70. Unless otherwise noted, all manipulations for purifying gp70 were conducted on ice or in cold rooms at 4°C. Viral pellets were resuspended at 1 mg/ml by vigorous pipetting to generate a homogeneous suspension in detergent solubilization buffer [DSB; 0.25% Triton X-100 (Sigma), 100 mM NaCl, 20 mM Tris-HCl (pH 7.6), 0.1 phenylmethyl sulfony/flouride (PMSF;Sigma), and 1 mM EDTA]. The resulting detergent suspension was centrifuged in a Beckman T 65 rotor at 25,000 rpm for 20 min to remove nondisrupted virion cores and debris, and the detergent supernate was retained. The pellet was then recycled once more through the above solubilization procedure to further extract virion proteins. The resulting second-cycle detergent supernate was pooled with the first for use in later steps.

2. Lectin Affinity Chromatography.

Lectin affinity-purified virion glycoproteins

were prepared from the solubilized virus supernate generated as described in Section II, D1. This detergent supernate was incubated with agitation on a gyratory shaker on ice for 1 hour with wheat germ agglutinin immobilized on agarose beads (P & L Biochemicals) using 5 mls of supernate per ml of packed beads. The beads were then pelleted and the resulting detergent supernate (referred to as 'unadsorbed sample') was retained. Finally, the beads were resuspended in DSB and transferred to a small syringe column. Once in the column, the lectin was first rinsed with DSB, then washed with DSB lacking Triton X-100 but including 300 mM NaCl. The adsorbed glycoproteins were then eluted at a flow rate of 3-5 mls/hr in the second wash buffer containing 0.2 M N-acetyl-D-glucosamine. After elution the lectin column was re-equilibrated by washing with DSB, and the unadsorbed sample was allowed to adsorb with the lectin a second time as described above. These two eluants were routinely combined for subsequent manipulations, although yields of specifically eluted glycoproteins obtained on the second cycle were approximately 30% or less than the yields obtained from the first cycle.

3. Sephadex G-150 Gel Filtration.

Glycoproteins specifically eluted from the wheat germ agglutinin affinity column (section II,D2) were then chromatographed on a Sephadex G-150 column in the presence of reducing agents to separate gp70 from p15(E)

and other contaminants. First, the glycoprotein-containing sugar eluant was prepared for loading onto the Sephadex column by concentrating the sample down to a minimum volume. This concentration step was done by placing a dialysis bag containing the sample into dry Biogel P-100 beads (Biorad) and allowing moisture to flow out of the sample. The reduction of volume from 3 mls to 1 ml took approximately 4 hours and required that every 30 min moist beads be removed from around the dialysis bag and re-covered with more dry beads. Second, the disulfide bridge between p15(E) and gp70 (97,105,106) was reduced so that gel filtration chromatography would resolve these two components. For this reduction, a '10x' solution of dithiothreitol (Sigma) prepared in sugar elution buffer was added to the sample to make a final concentration of 10 mM before it was concentrated in a dialysis bag against dry Biogel beads. Finally, the reduced and concentrated sample was applied to a Sephadex G-150 column (90 cm height, 102 ml bed volume) and chromatographed at a flow rate of 0.25 ml/min in 10 mM sodium phosphate (pH 6.5), 300 mM NaCl and 0.1 mM PMSF. The Sephadex G-150 column was re-run with molecular weight markers (bromphenol blue, cytochrome c and blue dextran 2000) to evaluate the proper operation and resolving power of the column (see section III, figure 3).

4. Iodination of gp70.

It was observed upon polyacrylamide-SDS gel

electrophoresis (section III, figure 1) that electrophoretic homogeneity of our sample was not improved by gel filtration (see Results, section III). Therefore, the gp70 sample used for iodonation was the lectin affinity-purified material and not the peak fractions obtained from Sephadex G-150 chromatography. gp70 was iodonated with carrier-free [^{125}I]-sodium iodide (ICN) according to a modification (236) of the chloramine-T method (184) using 1 mCi per 20 ug protein. After iodonation, unreacted free iodine was separated from [^{125}I]-labeled protein using a Sephadex G-25 desalting column as described by Greenwood et al. (184). Aliquots of three consecutive fractions were then diluted 1:1 in electrophoresis sample buffer and analysed for homogeneity by electrophoresis on polyacrylamide gels.

5. gp70 Receptor Binding Assay.

[^{125}I]-labeled gp70 (prepared in section II, C4) was used to probe the surface of F-MuLV infected NIH 3T3, Balb/c and NRK fibroblasts for gp70-binding receptors according to DeLarco and Todaro (117).

E. Extraction of Labeled Cells.

Metabolically labeled cells were routinely extracted by lysis at 4°C for 30 min in immunoprecipitation buffer (IPB, 20 mM Tris-HCl [pH 7.5], 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 M NaCl, 1 mM EDTA, 0.2% sodium azide) using 1 ml per 25 cm^2 culture flask of fibroblast monolayer or 1 ml per 4 x 10^6

suspension cells. The extracts were then clarified by centrifugation at 140,000 xg for 35 min in a Beckman T 65 rotor. Cells iodonated for two-dimensional analysis on O'Farrell gels were lysed in a minimal volume (0.3 ml/ 25 cm^2 culture flask) of lysis buffer (185), and aliquots containing equal quantities of trichloroacetic acid-precipitable counts (section II, M) were loaded onto gels. The extraction procedures for the cell surface protein detection method are described in section II, H4.

F. Immunoprecipitation Procedures.

Immunoprecipitation was performed by using a modification (186) of the fixed Staphylococcus aureus (Pansorbin, Sigma) procedure described by Kessler (187) but omitting bovine serum albumin from the buffers. The Pansorbin was suspended (10%) in IPB and centrifuged through 1 M sucrose in IPB without NaCl prior to use. The radioactive cell extracts were first preadsorbed with normal goat serum to reduce nonspecific immunoprecipitation, followed by specific immunoprecipitation with various antisera. Specific immunoprecipitations routinely used 1 ul serum per ml of cell lysate incubating for 1 hour at 4°C followed by 20 ul Pansorbin per microliter serum for 1 hour at 4°C. However, for cell extracts generated by the cell surface protein detection method (described in section II, H4), only 5 ul Pansorbin was used for each 1 ml sample to minimize non-specific immunoprecipitation.

G. SDS-Polyacrylamide Gel Electrophoresis

Proteins were separated on 10-20% polyacrylamide gradient slab gels containing 0.1% SDS using the system described by Laemmli (188). 10% polyacrylamide slab gels containing 0.1% SDS and 8 M Urea were prepared using the Lzemmli buffers with minor modifications as described by Evans et al. (46). Two-dimensional polyacrylamide gel electrophoresis was performed essentially as described by O'Farrell (185), with minor modifications as described (72), with the generous help of S. Clarke.

Antigen-antibody complexes were disrupted by heating the samples in Laemmli electrophoresis buffer (0.05 M Tris-HCI [pH 6.8], 1% SDS, 1% β-mercaptoethanol, 20% glycerol) at 100°C for 5-10 min. The Pansorbin was then pelleted by centrifugation (Beckman JS 7.5 rotor, 7500 rpm, 10 min.), and the supernates were carefully transferred to individual lanes on the gels using a 50 ul Hamilton syringe. All gels containing radioactive proteins were processed for flourography according to Bonner and Laskey (189) or by using EN^{3}HANCE (New England Nuclear), and exposed at -70°C to Kodak XR-5 X-ray film. Gels containing [^{125}I]-labeled proteins were exposed to X-ray film as above except that the gel and film were sandwiched between intensifying screens ('lightning plus' Cronex screens, Dupont). Gels used for quantitation of bands were preflashed (190) before exposure to X-ray films, and were scanned with a Transidyne General

integrating densitometer, as described previously (72). Gels to be stained for protein were fixed overnight in 12.5% tri-chloroacetic acid at room temperature and stained in a 0.2% coomassie brilliant blue solution in 10% acetic acid; 20% methanol for 10-12 hours. The unbound stain was removed by immersion in 10% acetic acid; 20% methanol for 24 hours with several changes. [^{14}C]-labeled molecular weight standards (New England Nuclear) with molecular weights of 92,500, 69,000, 46,000, 30,000 and 12,300 daltons were routinely electrophoresed in parallel with the samples.

H. Detection of Cell Surface Proteins

1. Complement - and Antibody-Dependent Cytolysis

Killing of cultured fibroblasts by cytotoxic antibody in the presence of rabbit complement has been previously described (172). This killing was monitored microscopically by observing the rounding up of cells and the condensation of their nuclei, followed by the loss of their adherence to the substratum. In addition, this killing is quantitatively analysed by measuring the release of incorporated [^{35}S]-methionine from the monolayer using a previously described procedure (172) with minor modifications. Cells were pulse labeled for 2 hours with [^{35}S]-methionine as described in section II, C4. After this incorporation period, the monolayers were thoroughly washed three times with PBS at 25°C. Each flask was then given 1.0 ml of prepared killing medium

containing cell culture medium, rabbit complement and cytotoxic antiserum in a ratio of 1100:10:3. The flasks were incubated at 25°C and 50 ml samples of media were taken at the indicated time points. The radioactivity present in samples taken was counted in 3.0 mls "Scint-A" scintillation flour (Packard) on a Beckman liquid scintillation spectrometer. The amount of radioactivity released was expressed as a percentage of the total amount of incorporated radioactivity which was determined by adding the released radioactivity to that which could be recovered by lysing the monolayers with 0.1% SDS at the end of the killing period.

2. Rosette Assay for Cell Surface Antigens. Sheep erythrocytes were coupled with S. Aureus protein A (Sigma) by the $CrCl_3$ method of Goding (181). Briefly, 100 ul (sedimented volume) of sheep erythrocyctes are washed five times in 0.9% NaCl and suspended at 25°C in 700 ul 0.9% NaCl containing 100 ug protein A (Sigma Chemical Co.). Then, 500 ul of 0.004% $CrCl_3$ (prepared as described by Goding (181) and diluted immediately before use) is added dropwise while continuously vortexing the erythrocyte suspension. After 10 min, the suspension is diluted fivefold with PBS and washed four times by sedimentation with PBS. The coated erythrocytes are then suspended in 6 mls of Dulbecco-modified Eagle medium containing 10% heat-inactivated fetal calf serum and are ready to use after 24 hrs storage

at 4°C. Fetal calf serum seems to be essential for coupled erythrocyte stability. For rosette analysis, fibroblast monolayer cultures in 25 cm^2 T-flasks were washed and then provided with 5 ml of fresh culture medium containing 10% heat-inactivated fetal calf serum. Cultures were then supplemented with 4 ml of antiserum or normal goat serum. After 15 min at 37°C, 0.2 ml of the coupled erythrocyte suspension was added to each culture. After 2 h at 37°C, the monolayers were washed with fresh culture medium and examined under a microscope.

3. Cell Surface Radioiodonation

Lactoperoxidase-catalysed cell surface radioiodonation of fibroblast monolayers with [^{125}I]-iodine was performed as previously described (172). Cells iodonated for two-dimensional electrophoretic analysis were lysed in a minimal volume (0.3 ml/25 cm^2 culture flask) of lysis buffer (185), and aliquots containing equal quantities of trichloroacetic acid-precipitable counts were loaded onto the gels.

4. Endogenous Labeling of Cell Surface Proteins

The selective binding and extraction of cell surface antigens with specific antisera was performed as described (191,192) with some modifications. Eveline cells (10^8 cells) were pulse-labeled with L- [^{35}S]-methionine and then chased with cold methionine as described in section II, C4 for the six time points indicated in figure 14 (section V). At the end of each

labeling period, one sixth of the total culture was removed and chilled to 0°C to terminate protein synthesis, processing and transport. The cells were then collected by low speed centrifugation and the culture medium was retained. Each cell pellet was resuspended in PBS and divided four ways equally to be analysed for virus-encoded proteins immunoprecipitable (1) from whole cell extracts (as described in Section II, E) or from the cell surface as described below (2) with anti-gp70 antiserum, (3) with anti-p12 plus p30 antisera or (4) with normal goat antiserum. From the retained cell-free culture medium Eveline virus was pelleted as previously described

To conduct the selective binding and extraction of MuLV-encoded antigens on Eveline cells, 4 x 10^6 cells from each time point were incubated for 30 min at 0°C in 1 ml of PBS containing either anti-gp70 antiserum (4 ul), anti-p12 plus anti-p30 antiserum (2 ul each, combined) or normal goat serum (4 ul). Cells were then washed twice with PBS at 0°C to remove unbound antibodies and lysed with 1 ml of IPB detergent lysate containing a ten-fold excess of an unlabeled Eveline cell extract prepared as described below. These lysates were then centrifuged on a Beckman T65 rotor at 2000 rpm for 20 min, and the supernates were recovered for immunoprecipitation (Section II, F).

The preparation of a ten-fold excess of unlabeled cell extract was modified because when applied

to Eveline cells it produced extracts of excessive viscosity upon nuclear lysis with IPB. This viscosity interfered with the aspiration of wash supernates in the immunoprecipitation procedure. To prepare this extract a ten-fold excess of Eveline cells (4×10^7 cells) were pelleted from growth medium and resuspended in 1 ml of nuclei separation buffer (NSB; 20mM Tris-HCL [pH 7.4], 0.25% Triton X-100, 0.1 M NaCl) and allowed to swell at 0°C. The swollen cells were disrupted with 20-40 strokes in a homogenizer using a rotating teflon plunger. The progress of disruption was monitored microscopically until the cells were broken but greater than 95% of the nuclei remained intact. The nuclei were then pelleted on a Beckman JA-20 rotor at 1000 rpm for 10 min and the supernate was reconstituted to an IPB solution by the addition of concentrated detergents and salts.

I. Immunoselection of Resistant Cell Lines

The immunoselection procedure, which utilizes the killing of cloned cultured fibroblasts by cytotoxic antibody in the presence of rabbit complement, has been previously described (172). F-MuLV clone F-12 infected NRK cells (F12 NRK) were used as the parental population. Immunoselection was with highly cytotoxic monospecific antiserum to Friend MuLV gp70 and with antiserum to Rauscher MuLV p30 and p12. Following selection of immunoresistant cell populations, cells were cloned to

obtain the individual cell lines. The cell lines studied were the H-4 cells isolated with antibody to gp70, the p30-2, p30-5 and p30-12 cell lines isolated with antibody to p30, the p12-12 cell line isolated with antibody to p12, and the M-13 cell line isolated with antibody to p12 after F12NRK had been mutagenized with NTG as described in section II, B. All of the immunoresistant variants of the MuLV-infected NRK cells have glycoprotein processing defects that have been stable during culturing for many months in the absence of antisera. However, some resistant cell lines grow only relatively slowly and tend to be overgrown by secondary variants that proliferate more rapidly. Nevertheless, morphological observations and analyses of specific membrane processing abnormalities as described in section IV suggest that the secondary variants contain suppressor-type alterations and are not true revertants. Thus the glycoprotein processing abnormalities in these cell lines are stably inherited despite the morphological and growth rate progression that is sometimes observed. Even highly abnormal and slowly growing cell lines such as those shown in Figure 5 have been maintained by occasional recloning to eliminate secondary variants. Because these immunoresistant cell lines have stably inherited abnormalities, we refer to them in the text as mutants. However, we recognize that some cellular variation may be caused by chromosome loss (193).

J. Cell Fusion

Cell fusion of monolayers was induced with polyethylene glycol (PEG), as previously described (194), with some modifications. Cells or equal ratios of two different types of cells were plated at low density (10% confluency) and cultured for 24 hr. The culture medium was thoroughly drained off, and 50% (w/v) PEG 6000 (J.T. Baker) in complete medium was added. After exactly 1 min, the PEG solution was rapidly aspirated with a wide-mouthed pipette, and the monolayer was washed vigorously four times with serum-free media. After a fifth wash with complete medium, the monolayer was incubated in complete medium until needed.

K. Reverse Transcriptase Assay

Reverse transcriptase released into the culture media was assayed in pellets of virus harvested from the culture fluid by high-speed centrifugation (Beckman T65 rotor, 40,000 rpm, 35 min) after low speed centrifugation to remove cells and debris (Beckman SS34 rotor, 10,000 rpm, 10 min). The enzyme assay was conducted at 30°C for 30 min as described (195) for a synthetic template-primer reaction in a final reaction mixture that contained: 10% glycerol, 30 mM Tris-HCl, 5 mM $MnCl_2$, 2 mM DTT, 80 mM KCl, 1.5 ug poly(dA)-oligo(dT) (P-L Biochemicals), 0.2 mM dATP, 2 uCi[^{3}H]-dTTp (NEN, 60 Ci/mM) and sufficient unlabeled dTTP for a final concentration of 2 uM in a total volume of 30 ul.

L. Electron Microscopy

Thin sections of monolayers growing in log phase were examined for budding virus according to standard electron microscopy methods (176) with the generous help of M. Webb.

M. Scintillation Procedures

The radioactivity present in various metabolically labeled protein preparations was determined by precipitating a small aliquot (2-5 ul) of the sample with tricholoracetic acid (TCA) after two drops of 2% bovine serum albumin had been added as carrier. The precipitate was collected on a glass fiber filter (Whatman) and digested with Protosol (New England Nuclear): toluene (1:2) for 30 min in a scintillation vial. After the addition of 5 ml of toluene-acetic acid scintillation fluid (Baker scintillation grade toluene containing 0.3% 2,5-diphenyloxazole, 0.03% p-Bis [2-(5-phenyloxazoly)]-benzene, and 0.1% glacial acetic acid), the sample was counted in a Packard Tri-Carb liquid scintillation spectrometer. Protein preparations labeled with [^{125}I]-sodium iodide were precipitated as described above, but the resulting precipitate was counted directly on the glass fiber filter in a Beckman 'Biogamma' counter set for [^{125}I].

N. Protein Determinations

Protein was assayed by a modification of the procedure of Schaffner and Weissman (196). Protein

samples were adjusted to 0.25 ml with water. Fifty microliter of 0.6 M Tris-HCl, pH 8.0, 0.6% SDS was added to each sample and mixed. One hundred microliter of 50% trichloracetic acid (TCA) was added to each sample and incubated on ice for 5 minutes. The precipitates were filtered on 0.45 um Millipore filters and washed with 5% TCA. The filters were placed in scintillation vials and stained for 10 minutes with a 0.2% solution of Amido-Schwartz 10B (Allied Chemical) in 45% methanol and 10% acetic acid. Then the stain was removed and the filters rinsed twice with water. The filters were destained with three rinses of 90% methanol, 2% acetic acid solution. The destaining procedure should leave the borders of the filter white. The filters were then rinsed twice with water and drained completely. The stain remaining bound to the protein precipitate on the filter was eluted with 1 ml of 0.025 M NaOH, 0.00005 M EDTA, 50% ethanol for 10 minutes or until all the stain had eluted. The scintillation vials were capped during the elution step. The protein in each sample was quantitated from the absorbance at 630 nm of the eluted stain relative to a bovine serum albumin standard Protein samplecurve.

O. Subcellular Fractionation

The subcellular fractionation of F4-6 cells (section V) was conducted as described (197) with the generous assistance of David Kabat and Suzanne Clarke.

III. gp70 PURIFICATION

A. General Introduction

gp70[1] is the major envelope glycoprotein of murine leukemia viruses. It plays an essential role in virus infectivity, host range, interference to superinfecting virus and even host immune response to infection as described in the Introduction (section I, F2). The manner in which gp70 participates in these various biological interactions is not clearly understood and this has motivated us to purify gp70 from whole Eveline virus in order to pursue several specific goals:

1. To use gp70 as a ligand to probe for receptor-mediated interactions between infectious virus and susceptible host cells. The specificity of the gp70 interaction with cell surface receptors is interesting because it defines the tropism of the infecting virus and generates the interference phenonmenon (117).

2. To adapt this receptor binding property of gp70 (117,128,131-133) so that an assay would be available to monitor the purification of gp70-binding receptors from susceptible host cell membranes.

[1]The term "gp70" is used here, generically, to mean the viral envelope glycoprotein of the MuLV. In Eveline cells this protein occurs on SDS gels as a doublet of apparent Mr of 69,000 and 71,000 daltons (as can be seen in Fig. 1). gp69/70 doublets occur in many strains of MuLV (42,200) and have been shown in Eveline cell cultures to be caused by the presence of multiple viral genomes that may be incorporated in a single heterozygous virus particle (116). For simplicity, in the text gp70 and gp69/71 will be considered synonomous.

3. To generate anti-gp70 antiserum for use in immunoprecipitation and other immunochemical techniques for mapping the sub-cellular localization and processing pathways of gp70.

Many gp70 purification schemes have been previously reported (107,124,198,199) from which we could select a methodology appropriate to our goals. They vary, however, in strategy and yield. Methods for the isolation of crude gp70 include the selective release of gp70 from intact virions by freeze-thawing (124), selective detergent solubilization of membrane glycoportein aggregates called "rosettes" which contain gp70 and p15(E) (107) and more harsh extractions using chaotropic agents (e.g., 3.5 M KBr) (199). Further purification of gp70 was accomplished by ion-exchange chromotography on phosphocellulose P-11 (124) or by using the lectin, concanavalin-A, in affinity chromatography (198).

In this chapter a protocol for the purification of gp70 is presented. This protocol combines the use of detergents to solubilize the membrane glycoprotein with the selectivity of lectin affinity chromatography.

B. Results.

1. Harvesting of Eveline Virus.

Eveline virus was harvested from suspension cultures of Eveline cells which were grown as described in Materials and Methods (section II, C2). gp70 was then

purified from the Eveline virus. Two methods of harvesting Eveline virus were used to compare yields and purity. (1) "Sucrose-banded virus" was prepared by pelleting virus from culture medium, resuspending the viral pellet in buffered TSE (see section II, B) and layering the virus onto a sucrose gradient for isopycnic banding. The banded virus was then collected with a fraction collector and repelleted. (2) "Pelleted virus" was prepared simply by pelleting virus from culture medium and omitting all subsequent steps detailed above for sucrose-banded virus. Pelleted virus was found to have higher yields of gp70 than sucrose-banded virus presumably because the osmotic shock of sucrose-banding can cause release of the gp70 from virions (124,183,198). On the other hand, sucrose-banded virus had fewer contaminants originating from the culture medium, particularly serum albumin which migrates with, and slightly above, gp70 on SDS gels (compare Fig. 1A, 1B).

2. Solubilization of gp70

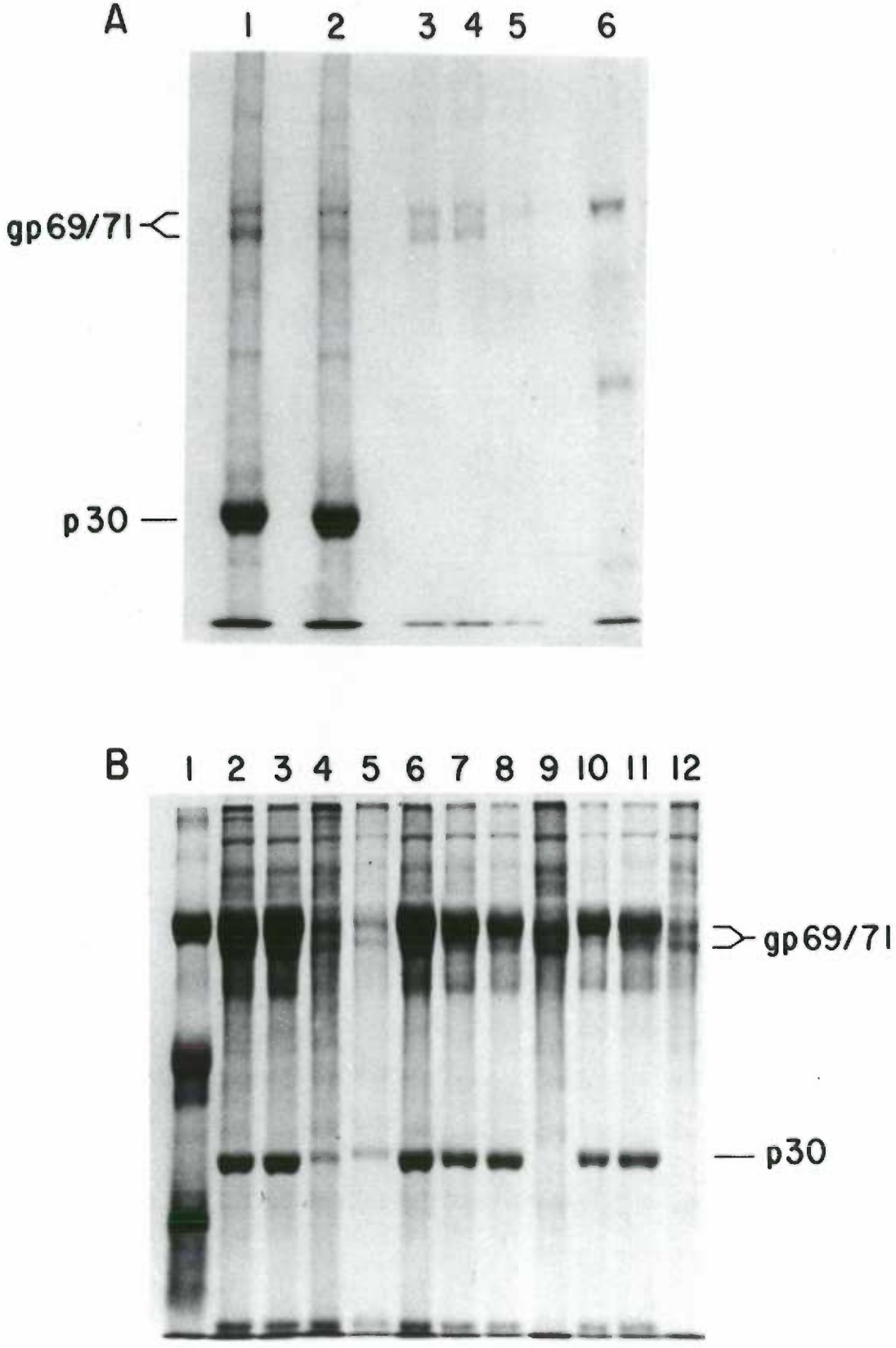
To solubilize gp70 for purification from intact virions, purified virus was disrupted using a buffered 0.25% Triton X-100 solution. We chose detergent solubilization because our yields of gp70 using the freeze-thaw method (124) were less than 30% (data not shown). Triton X-100 was selected to solubilize gp70 for two reasons. (i) Sucessful purifications of other membrane glycoproteins were reported using Triton X-100 (201)

Figure 1. Polyacrylamide Gel Electrophoresis of Protein Samples at Various Stages of gp70 Purification.

