BIOCHEMICAL AND GENETIC CHARACTERIZATION OF THE MURINE LEUKEMIA VIRUS PROTEINS

by

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A THESIS

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TABLE OF CONTENTS

		Page
I.	Introduction	1
	A. Structure and Morphology of Retroviruses.	3
	B. The Murine Leukemia Virus Genome.	4
	C. Murine Leukemia Virus Protein Nomenclature.	4
	D. General Methodology.	5
	E. Expression of the *gag* Gene.	5
	1. Biosynthesis of the Murine Leukemia Virus Core Proteins.	5
	2. Proteins of the Murine Leukemia Virus Core.	6
	3. Murine Leukemia Virus Tropism.	7
	F. Expression of the *pol* Gene.	9
	1. Biosynthesis of Reverse Transcriptase.	9
	2. Reverse Transcription.	9
	G. Expression of the *env* Gene.	10
	1. Biosynthesis of the Murine Leukemia Virus Envelope Proteins.	10
	2. The Biology of gp70.	14
	3. The Biochemistry of gp70.	19
	H. Thesis Objective and Experimental Rationale.	21
	I. Organization of the Thesis.	23
II.	Materials and Methods	25
	A. Materials.	25
	B. Cell Culture.	25
	C. Virus Methodology.	27
	1. Assays of Murine Leukemia Virus Components.	27
	2. Virus Infections and Cloning.	28
	3. Harvest and Purification of Virus.	30
	4. Radioactive Labelling of Virus Proteins.	30

D. Murine Leukemia Virus Protein Purification. 32

1. Phosphocellulose Column Chromatography. 32
2. Guanidine Hydrochloride Column Chromatography. 34

E. Immune Precipitation Procedures and Cellular Labelling. 34
F. Polyacrylamide Gel Electrophoresis. 35

1. One-Dimensional Sodium Dodecyl Sulfate-Urea Electrophoresis. 36
2. Two-Dimensional Isoelectric Focusing/Sodium Dodecyl Sulfate Electrophoresis. 36

G. Cellulose Acetate Electrophoresis. 42
H. Neuraminidase Treatment. 42
I. Tryptic Glycopeptide Analysis. 43

1. DEAE-Sephadex A25 Column Chromatography. 43
2. High Voltage Paper Electrophoresis. 43

III. Murine Leukemia Virus-Specific Proteins: Identification by Two-Dimensional Polyacrylamide Gel Electrophoresis 45

A. Two-Dimensional Map of Murine Leukemia Virus-Specific Proteins. 46
B. Identification of Known Murine Leukemia Virus Proteins. 52
C. The Viral Determinant of Tropism-p30. 59
D. Discussion. 60

IV. Genetic and Sialylation Causes for Heterogeneity of Murine Leukemia Virus Envelope Glycoproteins gp69/71. 67

A. Genetic Heterogeneity of Eveline II Virus gp69/71. 67
B. Heterogeneous Sialylation of Envelope Glycoproteins. 73
C. Eveline II MuLV Differs from the Original Strain of Friend MuLV. 87
D. Discussion. 90

V. A Murine Leukemia Virus Mutant with a Temperature-Sensitive Defect in Glycoprotein Synthesis. 96

A. Previous Work with Rauscher MuLV ts26. 96
B. Absence of Plasma Membrane gp70 in ts26 Infected Cells at Restrictive Temperature. 99

C. Release of Virus Particles from ts26 Infected Cells at Restrictive Temperature. 99
D. Discussion. 114

VI. Summary and Conclusions. 119

LIST OF FIGURES

		Page
1	Calibration of the Two-Dimensional Polyacrylamide Gels.	41
2	Two-Dimensional Polyacrylamide Gel Electrophoresis of Clone B4Sc-1 Virus.	49
3	Two-Dimensional Polyacrylamide Gel Electrophoresis of Clone B4NRK Virus and Proteins Harvested from Uninfected Sc-1 Cells.	51
4	Schematic Two-Dimensional Map of Virus-Specific Proteins in Murine Leukemia Virus Preparations.	54
5	Guanidine Hydrochloride Column Chromatography of Radioactively Labelled Murine Leukemia Virus Proteins.	57
6	The p30s of N-tropic, B-tropic and NB-tropic Murine Leukemia Virus Clones.	62
7	Pulse-Chase Analysis of gp69/71-Specific Proteins of Eveline II Virus in Sc-1 Cells and Clone B4 Virus in Sc-1 and NRK Cells.	70
8	Two-Dimensional Polyacrylamide Gel Electrophoresis of Eveline II Virus gp69/71 and Clone B4 Virus gp71.	72
9	Two-Dimensional Polyacrylamide Gel Electrophoresis of Control and Neuraminidase-Treated Eveline II Virus gp69/71.	75
10	Immune Precipitation Analysis of gp69/71-Specific Proteins in Cells Infected with Eveline II Virus Clones.	78
11	Cellulose Acetate Electrophoresis of Control and Neuraminidase-Treated Eveline II Virus gp69/71.	80
12	DEAE-Sephadex Chromatography of Tryptic Glycopeptides of Eveline II Virus gp69/71.	83
13	High Voltage Paper Electrophoresis of Tryptic Glycopeptides of Eveline II Virus gp69/71.	85

14 Cell Surface Glycoproteins of Eveline II Cells and Friend Erythroleukemia Cells, as Revealed by Lactoperoxidase-Catalyzed Iodination of Cells with [^{125}I]iodine. 89

15 Pulse-Chase Analysis of gp70-Specific Proteins from Wild-Type and ts26 Rauscher Murine Leukemia Virus Infected Cells Grown at 39°. 98

16 Cell Surface Glycoprotein of ts26 Infected Cells, as Revealed by Lactoperoxidase-Catalyzed Iodination of Cells with [^{125}I]iodine. 101

17 Immune Precipitation of gp70 from Particles Released at 39° by Cells Infected with Rauscher Murine Leukemia Virus Wild-Type and Temperature Sensitive Mutants. 105

18 Two-Dimensional Polyacrylamide Gel Electrophoresis of Wild-Type Rauscher Murine Leukemia Virus Formed at 39°. 109

19 Two-Dimensional Polyacrylamide Gel Electrophoresis of the ts26 Mutant of Rauscher Murine Leukemia Virus Formed at 39°. 111

20 Electron Microscopic Examination of Wild-Type and ts26 Rauscher Murine Leukemia Virus Particles Produced at 39°C. 113

21 Electron Microscopic Examination of Wild-Type Rauscher Murine Leukemia Virus Particles Budding from Infected Cells. 116

I. INTRODUCTION

The retroviruses (also called RNA tumor viruses or oncornaviruses) are a group of enveloped animal viruses, some members of which are capable of causing cancer in animals. This group of viruses is characterized by having ribonucleic acid as genetic material and an enzyme capable of reverse transcribing this RNA into a double stranded DNA that subsequently becomes integrated into the host cell genome. The chromosomally integrated DNA form of the viral genome is commonly called the "provirus" or the "proviral DNA".

There are several good introductory references concerning these viruses. A thorough description of the historical development of this field by one of its pioneers can be found in Oncogenic Viruses (1). A modern account of the biology of these viruses is detailed by a group of authors in The Biology of Animal Viruses (2). The molecular biology of this field is introduced by John Tooze in The Molecular Biology of Tumor Viruses (3). A major updating of the molecular biology of tumor viruses took place at the Cold Spring Harbor Symposium on the subject in 1974 (4). David Baltimore's summary of this meeting nicely integrated the prevailing concepts (5). The field was updated again in 1976 at the ICN-UCLA symposium (6,7).

The proteins of the murine leukemia viruses are the subject of several reviews. Recent reviews by Strand et al. (8) and Bolognesi et al. (9,10) are particularly useful. The work on proteolytic processing of precursors to mature virus proteins has been reviewed by Shapiro and August (11). The literature on the

various antigenicities found in viral proteins has been reviewed by Lilly and Steeves (12).

Retroviruses have been associated with malignant neoplasia in numerous vertebrate species and can be isolated from the tissues of these species (1,13-16). Indeed, retrovirus-specific proviral DNA sequences appear to be present in all vertebrate genomes and are inherited by offspring from their parents (13-16). When viral nucleic acid sequences from different species are examined for homology by molecular hybridization, they can be arranged in a phylogeny which parallels the taxonomic phylogeny of those species (13-16). This result indicates that the viral sequences are ancient occupants of animal genomes and not the result of horizontal transmission between species. However there are several exceptions (16).

Much of the genetics and biochemistry of the retroviruses has been elucidated with the avian viruses. The work in the avian system has been facilitated by the availability of large amounts of virus for biochemical and immunochemical analyses and numerous conditional lethal and deletion mutants for genetic analyses (17). Some of these tools are now available in the murine system. Present information indicates that there are several important differences between the avian and mammalian retroviruses. The need to know how the mammalian viruses operate is of obvious importance and justifies a more thorough analysis of the murine system. This thesis deals solely with the murine leukemia viruses (MuLVs) which are members

of the retrovirus group.

A. Structure and Morphology of Retroviruses.

The murine leukemia viruses are chemically composed of 60-70% protein, 20-30% lipid, 2% carbohydrate and 1% RNA (18).

The major RNA component of these viruses is a single stranded molecule which sediments at 60-70S (5,19-22). This 60-70S RNA denatures into two 35S subunits which comprise the viral genome. The 35S subunits are thought to be identical, which suggests that the viral genome is diploid (5,20-22). The 35S subunits also possess features which are characteristic of eukaryotic messenger RNA (mRNA), i.e., they are polyadenylated at the 3' end and have the 7-methyl guanosine "capped" 5' end. In addition, they can be directly translated by ribosomes (section I,E).

In virus producing cells, viral mRNA is transcribed from the integrated proviral DNA and viral proteins are synthesized in the cytoplasm on the host protein synthetic machinery. The viral RNA genome and core proteins are partially assembled at or near the cellular plasma membrane. The maturing virion "buds" from the cell surface, acquiring its envelope from the cell membrane (18). Structural maturation is completed in the virion after it has left the cell (23-25).

By virtue of their morphology as seen with the electron microscope, retroviruses can be classified as type-A, type-B, type-C or type-D particles (18,26,27). The murine leukemia viruses are type-C particles. The envelope of the type-C virion consists of a

unit membrane, the outer surface of which is lined with knob-like structures. Immediately within the envelope is a hexagonal core shell, with an outer capsid made up of subunits. Within the capsid layer of the core shell lies a core membrane (28). Within the capsid and the core membrane is the ribonucleoprotein nucleoid which appears as a spherical, centrally located, filamentous structure (18).

B. The Murine Leukemia Virus Genome.

The MuLVs have three known genes which are called "*gag*", "*pol*" and "*env*" (5). The current genetic and physical maps of the genome, order the genes as follows: 5'-*gag*-*pol*-*env*-c-polyA-3' (29-31). The region designated "c" is common to both leukemia and sarcoma viruses but its function is not known (32,33).

Genetic complexity analysis (hybridization kinetics) indicates that the 35S RNA subunits are about $3x10^6$ daltons (5,20-22). This would mean that the total information content of the genome is sufficient to encode only about 300,000 daltons of protein. The total molecular weight of the known viral encoded proteins is approximately 230,000. Therefore, most of the coding capacity of the genome has already been accounted for in virion proteins (8-10).

C. Murine Leukemia Virus Protein Nomenclature.

In the sections to follow (E, F and G) the literature on the biosynthesis and subsequent function of the viral proteins is surveyed. The nomenclature is that suggested by a committee of the National Cancer Institute (34). The proteins are designated "p";

the glycoproteins "gp", followed by a number indicating the apparent molecular weight in daltons. The precursors to viral proteins are designated "Pr" or "gPr", followed again by the apparent molecular weight with a superscript indicating the viral gene region which encodes the precursor. The products of the *gag* and *pol* regions are described briefly (sections E and F) as background to discussion of these proteins in chapter III. The major portion of the work presented in this thesis involves the product of the *env* gene and this is reviewed thoroughly in section G.

D. General Methodology.

Host protein synthesis is not inhibited when cells are infected with RNA tumor viruses and the synthesis of viral proteins constitutes only a very small percentage of the total. For this reason, studies of viral protein metabolism require the use of antisera to selectively precipitate viral proteins from the vast excess of cellular proteins. The mechanisms of virus protein precursor synthesis and processing have been revealed largely by immune precipitating viral products from pulse-labelled host cells and by cell-free translation of viral mRNAs. The immune precipitated viral products are generally analyzed by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS).

E. Expression of the *gag* Gene.

1. Biosynthesis of the MuLV Core Proteins.

The *gag* gene encodes the proteins of the virus core, p30, p15, p12 and p10. These proteins possess many of the group-

specific antigens of the virus, historically giving rise to the name "*gag*". Translation of full genome length 35S mRNA yields 65,000 dalton ($Pr65^{gag}$) and 75,000 dalton ($Pr75^{gag}$)polyproteins (11,35-47). In some MuLVs $Pr75^{gag}$ is subsequently glycosylated and appears on the cell surface (48), whereas in other viruses it may be degraded or converted to $Pr65^{gag}$ (37). It's function is not clear. $Pr65^{gag}$ is processed, via successive intermediate proteolytic steps, to form the core proteins. Apparently, $Pr65^{gag}$ is assembled directly into budding virions and cleavage to form the mature core proteins takes place predominantly in extracellular virions (23-25). Virus particles are reported to contain a specific proteolytic factor which carries out this cleavage (49). Analysis of processing intermediates has led to the ordering of the protein sequences in $Pr65^{gag}$ as follows: NH_2-p15-p12-p30-p10-COOH (50,51).

2. The Proteins of the Murine Leukemia Virus Core.

p15 is located in the exterior core of the virus (23, 52-54). A recent model for virus assembly suggests that p15, by virtue of its location at the amino-terminus of $Pr65^{gag}$, may be involved in recognition of the virus glycoprotein in the cell membrane and contribute to the organization of a virus bud (23).

p12 is a phosphoprotein located in the virus core that binds specifically to homologous viral RNA. There is no binding demonstrable between heterologous p12 and viral RNA. The stoichiometry of the interaction indicates that each RNA molecule binds fewer than fifteen molecules of p12. The phosphorylation of

p12 influences the extent but not the specificity of the binding (55-58).

p30 is the major core protein of the virion, constituting 10-20% of the total virus protein (8). Amino terminal sequence analysis of several MuLV p30s showed complete homology through twenty-four residues with the exception of position four (59,60). The role of p30 in MuLV host range is discussed in section E3 below.

p10 is located in the virus core and has a non-specific single strand nucleic acid binding activity. In vitro binding is strongly affected by salt concentration (61). Its position at the C-terminus of the $Pr65^{gag}$ suggests a role for p10 in organizing the viral RNA genome into the assembling virus bud (23).

3. Murine Leukemia Virus Tropism.

p30 appears to have a role in determining some of the host range properties of the ecotropic MuLVs (ecotropic MuLVs are those able to infect only mouse cells). The ecotropic MuLVs can be divided into three categories based upon their ability to grow on NIH Swiss cells (N-type) or Balb/c cells (B-type). N-tropic viruses initiate infection more efficiently on N-type cells, whereas B-tropic viruses show a reciprocal pattern. NB-tropic viruses infect both cell types equally well (62). MuLVs are genetically stable in terms of their tropism. However by forced passage through resistant host cells, N and B-tropic viruses can be adapted to become NB-tropic. The N to NB adaptation was made in vivo with Friend virus (63), whereas

B to NB adaptation has only been accomplished in vitro (64,65). The mechanism of this adaptation is not clear. Adaptation of N to B or vice versa has not been reported. A single mouse gene (called Fv-1) controls the susceptibility of host cells to infection by the different classes of ecotropic MuLVs. The Fv-1 gene has two alleles termed Fv-1^n and Fv-1^b. N and B-type cells are homozygous (Fv-$1^{n/n}$ and Fv-$1^{b/b}$) at this locus and resistance to infection is dominant (64). Several lines of evidence indicate that the tropism restriction occurs after virus penetration of the cell and before integration of the provirus (66-68).

