PLASMA MEMBRANE LOCALIZATION OF PROTEINS ENCODED BY MURINE RETROVIRUSES: RELATIONSHIP TO VIRAL LEUKEMIA

by

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Dedication

This dissertation is dedicated to Jesus Christ, who provided me with the direction, encouragement, and perseverance needed to complete this work.

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I. Introduction

A. Perspectives

The cellular plasma membrane performs critical functions as a communicator between the cell interior and its environment. The plasma membrane contains a multitude of different components which are involved in important cellular processes, such as cell-cell interactions, cellular differentiation, cellular locomotion, and cellular immunity (176). Abnormalities in plasma membrane structure and function are usually manifestations of aberrant intracellular processes. Such plasma membrane abnormalities have long been noted in established cancers and it has been speculated that they may play important pathogenic roles in the initiation of neoplasia (176).

The retroviruses or RNA tumor viruses provide simple model systems to investigate the role of the plasma membrane in neoplastic transformation. They also provide an opportunity to analyze the structure and function of membranes and the mechanisms of membrane synthesis. Such information is needed before a critical evaluation of the role of the plasma membrane in neoplasia can be achieved. The retroviruses encode different membrane components that can be readily analyzed by a combined experimental approach, utilizing various tools of cellular and molecular biology and immunology. We have focused on several retroviral systems in which membrane components have been implicated as playing a critical pathogenic role. Three retroviral systems are described in this dissertation: the replication-competent murine leukemia virus (MuLV) and two highly oncogenic derivatives, the spleen focus-forming virus (SFFV) and the Abelson murine leukemia virus

(A-MuLV). These systems have been utilized to specifically investigate 1) the processing mechanisms of retroviral-encoded proteins to the cell surface and 2) the role of retroviral and cellular-encoded membrane proteins in transformation and in tumor rejection.

The following introduction will review in detail the molecular properties of the murine leukemia viruses and its derivatives, the spleen focus-forming virus and the Abelson murine leukemia virus. For purposes of clarity, the review of each retrovirus will be conducted separately. In addition, where appropriate, each review will contain additional information concerning the origin and biological characterization of the virus, the plasma membrane localization of encoded gene products, and the significance of this localization to viral infectivity and morphogenesis, transformation, and tumor rejection. A historical account of the RNA tumor viruses is provided by Gross (59). The molecular biology of retroviruses have been further described by Stephenson (168) and updated recently in Cold Spring Harbor Symposium monographs (188).

B. The Murine Leukemia Virus (MuLV)

1. Viral Host Range and Life Cycle

The murine leukemia viruses (MuLVs) are members of the retrovirus group, many of which cause malignant neoplasia in a variety of vertebrate hosts (1,15,59). The MuLVs are functionally divided into two categories depending on their competency for independent replication (1,83). The replication-competent murine leukemia viruses or helper MuLVs replicate to high titer in susceptible embryo fibroblast cultures, but cause no obvious morphological alteration of the infected cells. Upon infection into appropriate adult hosts, such viruses frequently

induce lymphatic leukemia and thymic lymphoma after a long latency period. The replication-defective murine leukemia viruses, on the other hand, require the presence of helper virus for their multiplication, induce tumors of a variety of histologic types in vivo and frequently cause morphologic transformation of embryo fibroblasts in vitro. These defective viruses usually arise as a result of genetic recombination between a replication-competent helper virus genome and host cell genetic sequences (15,168,182). Two defective viruses, the spleen focus forming virus (SFFV) and the Abelson murine leukemia virus (A-MuLV) will be discussed in greater detail in later sections of this dissertation.

There are four classes of murine leukemia viruses, distinguished on the basis of their host range, neutralization, and interference properties (1,168). These classifications primarily reflect differences in the antigenicity of the virus' envelope glycoproteins and the abilities of these glycoproteins to bind to specific receptors on the host cellular membrane. These classes are: 1) ecotropic, 2) xenotropic, 3) dual-tropic (or poly-tropic), and 4) amphotropic. The ecotropic viruses can infect and replicate efficiently only in murine cells, while the xenotropic viruses infect and replicate only in nonmurine cells. The dual-tropic or poly-tropic viruses can infect and replicate efficiently in both cell types. The amphotropic viruses, found only in wild mice, can also infect both murine and nonmurine cells, but differ from the dual-tropic viruses in their neutralization and interference properties.

The murine leukemia viruses are enveloped viruses which contain a single-stranded ribonucleic acid genome and an RNA-dependent, DNA-polymerase or reverse transcriptase enzyme (1,8,15,59,176). The

reverse transcriptase, an enzyme essential to the replicative cycle of the virus and unique to the retrovirus group, transcribes the viral RNA into a double-stranded DNA molecule or proviral DNA, which subsequently integrates into the host cell genome (8,15,168). Current evidence indicates that integration can be accomodated at different sites on the host cell DNA, but only at a single site on the proviral DNA (15,168). This integrated sequence then undergoes transcription by host polymerases into single-stranded viral RNA molecules, which serve as either messenger RNAs to direct the synthesis of virus-specific proteins or become incorporated into newly synthesized virions (15,168).

Morphologically, the murine leukemia viruses are classified as type C virus particles, a designation established by early electron microscopy studies denoting the configuration and placement of the electron dense inner core within the virion structure (1,176). These particles, roughly spherical in shape and about 100 nm in diameter, contain a lipoprotein outer envelope, a hexagonal capsid, and a core membrane, in addition to a ribonucleoprotein inner core (14). This core structure contains the viral RNA genome, several different proteins, and the reverse transcriptase molecule (1,15,168).

The replicative cycle of the murine leukemia virus begins with the attachment of the virus' envelope glycoprotein to a specific cellular receptor protein (33). The virus then penetrates into the cell by either membrane fusion or engulfment (168). The uncoating of the virion core occurs either intracellularly or as a consequence of penetration mediated by membrane fusion. Once within the cell, the viral RNA genome is reverse transcribed and a double-stranded DNA intermediate or provirus is synthesized. The proviral DNA then

integrates into the host chromosome (15,168). As mentioned previously, the integration of the provirus is site specific. The proviral genes are then transcribed into messenger RNAs. These mRNAs are then translated on host ribosomes to form various polyprotein precursors, which are subsequently processed by proteolytic, phosphorylation, or glycosylation events (15,168). Some of these components are either assembled directly on the cytoplasmic face of the plasma membrane or inserted into the membrane by vesicles originating from the subcellular membranous organelles (15,168). The assembled virus particles incorporate genomic length viral mRNA and bud from the cell, acquiring a host-derived lipid envelope containing the viral envelope glycoproteins (15,168). The newly released viral particles then undergo maturation during which the virion core polyproteins are cleaved. Certain aspects of the virus life cycle are described in more detail below.

2. The Retroviral Genome and Proviral Synthesis

The major genetic component of the MuLVs is a single stranded RNA, a molecule with an apparent molecular weight of 6×10^6 that sediments at 60-70S and is denatured into two identical 35S subunits (37,91). These subunits, possessing features characteristic of eukaryotic messenger RNA, have positive messenger polarity (98,111) and are polyadenylated at their 3' end and capped with 7-methyl guanosine at their 5' end (77,130). Each 35S mRNA subunit contains three genes. These include the gag gene coding for the major nonglycosylated virion core proteins, pol coding for the RNA-dependent, DNA polymerase or reverse transcriptase enzyme, and env coding constituents of the viral membrane envelope (15,168). The arrangement of individual genes within

the retroviral genome has been established as 5'-7^{m}G-gag-pol-env-polyA-3' (15,168).

The MuLV 35S RNA is transcribed into a double stranded DNA provirus by the viral enzyme reverse transcriptase (8,54,55,187,191). The reverse transcription process initiates near the 5'-end of the 35S RNA molecule at a site where a host tRNA primer (tRNA^{pro}) is hydrogen bonded to complementary viral sequences (54,55). Reverse transcription proceeds from this host primer to the 5'-end of the viral RNA, synthesizing a short complementary (minus-strand) DNA (187). Due to a terminal redundancy of sequences at both the 5'-and 3'-ends of the 35S RNA molecule (28), the short complementary DNA can also hybridize to sequences at the 3'-end of the viral RNA and in fact apparently "jumps" to this terminus (69,92,172). The remainder of the 35S RNA is then reverse transcribed into minus-strand DNA using this short cDNA sequence as a primer. The positive-strand DNA is then synthesized, completing the double-stranded DNA provirus. After completion of proviral synthesis, the provirus then integrates into the host cell DNA (191).

The DNA provirus is longer than the 35S RNA molecule and contains long terminal repeats (LTRs) generated as a result of the reverse transcription process (69,92,172). These LTRs flank the viral structural genes at the 5'- and 3'-ends of the integrated provirus and vary in length in different retroviruses from 300-1200 bp (69,92,172).

It is currently believed that 5' LTRs contain a promoter from which transcription of the proviral genes is initiated (69,92,172). Consistent with this hypothesis, messenger RNAs transcribed from proviral genes are found to contain leader sequences which are complementary to sequences found in the LTR (60,101,110). Furthermore,

the LTR contains a sequence similar to the "Hogness box", a promoter used by RNA polymerase II for the transcription of eukaryotic genes (69,92,172). Interestingly, there is also evidence that the LTR at the 3'-end of the integrated provirus serves as a transcriptional promoter of adjacent cellular genes that may be critical for the initiation of neoplastic transformation (61,110).

3. The Viral Proteins

The murine leukemia viruses contain 3 genes designated gag, pol, and env that respectively encode the viral core proteins, the reverse transcriptase enzyme, and the viral envelope proteins (1,15,168). The proteins encoded by these genes are all necessary components for viral replication (1,15,168).

Host protein synthesis is not inhibited in MuLV-infected cells. The viral proteins synthesized in infected cells represent only a very small percentage of the total proteins synthesized. The post-translational processing mechanisms of retroviral-encoded proteins have been revealed primarily with the use of monospecific antisera directed toward these viral components in immunoprecipitation studies (1) and by cell-free translation of viral mRNAs (98,111).

The nomenclature used in this dissertation has been recommended by the National Cancer Institute (67). According to this nomenclature, the symbols "p", "gp", "Pr", and "gPr" respectively indicate protein, glycoprotein, precursor protein and precursor glycoprotein. The number which follows these designations represents the apparent molecular weight of the molecule in thousands as determined by gel electrophoresis or gel filtration. The superscripts indicate the gene of origin.

a. The gag Proteins

Translation of MuLV 35S mRNA generates two gag precursor polyproteins, molecules with apparent Mr's of 65,000 and 75,000 designated $Pr65^{gag}$ and $Pr75^{gag}$ respectively (41,81). Each of these gag precursors is processed along a separate pathway to respectively form the viral core proteins and a cell-surface glycoprotein (41,81).

$Pr65^{gag}$ is synthesized in the cytoplasm and is phosphorylated and processed through numerous, poorly defined proteolytic intermediates to form the core proteins, p15, phosphorylated p12, p30, and p10 (16,89,168). This polyprotein molecule accumulates at the cytoplasmic surface of the plasma membrane and is assembled directly into budding virus particles (16,81). The $Pr65^{gag}$ molecule is cleaved in the extracellular virion to form the mature core proteins (16,168) by a cellular protease that is also incorporated into the virus particle (89). Analysis of the processing intermediates has led to the ordering of protein sequences in $Pr65^{gag}$ to be: NH_2-p15-p12-p30-p10-COOH (11). This order has been recently confirmed with the nucleotide sequencing of the Moloney MuLV genome (172).

The viral core proteins have different structural and functional characteristics (11,168). p15, a hydrophobic protein, is located at the amino terminal of the $Pr65^{gag}$ molecule and is believed to interact with plasma membranes during the assembly of virion cores. p12 is a type-specific RNA-binding phosphoprotein, which specifically binds to the RNA of homologous viruses, but not to the RNA of heterologous viruses. p30 is the major structural protein of the virion core and has been implicated as a determinant of growth restriction that exists between classes of MuLV and different inbred mouse strains. p10 is a

highly basic protein that associates with the viral RNA to form a ribonucleoprotein complex within the virion core.

$Pr75^{gag}$ contains an amino terminal leader peptide (11,154), in addition to the sequences found in $Pr65^{gag}$. Cell-free translation studies (38), as well as nucleotide sequencing determinations of the Moloney MuLV genome (172) provide evidence that $Pr75^{gag}$ is generated from a spliced mRNA. Unlike cytoplasmically-synthesized $Pr65^{gag}$, the $Pr75^{gag}$ molecule is translated on membrane-bound ribosomes and is inserted into the lumen of the rough endoplasmic reticulum (38). $Pr75^{gag}$ is glycosylated with the addition of high-mannose oligosaccharides to form the processing intermediate $gPr80^{gag}$ (38). $gPr80^{gag}$ then migrates to the Golgi apparatus where it is modified by removal of some mannose residues and by addition of the terminal sugars fucose and sialic acid to form $gp93^{gag}$ (38). The latter molecule is then transported to the cell surface (38,49). This cell surface molecule performs no known function in viral morphogenesis (49), but is believed to interact with the host immune system (107) and is therefore probably important in the host response to virus-induced cancers.

b. The pol Proteins

The pol gene encodes an RNA-dependent DNA polymerase, otherwise known as reverse transcriptase (8,187). This molecule has an apparent Mr of 70,000 ($p70^{pol}$) and catalyzes the synthesis of the proviral DNA from the viral 35S RNA (8,187). In addition to its polymerase activity, $p70^{pol}$ contains an RNAse H activity, capable of digesting the RNA of a DNA:RNA hybrid (187).

The pol gene product is derived from the 180,000 dalton precursor protein $Pr180^{gag\text{-}pol}$, which contains both gag and pol gene-encoded polypeptide sequences (98,111,156). This precursor molecule is generated at low frequencies by a translational read-through of an inefficient termination signal located at the 3' end of the retroviral gag gene (168). The $p70^{pol}$ molecule is generated from this precursor molecule into its final form by a cellular cleavage event (98,111).

c. The env Proteins

The env gene encodes the protein components $gp70^{env}$ and p15E, translated from a subgenomic 22S messenger RNA transcript (53,187). This 22S RNA transcript is believed to be formed by a splicing event linking regions from the 5' end of the 35S mRNA to the 3' env gene region (53,187). Translation of the 22S mRNA generates a polypeptide which is immediately inserted into the lumen of the rough endoplasmic reticulum (157). This polypeptide is glycosylated with the addition of high mannose-type oligosaccharides, resulting in the formation of the polyprotein precursor molecule, $gPr90^{env}$. $gPr90^{env}$ subsequently migrates from the rough endoplasmic reticulum to the Golgi apparatus as a membrane-bound glycoprotein (157). Just before transit to or shortly after reaching the latter organelle, the env precursor is proteolytically cleaved to form the $gp70^{env}$ and p15E molecules (48,100,157,200). Once within the Golgi apparatus, the high mannose-type oligosaccharides present on the $gp70^{env}$ molecule are modified by removal of some mannose residues and by addition of terminal sugars fucose and sialic acid to form complex-type oligosaccharide

structures. The processed gp70$^{\underline{env}}$ and p15E molecules are then transported to the cell surface by membrane vesicles.

With the use of pactamycin, an inhibitor which blocks initiation of translation, the arrangement of the two major constituents, gp70$^{\underline{env}}$ and p15E within gPr90$^{\underline{env}}$ has been deduced as NH_2-gp70-p15E-COOH (168). Further analysis of gPr90$^{\underline{env}}$ by use of tunicamycin to inhibit glycosylation and by specific cleavage of sugars from the polypeptide chain by endoglycosidase H has shown that it contains an apoprotein of approximately 70,000 daltons (200). Additionally, the env precursor molecule has been shown to contain 6-7 oligosaccharide attachment sites (113,137).

Both gp70$^{\underline{env}}$ and p15E are present in the virus envelope and on the surface of the infected cell as a disulfide-bonded complex (43,200). The precursor molecule is not found on the cell surface, implying that cleavage of gPr90$^{\underline{env}}$ efficiently occurs before appearance of gp70$^{\underline{env}}$ and p15E on the plasma membrane. Whether disulfide linkage occurs prior or subsequent to post-translational cleavage of gPr90$^{\underline{env}}$, however, remains unresolved. Pulse chase and peptide mapping data also indicate that p15E is subject to cleavage giving rise to a smaller protein, p12E (168).

4. Envelope Glycoprotein Recognition of Cell Membrane Receptors

The envelope glycoprotein gp70 of the murine leukemia virus is believed to serve as the major determinant governing the virus' penetration into susceptible cells, its host range, and the ability of infected cells to resist exogeneous superinfection by a process called interference (15,168). The gp70 molecule has been shown to represent a major constituent of the viral envelope and to constitute the spikes or

surface projections which are present on the virion surface (201). The gp70 molecule is believed to control the virus' infectivity and host range properties by its ability to interact with specific cellular receptor molecules (33). Interference to superinfecting MuLV occurs when the target cell expresses gp70 on its surface as a result of endogeneous synthesis, thereby blocking these critical receptor sites (33).

With the availability of purified gp70, studies of cell receptor site recognition at a more molecular level has been possible. DeLarco and Todaro (33) used [^{125}I]-labeled gp70 to determine the binding kinetics of this molecule to the cell surface and have provided evidence for the specificity of receptor recognition by the viral envelope glycoproteins. These investigators found that iodinated gp70 from Rauscher MuLV, an ecotropic virus, binds receptors restricted to cell lines of mouse origin. In contrast, studies by Moldow et al. (97) have shown that [^{125}I]-labeled gp70 from amphotropic MuLV, a virus capable of infecting both mouse and non-mouse cells, recognizes cell receptors of a wide range of mammalian species.

Receptor recognition studies have been extended by Moldow's group to include an analysis of the gp70s from the RD-114 feline and M7 baboon type C viruses, and the squirrel monkey retrovirus, (SMRV) a representative type-D mammalian virus. These studies have shown that both type-C viruses recognize a common membrane receptor site distinct from those recognized by amphotropic MuLV gp70. Both type C gp70 molecules, not only exhibited complete reciprocal cross-interference in receptor site binding, but interfered to a much lesser degree with the representative type D retrovirus, SMRV. In contrast, minimal

cross-interference was observed between glycoproteins of the murine amphotropic virus, 4070-A, and either of the type-C viruses and none between glycoproteins of 4070-A and the type D isolate. These investigators concluded that the type-C and type-D retroviruses studied apparently recognize the same surface receptor molecules, but with slightly different specificities. Their results are consistent with serological studies performed by Stephenson et al (169) and Devare et al (34), who demonstrated a class of interspecies gp70 antigenic determinants shared by all type-C retroviruses and a second group of determinants specific to envelope glycoproteins of primate-derived type-C and type-D isolates.

Efforts to isolate the MuLV envelope glycoprotein receptor have intensified in recent years. Kalyanaraman et al.(71) characterized the binding interactions between Rauscher MuLV gp70 and receptors obtained from plasma membrane preparations of KA31 mouse cells. This binding, demonstrated by gel filtration of a mixture of the microsomal fraction of the cells and [^{125}I]-labeled gp70, was shown to be a noncooperative, saturable, yet reversible process with an association constant of $3.5 \times 10^{8} M^{-1}$. Landen and Fox (78) have furthermore purified a protein from the culture medium used for growth of Balb/C 3T3 mouse cells, that avidly binds Rauscher MuLV gp70. Gel filtration chromatography in the Landen and Fox study revealed the presence of a single gp70 binding component, BPgp70, with an apparent Mr of 10,000. This binding component was shown to be efficiently labeled when the cells were incubated with [^{3}H]leucine, indicative of the molecule's cellular origin. BPgp70 was also shown to be precipitable only with a mixture of gp70 and antibodies recognizing the gp70 molecule, an

indication that prior complex formation was required for immunoprecipitation of BPgp70 to occur. In addition, Robinson et al. (129) have reported the isolation of a possible receptor of Friend MuLV gp70 from C57BL/6 mouse spleen leukocytes, using an immunoprecipitation technique. The putative receptor protein isolated by Robinson et al. (129), has an apparent Mr of 14,000 and was shown not to comprise the mouse H-2 histocompatibility antigens, proteins known to be the receptors for Semliki Forest virus. Schaffar-Deshayes et al. (152) have additionally reported the isolation of the thymus cell surface receptor for Moloney MuLV gp70. A molecule with an apparent Mr of 190,000 was identified in this study as the putative gp70 receptor. Analysis in nonreducing conditions also indicated that this receptor might be composed of at least two subunits of 190,000 daltons.

C. The Spleen Focus Forming Virus (SFFV)

The spleen focus forming virus (SFFV) is a replication-defective retrovirus, whose genome was formed by genetic recombination between portions of the replication-competent MuLV genome and sequences highly related to the env gene of murine xenotropic and dual tropic mink cell focus (MCF)-inducing type C viruses (36,182). SFFV causes a rapidly fatal erythroleukemia in mice (182) and was originally isolated from tumor cells as a virus complex with a replication-competent helper MuLV (51,121) Two strains of SFFV have been independently isolated, the Friend and Rauscher isolates, and both encode an env molecule believed to be a pathogenic factor in causing erythroleukemia (36,146,182).

1. Origin and Biological Characterization of Friend and Rauscher Erytholeukemia Viruses

The Friend erythroleukemia virus was isolated in 1957 by C. Friend during passage of cell-free extracts of Erlich ascites tumor cells in newborn Swiss mice (51). Subsequent passaging of leukemic tissues obtained from these animals into adult Swiss mice produced a disease characterized by marked hepatosplenomegaly and culminating in death one to three months following inoculation. Initial histologic examination of tissues obtained from the afflicted animals revealed extensive infiltration of the spleen, liver, and bone marrow by large mononuclear cells and a predominance of nucleated red blood cells in the peripheral blood. A much more extensive analysis of Friend virus pathology was conducted by Metcalfe et al. (94), who noted that the neoplastic proliferation of mononuclear cells was accompanied by marked erythroblastosis and terminal lymphocytosis.

The uncertainty of the hematopoietic cell type involved in Friend disease was resolved with the observation that leukemic cells established either in cell culture (52) or as splenic foci in irradiated mice (138) differentiated along the erythroid cell pathway. The identification of the hematopoietic lineage as erythroid was established by both morphologic and functional criteria, involving ^{59}Fe uptake, hemoglobin synthesis, and induction of hematopoiesis following treatment with dimethyl sulfoxide or other chemicals (52,138).

Mirand and co-workers (96) and others (6) have distinguished strains of Friend virus, based on observed differences in the biologic properties of different stocks of the virus. Certain preparations of Friend virus which induced a marked polycythemia in adult mice were

pathologically distinguished from the anemia-inducing strains (95,96). Both strains, however, are capable of inducing rapidly fatal erythroleukemia in Swiss mice (95,96). In spite of the differences in the hematopoietic changes induced by these different strains, both virus types exhibit properties by which they can be distinguished from other previously characterized murine leukemia viruses: 1) a short 2-4 week latent period following virus inoculation, 2) fatal erythroleukemia in adult as well as newborn mice, 3) lack of thymic or lymph node involvement, and 4) predominant involvement of cells of the erythroid lineage (6,95,96).

A second isolate of murine erythroleukemia virus was discovered by F. Rauscher in 1961 during passage of extracts from Schoolman-Schwartz ascites tumor cells in Balb/c mice (121). Like the Friend isolate, Rauscher virus can cause a rapidly fatal erythroleukemia in mice, characterized by massive hepatosplenomegaly, induction of neoplastic splenic foci, and moderate to acute anemia in the terminal stages (121). A histologic examination of spleens obtained from infected animals shows infiltration of nucleated erythrocytes, erythroblasts, and reticulum cells in the red pulp. Thus, the independently isolated Friend and Rauscher erythroleukemia viruses both induce similar disease pathologies in mice and contain spleen focus forming activity.

The Friend and Rauscher erythroleukemia viruses have been utilized as model systems for understanding erythroid differentiation (35,50,93,122) Since the research described in this dissertation is primarily directed toward characterizing the proteins encoded by the

virus, this dissertation review will emphasize a discussion on the virus' molecular properties.

2. Viral Components of the Erythroleukemia Virus

The Friend and Rauscher erythroleukemia viruses are viral complexes containing at least 2 biologically active components. One is the highly oncogenic, replication-defective spleen focus forming virus (SFFV) which is responsible for the rapid transformation of erythroid precursor cells in vivo (6,167) or in tissue culture (27,74). The other component is the replication-competent murine leukemia virus (MuLV), which can elicit a lymphatic leukemia of thymus cells after a long latent period (32,47). The MuLV functions as a helper virus, providing components to the replication-defective SFFV that are necessary for spreading the infection to other cells.

These two components were initially distinguished by genetic studies, which had shown the restriction of either the MuLV or SFFV component in genetically resistant mice (Section I.B.3). More recent studies have resulted in the biological cloning of the Friend and Rauscher viral components into mouse and rat fibroblasts (90,146,181). These cloned virus isolates have been extremely important in the elucidation of the molecular and biological properties of SFFV.

3. Genetic Factors Affecting Susceptibility to Erythroleukemia Virus Infection

Host genetic determinants that influence leukemia virus replication and expression are known to play an important role in deciding the outcome of virus-host interactions (168,182). Several genetic loci have been identified that affect and control host susceptibility to Friend SFFV (5,104,112). Three of these genetic loci,

Fv-1 and Fv-2 and the mouse histocompatibility complex H-2 play a prominent role in determining host resistance or susceptibility to virus-induced erythroleukemia (5,104,112).

The exact mechanisms of resistance to erythroleukemia induction by these loci are not known. However, it seems likely that these genetic determinants act by influencing one or more of the following steps in viral replication and transformation: (1) inhibition of the replication of the transforming or helper viruses, (2) reduction in the number of target cells available for viral transformation or replication, (3) suppression of the proliferation of the infected or transformed target cells and (4) regulation of the number of target cells by the immune system of the host.

Genetic control of Friend virus infection was initially described by Odaka and Yamamoto (104) and Axelrad (5) and provided the initial basis for suggesting that the erythroleukemia virus complex might contain two distinct components. Resistance to Friend virus is controlled in at least three murine loci. The first locus, Fv-1, controls the intracellular replication of the ecotropic MuLV component of the Friend virus complex and apparently involves processes regulating the integration of the provirus into the host chromosome (63,68,174). The second genetic locus, Fv-2, confers resistance to the SFFV component by its regulation of the proliferation of the erythroid target cell (6,173). The third genetic locus, the major histocompatibility complex H-2 influences later events in murine leukemogenesis, subsequent to virus attachment (23,84). H-2 molecules appear to interact with virion determinants at the cell surface (23,84) and control of viral antigen expression by the H-2 locus may occur. A complete description of the

genetic studies of these murine loci and of other genetic loci known to affect hematopoiesis is beyond the scope of this dissertation. A review of the genetic control of erythroleukemia virus infection by the Fv-1 and Fv-2 loci and by the major histocompatibility locus H-2 is provided by Pincus (112). Other genetic loci affecting host susceptibility to erythroleukemia viruses have been described by Chesebro et al. (24,25) and others (75,177).

4. Isolation of Spleen Focus Forming Virus Component

The isolation of SFFV from MuLV by limiting dilution cloning into fibroblast cells was important for defining the molecular characteristics of SFFV. The technique which was originally devised to clone SFFV free of MuLV was simply to infect murine fibroblasts cells at a low multiplicity with a virus complex containing SFFV and MuLV in an approximately equivalent titer and to immediately clone individual cells, resulting in clonal cell lines which contained (1) helper virus alone, (2) helper virus plus SFFV, (3) SFFV alone, or (4) no virus. The presence of SFFV in single cell clones which did not contain helper virus could be demonstrated by superinfection of an aliquot of such cells with MuLV and the inoculation of the resultant virus complex into adult mice. Nonproducer cells containing SFFV have been derived using this technique from Balb/c 3T3 and NIH 3T3 murine fibroblasts, as well as normal rat kidney and Fischer rat embryo cells (178,180).

Nonproducer cell lines infected with SFFV are morphologically normal, unlike nonproducer cells containing the highly oncogenic, replication-defective murine sarcoma viruses (168,182) and Abelson murine leukemia viruses (9). The SFFV nonproducer cells do not release virus into the extracellular medium (180). The defective virus can be

rescued from these SFFV nonproducer cells by superinfection with a variety of replicating type-C viruses, including nonmurine retroviruses such as the Woolly leukemia virus (182).

The biological cloning of SFFV components by limiting dilution analysis has been utilized extensively in this study and has resulted in the isolation of spontaneous SFFV mutants with decreased leukemogenic potential (Section IV).

5. Molecular Properties of Spleen Focus-Forming Virus (SFFV)

a. SFFV Genome

Electrophoretic analysis of RNA obtained from virus rescued from Friend SFFV (F-SFFV) infected cells resulted in the determination of the F-SFFV genome size as approximately 5.5-6 kb (86). The Rauscher SFFV (R-SFFV) genome (7.8 kb) is substantially larger than that of F-SFFV (5.5 kb) and is only 0.7 kb smaller than that of Rauscher MuLV (R-MuLV) (8.5 kb) (R. Bestwick and D. Kabat, unpublished observations). However, F-SFFV and R-SFFV have the same sized subgenomic env messenger RNAs (2.1 kb), and these are 0.7 kb smaller than that of R-MuLV (2.8 kb) (R. Bestwick and D. Kabat, unpublished observations).

Molecular hybridization studies imply that the SFFV genome was formed by genetic recombination between portions of the replication-competent MuLV genome and sequences highly related to the env gene of murine xenotropic and mink cell focus(MCF)-inducing type C viruses (179,182). Like SFFV, the MCF viruses are recombinants between ecotropic MuLV and env sequences related to those found in xenotropic viruses (23). Tryptic analysis of viral gp70 molecules encoded by the MCF viruses originally suggested that these proteins were part ecotropic

and part xenotropic and led to the hypothesis that these gene products were hybrid molecules (39). However, Chattopadhyay et al. (23) have recently detected endogeneous MCF-like env sequences that closely resemble those found in MCF viruses. This finding supports an alternative hypothesis that the gp70 molecules encoded by some MCF viruses may be derived entirely from these endogeneous viral sequences. It is possible that the env gene of SFFV may have also originated from similar endogeneous env sequences.