(Panel A) The source of gp70 is sucrose-gradient banded Eveline virus, and is purified as described in Materials and Methods. (1) Detergent supernates containing soluble proteins pooled after two cycles of solubilizing virus in Triton X-100, (2) Unadsorbed material after lectin adsorption, (3) sugar specific lectin eluant, (4) sugar specific lectin eluants from two cycles of adsorption after being pooled and concentrated for loading onto gel filtration column, (5) peak protein fraction off of Sephadex G-150, (6) protein standards: bovine serum albumin (70,000), ovalbumin (45,000), chymotrypsinogen A (25,000), and ribonuclease A (13,700).

(Panel B) The source of gp70 is Eveline virus pelleted directly from culture medium, and is purified as described in Materials and Methods. (1) Protein standards as in lane 6, Panel A, (2 and 4) detergent suspension, cycle 1 (2) or cycle 2 (4), (3 and 5) detergent supernates, cycle 1 (3) or cycle 2 (5), (6) detergent supernate, pooled samples from cycles 1 and 2, (7 and 10) unadsorbed materials after lectin adsorption, cycle 1 (7) or cycle 2 (10), (8 and 11) run off of lectin column rinse, cycle 1 (8) or cycle 2 (11), (9 and 12) sugar specific eluant, cycle 1 (9) or cycle 2 (12).

Electrophoresis was conducted on 10% polyacrylamide slab gels containing 1.0% SDS, 8 M urea in the Laemmli buffer system as described in Materials and Methods. Gels were stained for protein in 0.2% coomassie brilliant blue solutions as described in Materials and Methods.


A
1
2
3
4
5
6
gp69/71
p30
B
1
2
3
4
5
6
7
8
9
10
11
12
gp69/71
p30

including the insulin and acetylcholine receptors and, (ii) a recent report described that the specific affinity of the lectin, wheat germ agglutinin, was not lowered by Triton X-100 (202).

Fig. 1A and 1B show the preparation of gp70 through various stages of purification. Typically, 20 mg of sucrose-banded or pelleted virus was solubilized in detergent to generate a suspension (Fig. 1B, lane 2). From this suspension unsolubilized cores and debris were then pelleted to generate a Triton X-100 supernate (Fig. 1A, lane 1, or Fig. 1B, lane 3). Routinely this pellet was resuspended in detergent to obtain the gp70 that was not solubilized in the first cycle (Fig. 1B, lane 4). This suspension was then repelleted (Fig. 1B, lane 5), and Triton X-100 supernates from cycle 1 and cycle 2 were pooled (Fig. 1A, lane 1, or Fig. 1B, lane 6). A comparison of Fig. 1A, lane 1, and Fig. 1B, lane 6, shows that sucrose-banding eliminates a major contaminant at 70,000 daltons apparent molecular weight, presumably serum albumin.

The efficiency of protein solubilization by Triton X-100 was determined by various observations. A visual inspection of Fig. 1A, lane 1, shows that two major proteins were released into detergent supernates which are identified as gp69/71 and p30 on the basis of their electrophoretic mobility. Furthermore, we see that a majority of the total virion protein was released by the

first cycle of solubilization and significantly less protein was released into the detergent supernate in the second cycle of solubilization (compare lanes 3 and 5, Fig. 1B, in which equal volumes of detergent supernate were applied to the gel in order to compare the extraction efficiencies). Since gp70 is the only major MuLV-encoded glycoprotein detectable in virus preparations by labeling with [^{3}H]-glucosamine (97) (unpublished observations), we can use the specific activity of incorporated [^{3}H]-glucosamine as a specific marker to monitor the enrichment of gp70 as it is purified from sucrose-gradient banded virus (Table 1). 62% of the [^{3}H]-glucosamine labeled gp70 was solubilized into supernates after two cycles of detergent solubilization. Furthermore, 1.4X enrichment of gp70 was occurring here because only 43% of the total protein was released in these same two cycles of solubilization.

3. Affinity Chromotography on Wheat Germ Agglutinin

Detergent-solubilized gp70 was then purified away from non-glycosylated virion proteins and contaminants by affinity chromatography using the lectin, wheat germ agglutinin (WGA), obtained from Triticum vulgaris. WGA specifically binds to the sugar, N-acetyl-D-glucosamine (2), which is commonly found on the peripheral region of complex oligosaccharides (section I, G) (100). Binding of gp70 to the lectin was conducted batchwise at 0° C for

Table 1

Purification[a] of Friend Murine Leukemia Virus gp70

Purification Step	Protein[b]		[^{3}H]-Glucosamine[c]		Specific Activity	Factor of Enrichment
	mg.	% Total	cpm	% Total	cpm/mg	
Detergent suspension	22.30	100	907,000	100	40,700	1.0
Detergent supernate	9.65	43	563,600	62	58,400	1.4
Lectin eluant	0.51	2.3	209,400	23	407,400	10.0
Sephadex peak fractions	0.29	1.3	82,000	9	282,800	7.0

[a]The purification of gp70 from F-MuLV is descrbed in the text and involves harvesting virus from Eveline cells by sucrose-gradient banding (method 1), generating a detergent suspension by solubilizing virus in 0.25% Triton X-100, pelleting suspended debris to obtain a soluble protein detergent supernate, enriching for gylcoproteins by use of the lectin, wheat germ agglutinin, and performing gel filtration on Sephadex G-150.

[b]Protein was assayed by a modification of the method of Schaffner and Weissman (196).

[c][^{3}H]-glucosamine was assayed by determining trichloroacetic acid precipitable counts as described in Materials and Methods. gp70 is the only protein in released virion particles which incorporates [^{3}H]-glucosamine during an 18 hr labeling period (97). Therefore incorporated [^{3}H]-glucosamine serves as a specific marker for gp70 during its purification away from contaminating protein.

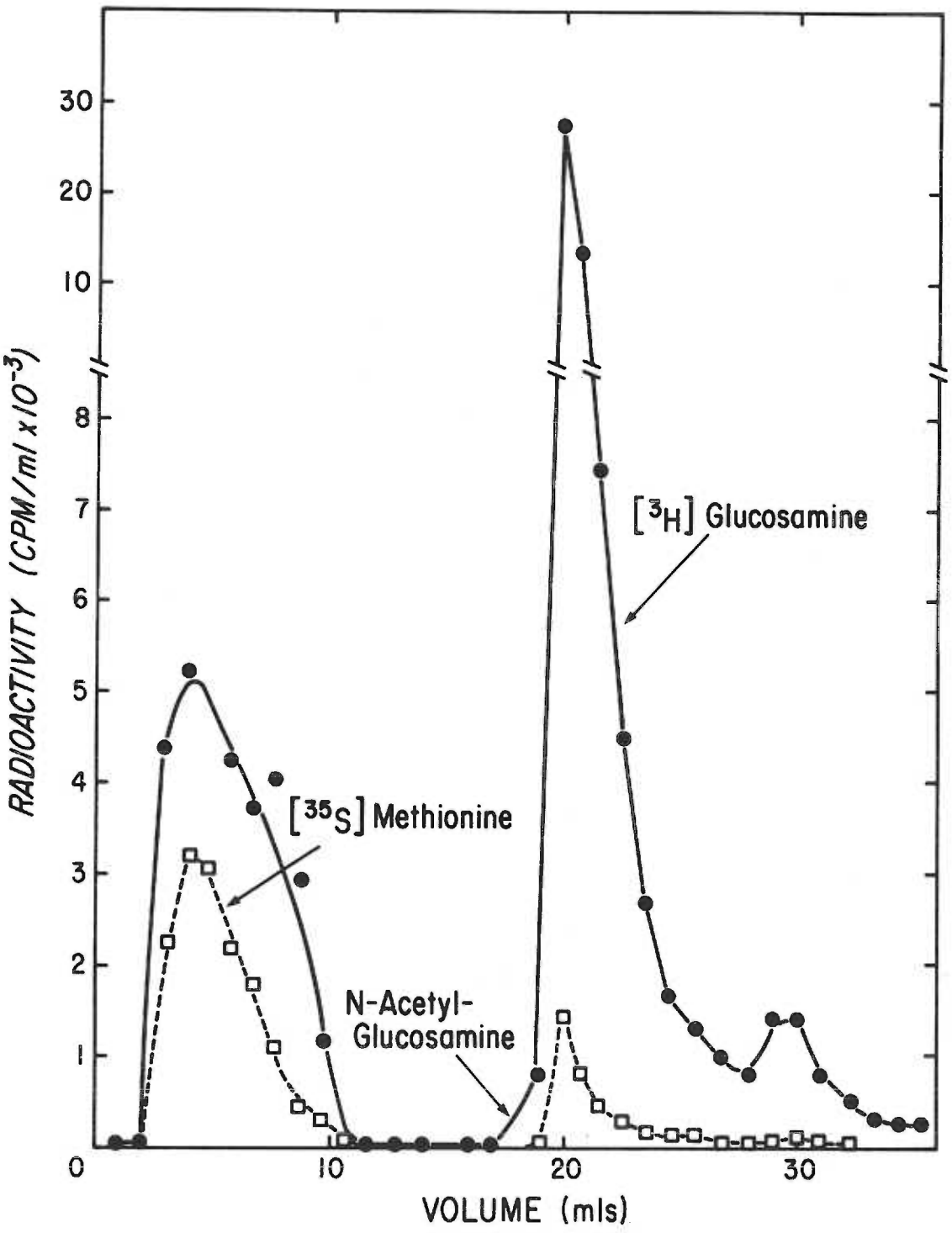
hour. Then the agarose beads containing immobilized WGA and adsorbed glycoproteins were transferred to a small column, washed and specifically eluted with 0.2 M N-acetyl-D-glucosamine as described in Materials and Methods (section II, D2).

The use of WGA to purify gp70 resulted in significant enrichment of gp70 away from p30 (see Fig. 1A, lane 3 or Fig. 1B, lanes 9 and 12). Furthermore, WGA chromatography of gp70 effectively removed contaminating serum albumin present in virus preparations that were not sucrose-banded (compare lanes 8 and 9 or lanes 11 and 12, Fig. 1B). When a second cycle of lectin adsorption was conducted on the detergent supernate, more gp70 was obtained (Fig. 1B, lane 12). However, the second cycle yield of gp70 was less than 10% of the total yield, indicating that the lectin had not been overloaded on the first cycle.

To monitor the enrichment of gp70 directly during the course of the affinity chromatography, Eveline virus was double-labeled prior to harvesting with [^{35}S]-methionine and [^{3}H]-glucosamine. Sugar specific elution (Figure 2) significantly increased the ratio of [^{3}H]-glucosamine to [^{35}S]-methionine above the ratio found in the original detergent supernate. As shown in Table 1, the total yield of gp70 recoveries, as measured by [^{3}H]-glucosamine, was 23% of the input radioactivity after WGA purification. This gp70 yield is superior to a net yield

Figure 2. Lectin Affinity Chromatography of Detergent Solubilized gp70 on Wheat Germ Agglutinin

Eveline virus was harvested by the sucrose-gradient banding method from Eveline cells that were double-labeled with [^{3}H]-glycosamine and [^{35}S]-methionine continuously for 18 hours as described in Materials and Methods. The resulting virus was solubilized in 0.25% Triton X-100 and supernate containing soluble proteins were collected by centrifugation as described in Materials and Methods. Batchwise adsorption of glycoproteins to agarose-immobilized wheat germ agglutinin was conducted at 4°C for 1 hour, after which the agarose beads were transferred to a small column, rinsed to remove unbound proteins (seen running off at 0-10 mls effluent) and eluted with 0.2 M N-acetyl-D-glucosamine beginning at 18 mls effluent volume. [^{35}S]-methionine (--□--) represents total viral and contaminating protein whereas [^{3}H]-glucosamine (---●---) labels only gp70 in virus preparations (97) (unpublished observation) and therefore is a specific marker for gp70.


RADIOACTIVITY (CPM/ml x10⁻³)
30
20
10
8
7
6
5
4
3
2
1
0
10
20
30
VOLUME (mls)
[³H] Glucosamine
[³⁵S] Methionine
N-Acetyl-
Glucosamine

of 12% for a previously reported purification protocol in which the lectin, concanavalin-A, was used after sucrose-gradient banding (198). Our enrichment in specific activity from the starting virus preparation to the gp70 obtained after the WGA step is approximately 10 fold and is comparable to the 11 fold enrichment previously reported (198). These values of net enrichment suggest that gp70 represents about 10% of the total virion protein.

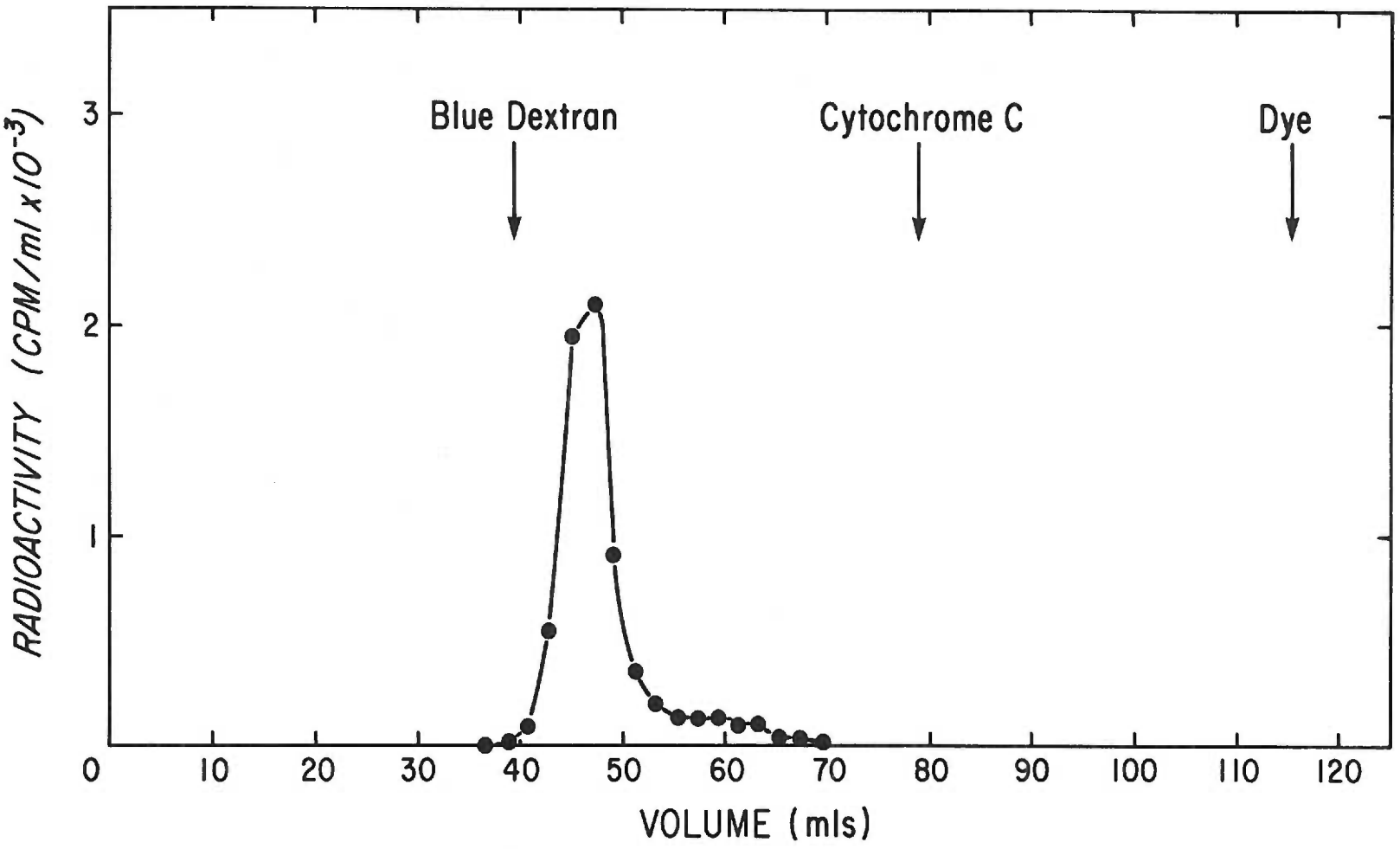
4. Sephadex Chromatography of gp70

A final stage of purification designed to remove gp70 from trace high molecular weight contaminants and disulfide-bridged p15(E) employed the use of gel filtration chromatography on Sephadex G-150 in the presence of reducing agents. Sugar-eluted fractions from the lectin column were pooled and concentrated to a suitable sample volume by moisture adsorption with Sephadex beads as described in Materials and Methods. The concentrated sample is shown in Figure 1A, lane 4, prior to loading onto the Sephadex column. This sample appears identical to the initial sugar eluant (lane 3) indicating that degradation had not occurred during concentration.

Fig. 3 shows the Sephadex G-150 elution profile in which lectin-purified gp70 had been chromatographed. Although a single peak of approximately 70,000 daltons molecular weight appears on the gel

Figure 3. Gel Filtration of Lectin Eluant on Sephadex G-150

[^{3}H]-glucosamine labeled glucoproteins which were specifically eluted from the lectin, wheat germ agglutinin (Fig. 2) were prepared for gel filtration by concentrating the sample volume and reducing the disulfide bridge present between gp70 and p15(E) (97,105,106) by the addition of dithiothreitol to make 10 mM as described in Materials and Methods. This sample (1 ml) was then applied to a Sephadex G-150 column (102 mls bed volume) equilibrated with 10 mM sodium phosphate (pH 6.5), 300 mM NaCl and 0.1 mM PMSF and chromatographed at a flow rate of 0.25 ml/min as described in Materials and Methods. 1 ml samples were collected and the contents monitored by determining radioactivity/fraction (Section II, M) and by analysis with polyacrylamide gel electrophoresis (Fig. 1A, lane 5).


Blue Dextran
Cytochrome C
Dye
RADIOACTIVITY (CPM/ml x10⁻³)
3
2
1
0
10
20
30
40
50
60
70
80
90
100
110
120
VOLUME (mls)

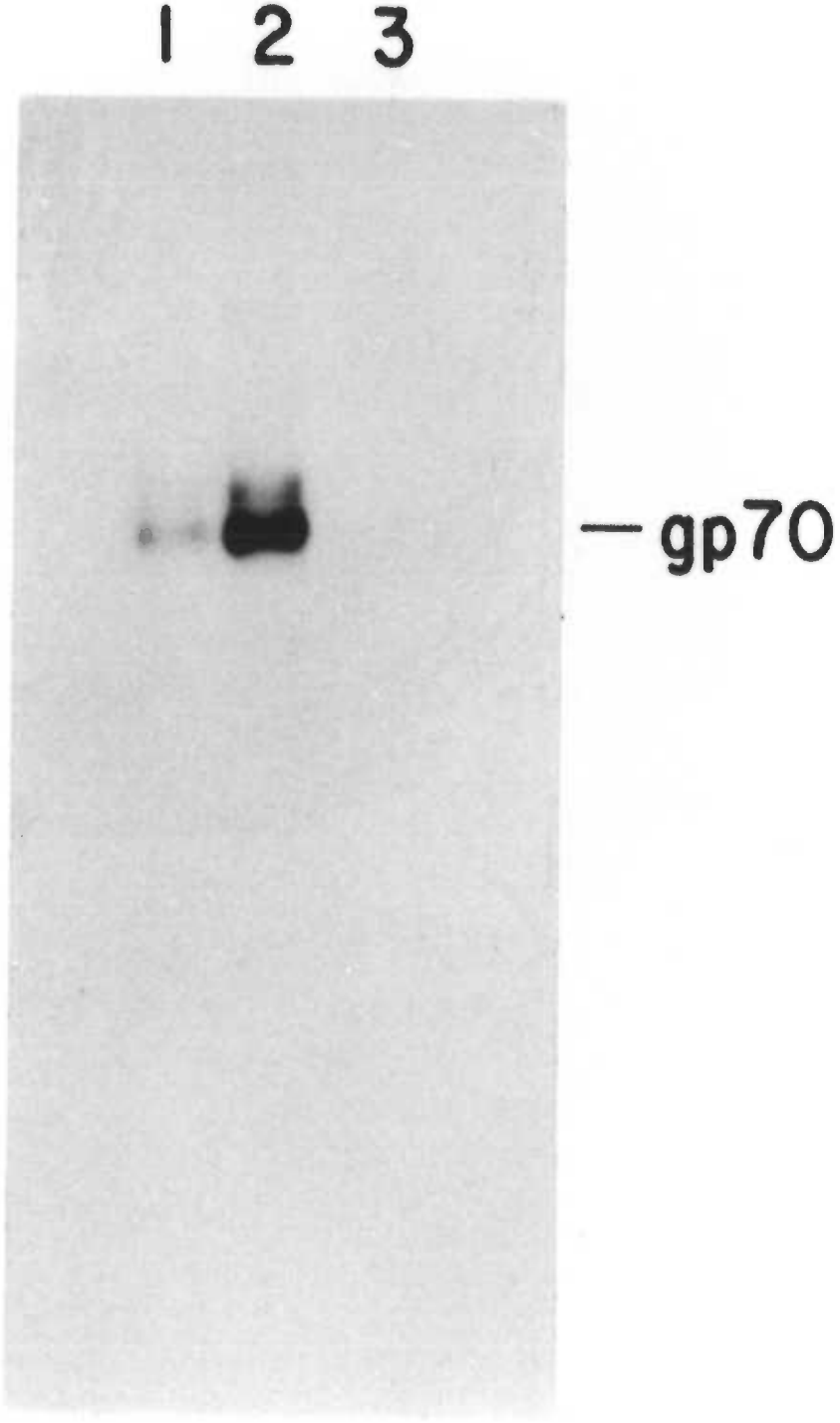
filtration profile, subsequent analysis using polyacrylamide gel electrophoresis (Fig. 1A, lane 5) reveals persistent minor contaminants and an unexplained enrichment of the higher (71,000 dalton) species of the gp69/71 doublet normally seen in Eveline cells (116). Furthermore, a poor yield and slight loss, rather than an enrichment, of [^{3}H]-glucosamine specific activity in the putative gp70 occurred at this stage of purification (Table 1). Therefore, the use of gel filtration is not recommended after successful WGA chromatography.

C. Evaluation of Purified F-MuLV gp70

As stated in the introduction to the purification protocol, a primary use for gp70 was in a biologically interacting receptor binding assay. This necessitates a pure and native preparation of gp70. The electrophoretic analysis of WGA- purified gp70 shown in Fig. 1A, lane 3 indicated a substantially homogeneous preparation of the envelope glycoprotein doublet known as gp69/71 in Eveline virus (116,200). Therefore, WGA-purified gp70 was prepared for binding assays by iodination with [^{125}I]-iodine using chloramine T as described in Materials and Methods. Following standard iodination procedures, free, unreacted iodine was then separated from the labeled protein on a Sephadex G-25 desalting column. Fractions containing gamma emmissions were then loaded onto polyacrylamide gels for electrophoretic analysis (Figure 4). A single, iodinated

Figure 4. Electrophoretic Analysis of [^{125}I]-Labeled gp70.

gp70 present in sugar specific eluants obtained from wheat germ agglutinin affinity chromatography (conducted as shown in Fig. 2 and as described in Materials and Methods) was iodonated with [^{125}I]-iodine using the Chloramine-T method as described in Materials and Methods, section II, D4. After iodonation, labeled sample was applied to a Sephadex G-25 desalting column to separate the iodonated proteins from the free, unreacted [^{125}I]-iodine (184). Three sequential fractions containing gamma-emmitting substance in the run-off of the G-25 column were diluted 1:1 in electrophoresis sample buffer and loaded directly onto 10% polyacrylamide slab gels for analysis as described in Materials and Methods.


1 2 3
— gp70

protein of approximately 70,000 daltons apparent molecular weight appears on our fluorograms. However, repeated attempts to get specific binding as described on susceptible cells (117) using this [^{125}I]-labeled gp70 were unsuccessful (data not shown) suggesting that this preparation of gp70 did not have sufficient biological activity for native interaction with receptor.

We immunoprecipitated the [^{125}I]-gp70 using anti-gp70 antiserum to investigate the possibility that our gp70 preparation had lost biological activity. After preclearing nonspecific immunoprecipitates with normal goat serum, 37% of the remaining radioactivity immunoprecipitated with anti-gp70 antiserum and 63% of the radioactivity did not bind to the antiserum. A second cycle of specific immunoprecipitation on the unbound portion (i.e., 63%) yielded only 4% more anti-gp70 binding activity. This indicates that most of the immunologically reactive gp70 had adsorbed in the first cycle. The positive cross-reaction with anti-gp70 antiserum strongly suggests that the homogeneous band in Figure 4 represents pure gp70. However, the inability of antibody to bind a net 59% of the iodinated protein suggests that damage to native gp70 had occurred either by oxidative damage of the chloramine- T catalysed iodination (203) or by some manipulation in the purification procedure.

D. Discussion

The gp70 purification protocol presented here improves previous methods because procedural steps have been condensed and simplified, and yet continue to maintain high yields. To help minimize gp70 losses, the conventional sucrose-gradient banding of virus has been eliminated. We attribute gp70 losses at this stage to a sensitivity of gp70 to the osmotic shock when suspending virus in sucrose (124,183,198). Furthermore, gp70 yields have been maximized because the viral proteins were then solubilized from intact virus by using a Triton X-100 solution before lectin affinity chromatography.

Affinity chromatography with wheat germ agglutinin (WGA) provides several advantages in the purification of gp70. The unique oligosaccharide specificity of WGA selectively removes gp70 from the contaminating serum albumin (Fig. 1B, lanes 9 and 12). This circumvents the problem of increased contamination that occurs when sucrose banding is eliminated. Previous gp70 purifications using concanavalin-A yielded preparations contaminated with glycosylated serum proteins (198) which would require subsequent gel filtration. Thus, the use of WGA also reduces the need for gel filtration. Furthermore, the use of gel filtration after WGA affinity chromatography does not further enrich our gp70 preparation (Table 1), but rather contributes to

additional losses. Therefore, it should be possible to eliminate gel filtration altogether since gp70 and p15(E) can be separated during the lectin purification stage on the basis that p15(E) is unglycosylated (204).

Our studies to evaluate the gp70 interaction with cell surface receptor were not successful. Although the electrophoretic homogeneity (Fig. 4) and the immunoprecipitation with anti-gp70 antiserum suggests that our gp70 preparation is pure, the presence of large (59%) amounts of gp70 which do not bind to specific antiserum suggest that its antigenicity, and presumably its receptor binding activity, have been damaged. Chloramine-T iodinations may damage receptor binding activity by over-iodination or by the strong oxidative environment which the method provides (205). Solid-phase lactoperoxidase catalysed-iodination may solve this problem because of the milder, controlled oxidation reaction which lactoperoxidase provides (203).