A class of MuLV with dual sensitivity to Fv-1 restriction has recently been described (69). Rein et al. used a line of cells which shows no host range restrictions. They dually infected these cells with both N- and B-tropic viruses. The progeny from such a mixed infection were sensitive to Fv-1 restriction in both N-type and B-type cells. Upon cloning, the dually restricted progeny virions were shown to be genetically either N- or B-tropic. Therefore Rein et al. concluded that the dual sensitivity was a phenotypic property endowed by a factor (possibly a protein of the virus) which is not an integral part of the viral genome. In other words, the individual virions released from the mixedly infected cells each contained a mixture of proteins encoded by the two different viral genomes. These "phenotypically mixed" virus particles were unable to efficiently infect either N-type or B-type cells. Therefore, it was concluded that the host-cell restriction mechanism involves

interaction between a cellular inhibitor and one of the viral proteins (69,70).

Hopkins and her co-workers have isolated NB-tropic variants from B-tropic MuLV clones and have examined the p30s (65,71). They found slightly smaller p30s in the NB-tropic viruses. They were also able to distinguish the size of p30s from N and B-tropic viruses (72). In recombinants between N and B-tropic viruses, selected for N-tropism, they found that the p30 always segregated with the tropism marker (72). These experiments strongly suggest that p30 is a determinant in the tropism of ecotropic MuLVs.

F. Expression of the *pol* Gene.

1. The Biosynthesis of Reverse Transcriptase.

The "*pol*" gene encodes the viral polymerase, an RNA dependent DNA polymerase (RDDP). The polymerase is derived from a 200,000 dalton precursor which contains "*gag*" and "*pol*" antigens ($Pr200^{gag-pol}$) (40,45,47). An in vitro translation system programmed by 35S viral RNA can be made to enhance production of $Pr200^{gag-pol}$ by addition of suppressor tRNA's (47). This implies that $Pr200^{gag-pol}$ is formed intracellularly by read-through of an inefficient termination signal situated between *gag* and *pol* sequences on the 35S mRNA.

2. Reverse Transcription.

The murine reverse transcriptase is a single polypeptide of about 70,000 daltons (5,73). In addition to its DNA polymerase activity, the protein has a ribonuclease H activity which degrades the RNA moiety of an RNA-DNA hybrid (74,75). The

murine polymerase is characterized by specific primer, template and cation requirements (73-75). The reverse transcriptase synthesizes a single strand DNA complement of the viral genome, and then degrades the RNA in the hybrid and synthesizes the second strand of the DNA provirus (76). The provirus is then integrated into the host cell genome (77,78). Current evidence indicates that there is a single site on the provirus where integration occurs but many sites in the host cell DNA which can accommodate integration (79,80). The precise details of this process are not yet known.

There is a report in the literature that p30 can complex with reverse transcriptase and stimulate polymerase activity. This is intriguing in light of the probable role of p30 in viral tropism (81).

G. Expression of the *env* Gene.

1. Biosynthesis of the Virus Envelope Proteins.

The "*env*" gene encodes the protein components of the viral envelope gp70* and p15E (the suffix E is used to differentiate this protein from the core protein p15).

Evidence indicates that the envelope proteins are derived from a 90,000 dalton precursor ($gPr90^{env}$) which is translated from a subgenomic 22S mRNA. Van Zaane et al. (46) have isolated

*The term gp70 is used here, generically, to mean the viral envelope glycoprotein. In section I,G3 the term gp69/71 will be introduced. For purposes of this discussion they can be considered synonymous.

polyA containing mRNA from Rauscher MuLV infected cells and separated it according to size on sucrose gradients. The gradients were fractionated and each fraction was injected into *Xenopus laevis* oocytes which are able to faithfully translate exogenous mRNAs. The resulting virus-specific translation products were analyzed by immunoprecipitation followed by electrophoresis in polyacrylamide gels in the presence of SDS. Envelope related products, $gPr90^{env}$, gp70 and p15E, were all synthesized in response to 22S mRNA only. The 35S mRNA fractions from the same gradients only directed the synthesis of *gag* proteins and of *gag-pol* (section I,E). These results also demonstrate that *Xenopus* oocytes are able to carry out the appropriate glycosylation and processing reactions. Previous work by this group demonstrated a 70,000 dalton "*env*" related product, in a reticulocyte cell-free protein synthetic system directed by 22S mRNA, which may have been the unglycosylated form of $gPr90^{env}$ (44). The avian retroviruses also use a 22S mRNA for envelope protein synthesis (82,83).

The derivation of the 22S mRNA is a fascinating problem. The integrated provirus is co-linear with full genome length 35S viral RNA (79) and direct transcription of the provirus yields the 35S mRNA. Recently, an avian 22S RNA has been shown, by RNAse T_1 oligonucleotide mapping, to have oligonucleotides from its 5' end in common with those from the 5' end of the 35S RNA (84). These 5' oligonucleotides are not found anywhere else in the RNAs. The remaining oligonucleotides in the 22S RNA come from the 3' end

of the 35S RNA. This indicates that two non-contiguous regions of the viral genome are joined in the 22S RNA. The RNA "splicing" phenomenon now observed in numerous eukaryotic systems (85-89) seems to be operating here as well. The indications are that the 22S RNA is derived from the 35S RNA by splicing out the *gag* and *pol* regions while retaining some portion of the 5' terminus.

The primary translation product of the 22S mRNA is a glycosylated polyprotein precursor ($gPr90^{env}$) which is subsequently processed to yield gp70 and p15E (90). The details of this process have been revealed by immunoprecipitation of pulse-labelled cell extracts with monospecific antiserum to gp70. The in vivo labelling results are consistent with the in vitro translation data (37,46,91, 92).

The biosynthesis and glycosylation of this viral glycoprotein precursor ($gPr90^{env}$) appears to be typical of N-glycosylated membrane glycoproteins in general and is carried out by the host cell synthetic machinery. Glycoproteins are synthesized on membrane bound polyribosomes (93-95) of the rough endoplasmic reticulum (RER). Radiolabelled $gPr90^{env}$ is found in cells after very brief labelling periods with radioactive amino acids and it is believed that the nascent polypeptide chain is glycosylated while it is still bound to ribosomes. Unglycosylated gp70 related products are only found when cellular glycosylation is inhibited with 2-deoxy-glucose (94, 95). During synthesis the nascent polypeptide chain is passed through the membrane to the extracytoplasmic side of the rough

endoplasmic reticulum. The presence of an amino-terminal "signal" peptide, rich in hydrophobic amino acids, enables the nascent polypeptide to cross the membrane (94-96). On the extracytoplasmic side of the RER the "signal" peptide is removed and "core" carbohydrate structures are added to the polypeptide (97,98). The "core" carbohydrate structure is an oligosaccaride consisting of N-acetylglucosamine and more than five mannose residues. This core is enzymatically transferred (99,100) *en bloc* from a lipid carrier (e.g., dolichol phosphate or retinyl phosphate) to the glycosylation site on the protein. The structure of the site on the protein is typically NH_2-Asn-X-$\overset{Ser}{Thr}$-COOH (X is a variable amino acid) (93,99). N-acetylglucosamine of the core structure is joined in an N-glycosidic linkage to the asparagine side chain (93). A competent glycosylation site requires either a serine or a threonine residue two positions to the carboxyl side of the asparagine. Recently, in vitro membrane protein synthetic systems have been developed which are able to glycosylate and sequester nascent polypeptide chains in membrane vesicles (94,95,101,102). These systems should allow molecular dissection of these events.

Processing of the glycoprotein precursor gPr90^{env} involves further glycosylation as well as proteolysis. The newly formed glycoprotein, now associated with the membrane, migrates via the smooth endoplasmic reticulum to the Golgi apparatus (103). During this transit, sugar residues may be added or removed from the glycoprotein (97). However, terminal sugar addition occurs in

the Golgi apparatus (93,104). While the mature gp70 molecules contain the terminal sugars fucose and sialic acid (N-acetylneuraminic acid), these are absent from $gPr90^{env}$ (92). At some point during these glycosylation events $gPr90^{env}$ is proteolytically cleaved to yield gp70 and p15E which contains no carbohydrate. These two components are found disulfide bonded together in the virus envelope and on the cell surface (105,106). The uncleaved precursor is not found on the cell surface, so the cleavage is a requirement for appearance on the plasma membrane (107). The relationship of the carbohydrate processing and the proteolytic event(s) is not known.

The glycoproteins also enter newly formed lipoprotein membrane in the Golgi apparatus. This new membrane buds off the Golgi as small vesicles which travel to the plasma membrane and fuse with it, causing the new glycoproteins to be incorporated into the plasma membrane with their carbohydrate moieties facing the extracellular side (108).

2. The Biology of gp70.

The envelope glycoprotein component of mammalian RNA tumor viruses plays a major role in MuLV infections and possibly in malignant transformation.

Interaction of the glycoprotein with a cell surface receptor is required for penetration of MuLV into cells (109). DeLarco and Todaro (110) investigated this interaction with Rauscher MuLV (R-MuLV) gp70. They developed an assay to detect the binding of iodinated gp70 (^{125}I-gp70) to cultured cells and observed that

R-MuLV ^{125}I-gp70 bound specifically to murine cells and not to other mammalian cells tested. Monospecific antiserum to gp70, pre-incubated with the ^{125}I-gp70, prevented binding to cells. The ^{125}I-gp70 binding to murine cells was time-dependent, saturable and exhibited first order kinetics at either receptor or gp70 excess. Calcium ions and low pH enhanced the binding. Calculations based on the specific activity of the ^{125}I-gp70 indicated that there were approximately $5x10^5$ binding sites per cell (110,111). Murine cells actively producing ecotropic MuLVs related to Rauscher were unable to bind the ^{125}I-gp70, presumably because the appropriate receptors were already occupied. In other words the receptor sites were no longer accessible when the cell was infected with a related MuLV.

The host range and interference properties of the MuLVs are also determined by the gp70 interaction with cell surface receptors. Ecotropic MuLVs are able to infect only murine cells while MuLVs termed amphotropic can infect cells of other species as well. DeLarco and Todaro showed that murine cells infected with amphotropic MuLV were able to efficiently bind ecotropic ^{125}I-gp70, suggesting that ecotropic and amphotropic gp70s bind to different sets of receptors on murine cells and that binding to one set does not interfere with binding to the other (110). Furthermore, ecotropic and xenotropic MuLVs do not interfere with infection of cells by one another. Xenotropic MuLVs only infect cells of other species. Besmer and Baltimore (112) made pseudotypes of vesicular stomatitis virus (VSV) which had the VSV genome encapsulated in the envelope

of either ecotropic or xenotropic MuLVs. Studies with these pseudotypes showed that the interference patterns were entirely determined by their envelope glycoprotein (112,113). Elder and his co-workers (114) compared the tryptic peptides of ecotropic and xenotropic MuLV gp70s and observed that the different ecotropic gp70s were closely related to one another but were distinct from the xenotropic gp70s. In summary, the limitations on the host range of MuLVs are governed by the interaction of the viral glycoprotein with an appropriate cell surface receptor. Ecotropic MuLV host range is further limited by viral tropism (section I,E3).

As mentioned above, gp70 is a component of the MuLV membrane envelope and it is also a plasma membrane constituent of cells which are producing virus (37,115-119). It has been proposed that gp70 is an integral membrane protein which interacts with the virus core structures on the cytoplasmic side and in this way plays a necessary role in formation of a virus bud (23,120-123). However, evidence concerning the ease with which gp70 is removed from the virus membrane suggests that it may not be an integral membrane protein (54,124-126). As described above, cell surface gp70 also may bind to MuLV receptor sites and thereby block superinfection of cells with related viruses.

Uninfected mouse cells also have gp70-like molecules on their surfaces (127-129). Furthermore, normal uninfected mice appear to harbor a family of genes for glycoproteins closely related to the MuLV envelope gp70. Such glycoproteins can be found in mouse

serum, in epididymal fluid associated with the surface of sperm and on the surface of normal and leukemic thymocytes (114,130-133). Elder and his co-workers (114) compared several of these gp70s by two-dimensional tryptic peptide analysis. This analysis enabled them to directly compare the primary structure of these gp70s and to determine relationships between them. The serum gp70s from several different strains of mice were closely related. They were, in fact, more closely related than were the gp70s from different tissue sources within a single mouse strain. These results are indicative of a multi-gene family in mice which encodes for a polymorphic population of gp70s. In addition, these endogenous gp70s are expressed in different tissues at specific stages of differentiation (134-136). The mouse differentiation antigen G_{IX} is now known to reside on the gp70 molecule (137-139). Furthermore, mice may produce autogenous antibodies against these glycoproteins (140-144). Both T and B lymphocytes of mice have been reported to express a gp70-like antigen on their surfaces when antigenically stimulated (136). This data implicates viral gene sequences, in particular the *env* gene, in normal mouse development.

The MuLV gp70 molecule also appears to play an important role in leukemogenesis. The AKR mice are a "high leukemic" strain of mice with over 90% of them dying of leukemia by five to six months of age. The fatal leukemia is closely associated with the expression of endogenous AKR-MuLV (145). Administration of anti-gp70 serum to AKR mice significantly reduced the incidence of fatal leukemia with

the greatest effect observed when mice were treated at a very early age (146).

Recent examination of some of the most highly leukemogenic strains of MuLV indicates that they are recombinants between less leukemogenic MuLVs and furthermore that the recombination has occurred in the *env* gene. An example of such a high leukemogenic virus is the MCF virus isolated by Staal et al. (147). This virus has host range and interference characteristics of both ecotropic and xenotropic MuLVs. Hopkins and her co-workers (148) examined the ribonuclease T_1 oligonucleotide map of the MCF MuLV and concluded that it contained oligonucleotides characteristic of the endogenous AKR ecotropic virus and an unknown xenotropic virus. Elder et al. (149) applied two-dimensional tryptic peptide analysis to the gp70 of the MCF virus and found that the MCF gp70 was a hybrid which contained sequences characteristic of both the ecotropic and the xenotropic gp70s. These results suggest that the highly leukemogenic MCF virus arose by recombination between two relatively low leukemogenic viruses in the *env* gene. The Friend spleen focus forming virus (F-SFFV) is another example. It causes rapid splenomegaly and leukemia (150). F-SFFV is able to transform hematopoetic cells in the spleens of susceptible mice within forty-eight hours of injection. Using nucleic acid hybridization techniques, Troxler et al. (151,152) showed that F-SFFV was a recombinant, in the *env* gene, between an ecotropic and a xenotropic MuLV.

The realization that some of the highly oncogenic

murine leukemia viruses are *env* gene recombinants has led to speculation concerning how these recombinant gp70s might be related to pathogenesis. Some possibilities suggested by the data are that altered MuLV gp70 expression on cell membranes may be directly involved in leukemogenesis by interfering with cell surface differentiation signals, by changing the properties of cell membranes, or by altering the cellular host range properties of the virus (149).

3. The Biochemistry of gp70.

Despite their biological importance, the molecular properties of MuLV glycoproteins are poorly understood. Methods have been developed to isolate and purify gp70 in order to study its biochemistry and immunology. Simple detergent (SDS, Triton X-100, Nonidet P-40) disruption is commonly used to dissociate all the viral proteins including gp70. A gp70 fraction can be obtained when whole virus is denatured in 6M guanidine hydrochloride and fractionated on a gel filtration column (153). Less severe procedures like freezing and thawing or osmotic shock will liberate much of the gp70 from the virus envelope without denaturation (124-126,141). Isolated gp70 can be further purified on phosphocellulose columns (124,126,154) or on lectin affinity columns (125) which bind its carbohydrate moieties.

Purified gp70 has been biochemically and immunologically analyzed using traditional techniques. N-terminal analysis reveals that alanine is the amino-terminal residue in Rauscher MuLV gp70 (124). The glycoprotein has not been sequenced, but an amino acid

analysis has been reported (124). The carbohydrate composition of gp70 has been determined by labelling with radioactive sugars and by direct quantitation on the amino acid analyzer (92,106,124). The major components are mannose, galactose, glucosamine and sialic acid; small amounts of fucose and galactosamine are also found (124). The total carbohydrate content of gp70 has been estimated to be approximately thirty-two percent of its mass (124), but the structure of the carbohydrate portion is not known. Current evidence on the role of the carbohydrate moieties in the antigenicity of gp70 is in conflict. One group claims no involvement (155-157) while another claims that the carbohydrate is responsible for the interspecies reactivity of gp70 (158).