In initial molecular studies, Troxler et al. (179) synthesized radioactively labeled cDNA probes from a virus complex containing both SFFV and Friend MuLV (F-MuLV) and, by hydroxyapatite chromatography, subsequently isolated a cDNA fraction that was SFFV-specific. This probe did not hybridize to RNA of ecotropic MuLV, but did hybridize to RNA from SFFV-infected nonproducer cells. In addition, this same probe hybridized significantly to genomic RNA of three separate isolates of murine xenotropic virus, as well as those contained in several strains of MCF viruses. Based on the recombinant nature of the MCF viruses, Troxler et al. (179) concluded that SFFV was a recombinant containing MuLV genetic sequences and sequences highly related to the env gene of murine xenotropic viruses.

These observations were consistent with the oligonucleotide mapping of the SFFV genome conducted by Evans et al. (42). These investigators analyzed 23 large ribonuclease T_1-resistant oligonucleotides found in SFFV RNA. Sixteen of the 23 oligonucleotides found in the SFFV genome were either identical or closely related to genetic sequences found in F-MuLV. The remaining 7 oligonucleotides were clustered in a region located 1.5 to 2.7 kilobases from the 3' end

of SFFV RNA. The 3' location of these SFFV-specific oligonucleotides would be consistent with the approximate genetic region of the env gene of the helper-independent MuLVs. In addition, several of these SFFV-specific oligonucleotides were shown to be identical in base composition to oligonucleotides found in several MCF virus isolates (42). Thus, oligonucleotide studies of the SFFV genome were consistent with the hypothesis that SFFV arose by recombination between MuLV and sequences highly related to the env gene of xenotropic viruses. Electron microscopic analysis of heteroduplexes between genomic RNA of F-SFFV and cDNA transcripts obtained from F-MuLV was in concordance with all other molecular data. Bosselman et al (19) had shown that the F-SFFV genome incurred deletions in the env and pol gene regions and had undergone sequence substitutions within the env and gag genes.

Linemeyer et al. (86) have recently molecularly cloned the F-SFFV genome into the plasmid vector pBR322. Biologically-active SFFV can be recovered by releasing the SFFV DNA from the vector, transfecting the released DNA onto NIH-3T3 cells, and rescuing the SFFV either by superinfection with helper virus or by cotransfection with molecularly cloned infectious helper viral DNA. Subsequent work by these investigators (87) has resulted in the molecular cloning of a biologically-active DNA fragment of F-SFFV. This DNA fragment, containing the env-related gene of SFFV, includes sequences 2.0 kb from the 3' end of the SFFV genome, the long terminal repeat sequences of SFFV, and sequences 0.4 kb from the 5' end of the SFFV genome.

Recently, Linemeyer et al. (85) have also constructed a series of insertion-deletion mutants in a molecularly cloned DNA copy of F-SFFV. The mutants were produced by inserting a synthetic

oligonucleotide containing the SalI endonuclease recognition sequence into several different locations of the SFFV DNA. Insertion-deletion mutants were generated in the 5' half of the SFFV genome, in the LTR of the SFFV genome, and in the env gene of the SFFV genome. From analyses of the biological activities of the various mutants, these investigators have deduced that the genetic sequences encompassing the env gene of SFFV is required for biological activity. These results, however, are not entirely definitive in light of the limitations of the cotransfection assays used in generating these virus particles (87). The complementary effects of the MuLV DNA in the cotransfection assay are not clearly known. For this reason, our laboratory has initiated studies characterizing the genomes and gene products of several spontaneous Friend and Rauscher SFFV mutants and defining the role of these genetic components in leukemogenicity (Section IV; also additional work by R. Bestwick and D. Kabat).

b. SFFV Gene Products

Evidence described in the previous section implies that the spleen focus-forming virus may be a genetic recombinant between an ecotropic murine leukemia virus and sequences highly related to the env gene of murine xenotropic and MCF viruses. Ruscetti et al. (142) initially analyzed SFFV nonproducer cells for the expression of potential env gene products utilizing type-specific gp70 competition radioimmunoassays. Consistent with the data obtained from molecular hybridization studies, Ruscetti et al.(142) found that extracts of F-SFFV infected cells contained an antigen that was highly cross-reactive to the gp70 molecules encoded by the MCF viruses.

Subsequent to these initial findings, Dresler et al. (36) and Ruscetti et al. (144) utilized immunoprecipitation techniques to identify the SFFV env gene component as a glycoprotein with an apparent Mr of 55,000 (gp55). This molecule is both immunologically and structurally related to the MuLV envelope glycoprotein gp70 and with the gp70 of dual tropic MuLVs. (36,144). gp55 is synthesized at high levels within SFFV nonproducer fibroblast cells, as well as in erythroleukemia cell lines and in the spleens of mice with SFFV-induced disease (116,117,182). A closely related env gene product with an apparent Mr of 54,000 (gp54) has also been identified in Rauscher SFFV nonproducer cells (146). There is evidence that some but apparently not all leukemogenic F-SFFV genomes encode a large gag-related polypeptide that is immunologically cross-reactive with antiserum made to F-MuLV $p12^{gag}$ (142,144). In addition, there have been reports that some strains of F-SFFV encode a 15,000 dalton protein that may be identical to the $p15^{gag}$ protein encoded by F-MuLV (144). The gp55/gp54 molecules appear to be the only viral proteins consistently detected in all cell lines infected by various leukemogenic strains of SFFV (12,36,66,146,182). Consequently, these env gene-encoded molecules have been implicated as potential pathogenic factors in causing erythroleukemia.

c. Subcellular Localization of SFFV-encoded gp55

Work conducted by Kabat and coworkers (70,145) has shown that a small percentage of gp55 (3-5%) which has a larger size than the major component and is termed $gp55^{p}$ is located on the outer surface of plasma membranes. The major component is irreversibly situated in membranous intracellular organelles (70,145) and contains small high mannose oligosaccharides of the type formed in the endoplasmic reticulum

(70,145). The plasma membrane component, $gp55^{p}$, contains larger complex oligosaccharides with galactose, fucose, and sialic acid, indicative of transit through the Golgi apparatus (70,145). Neither gp55-related component has been detected in virus particles released into the extracellular medium of SFFV infected cells superinfected with helper MuLV (70). The Rauscher SFFV $gp54^{p}$ molecule appears to be produced in smaller quantities than the Friend SFFV plasma membrane glycoprotein (145). In addition, there is evidence reported by others (182) that gp55 may be associated with the cell nucleus, possibly migrating to this organelle after synthesis in the rough endoplasmic reticulum.

D. The Abelson Murine Leukemia Virus (A-MuLV)

Abelson murine leukemia virus (A-MuLV) is a replication-defective retrovirus that can induce a rapidly fatal nonthymic lymphosarcoma in mice and can transform certain bone marrow and fibroblast cultures in vitro (2,3,9). The tumorigenic potential of A-MuLV has been generally attributed to the expression of the virus' only known gene product, p120 (9), a recombinant-type molecule containing serological determinants related to the Moloney murine leukemia virus (M-MuLV) amino terminal gag gene products, p15 and p12 (123,198), and also containing determinants related to a protein encoded by the abl gene found in the normal mouse genome (159,196).

1. Origin and Biological Characterization of the Abelson Murine Leukemia Virus

The Abelson murine leukemia virus arose from a steroid-treated Balb/c mouse infected with replication-competent M-MuLV (2,3,9). A-MuLV has unique biological properties that distinguish it from its parental helper virus. A-MuLV appears to transform hematopoietic target cells of

pre-B cell origin, distinctly different from the T cell targets known to be affected by the replication-competent MuLV (9). In addition, A-MuLV induces a rapidly fatal nonthymic lymphosarcoma 3 to 4 weeks after injection into neonatal mice and is capable of transforming fibroblast cells *in vitro* (9). The latency period for thymic leukemia induced by M-MuLV on the other hand is 4-6 months. The replication-competent virus also is incapable of transforming fibroblast cells *in vitro*.

2. Molecular Properties of the Abelson Murine Leukemia Virus (A-MuLV)

a. A-MuLV Genome

The length of the A-MuLV genome is 5.5 kb, substantially smaller than the 8.3 kb genomic size of M-MuLV (10,159). Hybridization experiments conducted by Parks et *al.*(109) initially suggested that about 25% of the M-MuLV genome was present in the A-MuLV genome. More recent work utilizing heteroduplex and S1 nuclease mapping (10,159) has shown that the A-MuLV RNA genome contains 2 regions of homology with M-MuLV: a 730-base region at the 3' end and a 1320-base region at the 5' end. Inserted between these two terminal fragments is a region of 3.6 kb derived from a portion of the Balb/c mouse genome from which A-MuLV was originally isolated. Shields et al. (159) constructed a cDNA probe against A-MuLV specific nucleotide sequences and subsequently showed by Cot analysis that a portion of the A-MuLV genome is closely related to the *abl* gene found in the genome of normal mouse cells. This probe hybridized to A-MuLV RNA and to normal DNA derived from either uninfected Balb/c or NIH mice, but not to the RNA of murine sarcoma viruses.

b. A-MuLV Gene Products

A-MuLV encodes as its major gene product an Mr 120,000 hybrid protein (p120) containing serological determinants related to M-MuLV amino terminal gag gene products and determinants related to a protein encoded by the abl gene found in the normal mouse genome (123,124,196-198). Immunoprecipitation studies have shown that p120 contains p15, p12, and possibly a limited portion of the p30 gag M-MuLV polypeptide sequence and that the remainder of the p120 molecule consists of a host cell derived portion (123,124,196-198). Van de Ven et al. (185) have furthermore shown that A-MuLV p120 consists of 12 major L-[^{35}S]methionine labeled tryptic peptides, only one of which corresponds to a peptide found in Moloney-MuLV encoded proteins Pr65^{gag} and Pr180$^{gag\text{-}pol}$. In addition, of the three M-MuLV Pr80^{env} peptides resolved by these investigators, none were present in A-MuLV p120.

A mouse tumor regressor serum (Abelson tumor serum) reactive against the host-cell derived portion of p120 was produced by injecting syngeneic bone marrow cells transformed by A-MuLV into C57L mice (196). Almost all other inbred strains of mice were unable to reject syngeneic bone marrow cells transformed by A-MuLV. This antiserum, in addition to precipitating p120, precipitates an Mr 150,000 protein (NCP150) from normal mouse tissues. NCP150 is believed to be a normal cellular protein with shared antigenicity to the host-derived portion of the Abelson protein (197) and is found in normal thymocytes, bone marrow, and spleen cells of uninfected mice at a concentration 50-100 fold lower than the average concentration of p120 found in A-MuLV transformed cells (197).

Both A-MuLV p120 and NCP150 are phosphoproteins and have been shown to become phosphorylated *in vitro* by [γ-^{32}P]ATP (193,199). The phosphate linked to p120 is at a tyrosine residue, a very rare linkage previously reported for the pp60^{src} protein kinase encoded by avian sarcoma viruses (29,66,82) and for the transforming proteins encoded by feline sarcoma virus and Fujinami virus (15,168). Furthermore, this protein kinase activity copurifies with the A-MuLV protein in immunoprecipitates, implicating p120 as the molecule responsible for tyrosine phosphorylation (193,199). Deletion and premature termination mutations that affect the carboxyl-terminal *abl*-encoded region of the p120 molecule show parallel changes in the virus' transforming ability and *in vitro* phosphotyrosine kinase activity (58,125,194) (Section I.C.4). These observations strongly suggest that p120 contains the transforming function for A-MuLV. Sefton et al. (155) have furthermore shown that A-MuLV transformed cells contain elevated phosphotyrosine levels, providing evidence that p120 may function *in vivo* as a tyrosine-phosphorylating protein kinase. These investigators have found that the A-MuLV protein is phosphorylated *in vivo* at two sites. Alternatively, Witte and co-workers (195) have found that p120 phosphorylated *in vitro* contains up to 12 tyrosine-phosphorylation sites, most of them restricted to the amino-terminal region of the Abelson protein. Both groups believe that the *in vitro* autophosphorylation reactions probably correlates with an activity important in transformation, but that the specific *in vitro* end products are artifically trapped intermediates that would not normally be observed in an *in vivo* reaction (155,195).

Studies characterizing the p120 molecule suggest that it is not a glycoprotein (196). The p120 molecule is not labeled with radioactive sugars and its molecular weight is not affected by endoglycosidase H, an enzyme known to degrade the inner core structure of high mannose-type oligosaccharides (196). The Abelson protein is also not labeled by lactoperoxidase-catalyzed [^{125}I]-iodination of intact cells, implying either that p120 is absent from the cell surface or that it does not have an exposed tyrosine or histidine residue (196). This protein is structurally related to the gene products of other defective leukemia/sarcoma viruses in the retrovirus group. The avian virus MC29 p120 and feline sarcoma virus p110 molecules are similar to A-MuLV p120 in that all are hybrid type proteins, containing both gag-related polypeptide sequences and sequences related to those found in host-encoded proteins (168,196).

c. Plasma Membrane Localization of the A-MuLV Protein

Witte and co-workers have obtained evidence that the A-MuLV protein is localized on the surface of infected cells, using both cell fractionation and indirect immunofluorescence techniques (15,196). Their immunofluorescence data shows that the Abelson tumor serum, containing antibodies reactive against the abl gene-encoded portion of the A-MuLV protein, binds to the surface of Abelson transformed nonproducer fibroblasts. No fluorescence was obtained using anti-Moloney MuLV sera in their indirect immunofluorescence procedure. These experiments suggest that p120 may be a transmembrane protein, in which the abl portion, but not the corresponding gag determinants, is exposed on the cell surface.

3. A-MuLV Strain Variants and Effects on Transformation Potential

Several variants of A-MuLV which produce proteins of sizes different from that of the prototype p120 molecule have been recently isolated (135). These strain variants encode Abelson-specific proteins with apparent Mr's 90,000, 100,000 and 160,000 (p90, p100 and p160 respectively) (135). The A-MuLV strains which encode proteins smaller than the p120 molecule, contain genomes indistinguishable in size from that contained in the prototype strain, implying that the shorter proteins are a result of early translational termination (58). These two strains, originally derived from A-MuLV infected cells treated with the drug fluorodeoxyuridine (135), transforms lymphoid cells very poorly both *in vitro* and *in vivo*. The reduced oncogenic potential of these isolates were correlated with a high level of synthesis of unstable p90 and p100 and the inability of these molecules to function efficiently in protein kinase assays (133). The correlation of abnormal metabolism and deficient protein kinase activity with the reduced oncogenic potentials of these virus strains support a direct role for these proteins and the kinase activity in transformation (133). The third A-MuLV variant, which encodes a p160 molecule, contains a genome that is 0.8 kilobase larger than the prototype genome in the *abl*-specific region (58). This strain variant contains biological properties identical to the prototype A-MuLV. The geneology of this strain and the prototype strain is obscure. Goff *et al.* (58) believe that the p160-encoding genome was the original isolate which subsequently suffered a deletion of 800 kb to give rise to the shorter p120-encoding genome. However, another possibility is that the p120 strain was the original virus which gave

rise to the larger genome by a duplication or insertion of 800 kb (58). Preliminary mapping studies (58) places the difference between these two strains near the center of the viral genome, within the abl region. The differences in the sizes of these two A-MuLV genomes roughly accounts for the difference in the size of the proteins, implying that the extra sequences of the p160 strain are translated in phase and that termination occurs at the same point as in the p120 strain (58).

In addition to the above 3 A-MuLV strain variants, Witte et al. (194) have isolated a spontaneous transformation-defective mutant designated A-MuLV-P92td. This mutant encodes an A-MuLV protein with an apparent Mr of 92,000 (p92), but lacks the ability to transform either fibroblasts or bone marrow lymphoid cells. In contrast to the proteins made by transforming strains of A-MuLV, the protein made by A-MuLV-P92td does not become phosphorylated in an in vitro protein kinase assay, but can serve as an acceptor for phosphate if mixed with a wild-type A-MuLV protein (194). The genome which codes for p92 has a 700-base pair deletion internal to the region normally encoding the p120 molecule. The size of the deletion accounts for the smaller size of the p92 molecule. The deletion therefore probably does not disturb the translational reading frame and allows read-through to a termination codon at the normal position (194). The region deleted corresponds to a stretch of amino acids 50,000 to 78,000 from the N-terminus of p120. Whereas the C-terminal portion is apparently dispensible for at least some of the p120 function, this internal region cannot be removed without complete loss of function (194). Reynolds et al. (125) have reported a similar A-MuLV transformation-defective mutant that encodes an Mr 80,000 fusion protein with impaired protein kinase activity.

Their findings (125) corroborate the contention of Witte et al.(194) that the A-MuLV-encoded p120 molecule and its associated protein kinase activity are involved in Abelson virus tumorigenesis.

4. Surface Markers on the A-MuLV Target Cell

The Abelson murine leukemia viruses are of special interest to immunologists because of their ability to transform cells of the lymphocyte lineage into permanently growing cell lines (9). This virus is able to generate cell lines with properties that resemble cells at various stages of B-lymphocyte differentiation (9). Studies of the phenotype of transformed hematopoietic cells have proven useful in confirming the cell lineage of the transformants in the case of both thymic lymphomas and Friend virus erythroleukemia (9,182,188). In addition, studies with Friend cells have supplied valuable insights into some of the steps of erythrocyte differentiation (35,50,93).

The principal cell type transformed by A-MuLV either in vivo or in vitro is lymphoid. A relationship of Abelson lymphosarcoma to cells of the B-lymphocyte lineage was initially suggested by the absence of thymic pathology in A-MuLV-induced disease (2,3,115,164) and the observation that athymic nude mice were susceptible to Abelson disease (118,120). These tumor cells do not express T lymphocyte markers, such as Thy1, lyt antigens and TL antigens, that are usually present on thymus-derived lymphomas (20,106,118,165,166).

Since no immunoglobulin is detected on the surface of A-MuLV transformed lymphocytes, the immunocompetent B cell seems not to be the target of A-MuLV (9). Lymphocytes expressing only cytoplasmic immunoglobulin chains are found within the normal cell population, especially in the bone marrow and fetal liver where the early stages of

B-lymphocyte differentiation predominate (30,108). Since these tissues contain the highest percentage of cells susceptible to transformation, the most consistent interpretation is that A-MuLV transforms an early stage of the B lymphocyte lineage (9).

Further molecular evidence substantiates this contention. Recent work by Siden et al. (163) and Boss, Greaves and Teich (18) demonstrates that approximately 60% of early transformants synthesize IgM or μ heavy chains and that a few of these cell lines can be induced by the B lymphocyte mitogen lipopolysaccharide to synthesize K chains and assemble 7-8 S IgM molecules. In addition, approximately 90% of these in vitro transformed A-MuLV-derived cell lines contain low levels of terminal deoxynucleotidyl transferase (TdT) (135). This enzyme has been classically associated with thymocytes, but more recently TdT has been detected in immature lymphocytes in the bone marrow (165). Furthermore, the expression of lyb-2, a surface antigen found on normal B lymphocytes (150), on in vitro transformed A-MuLV cells supports an association with the B cell lineage (165).

While the cellular phenotypes of the in vitro transformed cell lines described above suggest that a bone marrow cell early in the lymphocyte lineage might be a preferred target of the virus, tumors with properties of mature leukocytes have occasionally arisen in A-MuLV-injected mice (114,119,120). Studies by Potter et al. (114) demonstrated that A-MuLV administered to Balb/c mice previously injected intraperitoneally with pristane gave rise to tumors of which 20-25% were immunoglobulin-secreting plasmacytomas. In addition, Raschke et al. (119) have established 3 clonal cell lines from murine tumors induced with A-MuLV that express properties of macrophages. Secretion of

infectious A-MuLV by two of the macrophage lines, RAW 309Cr and Wr19M, provided conclusive evidence that A-MuLV is capable of productively infecting the macrophage cell line. This raises the possibility that a less differentiated cell with multiple differentiative potential might serve as the target for A-MuLV. One possible candidate for such a cell could be the pluripotent hematopoietic stem cell or spleen colony forming unit (CFU), a progenitor of the monocytic, granulocytic, erythrocytic, megakaryocytic and possibly lymphocytic lines of blood cells (4,203). Silverstone et al. (165) have shown that a xenoantiserum made against mouse brain detects antigens expressed both on A-MuLV transformed lymphoid cell lines and on the pluripotent CFU. In addition, Risser et al. (126-128) have described an alloantigen expressed on all cells transformed by A-MuLV, on uninfected cells from Balb/c bone marrow, spleen and fetal liver, and on 50% of Balb/c CFU cells.

E. Thesis Objectives and Organization

The primary objectives of this dissertation are to investigate the surface membrane phenomena that occur at distinct stages of murine leukemia virus infection. Three distinct and independent retroviral systems have been utilized to investigate 1) the processing mechanisms of retroviral-encoded proteins to the cell surface and 2) the role of retroviral and cellular-encoded membrane proteins in transformation and in tumor rejection.

For purposes of clarity, the experimental results are organized into three sections. The first section (section III) examines the abnormally processed plasma membrane and virion glycoproteins encoded by env gene mutants of the replication-competent Rauscher murine leukemia

virus (R-MuLV). The protein processing characteristics of three R-MuLV mutants will be presented and will be discussed in relation to a general model that will illustrate how structural abnormalities of the env gene products can differentially affect various parameters of their intracellular transport and function.

The second section (section IV) describes the isolation and biochemical characterization of spontaneous env gene mutants of Rauscher spleen focus-forming virus (R-SFFV). These R-SFFV mutants are weakly leukemogenic and encode abnormally sized proteins which do not appear to be processed like the wild-type counterpart to the surface membranes of infected cells. The analysis of these weakly leukemogenic R-SFFV mutants would be useful in identifying and studying the critical features of the structure and membrane processing of the virus' transformation protein and its role in erythroleukemia.

The third section of this dissertation (section V) examines the plasma membranes of an Abelson murine leukemia virus (A-MuLV) transformed lymphoid cell line, the L1-2 of C57L origin. The goals of this study were to analyze the metabolism and membrane processing of the gag-abl fusion proteins and to identify plasma membrane targets of the immune response that allow this specific tumor cell to become uniquely rejected in the C57 L mouse.

Due to the distinctive nature of the three retroviral systems presented in this dissertation, the results of the respective sections are discussed independently at the end of each section. A general summary (section VI) is presented at the end of the dissertation.

II. Materials and Methods

A. Cells and Viruses

A stock of wild-type Rauscher murine leukemia virus (R-MuLV) and NIH-3T3 cells were obtained through the courtesy of S.A. Aaronson (Laboratory of Tumor Virus Genetics, National Cancer Institute, Bethesda, MD). The NIH-3T3 cells, as well as Balb 3T3 fibroblasts were maintained as monolayers in Dulbecco's Modified Eagle Medium (DMEM, Grand Island Biological Company, Grand Island, NY), supplemented with 10% complement-inactivated calf serum and antibiotics. Sc-1 cells derived from a fetal mouse embryo were provided by J. Weaver (Cell Culture Laboratory, University of California School of Public Health, Oakland, CA) and maintained as a monolayer culture using McCoy's modified 5A medium (Grand Island Biological Company) supplemented with 10% calf serum and antibiotics. Eveline II cells, which are chronically infected with Friend murine leukemia virus (F-MuLV), were kindly provided by D. Bolognesi (Duke University Medical Center, Durham, NC) and grown as suspension cultures in DMEM supplemented with 10% fetal calf serum and antibiotics.

The R-MuLV variants, R7 clone 1, R7 clone 61 and R Balb clone 29, were isolated by limiting dilution cloning of R-MuLV (Section II.D) into either NIH-3T3 (as in the case of R7 clone 1 and R7 clone 61) or Balb 3T3 (as in the case of R Balb clone 29) fibroblasts. These R-MuLV infected cell lines were maintained as monolayer cultures in DMEM supplemented with 10% complement-inactivated calf serum and antibiotics.

The Rauscher spleen focus forming virus (R-SFFV)-infected NIH-3T3 cell line, RV NIH clone 6, has been previously described by Ruta and

Kabat (146). Virus from the RV NIH clone 6 nonproducer cell line was rescued by superinfection with R-MuLV and injected into the tail vein of 4-6 week old NIH/Swiss female mice. Two weeks following the initial injections, cell-free extracts of spleens obtained from these infected animals were prepared and injected into fresh mice (Section II.N). Virus was transferred sequentially in this manner into new mice for a total of 3 passages. Virus collected after the third in vivo passage was used to infect Sc-1 cells to prepare a new virus stock. Virus released from this infected Sc-1 culture, termed passaged Rauscher virus (PRV), was then cloned by limiting dilution into NIH-3T3 fibroblasts. The R-SFFV-infected cell lines, PRV clone 3-32, PRV clone 3-25, PRV clone 4-3, and PRV clone 4-10 were derived from a subcloning of virus stocks obtained from cells that arose from the initial cloning experiment. All R-SFFV infected NIH-3T3 cell lines were maintained as monolayers in DMEM, supplemented with 10% complement-inactivated calf-serum and antibiotics.

The C57 L derived, Abelson murine leukemia virus (A-MuLV) transformed lymphoid cell line, L1-2, was obtained from N. Rosenberg (Cancer Research Center, Tufts University School of Medicine, Boston, MA) and grown in RPMI 1640 medium (Grand Island Biological Company) supplemented with 10% heat-inactivated fetal calf serum and 2×10^{5} M β-mercaptoethanol. The SWR/4, 230-23-8, and 230-37 A-MuLV transformed lymphoid cell lines were kindly provided by S. Goff (Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA) and described previously (131,132). The ANN-1 cell line, an A-MuLV transformed nonproducer cell line of NIH-3T3 origin, was kindly provided by J. Stephenson (Laboratory of Viral Carcinogenesis, National Cancer

Institute, Bethesda, MD) and grown in McCoy's modified 5a medium containing 10% calf serum and antibiotics.

B. Viral Assays

Infectious MuLV titers were measured by using a variation of the S+L- method of Bassin et al. (13).

SFFV was assayed by the macroscopic spleen focus assay of Axelrad and Steeves (6). Virus samples were assayed for SFFV by inoculation into the tail vein of 3 different 4-6 week old NIH/Swiss female mice. Spleens not exhibiting extensive enlargement or development of macroscopic foci, indicators of SFFV-induced disease, after 21 days were examined histologically. Histologic diagnosis was based on the presence of foci of large cells with large nuclei having a distinct nuclear membrane with one or two nucleoli and a moderate amount of pale eosinophilic cytoplasm.

C. Virus Infections and Purification

Virus samples collected from the culture medium of infected cell lines were clarified by centrifugation at 10,000 x g for 10 min with a Sorvall SS-34 rotor, prior to infection upon recipient cell monolayers. The virus suspension was adjusted to 8 μg per ml polybrene, and 1.0 ml was overlaid onto cell monolayers plated 24 h previously at 5×10^4 cells per 25 cm^2 culture flask. After 2 h at 37°C, 4 ml of fresh medium containing polybrene was added. The cells were grown to confluency and passaged over a 1.5-2 week period to ensure that the infection had spread to all of the cells in the culture.

MuLV was purified from the culture medium of logarithmically growing cells by isopycnic banding in sucrose gradients essentially as described by Evans et al. (42). Basically, culture supernatants were

clarified of cells and cell debris by sequential centrifugation at 3000 rpm for 10 min and 10,000 rpm for 10 min in a Sorvall GSA rotor. Virus contained in the clarified culture medium was then pelleted by centrifugation in a Beckman type 30 rotor for 1 h at 27,000 rpm. The virus pellet was resuspended in a minimal volume of TSE buffer [0.01 M Tris-HCl (pH 7.4), 0.001 M EDTA, and 0.01 M NaCl] and subsequently applied to a 15-60% sucrose density gradient in TSE. The gradient was centrifuged for 3 h at 25,000 rpm in a Beckman SW27 rotor. The virus particles which banded at a density of 1.14-1.16 were collected, diluted in 10 volumes of TSE buffer and pelleted by centrifugation at 27,000 rpm for 60 min.

D. Virus Cloning

Virus was cloned by using a modification of previously described methods (146). Essentially, a monolayer of uninfected cells was infected with a 1.0 ml virus suspension at a multiplicity of infection of 0.25 based on the S+L- assay. After 2 h at 37°C, the cells were trypsinized, diluted to 1.3 cells/ml in medium and 0.2 ml of the diluted cell suspension was seeded into each of the 96 wells of a Falcon Microtiter II dish. Wells containing single cells were grown to form clonal cell lines.

E. Radioactive Labeling and Extraction of Cells

Virus-infected cells were pre-incubated for 10 min in methionine-free minimal essential medium (MEM, Grand Island Biological Company) and then pulse-labeled for 2 h in methionine-free MEM supplemented with 50 to 500 uCi/ml L-[^{35}S]methionine (New England Nuclear, Boston, MA). After removing the labeling medium, cells were routinely extracted with 1 ml of immunoprecipitation-lysis buffer [IPB, 20 mM Tris-HCl (pH 7.5), 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 M NaCl, 1 mM EDTA, 0.2% sodium azide, and 5 mM phenylmethyl sulfonyl fluoride] at 4°C for 30 min. The cell extracts were centrifuged at 140,000 x g for 35 min in a Beckman T65 rotor and the supernatants were recovered.

Cells were labeled with D-[^{3}H]glucosamine (New England Nuclear) by incubation for 6 h in glucose-free MEM supplemented with 100 uCi/ml of the radioactive sugar.