Other investigators are characterizing and isolating the gp70 receptor more extensively. Numerous reports on the tissue specifities, binding kinetics, affinities and number of receptor molecules per cell for different cell types have been published (117,128-133). Furthermore, several laboratories have isolated gp70-binding proteins from susceptible host cells during the same recent period of time as our efforts described above

(134-137). However, these putative receptors range in molecular weight from 10,000 to 190,000 daltons per monomeric (reduced) subunit which suggests that the gp70 receptor has not yet been unambigously identified.

IV. MUTANT CELLS THAT ABNORMALLY PROCESS PLASMA MEMBRANE GLYCOPROTEINS ENCODED BY MURINE LEUKEMIA VIRUS

A. General Introduction

Cells infected with replication-competent murine leukemia viruses (MuLVs) contain two major virus-encoded glycoproteins in their surface membranes: gp70 encoded by the viral *env* gene, and gp93^{gag} encoded by the viral *gag* gene (45,78,80). gp70 performs important but poorly understood functions in virus attachment to susceptible cells and in virus interference (117). Furthermore, gp70 has been implicated in other important processes such as differentiation of lymphocytes (118-121) and in leukemogenesis (122,123). The functions of membranous *gag* glycoproteins, however, are almost completely unknown. Nevertheless, both of these membrane glycoproteins interact with the host immune system (85) and therefore are probably important in the host response to virus-induced cancers.

We have begun to develop genetic techniques for analyzing the plasma membrane components that are encoded by MuLVs. An important element of our approach is the immunoselection of infected cell mutants that lack virus-encoded antigens on their surface membranes (171-173). This technique involves the efficient killing of infected cells with specific antisera in the presence of complement and the isolation of immunoresistant subclones (section II, I). In this chapter, we describe our methods for

isolation and analysis of immunoselected mutants with cellular defects that prevent gp70 and gp93^{gag} processing into cellular plasma membranes. These analyses provide evidence that MuLVs may be able to bud efficiently through membranes that lack any known MuLV-encoded constituents.

B. General Properties of Immunoresistant Cell Lines

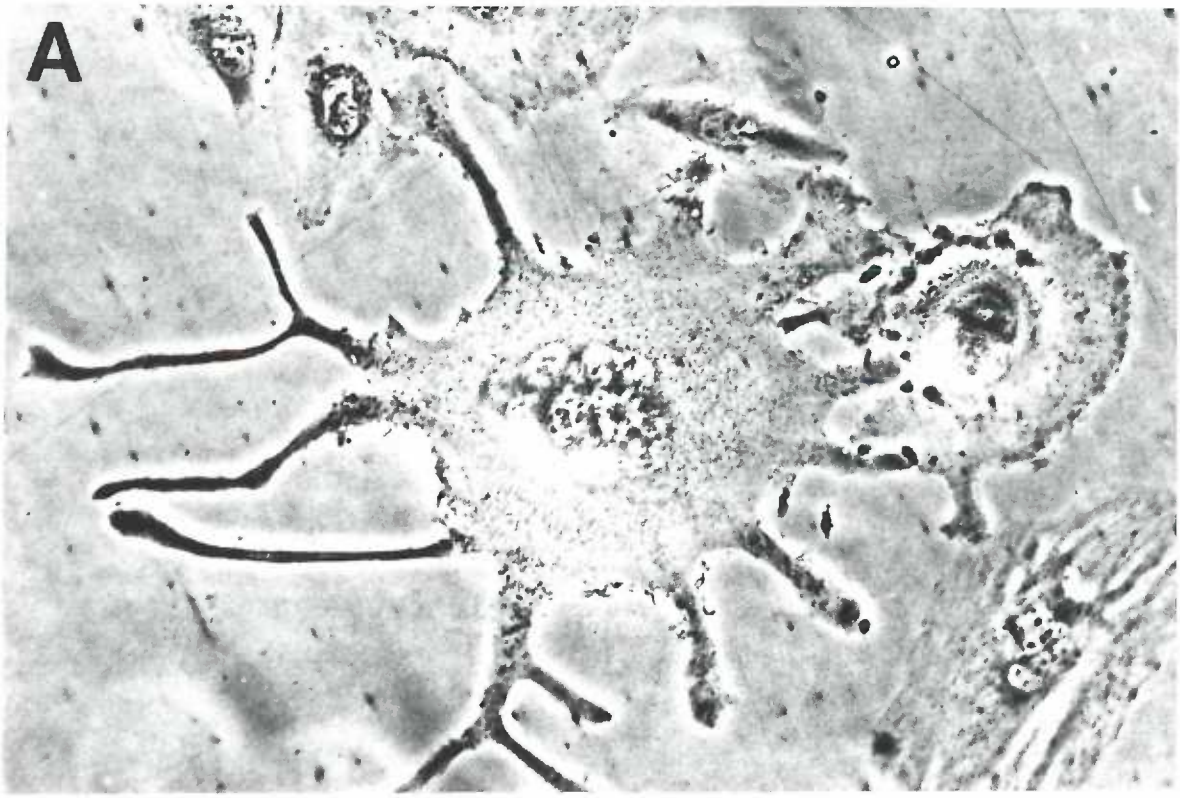
Cytotoxic immunoselection of MuLV infected normal rat kidney (NRK) fibroblasts which antisera to gp70, p30 or p12 in the presence of complement has enabled us to isolate stable resistant variants which appear to lack these antigens in their surface membranes (see Materials and Methods, section II, I). As shown below, the immunoresistant variants contain defects in cell-encoded components that are required for processing the virus glycoproteins into plasma membranes. In contrast, immunoresistance of infected BALB/c fibroblasts was relatively frequent and was caused by loss or decrease of virus gene expression, perhaps by provirus elimination (206) or by repression (207). These results suggest that heritable loss of virus-encoded antigens from plasma membranes of infected cells can occur by several mechanisms, which occur at different relative frequencies in different cell lines. Furthermore, we have observed that some cell lines are unsuitable for immunoselection by our procedure. For example, infected NIH/3T3 fibroblasts are killed inefficiently by antisera compared with many other cell lines, and they are highly sensitive to

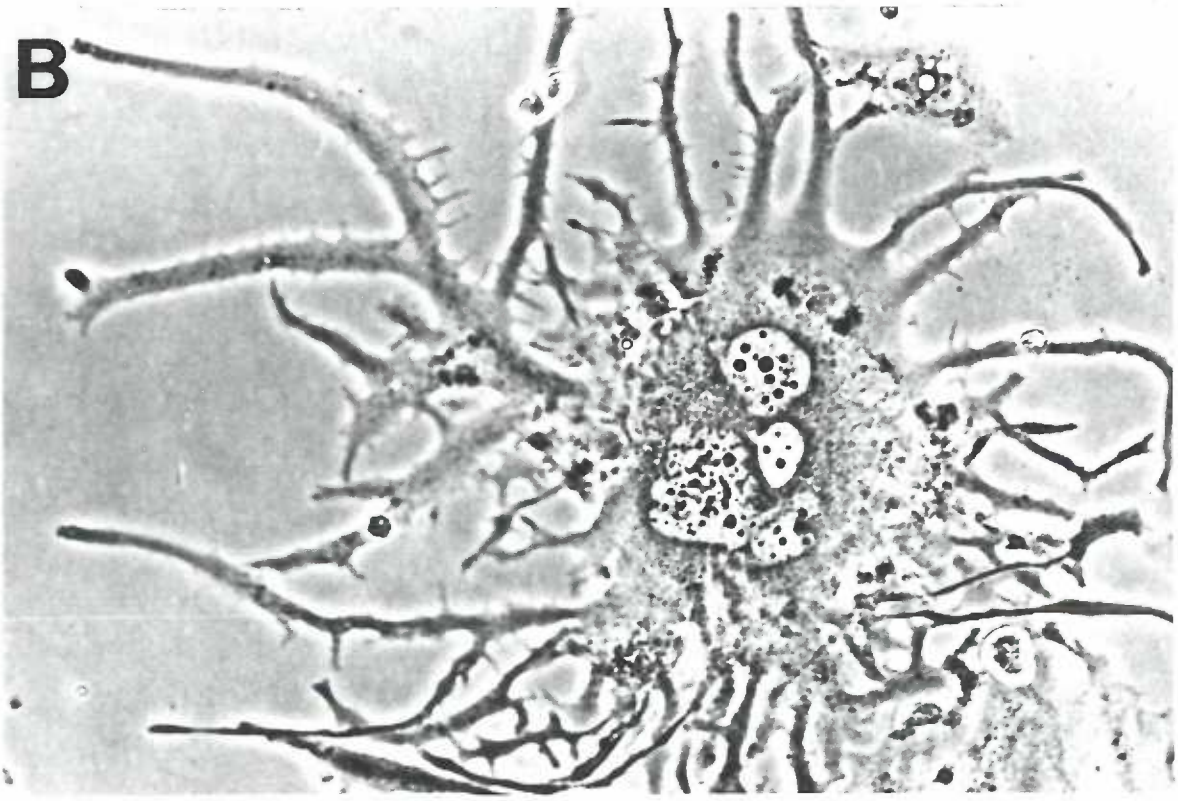
nonspecific cytolysis and to loss of adherence caused by complement. Consequently, the immunoselection procedure could select these cells for a complex set of membrane changes rather than simply for the absence of a target viral antigen. Because such extraneous membrane alterations could influence virus metabolism indirectly, we decided not to use this cell line.

As would be expected of cellular mutants with abnormalities of membrane synthesis, the immunoresistant MuLV-infected NRK cells often have abnormal morphologies and behaviors. Although the morphological alterations are usually minor, approximately 5% of the immunoresistant subclones have substantial anomalies. For example, the cell line shown in Fig. 5A, which was selected using antiserum to p12 (cell line p12-12), contains a large proportion of giant cells with large pseudopodia and with several nuclei of different sizes. Approximately 50% of the nuclei in these cultures occurs in multinucleated cells. These multinucleated cells seem to be formed by failure of plasma membrane division at the time of mitosis. Surprisingly similar cell lines were also obtained by use of antiserum to p30 (cell line p30-12, Fig. 5B). These morphologically abnormal cell lines lack plasma membrane MuLV antigens but contain the MuLV-encoded components intracellularly in normal amounts.

Figure 5. Morphologically Abnormal MuLV Infected NRK Fibroblasts Obtained After Immunoselection.

Cell line p12-12 (Panel A) was immunoselected with antisera directed against Rauscher-MuLV p12. Cell line p30-12 (Panel B) was immunoselected with antisera directed against Rauscher-MuLV p30. Immunoselection was conducted as described in Materials and Methods (section II, I) with rabbit complement and cytotoxic antisera directed against the cell surface membrane glycoprotein, $gp93^{gag}$, which contains both p12 and p30 antigenic determinants (45,78). Bar=60u.


A


B

C. A Mutant Resistant to Antiserum to gp70 Which is Deficient in Cell Surface gp70 and gp93

The H-4 cell line was isolated as a clone from a population of MuLV-infected NRK cells resistant to killing with antiserum to gp70 (172). To analyze the synthesis and processing of viral proteins, we pulse labeled the cells with [^{35}S]-methionine and subsequently gave them a cold chase with an excess of unlabeled methionine. At intervals throughout this procedure, radioactive gp70 and p30 antigens were prepared for electrophoresis in polyacrylamide gels containing SDS. Fig. 6 shows an electrophoretic comparison of gp70 synthesis in wild-type cells (lanes 1-5) to its synthesis in H-4 cells (lanes 6-10). Although H-4 cells synthesize gPr90$^{\underline{env}}$, they do not process this precursor to form gp70 or p15(E).

The processing of proteins encoded by the viral gag gene is also altered in H-4 cells, as shown in Fig. 7. It is believed that the cytoplasmic polyprotein Pr65$^{\underline{gag}}$ in wild-type cells is cleaved to form p30 and other virion internal core proteins and that this cleavage is often inhibited as a secondary consequence of various abnormalities of MuLV metabolism (72,76). Furthermore, gPr80$^{\underline{gag}}$ is believed to occur in the endoplasmic reticulum and to be further glycosylated to form gp93$^{\underline{gag}}$ (46,47,79). As shown in Fig. 7, the kinetics of Pr65$^{\underline{gag}}$ processing to form p30 is reduced in H-4 cells (lanes 6-10) compared with wild-type cells (lanes 1-5). In addition, the

Figure 6. Pulse-Chase Analysis of Synthesis and Processing of F-MuLV *env* Gene Products in Wild-Type F12 NRK and Immunoresistant H-4 Cells.

Wild-type or anti-gp70 immunoresistant H-4 cells were labeled at 50% confluency for either 5 or 15 min with [^{35}S]-methionine (50 uCi/ml). After labeling for 15 min, the appropriate flasks were chased for 30 min, 2 or 4 hr using unlabeled complete medium. At the end of the appropriate chase interval, the flasks were drained, and lysates were made and immunoprecipitated with antiserum to gp70. The immunoprecipitates were electrophoresed in 10-20% polyacrylamide gradient gels containing 0.1% SDS, and the bands were visualized by flourography. The unlabeled bands are nonspecific contaminants. Lanes 1-5 contain immunoprecipitates from wild-type cells; lanes 6-10 contain immunoprecipitates from H-4 cells (1 and 6) 5 min pulse, (2 and 7) 15 min pulse, (3 and 8) 30 min chase, (4 and 9) 2 hr chase, (5 and 10) 4 hr chase. "gPr90" is synonomous with gPr90^{env} discussed in the text.

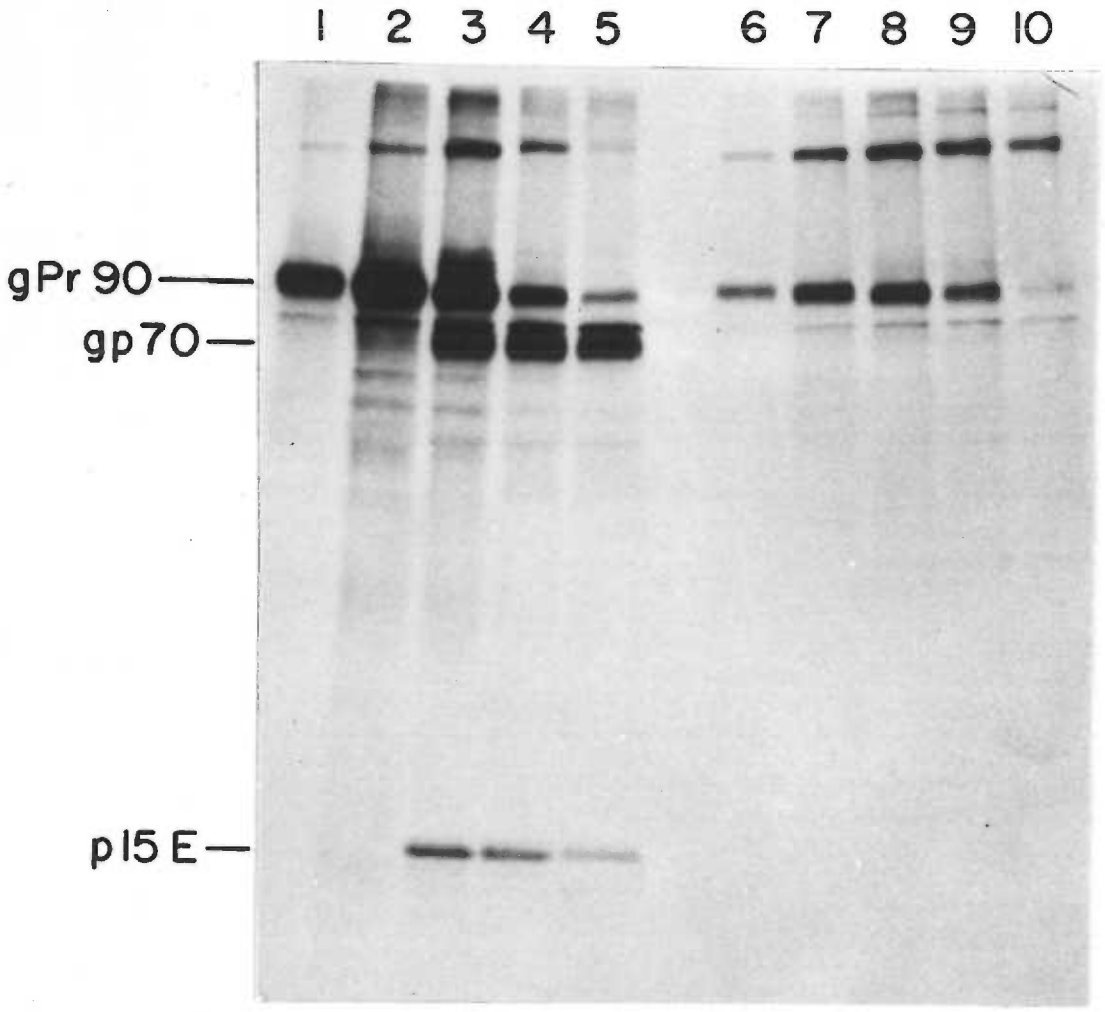
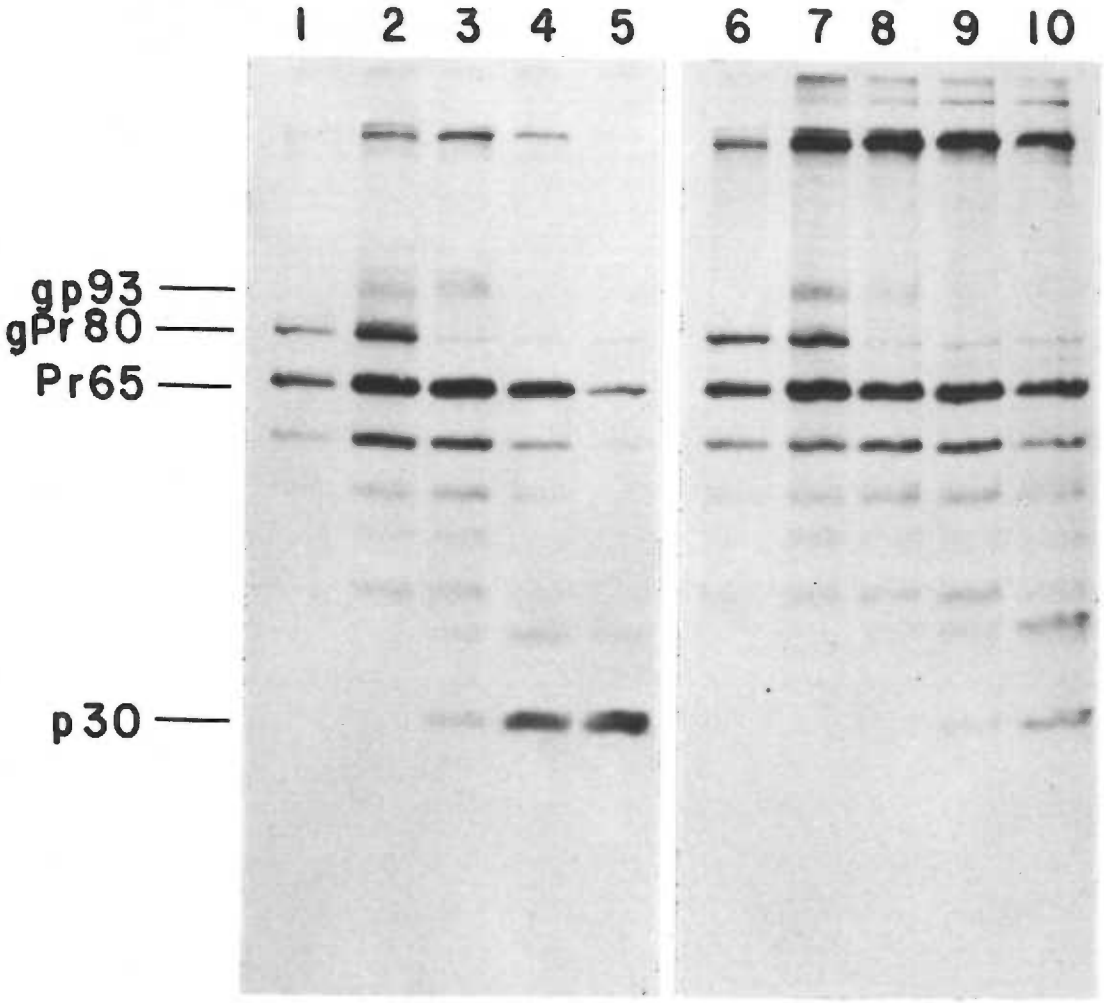

1 2 3 4 5 6 7 8 9 10
gPr 90
gp70
p15 E

Figure 7. Pulse-Chase Analysis of Synthesis and Processing of F-MuLV gag Gene Products in Wild-Type F12 NRK and Immunoresisant H-4 Cells.

Wild-type or anti-gp70 immunoresistant H-4 cells were labeled for either 5 or 15 min with [^{35}S]-methionine (50 uCi/ml) at 50% confluency. After labeling for 15 min, the appropriate flasks were chased for 30 min, 2, or 4 hr using unlabeled complete medium. At the end of the appropriate chase interval, the flasks were drained, and lysates were made and immunoprecipitated with antiserum to p30. The immunoprecipitates were electrophoresed in 10-20% polyacrylamide gradient gels containing 0.1% SDS, and the bands were visualized by flourography. The unlabeled bands which migrate above gp93^{gag} are nonspecific and precipitate also with normal goat serum. Lanes 1-5 contain immunoprecipitates from wild-type cells; lanes 6-10 contain immunoprecipitates from H-4 cells (1 and 6) 5 min pulse, (2 and 7) 15 min pulse, (3 and 8) 30 min chase, (4 and 9) 2 hr chase, (5 and 10) 4 hr chase. "Pr65", "gPr80" and "gp93" are synonomous with Pr65^{gag}, gPr80^{gag} and gp93^{gag} discussed in the text.

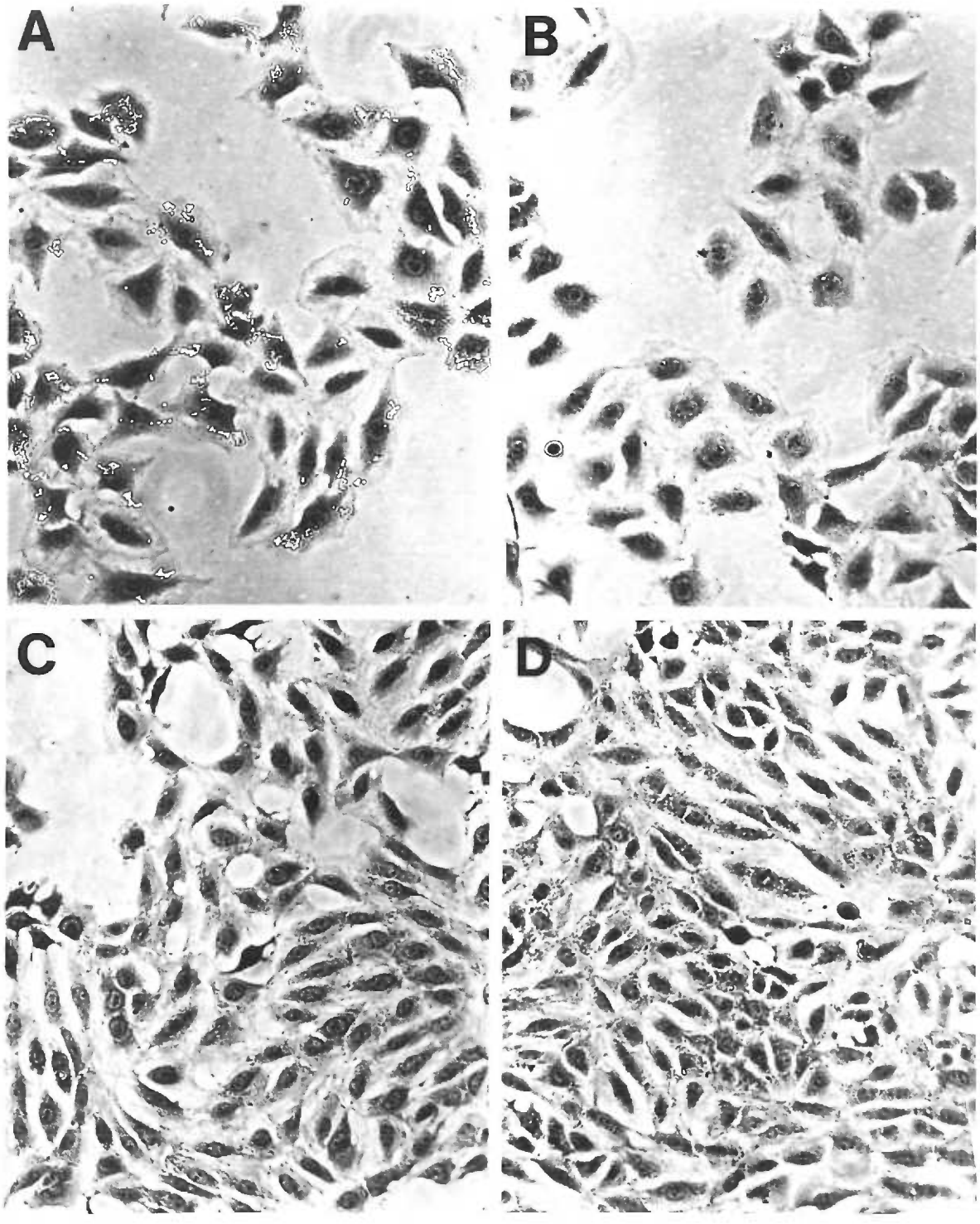

1
2
3
4
5
6
7
8
9
10
gp93
gPr80
Pr65
p30

gp93$^{\underline{gag}}$ that is made in H-4 cells appears to be reproducibly chased away more rapidly than in wild-type cells. Thus, from densitometric scans of exposed autoradiograms (see Materials and Methods, section II, G), we estimate that 82% of the radioactivity in gp93$^{\underline{gag}}$ is lost from H-4 cells during a 30 min cold chase, whereas only 20% is lost from wild-type cells during this time.