A second minor glycoprotein found in some MuLV preparations is gp45 which is believed to be a subglycosylated form of gp70. The amino acid compositions of gp45 and gp70 are very similar and the N-terminal alanine is the same, but the carbohydrate content of gp45 is estimated to be only 6% (124). Furthermore, gp70 contains the major antigenic determinants of gp45 (158).

The viral envelope glycoprotein is termed gp70 because it has an apparent molecular weight of 70,000 when electrophoresed in polyacrylamide gels in the presence of SDS. It is recognized that the carbohydrate on the protein causes an erroneously high molecular weight estimate in the presence of SDS (159). The actual molecular weight of gp70 is about 58,000 when determined by analytical sedimentation velocity (125). The molecular weight of the polypeptide

chain alone is thought to be about 45,000 (124).

The envelope glycoprotein of MuLVs and of other mammalian type-C retroviruses frequently separates into two polydisperse components when analyzed by polyacrylamide gel electrophoresis in the presence of SDS (8,126,160). These components have been termed gp69/71 because they migrate in the gels with apparent molecular weights of 69,000 and 71,000 respectively (160). The nature of this doublet has been an important unsolved problem. Previous studies have indicated that these two components could not be distinguished serologically or by tryptic peptide analysis. Elder et al. (114) performed a two-dimensional analysis of the tyrosine containing tryptic peptides after iodination of the gp69 and gp71 components from Moloney MuLV (M-MuLV). They were unable to distinguish the two. It should be noted, however, that the glycopeptides did not migrate in the apolar solvent used for the chromatography dimension and were not resolved. Krantz et al. (161) performed a similar analysis for Rauscher MuLV gp69 and gp71 with the same result. Additionally, Krantz et al. found gp69 and gp71 to have similar immunological reactivities. Based on these results it has been suggested that gp69 and gp71 may contain identical polypeptide chains but different amounts of carbohydrate (91). Work presented in this thesis indicates that this idea is not correct (section IV,A).

H. Thesis Objective and Experimental Rationale.

The objective of the work described in this dissertation was to develop a system which could use the viral proteins as probes

for viral gene functions. At present, a major obstacle to the understanding of mammalian retrovirus genetics is the paucity of conditional lethal mutants in the murine system. What is needed is a way to perform biochemical genetic analyses without the need for a large repertoire of mutants.

The emergence of a two-dimensional electrophoretic system capable of resolving large numbers of proteins provided a powerful tool for this work. The system, developed by Patrick O'Farrell (162, 163), separates proteins by two independent parameters; isoelectric point and molecular weight. In the first dimension, proteins are isoelectric focused under denaturing conditions (9M urea, Nonidet P-40, 2-mercaptoethanol). In the second dimension, proteins are electrophoresed in the presence of SDS and migrate as a function of their size. Both dimensions are performed in polyacrylamide gel. The result is a unique, highly reproducible, two-dimensional map of the protein sample in question. These maps are sensitive to any modifications in protein structure which will affect the net charge or size of the protein. This system allows direct comparison of the proteins of different virus strains as well as normal versus mutant proteins. Mutations in viral proteins which affect their mobility in this system are particularly useful because the altered biological function can be correlated with the altered protein seen in the two-dimensional separation. In this way viral functions can be attributed to viral gene products.

The murine leukemia virus system has additional important

features. Much effort is presently being expended in the study of so-called "tumor viruses" (DNA as well as RNA viruses). The idea, of course, is to understand the mechanisms responsible for malignant transformation of animal cells. We feel that the RNA tumor viruses are the pivotal experimental system because they are the only "tumor viruses" (with the possible exception of Herpes viruses) which clearly cause cancer in their natural host. Another important feature of this system is that it allows manipulation of eukaryotic genes. The MuLV packages its genes and proteins in a convenient particle which is easily isolated and purified. The viral genome can be cloned, mutagenized and subsequently inserted into the host cell DNA. Once integrated into the host genome, viral genes are subject to the normal control and expression mechanisms operating in eukaryotic cells. An example is the case of gp70, a membrane glycoprotein which is implicated in normal differentiation and in leukemogenesis. The expression of gp70 is probably typical of eukaryotic membrane proteins in general and can be used as a model for studying membrane glycoprotein synthesis in mammalian cells.

I. Organization of the Thesis.

The results presented in this thesis are organized into three chapters. The first chapter (section III) develops the use of the O'Farrell two-dimensional gel system to study murine leukemia virus proteins. The second chapter (section IV) describes biochemical and genetic characterization of the MuLV envelope glycoprotein gp70. The third chapter (section V) describes experiments with a temperature

sensitive mutant of the MuLV gp70. The respective results are discussed independently at the end of each chapter.

II. MATERIALS AND METHODS

A. Materials.

The following materials for cell culture were obtained from Grand Island Biological Company: cell culture medium, fetal calf serum, antibiotics, and trypsin-EDTA solution. The source of other materials used in this study are indicated in the text of this chapter.

B. Cell Culture.

Eveline II cells were kindly provided by D. Bolognesi, Duke University Medical Center, Durham, NC. These cells, which were originally derived by infection of STU mouse cells with Friend virus* (164), produce large amounts of F-MuLV and negligible amounts of spleen focus forming virus (F-SFFV) (41,164,165). The cells were grown as suspension cultures in Dulbecco's Modified Eagle Medium (dry powdered medium) containing 10% complement inactivated (30 min at 56°) fetal calf serum and 0.62 g $NaHCO_3$ per liter. Penicillin (100 units/ml), streptomycin (100 µg/ml) and sometimes gentamycin (Schering, 100 µg/ml) were added as antibiotics. Sixty milliliter cultures growing in 250 ml Erlenmeyer flasks (Corning) were passaged at two- to three-day intervals. The cell concentration was maintained between 6×10^5 and 3×10^5 cells/ml.

The NRK and Sc-1 cells were provided by Contract E-73-2001-N01 within the Special Virus-Cancer Program, National Institutes of Health,

*Friend virus is a complex of an MuLV (F-MuLV) and a replication defective spleen focus forming virus (F-SFFV). The nomenclature is modified from that of Steeves (150).

Public Health Service, through the courtesy of Jack Weaver at the Cell Culture Laboratory, University of California School of Public Health. The Sc-1 cell line was derived from a fetal mouse embryo (166). This cell line exhibits no Fv-1 restriction to N- or B-tropic murine leukemia viruses and is apparently free of endogenous MuLV expression. The cells were maintained in monolayer culture with McCoy's Modified 5A Medium supplemented with 10% complement inactivated fetal calf serum and antibiotics. The NRK cells (normal rat kidney) were maintained in monolayer culture with Minimal Essential Medium (Eagle) supplemented with 10% complement inactivated fetal calf serum and antibiotics.

S^+L^- cells were kindly provided by P. J. Fischinger, National Institutes of Health. The cells were grown in monolayer culture with McCoy's Modified 5A Medium supplemented with 10% fetal calf serum, antibiotics and 0.25 µg/ml fungizone (GIBCO).

NIH/3T3 cells chronically infected with wild-type Rauscher murine leukemia virus (R-MuLV) and a temperature-sensitive mutant of R-MuLV (ts26) were kindly provided by Stuart Aaronson, National Institutes of Health. These cells were grown in monolayer culture with Dulbecco's Modified Eagle Medium containing 10% complement inactivated fetal calf serum and antibiotics.

All the monolayer cell cultures were maintained in 25 cm^2 plastic T-flasks (Falcon). For transfers, the medium was decanted and the monolayer was rinsed twice with 0.7 ml of trypsin-EDTA solution (0.5 g/liter trypsin, 0.2 g/liter EDTA). Trypsin-EDTA

(0.7 ml) was added a third time and incubated at 37°C for approximately five minutes, after which 2.8 ml of fresh culture medium was added. The cells were disaggregated by vigorously drawing into a Pasteur pipette and were then counted in a hemocytometer. For regular transfers approximately 0.5 - 2 x 10^5 cells were seeded into 25 cm^2 flasks and adjusted to 5 ml with culture medium. The cultures were incubated at 37° in a humidified 5% CO_2 atmosphere for 24 hours, after which the caps were closed. The cultures were transferred in this manner once a week. MuLV infected cell lines were maintained in the same way as the uninfected cells. Large volumes of cells were grown in roller bottles (Corning) with the appropriate culture medium.

The Friend virus-induced erythroleukemia cells (line F4-6/K) (167-169) were generously donated by W. Ostertag, Max-Planck Institut fur Experimentelle Medizin, Gottingen, Germany.

C. Virus Methodology.

1. Assays of MuLV Components.

The titer of MuLV was measured using a variation of the S^+L^- assay of Bassin et al. (170). S^+L^- cells ($2x10^5$) were seeded into 25 cm^2 tissue culture flasks and the culture volume adjusted to 5 ml with McCoy's Modified 5A Medium supplemented as described above. After 24 hours of incubation in a humidified 5% CO_2 atmosphere at 37°C, the medium was removed and replaced with 1 ml of medium containing 25 µg/ml of DEAE-dextran (Sigma) and incubated for 30 minutes at 37°C. The DEAE-dextran solution was removed, a

0.5 ml virus sample was added, and the flasks were incubated for another 30 minutes. Finally, 5 ml of fresh culture medium was added and the flasks were incubated, tightly closed, at 37°C. After three days an additional 3 ml of medium was added. Macroscopic foci were counted under a dissecting microscope 5-7 days after sample application.

The titer of spleen focus forming virus (F-SFFV) was assayed in 6- to 8-week-old NIH Swiss female mice by the spleen focus assay of Axelrad and Steeves (171). Mice were injected intravenously in the tail vein with a virus sample adjusted to 0.5 ml in Eagle's Medium. Three dilutions of each virus sample were assayed in duplicate (six mice/sample). After nine days the mice were sacrificed, and the spleens were removed and fixed in Bouin's solution (0.015% picric acid, 9.25% formaldehyde, 5% acetic acid). The foci on the spleen surface measuring greater than 0.5 mm in diameter were counted.

2. Virus Infections and Cloning.

The Eveline II virus (EIIV) was cloned on Sc-1 cells. Medium from a logarithmically growing Eveline II cell suspension culture was centrifuged at 4,000 rpm to remove the cells and at 10,000 rpm to remove debris, and was then diluted (125 X) with fresh culture medium. The dilution reduced viral infectivity from 250,000 foci/ml to 2,000 foci/ml. Viral infectivity was determined using the S^+L^- assay (section I,C1). Then the medium was removed from an Sc-1 culture seeded the previous day with 2 x 10^5 cells (25 cm^2 T-flask) and the monolayer was overlayed with 1 ml DEAE-dextran (25 µg/ml) in culture medium and incubated at 37°C for 30 minutes. The DEAE-dextran

was removed and 0.5 ml of the dilute Eveline II virus inoculum was put onto the cells and incubated for 90 minutes at 37°C. Following the incubation period, the inoculum was removed and the cells were dispersed with trypsin-EDTA solution and diluted with medium to 200 cells/ml. Then 40 cells (0.2 ml) were seeded into each well of a microtiter plate (Falcon Plastics) and incubated at 37°C in a humidified 5% CO_2 atmosphere. When the cells in the microtiter wells were 60-70% confluent, the medium was removed for S^+L^- assay of viral infectivity and fresh medium was added to the cells. Cells were maintained in the wells an additional 5-6 days, until the results of the S^+L^- assay were known. Then cells from virus-producing wells were grown up to 25 cm^2 cultures and were maintained like the uninfected Sc-1 cells. These procedures were designed to give multiplicities of infection of 5×10^{-3}, although in practice they appeared to be somewhat lower (5×10^{-4}).

The Eveline II virus clones were assayed for the presence of F-SFFV using the spleen focus assay of Axelrad and Steeves (section II,C1). All the clones were found to be free of F-SFFV.

The EIIVSc-1 line was constructed simply by infecting Sc-1 cells with whole (uncloned) Eveline II virus essentially as described above. Medium from logarithmically growing Eveline II cells was clarified by centrifugation, adjusted to 8 µg/ml polybrene (Aldrich), and inoculated onto an Sc-1 monolayer. None of the steps designed to yield a clone were used, i.e., dilution of culture medium or

dispersal of infected cells.

The B4NRK cell line was constructed by infecting NRK cells with the virus produced by the B4Sc-1 line. B4Sc-1 is an Eveline II virus clone growing in Sc-1 cells.

3. Harvest and Purification of Virus.

Virus was harvested from logarithmically growing cell cultures (167,172). The procedures for harvesting and purification of virus have been described previously (41). Simply stated, the virus was pelleted from the culture medium by high speed centrifugation and was then centrifuged to equilibrium in a 15-60% sucrose density gradient. The virus banding at 1.15 to 1.16 g/cm^3 was collected and repelleted. Isotopically labelled MuLV was located in sucrose gradients by fractionating the gradients and quantitating the radioactivity in each fraction. A 10 µl sample of each 1 ml gradient fraction was added to 5 ml Aquasol (New England Nuclear) and counted in a Packard Tri-Carb scintillation spectrometer.

4. Radioactive Labelling of Virus Proteins.

Viral proteins were labelled with L-[^{35}S]methionine, L-[^{3}H]leucine or D-[^{3}H]glucosamine (New England Nuclear). Amino acid labelling was performed by incubating virus-producing cells in Minimum Essential Medium (GIBCO) containing 10% dialyzed fetal calf serum but lacking the appropriate amino acid and supplemented with the isotopically labelled amino acid. The labelled amino acids were included at concentrations of 15-20 µCi/ml. Before labelling, the cells were washed with phosphate buffered saline

(GIBCO). The cells were then incubated with the radioactive medium at 37°C in a humidified 5% CO_2 atmosphere for 2 hours, and the radioactive medium was then removed and replaced with fresh complete medium. The cultures were further incubated for 18-24 hours, and the virus harvested (section II,C3). D-[^{3}H]glucosamine labelling was accomplished simply by adding the label to complete medium (20 μCi/ml) and incubating the cells for 8-18 hours, after which time the virus was harvested. Two-dimensional electrophoretic (section II,F2) analysis of the virions labelled with D-[^{3}H]glucosamine showed that all detectable label (>95%) was incorporated into the envelope glycoproteins gp69/71. In agreement with previous studies, the Eveline II cells were relatively active in MuLV synthesis (41,164,165) and incorporation of D-[^{3}H]glucosamine into glycoproteins. In contrast, the B4Sc-1 cells incorporated only small amounts of D-[^{3}H]glucosamine into glycoproteins.

The radioactivity in a labelled virus preparation was determined by solubilizing the pellet in lysis buffer (section II,F2) and trichloroacetic acid precipitating an aliquot (2-5 μl) with two drops of 2% bovine serum albumin added as carrier. The precipitate was collected on a glass fiber filter (Whatman) and digested with Protosol (New England Nuclear):toluene (1:2) for 30 minutes in a scintillation vial. After addition of 5 ml of toluene-acetic acid scintillation fluid (Baker scintillation grade toluene containing 0.3% 2,5-diphenyloxazole, 0.03% p-Bis[2-(5-phenyloxazoly)]-benzene, and 0.1% glacial acetic acid), the sample was counted in

a Packard Tri-Carb scintillation spectrometer.

D. Murine Leukemia Virus Protein Purification.

1. Phosphocellulose Column Chromatography.

The virion proteins p15 and p30 were among those purified by a modification of the procedure of Strand and August (154). Approximately 45 mg of purified virus (section II,C3) was resuspended in 20 ml of 0.02 M Tris-HCl, pH 7.6, 0.001 M EDTA and 0.1 M NaCl buffer solution. The suspension was frozen and thawed and centrifuged at 230,000 x g for 45 minutes. This "freeze-thaw" pellet was suspended in 4 ml of a buffer solution containing 0.005 M Tris-HCl, pH 9.2, 0.001 M EDTA and 0.2 M KCl and sonicated. Triton X-100 was added to a final concentration of 1% and the suspension incubated at 37°C for 20 minutes. Following incubation, the suspension was centrifuged at 12,100 x g for 20 minutes. The supernatant from this extraction was saved and the pellet extracted a second time in 2 ml of 0.005 M Tris-HCl, pH 9.2, 0.001 M EDTA but lacking KCl. The supernatants from the (+) and (-) KCl extractions were combined and dialyzed against 0.01 M BES [N,N-bis(2-hydroxyethel)-2-aminomethane sulfonic acid], pH 6.5, 0.001 M EDTA and 0.4% Triton X-100. The dialyzed extract was then applied to a phosphocellulose (Whatman P-11) column which had been equilibrated with the dialysis buffer. The column was eluted with 200 ml of solution containing 0.01 M BES, pH 6.5, 0.001 M EDTA and a linear gradient of 0 to 1.0 M KCl. The p30 eluted in fractions containing 0.2 to 0.3 M KCl and the p15 eluted in the

0.35 to 0.65 M KCl region. The p30 and p15 containing fractions were pooled separately and precipitated by addition of $(NH_4)_2SO_4$ to 75% saturation. The precipitates were collected by centrifugation, suspended in 0.3 ml of 0.01 M BES, pH 6.5, 1.0 M NaCl and 5% glycerol and applied to a sephadex G-75 (Pharmacia) column equilibrated and eluted with the same buffer. The resulting protein components were judged to be homogeneous by polyacrylamide gel electrophoresis in the presence of SDS.