Lactoperoxidase-catalyzed surface iodination with [^{125}I]iodine was performed at 0°C by the method of Vitetta et al. (189). Essentially, cell monolayers were overlaid with 1 ml of phosphate buffered saline (pH 7.4) (Grand Island Biological Company) containing 100 ug of lactoperoxidase (Worthington Biochemical Company, Freehold, NJ) and 1 mCi of [^{125}I]iodine (New England Nuclear). Three additions of 0.06% hydrogen peroxide were made to the reaction mixture at 4 min intervals.

In pulse-chase experiments, cells were pulse-labeled with L-[^{35}S]methionine for the indicated time and subsequently placed in complete medium lacking the radioisotope for the appropriate chase period. Glycosylation inhibition studies were performed by treating

cells with medium containing 25 μg/ml of tunicamycin both prior to (3 h) and during (10 min) labeling with L-[^{35}S]methionine.

F. Antisera and Immunoprecipitation Procedures

Monospecific antisera to R-MuLV p30, p12, and gp70 were obtained from the Office of Program Resources and Logistics, National Cancer Institute, NIH, Bethesda, Maryland and have been previously characterized (41,146). Monoclonal serum recognizing the R-MuLV p15E determinant was kindly provided by R. Nowinski (Fred Hutchinson Cancer Center, Seattle, WA) and described previously (102).

A rat antiserum specific to SFFV gp55 was prepared by M. Ruta following the procedures of Ruscetti et al. (142). Briefly, Osborne-Mendel rats were inoculated subcutaneously with 5 x 10^5 F-SFFV infected NRK clone 1 cells and subsequently boosted with additional cells 3 weeks later. Antiserum prepared from these rats reacted with gp55 and with the gp70s of the dual tropic MCF and HIX viruses, but not with the gp70s of ecotropic MuLV. Secondary reactivity of this antiserum to MuLV gag determinants were removed by absorption to viral core proteins that were covalently linked to Sepharose 4B.

The Abelson tumor regressor serum was obtained from C57 L mice (Jackson Laboratories, Bar Harbor, ME) that successfully rejected tumor challenge induced by subcutaneous injections of 5 x 10^5 viable L1-2 cells. Immunization and bleeding schedules for obtaining this serum were by the procedure of Witte et al. (196).

Immunoprecipitations were performed using a variation of the fixed Staphylococcus aureus (Pansorbin, Sigma Chemical Company, St. Louis, MO) procedure described by Kessler (72). The Pansorbin was suspended (10%) in IPB and centrifuged through 1 M sucrose in IPB without NaCl prior to

use. Lysates were preadsorbed with either normal goat serum or normal C57 L mouse serum for 1 h at 4°C, followed by the addition of 20 µl Pansorbin per microliter of serum for an additional hour at 4°C before conducting precipitations with the principal antisera.

G. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated on 10-20% polyacrylamide gradient slab gels containing 0.1% sodium dodecyl sulfate (SDS) using the system described by Laemmli (76). Electrophoresis was conducted on a constant current mode at 20 mA with a voltage ceiling of 200 V for a minimum of 4 h. The gels were 0.75 mm thick, 14 cm wide and 11 cm high, with a 1 cm stacking gel. The apparent molecular weights of proteins were determined by comparison with ^{14}C-labeled protein standards (New England Nuclear) with apparent Mr of 92,500, 69,000, 46,000, 30,000, and 12,300.

Antigen-antibody complexes were disrupted by heating the samples in Laemmli electrophoresis buffer [0.05 M Tris-hydrochloride (pH 6.8), 1% sodium dodecyl sulfate, 1% β-mercaptoethanol, and 20% glycerol] at 100°C for 5-10 min. The S. aureus was then pelleted by centrifugation and the supernatants carefully removed. Gels were processed by fluorography (17) and exposed at -70°C to Kodak XR-5 X-ray film. Gels used for quantitation of bands were preflashed (17,79) before exposure to X-ray film, and were scanned with the Transidyne General integrating densitometer as previously described (147).

H. Endogeneous Labeling of Cell Surface Proteins

The selective binding and purification of cell surface antigens using specific antisera was performed essentially as described by Krangel et al. (74) with modifications specified by Fitting and Kabat (48). Briefly, cells (5×10^6) were pulse-labeled by incubation with L-[^{35}S]methionine (100 µCi/ml of medium) for 2 h at 37°C and subsequently incubated with specific antibody for 45 min at 4°C. These cells were then washed twice with cold phosphate buffered saline and extracted in the presence of a 10 fold excess of nonradioactive cellular lysate for 30 min at 4°C. In these extraction conditions, antibodies which may dissociate from their immune complexes are competitively inhibited from rebinding to other labeled immunologically reactive molecules (48). The lysates were subsequently clarified by centrifugation at 15,000 x g for 20 min. The antigen-antibody complexes were then collected with Pansorbin and the immunoprecipitates subjected to SDS-PAGE as described above (Section II.G).

To prepare nonradioactive cellular extracts, cells were suspended in nuclei separation buffer (20 mM Tris-HCl, 0.1 M NaCl, 0.25% Triton-X 100, pH 7.4) for 20 min at 4°C. The swollen cells were disrupted by 20-50 strokes of a Dounce homogenizer. The progress of disruption was monitored microscopically to ensure that all the cells were broken with at least 95% of the nuclei remaining intact. The nuclei were then pelleted at 500 x g for 10 min and the supernatants reconstituted to the composition of lysis buffer (IPB) with the addition of concentrated salts and detergents.

I. Complement- and Antibody-dependent Cytolysis

Killing of cultured fibroblasts by cytotoxic antibody in the presence of rabbit complement has been described previously (70). Briefly, cell monolayers were prelabeled with 5 μCi of L-[^{35}S]methionine for 2 h before treatment with 1.1 ml medium containing 0.1 ml rabbit complement and 4 ul of either specific or normal goat serum. Cell killing was monitored microscopically by observing the rounding of the cells and the condensation of their nuclei, followed by the loss of their adherence to the substratum (70). In addition, killing was analyzed quantitatively by measuring the release of trichloroacetic acid- precipitable radioactivity from the monolayer as a function of time (70).

J. Rosette Assay for Detection of Cell Surface Antigens

Sheep erythrocytes were coupled with S. aureus protein A (Sigma Chemical Company) by the chromium chloride method of Goding (56). Briefly, 50 μl of sedimented erythrocytes were washed twice with 0.9 M NaCl prior to resuspension of the erythrocytes in 400 μl of salt solution containing 25 μg of protein A. A volume of 0.01% chromium chloride equal to one half the volume of the erythrocyte suspension was added slowly and the suspension was incubated at 25°C for 10 min. The protein A-coupled sheep erythrocytes were washed twice with 0.9 M NaCl, prior to resuspension of the sedimented cells in 3 ml of DMEM.

In the rosette procedure, fibroblast monolayer cultures (in 25 cm^2 flasks) were provided with 5 ml of fresh DMEM containing 2-5 μl of either specific or nonimmune serum. After a 15 min incubation at 37°C, 0.2 ml of the coupled erythrocyte suspension was added to each culture. Attachment of protein A-coupled erythrocytes to specific cell surface

antigens forms "rosettes" that can be observed microscopically after incubating these cultures for 2 h at 37°C.

K. Protein Kinase Assay

A-MuLV proteins, immunoprecipitated from nonradioactive L1-2 lysates with antiserum prepared against the $p12^{gag}$ molecule, were phosphorylated in vitro with [γ-^{32}P]ATP using the method of Witte et al. (193). Briefly, immunoprecipitates were suspended in 20 mM Tris-HCl (pH 7.2) - 5 mM $MgCl_2$ buffer containing 1 µCi of [γ-^{32}P]ATP (New England Nuclear) and incubated for 10 min at 30°C. Reactions were terminated by addition of ice-cold 20 mM Tris-HCl (pH 7.2), and washed repeatedly by centrifugation. The immunoprecipitates were then resuspended in 15 µl Laemmli electrophoresis buffer, boiled for 5-10 min and then analyzed by SDS-PAGE as described above (see Section II.G).

L. Tryptic Peptide Analyses

Peptide maps were performed by a modification of a procedure described by Elder et al. (39). Proteins either metabolically labeled with L-[^{35}S]methionine or labeled in vitro with [γ-^{32}P]ATP were purified by immunoprecipitation and SDS-PAGE and subsequently digested by incubation of selected gel slices in 50 ug/ml of tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK) trypsin for 24 h at 37°C. Samples were then oxidized with performic acid for 4 h at 0°C and subsequently diluted in 2 ml of H_2O and concentrated to dryness. The peptide digest was then washed twice with distilled water and subsequently resuspended in electrophoresis solution (28% formic acid) and spotted on cellulose thin layer chromatography plates (Brinkman Instruments, Albany, NY, CEL 300, 20 x 20 cm, 0.25 mm thick). Electrophoresis was conducted for 4 h at 150 V in the first dimension

and ascending chromatography was performed in the second dimension in solution containing either isoamyl alcohol:pyridine:ethanol:glacial acetic acid:H_2O (70:70:40:10:60) for L-[^{35}S]methionine labeled peptides or N-butanol:pyridine:glacial acetic acid:H_2O (32:25:5:20) for [^{32}P]-labeled peptides. Detection of L-[^{35}S]methionine labeled peptides was enhanced by spraying plates with 7% diphenyloxazole in ether. Radiolabeled peptides were visualized by autoradiography using Kodak XR-5 film and intensified in the case of [^{32}P]-labeled peptides with the use of calcium tungstate intensifying screens (Eastman Kodak, Rochester, NY).

M. Phosphoamino Acid Analyses

[^{32}P]-labeled proteins, phosphorylated *in vitro*, purified by SDS-PAGE and collected by *in situ* trypsinization as described above, were heated to 110°C for 1 h in 200 µl of 6 N hydrochloric acid. Samples were washed repeatedly by concentration under vacuum and then resuspended in electrophoresis solution containing acetic acid:formic acid:H_2O (15:5:80) pH 1.9. Phosphoamino acid standards, *o*-phospho-D-serine and *o*-phospho-DL-threonine were purchased from Sigma Chemical Co. *o*-phospho-L-tyrosine was kindly provided by Tony Hunter (Salk Institute, San Diego, CA). Hydrolyzed samples containing 0.05 µg of each phosphoamino acid standard were applied to cellulose thin layer chromatography glass plates and analyzed by two-dimensional high voltage electrophoresis. Electrophoresis was conducted with pH 1.9 solution for 2.5 h at 1000 V in the first dimension and with pyridine:glacial acetic acid:H_2O (10:100:1890) pH 3.5 for 1 h at 1000 V in the second dimension. Unlabeled standards were detected by ninhydrin staining and

[^{32}-P]-labeled amino acids were visualized by autoradiography using calcium tungstate intensifying screens.

N. Leukemogenicity Testing in Mice

To ensure that the SFFV mutants were truly weak in their ability to induce disease in NIH/Swiss mice, cell-free extracts of spleens obtained from infected animals were injected into fresh mice and virus was transferred sequentially in this manner for a total of 3 passages. This procedure was performed to ensure that the absence or apparent lessening of the disease in these animals were due to defects in virus leukemogenicity and not due to low titers of virus in the sample preparation. Mutant virus samples were injected into the tail veins of ten 4-6 week old NIH/Swiss mice. Spleens not exhibiting gross enlargement or development of macroscopic foci after 14 days were crushed and extracted in a minimal volume of phosphate buffered saline (pH 7.4) (Grand Island Biological Company). The extract was then centrifuged to remove cell debris and subsequently filtered before injecting the filtrate into the tail vein of fresh mice. This procedure was repeated for a total of 3 passages per virus sample to ensure the validity of the leukemogenicity determination.

O. Scintillation Procedures

The radioactivity present in metabolically labeled protein preparations were determined by precipitating a small aliquot (5 µl) of the sample with 2 ml of a 25% trichloroacetic acid (TCA) solution for 30 min at 4°C. The precipitate was collected on a glass fiber filter (Whatman, Inc., Clifton, NJ) and digested with the addition of 1 ml of a toluene:Protosol (New England Nuclear) solution (2:1) for 1 h in a scintillation vial. Following addition of 2 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.

P. Subcellular Fractionation

Cells were fractionated into subcellular organelles following the procedure of Courtneidge et al. (31). Basically, cells (10^7) in suspension culture were labeled by incubation with 1 mCi of L-[^{35}S]methionine for 2 h at 37°C and subsequently washed and collected by gentle centrifugation in a clinical centrifuge. The cells were resuspended in cold buffer "A" (5 mM KCl, 1 mM $MgCl_2$, and 20 mM HEPES, pH 7.2), allowed to swell at 4°C for 10 min, and then broken with 10-30 strokes in a tight-fitting Dounce homogenizer. Inspection by phase-contrast microscopy indicated that over 90% of the cells were broken without apparent damage to the nuclei. The lysate was centrifuged at 1000 x g for 5-10 min and the resulting pellet or "nuclear fraction" was stored at 4°C for subsequent analysis. The supernatant fluids consisting of cytoplasmic and membrane constituents were then centrifuged in a Beckman Ti 50.2 rotor for 1 h at 38,000 rpm. The supernatant from this second centrifugation contained cytoplasmic material, while the pellet consisted of a crude membrane fraction. The membranes were then subjected to isopycnic density centrifugation in a 20-50% sucrose step gradient (prepared in buffer "B": 5 mM KCl and 20 mM HEPES, pH 7.2) at 25,000 rpm for 18 h in a Beckman SW 27 rotor. The fraction at the 20-30% sucrose interface was enriched in plasma membranes and in other smooth membranous organelles, while the material that banded at the 40-50% sucrose interface contained ribosomes and

highly purified rough endoplasmic reticulum. The intermediate fraction at the 35-40% sucrose interface contained relatively little material and appeared to consist primarily of a mixture of membranes from the adjacent fractions. Radioactively-labeled proteins from each of the fractions isolated in this procedure were immunoprecipitated with monospecific antiserum and analyzed by SDS-PAGE as described in previous sections (Sections II.F and II.G).

Q. Extraction of RNA from Virus-infected Cells

RNA was extracted from virus-infected cells using procedures described by Sharp et al. (158) and Holmes and Bonner (62). Essentially, cell monolayers were lifted from tissue culture surfaces with 0.2 M EDTA (pH 7.2) and subsequently washed and collected by centrifugation in a J2-21 rotor at 2000 rpm for 10 min. The cells were resuspended in 0.65% NP-40 in phosphate buffered saline (pH 7.2) (Grand Island Biological Company), allowed to swell for 10 min at 4°C, and then broken with 5-10 strokes in a Dounce homogenizer. The homogenization was monitored by phase-contrast microscopy to ensure that the majority of the cell nuclei remained intact. The lysate was then centrifuged at 1000 x g for 5 min and the nuclei pellet was discarded. The retained supernatant or "cytoplasmic fraction", transferred into a fresh siliconized glass tube, was then diluted with an equal volume of solution containing 7 M urea, 0.35 M NaCl, 0.001 M EDTA, 1% SDS, and 0.01 M Tris-HCl (pH 7.4) and a 2X volume of chloroform:phenol (1:1, pH 7.9). This mixture was then agitated vigorously for 5 min and then centrifuged in a J2-21 rotor at 4000 rpm for 15 min. The resulting upper aqueous phase containing the extracted RNA was collected and stored at 4°C. The lower chloroform:phenol phase was re-extracted twice

with an aqueous solution consisting of 0.1 M Tris-HCl (pH 7.9), 0.15 M NaCl, and 0.001 M EDTA to ensure quantitative removal of the RNA.

The aqueous phases were subsequently pooled and then diluted with a 25% sodium acetate solution (pH 6) to a final concentration of 2%. Three volumes of cold absolute ethanol were added to the sample and the RNA was allowed to precipitate overnight at -20°C. The ethanol-precipitated RNA was then pelleted by centrifugation in a J2-21 rotor at 7000 rpm for 10 min and dissolved in 1 ml of a solution containing 5 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.2% SDS. The resuspended RNA was then diluted with an equal volume of solution consisting of 0.5 M NaCl, 50 mM Tris-HCl, and 1 mM EDTA (pH 7.5) and subsequently mixed with 0.1 ml of oligo (dT) cellulose (Collaborative Research, Boston, MA). The oligo (dT) cellulose was collected by centrifugation in a clinical centrifuge and washed repeatedly with 150 µl volumes of sterile water to elute the bound, polyadenylated RNA. A_{260} absorbance readings were conducted on the pooled eluates and 10-20 µg of carrier tRNA was added to the samples if absorbances measured below 0.5. The RNA solution was then diluted with concentrated sodium acetate to a final concentration of 10% and the RNA subsequently precipitated by 3 volumes of cold absolute ethanol as described above.

R. Agarose Gel Electrophoresis and Northern Blotting Procedure

RNA samples were electrophoresed in horizontal 1% agarose gels containing 10 mM methyl mercuric hydroxide as described by Bailey and Davidson (7). Basically, RNA samples in water were mixed with an equal volume of Buffer "E" (5 mM sodium borate, 10 mM sodium sulfate, 1 mM EDTA, and 50 mM boric acid, pH 8.2) containing 10% glycerol, 0.06% bromphenol blue and 10 mM methyl mercuric hydroxide (Alfa Division,

Danvers, ME). Gels consisted of 1% agarose (electrophoresis purity, Bio-Rad Laboratories, Richmond, CA) in Buffer "E" containing 10 mM methyl mercuric hydroxide. The electrophoresis was conducted for 18 h at 20 V with slow buffer recirculation.

Gels were prepared for blot transfer to activated paper (for activation of paper, see below) by successive washes in 1) 50 mM NaOH, 5 mM betamercaptoethanol, 1 μg/ml ethidium bromide (20-40 min), 2) 200 mM sodium acetate, pH 4.0, containing 7 mM iodacetate (2 washes, 10 min each), 3) 200 mM sodium acetate, pH 4.0 (2 washes, 5 min each), and 4) 20 mM sodium acetate pH 4.0 (30 min).

The aminophenylthioether (APT) paper was kindly provided by C. Faust and R. Bestwick (Dept. of Biochemistry, Oregon Health Sciences University). The APT paper was diazotized by the addition of 120 ml of cold 1.2 N HCl and 3.4 ml of freshly prepared 10 mg/ml sodium nitrite. The APT paper was immersed in this reaction mixture for 30 min at 4°C.

The Northern blotting procedure was performed following methods described by C. Faust, using 20 mM sodium acetate (pH 4.0) as the blotting buffer. Pretreatment and hybridization of the blot (187) with [^{32}P]-labeled SFFV or MuLV specific probes were generously conducted by R. Bestwick. Hybridization probes were prepared by R. Bestwick using T4 DNA polymerase replacement synthesis labeling (105). Specific activities of the hybridization probes were between 5×10^7 and 2×10^8 dpm/μg. For detection of R-SFFV specific sequences, the 0.6 kb Bam H1 to Eco R1 fragment of F-SFFV cloned into pBr322 was used (23,86). MuLV sequences were detected using a plasmid containing the entire 8.5 kb F-MuLV genome inserted at the Eco R1 site of pBr322 (23,86).

III. Abnormally Processed Plasma Membrane and Virion Glycoproteins Encoded by Env Gene Mutants of Murine Leukemia Virus

A. General Introduction

The murine leukemia virus (MuLV) env gene encodes two structural proteins, a 70,000 dalton glycosylated component known as $gp70^{env}$ and a nonglycosylated protein with an apparent Mr of 15,000 termed p15E. These molecules are processed from a glycosylated polyprotein precursor, $gPr90^{env}$, that undergoes partial proteolysis and oligosaccharide modifications en route to the cell surface. It is known that $gp70^{env}$ performs important but poorly understood functions in virus attachment to susceptible cells and in virus interference (33). This molecule, in addition, has been implicated in other important processes such as differentiation of lymphocytes (22,39,103,181) and in leukemogenesis (39). Our laboratory has isolated several MuLV mutants that contain defects in the post-translational processing of the env components. One viral mutant, the R7 clone 1, has been extensively characterized. This mutant has been shown to encode an env precursor molecule, which is inefficiently cleaved by partial proteolysis. Both the cleaved and uncleaved forms of the glycoprotein are transported to the surface of infected cells. These mutant surface forms of the env glycoprotein are apparently ineffective in establishing interference to superinfection by wild-type MuLV. This mutant, as well as two other MuLV env gene mutants, the R Balb clone 29 and R7 clone 61, will be discussed in relation to a general model that will illustrate how structural abnormalities of the env gene products can differentially affect various parameters of their intracellular transport and function.

B. Characterization of the R7 clone 1 Mutant Virus

1. R7 clone 1 encodes an env-related glycoprotein with an apparent Mr of 80,000 ($gPr80^{env}$)

The R7 clone 1 mutant was isolated by limiting dilution cloning of R-MuLV into NIH-3T3 fibroblasts (145). As shown in Fig. 1, this spontaneous mutant encodes a gp70-related protein with an apparent Mr of 80,000 ($gPr80^{env}$)and $p30^{gag}$-related polyproteins which appear to be indistinguishable from those encoded by wild-type MuLV. Two observations indicate that the abnormal glycoprotein phenotype in the mutant cell line is caused by a viral rather than by a cellular mutation. First, as described below, it can be transferred with virus to uninfected wild-type cells. Second, the R7 clone 1-infected cells synthesize $gPr90^{env}$, gp70, and p15E after superinfection by wild-type MuLV. In addition, pulse-chase analysis using L-[^{35}S]methionine showed that $gPr80^{env}$ is rapidly labeled, suggesting that it is a primary translation product rather than a derivative of a larger glycoprotein (C. Machida and D. Kabat, unpublished observations).

2. $gPr90^{env}$ and $gPr80^{env}$ have different sized polypeptide chains

Addition of asn-linked oligosaccharides to glycoproteins can be blocked by addition of the glycosylation inhibitor tunicamycin (80,175). As shown in Fig. 2, cells preincubated with tunicamycin synthesize env-related polypeptide chains which are smaller than the complete glycoproteins. Furthermore, the polypeptide chain encoded by R7 clone 1 (Mr 63,000) (lane 4) is considerably smaller than that encoded by wild-type R-MuLV (Mr 70,000) (lane 3). Extensive labeling of the R7 clone 1 unglycosylated env gene product was not achieved because

Figure 1. Electrophoretic analysis of viral-encoded proteins found in the R-MuLV- and R7 clone 1-infected NIH-3T3 cell lines.

R-MuLV-infected (lanes 1 and 2) and R7 clone 1-infected (lanes 3 and 4) cell lines were pulse-labeled by incubation with 50 μCi of L-[^{35}S]methionine for 2 h at 37°C. The cells were subsequently lysed and the virus-encoded proteins in the lysates were immunoprecipitated with monospecific serum to p30 gag (lanes 1 and 3) or gp70 (lanes 2 and 4) proteins. Immunoprecipitated proteins were electrophoresed in 10-20% polyacrylamide gels containing 0.1% SDS.

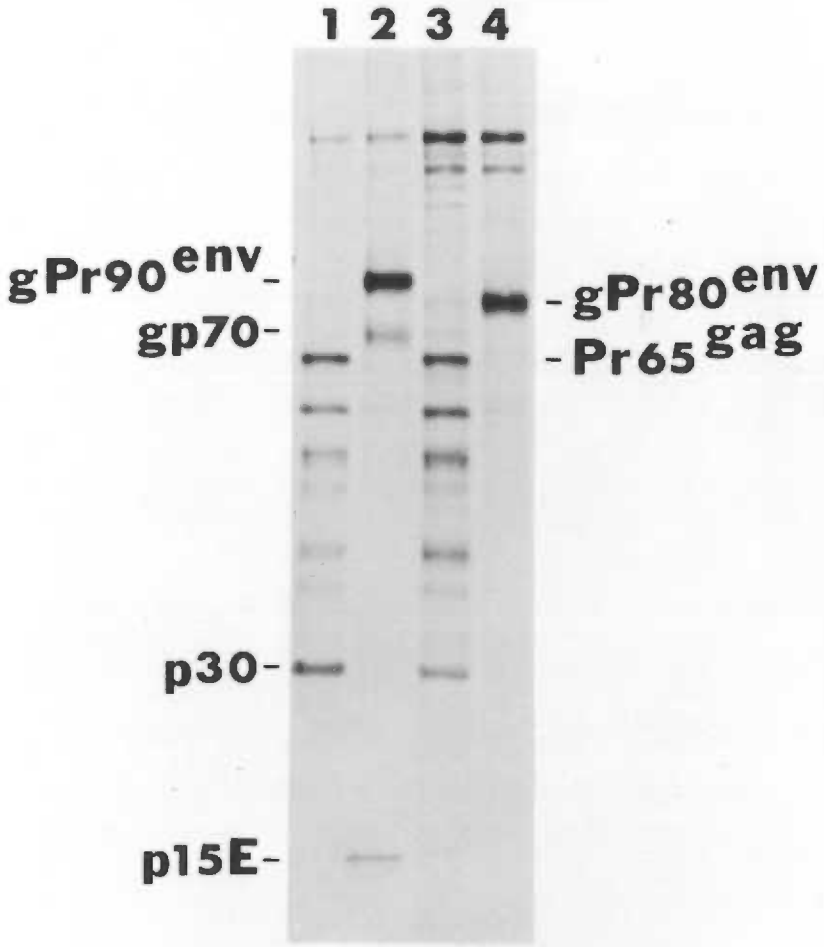
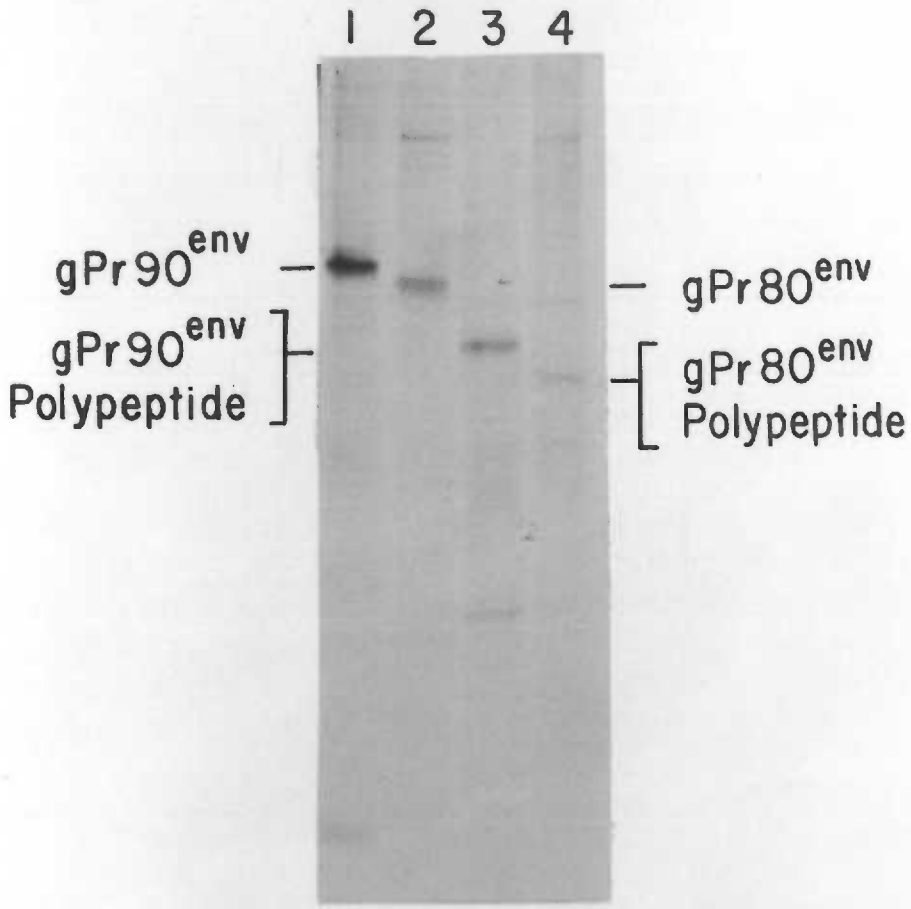

1 2 3 4
gPr90env
gp70
p30
p15E
gPr80env
Pr65gag

Figure 2. Electrophoretic analysis of nonglycosylated polypeptide chains of R-MuLV gPr90^{env} and R7 clone 1 gPr80^{env}.

R-MuLV-infected and R7 clone 1-infected NIH-3T3 cells were treated with 25 μg/ml tunicamycin both prior to (3 h) and during labeling (10 min) with 100 μCi of L-[^{35}S]methionine. These cultures, as well as labeled control cultures incubated without tunicamycin were lysed after labeling and the viral proteins in the lysates were immunoprecipitated with anti-gp70 serum. Labeled cells infected with R-MuLV and with R7 clone 1 are in lanes 1 and 2, respectively. These same cell lines labeled in the presence of tunicamycin are in lanes 3 and 4, respectively.


1 2 3 4
gPr90env
gPr90env
Polypeptide
gPr80env
gPr80env
Polypeptide

it was rapidly degraded intracellularly. Consequently, when the labeling times were longer than 10 min, a heterogeneous group of polypeptide chains consisting of intact plus partially degraded components was present in the immunoprecipitates.

Additional evidence consistent with the idea that the gPr80$^{\underline{env}}$ polypeptide may be smaller than the gPr90$^{\underline{env}}$ polypeptide was obtained by analysis of tryptic peptides of the L-[^{35}S]methionine labeled glycoproteins. As shown in Fig. 3, gPr80$^{\underline{env}}$ has a substantially simpler peptide pattern than gPr90$^{\underline{env}}$, suggesting that it has fewer methionine-containing peptides. For example, gPr80$^{\underline{env}}$ lacks the well resolved peptide 2. In addition, it lacks the microheterogeneous peptide spots 4 and 5. Whether this microheterogeneity is caused by the well known structural variability of the oligosaccharides in these envelope glycoproteins (99) or from other causes has not been ascertained.