Using an erythrocyte rosette assay (section II, H2), we can detect gp70 (see below) and gag (Fig. 8A) antigens on the surfaces of MuLV-infected wild-type cells. The gag antigens can be rosetted using antisera to p30, p15 or p12. However, p10 cannot be detected, suggesting that this portion of gp93$^{\underline{gag}}$ may be buried in the membranes. Furthermore, as shown previously (178), erythrocyte binding was always absent from control cell monolayers which were routinely incubated with normal goat serum instead of specific antisera. As expected, the rosetting that occurs with antiserum to p30 (Fig. 8A) is competitively blocked by adding 2 ug/ml of purified p30 (Fig. 8B). In contrast to these results, neither gag nor gp70 antigens could be detected on the surface membranes of H-4 cells either by rosetting (Fig. 8C and D) or with cytotoxic antisera. In agreement with these results, lactoperoxidase catalyzed cell surface iodinations with [^{125}I]-iodine indicated that gp70 is present on wild-type cells but is not detected on H-4 cells. However, the cell

Figure 8. Erythrocyte Rosette Assay for Detection of Virus-Coded Antigens on the Surface of F12 NRK and H-4 Cells.

The binding of erythrocytes coated with protein A to the fibroblast monolayers occurs only in the presence of specific antibodies which react with molecules on the fibroblast surface membranes. Frame (A) shows F-MuLV infected normal rat kidney fibroblasts (clone F-12) reacted with goat antibody to p30. Frame (B) shows the same cells reacted with goat antibody to p30 in the presence of 2 ug/ml of F-MuLV p30 that had been purified by phosphocellulose chromatography (124). The anti-gp70 immunoresistant H-4 cells are shown reacted with goat antibody to either gp70 (frame C) or to p30 (frame D). Bar = 60u.


A
B
C
D

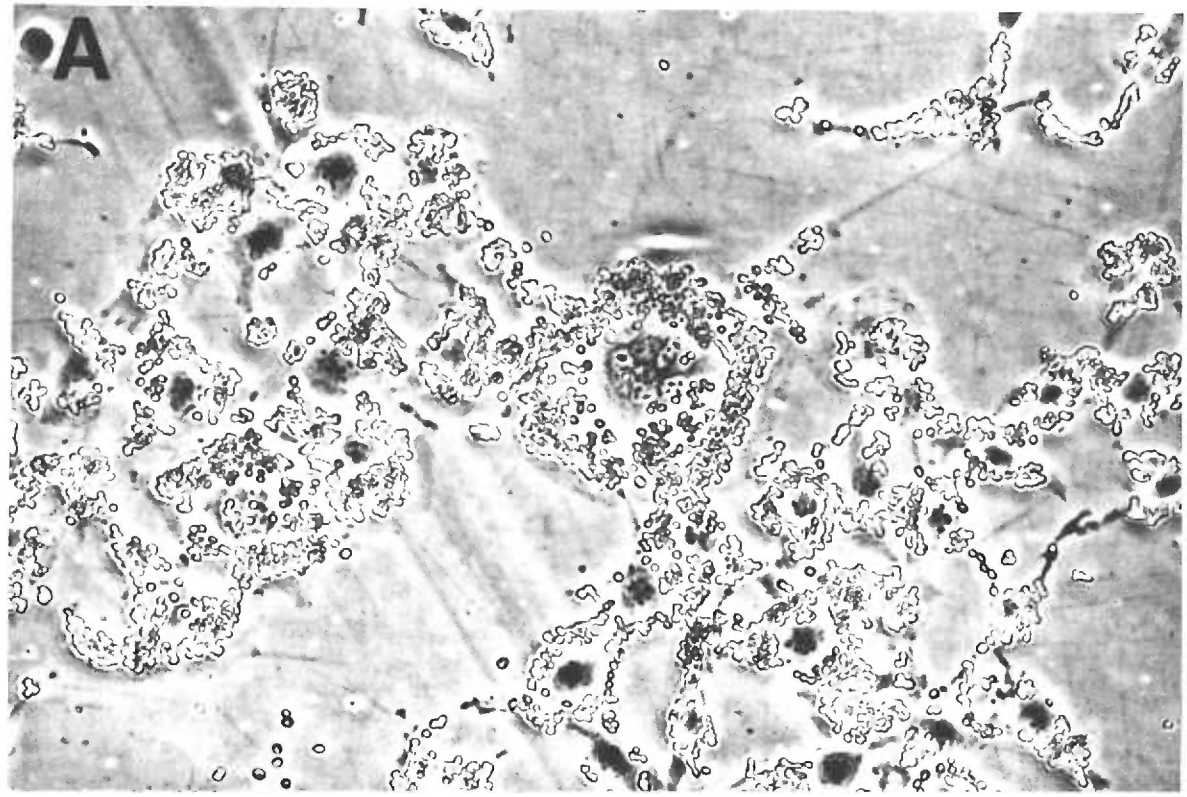
surface gag component encoded by Friend MuLV cannot be labeled by the iodination procedure, suggesting that this molecule may lack exposed tyrosines. These results suggest that H-4 cells have plasma membranes deficient in both gp70 and gag antigens.

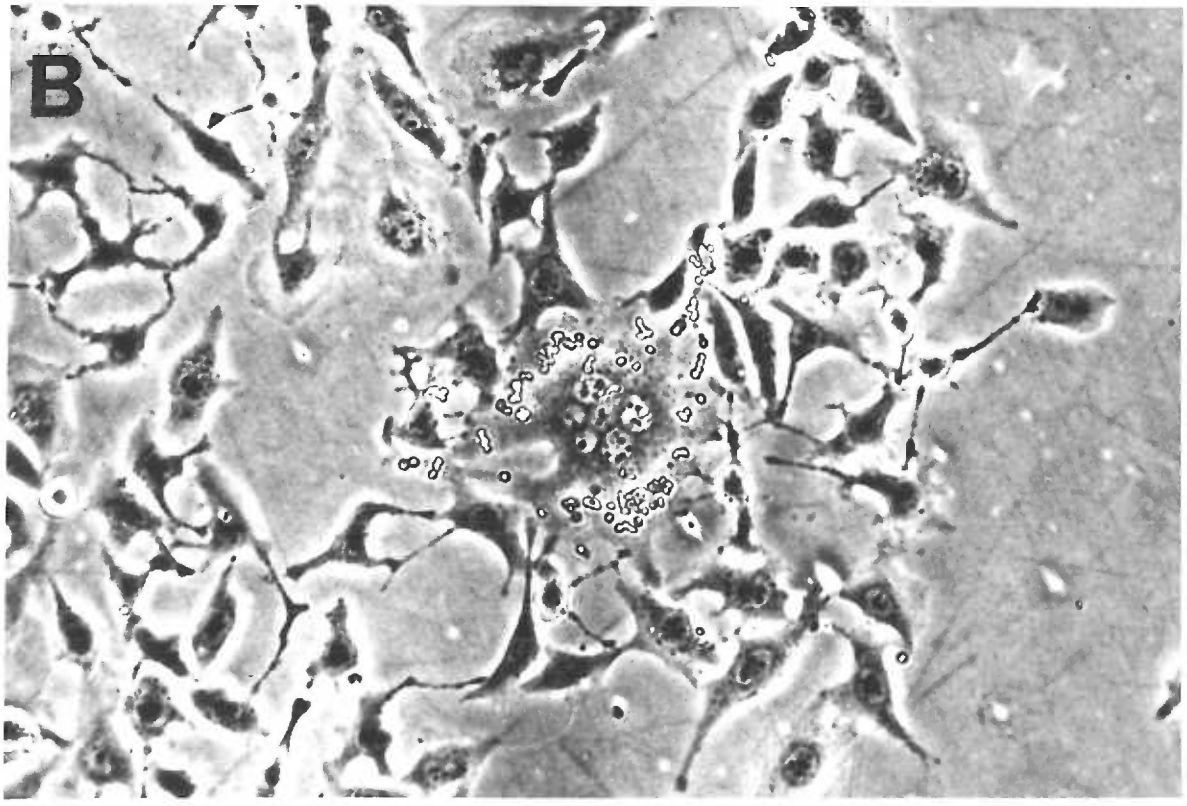
H-4 has a Cellular Encoded Defect. To determine whether the defect in H-4 cells is encoded by a viral or cellular gene, we fused the H-4 cells with normal uninfected NRK cells and used the rosette assay to examine the resulting heterokaryons for cell surface gp70 antigens. The control MuLV-infected NRK cells express gp70 on both single and multinucleated cells (Fig. 9A); single H-4 cells or NRK cells lack gp70, whereas their heterokaryons contain cell surface gp70 antigens. Fig. 9B shows a representative example of such a heterokaryon which is rosetted in the presence of antiserum to gp70. As expected, control fusion cultures made with either H-4 cells alone or NRK cells alone did not rosette with antiserum to gp70 (data not shown). Thus the processing defect in H-4 cells is complemented by a factor present in uninfected NRK cells.

Additional evidence that H-4 cells have a cellular gene-encoded defect was obtained by superinfecting H-4 cells either with wild-type MuLV or with a preparation of Friend virus complex which contains MuLV plus spleen focus-forming virus (SFFV) in a 1:2 ratio (177) and examining the superinfected cells for gp70 (Fig.

Figure 9. Complementation Rescue of Defective gp70 Processing in the H-4 Cells by Fusion of H-4 Cells to Uninfected NRK Cells.

Cell Fusion induced by polyethylene glycol (section II, J) was used to rescue by complementation the cellular defect in gp70 processing. Frame (A) shows wild-type MuLV-infected NRK fibroblasts (F12 NRK) fused to themselves; frame (B) shows H-4 cells fused to uninfected NRK cells. The surface expression of gp70 antigens was detected by the rosette assay (section II, H2) in which protein A coated erythrocytes bind in the presence of anti-gp70 antiserum to cells expressing cell surface gp70. Bar = 60u.


A


B

10). As expected, the H-4 cells (lane 1) differ from the wild-type cells (lane 2) in lacking gp70. Furthermore, H-4 cells superinfected with MuLV (lane 3) or with the Friend virus complex (lane 4) also lack gp70. The synthesis of gp55, which is encoded by SFFV (177,208), occurs in the H-4 cells superinfected with the Friend virus complex, indicating that the superinfections were successful and that wild-type gPr90$^{\underline{env}}$ cannot be processed to gp70 in H-4 cells.

D. Mutuant Cell Lines Resistant to Antiserum to p30

Two phenotypically distinct clones, p30-2 and p30-5, were isolated from a population of MuLV-infected NRK cells selected for resistance to killing during prolonged exposure to antiserum to p30. Figure 11 shows an electrophoretic analysis of [^{35}S]-methionine-labeled gag gene products synthesized during a pulse-label and cold-chase experiment in wild-type cells (lanes 1-5), in p30-2 cells (lanes 6-10) and in p30-5 cells (lanes 11-15). Although the mutant cell lines appear to process Pr65$^{\underline{gag}}$ to form p30 somewhat less rapidly than wild-type cells, their striking abnormality is an absence of gp93$^{\underline{gag}}$, which cannot be seen even in extensively overexposed autoradiograms. In addition, these variant cell lines contain relatively little gPr80$^{\underline{gag}}$ (the gp93$^{\underline{gag}}$ precursor), either because of reduced synthesis or enhanced degradation. Furthermore, the p30-2 cells

Figure 10. Analysis of gp70-Related Proteins in the MuLV-Infected Wild-Type and H-4 Variant Cells After Superinfection by Wild-Type Friend or by the Friend Virus Complex.

Cells were pulse labled by incubation with [^{35}S]-methionine for 2 hr. The lysates were immunoprecipitated with monospecific antiserum to gp70 and the immunoprecipitates were electrophoresed in 10-20% polyacraylmide gradient gels containing 0.1% SDS. Immunoprecipitates analyzed were from (lane 1) H-4 cells, (lane 2) wild-type F-MuLV infected NRK fibroblasts, (lane 3) H-4 cells superinfected with F-MuLV and (lane 4) H-4 cells superinfected as described in Materials and Methods (section II, C3) with a preparation of Friend virus complex contraining F-MuLV plus spleen focus forming virus (SFFV) in a ratio of 1:2 (177). "gPr90" is synonomous with gPr90$^{\underline{env}}$ discussed in the text.

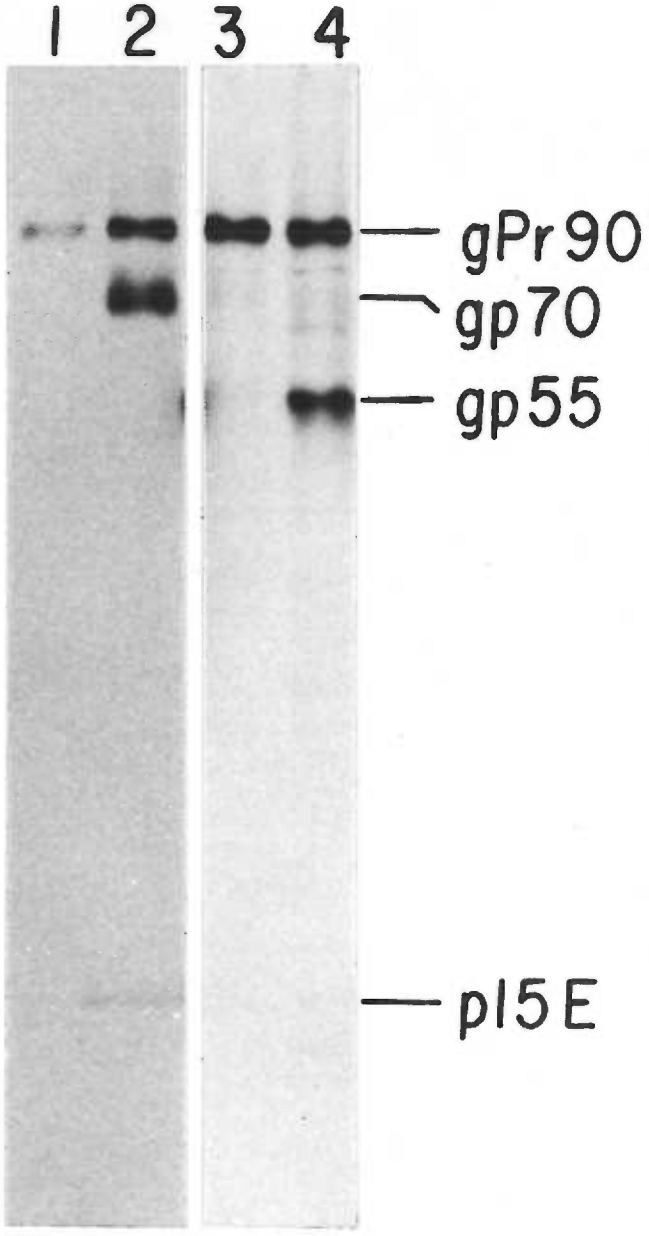
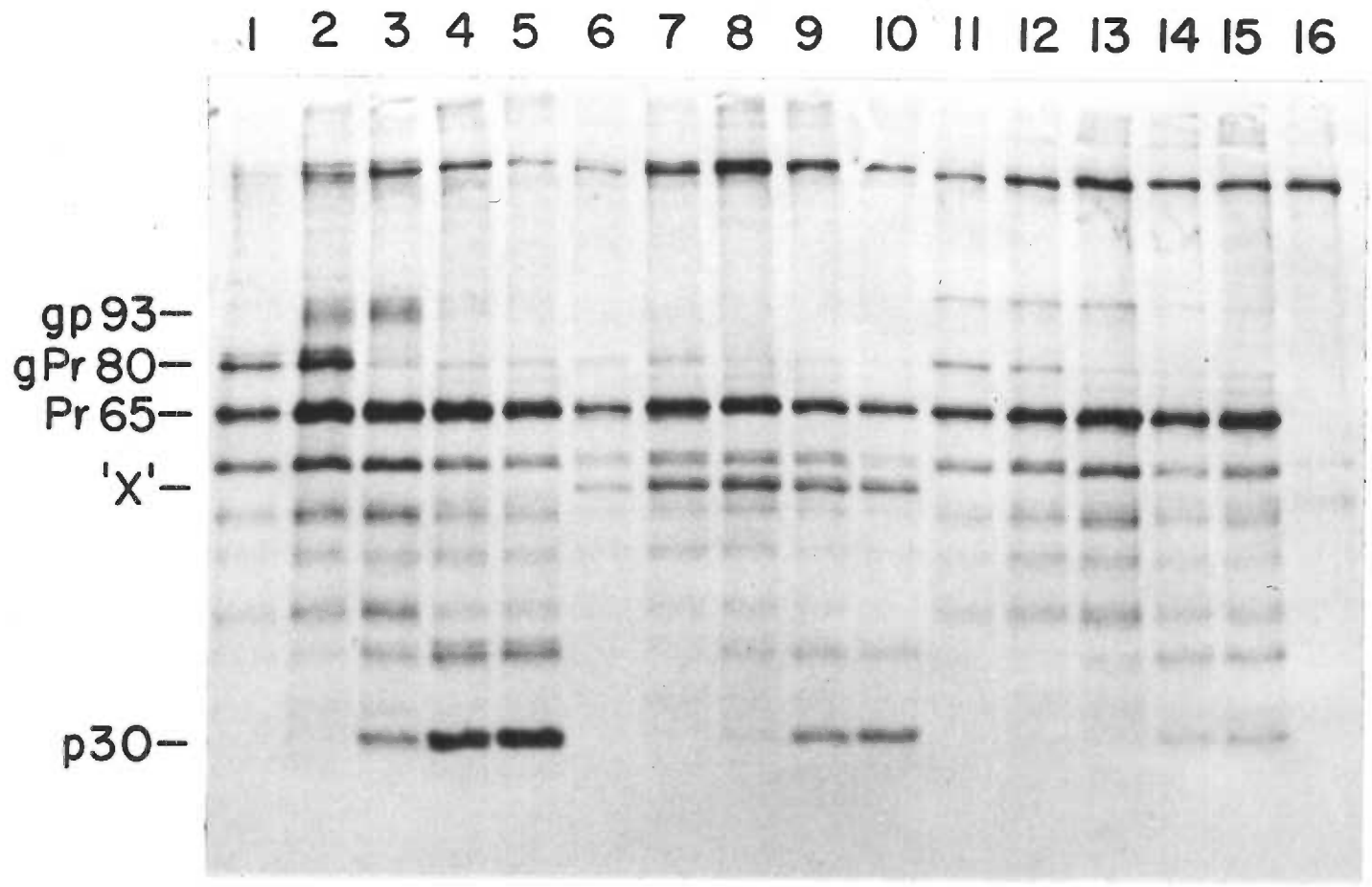

1 2 3 4
gPr90
gp70
gp55
p15E

Figure 11. Pulse Chase Analysis of Synthesis and Processing of F-MuLV gag Gene Products in Wild-Type F12 NRK and in the Anti-p30 Immunoresistant Cell Lines, p30-2 and p30-5.

Wild-type or anti-p30 immunoresistant cells were labeled for either 5 or 15 min with [^{35}S]-methionine (50 uCi/ml) at 50% confluency. After pulse labeling for 15 min, the appropriate flasks were chased for 30 min, 2, or 4 hr using unlabeled complete medium. At the end of the appropriate chase interval, the flasks were drained, and the lysates were made and immunoprecipitated with antiserum to p30. The immunoprecipitates were electrophoresed in 10-20% polyacrylamide gradient gels containing 0.1% SDS, and the bands were visualized by fluorography. The unlabeled bands which migrate above gp93^{gag} are nonspecific and precipitate also with normal goat serum. Lanes 1-5 contain immunoprecipiates from the wild-type cells; lanes 6-15 contain immunoprecipitates from the anti-p30 resistant lines, p30-2 (lanes 6-10) and p30-5 (lanes 11-15). (1, 6 and 11) 5 min pulse, (2, 7 and 12) 15 min pulse, (3, 8 and 13) 30 min chase, (4, 9 and 14) 2 hr chase, (5, 10 and 15) 4 hr chase. Lane 16 contains a normal goat serum immunoprecipitate of wild-type cells pulse labeled for 15 min. "Pr65", "gPr80" and "gp93" are synonomous with Pr65^{gag}, gPr80^{gag} and gp93^{gag} discussed in the text.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
gp93–
gPr80–
Pr65–
'X'–
p30–

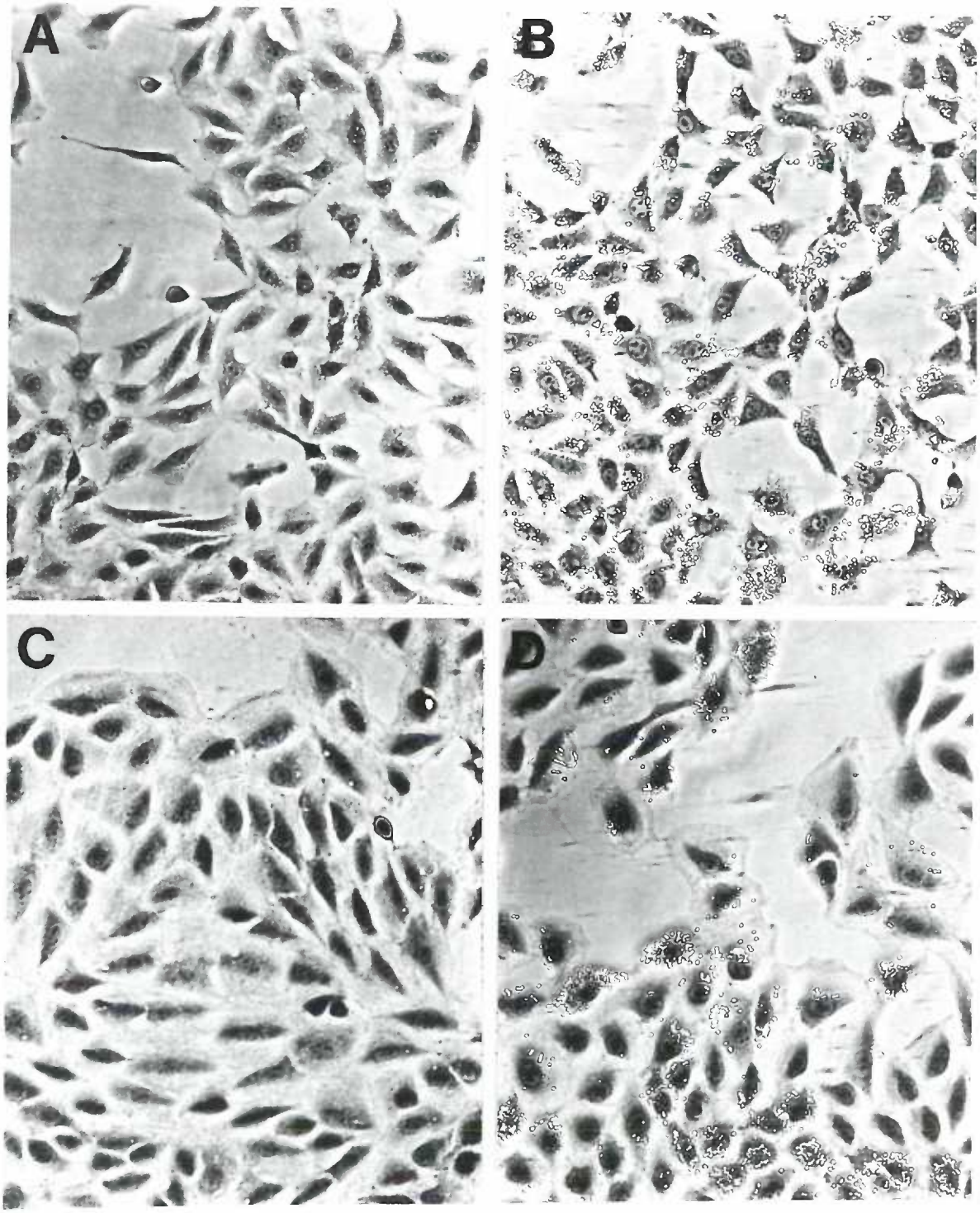
contain a new component "X" which contains p30 antigens (Fig. 11). In contrast, gp70 synthesis in p30-2 and p30-5 cells is indistinguishable from that described above for wild-type cells (Fig. 6, lanes 1-5).

Rosette analysis supports the immunoresistance results and indicates that p30-2 and p30-5 cells both lack cell surface gag antigens (Figures 12A and 12C). Although they both contain gp70 on their surface membranes, p30-2 cells rosette homogeneously (12B), whereas different cells in the p30-5 cultures rosette to differing extents (12D). Furthermore, 12 subclones of the p30-5 cell population rosette with gp70 antiserum in this heterogeneous fashion. Lactoperoxidase catalyzed iodination with [^{125}I]-iodine confirmed that these cell lines contain surface membrane gp70.

Mutants p30-2 and p30-5 have Cellular Gene Encoded Defects. Consistent with evidence described below, p30-2 and p30-5 cells both release infectious MuLV. Furthermore, wild-type NRK cells infected with MuLV released from these cell lines synthesize gp93^{gag} and rosette with antiserum to p30 (data not shown). The wild-type cells infected with virus released from p30-2 cells do not synthesize the abnormal component "X" described above. Thus the virus released from these cell lines appears to be wild-type MuLV in all of its properties.

Figure 12. Erythrocyte Rosette Assay for Detection of Virus-Coded Antigens on the Surface of p30-2 and p30-5 Cells.

The binding of erythrocytes coated with protein A to the fibroblast monolayers occurs only in the presence of specific antibodies which react with molecules on the fibroblast surface membranes. Frames (A) and (B) show the immunoresistant p30-2 cells reacted with goat antibody to p30 or gp70, respectively. Frames (C) and (D) show the immunoresistant p30-5 cells reacted with goat antibody to p30 or gp70, respectively. Bar = 60u.


A
B
C
D

E. Release of Virus Particles from Mutants Lacking Cell Surface gag or env Antigens.

Most of the mutant cell lines described in section IV release particles containing reverse transcriptase into their culture media (Table 2). These cell lines include the H-4 cells that appear deficient in surface membrane gp70 and gp93^{gag}, and the p30-2 and p30-5 cells that appear to lack gp93^{gag} but to contain gp70 on their surfaces. However, the morphologically abnormal cell lines shown in Fig. 5 do not release significant amounts of virion particles containing reverse transcriptase (Table 2). In addition, electron microscopy (section II, L) indicated the presence of budding type-C particles on the surfaces of H-4 and P30-2 cells (we did not examine p30-5 cells), but not on the morphologically abnormal p12-12 cells. The virus released from H-4 cells was noninfectious and lacks gp70, whereas that released from p30-2 and p30-5 cells contains gp70 and was infectious (see previous section). These conclusions were substantiated by analyzing the radioactive particles that banded in a sucrose density gradient at 1.13-1.17 g/cc and that were released from wild-type and H-4 cells during an 18 hour incubation with [^{35}S]-methionine. Although these virus preparations contained nearly identical amounts of radioactive p30, the H-4 virus preparation specifically lacked gp70 (data not shown). The results in Table 2 suggest that budding of MuLV particles from infected cells

Table 2. Reverse Transcriptase in Particles Released from Immunoslected Lines

Cell Lines	Radioactivity[a] (pmoles per 30' at 30°C)
F12 NRK Wild-Type	3.29
F12 NRK H-4	4.72
F12 NRK p30-2	2.02
F12 NRK p30-5	0.77
F12 NRK p12-12	0.08
F12 NRK p30-12	0.04

[a]The assays were performed in duplicate as described in Materials and Methods, section II, K. Blank sample values (0.05) have been subtracted from the radioactivity data.

can be reduced or blocked by cellular-gene-encoded abnormalities of plasma membranes. However, the presence of virus-encoded components in these membranes seems not to be essential for virus release.