During this procedure protein was assayed by a modification of the method of Schaffner and Weissman (173). Protein samples were adjusted to 0.25 ml with water. Fifty microliter of 0.6 M Tris-HCl, pH 8.0, 0.6% SDS was added to each sample and mixed. One hundred microliter of 50% trichloroacetic acid (TCA) was added to each sample and incubated on ice for 5 minutes. The precipitates were filtered on 0.45 µm Millipore filters and washed with 5% TCA. The filters were placed in scintillation vials and stained for 10 minutes with a 0.2% solution of Amido-Schwartz 10B (Allied Chemical) in 45% methanol and 10% acetic acid. Then the stain was removed and the filters rinsed twice with water. The filters were destained with three rinses of 90% methanol, 2% acetic acid solution. The destaining procedure should leave the borders of the filter white. The filters were then rinsed twice with water and drained completely. The stain remaining bound to the protein precipitate on the filter was eluted with 1 ml of 0.025 M NaOH, 0.00005 M EDTA, 50% ethanol for 10 minutes or until all the stain had eluted. The scintillation

vials were capped during the elution step. The protein in each sample was quantitated from the absorbance at 630 nm of the eluted stain relative to a bovine serum albumin standard curve.

2. Guanidine Hydrochloride Column Chromatography.

Gel filtration of viral proteins in guanidine hydrochloride (GuHCl) was performed according to the procedure of Nowinski et al. (153). Radiolabelled viral pellets (section II,C3) were solubilized in 1 ml of a saturated guanidine hydrochloride solution (pH 8.6) containing 2% 2-mercaptoethanol and 10% sucrose and were heated at 100°C for 3 minutes. Samples were then applied to an agarose (BioGel A-5m, 200 to 400 mesh) column (1 x 100 cm). The column was equilibrated and eluted with 6 M guanidine hydrochloride, 0.01 M dithiothreitol, pH 6.5, at room temperature and a 0.5 ml/hr flow rate. Fractions (0.5 ml) were collected and a 50 µl aliquot of each was counted in 5 ml Aquasol (New England Nuclear) in a Packard Tri-Carb liquid scintillation spectrometer.

Column fractions containing radioactive viral proteins were pooled and exhaustively dialyzed against 0.05 M NH_4HCO_3. Following dialysis, the samples were lyophilized and the residue solubilized in lysis buffer (II,F.2) for two-dimensional electrophoresis.

E. Immune Precipitation Procedures and Cellular Labelling.

Procedures for labelling cells with L-[^{35}S]methionine and for secondary immune precipitation of cell extracts with monospecific antisera to gp69/71 have been described previously (41). Antiserum

to Eveline II virus gp69/71 was generously donated by J. Collins, Duke University, Durham, NC, and was shown to be monospecific as described previously (41). Simply stated, labelled cells were extracted with immune buffer A (0.01 M NaH PO_4[pH 7.6], 0.001 M disodium EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate). An aliquot of the cell extract was incubated with primary antiserum for 1 hour at 4°C. Then the secondary antiserum (rabbit anti-goat IgG) was added and incubation continued overnight. The precipitates were collected by centrifugation and washed extensively. The radioactivity in the immune precipitates was quantitated during the final wash step. The pellet was resuspended in a known volume of wash buffer (usually 0.5 ml) and 20 µl was removed and counted in 5 ml Aquasol. The final precipitate was drained and dissolved in electrophoresis sample buffer as described below (section II,F1).

Cell surface labelling with [^{125}I]iodine was performed at 0°C by a modification (133) of the method of Vitetta et al. (174). Briefly, cells (5 x 10^7) were suspended in 1 ml of phosphate buffered saline (GIBCO) containing 100 µg of lactoperoxidase (Worthington) and 3 mCi of [^{125}I]iodine (New England Nuclear). Two additions of 0.06% hydrogen peroxide were made to the reaction mixture at 5 minute intervals. Growing cell cultures were used for the labelling. The labelled cells were extracted and immune precipitated as described (41).

F. Polyacrylamide Gel Electrophoresis.

1. One-Dimensional SDS-Urea Electrophoresis.

The procedures for one-dimensional sodium dodecyl sulfate-urea polyacrylamide gel electrophoresis have been described previously (41). The procedure is essentially a modification of the method of Laemmli (175). Immune precipitates were dissolved in sample buffer containing 0.05 M Tris-hydrochloride (pH 6.8), 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 20% glycerol, and 6 M urea and heated at 100°C for 2 minutes. The dissolved immune precipitates were then electrophoresed on slab gels. The slabs consisted of a 5% polyacrylamide, 0.1% sodium dodecyl sulfate, 6M urea stacking gel and a 10% polyacrylamide, 0.1% sodium dodecyl sulfate, 8 M urea separating gel. Following electrophoresis, the gels were fixed in 12.5% trichloroacetic acid for several hours and processed for fluorography according to the procedure of Bonner and Laskey (176).

2. Two-Dimensional Isoelectric Focusing/Sodium Dodecyl Sulfate Electrophoresis.

Virion proteins were analyzed in two-dimensional polyacrylamide gels (O'Farrell gels) using isoelectric focusing in the first dimension and sodium dodecyl sulfate (SDS) electrophoresis in the second dimension. Modifications made to the isoelectric focusing dimension are described below; the stock solution nomenclature is O'Farrell's (162,163). In all cases the urea (ultrapure, Schwarz/Mann) concentration was reduced from 9.5 M to 9 M. The Ampholines used, at a final concentration of 1%, were pH ranges 3.5-10, 9-11, 4-6,

5-7 in the ratio 14:2:1:1. This Ampholine composition was used in the lysis buffer, the sample overlay solution and in the isoelectric focusing gels. Five milliliters of isoelectric focusing gel mixture (Table 1) contained 2.7 g urea, 0.667 ml acrylamide stock solution, 1 ml Nonidet P-40 (Particle Data Lab) stock solution, 0.835 ml water and 0.116 ml of Ampholines mixed in the above ratio. This gel mixture was polymerized by addition of 6 µl of ammonium persulfate stock solution and 4 µl of TEMED (Eastman Kodak). The gels were poured to 11 cm in length and overlayed with 8 M urea. After 12 hours this overlay was removed and replaced with lysis buffer. The gels were used 18-24 hours after pouring. The anode electrode solution was 1 M H_3PO_4 and the cathode electrode solution was 1 M NaOH. These modifications produced a stable pH gradient from pH 3 to pH 10 (Figure 1A), substantially broader than that obtained by O'Farrell. Virus protein samples were isoelectric focused for 15 hours at 400 V followed by 2 hours at 800 V.

An exponential acrylamide gradient (10 to 20%) was used in the second dimension gel slab. The gradient slab is formed by mixing 10% and 20% acrylamide solutions (Table 2). Calibration of this dimension with protein molecular weight standards revealed a linear relationship between the logarithim of molecular weight and distance migrated (Figure 1B). After electrophoresis the gel slabs were fixed overnight in 12.5% trichloroacetic acid and were processed for fluorography (176).

All the two-dimensional gels presented in figures are

Table 1. Composition of First-Dimension Isoelectric Focusing Gels.

Stock Reagent[a]	Amount for 5 ml Gel Mixture	Final Concentration
solid urea	2.70 g	9 M
acrylamide (30%)	0.667 ml	4%
Nonidet P-40 (10%)	1.0 ml	2%
Ampholines[b]		
pH range 3-9.5	90 µl	
pH range 9-11	12 µl	
pH range 4-6	7 µl	
pH range 5-7	7 µl	
Total	116 µl[c]	2%
Water	0.985 ml	-
ammonium persulfate (10%)	6 µl	-
TEMED	4 µl	-

[a]The composition and nomenclature of the stock reagents are as described by O'Farrell (162,163).

[b]Ampholines are supplied by LKB as 40% solutions except pH range 9-11 which is 20%; they are used as supplied.

[c]It is most convenient to add 0.116 ml of an Ampholine mixture made from pH ranges 3-9.5/9-11/4-6/5-7 in the ratio 14/2/1/1.

Table 2. Composition of Second-Dimension Polyacrylamide Gradient Gel[a]

Stock Reagent[b]	10% Acrylamide Solution	20% Acrylamide Solution
lower gel buffer	4.5 ml	1.5 ml
acrylamide (30%)	6.0 ml	4.0 ml
glycerol	-	0.5 ml
water	7.5 ml	-
ammonium persulfate (10%)	30 µl	10 µl
TEMED	9 µl	3 µl

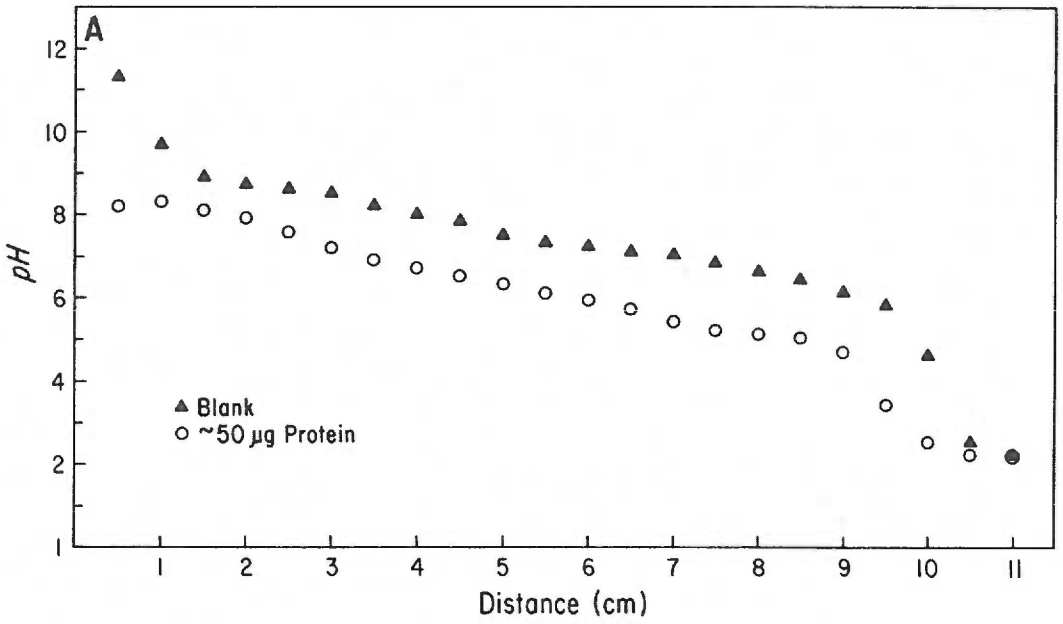
[a]The second-dimension slab gel is an exponential acrylamide gradient from 10 to 20% poured with a gradient maker.

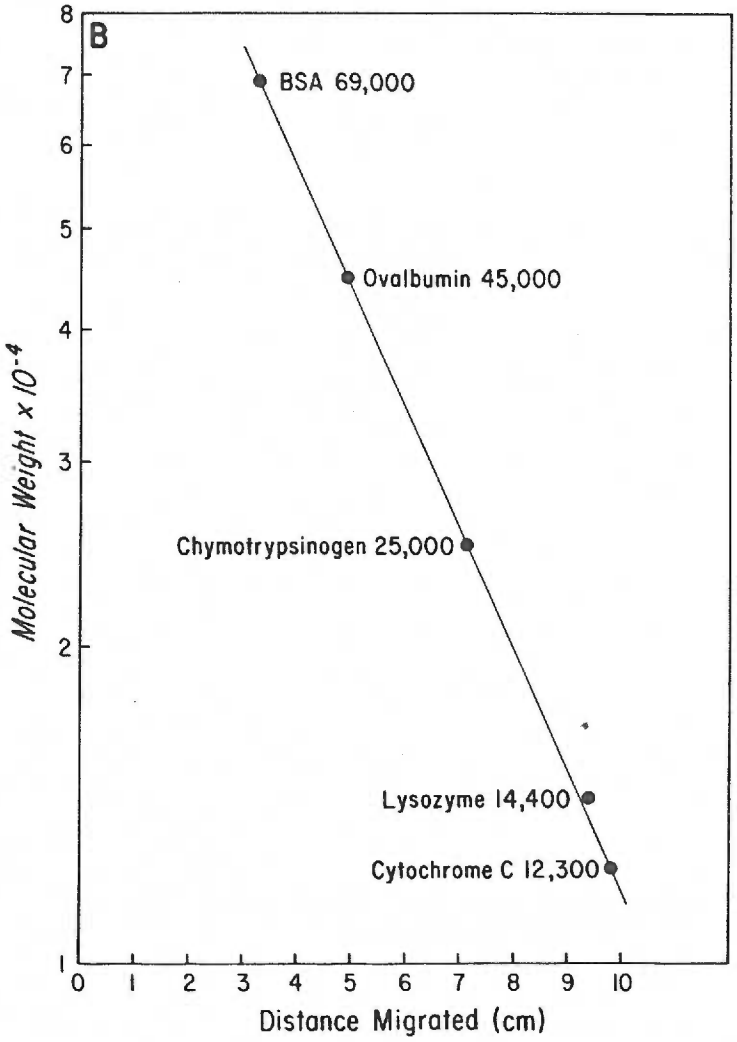
[b]The composition and nomenclature of the stock reagents are as described by O'Farrell (162,163).

Figure 1. Calibration of the Two-Dimensional Polyacrylamide Gels.

Panel A. The pH gradient developed in the first-dimension isoelectric focusing gels. The first-dimension isoelectric focusing gels were prepared as described by O'Farrell with the modifications described in section II,F2. Approximately 50 µg of a protein mixture was applied to one gel, while another was left "blank". Isoelectric focusing was carried out for 15 hr at 400V followed by 2 hr at 800V (7600Vhr total). The focused gels were sectioned into 0.5 cm pieces. Each section was placed into one of the wells of a microtiter plate (Falcon) with 40 µl of water and the microtiter plate was agitated for 1 hr. The pH of the solution in each well of the microtiter plate was then measured with a microelectrode. ▲, blank gel; O, gel with approximately 50 µg protein.

Panel B. Molecular weight calibration of the second-dimension gel. Standard proteins (5 µg of a standard mixture) of known molecular weight were electrophoresed in the second-dimension polyacrylamide gradient gel in the presence of 0.1% sodium dodecyl sulfate. Following electrophoresis, the gel was fixed in trichloroacetic acid and stained with Coomassie Blue as described by O'Farrell (162,163). The logarithm of the molecular weight of each standard protein is plotted as a function of the distance migrated in the gel.


A
pH
12
10
8
6
4
2
1
Blank
~50 µg Protein
1
2
3
4
5
6
7
8
9
10
11
Distance (cm)


B
Molecular Weight x 10⁻⁴
8
7
6
5
4
3
2
1
BSA 69,000
Ovalbumin 45,000
Chymotrypsinogen 25,000
Lysozyme 14,400
Cytochrome C 12,300
0
1
2
3
4
5
6
7
8
9
10
Distance Migrated (cm)

oriented with the high pH (basic) region to left and the low pH (acidic) to the right; the high molecular weights are at the top.