3. gPr80$^{\underline{env}}$ undergoes further glycosylation and inefficient cleavage to form cell surface molecules gp85 and gp68

Labeling the R7 clone 1-infected NIH-3T3 cell line with D-[^{3}H]glucosamine for increasing times, provided evidence that gPr80$^{\underline{env}}$ is processed to form two other glycosylated components with apparent Mr of 85,000 and 68,000 (gp85 and gp68) (Fig. 4). These processed glycoproteins are formed only slowly, and first appear after approximately two hours of labeling with D-[^{3}H]glucosamine. These components are labeled in higher proportion with D-[^{3}H]glucosamine than with L-[^{35}S]methionine, suggesting they may have more extensively processed oligosaccharides than gPr80$^{\underline{env}}$. Glucosamine and its

Figure 3. Tryptic peptide analysis of L-[^{35}S]methionine-labeled R-MuLV gPr90^{env} (Frame A) and R7 clone 1 gPr80^{env} (Frame B).

Purified radioactive glycoproteins were treated with TPCK-trypsin and analyzed on thin-layer plates as described in Section II. L. The origins were near the lower right corners. Electrophoresis in the first dimension was to the left; this was followed by chromatography to the top.

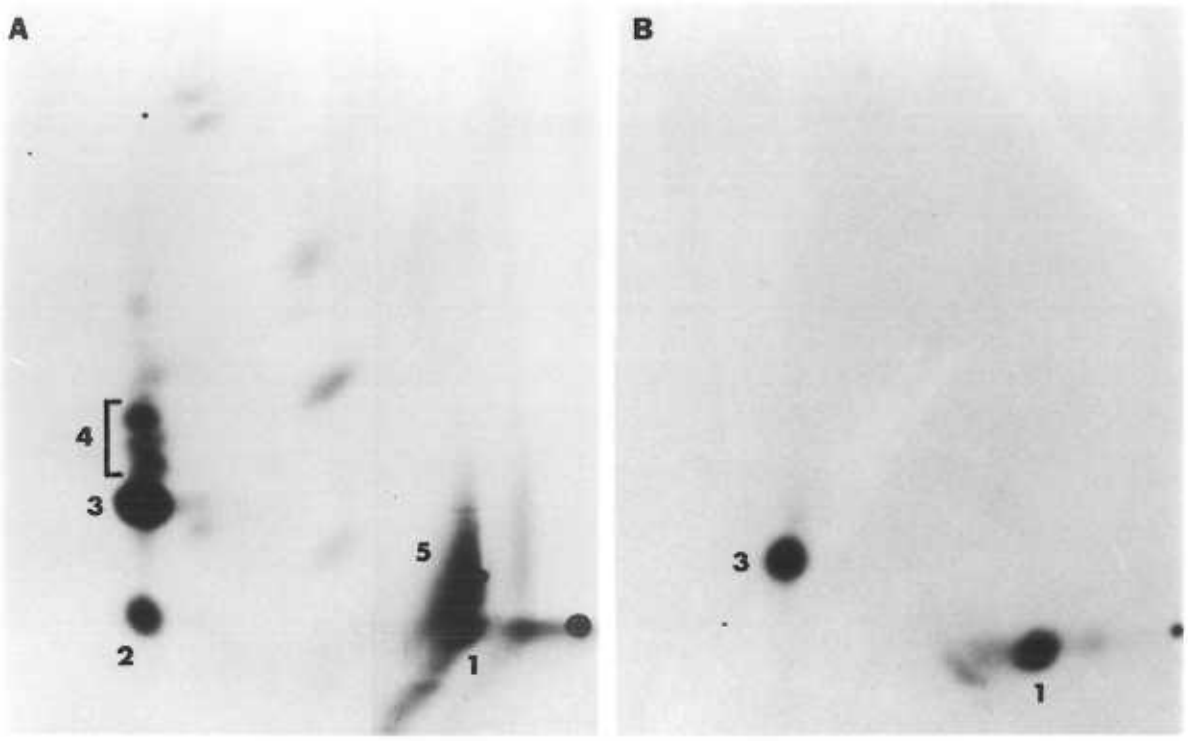
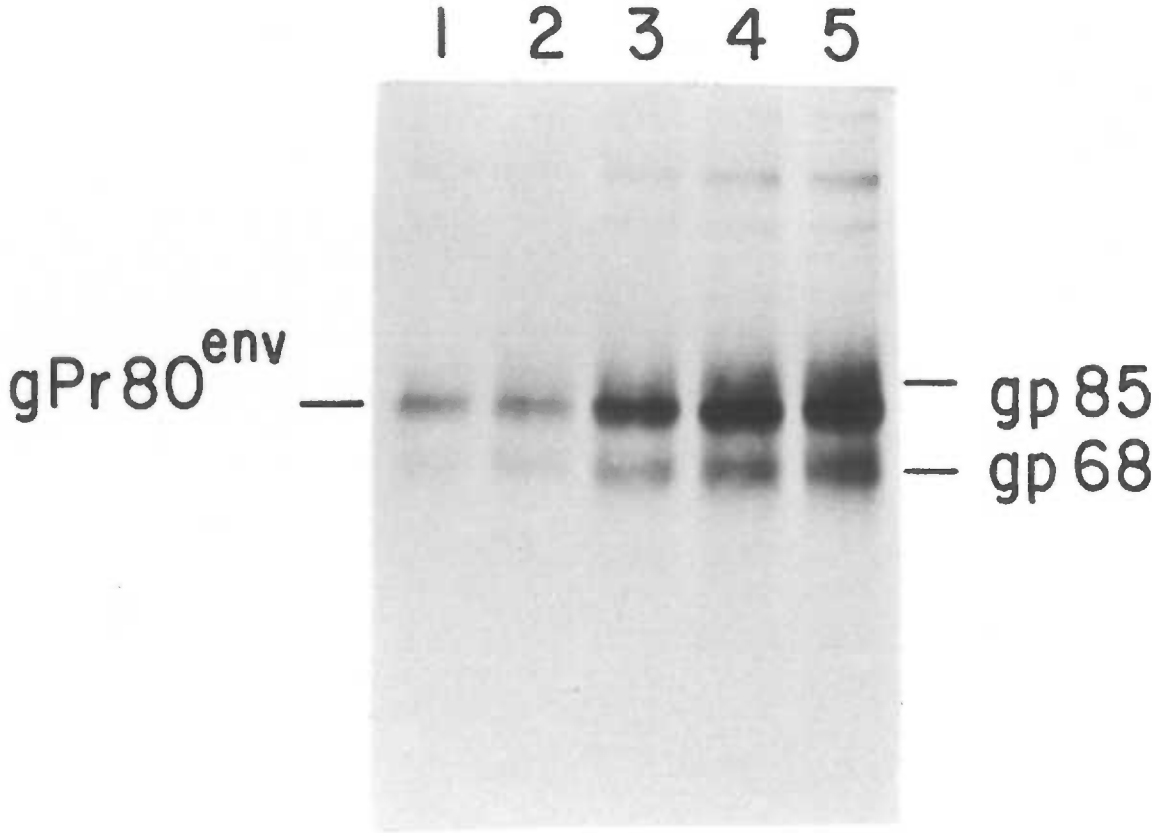

A
4
3
2
5
1
B
3
1

Figure 4. Kinetic analysis of gPr80$^{\underline{env}}$ processing in the R7 clone 1-infected NIH-3T3 cell line.

R7 clone 1-infected cells were labeled with 50 μCi of D-[^{3}H]glucosamine for 1.0, 1.5, 2.0, 2.5, and 3.0 h (lanes 1, 2, 3, 4, and 5 respectively) at 37°C, prior to cell lysis and immunoprecipitation of proteins with antiserum to gp70.


1 2 3 4 5
gPr80env
gp85
gp68

metabolic product sialic acid (151) both occur in relatively large amounts in complex oligosaccharides which have been processed in the Golgi apparatus (151,171). Consistent with the idea that they have been processed through the Golgi apparatus, gp85 and gp68 but not gPr80$^{\underline{env}}$ occur in the cellular plasma membranes as demonstrated by lactoperoxidase-catalyzed surface iodination with [^{125}I]iodine (Fig. 5, lane 5). The presence of env-related molecules on the surfaces of R7 clone 1 infected NIH-3T3 cells but not on uninfected cells was substantiated by complement-dependent cytolysis using cytotoxic antisera which react with R-MuLV gp70. Equivalent levels and kinetics of complement-dependent cytolysis were observed in both the R7 clone 1- and R-MuLV infected NIH-3T3 cell lines (Fig. 6).

As described above, gPr90$^{\underline{env}}$ is a polyprotein which is processed by glycosylation and proteolytic cleavage to form gp70 plus p15E. These processed molecules reach the cell surface and subsequently enter into budding virions (16). In order to learn whether similar phenomena are involved in gPr80$^{\underline{env}}$ processing, we analyzed the various glycoprotein components with a monoclonal antibody specific for p15E. As shown in Fig. 7, the p15E antigenic determinant occurs in gPr80$^{\underline{env}}$ and in gp85 but not in gp68. These results imply that gPr80$^{\underline{env}}$ is only inefficiently cleaved by proteolytic removal of its p15E region. Furthermore, both the cleaved and intact forms of the glycoprotein can undergo further glycosylation in the Golgi apparatus to produce gp68 and gp85, respectively, and both of these are then transferred to the cell surface. We cannot kinetically distinguish between the appearance of gp85 and gp68 (Fig. 4), consistent with the idea that the synthesis of complex oligosaccharides and cleavage of the p15E determinant are

Figure 5. Electrophoretic analysis of molecules found on the surface of R7 clone 1-infected NIH-3T3 cells.

Lactoperoxidase-catalyzed surface iodination of the R7 clone 1-infected cell line (lane 5) was conducted as described in Section II. E., prior to cell lysis and immunoprecipitation of viral proteins with antiserum to gp70. In addition, R-MuLV- and R7 clone 1-infected NIH-3T3 cell lines labeled with L-[^{35}S]methionine for 2 h (lanes 1 and 2, respectively) or D-[^{3}H]glucosamine for 6 h (lanes 3 and 4, respectively) were lysed and proteins in the lysate immunoprecipitated with anti-gp70 serum.

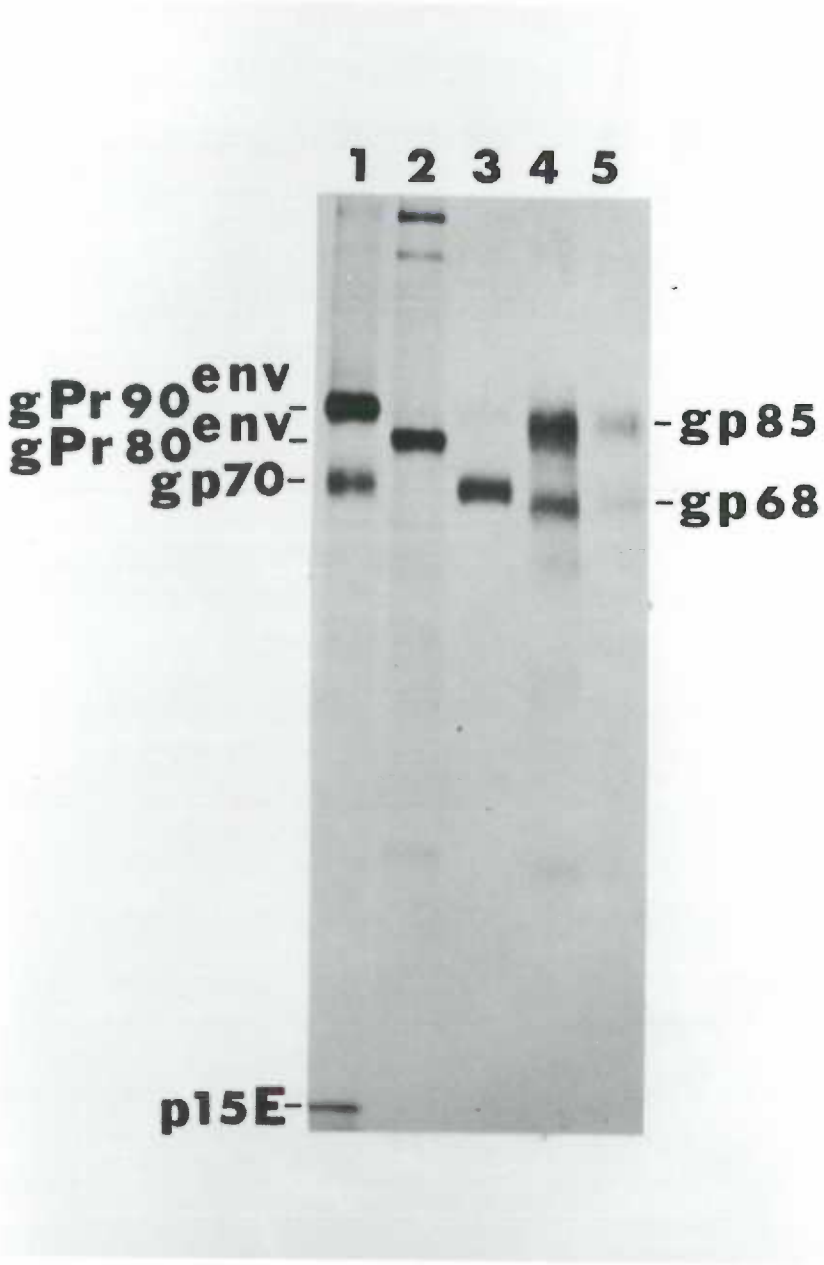

1 2 3 4 5
gPr90env-
gPr80env-
gp70-
p15E-
-gp85
-gp68

Figure 6. Detection of surface antigens on R7 clone 1-infected NIH-3T3 cells by antibody- and complement-dependent cytolysis.

Virus-infected cell cultures were labeled with L-[^{35}S]methionine for 2h at 37°C and subsequently treated with specific antibody and rabbit complement. TCA-precipitable radioactivity released from the cell lines were measured on 50 µl aliquots of the culture medium and expressed as a percentage of the total amount of incorporated radioactivity that could be recovered by adding 0.1% SDS. R-MuLV infected NIH-3T3 cells were treated with complement and either normal goat serum (Δ) or goat antiserum recognizing F-MuLV gp70$^{\underline{env}}$ (▲). R7 clone 1-infected cells were similarly treated with complement and normal goat serum (□) or anti-gp70$^{\underline{env}}$ serum (■). Controls using uninfected NIH-3T3 cells and specific serum and complement gave results that were indistinguishable from those of virus-infected cells treated with normal goat serum.

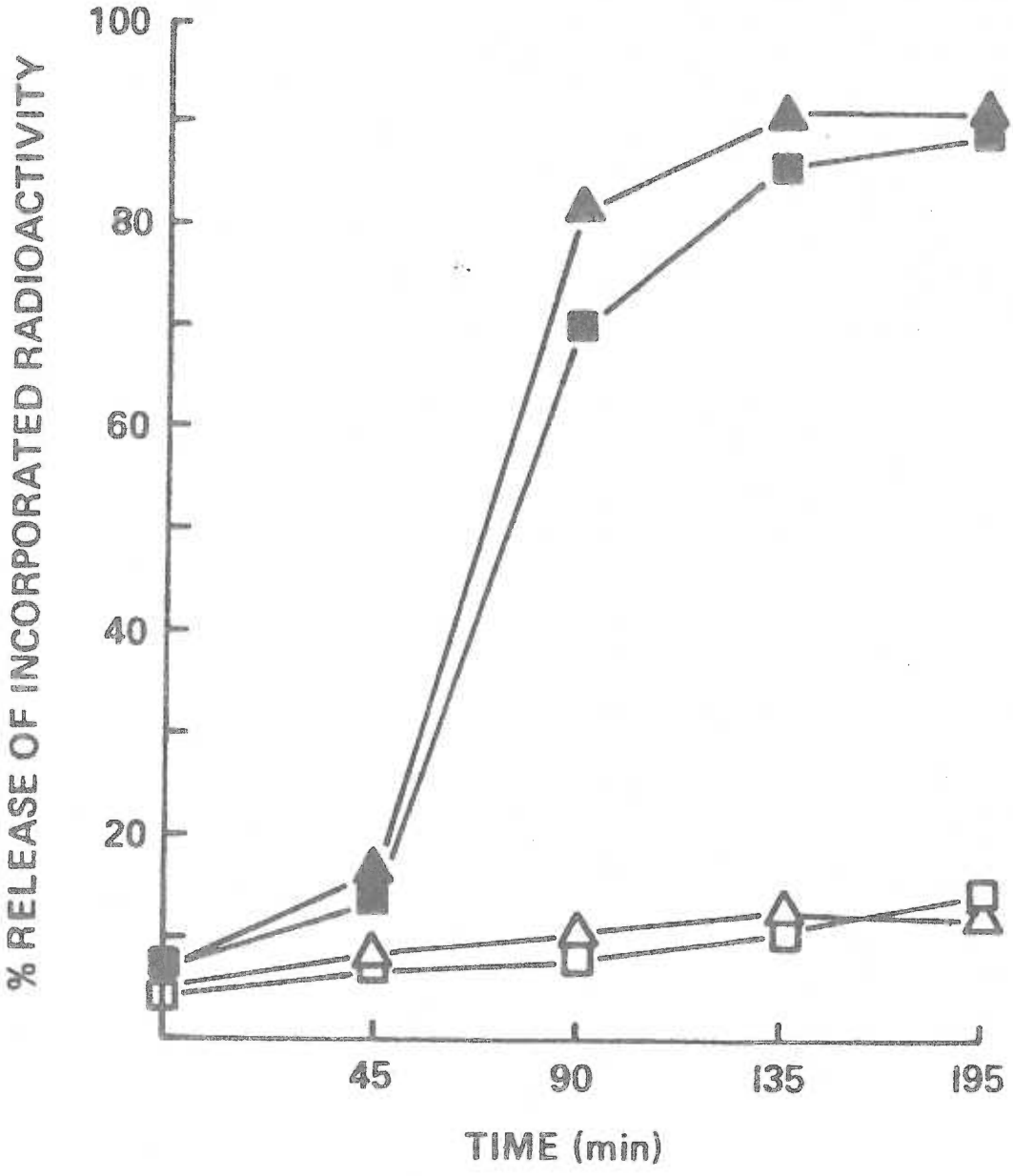
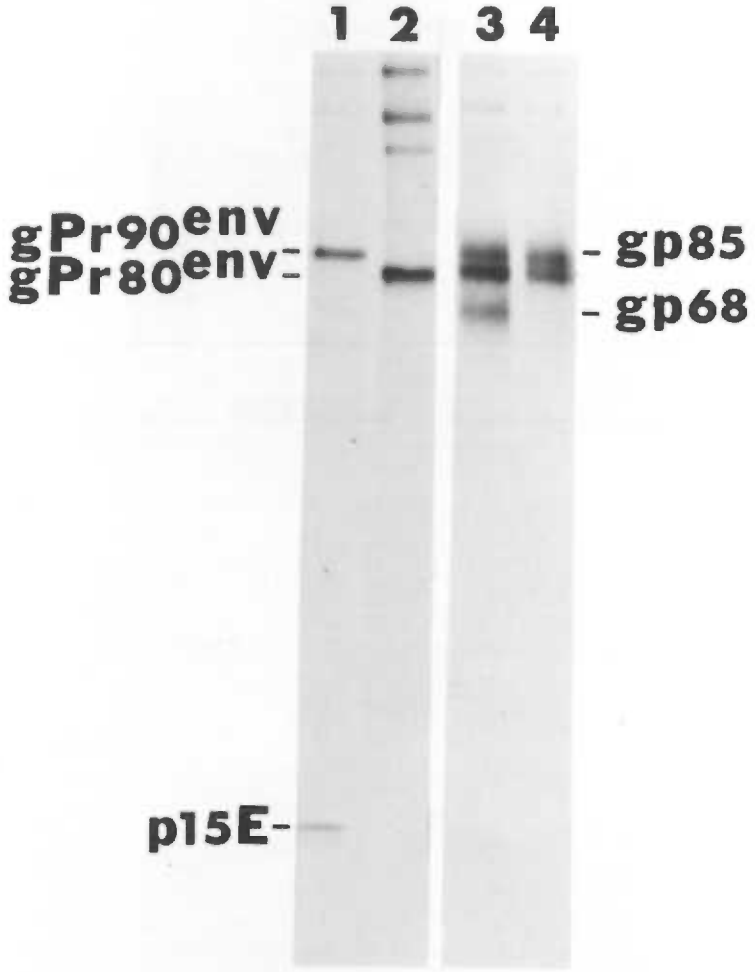

% RELEASE OF INCORPORATED RADIOACTIVITY
100
80
60
40
20
45
90
135
195
TIME (min)

Figure 7. Selective precipitation analysis of R7 clone 1 env gene products with monoclonal anti-p15E serum.

R-MuLV-infected and R7 clone 1-infected NIH-3T3 cell lines were labeled with L-[^{35}S]methionine for 2 h (lanes 1 and 2, respectively). The R7 clone 1 cell line was, in addition, labeled with D-[^{3}H]glucosamine for 6 h (lanes 3 and 4). These cultures were subsequently lysed and viral proteins in the lysates were immunoprecipitated with either monospecific anti-gp70 serum (lane 3) or with monoclonal p15E antiserum (lanes 1, 2, and 4).


1 2 3 4
gPr90env
gPr80env
gp85
gp68
p15E

temporally linked events (202). Since the relative proportions of gp85 and gp68 remain constant throughout the labeling period (Fig. 4), it is unlikely that either glycoprotein is a precursor of the other. Therefore, proteolytic cleavage of the polyprotein is a transient event during processing and the gp85 must become resistant to cleavage, presumably because it is transported away from the site of proteolysis.

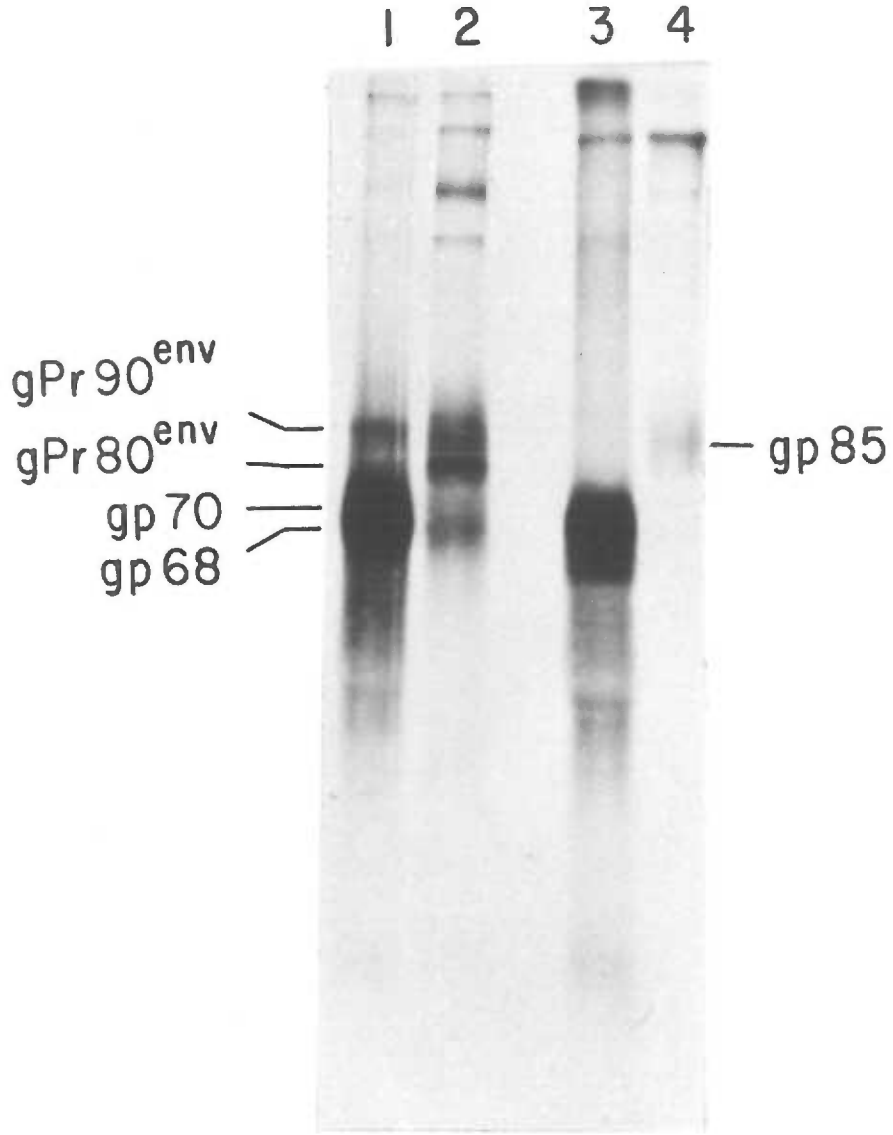
4. R7 clone 1-infected cells release virions which contain the env-related gp85 glycoprotein

Virus was collected from the culture media of R-MuLV- and R7 clone 1-infected NIH-3T3 cells which had been labeled with either L-[^{35}S]methionine or D-[^{3}H]glucosamine. The L-[^{35}S]methionine labeling data indicated that both the R-MuLV and R7 clone 1-infected cells release virions which contain the gag gene-encoded protein p30 (C. Machida and D. Kabat, unpublished observations). Analyses of D-[^{3}H]glucosamine-labeled virions indicated that R-MuLV contains gp70 and that the R7 clone 1 mutant virions contains gp85 but not gp68 or gPr80$^{\underline{env}}$ (Fig. 8, lanes 3 and 4).

These R7 clone 1 virions were also infectious since the mutant virus phenotype could be transferred to uninfected cells incubated with the virus preparation. The infectious virus titer, however, appeared to be very low and the infection failed to spread throughout the culture even when the cells were incubated in the presence of polybrene for several weeks. The gPr80$^{\underline{env}}$ component could therefore be detected in this newly infected culture only in trace amounts (C. Machida and D. Kabat, unpublished observations). We conclude that the mutant virions are weakly infectious and that they may also be released in only low titers from the R7 clone 1-infected cell line. Furthermore, the R7

Figure 8. Electrophoretic analysis of envelope glycoproteins found in virions released from the R-MuLV- and R7 clone 1-infected cell lines.

R-MuLV and R7 clone 1 virus particles were purified as described in Section II.C. after labeling virus-infected cell lines with D-[^{3}H]glucosamine for 18 h. Proteins contained in the lysates of R-MuLV (lane 3) and R7 clone 1 (lane 4) virions were then immunoprecipitated with anti-gp70 serum. gp70-related proteins obtained from D-[^{3}H]glucosamine-labeled cells (6 h) infected with R-MuLV (lane 1) and R7 clone 1 (lane 2) are shown for comparison.


1
2
3
4
gPr90env
gPr80env
gp70
gp68
gp85

clone 1 virions did not appear to cause leukemia in mice. Additionally, the R7 clone 1-infected cell line could be superinfected with wild-type R-MuLV and could then synthesize both wild-type gPr90$^{\underline{env}}$ and mutant gPr80$^{\underline{env}}$ molecules. These results suggest that the cells infected with the R7 clone 1 virus are susceptible to superinfection. Presumably, the mutant gp85 and gp68 molecules on the cell surface are unable to effectively block the viral receptor sites required for causing interference to superinfection.

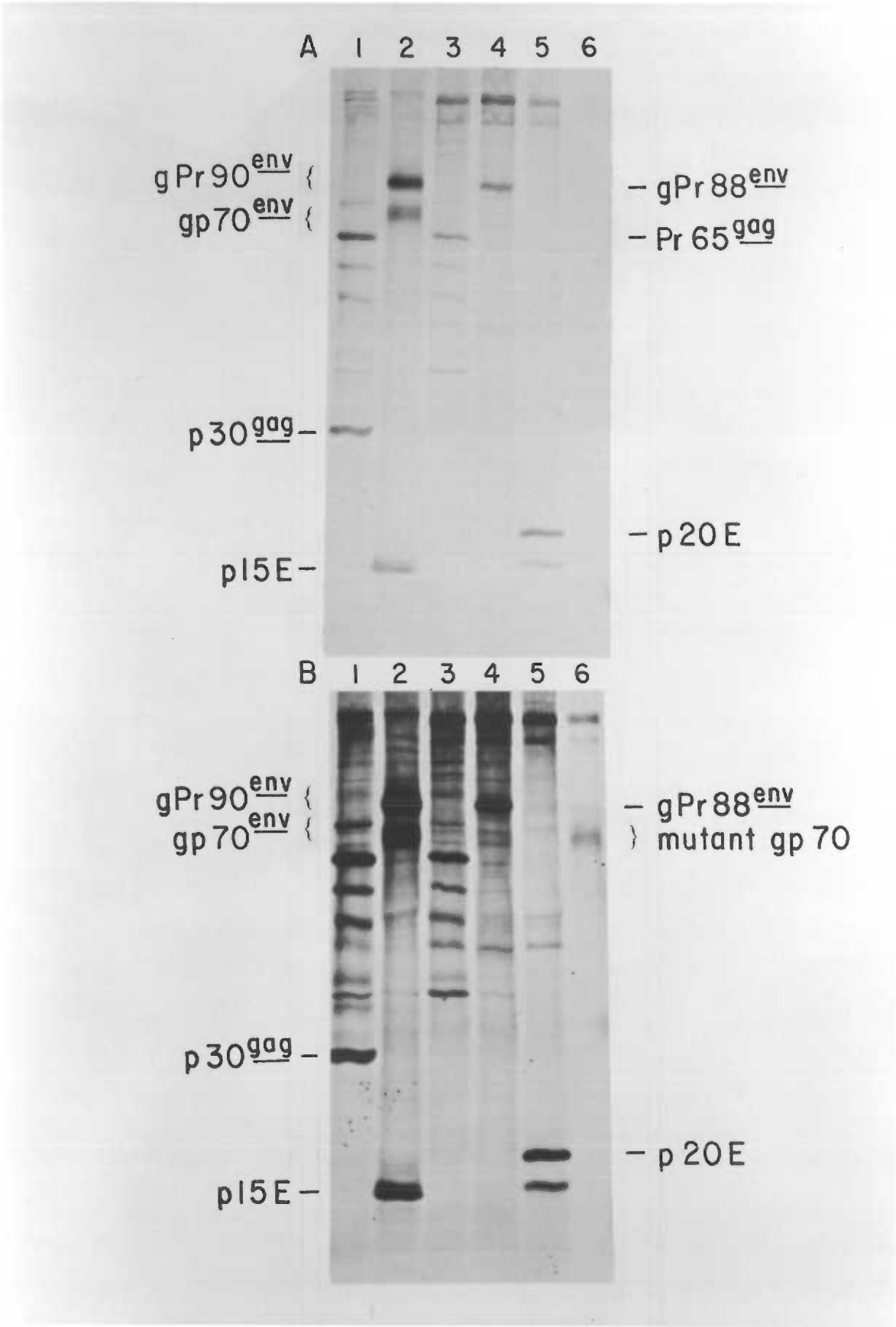
C. Preliminary Description of Two Other MuLV env Gene Mutants, the R Balb clone 29 and R7 clone 61 Viruses

1. The R Balb clone 29 MuLV mutant

The R Balb clone 29 is an R-MuLV mutant, cloned by limiting dilution of a wild-type virus stock into Balb-3T3 fibroblast cells. Like the R7 clone 1, this mutant encodes an env precursor molecule that is slightly smaller in apparent Mr than the wild-type glycoprotein. This spontaneous mutant encodes a gp70-related protein with an apparent Mr of 88,000 (gPr88$^{\underline{env}}$) and gag polyproteins similar to those encoded by wild-type MuLV (Fig. 9, Frame A, lanes 3 and 4). Although electrophoretically indistinguishable from the MuLV-encoded gag polyproteins, the gag polyproteins encoded by the R Balb clone 29 mutant do not appear to become processed like their wild-type counterparts to form the virion core molecule, p30 (Fig. 9, Frame A, lanes 2 and 4). Pulse-chase analysis using L-[^{35}S] methionine shows that gPr88$^{\underline{env}}$ is rapidly labeled, suggesting that it is primary translation product rather than a derivative of a larger protein (C. Machida and D. Kabat, unpublished observations). Although differences in the molecular weights of the wild-type and mutant env precursors are not easily

Figure 9. Electrophoretic analysis of viral-encoded proteins found in the R-MuLV and R Balb clone 29-infected cell lines.