F. Processing of Cellular Membrane Components in the Mutant Cells

The above experiments suggest that the immunoresistant NRK variants contain defects in cellular genes required for processing the target MuLV glycoproteins into their plasma membranes. Although such defects could influence the processing of cellular as well as viral glycoproteins, it seems apparent that viable cells cannot have substantial deficiencies in important plasma membrane glycoproteins. In agreement with this expectation, previously described plasma membrane synthesis mutants contain abnormalities which selectively block the processing of a small subset of plasma membrane glycoproteins (171,209).

We have obtained evidence consistent with this expectation by comparing the cell surface proteins on wild-type, H-4 and p30-2 cells which were labeled by lactoperoxidase catalyzed iodination with [^{125}I]-iodine and were subsequently fractionated by two-dimensional electrophoresis in polyacrylamide gels. Among approximately 50 prominent radioactive components in these gels, no qualitative differences between these cell lines were observed. However, there were several differences in

the apparent relative intensities of different components. Figure 13 shows a comparison of wild-type infected cells (13A) and H-4 mutant cells (13B). These cell lines appear to contain very similar plasma membrane proteins.

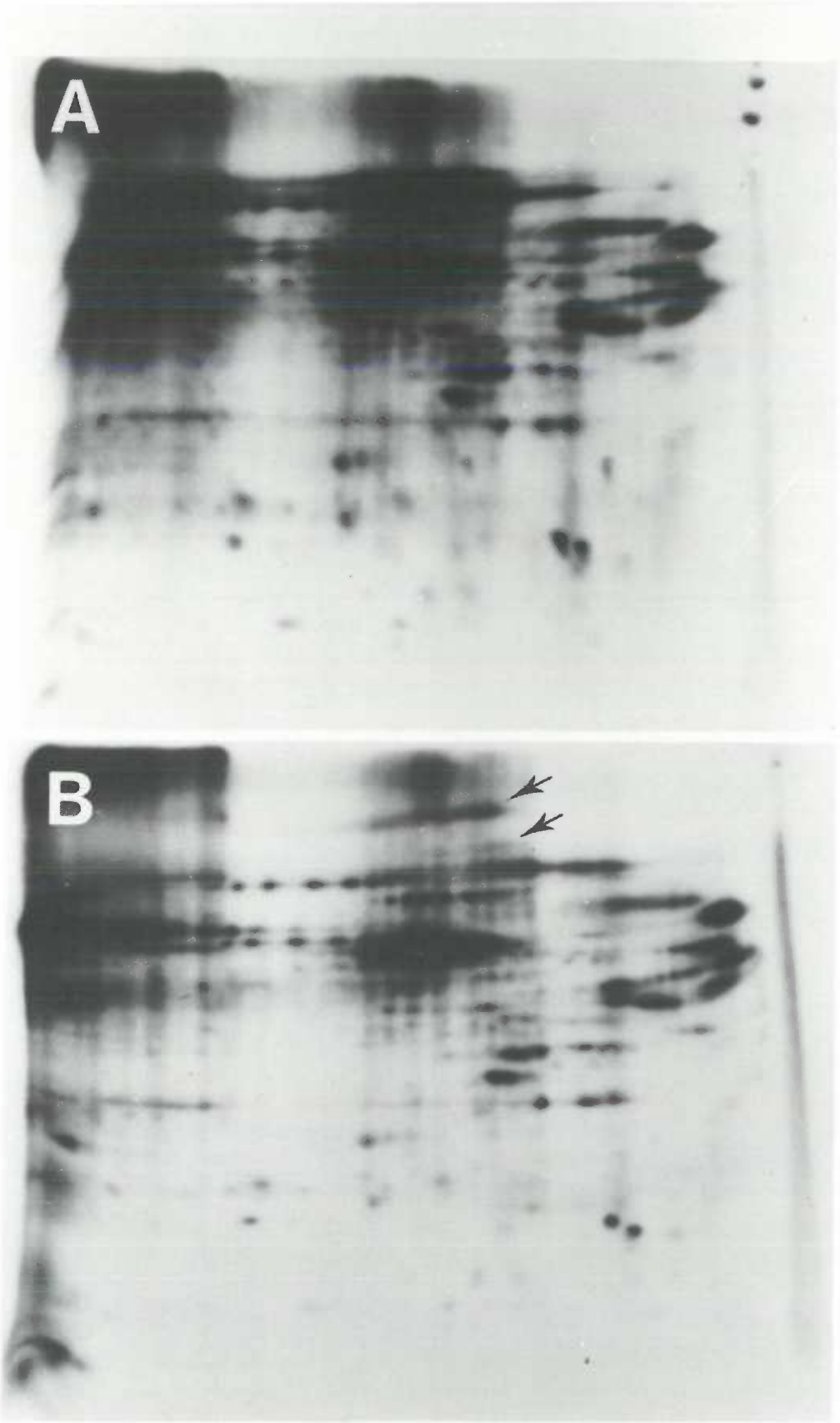
G. Discussion

1. Immunoselection of Mutants with Cellular Defects in Surface Expression of MuLV Encoded Antigens

Our results suggest that the immunoresistant MuLV-infected NRK cells contain cellular-gene-encoded defects in factors required for processing the MuLV plasma membrane glycoproteins. The resistance is stably inherited in the absence of antibody (See Materials and Methods, section II, I) suggesting that it is caused by heritable change rather than by antibody-dependent modulation (210,211). The variant cells contain the target antigens intracellularly but appear to lack them on their surface membranes. For example, the H-4 cell line that was selected with antiserum to gp70 contains a processing abnormality which prevents gp70 or p15(E) formation from the gPr90$^{\underline{env}}$ precursor (Fig. 6). In addition, the gp93$^{\underline{gag}}$ which is made in H-4 cells has a relatively short lifetime and may be degraded before it can reach the cell surface. Similarly, cell lines selected using antiserum to p30 specifically lack gp93$^{\underline{gag}}$ but contain the cytoplasmic forms of p30 (Fig. 11). In these respects, the resistant NRK cells have the properties expected of mutants, with processing defects

Figure 13. Autoradiogram of [^{125}I]-Labeled Cell Surface Proteins Resolved by Two-Dimensional Gel Electrophoresis.

Lactoperoxidase catalysed cell surface iodination with [^{125}I]-iodine of (A) wild-type F12 NRK cells and (B) anti-gp70 immunoresistant H-4 cells was conducted as described in Materials and Methods (section II, H3). Labeled cells were extracted and equal trichloroacetic acid precipitable counts were loaded and electrophoresed on O'Farrell two-dimensional polyacrylamide gels as described in Materials and Methods. Arrows in Figure B point to two examples of spots whose relative intensities differ in the two cell lines. Virus encoded surface proteins are minor and cannot be seen.


A
B

specifically related to the selection pressures that were applied. The processing defects we have characterized are a consequence of cellular-gene-encoded abnormalities because they can be complemented by fusion of the variant MuLV-infected cells with uninfected wild-type cells (Fig. 9) and because the processing defects affect the expression of wild-type MuLV when it is present in the variant cell lines (Fig. 10).

The specific glycoprotein processing abnormalities we have observed in the immunoresistant cell lines are in agreement with previous evidence that gp70 and gp93^{gag} are the MuLV encoded target molecules on the plasma membranes of infected cells (78,80). Unlike gp70, which can be labeled on the cell surface by lactoperoxidase catalyzed iodination with [^{125}I]-iodine, the Friend MuLV-encoded gag antigens were not detected by that technique, presumably because they lack exposed tyrosine residues.[1] Nevertheless, the cell surface gag antigens can be rapidly detected by cytotoxic antisera and by rosetting in the presence of antisera and erythrocytes that have been covalently coupled with protein A (Fig. 8). Furthermore, the gp95^{gag} encoded by Gross MuLV can be labeled on cell surfaces with [^{125}I]-iodine (78) and the gp94^{gag} encoded by Rauscher MuLV is selectively degraded

[1]Subsequent to the body of work reported here in Section IV, a new technique for the detection of cell surface gp70 and gp93^{gag} was developed in this lab and is described in Section V and in Materials and Methods (section II, H4).

by addition of extracellular protease (79). We conclude that the genetic techniques we have used can be useful for identification of cell surface molecules which may be difficult to directly label by chemical methods. We have also used this method to identify an electrophoretically minor form of gp55, which occurs on the surface membranes of cells infected with Friend spleen focus forming virus (172). In all cases, the immunoresistant variants specifically fail to synthesize this minor gp55 component.

2. Cellular Processing of Plasma Membrane Glycoproteins

It has become clear that certain cellular processing events which are essential for plasma membrane placement of particular glycoproteins may have negligible effect on the placement of other glycoproteins. For example, studies of Thy-1 antigen synthesis in mutant mouse lymphoma cells have established that specific oligosaccharide structures are essential for Thy-1 antigen processing into plasma membranes (171,212). Some other membrane glycoproteins are processed into plasma membranes even in the presence of tunicamycin, an antibiotic which blocks oligosaccharide additions to the asparagine side chains of glycoproteins (157,160,161). Similarly, the surface membranes of p30-2 and p30-5 cells appear to lack $gp93^{gag}$ but to contain gp70 (Fig. 12), whereas the surface membranes of H-4 cells are deficient in both glycoproteins (Fig. 8).

Our data seems inconsistent with the hypothesis of a purely passive flow of membrane components during processing from their site of synthesis in the endoplasmic reticulum into plasma membranes (147,213). Although there is evidence suggesting that membrane flow occurs in cells, the differential effect of the cellular mutations in H-4, p30-2 and p30-5 cells on the processing of gp70 and gp93^{gag} are difficult to reconcile with a completely passive transfer process. Rather, these results support the idea that correct placement of a particular plasma membrane glycoprotein requires specific interactions with a carrier or transport component(s). Therefore abnormalities within the subcellular organelles of glycosylation, pH, ionic millieu or of specific carrier components could interfere selectively with the processing of particular glycoproteins. We do not yet know the nature of the abnormalities in the mutants we have isolated.

3. Budding of Virus Particles from Cells Lacking gag or env Plasma Membrane Antigens

Our results suggest that virus particles are released from H-4 cells that appear to be deficient in surface membrane gp70 and gp93^{gag}, and from p30-2 and p30-5 cells that appear to lack gp93^{gag} but to contain plasma membrane gp70. Previous evidence has also indicated that gp70 and p15(E) are not required for budding of type-C MuLV (68,71,72). Furthermore, our conclusion that cell

surface gag antigens are not required for release of MuLV is consistent with electron microscopic evidence that gag antigens (specifically, the Gross cell surface antigen, GCSA) may be absent at sites of virus budding (70,86) and that gp93^{gag} molecules are not incorporated into released MuLV particles (70,78). Although we have described three distinct immunoresistant mutants that appear to lack cell surface gp93^{gag} and to release substantial amounts of MuLV, we emphasize that other cellular mutants which lack gp93^{gag} seem unable to release MuLV particles in significant amounts (Table 2). This is not surprising since cellular mutations often have pleiotropic effects.

We therefore propose that budding occurs when MuLV core components assembling in the cytoplasm bind to one or more cell-encoded constitutents on the inner surface of the plasma membrane. Presumably, these constituents could be lipids or proteins and they may be abnormal in the p30-12 and p12-12 mutants (Fig. 5), which do not release virus particles (Table 2). This binding reaction must somehow facilitate accumulation at these same sites of gp70 and p15(E) complexes, which are peripherally located on the opposite membrane surface (70,105,124). Although accumulation of gp70 and p15(E) at these budding sites must be energetically favorable to proceed, our data and previous results (68,71,72) suggest that it cannot provide a major driving force necessary for the continuation or completion of the virus release

process.

We believe that our results exclude any direct or necessary role for plasma membrane gp93^{gag} in MuLV release from cells. Furthermore, since gp93^{gag} seems to be absent from virions (78), it is unlikely that it could play any role in provirus synthesis in newly infected cells. Since MuLVs have evolved as endogenously inherited viruses, it is perhaps to be expected that some of their gene products might not function in the process of exogenous infection.

V. TWO PLASMA MEMBRANE GLYCOPROTEINS ENCODED BY MURINE LEUKEMIA VIRUS ARE TRANSPORTED INTRACELLULARLY WITH WIDELY DIFFERENT KINETICS

A. General Introduction

As described in the Introduction (section I, H), the study of the MuLV-encoded membrane glycoproteins can serve as a valuable model for eukaryotic membrane glycoprotein biosynthesis. This pathway of biosynthesis and processing is believed to proceed from synthesis in the rough endoplasmic reticulum via the Golgi apparatus to the cell surface and is more thoroughly described in the Introduction (section I, G). An important question in this field concerns the mechanism for selection and transfer of newly synthesized proteins between organelles. Although it is generally believed that transfer occurs in a linear manner as a cohort of simultaneously made proteins that are carried along by lipid flow (110,147), the literature does contain evidence to suggest that this model is oversimplified (170). Furthermore, Rothman has recently proposed a more complex process occurring in the Golgi apparatus in which proteins are fractionated by a reflux type mechanism (111).

In this chapter we describe kinetic studies of the synthesis and processing of the MuLV-encoded membrane glycoproteins, gp70 and $gp93^{\underline{gag}}$. These glycoproteins are processed into plasma membranes with widely different kinetics. Furthermore, there is a slow step in gp70

processing which appears to occur in the rough endoplasmic reticulum. Passage through this barrier occurs from a precursor pool in a random rather than a cohort fashion. Our data can be explained by a simple model - that transport between organelles involves interaction with specific carrier molecules that bind to different proteins with different affinities.

B. The Overall Approach Used

Cell cultures infected with MuLV were labeled with L-[^{35}S]-methionine by a pulse-chase procedure (see Materials and Methods, section II, C4 and H4) and the virus-encoded gene products were subsequently isolated by precipitation with antisera specific for gp70 and p30. The radioactive components were then analyzed by polyacrylamide gel electrophoresis. For the initial investigations, virus-encoded gene products were analyzed in three fractions: in whole cell lysates (Figs. 14A and 15A), on the outer surface of plasma membranes (Fig. 14B and 15B), and in released virions (Fig. 16). This data was consistently obtained in several independently conducted experiments. Careful comparison of these data reveals several important facts about the synthesis and transport to the cell surface of MuLV-encoded gylcoproteins.

1. Analysis of Whole Cell Lysates

As shown by the whole cell lysate data in Fig. 14A, after brief labeling times (lanes 1, 2 and 7, 8) the major viral gene products are gPr90$^{\underline{env}}$, the

Figure 14. Pulse-Chase Analysis of Synthesis, Processing and Cell Surface Expression of the F-MuLV-Encoded Membrane Glycoproteins.

Eveline cells were pulse-labeled with [^{35}S]-methionine in methionine-free minimum essential medium for 15 or 30 min and then chased by the addition of unlabeled methionine for 30 min, 1 hr, 2 hr or 24 hr as described in Materials and Methods (Section II, C4). After these labeling periods, equal aliquots of cells from each pulse-chase time point were split for analysis of viral proteins present in whole cell lysates (Panel A) or on the cell surface (Panel B) by immunoprecipitation using antisera directed against Rauscher MuLV p30 (lanes 1-6) or gp70 (lanes 7-12) as described in Materials and Methods (Section II, H4). These immunoprecipitates were then analysed by electrophoresis on 10-20% polyacrylamide gradient gels containing 0.1% sodium dodecyl sulfate as described in Materials and Methods (section II, G) (lanes 1 and 7) 15 min pulse, (lanes 2 and 8) 30 min pulse, (lanes 3 and 9) 30 min chase after 30 min pulse, (lanes 4 and 10) 1 hr chase after 30 min pulse, (lanes 5 and 11) 2 hr chase after 30 min pulse, (lanes 6 and 12) 24 hr chase after 30 min pulse. The fluorogram in Panel B was exposed for twice as long as the fluorogram in Panel A to make gp70 in Panel B readily visible compared to gp70 in Panel A. For accurate relative comparisons of the whole cell versus cell surface levels see Figure 15A and 15B.

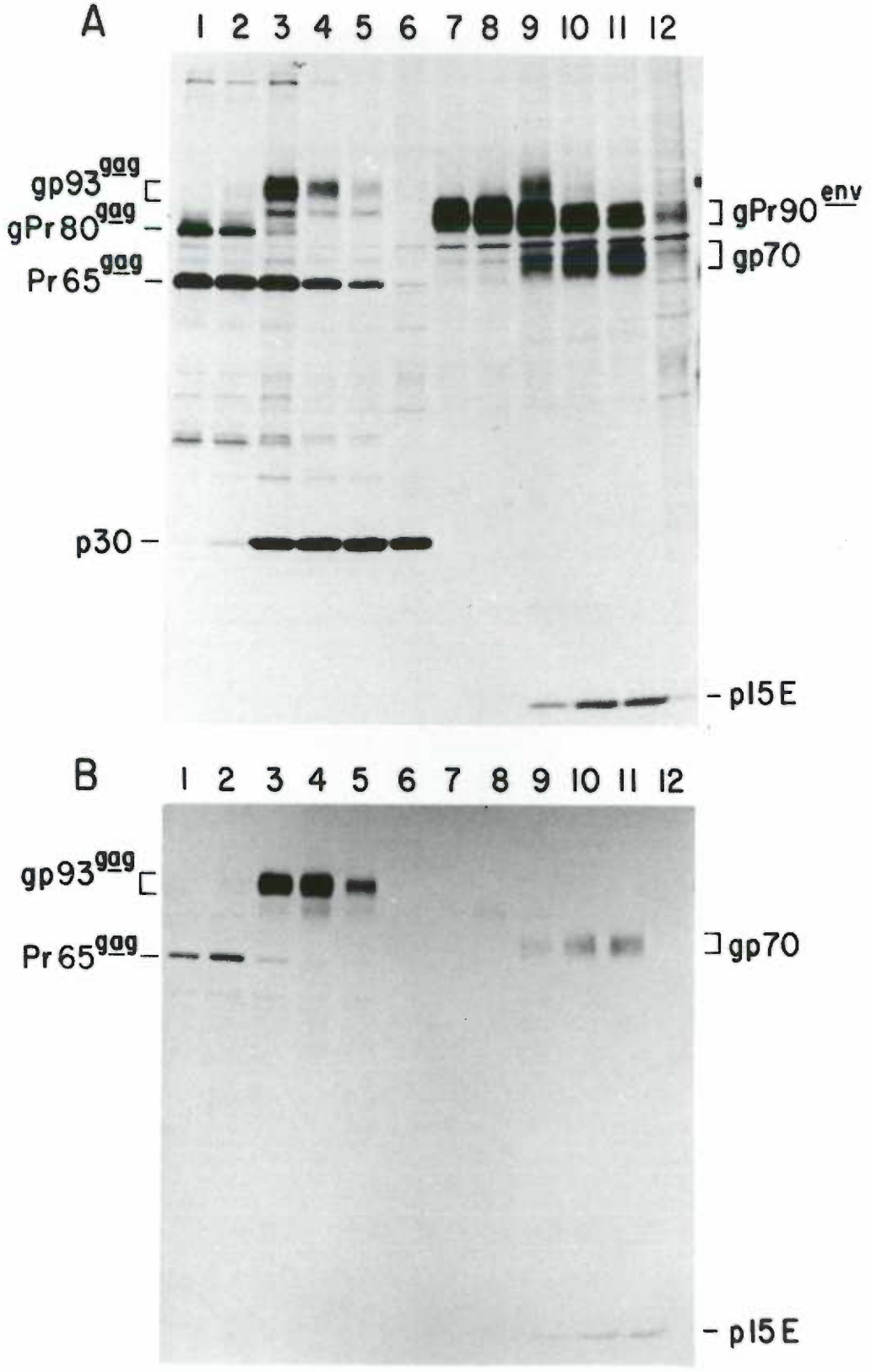

A
1 2 3 4 5 6 7 8 9 10 11 12
gp93gag
gPr80gag
Pr65gag
p30
gPr90env
gp70
p15E
B
1 2 3 4 5 6 7 8 9 10 11 12
gp93gag
Pr65gag
gp70
p15E

Figure 15. Relative Amounts of the F-MuLV-Encoded Membrane Glycoproteins Present Either in Whole Cell Lysates or On the Cell Surface During a Pulse-Chase Labeling Analysis of Eveline Cells.

Quantitive analysis of the relative intensities of MuLV-encoded proteins displayed in the pulse-chase analysis of Figure 14 was conducted by scanning the fluorograms with a Transidyne General integrating densitometer as described in Materials and Methods (section II, G). See legend to Figure 14 for a description of the [^{35}S]-methionine labeling conditions, viral protein immunoprecipitation and electrophoretic analysis. (●) gPr90$^{\underline{env}}$, (0) gp70, (■), gPr80$^{\underline{gag}}$, (□) gp93$^{\underline{gag}}$.

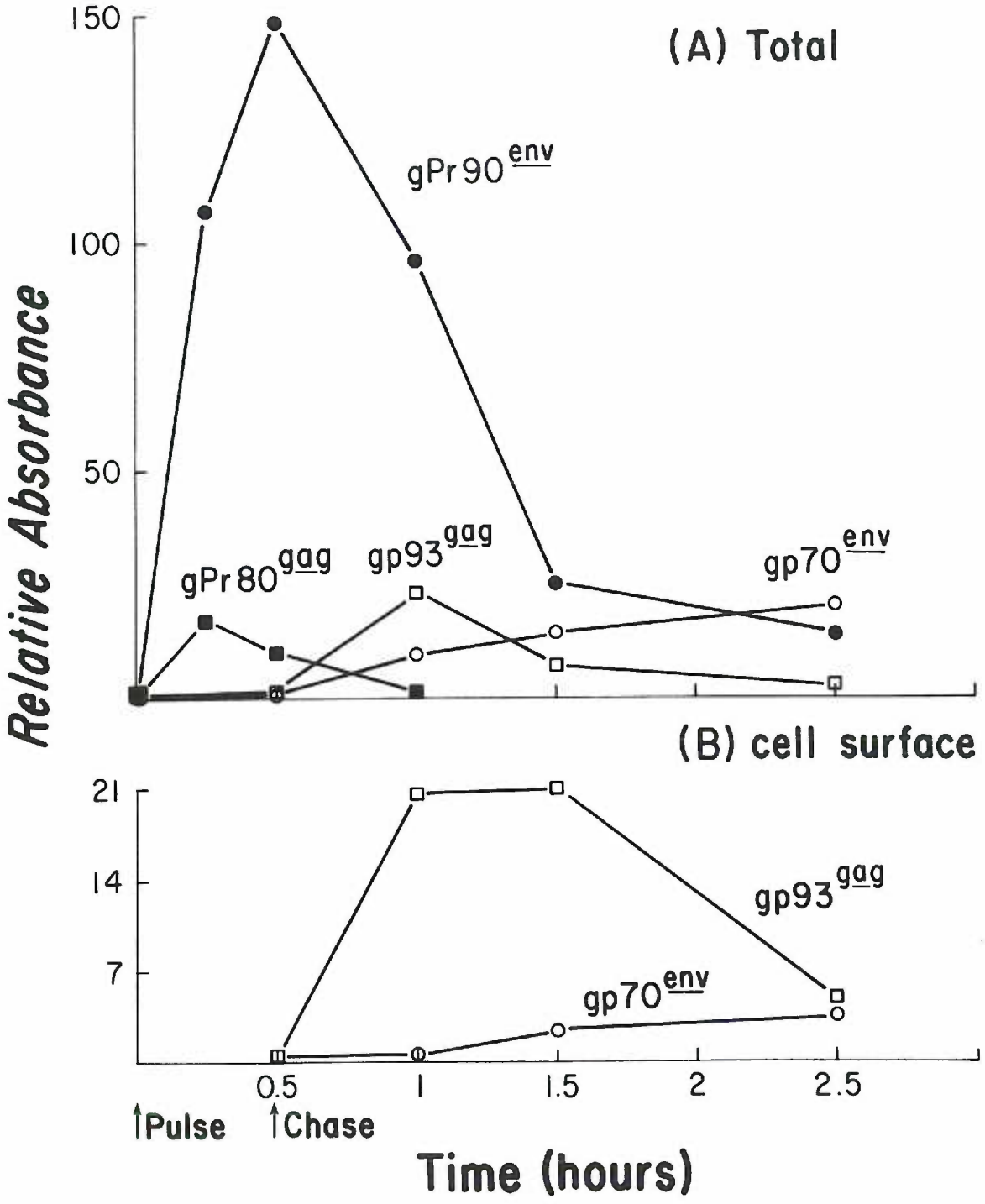
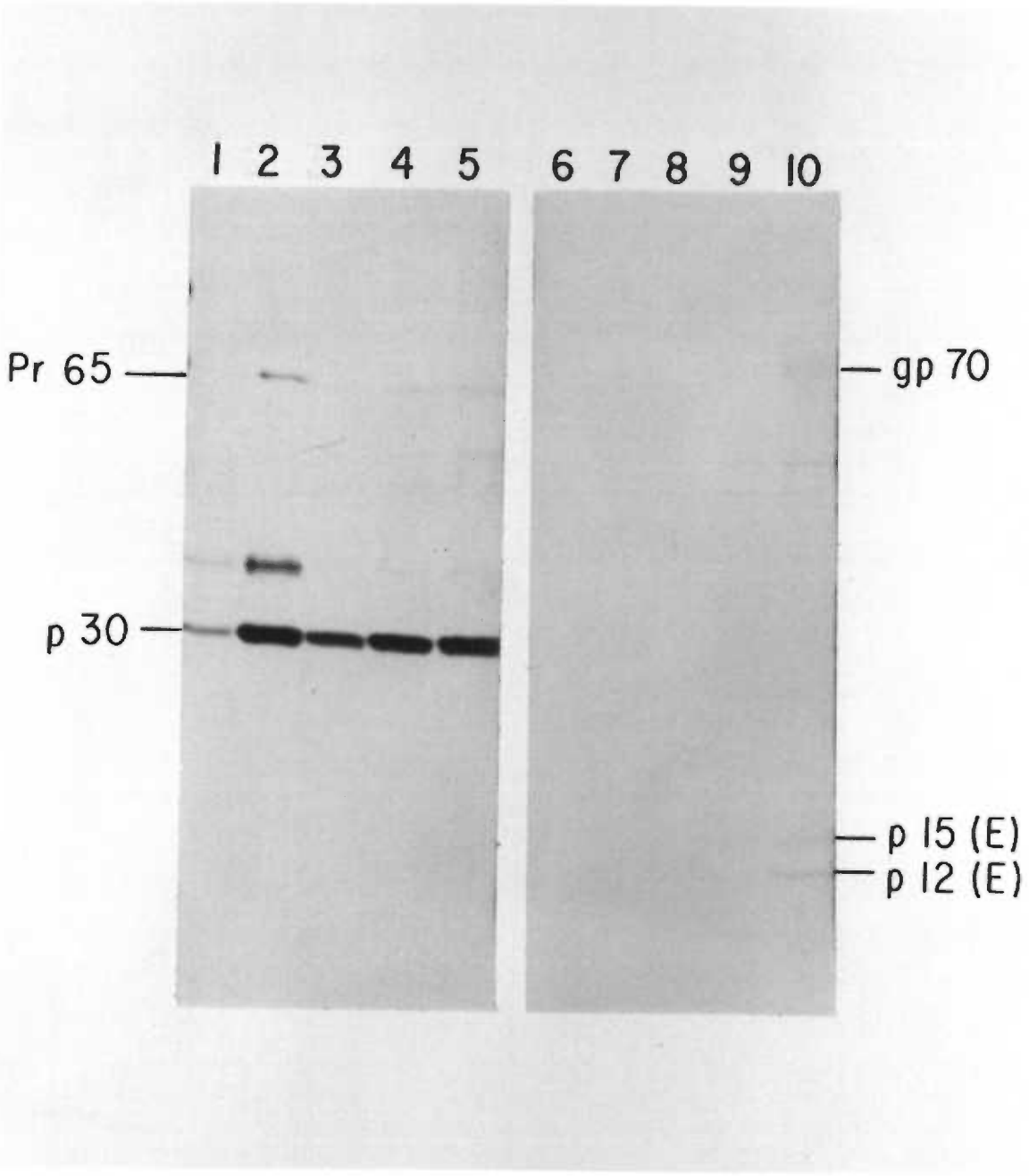

150
100
50
Relative Absorbance
(A) Total
gPr90 env
gPr80 gag
gp93 gag
gp70 env
(B) cell surface
21
14
7
gp93 gag
gp70 env
0.5
1
1.5
2
2.5
Pulse
Chase
Time (hours)

Figure 16. Pulse-Chase Analysis of Friend MuLV-Encoded Proteins Present in Virion Particles Released From Eveline Cells.