G. Cellulose Acetate Electrophoresis.

After neuraminidase treatment described below, the D-[^{3}H]glucosamine-labelled virus samples were adjusted to 8 M urea (deionized) and were electrophoresed on 2.5 cm x 18 cm cellulose acetate strips (Shandon) at 200 volts for 420 minutes. Approximately 1500 cpm in 3 µl was applied to the origin of each strip. The electrophoresis buffer was 0.0645 M Tris-HCl, 0.0016 M EDTA, 0.05 M boric acid, 8 M urea and 0.025% mercaptoethanol (v/v), pH 8.9. After electrophoresis the strips were treated with 5% trichloroacetic acid to fix the glycoproteins and to remove free sialic acid, and 0.5 cm sections were digested with Protosol (New England Nuclear): toluene (1:2) in scintillation vials. After addition of 5 ml of toluene-acetic acid scintillation fluid (Baker scintillation grade toluene containing 0.3% 2,5-diphenyloxazole, 0.03% p-Bis[2-(5-phenyloxazoly)]-benzene, and 0.1% glacial acetic acid), each section was counted in a Packard Tri-Carb liquid scintillation spectrometer.

H. Neuraminidase Treatment.

The protease-free neuraminidase [mucopolysaccharide N-acetylneuraminylhydrolase, (3·2·1·18) from *Vibrio Cholerae*] was obtained from Behringwerke (Marburg/Lahn, Germany). D-[^{3}H]glucosamine labelled virus (20,000-40,000 cpm) was suspended in 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4 and was incubated at 37°C for 2 hours in a shaking water bath with at least 10 units of enzyme. The samples

were then rapidly frozen. Control samples were incubated in parallel without neuraminidase. The percent of D-[^{3}H]glucosamine label in the radioactive glycoprotein which was incorporated after conversion to sialic acid (N-acetylneuraminic acid) was determined by quantitating the amount of radioactivity which became soluble in 5% trichloroacetic acid after neuraminidase treatment.

I. Tryptic Glycopeptide Analysis.

1. DEAE-Sephadex A25 Chromatography.

After the neuraminidase treatment described above, the D-[^{3}H]glucosamine-labelled samples were lyophilized and the residue was solubilized in 200 µl of 0.05 M ammonium bicarbonate, pH 8.5. Trypsin-TPCK (25 µg) (Worthington) was added and the samples were incubated at 37°C for 24 hours. The resulting digest was lyophilized and the residue was dissolved in 2 ml of 50 mM Tris-HCl, pH 8.5. The tryptic glycopeptide samples were applied to a DEAE-Sephadex A25 (Pharmacia) column (0.9 x 20 cm) and were eluted with a linear 200 ml 0-0.1 M NaCl gradient (177). The flow rate was approximately 1 ml/min and 1.8 ml fractions were collected. Each fraction was added to 15 ml Aquasol (New England Nuclear) and was counted in a Packard Tri-Carb liquid scintillation spectrometer. The large amount of water present reduced the counting efficiency by 25-30%. The peak which emerged from this column at 40-50 ml was identified as free sialic acid by co-chromatography with [^{14}C]-N-acetylneuraminic acid (Amersham-Searle).

2. Paper Electrophoresis.

After neuraminidase treatment 50 µg of carrier hemoglobin was added to the D-[^{3}H]glucosamine-labelled virus samples. The samples were precipitated with cold 5% trichloroacetic acid and the precipitates were washed with acetone. Trypsinization was carried out as described above (section II,I1). The lyophilized tryptic glycopeptides were solubilized in electrophoresis buffer (pyridine/acetic acid/water, 10/0.4/89.6) and were electrophoresed at 3000 volts for 3 hours on strips of Whatman 3 MM paper (3 x 60 cm).

III. MURINE LEUKEMIA VIRUS-SPECIFIC PROTEINS: IDENTIFICATION BY TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

As described above, there are seven proteins which are known to be encoded by the MuLV genome. These include the internal core (or *gag*) proteins p30, p15, p12 and p10, the membrane envelope proteins gp70 and p15E, and reverse transcriptase. The combined molecular weight of the polypeptide chains of these proteins is approximately 230,000. Since the MuLV genome is sufficiently large to encode approximately 300,000 daltons of protein (6,8) it is possible that there are additional MuLV encoded proteins which have not yet been identified. Conceivably such protein(s) could be incorporated into the MuLV particles or they could occur only within the infected cells.

In addition to these seven virus encoded proteins, it is believed that MuLV particles contain other proteins which are encoded by the host cell (8,24,178). For example, host cell enzymes occur in MuLV preparations (24). It is not known whether these are entrapped randomly from the host cell cytoplasm when the virions are formed or whether they are specifically incorporated into the virus particles. However, it is known that specific cellular transfer RNAs are selectively incorporated into virions and perform a crucial role in reverse transcription of the virion genomic RNA (179-181). Conceivably some of the host cell proteins also perform essential roles in the MuLV infection process.

In this section I describe an attempt to systematically

analyze the protein components of MuLV particles. The analysis is based on a high resolution two-dimensional protein separation technique in which the proteins are fractionated according to their isoelectric points (first dimension) and molecular weights (second dimension) in polyacrylamide gels. Theoretically, this technique should permit resolution of all virion proteins and provide some indication of their relative abundance in the virions. Furthermore, by comparing the proteins of virions grown in cells from different species we hoped to be able to distinguish between virus and cell encoded virion proteins. Since the position of a protein in these gels should be sensitively affected by many changes in the amino acid sequence of the protein, we also hoped to use this method to identify proteins altered in variant or mutant viruses. By correlating the protein altered in a mutant with the biological effect, we hoped to be able to determine the functions of the different virus-encoded proteins. Progress toward all of these goals is described in this section.

A. Two-Dimensional Map of Murine Leukemia Virus-Specific Proteins.

In order to use the O'Farrell (162,163) gel system for genetic analysis, it was necessary to generate a two-dimensional map of virion proteins and to determine which of these proteins are "virus-specific". To eliminate possible complications caused by multiple viral genomes, we employed a single clone of MuLV (clone B4) which was prepared from Eveline II virus (section II,C2).

The cloned virus was grown either in murine Sc-1 cells or in rat NRK cells. The resulting virus preparations are termed B4Sc-1 and B4NRK, respectively.

Figure 2 is a two-dimensional map of B4Sc-1 virion proteins. The B4Sc-1 culture was labelled with L-[^{3}H]leucine (section II,C4). The labelled virus was harvested, isopycnically banded in a sucrose gradient and electrophoresed on a two-dimensional gel. The gel was fluorographed and the resulting fluorogram* is shown in Figure 2. The predominant feature of the map is its complexity. There are approximately 80 to 100 proteins. This is many more than the known virus encoded proteins and is more than the MuLV genome can encode (6,8). The obvious question is which of these proteins is encoded by the virus genome and which by the host genome?

Two approaches were used to distinguish between host-cell and virus encoded proteins. The B4NRK virus produced by rat cells is shown in Figure 3 (top panel). There are many proteins in this map which migrate like those in the B4Sc-1 virus map. These similarities include spots which will be identified below as known viral proteins (section III,B). The second approach was to examine radioactive proteins shed from uninfected Sc-1 cells labelled with L-[^{3}H]leucine. The culture medium from these cells was taken through

*The second-dimension slab gels were processed for fluorography by the procedure of Bonner and Laskey (176). The process involves impregnating the polyacrylamide gel with PPO before overlaying it with X-ray film. The resulting exposure is termed a fluorogram.

Figure 2. Two-Dimensional Polyacrylamide Gel Electrophoresis of Clone B4Sc-1 Virus.

Virion proteins were radioactively labelled with L-[^{3}H]leucine as described in Materials and Methods (section II, C4). Purified B4Sc-1 virus was solubilized and electrophoresed in two-dimensions essentially as described by O'Farrell (162,163) with the modifications described in Materials and Methods (section II,F2). Following electrophoresis, the second-dimension slab gel was processed for fluorography (176). The resulting fluorogram is shown in this figure. The figure is oriented with the high pH end to the left and the low pH end to the right.

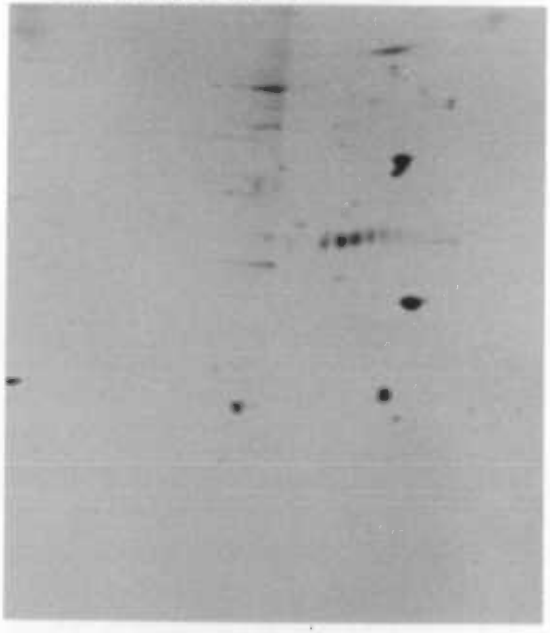
Clone B4Sc-1

Figure 3. Two-Dimensional Polyacrylamide Gel Electrophoresis of Clone B4NRK Virus and Proteins Harvested from Uninfected Sc-1 Cells.

Proteins were radioactively labelled with L-[^{3}H]leucine as described in Materials and Methods (section II,C4). Purified B4NRK virus (top panel) and material harvested from uninfected Sc-1 cells (bottom panel) were independently solubilized and electrophoresed in two-dimensions essentially as described by O'Farrell (162,163) with the modifications described in Materials and Methods (section II,F2). Following electrophoresis, the second-dimension slab gels were processed for fluorography (176). The resulting fluorograms are shown in this figure. The panels are oriented with the high pH end to the left and the low pH end to the right.

Clone B4NRK

Uninfected Sc-1



the harvest procedures used for MuLV (section II,C3) and a radioactive band was found in the sucrose gradient very close to where the MuLV bands. The material in this band was collected and analyzed on two-dimensional gels. The resulting fluorogram is shown in Figure 3 (bottom panel). Several prominent spots in the B4Sc-1 map are coincident with spots in this uninfected Sc-1 cell protein pattern. Spots very similar to these are also seen in the B4NRK virus map. Since these proteins can be harvested from uninfected Sc-1 cells, they must be encoded by the host cell. Furthermore, any other spots which are not coincident in the B4Sc-1 and B4NRK maps are not likely to be MuLV encoded. This argument is not airtight because the same MuLV gene product might be processed or modified differently in different host cells. However, our other results suggest that this is not the case (section IV,A).

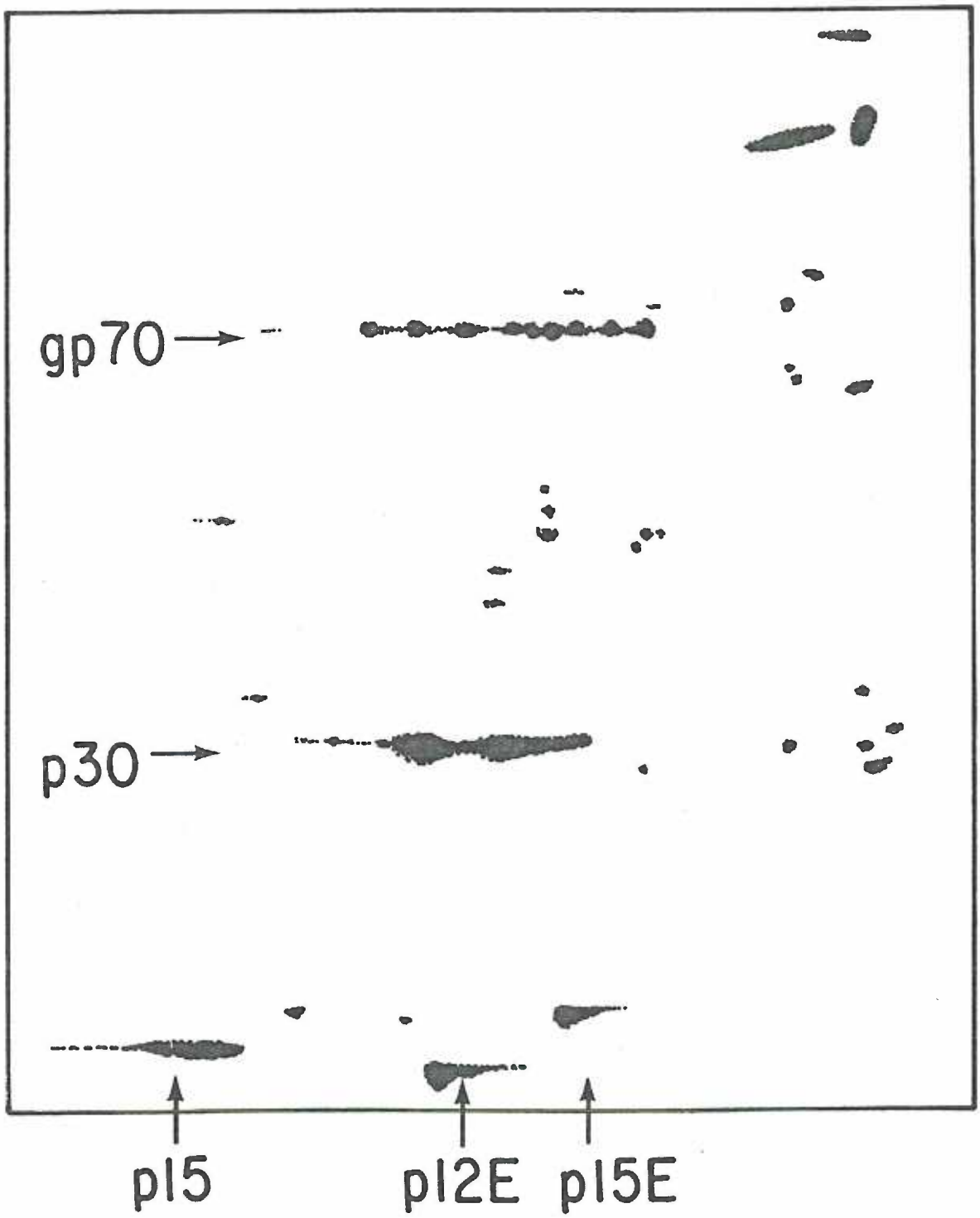
Figure 4 is a schematic map of the common denominator of "virus-specific" proteins. This map was derived by subtracting from the B4Sc-1 and B4NRK virus patterns those spots which could be attributed to the host cells by the above arguments. These remaining proteins can be considered "virus-specific" by the criteria employed.

B. Identification of Known Murine Leukemia Virus Proteins.

The correspondence between the known structural proteins of the virus and the spots we observed on the two-dimensional gels was determined in several ways. For the sake of brevity, all the data is not presented in figures but the results are indicated in the schematic figure 4.

Figure 4. Schematic Two-Dimensional Map of Virus-Specific Proteins in Murine Leukemia Virus Preparations.