R-MuLV infected (lanes 1 and 2) and R Balb clone 29 infected (lanes 3, 4, and 5) cell lines were pulse-labeled by incubation with 50 μCi of L-[^{35}S]methionine for 2 h at 37°C. The R Balb clone 29 infected cell line was additionally labeled with 100 μCi of D-[^{3}H]glucosamine for 6 h (lane 6). The cells were subsequently lysed and the viral proteins in the lysates were immunoprecipitated with monospecific antiserum recognizing p30^{gag} (lanes 1 and 3) or gp70^{env} (lanes 2, 4, and 6) or with monoclonal antiserum to p15E (lane 5). Immunoprecipitated proteins were electrophoresed in 10-20% polyacrylamide gels containing 0.1% SDS. Panel B represents a longer exposure of the fluorogram displayed in panel A.


A
1
2
3
4
5
6
gPr90env
gp70env
p30gag
p15E
gPr88env
Pr65gag
p20E
B
1
2
3
4
5
6
gPr90env
gp70env
p30gag
p15E
gPr88env
mutant gp70
p20E

distinguished in Fig. 9, other careful electrophoretic comparisons between these two molecules verifies that the mutant glycoprotein has a slightly smaller apparent Mr. Furthermore, studies using the glycosylation inhibitor tunicamycin reveal that the polypeptide chains encoded by the mutant env gene is smaller (Mr 68,000) than the env polypeptide chains encoded by the wild-type virus (Mr 70,000) (C. Machida and D. Kabat, unpublished observations).

Monoclonal antiserum recognizing the R-MuLV p15E determinant precipitated two components from L-[^{35}S]methionine labeled R Balb clone 29-infected cells (Fig. 9, Frame A, lane 5). One component appears to be the well-characterized p15E molecule (15,43). The second component has a slightly larger apparent Mr of 20,000 (p20E). In addition, labeling the R Balb clone 29-infected cell line with D-[^{3}H]glucosamine provided evidence that gPr88$^{\underline{env}}$ is processed to form a glycosylated molecule with an apparent Mr of 70,000 (Fig. 9, Frame B, lane 6), presumably the mutant virus correlate to R-MuLV gp70. Like the processed molecules encoded by the R7 clone 1 virus, the mutant gp70 of the R Balb clone 29 is labeled in a higher proportion with D-[^{3}H]glucosamine than with L-[^{35}S]methionine (Fig. 9, Frame B, lanes 4 and 6), suggesting it may have more extensively processed oligosaccharides than gPr88$^{\underline{env}}$.

Consistent with the idea that the mutant gp70 molecule has been processed through the Golgi apparatus, this glycoprotein occurs in the cellular plasma membranes as demonstrated by lactoperoxidase-catalyzed surface iodination with [^{125}I]iodine (C. Machida and D. Kabat, unpublished observations). Only an extreme over-exposure of the autoradiogram will reveal the iodination of this surface glycoprotein.

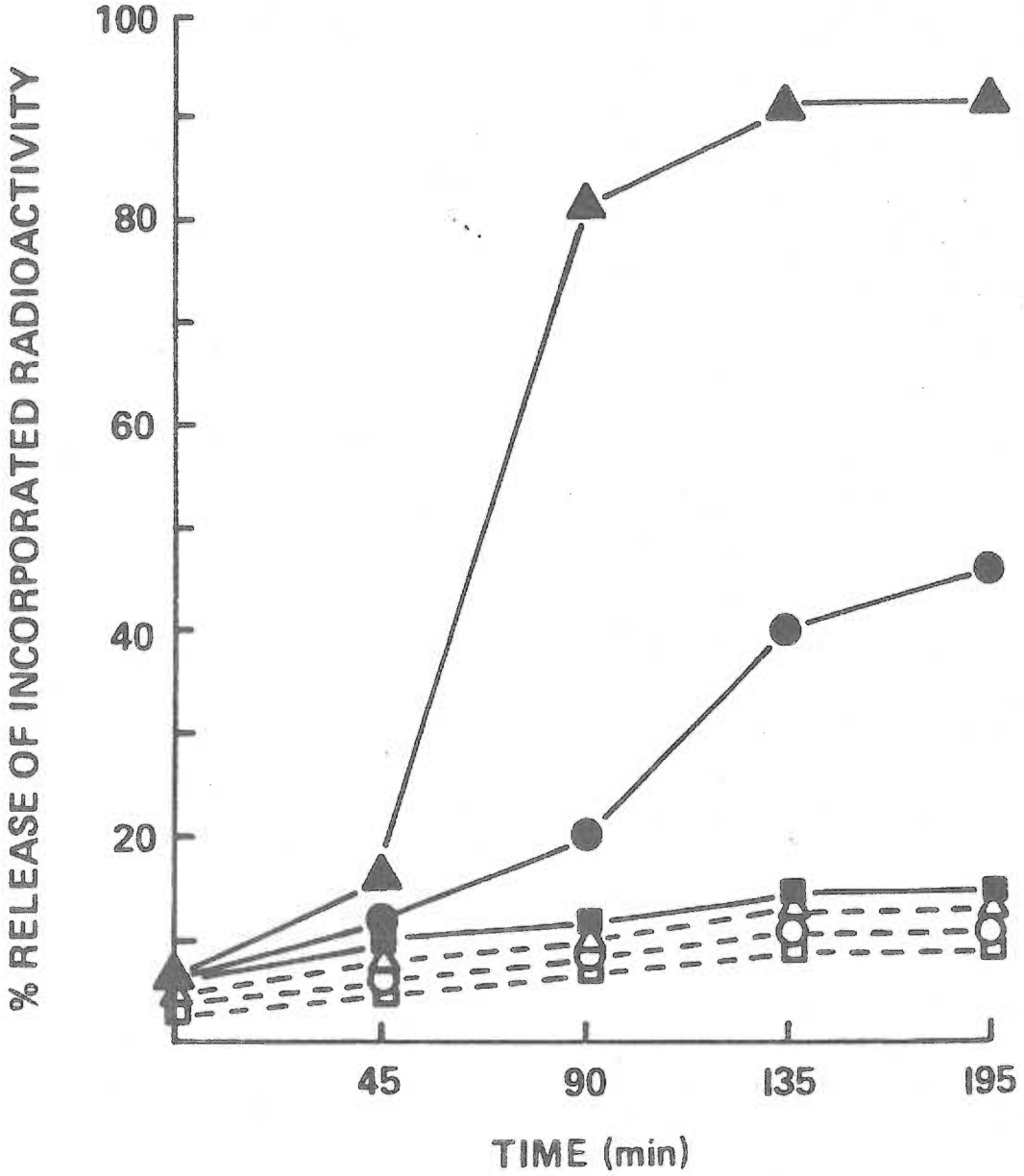
This suggests that either very few mutant gp70 molecules exist on the cellular plasma membranes or that the tyrosine residues of the mutant glycoprotein are sterically hindered from enzyme-catalyzed iodination. The presence of env-related molecules on the surfaces of R Balb clone 29 infected cells was substantiated by complement-dependent cytolysis using antiserum recognizing R-MuLV gp70 (Fig. 10). Lower levels of cytolysis were observed for the R Balb clone 29-infected cell line than for wild-type virus infected cells. This observation is consistent with the possibility that the mutant gp70 molecules encoded by the R Balb clone 29 are expressed at very low frequencies on the plasma membranes of infected cells. In addition, this mutant virus-infected cell line could be superinfected with wild-type MuLV (C. Machida and D. Kabat, unpublished observations). Presumably, the mutant gp70 molecules on the cell surface are unable to effectively block the viral receptors required for causing interference to superinfection.

2. The R7 Clone 61 MuLV Mutant

The R7 clone 61 is an R-MuLV mutant cloned in NIH-3T3 fibroblasts during the same virus cloning experiment that resulted in the isolation of the R7 clone 1. This spontaneous mutant, like the R7 clone 1 and R Balb clone 29, contains an abnormal env gene that encodes a molecule with an apparent Mr of 82,000 ($gPr82^{env}$). The ability of this molecule to become labeled with D-[^{3}H]glucosamine provides evidence that it is a glycoprotein. Furthermore, pulse-chase analyses using L-[^{35}S]methionine verifies that it is a primary translation product analogous to the $gPr90^{env}$ precursor of wild-type R-MuLV. This molecule does not appear to undergo further env processing and presumably becomes trapped in the rough endoplasmic reticulum. No env gene product has

Figure 10. Analyses of surface antigens on R Balb clone 29- and R7 clone 61-infected cells by antibody- and complement-dependent cytolysis.

Virus-infected cell cultures were labeled with L-[^{35}S]methionine for 2 h at 37°C and subsequently treated with specific antibody and rabbit complement. TCA-precipitable radioactivity released from the cell lines were measured on 50 µl aliquots of the culture medium and expressed as a percentage of the total amount of incorporated radioactivity that could be recovered by adding 0.1% SDS. R-MuLV-infected (Δ), R Balb clone 29-infected (○), and R7 clone 61-infected (□) cell cultures were treated with rabbit complement and either normal goat serum (▲, ○, □) or goat antiserum recognizing F-MuLV gp70$^{\underline{env}}$ (▲, ●, ■).


% RELEASE OF INCORPORATED RADIOACTIVITY
100
80
60
40
20
45
90
135
195
TIME (min)

been detected on the plasma membranes of R7 clone 61-infected cells by the antibody- and complement-dependent cytotoxicity procedure (Fig. 10).

D. Discussion

1. The Membrane Glycoprotein Processing Defects of the R7 Clone 1 Mutant

Our results show that the R7 clone 1 MuLV mutant encodes a glycosylated env precursor molecule, gPr80^{env}, analogous to the wild-type gPr90^{env}. Both of these glycoproteins contain p15E regions of similar size which can be removed by partial proteolysis during processing (Fig. 6). In addition, the wild-type and mutant env genes both generate products which appear to transfer via the Golgi apparatus to the cell surface (Fig. 5) and subsequently into virions (Fig. 7). A major difference between the wild-type and mutant env gene products is that the mutant gPr80^{env} molecule is inefficiently cleaved and can reach the cell surface without proteolytic removal of its p15E region, whereas gPr90^{env} is absent from the cellular plasma membranes.

There are several other differences between the mutant and wild-type encoded env glycoproteins. First, the mutant gPr80^{env} glycoprotein contains a smaller polypeptide chain (Fig. 2), suggesting that it may be encoded by a partially deleted env gene. Its smaller size is probably not caused by premature polypeptide chain termination because gPr80^{env} contains the p15E region which occurs at the carboxyl terminus of gPr90^{env}. Although, the highly processed gp68 and gp85 derivatives of gPr80^{env} both reach the cell surface, only gp85 is incorporated to a significant extent into infectious virions (Fig. 7). Furthermore, the mutant forms of the glycoprotein on the cell surface are apparently ineffective in establishing interference to

superinfection by wild-type ecotropic MuLV. Thus, structural abnormalities of env gene products can differentially affect the various parameters of their intracellular transport and function.

The occurrence of an uncleaved env gene product on the cell surface, although abnormal for ecotropic MuLV, has been reported previously for dual tropic mink cell focus inducing (MCF) MuLV (44-46). In that case, it was proposed that the resistance of the glycoprotein to cleavage was caused by its hybrid structure. Our results indicate that mutant forms of ecotropic MuLV can also have this phenotype.

2. A Comparison of the env Gene Mutations in the 3 R-MuLV Mutants: R7 Clone 1, R Balb Clone 29, and R7 Clone 61

In the preceding sections, three R-MuLV env gene mutants have been identified and characterized. Each mutant encodes an env precursor molecule with an apparent Mr that is smaller than the wild-type R-MuLV counterpart, $gPr90^{env}$. Studies using the glycosylation inhibitor tunicamycin reveal that both the R7 clone 1 and R Balb clone 29 envelope precursor molecules contain abnormally small polypeptide chains. This evidence suggests that these two mutant glycoproteins probably result from partially deleted env genes and are not due to abnormal glycosylation. Although the possibility exists that the smaller polypeptide chain lengths could be attributed to premature termination of env gene translation, this alternative appears highly unlikely. In the case of the R7 clone 1 mutant, the apparent Mr difference between the cleaved and uncleaved forms of the processed env glycoprotein approximates the 15,000 apparent Mr of p15E. This implies that nearly the entire coding region for the p15E molecule exists in the 3' end of R7 clone 1 env gene and that the presumptive missing genetic information

was deleted from an interior region of the gene. In addition, the R Balb clone 29 mutant encodes a p15E molecule which co-migrates electrophoretically with the wild-type counterpart. Both the wild-type and mutant p15E molecules are observed in an area of the gel where small apparent Mr differences can be readily detected. Hence, although these results are not totally conclusive, the envelope precursor molecules of the R7 clone 1 and R Balb clone 29 mutant viruses probably result from partially deleted env genes.

No evidence exists that identifies the defect in the R7 clone 61 env gene. Treatment of the R7 clone 61-infected cell line with the glycosylation inhibitor tunicamycin have failed to identify the nonglycosylated polypeptide core of gPr82^{env}. The polypeptide chain of R-MuLV gPr90^{env} is readily labeled with L-[^{35}S]methionine (Fig. 2) and is relatively resistant to degradation. Hence, the inability of the mutant gPr82^{env} core to be detected under identical conditions implies that it is an unstable molecule and presumably structurally different from the wild-type polypeptide chain of the env precursor molecule.

3. The "Signal" Model of Membrane Glycoprotein Transport

Data obtained from our laboratory have indicated that the processing of gPr90^{env} via the Golgi apparatus to the cell surface is very slow compared to the transfer and processing of gp93^{gag} or to other plasma membrane glycoproteins which have been analyzed previously by others (21,64,74,88,139,170). Furthermore, this slow step in envelope glycoprotein processing appears to precede or coincide with partial proteolysis and apparently occurs in the rough endoplasmic reticulum. In addition, gPr90^{env} molecules which have accumulated in the precursor pool transfer past this kinetic barrier in an apparently random fashion

rather than in the cohort order of their synthesis. Since gp93$^{\underline{gag}}$ and other glycoproteins are transferred more rapidly, Fitting and Kabat have proposed that there must be some structural feature or "signal" of newly-made membrane glycoproteins which is necessary for export from the rough endoplasmic reticulum. A simple model consistent with these observations is that intracellular transport requires binding to molecule(s) which interact with different glycoproteins with distinct affinities. Thus, some glycoproteins (e.g., gp93$^{\underline{gag}}$ or G of vesicular stomatitis virus) which bind strongly would be processed rapidly as an apparent cohort formed during a brief interval, whereas weakly binding glycoproteins would accumulate as a large pool in an organelle and would be transferred over a prolonged period in a stochastic rather than cohort fashion (48).

In the case of gPr90$^{\underline{env}}$, it is not possible to kinetically distinguish the proteolytic cleavage of this molecule from its transfer past the kinetic barrier in the rough endoplasmic reticulum (48). Furthermore, since neither of these two events occurs without the other, we suggested that they were causally related. Consequently, it is inferred that proteolytic cleavage of gPr90$^{\underline{env}}$ in a zymogen-like manner might be a prerequisite for its passage through the processing barrier. Alternatively, if the protease were located distal to the barrier, passage through the barrier could be a prerequisite for cleavage. In the latter case, cleavage would be an incidental consequence of passage through the barrier but would not necessarily be essential for subsequent steps of intracellular transport. It should be emphasized that either of these explanations for gPr90$^{\underline{env}}$ proteolytic cleavage

would be compatible with the basic signal-mediated transport model which has been proposed (48).

4. Application of the "Signal" Model to the Processing and Transport of the Mutant env Precursor Molecules

The results described here are consistent with the signal-mediated transport model described in the previous section. Like wild-type gPr90^{env}, the mutant R7 clone 1 encoded gPr80^{env} is processed very slowly rather than in a rapid cohort-like manner (Fig. 4). However, the R7 clone 1 mutant glycoprotein is cleaved inefficiently, suggesting that partial proteolysis cannot be a prerequisite for its passage to the cell surface. Nevertheless, these results do not exclude the possibility that proteolytic cleavage could be essential for the intracellular transport of the wild-type glycoprotein. The mutant gPr80^{env} glycoprotein might fold abnormally and therefore its carrier binding site (i.e., its "signal") might be at least partly accessible to the export system without proteolysis. On the contrary, the wild-type gPr90^{env} transport signal might become accessible only after proteolysis. Furthermore, our failure to detect cleaved derivatives of gPr80^{env} which have not been processed in the Golgi apparatus supports the idea that cleavage is associated with glycoprotein export from the rough endoplasmic reticulum. Thus, our studies imply that intracellular transport of the mutant glycoprotein is associated with but not dependent upon partial proteolysis.

Alterations in the structural configurations of the env precursor glycoproteins encoded by two other R-MuLV mutants, R Balb clone 29 and R7 clone 61, have been observed to limit or even block transport of these molecules to the cell surface. In the case of the R

Balb clone 29, the partial proteolysis of the mutant env precursor generates a gp70-like protein which contains an accessible but inefficient export "signal". This results in only marginal transport of the cleaved glycoprotein to the cell surface. The abnormal folding of this mutant precursor glycoprotein, in addition, appears to block the accessibility of the p15E determinant to antibody binding. The altered structure may also expose an additional site on the precursor molecule that is partially proteolyzed. Two cleaved molecules with p15E determinants are found in the R Balb clone 29-infected cell line, the p15E molecule itself and a second protein with an apparent Mr of 20,000 (p20E). On the other hand, the structural folding of the R7 clone 61 env gene product is presumed to completely cover its "signal" site necessary for export of this molecule to the cell surface. In this case, the env molecules are neither partially proteolyzed nor transported to the cellular plasma membranes. Alternatively, this gene product may resist transport as a secondary consequence of another structural deficiency such as insolubility or aggregation.

It is interesting to consider the possibility that the hybrid-type glycoproteins encoded by dual tropic MuLVs might also fold abnormally and thereby be capable of transfer without cleavage. Such considerations could also explain how specific covalent modifications (e.g., glycosylation or acylation) which can alter the folding of proteins might be essential for the intracellular transport of certain glycoproteins but not for others. For example, the intracellular transport of closely related immunoglobulins are affected differently by inhibition of glycosylation (61). The signal model for intracellular

transport of membrane and secretory glycoproteins offers a likely explanation for these diverse observations.

IV. Env Gene Mutants of Rauscher Spleen-Focus Forming Virus (R-SFFV): Effects on Leukemogenicity

A. General Introduction

The Rauscher spleen focus forming virus (R-SFFV) is a genetic recombinant containing portions of the replication-competent murine leukemia virus (MuLV) genome and sequences highly related to the env gene of murine xenotropic and mink cell focus (MCF)-inducing type C viruses (146,182). The R-SFFV env gene encodes a glycoprotein with an apparent Mr of 54,000 (gp54) that is immunologically and structurally related to the gp70 envelope glycoproteins of recombinant dual-tropic MCF viruses (146,182). The fact that gp54 has been consistently detected in all cell lines infected with R-SFFV has led to the proposal that it might be a pathogenic factor in causing erythroleukemia (146,182). The isolation and analysis of weakly leukemogenic or nonleukemogenic SFFV mutants would be useful for identifying and studying the mechanism of viral leukemogenesis. In this dissertation, the gene products of putative R-SFFV mutants are described and the role of these products in leukemogenicity will be discussed.

B. Isolation of Viral Mutants From Stocks of the Rauscher Virus Complex

Virus rescued from the R-SFFV nonproducer cell line, RV NIH clone 6 was injected into NIH/Swiss mice and subsequently amplified by passaging cell-free extracts of spleens obtained from these infected mice into fresh animals. Sc-1 cells were infected with virus generated from this in vivo amplification and the virus subsequently released from this infected cell line was cloned by limiting dilution into NIH-3T3

fibroblasts (Section II.D.). Each clonal cell line was labeled with L-[^{35}S]methionine, subsequently lysed with detergents, and the lysates then screened for the presence of molecules reactive to antiserum prepared against the MuLV gp70$^{\underline{env}}$ glycoprotein. Fig. 11 shows 28 out of approximately 100 clonal cell lines analyzed by this screening procedure. Of the 28 clones displayed in Fig. 11, 6 appear to contain replication-competent MuLV-encoded gPr90$^{\underline{env}}$ and its processed derivatives, gp70$^{\underline{env}}$ and p15E (panel A, lanes 4, 6 and 12; panel B, lanes 5, 13 and 14). Of the remaining 22 clonal cell lines, 4 appear to contain single env gene products that are immunologically related to the R-MuLV gp70$^{\underline{env}}$ molecule. One cell line contains the well-characterized R-SFFV encoded gp54 molecule (Panel A, lane 14) and has been designated PRV clone 3-32. The remaining 3 cell lines contain env-related molecules with apparent Mr's of 45,000 (gp45; Panel B, lane 1), 52,000 (gp52; Panel A, lane 8), and 60,000 (p60; Panel B, lane 8) and have been designated PRV clone 4-3, PRV clone 3-25, and PRV clone 4-10 respectively. The remaining 18 clonal cell lines displayed in Fig. 11 are apparently uninfected.

Evidence that the abnormal env gene products expressed in the three PRV clones were encoded by viral and not cellular genes was obtained by superinfecting these cell lines with R-MuLV and examining the superinfected cells for env components (Fig. 12). As expected, the PRV clone 3-32 cell line, which contains wild-type R-SFFV, can be successfully superinfected with R-MuLV. This superinfected cell line (fig. 12, lane 2) expressed R-MuLV gPr90$^{\underline{env}}$ and processed derivatives gp70$^{\underline{env}}$ and p15E. In addition, when culture medium from this super-

Figure 11. Electrophoretic analysis of gp70-related proteins in Rauscher virus infected clones.

Rauscher virus complex, amplified *in vivo* by passaging cell-free extracts of infected mouse spleens into fresh NIH/Swiss mice (Section II.N.) was cloned at a low multiplicity of infection into NIH-3T3 cells. The resulting cell clones were pulse-labeled with L-[^{35}S]methionine, subsequently lysed with detergents, and the lysates then screened for the presence of molecules reactive to antiserum prepared against the MuLV gp70^{env} glycoprotein. The following four clones were retained for subsequent analyses: 1) PRV clone 3-32 (Panel A, lane 14; gp54), 2) PRV clone 3-25 (Panel A, lane 8; gp52), 3) PRV clone 4-3 (Panel B, lane 1; gp45), and 4) PRV clone 4-10 (Panel B, lane 8; p60).

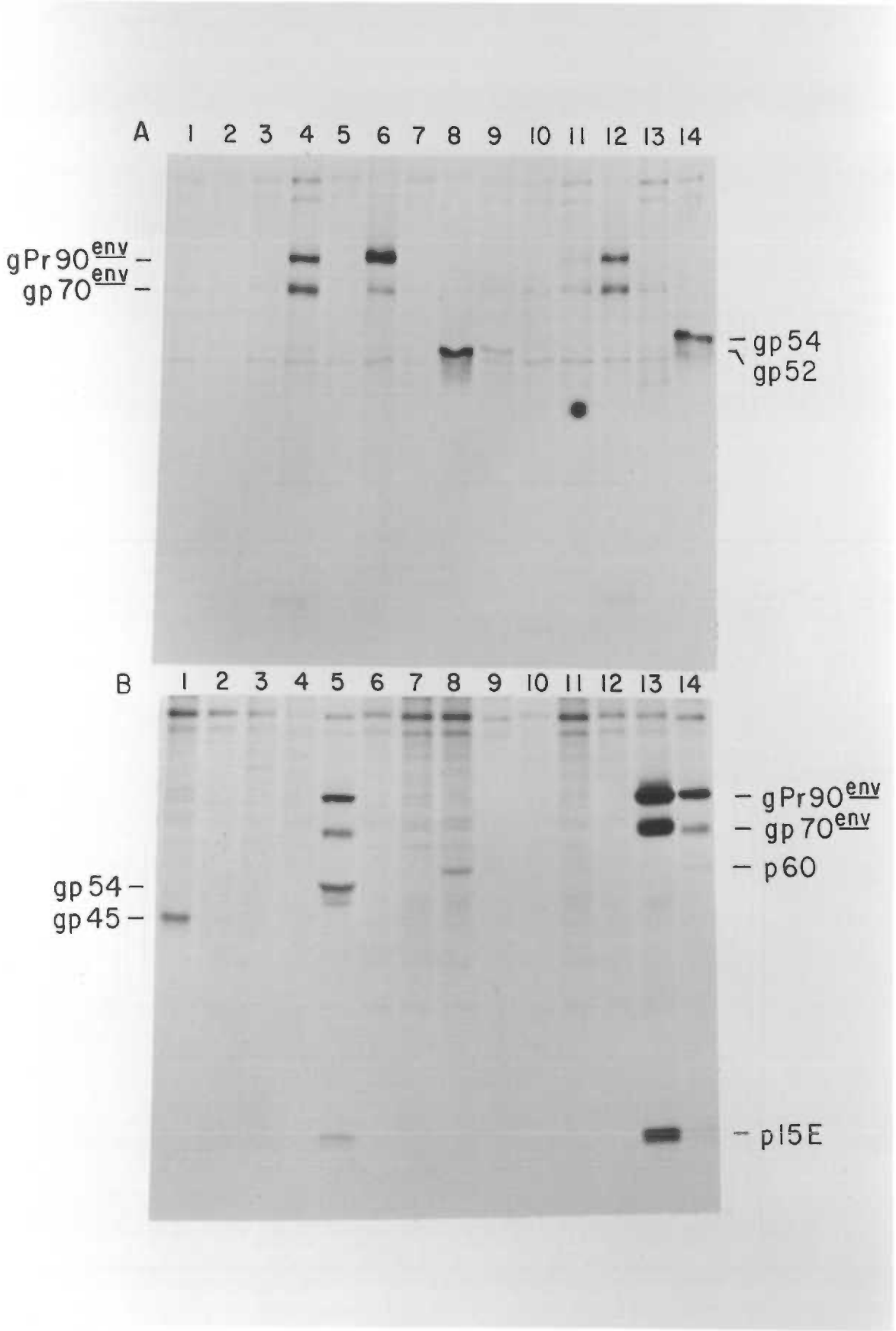
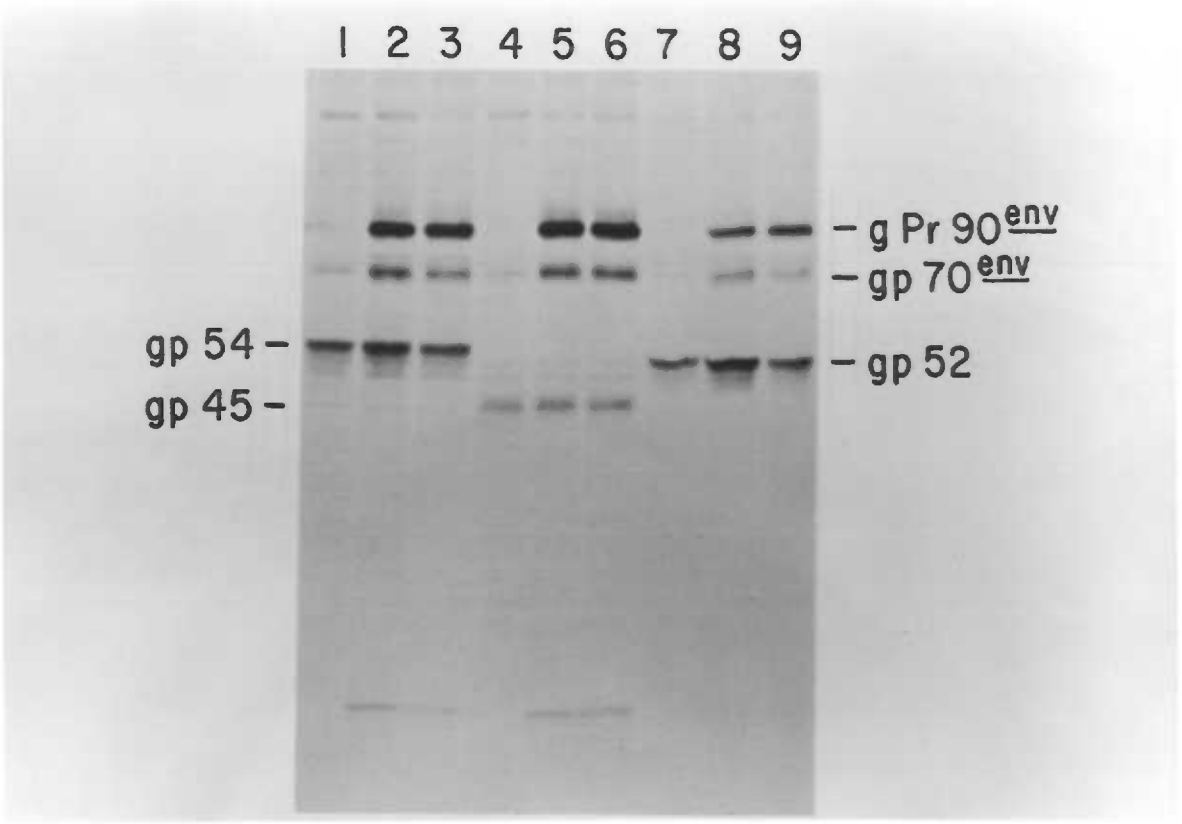

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
gPr90env
gp70env
gp54
gp52
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
gp54
gp45
gPr90env
gp70env
p60
p15E

Figure 12. Electrophoretic analysis of env glycoprotein processing in Rauscher virus-infected clones superinfected with R-MuLV.