Cells were labeled with [^{35}S]-methionine as described in the legend to Figure 14. Virus released into culture medium at the times indicated was harvested by centrifugation as described in Materials and Methods (section II, H4). The F-MuLV-encoded proteins in released virions were analysed by immunoprecipitating the virus with anti-p30 antiserum (lanes 1-5) or with anti-p70 antiserum (lanes 6-10) and electrophoresing the immunoprecipitates on polyacrylamide gels containing SDS as described in Materials and Methods. (lanes 1 and 6) 15 min pulse, (lanes 2 and 7) 30 min pulse, (lanes 3 and 8) 30 min chase after 30 min pulse, (lanes 4 and 9) 1 hr chase after 30 min pulse, (lanes 5 and 10) 2 hr chase after 30 min pulse. "Pr65" is synonomous with $Pr65^{gag}$ discussed in the text.


1 2 3 4 5 6 7 8 9 10
Pr 65
p 30
gp 70
p 15 (E)
p 12 (E)

precursor of gp70 and p15 E (98), Pr65^{gag}, the precursor of the virion internal core proteins (p15, p30, p12 and p10) (21), gPr80^{gag}, the precursor of gp93^{gag} (46,47), and Pr180$^{gag-pol}$, the precursor of reverse transcriptase (29,50). Furthermore, during a subsequent chase with an excess of nonradioactive methionine (lanes 3-6 and 9-12), these precursors are processed to form their expected products. In addition, there is a striking difference in the kinetics of synthesis of gp70 compared with gp93^{gag} whereas gPr80^{gag} is rapidly and quantitatively processed to form gp93^{gag} which is degraded or shed from the cells (47). During the chase period, the gPr90^{env} concentration decreases throughout the chase period and gp70 only slowly accumulates in the cells. A quantitative analysis of Fig. 14A is shown in Fig. 15A.

2. Transfer of Viral Glycoproteins to the Cell Surface

For the analysis of [^{35}S]-methionine labeled components in the plasma membrane (Fig. 14B and 15B), the antisera were adsorbed onto the cells before lysis, the unbound serum was removed, the cells were lysed, and the antigen-antibody complexes were then isolated. Consistent with previous evidence obtained by other methods (45,46,77,78), gp93^{gag} and gp70 are the major viral gene products on the cell surface. Furthermore, this method appears to be highly specific for detection of cell surface components. For example, the gp93^{gag}/Pr65^{gag}

ratio in lane 3 is at least 100 times higher in the surface preparation than in the whole cell lysate. Similarly, the gp70/gPr90$^{\underline{env}}$ ratio is at least 100 times higher in lanes 9-12 in Fig. 14B than in Fig. 14A. Presumably, therefore, the small amount of Pr65$^{\underline{gag}}$ and gPr90$^{\underline{env}}$ in Fig. 14B could have derived from a few broken cells.

Analysis of Fig. 14B and 15B clearly indicate that gp70 and gp93$^{\underline{gag}}$ are processed to the cell surface with different kinetics. Although both first reach the cell surface between 30-60 min after addition of [^{35}S]-methionine to cells (see lanes 3 and 9), gp93$^{\underline{gag}}$ is transferred efficiently as a cohort whereas gp70 accumulates on the plasma membrane only slowly. However, within the resolution of the sampling times used, both of these glycoproteins accumulate on the cell surface with the same kinetics that they accumulate in the whole cells (compare Fig. 14A with 14B). In other words, once gp70 and gp93$^{\underline{gag}}$ are formed intracellularly, they can be transported to the plasma membranes without a substantial lag (i.e., within 10-15 min). Clearly, therefore, the slow step in gp70 transfer to the cell surface precedes cleavage of the gPr90$^{\underline{env}}$ precursor. In addition, the amounts of gp93$^{\underline{gag}}$ isolated from the cell surface are similar to the total cellular amounts, suggesting that gp93$^{\underline{gag}}$ may be transferred efficiently to the cell surface. On the contrary, the gp70 which we detect on the

cell surface constitutes only approximately 16% of the total cellular quantity. This suggests either that gp70 is predominantly located at intracellular site(s) or that the cell surface antibody binding method does not efficiently detect plasma membrane gp70. For example, if the antibodies had a low avidity the gp70-antibody complexes might partially disassociate before they could be isolated.

3. Transfer of the MuLV Components into Virions

Our studies of virion release appear to be consistent with previous evidence. As is shown in Fig. 16, virion cores containing [^{35}S]-methionine labeled Pr65^{gag} and its products assemble from the cytoplasmic pool without a substantial lag period and are released into virions. The quantities of radioactivity observed in Fig. 16 are not directly comparable to those in Fig. 14 and 15 because the autoradiogram exposure time was ten times longer and the sample volume was four times larger. It is known that Pr65^{gag} is enriched in newly released virions which have a morphologically immature core structure and that its proteolysis coincides with core maturation (66). Comparison of Figs. 14 and 16 suggest that the cytoplasmic pool of Pr65^{gag} may be fairly large compared with the rate of virion release, so that [^{35}S]-methionine labeled Pr65^{gag} is only slowly removed from the

cells. In contrast to Pr65^{gag} incorporation, gp70 is only slowly released into virions (lanes 6-10). This is consistent with our observation that gp70 only slowly accumulates in the plasma membrane pool (Fig. 14 and 15) and with the fact that plasma membrane gp70 is a precursor of virion gp70 (97). A similarly slow entry of newly-made gp70 into virion was previously observed by Witte and Weissman (214,215). Throughout the time period studied, we did not detect any soluble radioactive gp70 antigens in the culture media (data not shown). Presumably, such soluble gp70 (97,216) derives from virions and may be formed only slowly. In addition, as reported earlier (78), gp93^{gag} does not occur in released virions.

C. In Which Subcellular Organelle Does gPr90^{env} Accumulate?

As will be discussed, the above data suggest that there is a slow step in gPr90^{nv} processing. Once past this barrier, however, transfer and completion of processing rapidly ensue. A key question, therefore, concerns the subcellular site for this barrier which must also be the site for accumulation of the gPr90^{env} precursor. We have approached this issue by subcellular fractionation of F4-6 erythroleukemia cells which are infected with both Friend MuLV and with the Friend strain of spleen focus forming virus. The latter cell line was used because gp70 synthesis kinetics appeared indistinguishable in these cells and in Eveline cells and

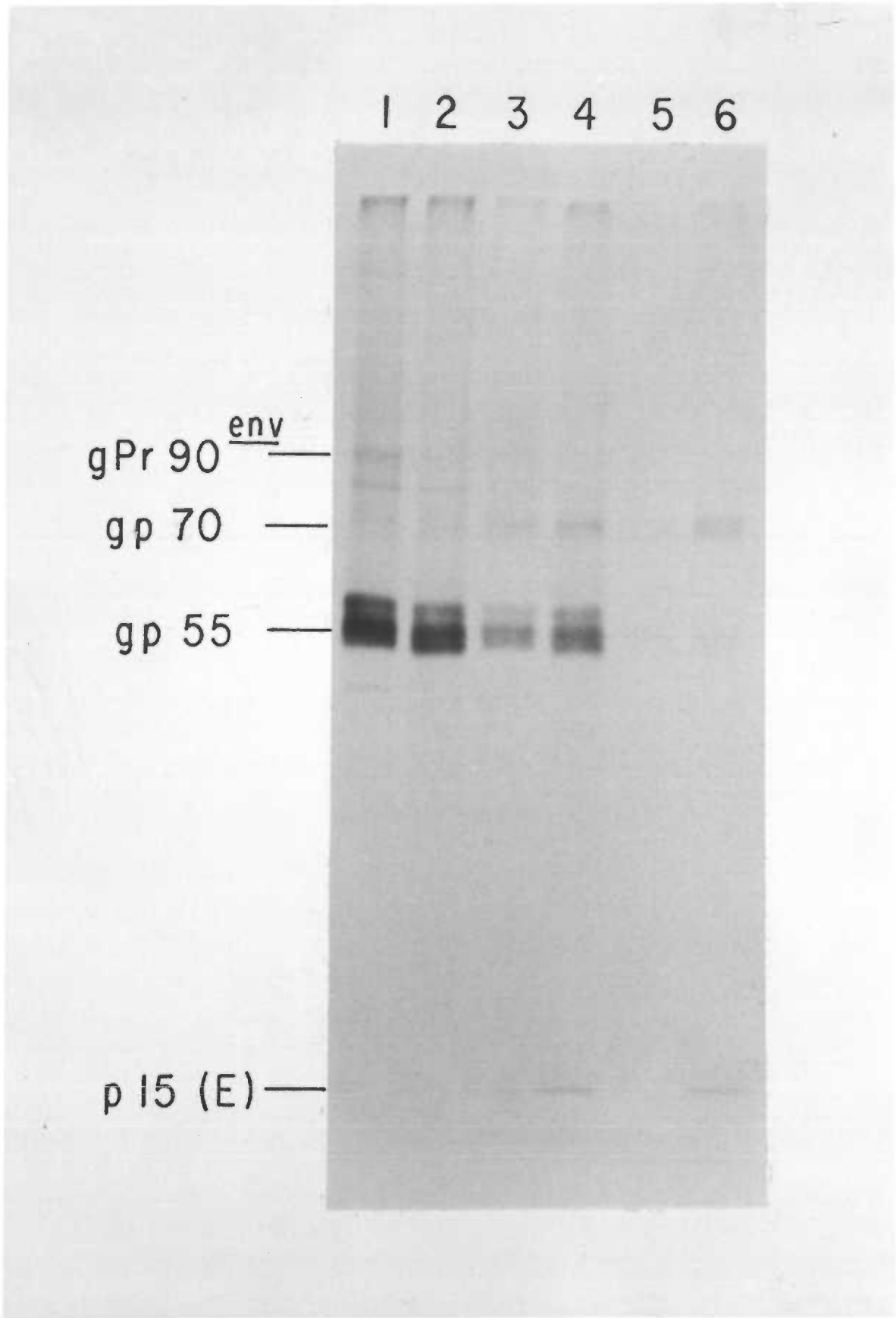
because we had previously developed methods for fractionation of its membranous subcellular organelles (217).

The latter studies had indicated that the membrane fraction which bands on discontinuous sucrose gradients at the interface between 40-50% sucrose contains ribosome and consists of highly purified rough endoplasmic reticulum (RER). Similarly, the fraction at the 20-35% interface is enriched in plasma membranes (197) whereas the intermediate fraction may contain Golgi membranes plus contaminating RER and plasma membranes. As observed by others (197), these preparations are enriched in their content of different membranous organelles, but they are not completely pure.

Figure 17 shows an analysis of virus encoded glycoproteins in different membrane fractions isolated from cells that had been pulse labeled with [^{35}S]-methionine for 30 min and then chased with unlabeled methionine for 1 and 2 hours. As expected, gp70 is substantially enriched in the plasma membranes whereas gPr90$^{\underline{env}}$ is nearly absent from this fraction (lanes 5 & 6). Furthermore, the gp55 glycoprotein, which is encoded by the spleen focus forming virus (177,208), seems to be largely confined to the rough endoplasmic reticulum (lanes 1 & 2) and to be nearly absent from plasma membranes (lanes 5 & 6), consistent with other evidence (217,218). Interestingly, [^{35}S]-labeled gPr90$^{\underline{env}}$ appears to be

Figure 17. Distribution of [^{35}S]-Methionine Labeled gp70 and gPr90$^{\underline{env}}$ Among Different Subcellular Organelles of F-MuLV-Infected Cells Fractionated on Discontinuous Sucrose Gradients.

F4-6 erythroleukemia cells were pulse-labeled with [^{35}S]-methionine in methionine-free minimum essential medium for 30 min and then chased by the addition of unlabeled methionine for 1 hr (lanes 1, 3 and 5) or 2 hr (lanes 2, 4 and 6) as described in Materials and Methods (section II, C4). Cells were then lysed and fractionated by differential centrifugation, and the membrane fraction was further resolved on discontinuous sucrose gradients (197) as described in Materials and Methods (section II, O). The sucrose interface fraction at 20%/35% contains plasma membranes (lanes 5 and 6), 35%/40% contains an intermediate mixture of plasma membranes, Golgi apparatus, and rough endoplasmic reticulum (lanes 3 and 4) and 40%/50% contains rough endoplasmic reticulum (lanes 1 and 2) according to Courtneidge et al., (197). Fractions were then immunoprecipitated with anti-gp70 antiserum and the immunoprecipitates were analysed by electrophoresis on 10-20% polyacrylamide gradient gels containing 0.1% SDS as described in Materials and Methods (section II, G). F4-6 cells express a complex of Friend virus containing both helper F-MuLV and spleen focus-forming virus (F-SFFV) in a ratio of 1:2 (177). The synthesis of gp55 is encoded for by F-SFFV (177,208), and is predominantly found in the rough endoplasmic reticulum (217,218).


1 2 3 4 5 6
env
gPr 90
gp 70
gp 55
p 15 (E)

present in largest amounts in the rough endoplasmic reticulum fraction and to remain in that fraction even two hours after initiating the cold chase with nonradioactive methionine (lane 2). As discussed below, the idea that the gPr90^{env} pool is confined to the rough endoplasmic reticulum is consistent with the fact that its oligosaccharides have a high-mannose type structure that is susceptible to digestion with endoglycosidase H (104,112,144).

D. Discussion

1. gp93^{gag} Is On The Surface Membranes of Cells Infected with the Friend Strain of MuLV

The antibody adsorption method for identifying molecules on the external surface of cellular plasma membranes (191,192) has been used to study the processing of H-2 alloantigens (192) and of HLA antigens (159,191) but has not been previously applied to virus infected cells. It has several advantages over other methods because the components are labeled by endogenous incorporation of radioactive amino acids, and it can therefore be used to study the kinetics of synthesis, intracellular transport, and turnover of the plasma membrane constituents. In addition, certain plasma membrane constitutents cannot be labeled by externally added reagents. For example, gp93^{gag} encoded by Friend MuLV cannot be labeled by lactoperoxidase catalyzed iodination, and we were previously unable to identify the

cell surface gag antigens by direct methods (73) despite evidence that the gp93^{gag}-type molecules encoded by Gross and Rauscher MuLVs are on the cell periphery (77,80). The role of gp93^{gag} in the virus life cycle is unknown (73). However, it apparently interacts with the host immune system (85) and therefore is probably important in the host response to virus-induced cancers.

2. gp70 Is Processed to the Cell Surface More Slowly than gp93^{gag}

gp70 is processed to the cell surface more slowly than gp93^{gag} (Fig. 14B and 15B), or other plasma membrane glycoproteins which have been previously described (97,165,191,237). This conclusion is strengthened by our analysis of two different glycoproteins in the same cells by the same technique.

An important result of this study is that there is a slow step in envelope glycoprotein processing and that after this slow step has been passed gp70 is processed quickly and without substantial hindrance to the cell surface. Furthermore, this processing barrier precedes proteolytic cleavage of gPr90^{env}. As a consequence, gPr90^{env} persists in the cells for a substantial time after its synthesis, and gp70 accumulates relatively slowly. However, the kinetics of gp70 appearance on the cell surface (Fig. 14B) is not significantly or reproducibly retarded compared to the appearance of total gp70 (Fig. 14A).

Several lines of evidence suggest that the slow step in gPr90$^{\underline{env}}$ processing occurs in the rough endoplasmic reticulum. First, our subcellular fractionation studies suggest that gPr90$^{\underline{env}}$ persists in the rough endoplasmic reticulum for a prolonged time after its synthesis (Fig. 17). Secondly, it is known that gPr90$^{\underline{env}}$ of Friend MuLV contains seven asn-linked oligosaccharides (219) which have a high-mannose type structure and are susceptible to cleavage with endoglycosidase H (104,144). However, gPr90$^{\underline{env}}$ lacks complex-type oligosaccharides with fucose and sialic acid which occur in gp70 (99,116,144). It is known that high-mannose type oligosaccharides with a $Glc_3Man_9GlcNAc_2$-structure are cotranslationally added onto glycoproteins in the rough endoplasmic reticulum (166,168). These are subsequently modified in the same organelle by removal of Glc and Man residues to produce a $Man_5GlcNAc_2$-structure (114,220). However, high-mannose type structures can only be built up to form large complex-type oligosaccharides after the glycoproteins have transferred into the Golgi apparatus (114,115).

3. Envelope Glycoprotein Transfer Past the Kinetic Barrier Occurs Randomly Rather Than in the Cohort Order of Synthesis

Our data strongly suggest that the transfer of envelope glycoprotein past the slow step in gPr90$^{\underline{env}}$ processing occurs randomly rather than in the cohort order

of glycoprotein synthesis. Thus, envelope glycoprotein which is synthesized during a brief 30 min pulse period arrived slowly at the cell surface at least throughout the subsequent 30 min - 2 hour period. Furthermore, some gPr90$^{\underline{env}}$ is still not processed after a 24 hour chase (Fig. 14A, lane 12). Consequently, it is apparent that some gp70 which was synthesized after the radioactive pulse period must have reached the cell surface before all of the radioactive gp70 had been transferred.

We therefore propose that gP90$^{\underline{env}}$ in the rough endoplasmic reticulum accumulates in a pool in front of this kinetic barrier. Furthermore, molecules are transferred past this barrier randomly rather than in the cohort order of their synthesis. According to this model, it would be expected that some newly made radioactive envelope glycoprotein would pass quickly through the barrier and that its products would rapidly reach the cell surface. This is consistent with the fact that radioactive gp70 and gp93$^{\underline{gag}}$ begin to reach the cell surface at the same time (Fig. 14B, lanes 3 and 9). The major difference is that the radioactive gp93$^{\underline{gag}}$ all reaches the cell surface at that time whereas the radioactive gp70 is transferred to the surface relatively slowly. According to our model, glycoproteins are not transferred in a cohort-type fashion. If there are no slow steps in their processing, however, they would appear to be transferred

as a cohort.

4. What is the Rate-Limiting Step for gp70 Processing?

Three events are known to be temporally closely related to the rate-limiting step in $gPr90^{\underline{env}}$ processing. These include the exit from the rough endoplasmic reticulum, partial proteolytic cleavage of $gPr90^{\underline{env}}$, and the oligosaccharide modifications which produce complex-type oligosaccharides. However, the oligosaccharide modifications occur in the Golgi apparatus (113-115) and are also not required for gp70 transport to the cell surface (234). Consequently, we believe that the rate-limiting step for $gPr90^{\underline{env}}$ processing may be its partial proteolysis or its exit from the rough endoplasmic reticulum. Presumably, either of these events could be a prerequisite for the other.

5. A Carrier-Mediated Model for Membrane Glycoprotein Transport Between Organelles

We believe that our results have important implications concerning the mechanism of membrane glycoprotein transport between organelles. Specifically, our data imply that glycoprotein exit from the rough endoplasmic reticulum probably occurs randomly from a precursor pool rather than in the cohort order of synthesis. Furthermore, the exit of different membrane glycoproteins can occur at widely differing rates.

These properties of the exit mechanism are precisely what would be expected of a simple binding reaction in which transport of any glycoprotein required its attachment to one or more carrier molecule(s). These carriers could be proteins or lipids and they would be expected to bind to different glycoproteins with different affinities. Furthermore, binding to the carriers could in some cases be facilitated by covalent modifications such as partial proteolysis or glycosylation. However, the critical binding sites on the substrate glycoproteins, at least for transfer to the cell surface, probably involve the amino acid sequence of the polypeptide chain because some proteins can be transferred efficiently to the cell surface in the presence of glycosylation inhibitors (157,159,235).

Although our observations would be compatible with the alternative hypothesis, that binding to a matrix is required for retention in the rough endoplasmic reticulum and that transport to the Golgi apparatus automatically follows release from this matrix, we consider this hypothesis inherently unlikely. For example, it cannot be readily reconciled with the fact that mutant forms of plasma membrane and secretory glycoproteins are frequently retained in the rough endoplasmic reticulum (217,218). Such evidence is fully compatible with the carrier mediated transport hypothesis.

VI. MURINE LEUKEMIA VIRUS MUTANTS DEFECTIVE IN SUBCELLULAR PLACEMENT OF *gag* GENE-ENCODED POLYPROTEINS

A. General Introduction

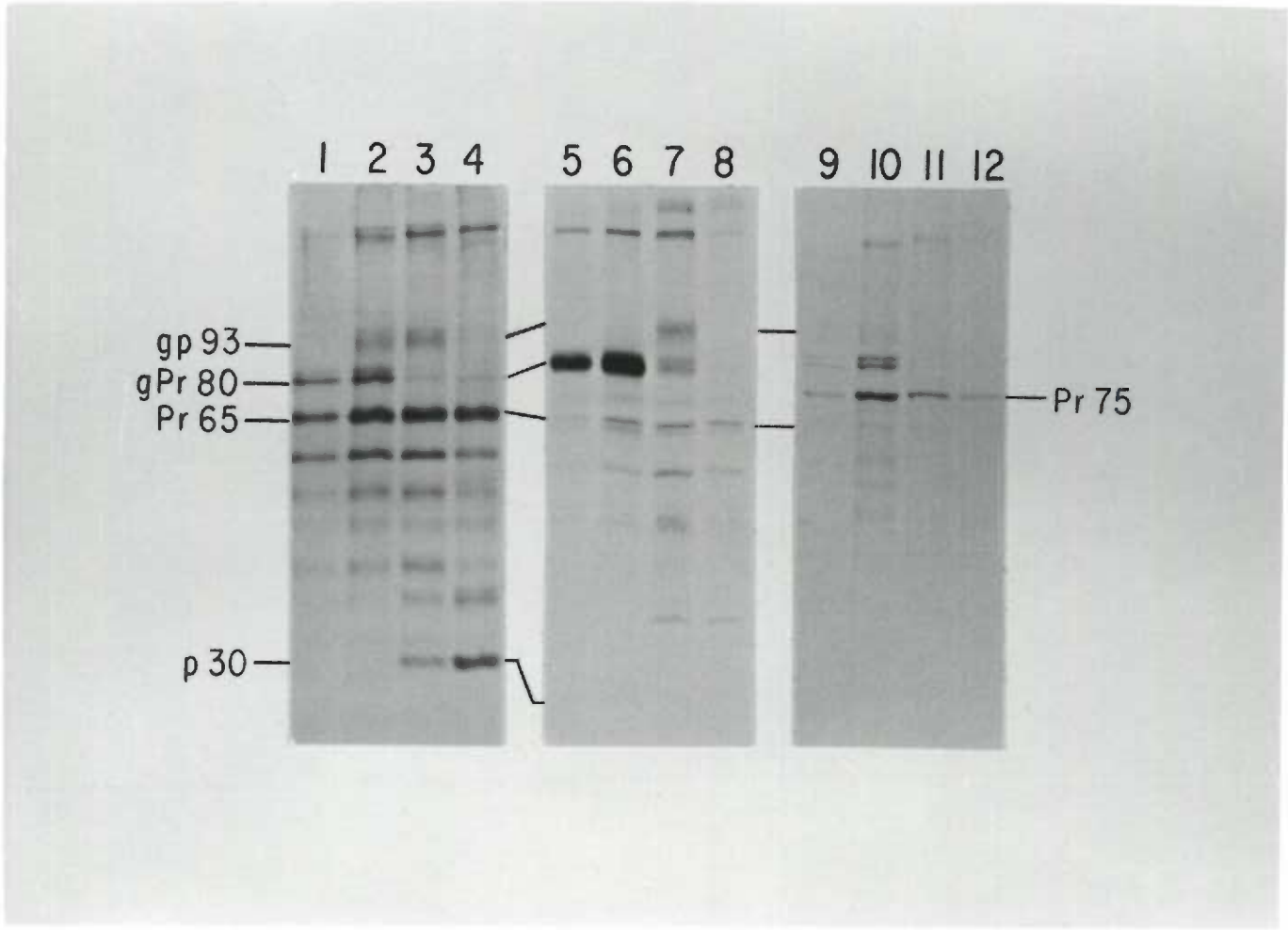
The *gag* gene of MuLV encodes a heterogeneous group of proteins which are processed in different subcellular regions (46,47,79). This synthesis and processing pathway is more thoroughly described in section I, D3. Briefly, $Pr75^{gag}$ is synthesized on the rough endoplasmic reticulum, cotranslationally glycosylated to form $gPr80^{gag}$, processed in the Golgi apparatus to form $gp93^{gag}$ and transported to the cell surface. Alternatively, $Pr65^{gag}$ is synthesized in the cytoplasm and processed to the virion core proteins p30, p15, p12, and p10 during core assembly, budding and maturation. Cell-free protein synthesis (47) and nucleic acid sequence analyses (27) suggest that $Pr75^{gag}$ contains an amino terminal "signal" sequence which is absent from $Pr65^{gag}$ and that the discrete mRNAs for these *gag* gene products must be produced by different splicings of the genomic MuLV RNA. We now describe two mutants which differentially affect the cytoplasmic versus membranous forms of *gag* gene products. The properties of these mutants are consistentwith the signal sequence RNA splicing model described above.