The protein components depicted are found in B4Sc-1 as well as B4NRK virus preparations. The derivation of this set of "virus-specific" spots is described in the text. The MuLV structural proteins gp70, p30, p15, p15E and p12E are designated by arrows and their identification is described in the text.


gp70
p30
p15
p12E
p15E

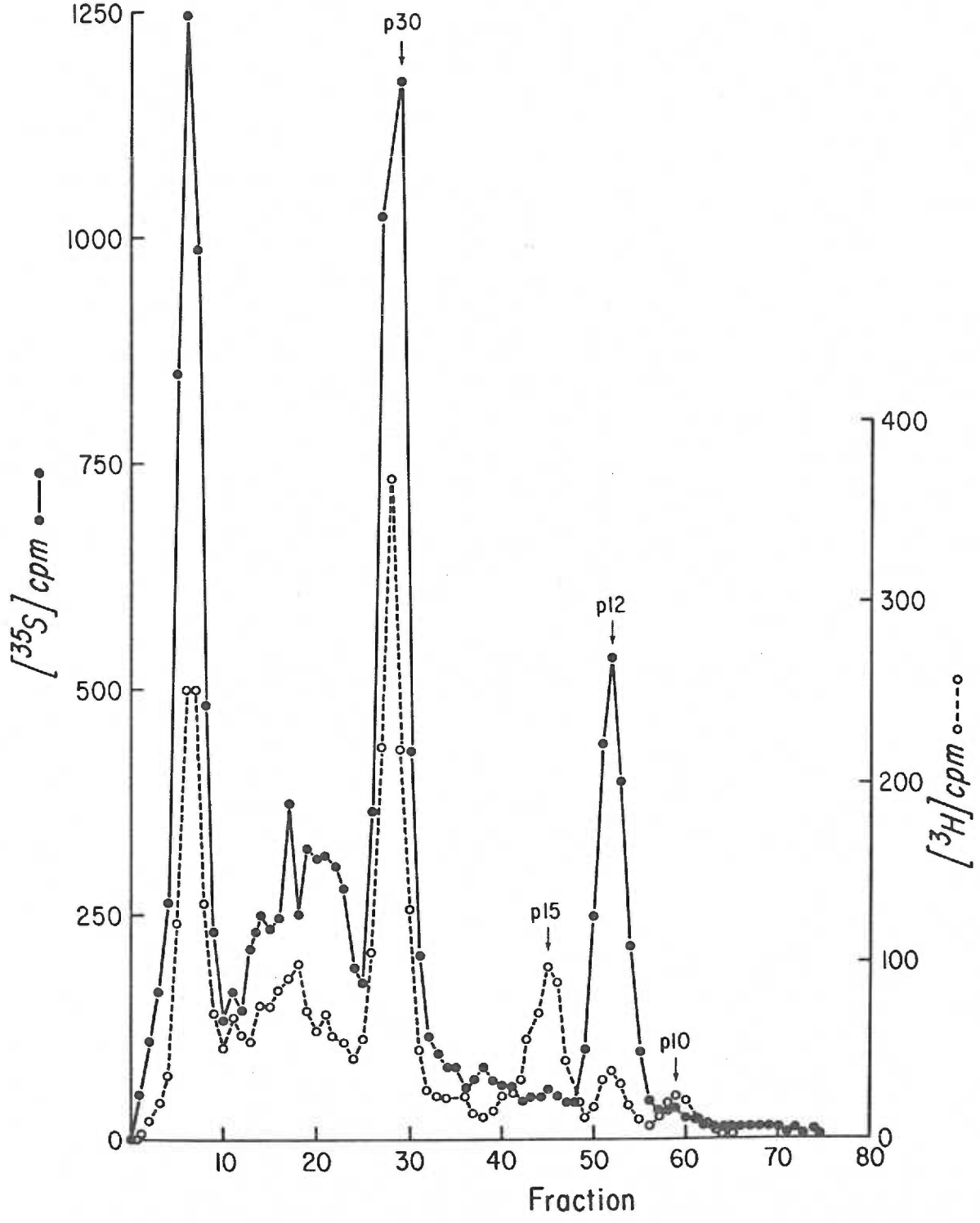
The recognized viral structural proteins have been named because of their apparent molecular weights (e.g., p30, p15, etc.). Therefore, size was the first criteria applied to the two-dimensional virus maps. The second dimension of the gel system is run in a 10 to 20% acrylamide gradient. Molecular weight standards were used to calibrate the gradient slab and the result indicated a linear relationship between the logarithim of the molecular weight and the distance migrated in the gel (Figure 1B).

The MuLV glycoprotein gp70 appears as a "string of beads", each the same size but differing in isoelectric point. This material was identified as gp70 by labelling virus with D-[^{3}H]glucosamine and by immune precipitation of MuLV protein extracts and virus infected cell extracts with monospecific antiserum to gp70. The D-[^{3}H]-glucosamine labelled virus and the immune precipitates were run on two-dimensional gels and the only component seen was the "string of beads". Furthermore, all the gp70 species (i.e., each bead on the string) were labelled with D-[^{3}H]glucosamine and precipitated with anti-gp70. No other D-[^{3}H]glucosamine labelled proteins were seen. Further characterization of gp70 is described in chapter IV, and an MuLV mutant with a temperature-sensitive defect in gp70 synthesis is described in chapter V.

The major structural protein of MuLVs is p30. p30 was identified by co-electrophoresis of immune precipitates from purified MuLV and virus infected cell extracts. Additionally, the p30 fractions from a guanidine hydrochloride column (Figure 5) were

Figure 5. Guanidine Hydrochloride Column Chromatography of Radioactively Labelled Murine Leukemia Virus Proteins.

A mixture of Eveline II virus preparations labelled separately with L-[^{3}H]leucine or L-[^{35}S]methionine was chromatographed on a BioGel A-5m (Bio-Rad) column in 6M guanidine hydrochloride (section II,D2) (153). The mixture contained 200,000 cpm [^{3}H] and 400,000 [^{35}S]. The column was eluted at a flow rate of 0.5 ml per hour and the fractions were counted for both isotopes. The MuLV proteins p30, p15, p12 and p10 chromatograph in the peaks designated as such (153).


1250
1000
750
500
250
0
[35S] cpm
400
300
200
100
0
[3H] cpm
p30
p12
p15
p10
10
20
30
40
50
60
70
80
Fraction

recovered and electrophoresed in two-dimensions, as was p30 purified from MuLV by phosphocellulose column chromatography (154). All these materials co-electrophoresed in two-dimensions with the material designated p30 on the MuLV maps (Figure 4). It should be noted that p30 also exhibits isoelectric point heterogeneity. The heterogeneity is not eliminated by cloning and is a feature common to all MuLV strains we have examined. We believe that it is due to an unknown post-translational modification (182) other than glycosylation or phosphorylation. p30 usually has a major basic component followed by two or three more acidic satellites. The role of p30 in viral tropism will be described in section III,C.

The identities of the low molecular weight MuLV proteins on the two-dimensional gels were deduced from several lines of evidence. These low molecular weight viral proteins have traditionally been analyzed on agarose columns in 6M guanidine hydrochloride (GuHCl) like the one shown in Figure 5. In this case a mixture of MuLV labelled with L-[^{3}H]leucine and L-[^{35}S]methionine was applied to the column and the eluted fractions were counted for both isotopes. The virus proteins p30, p15, p12 and p10 are found in the designated peaks (153). It should be noted that p12 labels with L-[^{35}S]methionine but p15 and p10 do not (91,92). The void volume (fractions 0 to 10) contains the viral proteins gp70, p15E and p12E (a derivative of p15E), all of which label with L-[^{35}S]methionine (91,92,183).

A two-dimensional comparison was made between L-[^{3}H]-leucine and L-[^{35}S]methionine labelled MuLV. This was followed by two-dimensional

analysis of the peaks from the guanidine hydrochloride column and p15 purified by phosphocellulose column chromatography (154). These data were considered along with the isoelectric points reported in the literature for these proteins (91,184-187). The results are indicated in Figure 4. p15E and p12E labelled with L-[^{35}S]methionine and were found in the GuHCl column void volume. p15 was identified by the lack of L-[^{35}S]methionine labelling and by co-electrophoresis with the material purified on phosphocellulose. p10 has a very high isoelectric point (>9) and is probably not resolved on these gels (184-186). The identity of p12 was not determined. It may be eclipsed by p12E, and it might be expected to have charge heterogeneity caused by a heterogeneous phosphorylation (55-58,91).

C. The Murine Leukemia Virus Determinant of Tropism-p30.

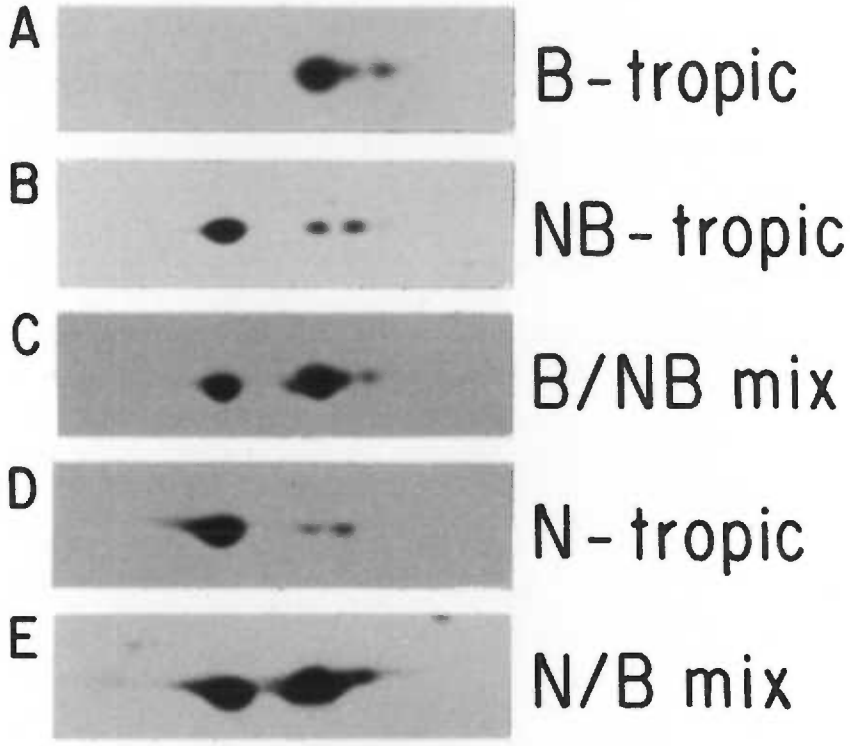
As described above (section I,E3) p30 has been implicated as a determinant in viral tropism. In order to further investigate this question we generated two-dimensional maps of N-tropic, B-tropic and NB-tropic MuLV proteins. The virus strains we examined were all derived from the endogenous MuLVs of Balb/c mice (65). From this source, Hopkins and her co-workers isolated several virus clones which were either N- or B-tropic and subsequently adapted some of the B-tropic clones to NB-tropic by "forced passage" through N-type cells (71). Hopkins (Massachusetts Institute of Technology) kindly provided us with several of these MuLV clones. We used each clone to infect Sc-1 cells and derived a MuLV producing cell line for each one. Each cell line was then labelled with L-[^{3}H]leucine and

the radioactive virus was harvested and analyzed on two-dimensional gels. We examined each MuLV clone alone as well as mixtures of N- and B-tropic virus and mixtures of B-tropic virus with the corresponding NB-tropic derivative. The mixtures contained equal amounts of radioactivity of the two components in the mix. Figure 6 shows the p30 region from five of these gels. The only differences observed were in the p30s. All the other protein spots on the gels were superimposable. All three B-tropic p30s (e.g., Panel A) were identical in terms of their mobilities on the two-dimensional gels, as were the three NB-tropic p30s examined (e.g., Panel B). Panels A and B demonstrate that when a B-tropic MuLV becomes NB-tropic by adaptation, the p30 becomes more basic (moves to the left). The adaptation to NB-tropic also results in a slightly smaller p30 which can be seen when the B- and NB-tropic viruses are mixed (Panel C). The major p30 component of the NB-tropic MuLV has almost the same isoelectric point as the N-tropic p30 (Panel D) but the NB-tropic p30 is smaller. The N-tropic and B-tropic p30s, while having different isoelectric points, are similar in size (Panel E). The p30 satellites also become more basic when B-tropic MuLV is adapted to NB-tropic, but they do not move as far as the major component. It is noteworthy that the p30s from three different B-tropic MuLVs undergo the same discrete alteration when independently adapted to become NB-tropic. These results provide strong evidence for the involvement of p30 as a determinant in MuLV tropism.

D. Discussion.

Figure 6. The p30s of N-tropic, B-tropic and NB-tropic Murine Leukemia Virus Clones.

Virion proteins were radioactively labelled with L-[^{3}H]leucine as described in Materials and Methods (section II,C4). The purified MuLV clones were solubilized in lysis buffer (section II,F2) containing 0.5% SDS and electrophoresed in two-dimensions essentially as described by O'Farrell (162,163) with the modifications described in Materials and Methods (section II,F2). Following electrophoresis, the second-dimension slab gels were processed for fluorography (176). Only the p30 region of the resulting fluorograms is shown. The mixtures (Panels C and E) contained equal amounts of radioactivity of the individual components.


A
B-tropic
B
NB-tropic
C
B/NB mix
D
N-tropic
E
N/B mix

Two-Dimensional Characterization of Murine Leukemia Virus-Specific Proteins. The two-dimensional analysis of MuLV proteins has revealed a degree of complexity previously not appreciated. This complexity manifests itself in the form of heterogeneous viral gene products (i.e., gp70 and p30) and numerous host cell proteins in virus preparations. The gp70 heterogeneity is analyzed further in the following chapter. The reason for the isoelectric heterogeneity of p30 has not been established. We propose that it is the result of a post-translational modification because it is a common feature of MuLVs and is not eliminated by cloning. In addition, all of the p30 components change their charge and size in a similar direction when B-tropic MuLV becomes adapted to NB-tropic.

Two approaches were used to derive a common denominator of "virus-specific" proteins. First, uninfected Sc-1 cells were found to shed several proteins which also occur in B4Sc-1 virus preparations. These proteins must be released from the cell by some mechanism independent of virus budding. The proteins shed from uninfected cells are perhaps packaged in a vesicle or aggregate because they pellet rapidly and band in sucrose density gradients very close to the MuLV particles. Proteins with mobilities very similar to these are also found in the B4NRK virus preparations. Apparently, NRK cells also shed particles containing proteins similar to those shed by Sc-1 cells.

In addition to the known MuLV proteins, designated in Figure 4, there are several proteins which are coincident in both

virus maps and are not found in the uninfected Sc-1 map. Some of these proteins might be viral gene products because they have the same size and isoelectric point whether they are produced in mouse or rat cells. Alternatively, these proteins might be host cell cytoplasmic or membrane proteins which are incorporated by the budding virus. If this is the case, the two-dimensional similarity of these proteins suggests that they are highly conserved from mouse to rat. Furthermore, the abundance of these proteins as revealed by their intensity in the gels suggests that they may be incorporated selectively into the virions rather than included merely by random engulfment of host cell proteins.

Future experiments will involve two-dimensional analysis of MuLV proteins when the B4 clone is propagated in hampster and monkey cells. This should provide more conclusive information concerning the virus-specific proteins.

p30 and MuLV Tropism. The work of Nancy Hopkins and her co-workers has suggested a role for p30 in MuLV tropism. They observed that B-tropic p30s were slightly larger than their NB-tropic derivatives and that the N-tropic p30s were intermediate in size (71,72). They also analyzed the ribonuclease T_1 resistant oligonucleotides of the B-tropic and NB-tropic MuLV genomes. They found a single oligonucleotide (about 17 nucleotides in size) that was different between the otherwise identical oligonucleotide maps (188). This oligonucleotide maps close to the 5' end of the viral RNA (i.e., in the p30 region) (189,190). Our examination of the

Hopkins MuLV clones confirmed the size difference they observed between the B- and NB-tropic p30s. Additionally, we have expanded that observation in three important ways. First, p30 reproducibly becomes a more basic protein as a result of the B- to NB-tropic adaptation. Secondly, our results suggest that there is likely to be a discrete region of the polypeptide chain that is reproducibly different in the p30s from viruses with different tropism. This "tropism region" of the p30 molecule appears to change in a reproducible fashion when the viral tropism is altered. We are currently testing this suggestion by peptide mapping. Third, no other component on the two-dimensional gels is altered when a MuLV changes its tropism, indicating that no alteration affecting size or isoelectric point has occurred in any other major viral protein.

Previously, p30 was considered to be simply a structural protein, involved somehow in packaging the virus core. Our work and that of Hopkins and co-workers (71,72,188-190) indicates that p30 has an important functional role as well. The idea of MuLV "structural proteins" having metabolic functions is appealing because the MuLVs carry out complicated biological functions with a small number of its own gene products.

When N-type cells are infected by B-tropic virus (or vice versa), the DNA provirus is synthesized but it is not efficiently integrated into the host cell chromosome (67,68), apparently because a cellular repressor (191) interferes with the function of a viral protein involved in integration. Our results implicate p30 in the

integration process and suggest that p30 may be the viral protein that interacts with the cellular repressor. This system has the elements of a model system for examining some aspects of host cell-virus interactions. There appears to be a discrete modification of the MuLV genome in response to hostile cellular surroundings leading to an altered gene product (p30) which influences the integration of the provirus during subsequent infections and alters the host range of the virus.

Future experiments in this laboratory will involve analyzing the tropism modifications which occur in the primary structure of the p30 molecule.