The PRV clone 3-32 (lane 1), PRV clone 4-3 (lane 4), and PRV clone 3-25 (lane 7) cell lines were superinfected with R-MuLV (lanes 2, 5, and 8 respectively) as described in Section II.C. Culture media obtained from each of these superinfected cultures were used to infect uninfected NIH-3T3 cells (transfer of culture fluids from PRV clone 3-32, PRV clone 4-3, and PRV clone 3-25 cell lines superinfected with R-MuLV correspond to lanes 3, 6, and 9 respectively). All of the above cell lines were labeled by incubation with L-[^{35}S]methionine, subsequently lysed and the viral proteins immunoprecipitated with antiserum recognizing MuLV gp70^{env}. The immunoprecipitates were then subjected to SDS-PAGE as described in Section II.G.


1 2 3 4 5 6 7 8 9
– g Pr 90env
– gp 70env
gp 54 –
– gp 52
gp 45 –

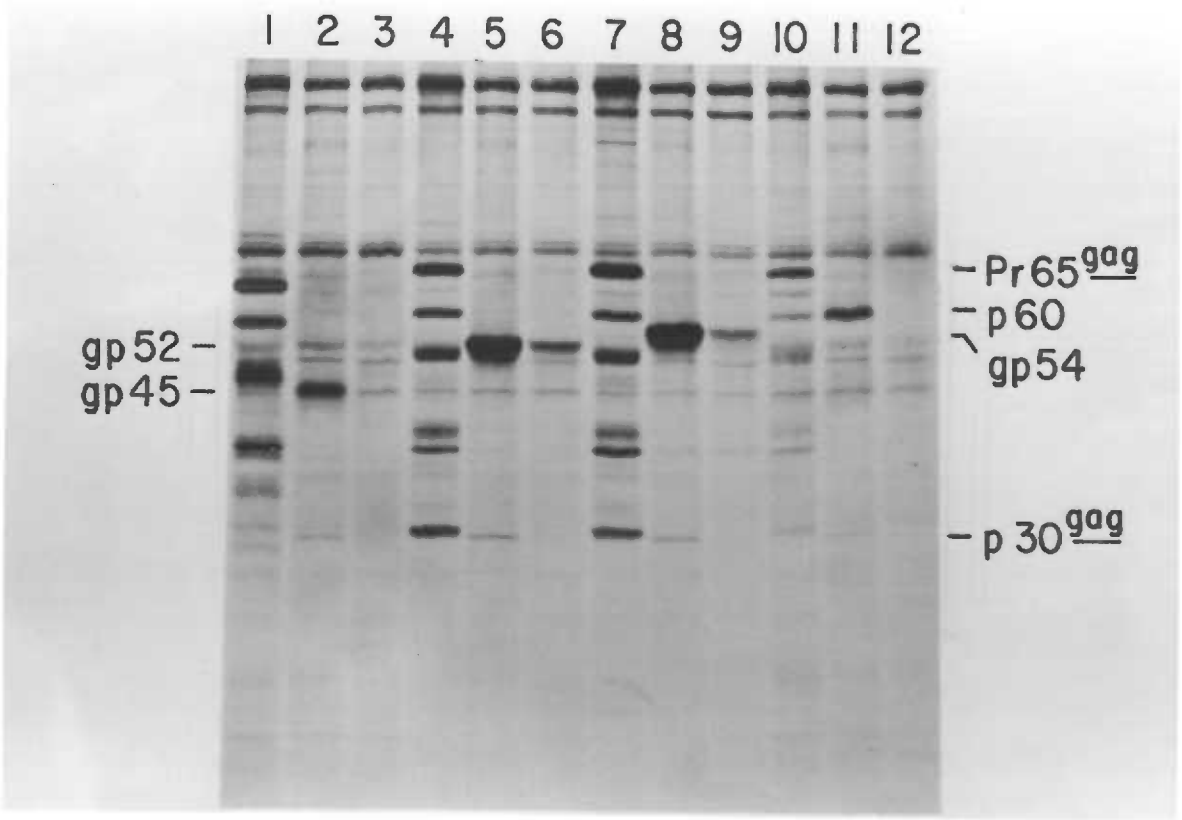
infected cell line is used to infect NIH-3T3 cells (Fig. 12, lane 3), both the R-MuLV and R-SFFV components are expressed in the newly-infected cell line. The other PRV clones, 4-3, 3-25, and 4-10, also express gPr90$^{\underline{env}}$ when superinfected with R-MuLV (Fig. 12, lanes 5 and 8; also unpublished observations). In addition, when culture medium from these superinfected cell lines are used to infect NIH-3T3 cells, the abnormal env phenotypes observed in the original PRV clones could be transferred with virus into the newly infected cell lines (Fig. 12, lanes 6 and 9; also unpublished observations). These observations provide evidence that the abnormal env products expressed in PRV clones 4-3, 3-25, and 4-10 are the result of viral and not of cellular gene mutations.

C. Viral Mutants Contain Defective gag and env Genes

As shown in Fig. 13, the wild-type R-SFFV contained in the PRV clone 3-32 cell line expresses an env gene component with an apparent Mr of 54,000 (gp54) (lane 7) and gag-related polyproteins which appear to be electrophoretically indistinguishable to those encoded by wild-type MuLV (lanes 8 and 9; also unpublished observations). Consistent with previous studies (146), the gp54 molecule can be precipitated either with monospecific antiserum prepared against MuLV gp70$^{\underline{env}}$ or with a rat antiserum that recognizes a determinant found in the gp70s of the dual tropic MCF and HIX viruses but not in the gp70s of ecotropic MuLV (Fig. 13, lane 8 and 9). The mutant virus contained in the PRV clone 3-25 cell line expresses an Mr 52,000 env molecule (gp52) that can be precipitated either with the monospecific gp70$^{\underline{env}}$ antiserum or with the rat antiserum that recognizes the dual tropic MCF gp70 determinant (Fig. 13, lanes 5 and 6). The mutant viruses contained in the PRV clone 4-3

Figure 13. Electrophoretic analysis of viral-encoded proteins found in the Rauscher virus-infected clones.

PRV clone 4-3 (lanes 1-3), PRV clone 3-25 (lanes 4-6), PRV clone 3-32 (lanes 7-9), and PRV clone 4-10 (lanes 10-12) cell lines were pulse-labeled by incubation with 100 μCi of L-[^{35}S]methionine for 2 h at 37°C. The cells were subsequently lysed and the viral-encoded proteins in the lysates were immunoprecipitated with monospecific serum to p30$^{\underline{gag}}$ (lanes 1, 4, 7, and 10) or gp70$^{\underline{env}}$ (lanes 2, 5, 8, and 11) proteins or with a rat serum that recognizes SFFV-specific determinants (lanes 3, 6, 9, and 12). Immunoprecipitated proteins were electrophoresed in 10-20% polyacrylamide gels containing 0.1% SDS.


1 2 3 4 5 6 7 8 9 10 11 12
gp52
gp45
Pr65gag
p60
gp54
p30gag

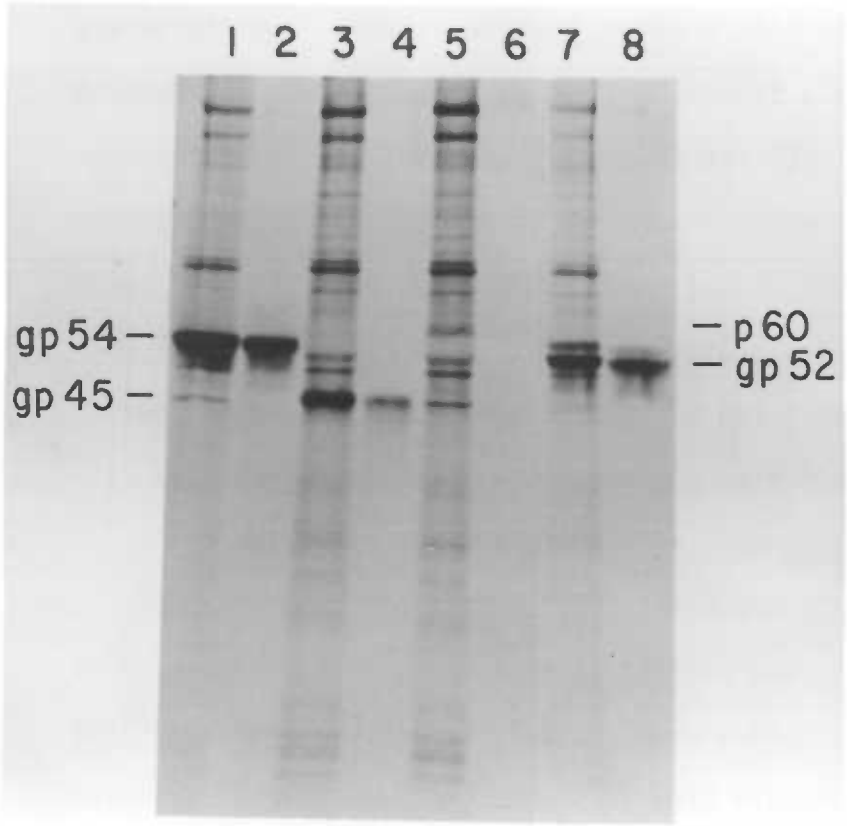
and 4-10 cell lines express env-related molecules with apparent Mr's of 45,000 (gp45; Fig. 13, lane 2) and 60,000 (p60; Fig. 13, lane 11) that are both readily precipitated with the monospecific gp70^{env} antiserum. Although the gp45 and p60 molecules do not appear to be precipitated with the rat antiserum in Fig. 13 (lanes 3 and 12) extensive over-exposure of the fluorogram reveals the weak precipitation of the former but not the latter molecule by this antiserum. In addition, the mutant viruses in both the PRV clone 3-25 and 4-10 cell lines contain gag-related polyproteins which appear to be electrophoretically indistinguishable from those encoded by the wild-type R-SFFV (Fig. 13, lanes 4, 7 and 10). On the other hand, the gag components encoded by mutant virus contained in the PRV clone 4-3 cell line (Fig. 13, lane 1) are electrophoretically different from those found in the R-SFFV infected PRV clone 3-32 (Fig. 13, lane 7). The evidence presented in this section indicates that the mutant viruses contained in the PRV clone 3-25 and 4-10 cell lines contain single mutations in their env genes, while the virus contained in PRV clone 4-3 contains mutations in both its gag and env genes.

D. Mutant env Molecules in the PRV clone 4-3 and 3-25 Cell Lines Are Glycosylated

Consistent with previous studies (146), the R-SFFV-encoded gp54 molecule is readily labeled with D-[^{3}H]glucosamine (Fig. 14, lane 2), providing evidence that the major env component is a glycoprotein. When the PRV clones expressing electrophoretically-abnormal env gene products are similarly incubated with radioactive sugar, the gp45 and gp52 molecules encoded by two of the mutant R-SFFVs are also readily labeled (Fig. 14, lane 4 and 8). The p60 component encoded by mutant virus in

Figure 14. Electrophoretic analysis of glycosylated components found in the Rauscher virus-infected clones.

PRV clone 3-32, PRV clone 4-3, PRV clone 4-10, and PRV clone 3 were labeled with either 100 μCi of L-[^{35}S]methionine (lanes 1, 3, 5, and 7 respectively) for 2 h or 100 μCi of D-[^{3}H]glucosamine (lanes 2, 4, 6, and 8 respectively) for 6 h. PRV clone 3 contains both the gp54 molecule encoded by wild-type R-SFFV and the gp52 component contained in the PRV clone 3-25 cell line. All of the above cells were subsequently lysed and the virus-encoded proteins in the lysates were immunoprecipitated with monospecific antiserum recognizing gp70$^{\underline{env}}$.


1 2 3 4 5 6 7 8
gp54 –
gp45 –
– p60
– gp52

the PRV clone 4-10 cell line is not readily labeled with D-[^{3}H]glucosamine (Fig. 14, lane 6).

E. Mutant env Molecules Have Different Sized Polypeptide Chains

Addition of asn-linked oligosaccharides to glycoproteins can be blocked by addition of the glycosylation inhibitor tunicamycin (80,175). As shown in Fig. 15, cells preincubated with tunicamycin synthesize env-related polypeptide chains which are smaller than the complete glycoproteins. Consistent with previous studies (36,66), the polypeptide chain encoded by the env gene of wild-type R-SFFV has an apparent Mr of 44,000 (Fig. 15, land 2 and 8). The env-related polypeptide chains encoded by the mutant viruses contained in the PRV clone 4-3 cell line (Mr 38,000) (lane 4) and in the PRV clone 3-25 cell line (Mr 44,000) (lane 6) are either smaller or nearly identical in size to the polypeptide chain encoded by the env gene of wild-type R-SFFV.

F. Mutant gp52 and Wild-type gp54 are Structurally Related Molecules and are Encoded by Identically Sized env Genes

To determine the structural relatedness of the mutant gp52 molecule to wild-type R-SFFV encoded gp54, tryptic peptide analyses of L-[^{35}S]methionine-labeled proteins were conducted. As shown in Fig. 16, wild-type gp54 (frame A) and mutant gp52 (frame B) molecules have nearly identical tryptic peptide maps. The gp52 molecule contains an additional peptide (see arrow) that is not observed in the wild-type R-SFFV encoded gp54 component.

To compare the sizes of the env genes encoding the mutant gp52 and wild-type gp54 molecules, poly (A)-containing RNAs were extracted from the infected cell lines and analyzed by the Northern blot technique. The size of the subgenomic env mRNA of R-SFFV has been previously

Figure 15. Electrophoretic analysis of env-specific proteins in Rauscher virus-infected clones grown in the presence of the glycosylation inhibitor tunicamycin.

PRV clone 3-32 (lanes 2 and 8), PRV clone 4-3 (lane 4), and PRV clone 3-25 (lane 6) cell lines were treated with 25 μg/ml tunicamycin both prior to (3 h) and during labeling (10 min) with 100 μCi of L-[^{35}S]methionine. These cultures, as well as labeled control cultures incubated without tunicamycin (PRV clone 3-32, lanes 1 and 7; PRV clone 4-3, lane 3; PRV clone 3-25, lane 5) were lysed after labeling and the viral proteins in the lysates were immunoprecipitated with anti-gp70^{env} serum.

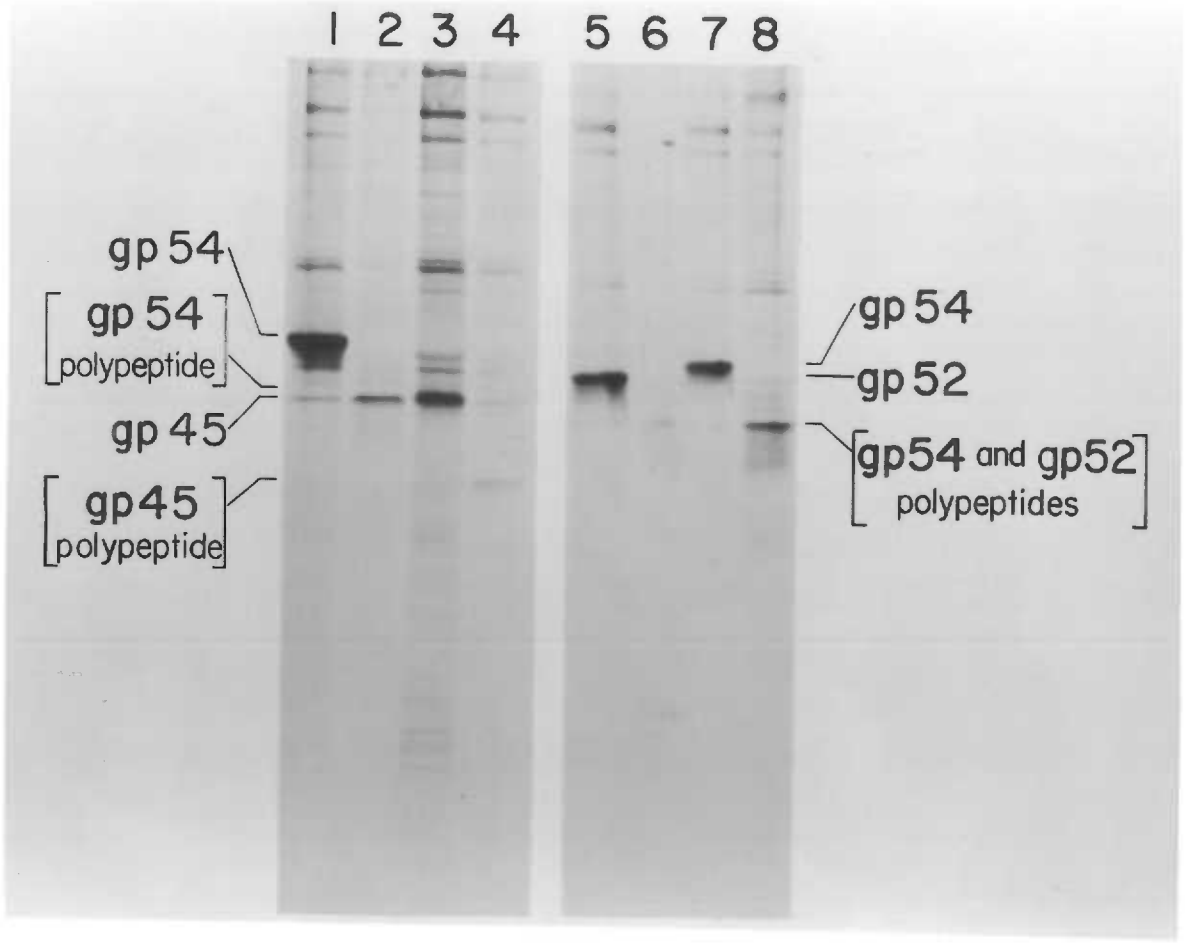
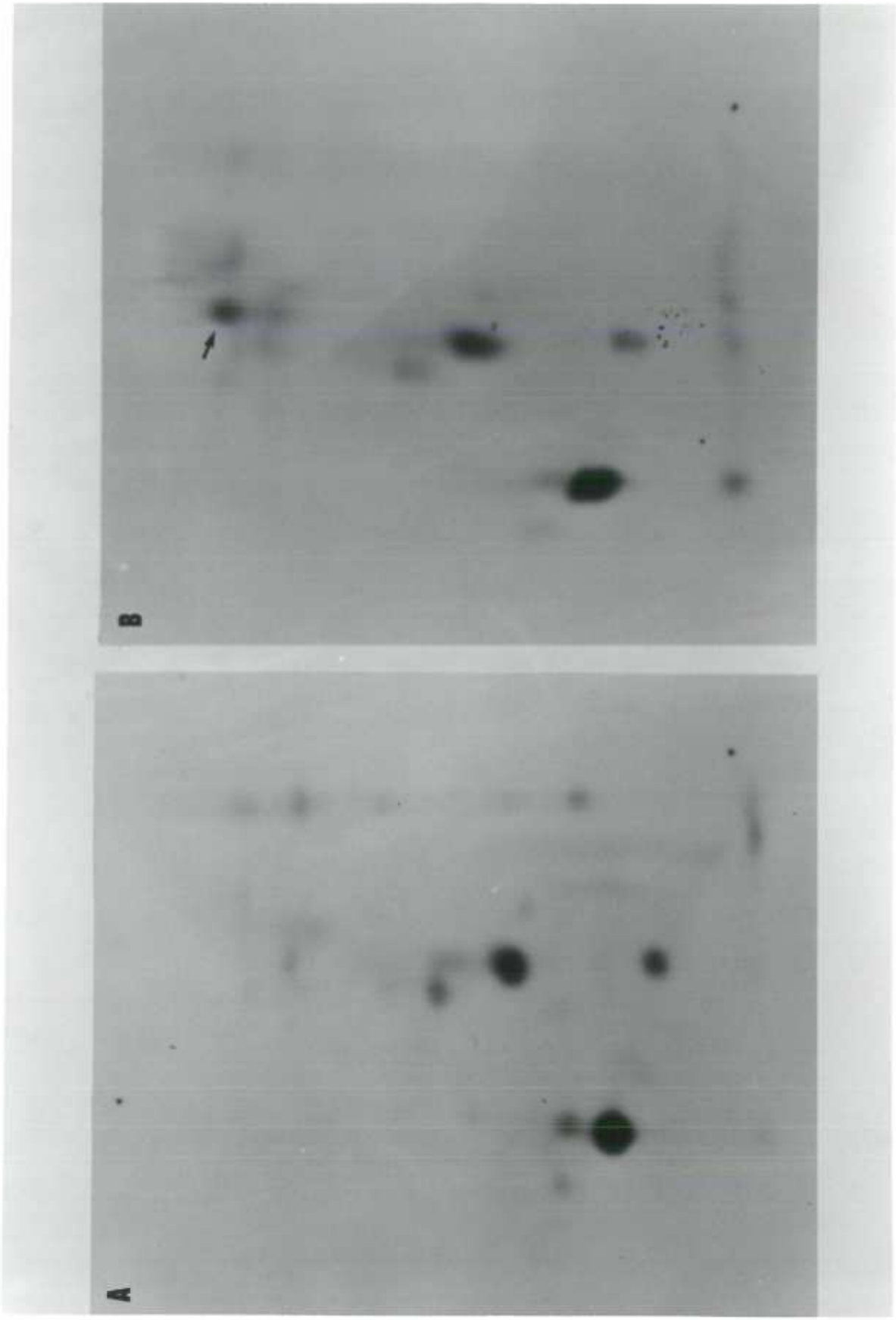

1 2 3 4 5 6 7 8
gp 54
gp 54
polypeptide
gp 45
gp45
polypeptide
gp 54
gp 52
gp54 and gp52
polypeptides

Figure 16. Tryptic peptide analyses of L-[^{35}S]methionine-labeled R-SFFV gp54 and mutant R-SFFV gp52.

L-[^{35}S]methionine-labeled proteins, purified by immunoprecipitation and SDS-PAGE, were digested by incubation of selected gel sections in TPCK-trypsin for 24 h at 37°C. Following performic acid oxidation, these samples were diluted in 2 ml of H_2O, concentrated under vacuum, resuspended in electrophoresis solution[2] (28% formic acid) and spotted on cellulose TLC plates. Electrophoresis was conducted at 150 v for 5 h in the horizontal dimension and chromatography was performed in solution containing isoamyl alcohol:pyridine:ethanol:glacial acetic acid:H_2O (70:70:40:10:60) in the vertical dimension. Detection of L-[^{35}S][2] methionine-labeled peptides was enhanced by spraying TLC plates with 7% diphenyloxazole in ether. Proteins analyzed included (A) wild-type R-SFFV gp54 and (B) mutant R-SFFV gp52 (obtained from the PRV clone 3-25 cell line). The arrow points to a peptide found in gp52 that is not observed in the gp54 molecule. The origin is at the lower right.


B
A

determined as 2.1 kb (R. Bestwick, C. Faust and D. Kabat, unpublished observations). Wild-type R-SFFV and the mutant virus contained in the PRV clone 3-25 cell line both contain identically sized env genes as indicated by coelectrophoresis of their subgenomic env mRNAs (C. Machida, R. Bestwick, C. Faust and D. Kabat, unpublished observations). Both of the above mRNAs hybridized to the SFFV-specific cDNA probe. Hybridization of the SFFV-specific probe to the poly(A)-containing RNAs extracted from the PRV clone 4-3 and 4-10 cell lines was not achieved in this study, possibly due to the low yields of viral RNA recovered from these cell lines.

G. Mutant env Molecules are not Processed to the Plasma Membrane

Previous studies have shown that a small proportion of R-SFFV encoded gp54 (3-5%) occurs on the outer surfaces of infected cells (145,146) while the majority of the gp54 molecules become irreversibly situated in the membranous intracellular organelle(s). Whereas the intracellular form of gp54 contains predominantly neutral oligosaccharides, the cell surface molecules are slightly larger and more heterogeneous in size (apparent Mr, 58,000 to 68,000) and they contain large complex sialylated oligosaccharides (145,146).

Presumably because they contain more carbohydrate and because D-[^{3}H]glucosamine serves as an efficient precursor to sialic acid, the surface molecules are labeled in a higher proportion with D-[^{3}H]glucosamine than with L-[^{35}S]methionine (145). Consequently, the plasma membrane form of gp54 is most readily observed when labeled with radioactive glucosamine. When the PRV clone 4-3 and 3-25 cell lines are incubated with D-[^{3}H]glucosamine, only the gp45 and gp52 molecules are labeled (Fig. 14; also unpublished observations). No additional env

glycoproteins are observed in either of these cell lines, implying that processing and transport of the env components to the cell surface does not occur. Furthermore, gp54-related surface molecules in the PRV clone 4-3 and 3-25 cell lines are not detected by either lactoperoxidase-catalyzed surface iodination with [^{125}I]iodine or by complement-dependent cytotoxicity assays employing anti-gp70^{env} serum (C. Machida and D. Kabat, unpublished observations).

H. Mutant Viruses in the PRV clone 3-25 and 4-3 Cell Lines are Weakly Leukemogenic

Mutant viruses rescued from the PRV clones by superinfection with R-MuLV generally did not induce SFFV-specific disease when injected into NIH/Swiss mice. In order to determine whether the apparent nonleukemogenicity of these mutants were due to insufficient amounts of virus injected or true decreases in leukemogenic potential, virus was amplified in vivo by passage in NIH/Swiss mice (Section II.N). Extracts of pooled, diseased spleens were subsequently injected into fresh animals. Virus samples were amplified sequentially from animal to animal for a total of 3 passages. The extent of SFFV-specific disease for each of the virus samples were observed at each stage of the amplification procedure. As shown in Table 1, amplification of wild-type R-SFFV obtained from the PRV clone 3-32 cell line resulted in a six-fold spleen enlargement and the development of readily-observed macroscopic splenic foci (Fig. 17). As expected, in vivo amplification of R-MuLV under identical conditions did not result in the development of any pathology characteristic of SFFV-induced disease (Table 1, also Fig. 17). When mutant viruses rescued from the PRV clones were subjected to the same amplification procedure, two of the mutant viruses

Table 1

Analysis of Spleen Weights of Mice Infected with Cloned Rauscher Virus at Various Stages of Passaging[a]

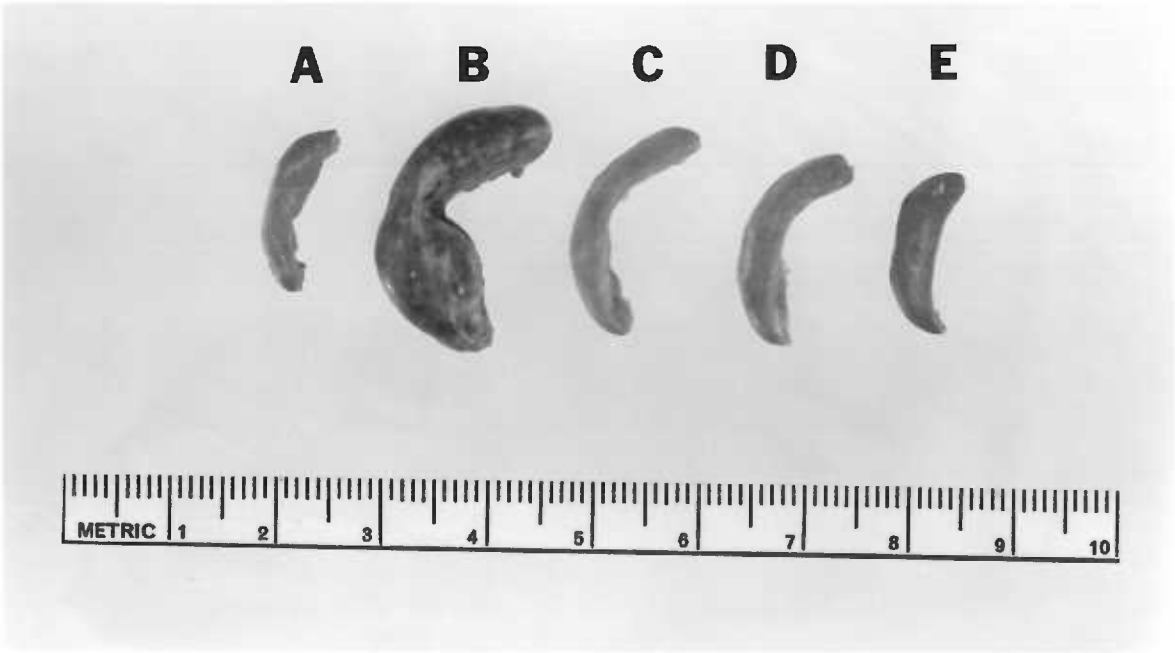
Virus Inoculated	Average Spleen Weight (grams)[b] ± Standard Deviation		
	At Passage 1	At Passage 2	At Passage 3
R-MuLV	0.12 ± 0.02	0.14 ± 0.02	0.13 ± 0.03
Wild-type R-SFFV (PRV clone 3-32)	0.12 ± 0.01	0.47 ± 0.05	0.75 ± 0.07
Wild-type R-SFFV (RV NIH clone 6)	0.13 ± 0.02	0.31 ± 0.08	0.68 ± 0.06
Wild-type R-SFFV (PRV clone 18)	0.14 ± 0.03	0.51 ± 0.10	0.75 ± 0.10
Mutant R-SFFV (PRV clone 3-25)	0.12 ± 0.02	0.21 ± 0.03	0.28 ± 0.05
Mutant R-SFFV (PRV clone 4-3)	0.12 ± 0.02	0.23 ± 0.03	0.24 ± 0.05
Mutant Virus (PRV clone 4-10)	0.14 ± 0.01	0.15 ± 0.02	0.13 ± 0.02

[a] Virus was rescued from R-SFFV-infected cells by superinfection with R-MuLV and subsequently injected into the tail veins of 10 NIH/Swiss mice. Two weeks later, cell-free extracts of pooled spleens obtained from these infected animals were prepared and then injected into fresh mice (passage 2). This procedure was repeated for a 3rd *in vivo* passage.