B. Two MuLV Mutants With Abnormal gag Gene Precursors

The precursor-product relationship of gag gene-encoded proteins in the two mutant MuLV-infected cell lines were analyzed by pulse labeling these cells with [^{35}S]-methionine and then subjecting them to a cold chase with nonradioactive methionine. The mutant M-13 cell line rapidly synthesizes two gag gene-encoded translation products with apparent Mr's of 80,000 and 75,000 (see Fig. 18) believed to represent the previously characterized gPr80^{gag} and Pr75^{gag} molecules (47). The minor gPr80^{gag} component is processed to form the surface molecule gp93^{gag} in both the wild-type (lanes 1-4) and mutant M-13 cell lines (lanes 9-12). Pr75^{gag} is degraded very slowly and represents the major gag gene-encoded component in this mutant M-13 cell line. The mutant R-61 cell line, on the other hand, rapidly synthesizes gPr80^{gag} and a second primary translation product with an apparent Mr of 65,000 (lanes 5-8), believed to represent Pr65^{gag}, the precursor to the virion core proteins (21). Mutant R-61 Pr65^{gag} is not processed to gag protein p30 and the other virion core antigens (lanes 6-8) in contrast to normal processing observed for the wild-type Pr65^{gag} molecule (lanes 2-4). In addition, gPr80^{gag} appears to be produced in unusually abundant amounts in this mutant compared to its wild-type MuLV correlate glycoprotein.

Figure 18. Pulse-chase Analysis of p30-Specific Proteins in MuLV-Infected Wild-Type, R-61 and M-13 Cells.

Wild-type F12 NRK, R-61 and M-13 cells were pulse labeled with [^{35}S]-methionine for various time intervals and chased by addition of unlabeled methionine for the times indicated. After labeling, cellular lysates were prepared, immunoprecipitated with antiserum to p30 and the innumoprecipitates were electrophoresed in 10-20% polyacrylamide gradient gels containing 0.1% SDS as described in Materials and Methods. In the fluorograph shown lanes (1-4) contain F12 NRK samples, lanes (5-8) contain R-61 samples, and lanes (9-12) contain M-13 samples. (1, 5 and 9) 5 min pulse, (2, 6 and 10) 15 min pulse, (3, 7 and 11) 30 min chase after 15 min pulse, (4, 8 and 12) 2 hr chase after 15 min pulse. The fluorographs shown do not align perfectly because the gels were run at different times. The relative mobilities were determined by including [^{14}C]-labeled molecular weight standards. "Pr65", "gPr80" and "gp93" are synonomous with Pr65^{gag}, gPr80^{gag} and gp93^{gag} discussed in the text.


1 2 3 4 5 6 7 8 9 10 11 12
gp 93
gPr 80
Pr 65
p 30
Pr 75

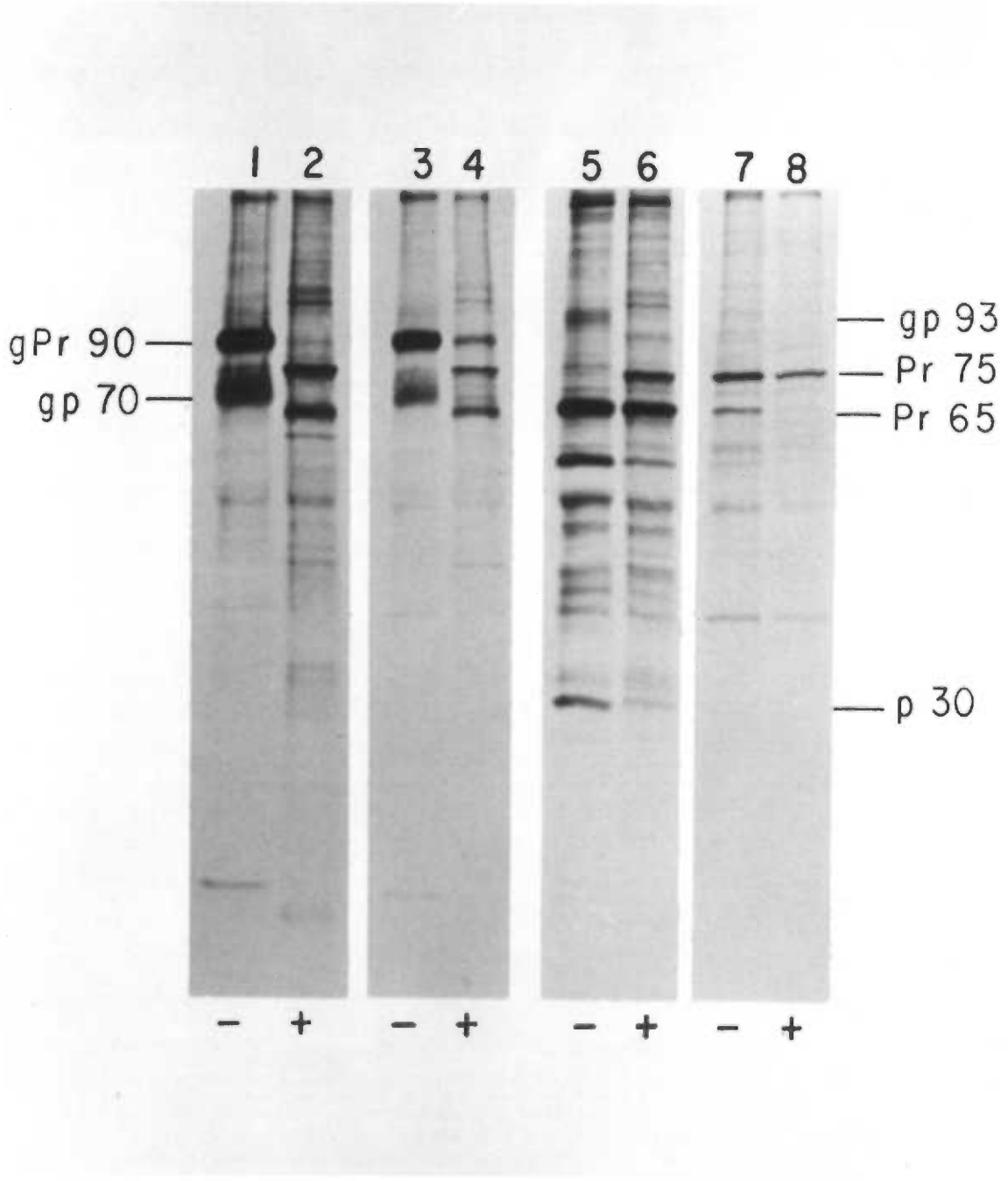
The identity of the 75,000 component observed in M-13 cells was confirmed as Pr75^{gag} by an analysis using cells labeled in the presence of the glycosylation inhibitor, tunicamycin. As shown in Fig. 19 (lanes 1,2 and 3,4), glycosylation was effectively blocked with tunicamycin in both the wild-type and M-13 gPr90^{env} and gp70 molecules. The deglycosylated cores of the proteins can be readily observed in both the tunicamycin-treated wild-type and mutant M-13 cells lines by an increase in the electrophoretic mobilities of these components (see Fig. 19, lanes 2 and 4). However, no shift in electrophoretic mobility occurred on the 75,000 dalton component found in M-13 cells when these cells were treated with tunicamycin (compare lanes 7 and 8), suggesting that this protein was not glycosylated. Furthermore, the 75,000 dalton component in M-13 cells (lane 7) co-migrates with the unglycosylated Pr75^{gag} present in wild-type infected cells treated under similar conditions of inhibition (lane 6). These results demonstrate that the mutant M-13 cells synthesize Pr75^{gag} but they do not glycosylate or process this precursor (as in wild-type infected cells).

C. Cells Lacking Normal Cytoplasmic Core Precursor Do Not Release Virions

Previous studies have established that Pr65^{gag} is the precursor to the major core proteins which make up the bulk of the core structure in released virion (21). The

Figure 19. Analysis of MuLV-Specific Proteins Synthesized in MuLV-Infected Wild-Type and M-13 Cells in the Presence of the Glycosylation Inhibitor Tunicamycin.

Wild-type F12 NRK and M-13 cells were preincubated for 3 hr in medium alone or containing 0.6 mg/ml tunicamycin prior to pulse-labeling with [^{35}S]-methionine in medium with or without tunicamycin. Cellular lysates were then prepared and immunoprecipitated with antiserum to gp70 (lanes 1-4) or to p30 (lanes 5-8). The immunoprecipitates were electrophoresed in 10-20% polyacrylamide gradient gels containing 0.1% SDS as described in Materials and Methods. The fluorograph corresponds to the following cells with (+) or without (-) tunicamycin: (1, 2, 5 and 6) F12 NRK, (3, 4, 7 and 8) M-13. "gPr90", "Pr65", "Pr75" and "gp93" are synonomous with gPr90$^{\underline{env}}$, Pr65$^{\underline{gag}}$, P75$^{\underline{gag}}$ and gp93$^{\underline{gag}}$ discussed in this text.


1 2 3 4 5 6 7 8
gPr 90
gp 70
gp 93
Pr 75
Pr 65
p 30
- + - + - + - +

above kinetic labeling studies suggest that mutants M-13 and R-61 express very little or no Pr65^{gag} and also lack the mature core proteins. Therefore, these mutants are expected to also lack significant virion particle release. To determine the levels of virus released from the mutants, culture supernates were assayed for reverse transcriptase activity present in virion particles. As shown in Table 3, both M-13 and R-61 cells do not release significant amount of virus particles containing reverse transcriptase activity.

D. Expression of the env Gene in M-13 and R-61 Cells

The synthesis and processing of env gene-encoded proteins in mutants M-13 and R-61 was analyzed by electrophoresis of [^{35}S]-methionine labeled env gene products synthesized during a pulse-label and cold-chase experiment. Fig. 20 shows that both wild-type (lanes 1-4) and M-13 cells (lanes 5-8) synthesize and process gPr90^{env} to gp70. Furthermore, the M-13 cells contain a transient gp70-related component of approximately 45,000 daltons (lanes 6-7) which may be the same component that appears in virus preparations by degradation of gp70 (221). In contrast, the R-61 cells synthesize a major env gene product of 80,000 daltons apparent Mr (p80^{env}) and a very small amount of gPr90^{env} (Fig. 20, lanes 9-12). Because the abnormal p80^{env} and the gPr90^{env} molecules are both present at the earliest labeling times, we infer that they

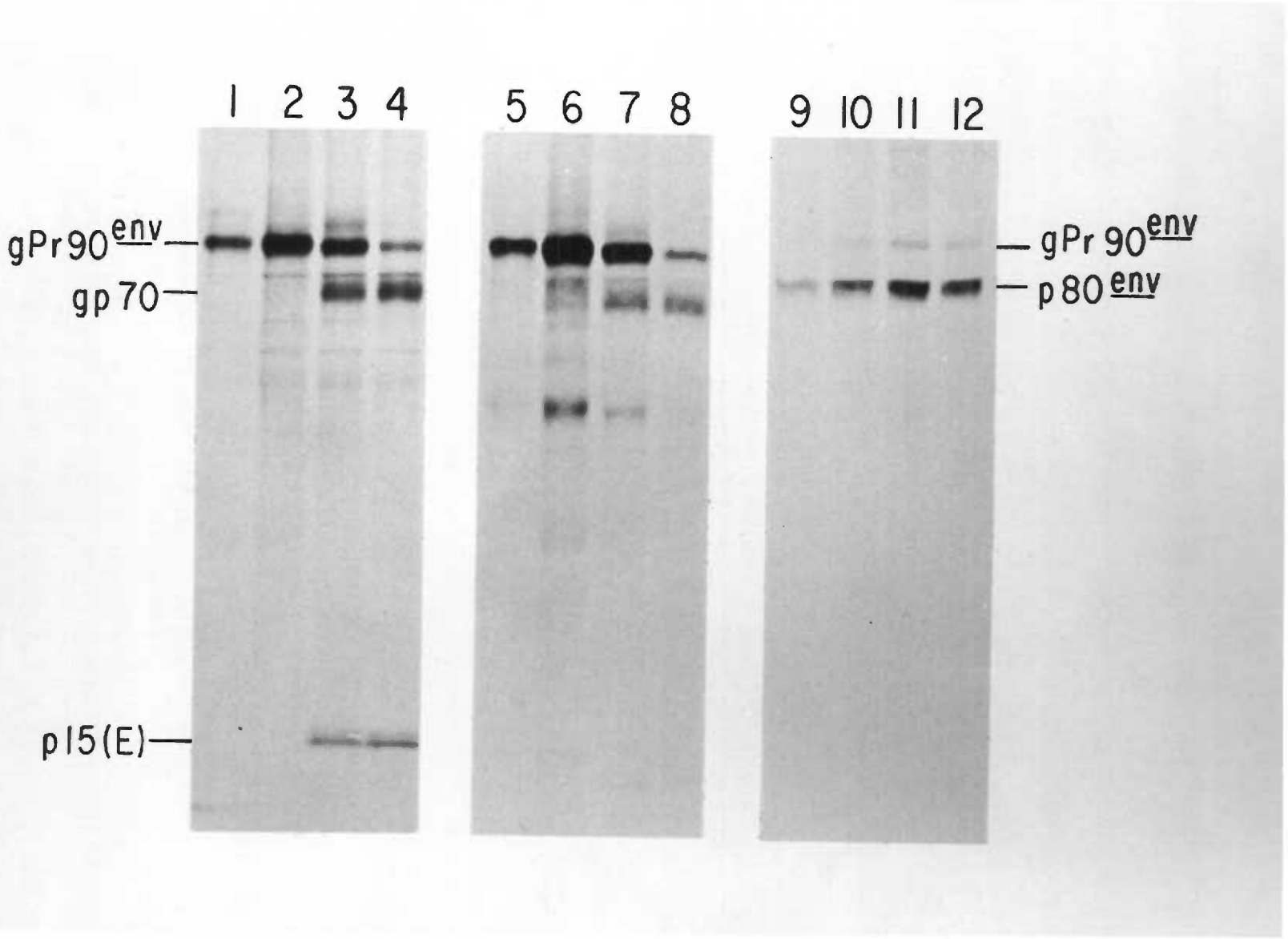
Table 3. Reverse Transcriptase in Particles Released from MuLV-Infected Cells

Cell line	Radioactivity[a] (cpm incorporated per 30' at 30°C)
F12 NRK	34,500
M-13	520
R-61	490

[a] Each assay was performed in duplicate as described in Materials and Methods. Blank sample values (912 cpm/30 min) have been subtracted from radioactivity data.

Figure 20. Pulse-Chase Analysis of gp70-Specific Proteins in MuLV-Infected Wild-Type, M-13 and R-61 Cells.

Wild-type F12 NRK, M-13 and R-61 cells were pulse labeled with [^{35}S]-methionine for various time intervals and then chased by addition of unlabeled methionine for the times indicated. After labeling, cellular lysates were prepared, immunoprecipitated with antiserum to gp70 and the immunoprecipitates were electrophoresed in 10-20% polyacrylamide gradient gels containing 0.1% SDS as described in Materials and Methods. In the fluorograph shown lanes (1-4) contain F12 NRK samples, lanes (5-8) contain M-13 samples and lanes (9-12) contain R-61 samples (1, 5 and 9) 5 min pulse, (2, 6 and 10) 15 min pulse, (3, 7 and 11) 30 min chase after 15 min pulse, (4, 8 and 12) 2 hr chase after 15 min pulse. The fluorographs shown do not align perfectly because the gels were run at different times. The relative mobilities were determined by including [^{14}C]-labeled molecular weight standards.


1 2 3 4
5 6 7 8
9 10 11 12
gPr90env
gp70
p15(E)
gPr90env
p80env

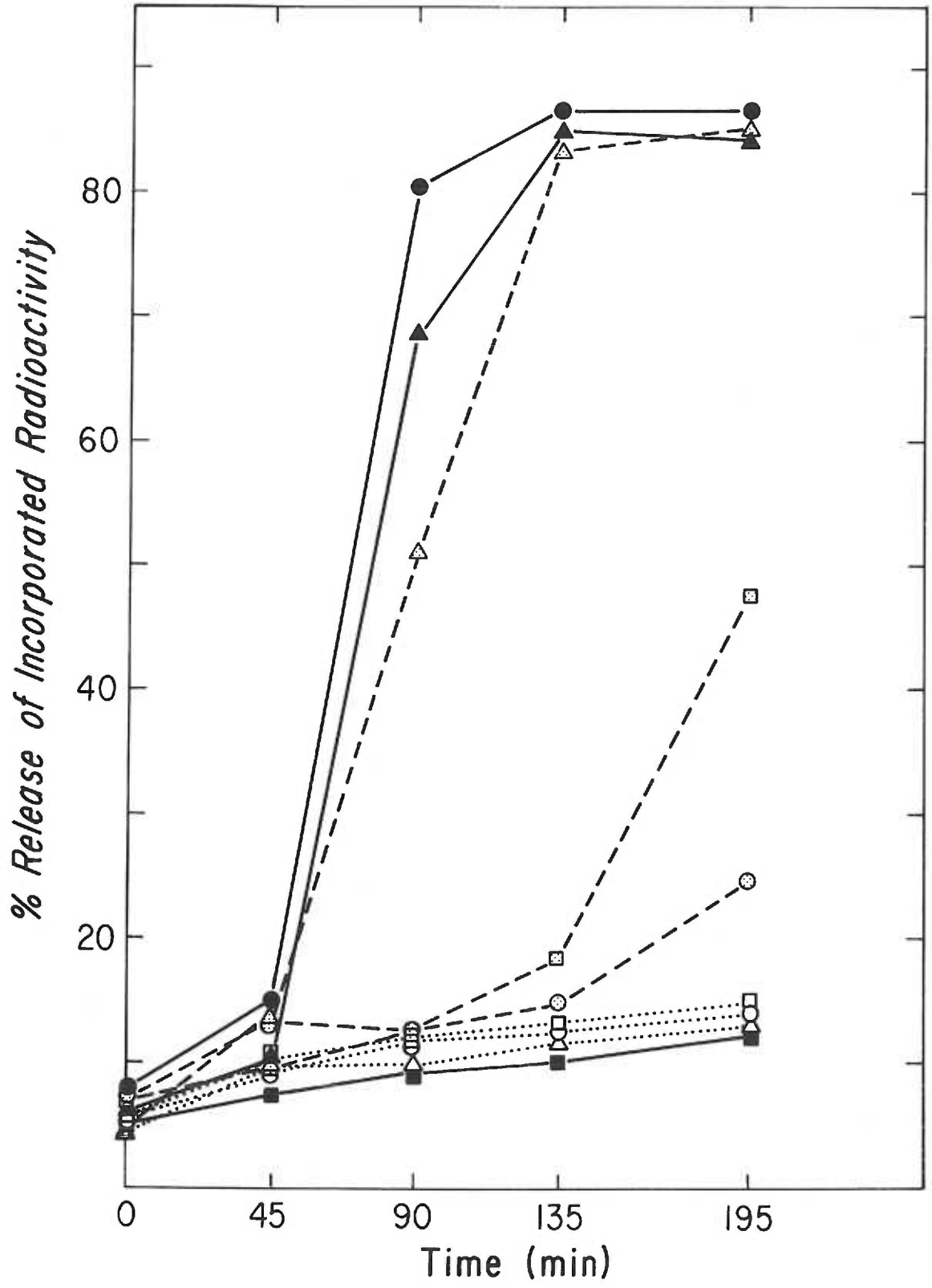
are both primary translation products, neither of which are processed further. This abnormal env precursor synthesis is consistent with a virus-encoded defect in which a defective viral 22S mRNA of R-61 cells produces an unglycosylated protein ($p80^{env}$) which very inefficiently enters the processing pathway that normally produces $gPr90^{env}$. The small amount of $gPr90^{env}$ that is formed by glycosylation of the $p80^{env}$ core is not processed further because the mutation which inhibits efficient glycosylation also prevents processing to gp70.

E. Cell Surface Placement of MuLV-Encoded gag and env Antigens

The intracellular labeling studies shown in Figs. 18, 19 and 20 demonstrate that the mutants M-13 and R-61 have abnormalities in the synthesis and processing of viral gene-encoded products. Therefore the surface expression of these molecules was expected to differ from wild-type MuLV-infected cells. We analysed the cell surface of mutants M-13 and R-61 for expressed antigens using antibody- and complement-dependent cytolysis (172) as shown in Fig. 21. The M-13 cells, which do synthesize and process env precursor (Fig. 20, lanes 5-8), are as sensitive to killing with anti-gp70 antiserum as the F12 NRK cells consistent with the interpretation that the mutant cell line expresses gp70 on its cell surface. However, these cells are only minimally sensitive to anti-p12 antiserum which is consistent with the intracellular

Figure 21. Cell Surface Antigen Measured by Antibody- and Complement-Dependent Cytolysis.

F12 NRK (△), M-13 (○) and R-61 (□) cells undergo cytolysis in the presence of rabbit complement and normal goat antiserum (----△----), goat serum made to Rauscher-nuclear-MuLV p12 (----▲----),or goat antiserum made to Friend-MuLV gp70 (----▲----), as described in Materials and Methods (section II, H1). Cytotoxic killing was monitored by the release of incorporated [^{35}S]-methionine into the culture medium and is expressed as a percentage of the total amount of incorporated radioactivity that could be recovered by adding 0.1% SDS.


% Release of Incorporated Radioactivity
80
60
40
20
0
45
90
135
195
Time (min)

data (Fig. 18) that $Pr75^{\underline{gag}}$ is not processed to the gag surface molecule, $gp93^{\underline{gag}}$. In contrast, data in Fig. 21 shows that the R-61 cells do exhibit anti-p12 sensitivity which we presume to be provided by the $gp93^{\underline{gag}}$ that was detectable intracellularly (Fig. 18, lane 7). Furthermore, the R-61 cells do not express gp70 antigens on their surface which suggests that $p80^{\underline{env}}$ is not transported to the cell surface.

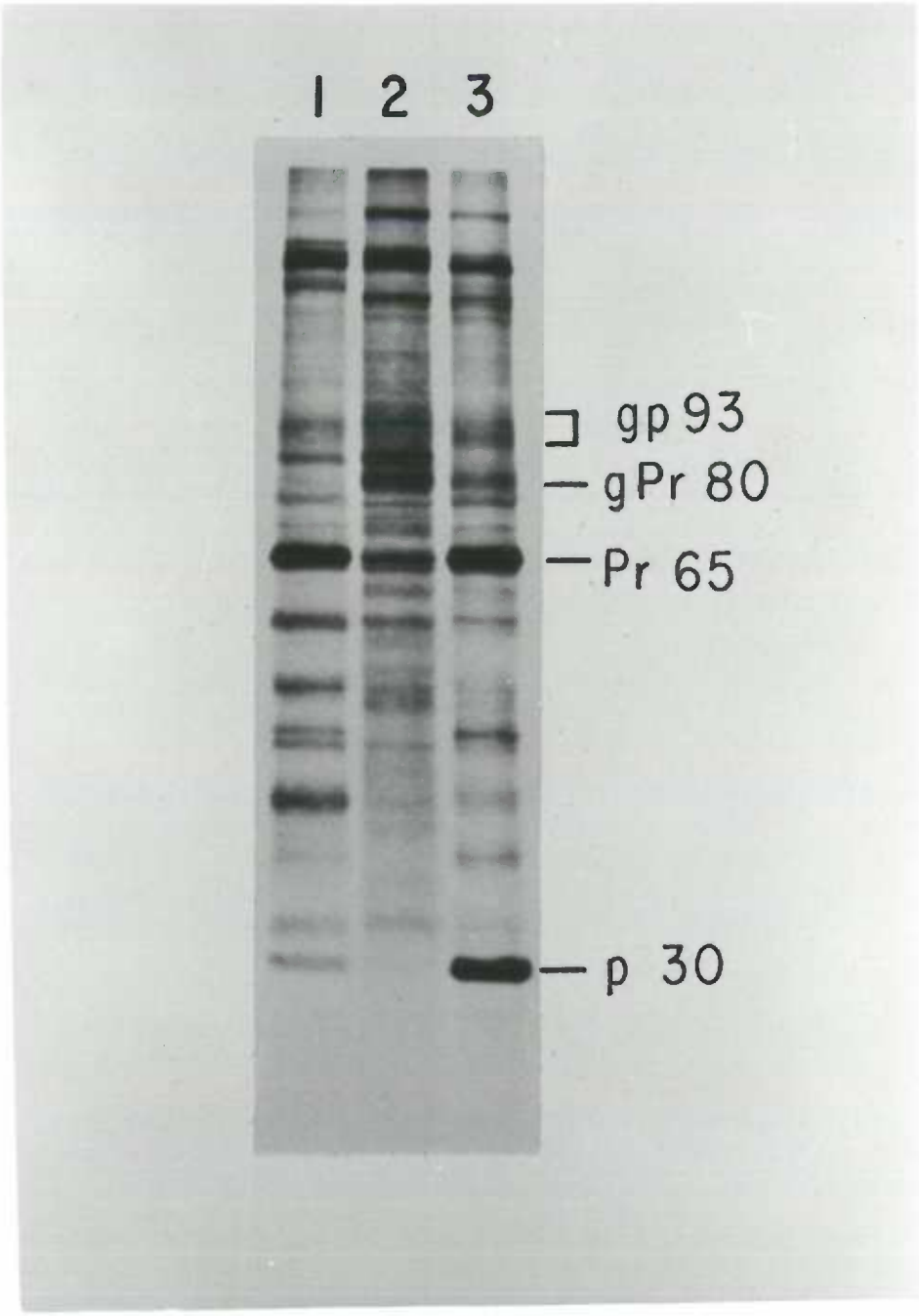
F. Do the Variant Cell Lines Contain Cellular or Viral Mutations?