IV. GENETIC AND SIALYLATION CAUSES FOR HETEROGENEITY OF MURINE LEUKEMIA VIRUS ENVELOPE GLYCOPROTEINS gp69/71

A. Genetic Heterogeneity of Eveline II Virus gp69/71.

Previous studies have indicated that the MuLV glycoprotein frequently has two components termed gp69/71 which are derived by processing of two larger precursors which have apparent molecular weights of 89,000 and 91,000 (37,39,41). The gPr89/91^{env} precursors contain core carbohydrates but lack the terminal sugars fucose and sialic acid which occur in gp69/71 (39,92,124). In order to study the synthesis of gp69/71, a suspension culture of Eveline II cells was pulse-labelled with L-[^{35}S]methionine and was subsequently chased with a large excess of nonradioactive methionine. Cell samples were collected at various times during this labelling procedure. Radioactive proteins were precipitated from the cell extracts with monospecific antibody to gp69/71 and were electrophoresed on polyacrylamide slab gels in the presence of SDS (data not shown). Our results are consistent with those in the literature (37,39,41) and show that gp89 and gp91 occur in an approximately constant ratio throughout the labelling period. This suggests that they are not related to each other in a precursor-product manner. Furthermore, they are rapidly formed and their unglycosylated precursor(s) were not detected. Thus, our results confirmed the doublet nature of gp69/71 in the Eveline II culture and demonstrated its derivation from a gp89/91 doublet precursor.

Evidence that the two components of gp69/71 are encoded

by different viral genomes was obtained using cloned Eveline II virus. Figure 7 shows an experiment in which one virus clone (B4) was grown in Sc-1 fibroblasts and was analyzed as described above. Sc-1 cells infected with uncloned Eveline II virus (lanes A-C) synthesize the gp89/91 and gp69/71 doublets as expected, but cells infected with the cloned B4 virus (lanes D-F) synthesize only the single glycoproteins gp91 and gp71. Normal rat kidney cells infected with the B4Sc-1 virus (lanes G-I) also synthesize only the gp91 and gp71 glycoproteins. These results suggest that gp91 and gp89 are precursors of gp71 and gp69, respectively. Furthermore, the two virion glycoproteins in Eveline II cells are apparently produced by different viral genomes which are separable by cloning and encode for the synthesis of distinct polypeptide chains. It is also apparent that the size heterogeneity of gp89/91 and gp69/71 is not a consequence of a processing heterogeneity (e.g., glycosylation) within the host cell.

An analysis of L-[^{35}S]methionine-labelled virion proteins by two-dimensional, isoelectric focusing/sodium dodecyl sulfate polyacrylamide gel electrophoresis (162,163) is shown in Figure 8. The gp69/71 glycoproteins separate in the isoelectric focusing (horizontal) dimension as a broad "string-of-beads". These proteins were identified as gp69/71 by labelling with D-[^{3}H]glucosamine and also by immune precipitation with antibody to gp69/71 (section III,B). Figure 8 shows that the virion glycoprotein from Eveline II virus (Panel A) separates in the sodium dodecyl sulfate electrophoresis

Figure 7. Pulse-Chase Analysis of gp69/71-Specific Proteins of Eveline II Virus in Sc-1 Cells and Clone B4 Virus in Sc-1 and NRK Cells.

Monolayer cultures (75 cm^2) of virus infected Sc-1 and NRK cells at 50-70% confluency were pulse-labelled for 30 min with 20 µCi/ml L-[^{35}S]methionine in 3 ml of methionine-free Minimal Essential Medium. Following the pulse period the cells were chased by addition of unlabelled methionine for 30 min or for 2 hr. Uninfected cells were pulse-labelled for 60 min. The labelled cells were then lysed and immune precipitated, using monospecific antiserum to gp69/71, and analyzed by electrophoresis in SDS-urea gels (41). Lanes A-C are immune precipitates from whole Eveline II virus infected Sc-1 cells (EIIVSc-1): (A) 30 min pulse; (B) 30 min pulse and 30 min chase; (C) 30 min pulse and 120 min chase. Lanes D-F are immune precipitates from B4 virus clone infected Sc-1 cells (B4Sc-1): (D) 30 min pulse; (E) 30 min pulse and 30 min chase; (F) 30 min pulse and 120 min chase. Lanes G-I are immune precipitates from B4 virus clone infected NRK cells (B4NRK): (G) 30 min pulse; (H) 30 min pulse and 30 min chase; (I) 30 min pulse and 120 min chase. Lane (J) is an immune precipitate from a 60 min pulse of uninfected Sc-1 cells. Lane (K) is an immune precipitate from a 60 min pulse of uninfected NRK cells. "gp89" and "gp91" are synonymous with $gPr90^{env}$ discussed in text.

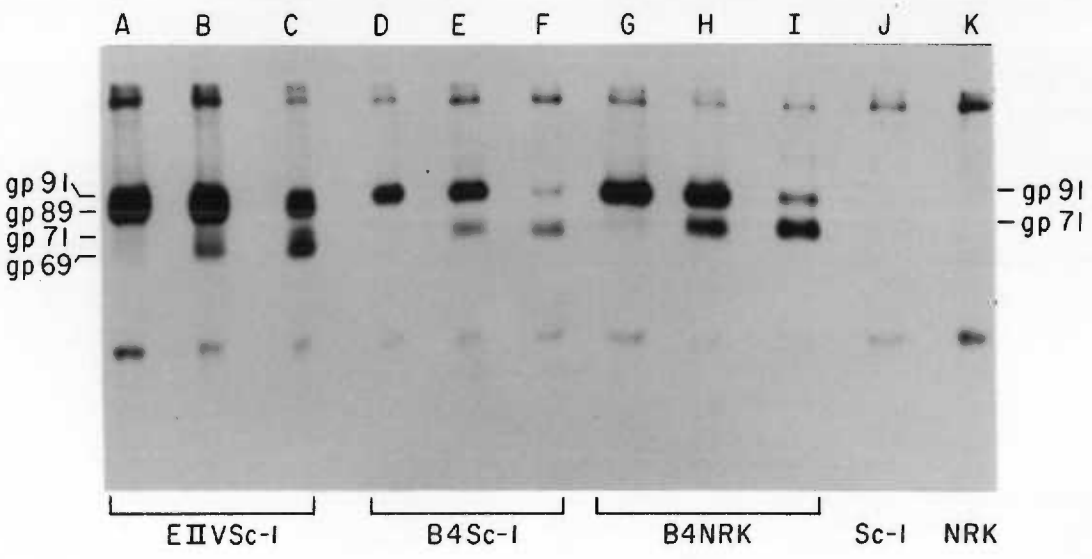
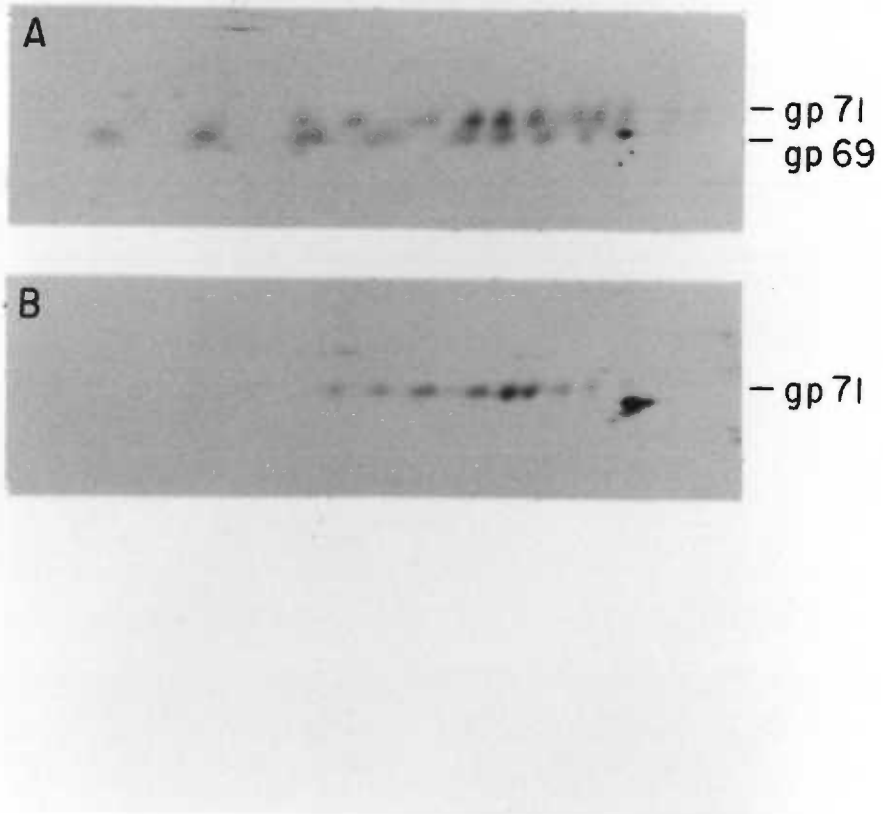

A
B
C
D
E
F
G
H
I
J
K
gp 91
gp 89
gp 71
gp 69
gp 91
gp 71
EIIVSc-1
B4Sc-1
B4NRK
Sc-1
NRK

Figure 8. Two-Dimensional Polyacrylamide Gel Electrophoresis of Eveline II Virus gp69/71 and Clone B4 Virus gp71.

Virion proteins were labelled with L-[^{3}H]leucine as described in Materials and Methods. Purified virus was solubilized and electrophoresed in two-dimensions essentially as described by O'Farrell (162,163). Only the gp69/71 region of the resulting fluorograms is shown. Panel (A) is Eveline virus and panel (B) is clone B4 virus. The panels are oriented with the high pH end to the left and the low pH end to the right. The region of the isoelectric dimension shown spans about pH 4-6.


A
gp 71
gp 69
B
gp 71

(vertical) dimension as two rows of spots which differ slightly in size, whereas the cloned B4Sc-1 virions (Panel B) contain only the larger size class of envelope glycoproteins. This supports the conclusions based on immunoprecipitation of intracellular proteins (Fig. 7).

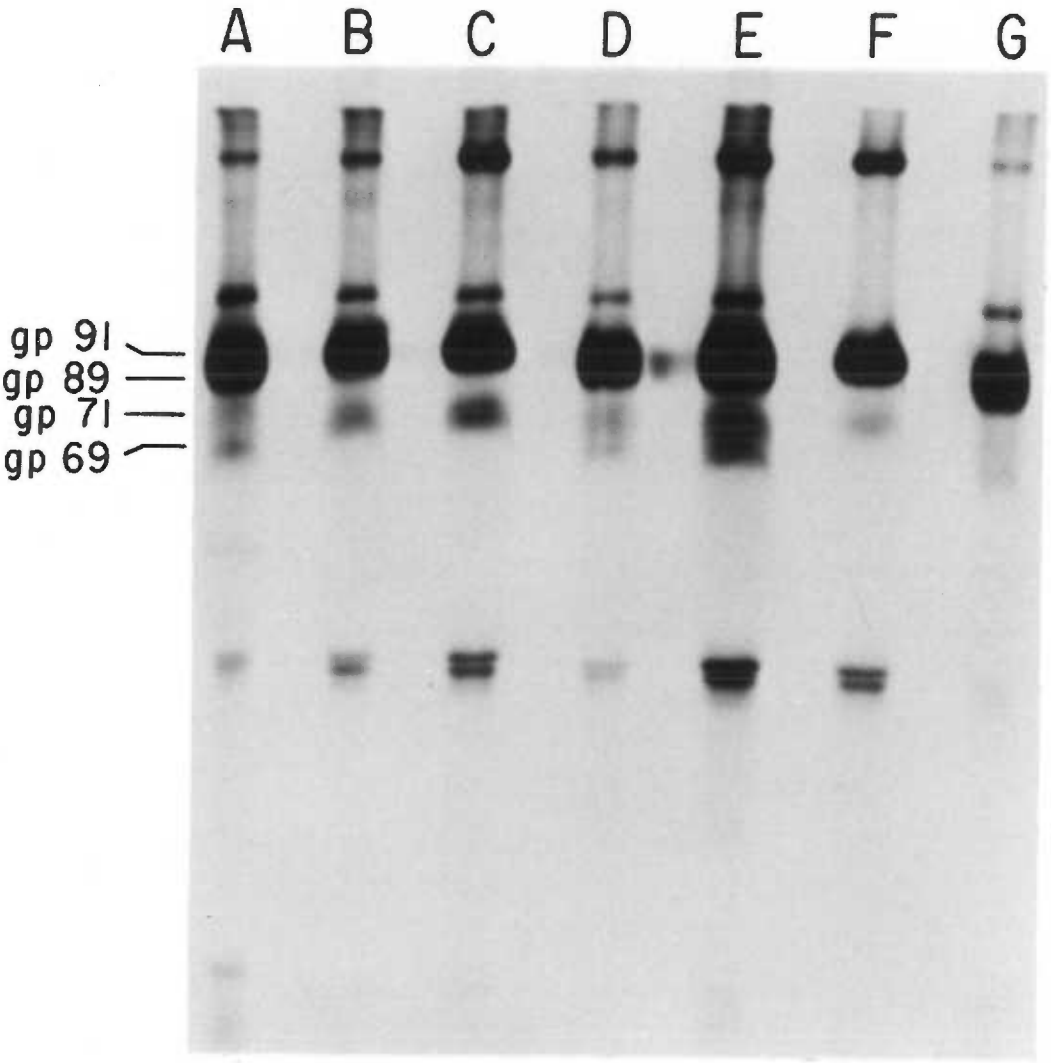
Using pulse-labelling and immune precipitation techniques we have also analyzed three other virus clones derived from the Eveline II virus, as well as a clone of Rauscher MuLV. The data is shown in Figure 9. One of these clones (lane B) was like the B4Sc-1 clone (lane C) and synthesized only the gp91 precursor and the gp71 envelope glycoprotein. This was also the case for the Rauscher MuLV clone (lane F). The other two Eveline II clones produced the doublets gp89/91 and gp69/71 (lanes D and E). Clones such as the latter could have occurred if virus aggregates were present in the preparation of Eveline II virus used for cloning. However, an electron microscopic examination of this virus did not show any aggregates (data not shown). Alternatively, such clones would occur if many of the virus particles released from Eveline II cells are heterozygotes which contain one genome coding for gp69 and a second coding for gp71. These possibilities will be discussed below.

B. Heterogeneous Sialylation of Envelope Glycoproteins.

In addition to their size heterogeneity, the envelope glycoproteins are clearly heterogeneous in their isoelectric point (Fig. 8). To investigate the possibility that negative charge

Figure 9. Immune Precipitation Analysis of gp69/71-Specific Proteins in Cells Infected with Eveline II Virus Clones.

Monolayer cultures (75 cm^2) of Sc-1 cells infected with Eveline II virus clones (section II,C2) at 50-70% confluency were pulse-labelled for 60 min with 15 µCi/ml L-[^{35}S]methionine in 3 ml of methionine-free Minimal Essential Medium. At the conclusion of the pulse, the cell monolayers were lysed and the cell extracts were immune precipitated by secondary antiserum precipitations using monospecific antiserum to gp69/71. The immune precipitates were then electrophoresed in SDS-urea gels (section II, F1). Lanes A and G are immune precipitates from Sc-1 cells infected with whole Eveline II virus (EIIVSc-1). Lanes B-E are immune precipitates from Sc-1 cells infected with Eveline II virus clones (lane C is clone B4). Lane F is an immune precipitate from NIH/3T3 cells infected with a Rauscher-MuLV clone.


A
B
C
D
E
F
G
gp 91
gp 89
gp 71
gp 69

supplied by sialic acids might contribute to the "string-of-beads" appearance of the glycoprotein in a relatively acidic (pH 4-6) region of the isoelectric focusing dimension, an L-[^{35}S]methionine labelled preparation of Eveline II virus was treated with neuraminidase to remove the terminal sialic acid residues. Figure 10 shows that the "string-of-beads" is eliminated by neuraminidase. Furthermore, a lightly labelled component in the control virions (Fig. 10A, arrow) is enriched following neuraminidase treatment (Fig. 10B, arrow). Because the asialoglycoprotein focuses at the high pH end of the isoelectric focusing dimension, where the pH gradient rises very steeply between pH 8-10 (Fig. 1A&B), its isoelectric point homogeneity cannot be evaluated. Although this result shows that sialic acids occur in gp69/71, consistent with previous reports (39,92,124), we cannot conclude that the isoelectric point microheterogeneity of the glycoprotein is caused by heterogeneous sialylation. We have therefore obtained additional information concerning the charge and sialylation heterogeneity of these envelope glycoproteins.