[b] Number of mice analyzed per sample at each passage: Passage 1: 10 mice; Passage 2: 6 mice; Passage 3: 4 mice.

Figure 17. Spleens of mice injected with rescued virus from Rauscher virus-infected clones.

Shown are the spleens of mice injected with (A) R-MuLV, (B) R-SFFV rescued with R-MuLV from the PRV clone 3-32 cell line, (C) mutant R-SFFV rescued with R-MuLV from the PRV clone 3-25 cell line, (D) mutant R-SFFV rescued with R-MuLV from the PRV clone 4-3 cell line, and (E) mutant virus rescued with R-MuLV from the PRV clone 4-10 cell line. Spleen (B) was obtained after the second in vivo passage. Spleens (A), (C), (D), and (E) were obtained after the third in vivo passage. Histological examination of spleen (C) and (D) confirmed the diagnosis of mild Rauscher disease.


A
B
C
D
E
METRIC
1
2
3
4
5
6
7
8
9
10

appeared to cause SFFV-specific disease to a limited degree in the later passages. Upon sequential passaging, virus from the PRV clone 3-25 and 4-3 cell lines were capable of eliciting slight splenic enlargement and development of macroscopic foci (Table 1, also Fig. 17). Mutant virus obtained from the PRV clone 4-10 cell line apparently does not induce erythroleukemia even after extensive in vivo amplification (Table 1; also Fig. 17). These studies provide evidence that the mutant R-SFFVs obtained from the PRV clone 3-25 and 4-3 cell lines may have decreased leukemogenic potential and implicate wild-type gp54 as a leukemogenic molecule.

I. Discussion

1. Characteristics of R-SFFV Mutants

Using simple cloning and screening procedures, we have isolated several spontaneous viral mutants that encode abnormal gp70-related proteins. The evidence presented in this dissertation indicates that at least two of these are R-SFFV mutants encoding abnormal gp54-related components. The mutant R-SFFV contained in the PRV clone 3-25 cell line encodes an env gene product with an apparent Mr of 52,000 (gp52; Fig. 13) and gag-related polyproteins which appear to be electrophoretically indistinguishable from those encoded by wild-type MuLV (C. Machida and D. Kabat, unpublished observations). Like the wild-type R-SFFV-encoded gp54 molecule, mutant gp52 contains antigenic determinants found in both the gp70s of ecotropic MuLV and in recombinant MCF and HIX viruses (Fig. 13). The wild-type gp54 and mutant gp52 components have identically sized polypeptide chains (Fig. 15) and nearly equivalent tryptic peptide maps (Fig. 16), implying that the mutant molecule results from abnormal glycosylation. This evidence

indicates that the mutant viral env gene in the PRV clone 3-25 cell line contains a point mutation at a region encoding an oligosaccharide attachment site on the gp52 polypeptide. This implication is consistent with evidence obtained from Northern blotting analyses that the wild-type and mutant subgenomic env mRNAs have identical sizes (C. Machida, R. Bestwick, C. Faust and D. Kabat, unpublished observations). Furthermore, studies using NIH/Swiss mice indicates that the mutant virus recovered from the PRV clone 3-25 cell line is weakly leukemogenic.

The R-SFFV mutant contained in the PRV clone 4-3 cell line encodes an env-related glycoprotein with an apparent Mr of 45,000 (gp45; Fig. 13) that is weakly precipitated by a rat serum which reacts specifically with gp54 and with dual tropic MCF gp70s (C. Machida and D. Kabat, unpublished observation). This virus, in addition, does not encode p30^{gag}-related proteins characteristic of wild-type R-SFFV. It seems reasonable to infer from these results that the PRV clone 4-3 cell line contains an R-SFFV mutant with mutations in both its gag and env genes. Studies using the glycosylation inhibitor tunicamycin reveal that the polypeptide chain encoded by the env gene of this mutant virus (Mr 38,000; Fig. 15) is smaller than the polypeptide chain encoded by the env gene of the wild-type R-SFFV (Mr 44,000; Fig. 15). This evidence indicates that the mutant gp45 molecule results from either a deletion or premature termination mutation of the virus' env gene. Interestingly, the mutant virus synthesized in the PRV clone 4-3 cell line is also weakly leukemogenic.

Finally, the PRV clone 4-10 cell line (Fig. 13) contains a viral mutant with an abnormal gp70-related component. This protein has

an apparent Mr of 60,000 (p60) and is viral encoded (C. Machida and D. Kabat; unpublished observations). However, p60 is not precipitated by the rat antiserum that is specific for gp54 and dual tropic MCF gp70s. In addition, the PRV clone 4-10 cell line does not release biologically active SFFV after superinfection with helper R-MuLV. Furthermore, poly(A)-containing RNA extracted from this cell line does not appear to hybridize to a SFFV-specific cDNA probe. On the basis of these preliminary results, we cannot unambiguously identify the nature of the virus which encodes the p60 molecule. It is possible that p60 is encoded by an MuLV mutant that has undergone alterations in its env gene region.

2. Wild-type R-SFFV gp54 is a Leukemogenic Molecule

The isolation of R-SFFV mutants containing a single mutation in the gp54 structural gene are important for defining the role of the gp54 molecule in leukemogenicity. The mutant R-SFFV found in the PRV clone 3-25 cell line contains only a single mutation in its env gene. The result of this single gene mutation significantly decreases the ability of the virus to elicit erythroleukemia in NIH/Swiss mice. These studies provide formal evidence that the gp54 molecule may be directly involved in the leukemogenic process and that even minor alterations in oligosaccharide attachment can significantly affect the ability of this molecule to elicit erythroleukemia.

3. Minor Structural Modifications of R-SFFV gp54 Block Processing to the Plasma Membrane

Previous studies have shown that R-SFFV gp54 is processed along a single pathway from the rough endoplasmic reticulum via the Golgi apparatus to the plasma membrane (145,146). However, the

oligosaccharide processing and transport of most gp54 molecules ceases *en route* and results in only the inefficient formation of the plasma membrane component (145,146). Interestingly, the *env* molecules encoded by mutant R-SFFVs in the PRV clones do not appear to undergo any further processing to the cell surface (Fig. 14; also unpublished observations). We hypothesize that the mutant glycoproteins fail to efficiently reach their appropriate ultimate destination because of abnormalities in their structures.

Our laboratory has previously described a temperature-sensitive *env* gene mutant of MuLV (147). At the nonpermissive temperature, infected cells accumulate the $gPr90^{env}$ precursor and only a small proportion of gp70 reaches the cell surface (147). Furthermore, $gPr90^{env}$ molecules which accumulate intracellularly are not processed when the infected cells are shifted to the permissive temperature (147). This example demonstrates that structural abnormalities of *env* gene products can reduce their processing efficiency and can cause irreversible intracellular accumulation. In all likelihood, the structural abnormalities of the *env* glycoproteins encoded by the R-SFFV mutants also result in blocked processing of these molecules to the cell surface.

V. Surface Membranes of Lymphoid Cells Transformed by Abelson Murine Leukemia Virus: Relationship to Tumor Rejection

A. General Introduction

Abelson murine leukemia virus (A-MuLV) is a replication-defective retrovirus that can induce a rapidly fatal nonthymic lymphosarcoma in mice (2,3) and can transform certain bone marrow and fibroblast cultures *in vitro* (120,132,153). The tumorigenic potential of A-MuLV has been generally attributed to the expression of the virus' only known gene product, p120 (123,195), a recombinant-type molecule containing serological determinants related to the Moloney murine leukemia virus (M-MuLV) amino terminal *gag* gene products, p15 and p12 (123,149,198), and also containing determinants related to a protein encoded by the *abl* gene found in the normal mouse genome (10,57,159).

Recent evidence has indicated that p120 is a tyrosine phosphorylating protein kinase (193,199). In addition, analysis of several A-MuLV strain variants encoding *gag-abl* fusion proteins with apparent Mr's between 90,000 and 160,000 have demonstrated a direct correlation between the molecules' protein kinase activity and its ability to transform lymphoid or fibroblast cultures (133,194). Furthermore, *abl* antigens have been detected on the outer surfaces of cells infected with A-MuLV (196). However, p120 occurs predominantly in intracellular site(s) (140) and it is not known whether the cell surface component is p120 or a processed derivative.

When cells transformed by A-MuLV are injected into syngeneic mice, massive tumors form rapidly and cause death within 1-2 months (196). Only one Abelson transformed lymphoid cell line, the L1-2 of C57 L

origin, is reproducibly rejected by its syngeneic host (196). Furthermore, the serum from recovered mice (Abelson tumor serum) has provided the only reliable source of antibody reactive with abl antigens (141,196).

For these reasons, we initiated a study of the plasma membranes of L1-2 cells. The goals were to analyze the metabolism of gag-abl fusion protein(s) and to identify plasma membrane targets of the immune response in the recovered C57 L mice.

B. Virus-Related Proteins in L1-2 Cells

Extracts of L-[^{35}S]methionine-labeled L1-2 cells contain two proteins which can be precipitated either with antiserum recognizing the M-MuLV gag gene products p15 and p12 or with serum collected from C57 L mice that have rejected A-MuLV induced tumors (Abelson tumor serum). These proteins include the well characterized A-MuLV p120 molecule (123,198) and a second minor component with an apparent Mr of 95,000 (p95) (Fig. 18, lanes 1 and 5). Preadsorption of the antisera to gag proteins with concentrated Friend murine leukemia virus (F-MuLV) quantitatively eliminates this serum's ability to precipitate p120 and p95 from L-[^{35}S]methionine-labeled L1-2 extracts (Fig. 18, lane 2 and Table 2). Similar preadsorption of the Abelson tumor serum with this concentrated virus preparation does not appreciably affect the precipitation of either of these two molecules (Fig. 18, lane 6 and Table 2). These observations imply that this tumor serum precipitates p120 with antibodies reactive with abl antigens rather than with gag antigens. Therefore, based on this immunological criterion, both p120 and p95 are fusion proteins containing gag and abl determinants.

Figure 18. Identification of viral proteins in the L1-2 cell line.

L1-2 cells were pulse labeled with L-[^{35}S]methionine (2 h, 50 μCi/ml) and subsequently extracted with detergents. Viral proteins in the lysates were then immunoprecipitated with anti-p12 (lane 1), anti-gp70 (lane 3), or an Abelson tumor serum obtained from C57 L mice (lane 5). Immunoprecipitations were also conducted with serum that had been preadsorbed with 100 μl of concentrated F-MuLV (preadsorption of anti-p12, anti-gp70, and tumor serum conducted in lanes 2, 4, and 6 respectively).

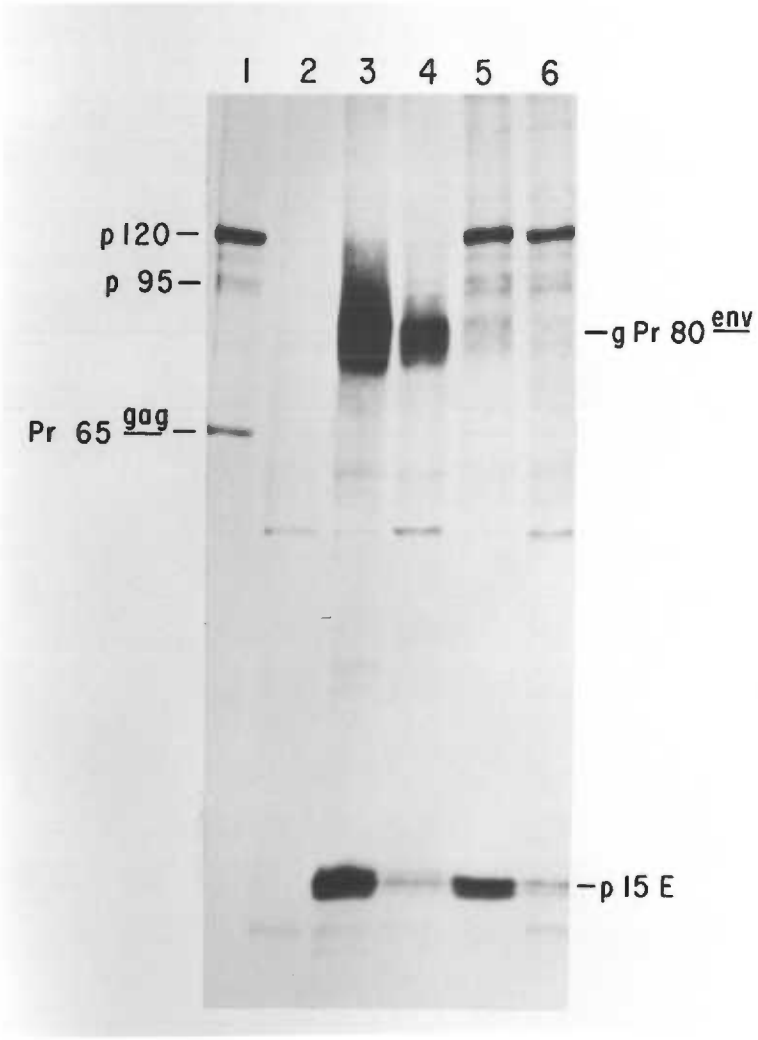

1
2
3
4
5
6
p120
p 95
Pr 65 gag
gPr 80 env
p15 E

Table 2

Relative Amounts of Viral Proteins Precipitated by Various Antisera from L-[^{35}S]methionine Labeled L1-2 Cells

	Relative Quantity of Protein Precipitated With					
Protein	Anti-p12 Serum	Anti-p12 Serum Preadsorbed With MuLV	Anti-gp70 Serum	Anti-gp70 Serum Preadsorbed With MuLV	Tumor Serum	Tumor Serum Preadsorbed With MuLV
p120	11.9	0.1	0	0	11.3	10.8
p95	1.5	0.1	0	0	1.4	1.4
gPr80^{env}	0	0	80.0	14.3	1.0	0.6
Pr65^{gag}	5.1	0.1	0	0	0	0
p15E	0	0	19.1	1.0	17.4	1.2

L1-2 cells were pulse-labeled with L-[^{35}S]methionine (100 µCi/ml) for 2 h at 37°C. Cell lysates were prepared as described in the Materials and Methods and viral proteins immunoprecipitated with either unadsorbed anti-p12, anti-gp70, or Abelson tumor serum or sera preadsorbed with 50 µl of concentrated F-MuLV for 45 min at 37°C. The immunoprecipitates were subjected to SDS-PAGE and the resulting gel developed by fluorography. This fluorogram is shown in Fig. 18. Fluorograms at appropriate exposure levels were scanned using a Transidyne General integrating densitometer (147). In all cases, comparisons of band intensities between proteins precipitated with the same serum were conducted with the same exposure. In addition, integrated band intensities were normalized to allow comparisons between proteins precipitated with different serum.

In addition to the A-MuLV p120 and p95 molecules, the L1-2 cell line contains other viral proteins encoded by the M-MuLV gag and env genes. Sera recognizing determinants encoded by these two genes precipitate the previously characterized M-MuLV proteins (38,202), $Pr65^{gag}$, $Pr75^{gag}$, $gPr80^{env}$, $gp70^{env}$ and p15E from L-[^{35}S]methionine-labeled L1-2 extracts (e.g., Fig. 18, lanes 1 and 3). The precipitation of the env related molecules p15E and its precursor, $gPr80^{env}$,by the Abelson tumor serum (Fig. 19, lane 5) implies that this serum contains a p15E antibody subpopulation. The precipitation of these two env proteins by the tumor serum can be efficiently reduced by preadsorption with concentrated F-MuLV (Fig. 18, lane 6 and Table 2).

C. p120 and p95 are Structurally Related Molecules and Contain Apparently Identical Tyrosine-Containing Phosphorylation Sites

To determine the structural relatedness of the p95 molecule to A-MuLV p120 and to M-MuLV $Pr65^{gag}$, tryptic peptide analysis of L-[^{35}S]methionine-labeled proteins were conducted. A-MuLV p120 contained 16 major and several minor methionine peptides (Fig. 19). Of the 16 major peptides found in p120, all but two appear to be present in the p95 molecule. These two missing peptides (see arrows in Fig. 19) do not appear in the tryptic peptide map of the M-MuLV $Pr65^{gag}$ protein. We infer that the portion of p120 which is absent from p95 occurs in the abl region, consistent with our immunological evidence that p95 contains the M-MuLV p15 (data not shown) and p12 gag regions found in p120.

Both of these gag-abl fusion proteins could be labeled by incubation of nonradioactive immunoprecipitates with [γ-^{32}P]ATP in an in vitro protein kinase assay (Fig. 20). Two dimensional electrophoretic analyses of acid hydrolysates of proteins phosphorylated in the L1-2

Figure 19. Tryptic peptide analyses of L-[^{35}S]methionine-labeled p120, p95, and M-MuLV Pr65^{gag}.

L-[^{35}S]methionine-labeled proteins, purified by immunoprecipitation and SDS-PAGE, were digested by incubation of selected gel sections in TPCK-trypsin for 24 h at 37°C. Following performic acid oxidation, these samples were diluted in 2 ml of H_2O, concentrated under vacuum, resuspended in electrophoresis solution^2 (28% formic acid) and spotted on cellulose TLC plates. Electrophoresis was conducted at 150 v for 5 h in the horizontal dimension and chromatography was performed in solution containing isoamyl alcohol:pyridine:ethanol:glacial acetic acid:H_2O (70:70:40:10:60) in the vertical dimension. Detection of L-[^{35}S]^2 methionine-labeled peptides was enhanced by spraying TLC plates with 7% diphenyloxazole in ether. Proteins analyzed included (A) A-MuLV p120, (B) A-MuLV p95, and (C) M-MuLV Pr65^{gag} Arrows point to some representative peptides found in p120 that are absent in p95 and Pr65^{gag}. The origin is at the lower right.

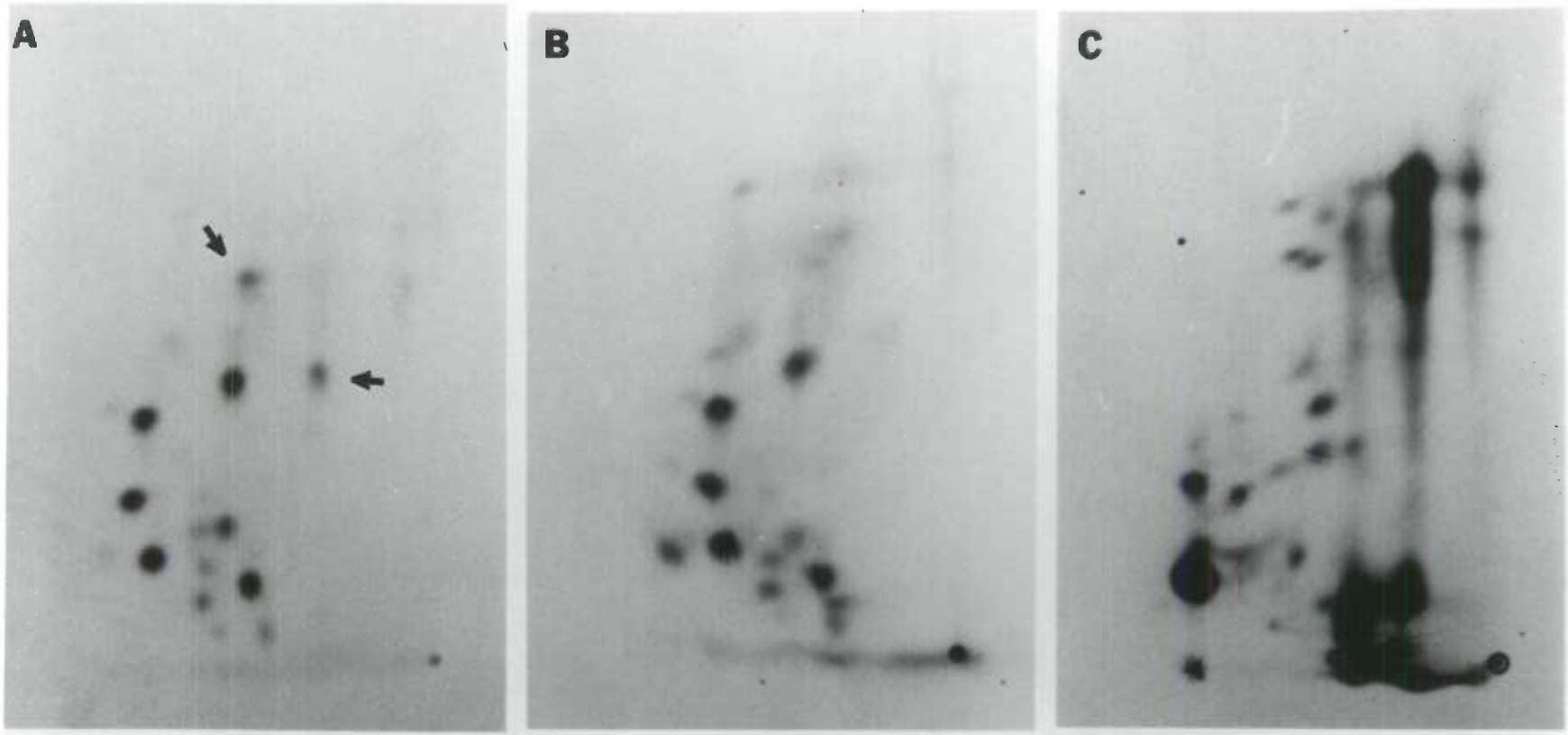
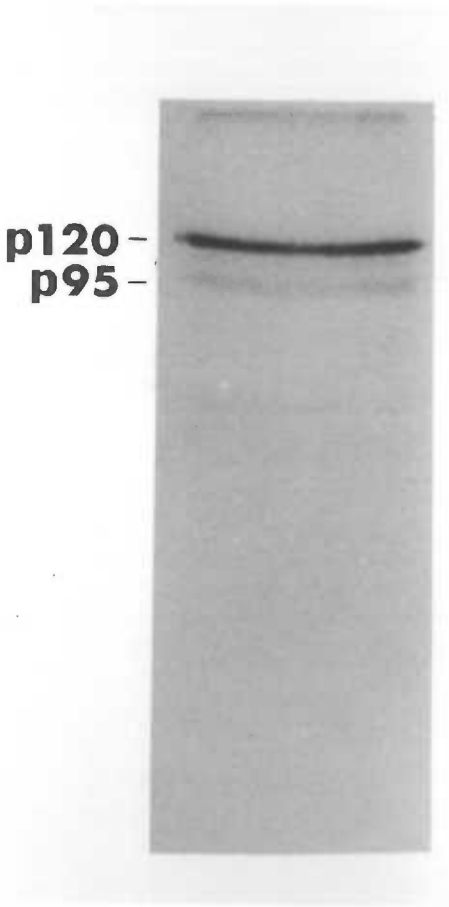

A
B
C

Figure 20. Electrophoretic analysis of proteins phosphorylated by [γ-^{32}P]ATP in the L1-2 immunoprecipitate.

Virus-specific proteins from nonradioactive L1-2 lysates were immunoprecipitated with anti-p12 serum. In vitro phosphorylation was conducted by suspending immunoprecipitates in 20 mM Tris-HCl (pH 7.2) - 5 mM $MgCl_2$ buffer containing 1 µCi of [γ-^{32}P]ATP and incubating them for 10 min at 30°C. Reactions were terminated by addition of ice-cold 20 mM Tris-HCl (pH 7.2) and after repeated washings were analyzed by SDS-PAGE as described in Section II.K.


p120
p95

immunoprecipitate, shows that the amino acid acceptor for phosphorylation on p95 is at tyrosine residues (Fig. 21), consistent with previously described results for the A-MuLV p120 molecule (193). In addition, phosphorylated p120 and p95 have nearly identical phosphotryptic peptide maps (Fig. 22), each containing one major and several minor phosphopeptides.

D. p120 and p95 Lack Precursor-Product Relationship.

Metabolic pulse chase studies utilizing L-[^{35}S]methionine show that both p120 and p95 are rapidly labeled after addition of the radioisotope, implying that these two molecules do not have a simple precursor-product relationship (Fig. 23).

E. Detection of abl Antigens on the Surface of Cells Transformed by A-MuLV

Evidence that the surface of Abelson transformed cells contains abl antigens was provided by an antibody-dependent rosetting assay. In this rosetting assay, sheep erythrocytes coated with protein A are incubated with cell monolayers in the presence of specific antisera (see Section II.J.). Binding of erythrocytes onto the surface of the Abelson nonproducer cell line ANN-1 occurred in the presence of tumor serum, but not in the presence of either preimmune mouse serum or of antiserum to p12^{gag} (see Fig. 24). The ANN-1 cell line was chosen for this study because it contains only A-MuLV and not M-MuLV encoded proteins. The use of this cell line obviated the necessity of absorbing the tumor serum's p15E antibody subpopulation prior to its application in the rosetting assay. The results in Fig. 24 imply that abl antigens occur on the surfaces of the A-MuLV transformed cell line, ANN-1.

Figure 21. Phosphoamino acid analysis of A-MuLV p120 and p95 phosphorylated *in vitro* with [γ-^{32}P]ATP.

A-MuLV p120 and p95, labeled *in vitro* with [γ-^{32}P]ATP, were purified by immunoprecipitation and SDS-PAGE, hydrolyzed with 6 N HCl, and subjected to two dimensional electrophoretic analyses, following procedures outlined in Section II.M. (pH 1.9 solution in horizontal dimension, pH 3.5 solution in vertical dimension). Analyses of p120 and p95 are shown in panels A and B respectively. Unlabeled phosphoamino acid standards (phosphoserine, phosphothreonine, and phosphotyrosine) detected by ninhydrin staining are shown in panel C. The origin is at the lower right.