To determine if the abnormalities demonstrated above reside in cellular or viral genes, mutant cells were superinfected with wild-type virus and the cells were then analysed for processing of MuLV-encoded products. For example, Fig. 22 shows an analysis of the gag gene-encoded proteins expressed in wild-type MuLV-infected cells (lane 1), in R-61 cells (lane 2) and in R-61 cells superinfected with wild-type F-MuLV (lane 3). After superinfection the mutant R-61 cells begin to express normal $Pr65^{\underline{gag}}$ and p30 levels. This result suggests that the cellular processing of normally expressed $Pr65^{\underline{gag}}$ is not defective, but rather that the proviral MuLV genome responsible for the R-61 phenotype is deficient in the expression of $Pr65^{\underline{gag}}$ and elevated in the expression of $gPr80^{\underline{gag}}$.

In contrast to mutant R-61 cells, attempts to ascertain the site of genetic lesion in the mutant M-13 cells were unsuccessful. For example, the M-13 cells were

Figure 22. Analysis of p30-Specific Protein Processing in MuLV-Infected Wild-Type and R-61 Cells after Superinfection by Wild-type Friend Virus.

The R-61 cell line was superinfected with wild-type Friend-MuLV (clone F12). The wild-type F12 NRK, R-61 and F-MuLV-superinfected R-61 cell lines were then pulse-labeled with [^{35}S]-methionine for 2 hr. Cellular lysates were prepared, immunoprecipitated with antiserum to p30 and the immunoprecipitates were electrophoresed on 10-20% polyacrylamide gradient gels containing 0.1% SDS as described in Materials and Methods. In the fluorograph shown, immunoprecipitates analysed were from F12 NRK cells (lane 1), R-61 cells (lanes 2) and R-61 cells superinfected with F-MuLV (lane 3). "Pr65", gPr80" and "gp93" are synonomous with Pr65$^{\underline{gag}}$, gPr80$^{\underline{gag}}$ and gp93$^{\underline{gag}}$ discussed in the text.


1 2 3
gp 93
gPr 80
Pr 65
p 30

resistant to superinfection with ecotropic MuLV. Presumably the gp70 detectable intracellularly (Fig. 20, lane 8) and on the cell surface (Fig. 21) interferes with superinfection as discussed in the Introduction (section I, F2). Furthermore, reverse transcriptase release data (Table 3) indicate that the mutant M-13 cells do not release virus particles into the extracellular medium. These release data are consistent with our observation that these cells do not process the intracellular viral core proteins which are believed to be essential components for viral budding to occur (68.69).

G. Discussion

This section describes the analysis of two MuLV mutants containing defects in the synthesis and processing of both intracellular and cell surface gag gene-encoded molecules. Kinetic labeling with [^{35}S]-methionine showed that both the mutant M-13 and R-61 cells do not significantly process the gag precursor molecules to the viral core proteins (Fig. 18). The absence of core proteins in these cells correlates with a lack of virus release, as measured by reverse transcriptase (Table 3). These data confirm the previously suggested idea that the presence of viral core proteins is requisite for release of virus particles (68,69).

Other studies with mutants lacking cell surface gp70 or gp93^{gag} (70,71,73) or reverse transcriptase (66,222) have found that virus release does occur from

cells lacking these virus-encoded components. Furthermore, virus particles lacking virus-specific 70S RNA were released from cells in the presence of actinomycin D (74,76). These results taken together suggest that virus budding and release is solely dependent on the internal core proteins. However, the possibility has not been sufficiently ruled out that unidentified cellular proteins and non-specific RNA fill essential roles in virion release in place of the normal viral components.

The processing of MuLV gag gene-encoded proteins had been proposed to occur in separate cellular pathways (46,47,79). $Pr65^{gag}$ is the primary translation product of the gag gene that acts as the polyprotein precursor to the core proteins, p15, p12, p30 and p10 (21). In contrast, the membranous gag glycoproteins, $gPr80^{gag}$ and $gp93^{gag}$, are synthesised and processed in a manner presumed analogous to normal eukaryotic membrane glycoproteins because of their cell surface location (77,97) and the associated sugars (45,46,77) typical of normal eukaryotic membrane glycoproteins (100). In this context, the proposal that core precursor synthesis is independent from gag surface glycoprotein synthesis (46,47,79) is substantiated by our present isolation of mutants with defects in one but not the other pathway of gag gene expression. For example, the synthesis of core proteins and subsequent virus release occurs in the mutant cell

lines p30-2 and p305 which have abnormal gp93^{gag} synthesis (described in section IV). Conversely, the mutant R-61 and M-13 cell lines synthesize gag surface glycoprotein precursors in the absence of significant core protein synthesis.

The imbalance in the ratio of expression of core precursor to surface glycoprotein precursor which is exhibited by both mutants is of particular interest in the context of current ideas for cell surface gag glycoprotein biogenesis. Pr75^{gag} is an unglycosylated precursor to membranous glycoproteins, and is distinguished from Pr65^{gag} by additional amino acids at the amino termini (82) of approximately 7,000 daltons (81). This additional peptide confers to Pr75^{gag} a presumptive signal sequence (83) that facilitates co-translational insertion of the nascent Pr75^{gag} into the endoplasmic reticulum and nascent glycosylation by "en bloc" transfer of preassembled lipid-linked oligosaccharide as describedin other systems (165,167,224,225). This structural difference at the amino termini of Pr65^{gag} and Pr75^{gag} controls their different paths of processing and has been proposed to arise by a splicing event of the provirus-encoded RNA to generate two distinct mRNAs as discussed in the Introduction (section I, D3).

Therefore we propose that mutant R-61 cells contain a virus-encoded defect which predominantly inhibits the expression of Pr65^{gag} and subsequently

results in very depressed levels of processed core proteins. This inhibition could result from a defective initiation of $Pr65^{\underline{gag}}$ translation from the 35S mRNA, or a defective splicing that would normally differentiate $Pr65^{\underline{gag}}$ mRNA from $Pr75^{\underline{gag}}$ mRNA. In either case, abundant $gPr80^{\underline{gag}}$ would be made because we propose that $Pr75^{\underline{gag}}$ is preferentially expressed and processed. In addition, we propose that mutant M-13 cells contain a defect in the signal or leader portion of the $Pr75^{\underline{gag}}$ polypeptide which prevents the proper insertion into the endoplasmic reticulum and its nascent glycosylation which would normally generate $gPr80^{\underline{gag}}$. In this model, the defect would also interfere with the splicing necessary to produce an mRNA encoding for $Pr65^{\underline{gag}}$. In this case, the defect in M-13 cells must be viral because a cellular defect should have also affected the insertion and glycosylation of $gPr90^{\underline{env}}$, which it did not (Fig. 19). Because the ratio of $Pr65^{\underline{gag}}$ to $Pr75^{\underline{gag}}$ is controlled at the level of splicing and because these two different viral polyproteins lead to distinct biological functions, an understanding of the controlling mechanism would be useful both from the view of MuLV biology and the control of gene expression.

VII. PROTEIN A COATED ERYTHROCYTE BINDING TO CELL SURFACE ANTIGENS: APPLICATION TO QUANTITATE RETROVIRUS INFECTIVITY IN VITRO

A. General Introduction

Methods to quantitate mammalian retroviruses by *in vitro* plaque or focus assays have largely supplanted relatively costly and cumbersome methods which depend on *in vivo* infectivity or on measurement of virus antigens snythesized by cultured cells (226-228). However, many retroviruses do not cause cytopathic changes in cells and will not rescue defective transforming viruses [as in the S^+L^- technique (180)] or induce syncytia with XC cells (229). Furthermore, it is important to realize that the virus titres obtained with any of these tissue culture assays are only relative numbers which are functions of the adsoprtion time and temperature, the culture conditions (e.g., the presence of DEAE-dextran) and the target cells employed. For example, we have observed that MuLV titres measured in S^+L^- cells cannot be accurately employed for estimating multiplicities of virus infection into BALB/c or normal rat kidney fibroblasts. For these reasons it would be extremely useful to have a generally applicable retrovirus assay method which could utilize any target cell which is most relevant to a particular investigation.

Immunological methods for detecting cell surface antigens that are expressed by infected cells have been

adapted to titre virus and are, in principle, applicable to many different virus-host culture systems (230,231). These approaches utilize "tagging" strategies which depend on having an antibody specific for a virus-encoded cell surface antigen.

Recently, an erythrocyte rosette method for detection of cell surface antigens expressed by MuLV-infected fibroblasts was described (178). In this method, erythrocytes coated with protein A bind to fibroblast monolayers only in the presence of antibodies specific for cell surface antigens. Furthermore, adherence of erythrocytes to fibroblasts in the absence of antibodies is extremely low so that ratios of specific to nonspecific binding are routinely greater than 10^3. In this section, which has been published as a short communication (232), we describe an application of this technique in a focus assay for ecotropic MuLV and for assay of a xenotropic MuLV grown in mink lung fibroblasts.

B. Results and Discussion

Conducting this rosette-focus assay requires (1) the preparation of protein A-coupled erythroyctes for the rosette assay and (2) a series of monolayers infected with various dilutions of a virus stock. Both the preparation of protein A-coupled erythrocytes and the preparation and rosetting of virus-infected culture flasks are described in Materials and Methods, sections II, H2 and II, C1, respectively.

Macroscopic rosette foci are visible to the unaided eye (Fig. 23A), but counting accuracy is improved with a dissecting microscope (10x). Foci are defined unambiguously by clumps of attached erythrocytes (Fig. 23B) when viewed under phase contrast or bright field microscopy. The dose response for F-MuLV in Sc-1 cells is linear over a range normal for other plaque assays (Table 4) and yields numbers and variances among replicate samples comparable to measurements made with the S^+L^- method. The rosette assay is applicable to other virus-host systems as shown by the foci observed with xenotropic Balb-2 virus in CCL64 mink lung fibroblasts (Fig. 23B and Table 5).

Figure 23. Macro- and Microscopic Views of Rosetted Foci Observable in a Plaque Assay for Infectious MuLV.

(A) A macroscopic view of erythrocyte rosette foci observed after rosetting Sc-1 cells (177) infected with the Friend strain of ecotropic MuLV (116). Rosetting of erythrocytes coated with protein A occurred in the presence of antiserum specific for gp70 (178). This flask contains approximately 200 foci. Bar = 7 mm. (B) An erythrocyte rosette focus of xenotropic MuLV growing in mink lung fibroblasts. Xentropic BALB virus-2 (BC117) (Electro-Nucleonics Laboratores, Inc., Bethesda, Md.) was infected into CCL64 mink lung fibroblasts. Rosetting of erythrocytes coated with protein A occurred in the presence of antiserum specific for BALB virus-2 gp70 (National Cancer Institute, NIH, Bethesda, Md.). Bar = 66 um.

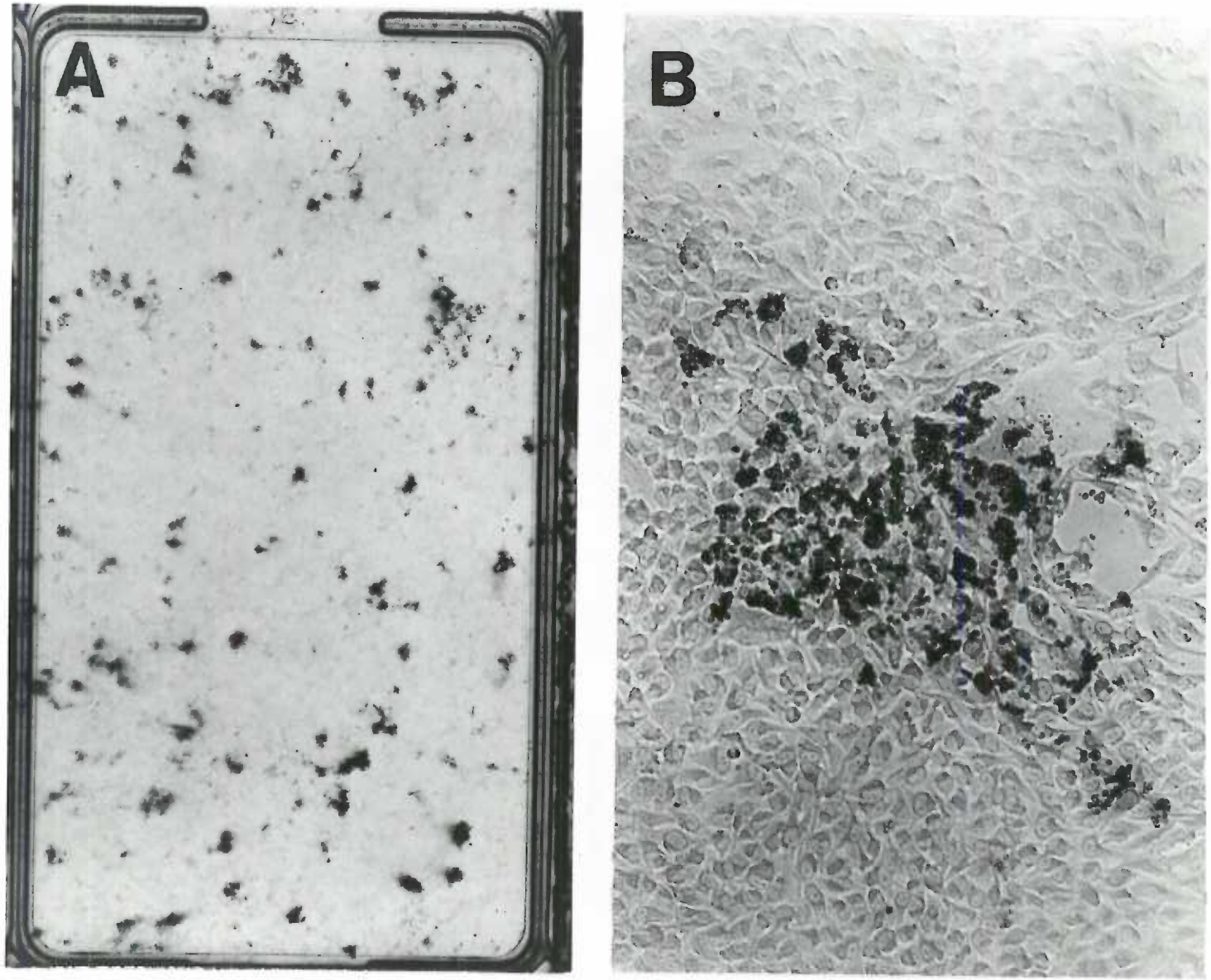

A
B

Table 4

FOCI OF INFECTIOUS CENTERS INDUCED BY F-MuLV IN SC-1 CELLS[a]

Virus dilution (1.0 ml/flask)	Calculated titer from S^+L^- assay[b] (FFU/ml)	Observed foci/flask	Titer estimates (FFU/ml)
Control	0	0	0
1/1600	25	16	2.6×10^4
1/800	50	30	2.4×10^4
1/400	100	65	2.6×10^4
1/266	150	90	2.4×10^4
1/200	200	127	2.5×10^4
1/160	250	145	2.3×10^4

[a] Infectious focus assay performed as described in the text (section VII).

[b] Calucalted titers are based on F-MuLV titer of 40,000 FFU/ml obtained on S^+L^- cells by a variation (233) of the method of Bassin et al. (180) as described in Materials and Methods (section II, C1).

Table 5

FOCI OF INFECTIOUS CENTERS INDUCED BY XENOTROPIC BALB V-2 VIRUS IN CCL64 CELLS[a]

Virus dilution (1.0 ml/flask)	Observed foci/flask	Titer estimates (FFU/ml)
Control	0	0
1/1,600,000	15	2.4×10^7
1/800,000	42	3.4×10^7
1/400,000	50	2.0×10^7
1/267,000	118	3.2×10^7
1/200,000	121	2.4×10^7
1/100,000	333	3.3×10^7

[a]Infectious focus assay performed as described in the text (section VII).

VIII SUMMARY

We have studied the structure, synthesis and processing of the membrane glycoproteins encoded by the murine leukemia virus (MuLV). These glycoproteins include gp70 and gp93^{gag} which are the final processed products of two distinct retroviral genes, *gag* and *env*. Because the MuLV genome only contains three genes and can readily be transferred between cells, it has provided a simple membrane glycoprotein system which we have used to isolate mutants with defects in synthesis and processing. Our analysis of these mutants has helped to elucidate both the biochemical events in the processing of retroviral membrane glycoproteins and the role that these glycoproteins play in MuLV biogenesis. As a result, this project has yielded several different experimental observations on the MuLV glycoproteins.

A protocol was developed for the purification of the envelope glycoprotein, gp70, from whole Eveline virus. In our approach, gp70 yields were improved by 1) reducing gp70 losses caused by the osmotic shock of isopycnic sucrose-gradient banding during the virus harvesting procedure and 2) efficiently extracting gp70 with the non-ionic detergent, Triton X-100. Detergent extracted gp70 was then purified in one step using highly selective affinity chromatography to the lectin, wheat germ agglutinin. Thus, this method has fewer steps than other procedures and provides a quick and high yield of purified

gp70.

A cell surface antigen detection method has been adapted as a plaque assay to quantitate infectious MuLV. Fibroblasts infected with MuLV bind erythrocytes precoated with protein A to form rosettes in the presence of MuLV-specific antisera. For example, Friend-MuLV-infected cells can be rosetted using antiserum directed against gp70 or $gp93^{gag}$. We have applied this rosette-focus assay to both eco- and xenotropic MuLV but the method is potentially applicable to many different virus-host culture systems in which virus specific antisera are available.

To study the synthesis, processing and functions of the MuLV-encoded membrane glycoproteins, we used the genetic technique of isolating viral and cellular mutants defective in processing these glycoproteins into their plasma membranes. Friend-MuLV-infected cells were immunoselected by incubation with antiserum to gp70 in the presence of complement to obtain mutants with defects in gp70 processing, or with antiserum to the viral core proteins, p30 and p12, in the presence of complement to obtain mutants with defects in the processing of gag gene-encoded polyproteins. Cells which survived this cytotoxic immunoselection were subjected to repeated treatments until fully resistant populations developed. These resistant cell lines were then analysed for defects which caused their immunoresistance.

The immunoresistant cell lines specifically lack the cell surface molecules which contain the target antigens, but may contain intracellular precursors to these cell surface molecules which are abnormally synthesized or processed such that their cell surface placement is blocked. Furthermore, these defects which block the surface placement of these membrane glycoproteins may reside in either cellular or viral genes. For example, the H-4 cell line which was selected with antiserum to gp70 contains a cellular defect which results in failure to cleave the gp70 precursor. Therefore, the H-4 cell line lacks gp70 in its plasma membrane. In addition, the p30-2 and p30-5 cell lines which were selected with antiserum to p30 contain cellular abnormalities in gag precursor processing which prevents the expression of cell surface $gp93^{gag}$. Alternatively, immunoselection of mutagenized MuLV-infected cells with antiserum to p12 generated the M-13 cell line which exhibits viral defects in the splicing of the mRNA encoding the gag-gene products. As a result, the M-13 cell line does not produce core precursor or processsed core proteins and does not insert the cell surface glycoprotein precursor, $Pr75^{gag}$, into the rough endoplasmic reticulum for glycosylation and processing.

The isolation of mutants which lack MuLV-encoded products either on the cell surface or intracellularly provides a system to determine what role these proteins

play in virus biosynthesis, assembly, particle release and infection. The mutants H-4, p30-2 and p30-5 lack cell surface gp70 or gp93^{gag} but do contain the intracellular core precursor Pr65^{gag} and release virus particles. Conversely, the mutants M-13 and R-61 lack virion core precursor but do express cell surface gp70 or gp93^{gag} and do not release virus particles. Taken together, these results suggest that MuLV particles bud efficiently from cells without the participation of gp70 or gp93^{gag} but require the cytoplasmic virion core precursor, Pr65^{gag}. Thus, budding of virions through the plasma membrane does not seem to require any known virus-encoded plasma membrane components.

We have analysed and compared the synthesis and intracellular transport of the two MuLV-encoded plasma membrane glycoproteins, gp70 and gp93^{gag}. The occurrence of these glycoproteins on the surface of infected cells was confirmed by a method in which extracellular antibodies were adosrbed onto [^{35}S]-methionine labeled cultured cells prior to cell lysis and immunoprecipitation. This technique was advantageous because the glycoproteins were endogenously labeled and their synthesis, arrival at the cell surface and turnover could be kinetically studied. In these studies we observed that the kinetics of processing and transport of gp70 and gp93^{gag} were widely different. Furthermore, there is a slow step in gp70 processing which appears to

occur in the rough endoplasmic reticulum. Passage through this kinetic barrier occurs from a precursor pool in a random rather than a cohort fashion. Our findings suggest that transport of membrane glycoproteins between organelles may involve interaction with specific carrier molecules that bind to different proteins with different affinities. Furthermore, our mutants which express one but not the other of the two viral membrane glycorpoteins support a similar notion that certain processing events which are essential for plasma membrane placement of a particular glycoprotein may have negligible effect on the placement of other glycoproteins.

A major focus of our investigation and of other researchers in the field has been on the controlling mechanism for the synthesis of the core and cell surface precursor polyproteins, $Pr65^{\underline{gag}}$ and $Pr75^{\underline{gag}}$, which derive from the same gag gene. It is believed that $Pr65^{\underline{gag}}$ and $Pr75^{\underline{gag}}$ are translated and processed independently to produce either the core proteins or the gag glycoproteins, respectively. Furthermore, the precursors are probably synthesized from two different mRNA's which differ due to a splicing event that removes those nucleotides which encode the small amino terminal leader portion of the $Pr75^{\underline{gag}}$ polypeptide. Several of our mutants differentially express one or the other of the gag gene precursors, supporting the idea that these processing pathways are independent. For example, the M-13 and R-61

viral mutants do not synthesize $Pr65^{\underline{gag}}$ but do synthesize $Pr75^{\underline{gag}}$ and yet are both defective in cell surface $gp93^{\underline{gag}}$ formation. Thus both viral and cellular defects can differentially affect the two pathways of gag gene product expression.

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AN ABSTRACT OF THE THESIS OF

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ABSTRACT

We have studied the biosynthesis and function of the membrane glycoproteins encoded by the murine leukemia virus (MuLV). These glycoproteins include gp70 and gp93^{gag} which are the final processed products of two distinct retroviral genes, gag and env. Because the MuLV genome only contains three genes and can readily be transferred between cells, it has provided a simple membrane glycoprotein system which we have used to isolate mutants with defects in synthesis and processing. Our analysis of these mutants has helped to elucidate both the biochemical events in the processing of retroviral membrane glycoproteins and the role that these glycoproteins play in MuLV biogenesis. As a result, this project has yielded a diverse body of experimental observations on the MuLV glycoproteins.

A protocol was developed for the purification of the envelope glycoprotein, gp70. Whole Eveline virus was harvested as a source of gp70 and was detergent solubilized with the non-ionic detergent, Trixton X-100. Detergent extracted gp70 was then purified in one step using highly selective affinity chromatography to the lectin, wheat germ agglutinin.

A cell surface antigen detection method has been adapted as a plaque assay to quantitate infectious MuLV. Fibroblasts infected with MuLV bind erythrocytes precoated with protein A to form rosettes in the presence of MuLV-

specific antisera. For example, Friend-MuLV-infected cells can be rosetted using antiserum directed against gp70 or gp93^{gag}. We have applied this rosette-focus assay to both eco- and xenotropic MuLV but the method is potentially applicable to many different virus-host culture systems in which virus specific antisera are available.

To study the synthesis, processing and functions of the MuLV-encoded membrane glycoproteins, we used the genetic technique of isolating viral and cellular mutants defective in processing these glycoproteins into their plasma membranes. Friend-MuLV-infected cells were immunoselected by incubation with antiserum to gp70 in the presence of complement to obtain mutants with defects in gp70 processing, or with antiserum to the viral core proteins, p30 and p12, in the presence of complement to obtain mutants with defects in the processing of gag gene-encoded polyproteins. Cells which survived this cytotoxic immunoselection were subjected to repeated treatments until fully resistant populations developed. These resistant cell lines were then analysed for defects which caused their immunoresistance.

The immunoresistant cell lines specifically lack the cell surface molecules which contain the target antigens, but may contain intracellular precursors to these cell surface molecules which are abnormally synthesized or processed such that their cell surface placement is

blocked. The defects which result in these abnormalities may reside in either cellular or viral genes. Furthermore, the isolation of mutants which lack MuLV-encoded products either on the cell surface or intracellularly provides a system to determine what role these proteins play in virus biosynthesis, assembly, particle release and infection.

For example, several mutants lack the virus-encoded cell surface membrane glycoprotein but synthesize the normal virion core proteins. These same mutants release virion particles, which suggests that the budding of virus particles through the plasma membrane does not require any known virus-encoded plasma membrane components. Alternatively, several different mutants have defects in the normal synthesis of the virion core proteins and do not release virion particles. This supports the notion that the core proteins are required for virus budding.

We have analysed and compared the synthesis and kinetics of intracellular transport of gp70 and $gp93^{gag}$. The occurrence of these glycoproteins on the surface of infected cells was confirmed by a method in which extracellular antibodies were adsorbed onto [^{35}S]-methionine labeled cultured cells prior to cell lysis and immunoprecipitation. This technique was advantageous because the glycoproteins were endogenously labeled and their synthesis, arrival at the cell surface and turnover could be kinetically studied. In these studies we observed that the kinetics of processing and transport of

gp70 and gp93^{gag} were widely different. Furthermore, there is a slow step in gp70 processing which appears to occur in the rough endoplasmic reticulum. Passage through this kinetic barrier occurs from a precursor pool in a random rather than a cohort fashion. Our findings suggest that transport of membrane glycoproteins between organelles may involve interaction with specific carrier molecules that bind to different proteins with different affinities.

A major focus of our investigation and of other researchers in the field has been on the controlling mechanism for the synthesis of the core and cell surface precursor polyproteins which derive from the same gag gene. It is believed that these two precursors are translated and processed independently to produce either the core proteins or the gag glycoproteins, respectively. Furthermore, the precursors are probably synthesized from two different mRNA's which differ due to a splicing event that removes those nucleotides which encode the small amino terminal leader portion of the polypeptide destined for the cell surface. Several of our mutants differentially express one or the other of the gag gene precursors, but not both, supporting the idea that these processing pathways are independent. Because it is likely that the synthesis of these two precursors is controlled by RNA splicing events, the isolation of these putative splicing mutants will be useful to study the controlling

mechanisms for the synthesis of these two important viral gene products and for the general topic of expression of genes destined for two cellular locations, the cytoplasm and the plasma membrane.