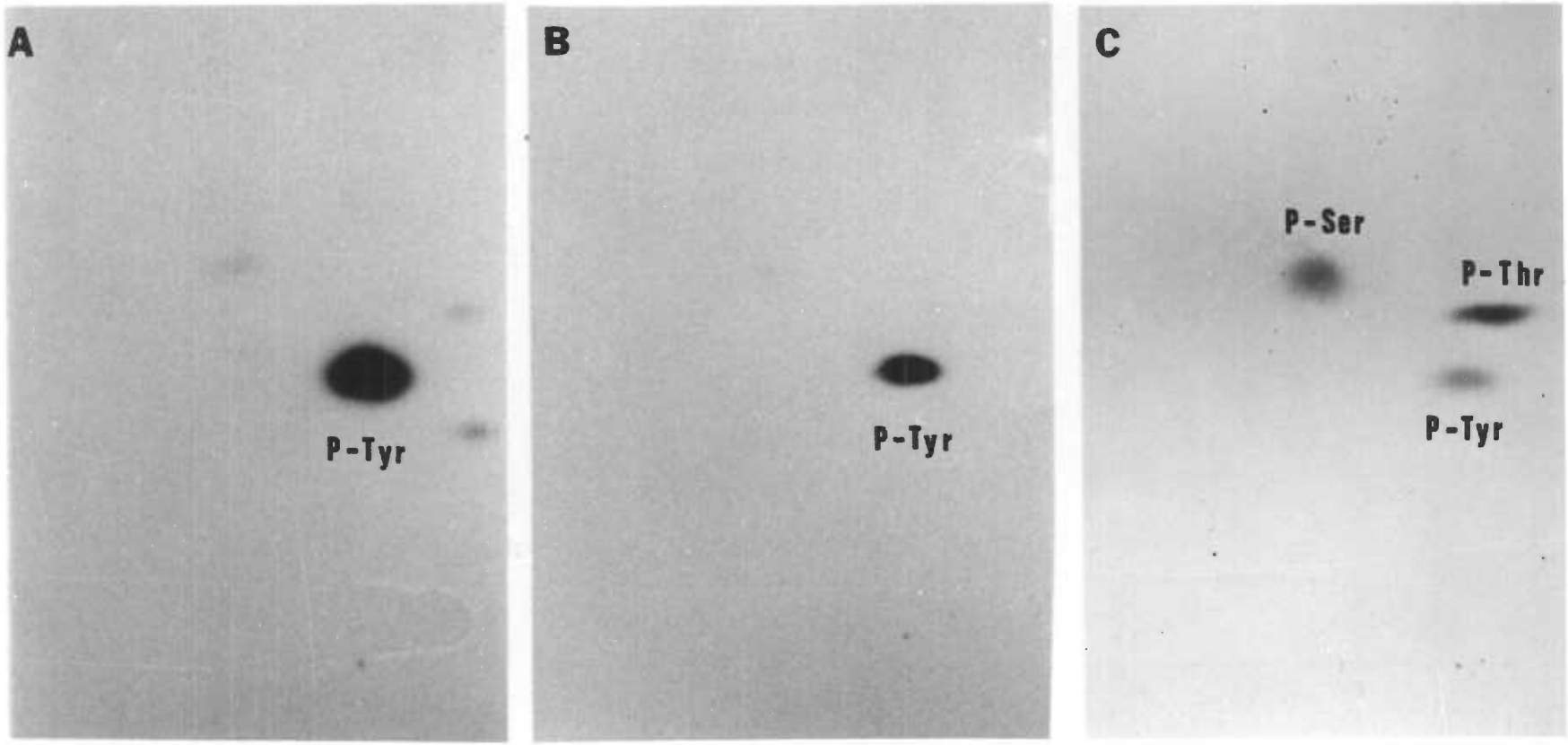
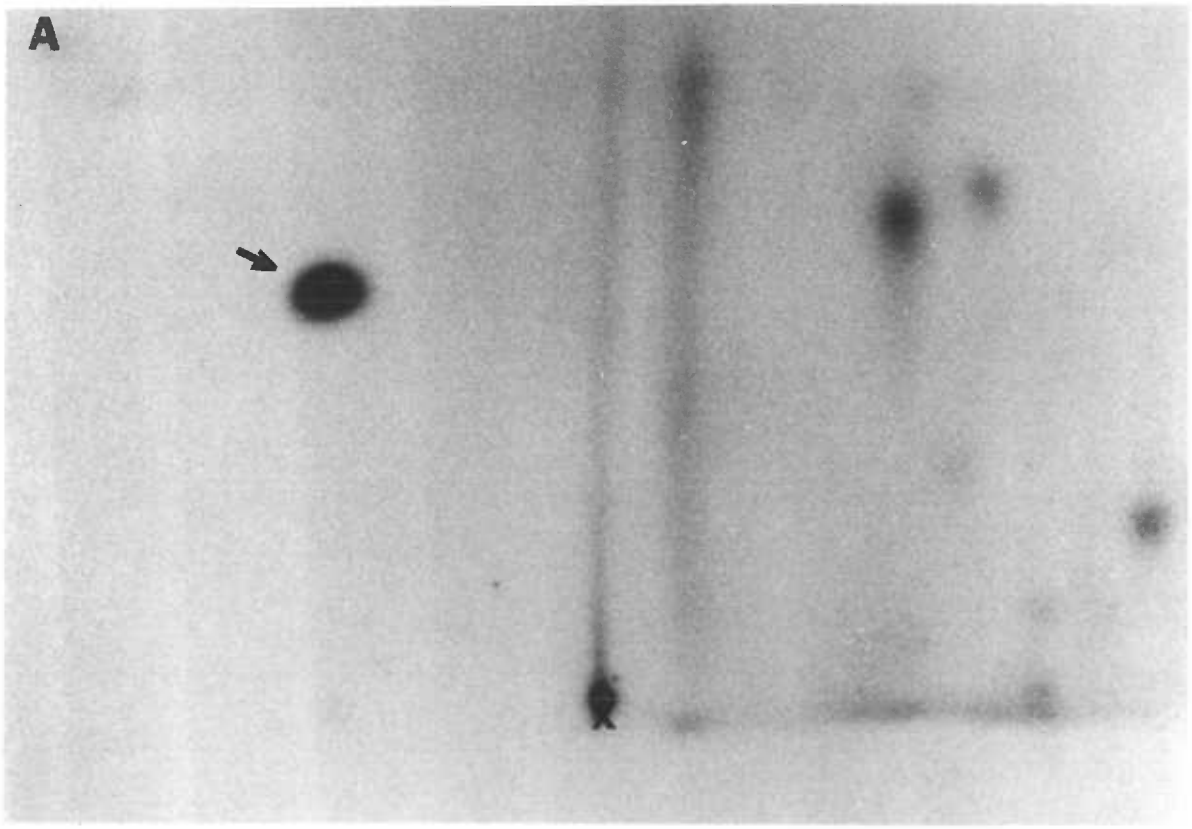

A
P-Tyr
B
P-Tyr
C
P-Ser
P-Thr
P-Tyr

Figure 22. Tryptic peptide analysis of A-MuLV p120 (A) and p95 (B) phosphorylated *in vitro* with [γ-^{32}P]ATP.

Proteins were labeled *in vitro* with [γ-^{32}P]ATP, purified by immunoprecipitation and SDS-PAGE, and subjected to tryptic proteolysis as described in Section II.L. Electrophoresis was conducted in the horizontal dimension and chromatography in the vertical dimension. The arrows point to the major phosphotryptic peptide found in both phosphorylated p120 and p95. This phosphopeptide comprises more than 75% of the total radioactivity found in the digest. The other minor phosphopeptides can only be detected after prolonged exposures of the film. The origin is denoted by the symbol "x".


A
X

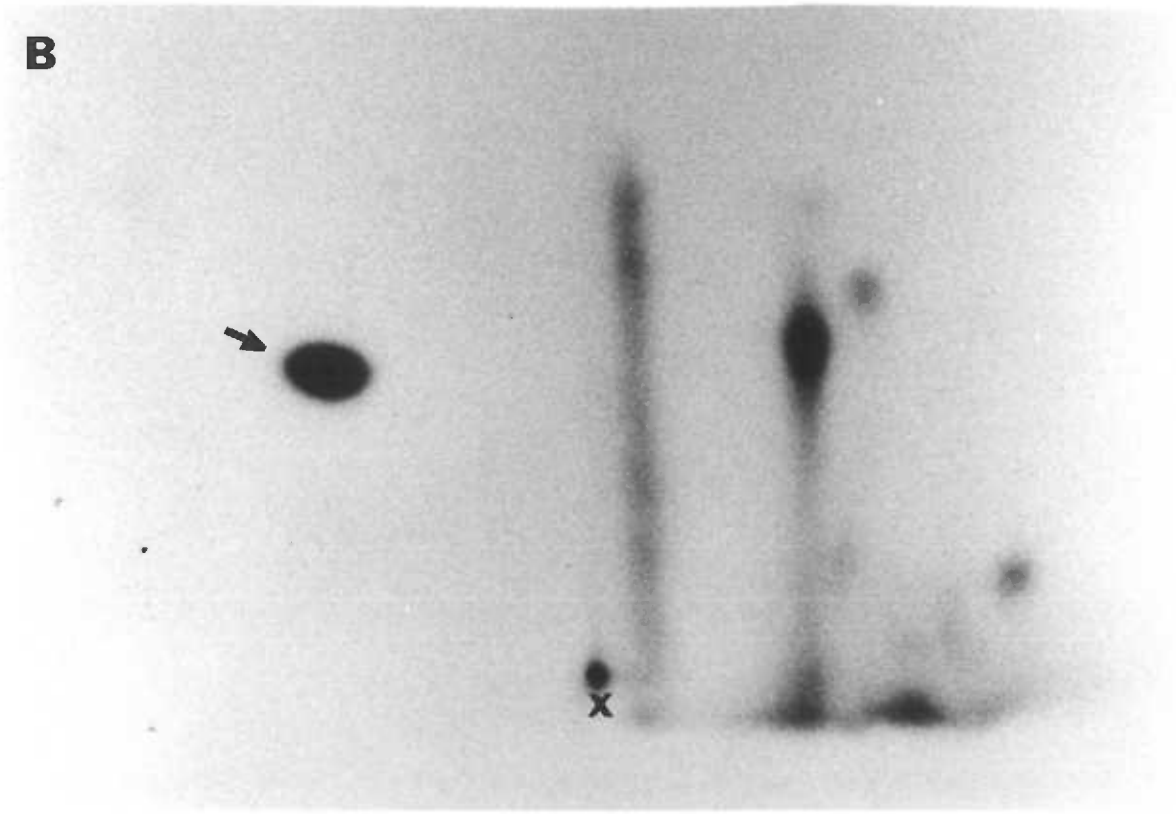

B
X

Figure 23. Pulse-chase analysis of viral proteins in L1-2 cells.

L1-2 cells (5×10^6) were pulse labeled with L-[^{35}S]methionine for various time intervals and chased by addition of unlabeled methionine. Cell samples taken during the experiment were lysed and viral proteins immunoprecipitated with anti-p30 (lanes 1-4), anti-p12 (lanes 5-8), anti-gp70 (lanes 9-12) and the Abelson tumor serum (lanes 13-16). The samples correspond to immunoprecipitates obtained from cells after a 10 min pulse (lanes 1, 5, 9, and 13), 30 min pulse (lanes 2, 6, 10, and 14), 30 min pulse and 1 hr chase (lanes 3, 7, 11, and 15), and 30 min pulse and 4 h chase (lanes 4, 8, 12, and 16).

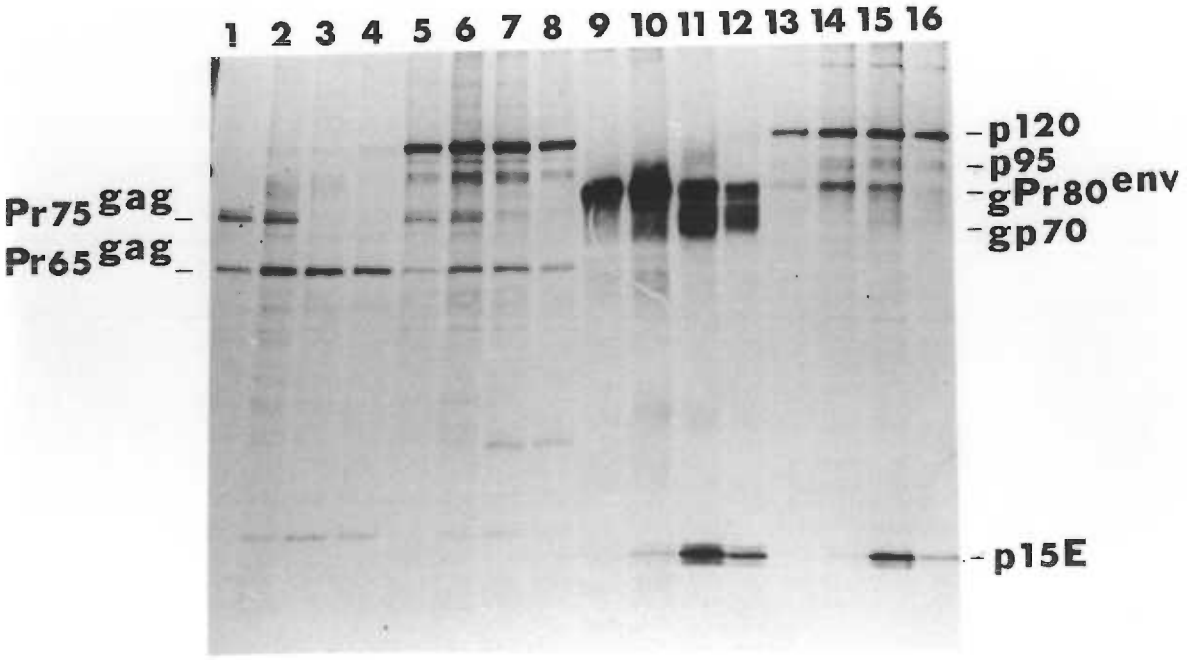
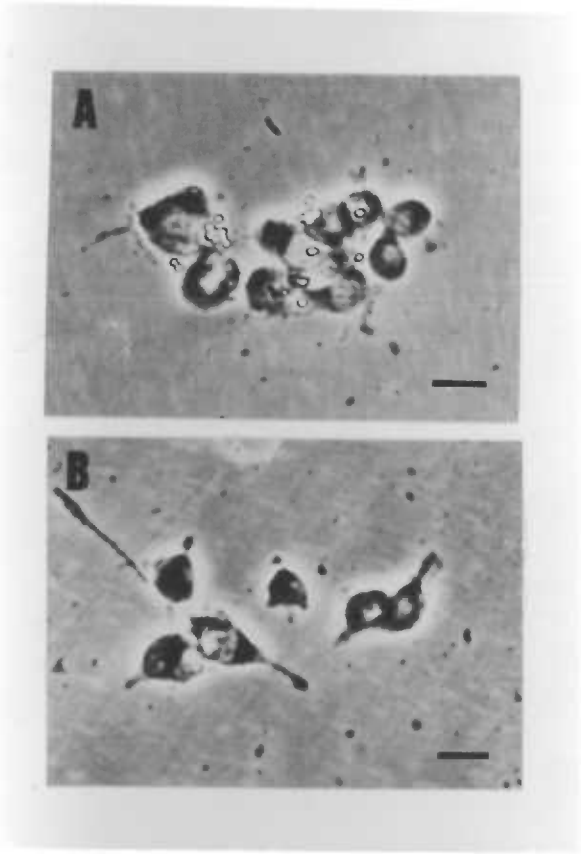

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Pr75gag_
Pr65gag_
p120
p95
gPr80env
gp70
p15E

Figure 24. Erythrocyte rosette assay for detection of A-MuLV encoded cell surface antigens.

The binding of sheep erythrocytes coated with protein A to the A-MuLV transformed non-producer fibroblast, ANN-1, occurred only in the presence of tumor serum containing antibodies reactive to the abl portion of the Abelson-encoded protein (A). No binding of erythrocytes were observed with either nonimmune mouse (B) or anti-p12 sera (data not shown). Bar corresponds to 20 μm in length. The erythrocytes in A are seen as highly refractile small white circles.


A
B

Further evidence to support the presence of abl antigens on the surface of A-MuLV transformed cells was provided by serum absorption experiments. As shown in Fig. 25, Abelson tumor serum preadsorbed with 10^6 ANN-1 cells (lane 3) cannot precipitate p120 from L-[^{35}S]methionine-labeled L1-2 cellular lysates. Alternatively, tumor serum preadsorbed with 10^6 or even 10^7 NIH-3T3 cells (lanes 6 and 7) precipitates p120 efficiently.

F. Use of Abelson Tumor Serum to Detect an Mr 95,000 Protein on the Surface of L1-2 Cells

We attempted to identify specific gag-abl surface molecules on the L1-2 cell line by a method that involves direct absorption of antibodies onto L-[^{35}S]methionine-labeled intact cells, followed by cell washing, lysis with detergents in the presence of an excess of nonradioactive cellular extract, and rapid isolation of antigen-antibody complexes (Section II.H.). The M-MuLV gp70^{env} and p15E molecules were detected in these surface preparations when antiserum to gp70^{env} was employed (Fig. 26, lane 3). The p15E protein binds weakly to gp70^{env} and is partially coprecipitated with the surface gp70^{env}-antibody complexes. When the Abelson tumor serum was used to isolate antigenically reactive molecules from the L1-2 cell surface, a diffuse band with an apparent Mr of 95,000 was observed in SDS-PAGE (Fig. 26, lane 4). This molecule was detected on the L1-2 surface only with tumor serum and not with preimmune mouse serum or antiserum recognizing p12^{gag} or gp70^{env} (Fig. 26; also unpublished observations). The A-MuLV p120 molecule was not detected by this method (Fig. 26, lane 4).

Figure 25. Absorption of tumor serum with ANN-1 or NIH-3T3 cells.

Tumor serum was preadsorbed with either 10^5, 10^6, or 10^7 ANN-1 cells (lanes 2, 3, and 4 respectively) or the same numbers of NIH-3T3 cells (lanes 5, 6, and 7 respectively), prior to the serum's application in the immunoprecipitation of p120 from L-[^{35}S]methionine-labeled L1-2 lysates. Tumor serum which did not undergo preadsorption with intact cells (lane 1) was also used to precipitate p120 from the radioactive lysates. The antigen-antibody complexes were then collected with S. aureus protein A and subjected to SDS-PAGE.

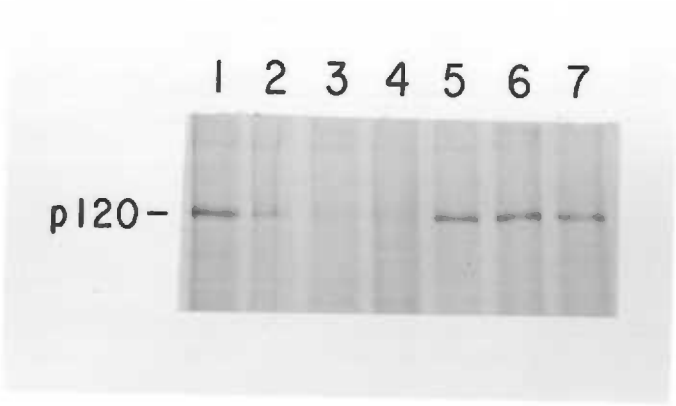

1 2 3 4 5 6 7
p120-

Figure 26. Electrophoretic analysis of MuLV-encoded proteins on the surface of L1-2 cells.

L1-2 cells were pulse labeled by incubation with L-[^{35}S]methionine (2 h, 100 μCi/ml). The labeled cells were subsequently extracted and viral proteins were immunoprecipitated from the lysates with either 5 μl of anti-gp70 (lane 1) or Abelson tumor serum (lane 2). Alternatively, the L1-2 cells, similarly labeled, were incubated directly with either 5 μl of anti-gp70 (lane 3) or Abelson tumor serum (lane 4) for 45 min at 4°C. After washing to remove unbound antibodies, lysates were prepared as described in Section II.H. Surface antigen-antibody complexes were then collected with S. aureus protein A and subjected to SDS-PAGE.

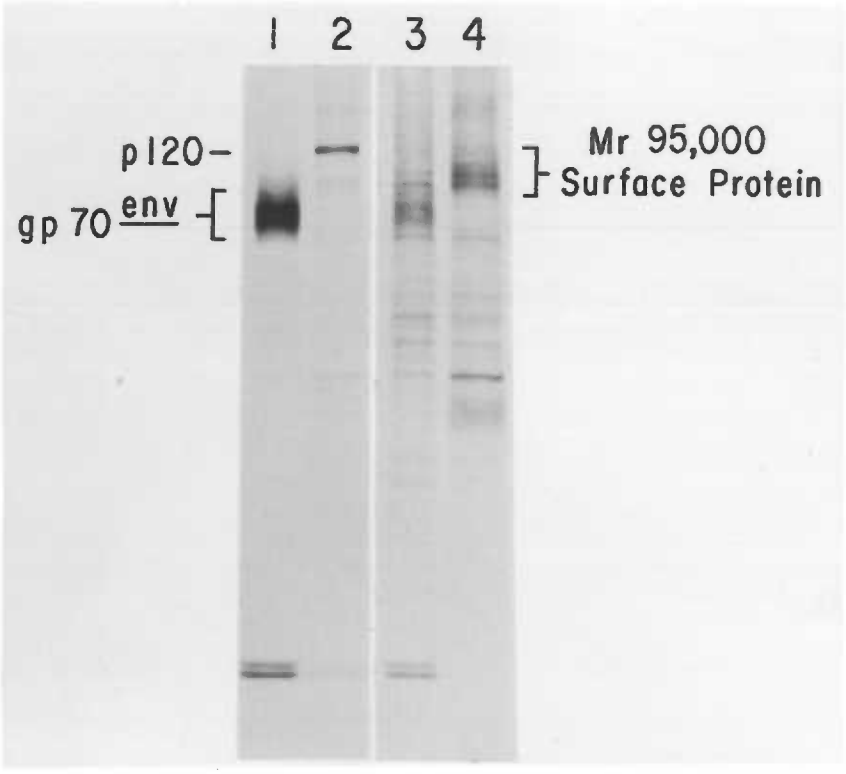

1
2
3
4
p120–
gp 70 env
Mr 95,000
Surface Protein

Figure 27. Electrophoretic analysis of Mr 95,000 surface molecule on A-MuLV transformed cells.

The surface membranes of L1-2 cells were analyzed by the extracellular antibody adsorption technique (Section II.H.) using normal mouse serum (lane 1), Abelson tumor serum (lane 2), or tumor serum preadsorbed with either 10^7 NIH-3T3 or ANN-1 cells (lanes 3 and 4 respectively). The same extracellular antibody adsorption technique using tumor serum as the probe was employed with two other A-MuLV transformed lymphoid cell lines, 230-23-8 (lane 5) and SWR/4 (lane 7). Surface preparations from the L1-2 cell line, illustrating the Mr 95,000 molecule, are shown in lane 6 for comparison.


1 2 3 4 5 6 7
Mr 95,000
Surface Protein

consistent with the previously described inability of these cell lines to absorb antibodies which react with this determinant.

H. Discussion

1. Regression of L1-2 Tumor Transplants in Syngeneic C57 L Mice

A major advance in understanding the molecular biology of A-MuLV and for defining the virus' transforming protein was the development of a mouse tumor serum reactive to the host-encoded abl determinant found in the virus' major gene product, p120 (196,198). This serum can only be produced in the C57 L mouse by the repeated injection of only one A-MuLV transformed syngeneic lymphoid cell line, the L1-2 (141,196). Rotter et al. (141) have established that the uniqueness of tumor regression in this one system lies in the properties of the cell line and not in the genetics of C57 L animals. Other A-MuLV transformed lymphoid cell lines of C57 L origin have been found to be highly lethal to their syngeneic hosts, consistent with studies of Abelson tumorigenesis in other mouse strains (196). This evidence suggested that L1-2 cells differ from other A-MuLV infected cells in their immunological interactions with the host.

2. abl Antigens Occur in Plasma Membranes

The extracellular antibody adsorption procedure described in this study has been used previously to investigate the membrane processing of the human histocompatibility antigens (74) and the surface molecules encoded by the gag and env genes of the Friend murine leukemia virus (48). The direct identification of p120 or an antigenically-related derivative on the plasma membranes of A-MuLV transformed cells was not ascertained in our study by either the

extracellular antibody adsorption technique (Fig. 26) or by subcellular fractionation (C. Machida and D. Kabat, unpublished observations). However, observations obtained by other techniques are consistent with the model proposed by Witte et al. (196) for the orientation of Abelson-encoded gag-abl molecules into cellular membranes. Thus, our results are consistent with the idea that abl antigen(s) may be a transmembrane protein with its amino-terminal gag related region embedded in the plasma membrane and its host-encoded abl determinant protruding to the exterior of the cell. Our studies have shown that intact ANN-1 cells can absorb antibodies recognizing the abl antigen from the tumor serum (Fig. 25). Furthermore, binding of protein A coupled-sheep erythrocytes to the surface of the A-MuLV transformed nonproducer fibroblast, ANN-1, was achieved only when the tumor serum was employed and not with serum recognizing $p12^{gag}$ antigens (Fig. 25).

The apparent paradox of why the extracellular antibody adsorption technique is able to detect M-MuLV $gp70^{env}$ on the surface of the L1-2 cell and not A-MuLV gag-abl fusion proteins is presumably related to the different antisera employed. Isolation of membrane proteins using this procedure requires highly avid antibodies (48). Antisera with a low avidity can dissociate from their antigens before the immune complexes can be isolated.

3. Significance of Minor gag-abl Components

There are several possible explanations for the appearance of the minor p95 gag-abl component in L1-2 cellular lysates. Similar minor components with heterogeneous Mr between 80,000 and 110,000 have been noted by previous investigators (198) in various clonally derived A-MuLV transformed cell lines. First, p95 might be a proteolytic fragment of

p120, artifactually produced during the extraction of L-[^{35}S]methionine-labeled L1-2 cells. This explanation seems unlikely because the concentration of this molecule was unaffected by the use of different lysis procedures or protease inhibitors. A second possibility which has been suggested previously for other cell lines (198) is that the different gag-abl components may be encoded by distinct A-MuLV proviruses which might co-exist in the same cells. A third possibility is that the processing or structural modification of gag-abl fusion proteins might occur intracellularly and that different components occur in distinct subcellular sites. This possibility is intriguing because MuLV encoded gag gene products are heterogeneously processed in both the cytoplasm and in subcellular membranous organelles (38,154). In this context, it is interesting to note that abl antigens also occur in both cytoplasmic and plasma membrane locales (140,196). Presumably, similar heterogeneous processing of gag-abl fusion proteins might be involved in determining the subcellular disposition of these molecules.

4. The Mr 95,000 Protein on the Surface Membranes of L1-2 Cells

The Mr 95,000 protein that occurs on the surface of L1-2 cells seems highly significant because it reacts strongly with antibodies present in the serum of mice which have rejected L1-2 tumor challenge. Furthermore, this molecule seems to lack abl antigens (Fig. 27) and to be absent from the surfaces of other A-MuLV transformed lymphoid and fibroblast cell lines, including those of C57 L origin. Unlike L1-2 cells, the latter transformed cell lines are highly tumorigenic when transplanted onto C57 L mice. These results suggest that the Mr 95,000 cell surface protein might be an important target for the immune

response of host C57 L mice to the L1-2 tumor challenge. Presumably, the Mr 95,000 cell surface protein is distinct from the surface antigen described by Risser et al. (126-128). The latter antigen is common to different A-MuLV transformed lymphoid tumors (126-128), whereas the Mr 95,000 protein occurs only or in substantially higher concentrations on L1-2 cells.

Although our results are consistent with the possibility that the Mr 95,000 protein plays an important role as a target for immune rejection of L1-2 tumor cells in C57 L mice, many aspects of its structure and function remain unknown. For example, immune attack against this component and against L1-2 cells might involve cellular as well as humoral mechanisms. Furthermore, the observed immune response to this molecule suggests that the host mice are not tolerized to all of its antigens. Therefore, we suggest that it might be encoded by a derepressed host gene or by a passenger virus which has somehow infected the L1-2 tumor cells. If these ideas are correct, passenger virus infection of other tumor cell lines might enhance their rejectability and thereby facilitate preparation of tumor specific antisera.

VI. Summary

Three distinct and independent retroviral systems have been utilized to investigate 1) the post-translational processing mechanisms of retroviral-encoded proteins to the cell surface and 2) the role of retroviral- and cellular- encoded membrane proteins in transformation and in tumor rejection.

The first system investigates the membrane protein processing characteristics of three env gene mutants of Rauscher murine leukemia virus (R-MuLV). One viral mutant, R7 clone 1, encodes an env precursor molecule that undergoes only inefficient partial proteolysis, resulting in the transport of both the cleaved and uncleaved forms of the glycoprotein to the surface of infected cells. These mutant surface forms of the env glycoprotein are apparently ineffective in establishing interference to superinfection by wild-type MuLV. The other two R-MuLV mutants, R7 clone 61 and R Balb clone 29, encode abnormally sized env precursor molecules. The structural abnormalities that are presumed to exist in these molecules either block or severely restrict the post-translational modifications that normally ensue for these proteins, in its migration from the subcellular membranous organelles to the cell surface. It is proposed that a critical structural feature or "signal" of newly-made membrane glycoproteins is necessary for export of these env precursor molecules from the rough endoplasmic reticulum to the Golgi apparatus. It is believed that the structural abnormalities of the mutant env glycoproteins obscure the "signal" that must be recognized before processing and transit of these molecules to the cell surface can be achieved. Such considerations could also explain how

specific covalent modifications (e.g., glycosylation and acylation) which can alter the folding of proteins might be essential for the intracellular transport of certain glycoproteins but not for others.

The second system examines the env-related proteins encoded by weakly leukemogenic Rauscher spleen focus-forming virus (R-SFFV) mutants, in an attempt to identify and study the critical features of the structure and membrane processing of the virus' transformation protein. Mutant R-SFFV contained in the PRV clone 3-25 cell line encodes an env gene product with an apparent Mr of 52,000 (gp52) that differs from the wild-type gp54 molecule by a single oligosaccharide. The R-SFFV mutant contained in the PRV clone 4-3 cell line encodes an env-related glycoprotein with an apparent Mr of 45,000 (gp45), generated from either a deletion or premature translational termination of the virus' env gene. It is interesting to note that both R-SFFV mutants are weakly leukemogenic when injected in NIH/Swiss mice. In addition, abnormalities in viral protein structure apparently block further processing of the SFFV glycoproteins to the plasma membranes of infected cells. The absence of SFFV-encoded glycoproteins on the cell surface may be partially responsible for the decreased leukemogenicity of these viruses.

The third system studies the plasma membranes of an Abelson murine leukemia virus-transformed lymphoid cell line L1-2 and analyzes the membrane processing of the gag-abl fusion proteins encoded by these recombinant-type viruses. This cell line contains the well-characterized A-MuLV p120 molecule and a second minor component with an apparent Mr of 95,000 (p95). Based on both immunological and structural criteria, p95 is an A-MuLV-encoded gag-abl fusion protein and

appears to lack the 25,000 dalton carboxyl terminal end of the p120 molecule. Our studies confirm that abl antigens are present on the outer surfaces of cells infected with A-MuLV. However, p120 occurs predominantly in intracellular sites and it is not known whether the cell surface component is p120 or a processed derivative. The appearance of a minor component such as p95 is significant in light of the possibility that it may represent a processed membrane form of the p120 molecule. In addition to A-MuLV p120 and p95, the L1-2 cell line contains an Mr 95,000 surface protein. This surface molecule appears to be absent from the surfaces of other A-MuLV transformed lymphoid and fibroblast cell lines and may play an important role as a target for immune rejection of L1-2 tumor cells in C57L mice.

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