Figure 11 shows an analysis of D-[^{3}H]glucosamine labelled envelope glycoproteins from Eveline II virus by electrophoresis on cellulose acetate membranes in 8 M urea and 1% 2-mercaptoethanol at pH 8.5. The radioactive glycoprotein migrated as a broad envelope of material which became increasingly polydisperse with further electrophoresis. In contrast, at this pH the neuraminidase treated glycoprotein remained as a narrow band near the origin. These results are consistent with the idea that heterogeneous sialylation

Figure 10. Two-Dimensional Polyacrylamide Gel Electrophoresis of Control and Neuraminidase Treated Eveline II Virus gp69/71.

Virion proteins were labelled with L-[^{35}S]methionine as described in Materials and Methods. Purified virus was suspended in 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4 and was incubated with neuraminidase for 2 hr at 37°. Control samples were incubated in parallel without the enzyme. Control and neuraminidase treated virus was denatured and electrophoresed in two dimensions essentially as described by O'Farrell (162,163). Only the gp69/71 region of the resulting fluorograms is shown. Panel (A) is control Eveline virus. Panel (B) is neuraminidase treated Eveline virus. The gels are oriented with the high pH end to the left and the low pH end to the right. The region of the isoelectric focusing dimension shown spans about pH 4 to pH 10. The arrows indicate the position of the asialoglycoprotein.

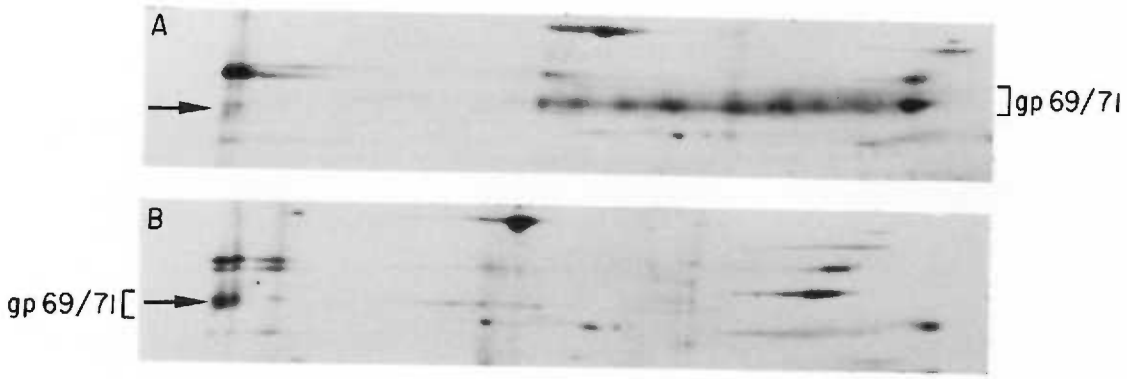
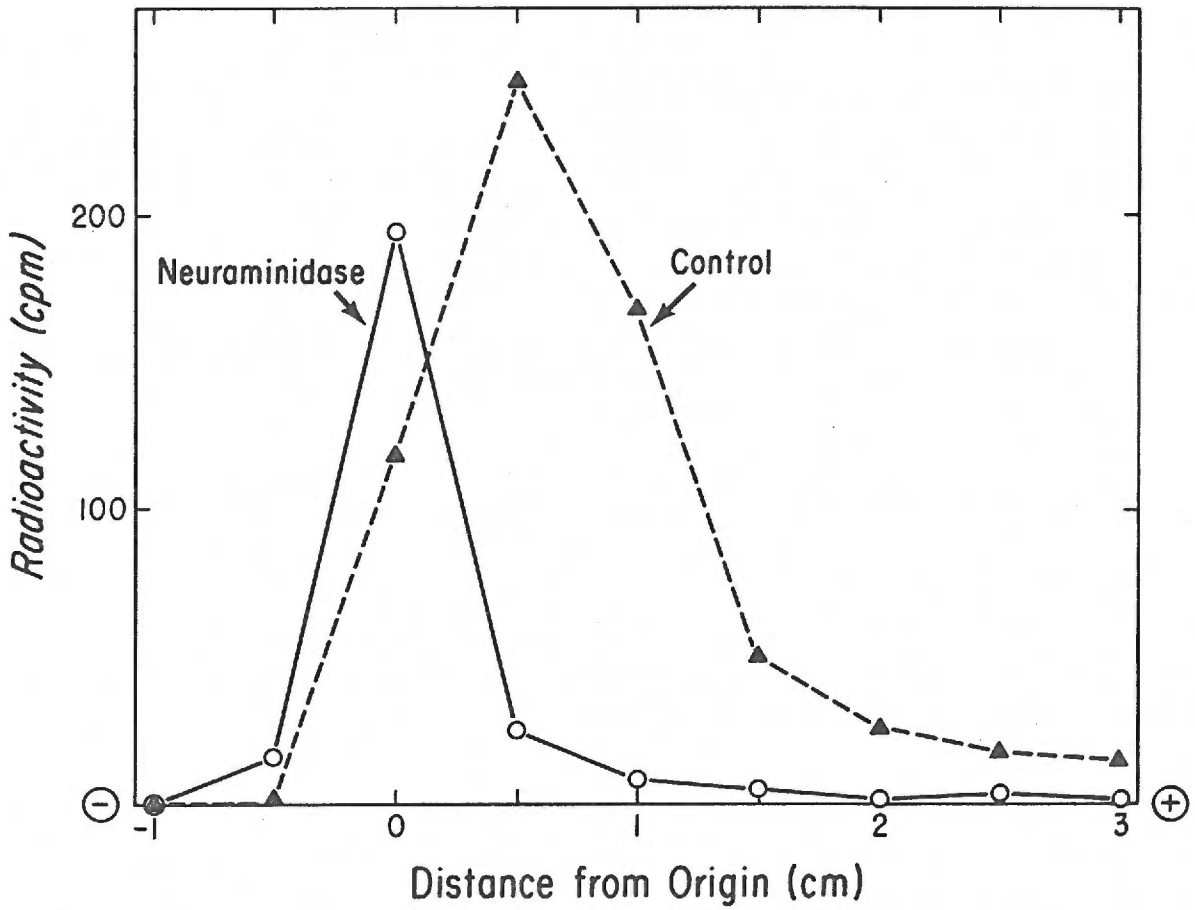

A
]gp 69/71
B
gp 69/71[

Figure 11. Cellulose Acetate Electrophoresis of Control and Neuraminidase Treated Eveline II Virus gp69/71.

Virion proteins were labelled with D-[^{3}H]glucosamine and were digested with neuraminidase as described in Materials and Methods. Control and neuraminidase treated virus samples were denatured and electrophoresed as described in Materials and Methods. The same amount of total radioactivity was electrophoresed on each cellulose acetate strip. However, the radioactive sialic acid in the neuraminidase treated sample was removed when the strips were fixed in 5% trichloroacetic acid. (▲---▲), untreated control; (0——0), neuraminidase treated sample.


Radioactivity (cpm)
200
100
Neuraminidase
Control
⊖
⊕
-1
0
1
2
3
Distance from Origin (cm)

is the major cause of the envelope glycoprotein charge heterogeneity.

A DEAE-Sephadex chromatograph of tryptic peptides from D-[^{3}H]glucosamine-labelled Eveline II virus glycoproteins is shown in Figure 12. The control glycopeptides chromatograph as a runoff peak followed by a heterodisperse spectrum of glucosamine labelled material which binds to the column with variable affinity. Emerging from this broad spectrum is a single prominent component which elutes at 175 ml. Neuraminidase treatment alters this profile. In this case the runoff is closely followed by a peak (40-50 ml) of free sialic acid (177) which was released from the glycoprotein by neuraminidase and which co-chromatographed with [^{14}C]N-acetylneuraminic acid. The broad heterogeneous spectrum of material which was present in the control chromatogram is eliminated by neuraminidase but the single component which elutes at 175 ml is unaffected by the enzyme treatment. These results imply that the glycoprotein contains a carbohydrate side chain which lacks sialic acid (i.e., the glycopeptide emerging at 175 ml) and at least one other carbohydrate side chain(s) which is heterogeneously sialylated. Desialylation of the latter glycopeptides presumably causes them to elute in the column runoff peak.

Figure 13 shows an analysis of the D-[^{3}H]glucosamine-labelled tryptic glycopeptides from Eveline II virus glycoproteins by high voltage paper electrophoresis. In this experiment the free sialic acid was removed from the glycoprotein sample after neuraminidase treatment by cold trichloroacetic acid precipitation and the

Figure 12. DEAE-Sephadex Chromatography of Tryptic Glycopeptides of Eveline II Virus gp69/71.

Virion proteins were labelled with D-[^{3}H]glucosamine as described in Materials and Methods. Neuraminidase and trypsin digestions and column chromatography procedures are described in Materials and Methods. The column procedure was essentially that of Robertson et al. (177). (●), untreated control glycopeptides; (○), neuraminidase treated glycopeptides. A 200 ml linear NaCl gradient (0 to 0.1 M) was started at 36 ml. The drop-off in the control radioactivity at about 70 ml is unexplained and was not reproducible.

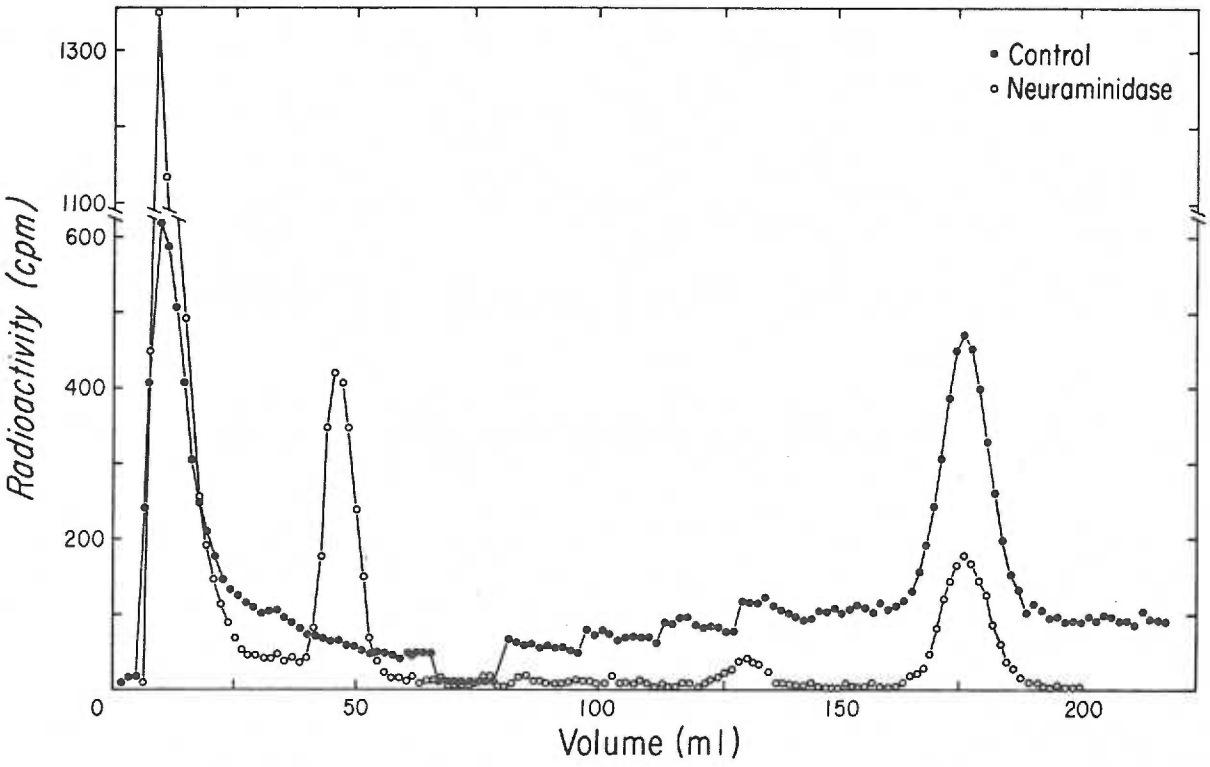
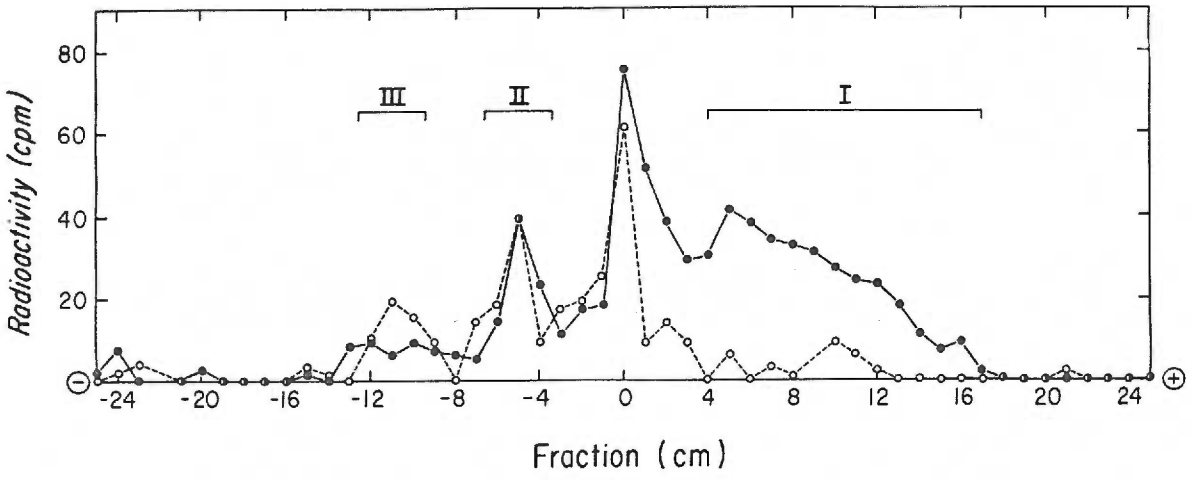

Control
Neuraminidase
Radioactivity (cpm)
1300
1100
600
400
200
0
50
100
150
200
Volume (ml)

Figure 13. High Voltage Paper Electrophoresis of Tryptic Glycopeptides of Eveline II Virus gp69/71.

Virion proteins were labelled with D-[^{3}H]glucosamine and were treated with neuraminidase as described in Materials and Methods. The neuraminidase and control samples were precipitated with cold 5% trichloroacetic acid before trypsin digestion (Materials and Methods). (●——●), untreated control sample; (O---O), neuraminidase treated sample. Regions designated I, II, III are described in text.


Radioactivity (cpm)
80
60
40
20
III
II
I
⊖
⊕
-24
-20
-16
-12
-8
-4
0
4
8
12
16
20
24
Fraction (cm)

glycoproteins were then digested with trypsin. The D-[^{3}H]glucosamine-labelled material which remained at the origin consists of insoluble core material that was not solubilized by trypsin and could be removed from the tryptic digests by centrifugation. The control glycopeptides have two major migrating components. The component labelled "I" migrates as a heterogeneous envelope toward the anode. Component "II" migrates toward the cathode as a single peak. Neuraminidase treatment eliminates component I but does not affect component II. The neuraminidase treated glycopeptides also appear to have a third, cathode migrating component (III) which is present to a lesser extent in the control sample. In agreement with Figure 12, these results suggest that at least one carbohydrate side chain is heterogeneously sialylated (component I) and that a second carbohydrate side chain lacks sialic acids (component II). Component III may contain desialylated component I.

In the experiments described above (Figs. 10, 11 and 13), there does not appear to be quantitative transfer of D-[^{3}H]glucosamine label from the sialylated to the corresponding asialylated material upon neuraminidase treatment. This was expected because D-[^{3}H]-glucosamine is generally incorporated into glycoproteins as either N-acetylglucosamine or as sialic acid (104). We have determined that approximately 45% of the radioactivity present in D-[^{3}H]glucosamine labelled virion envelope glycoproteins is converted by neuraminidase treatment into a form that is soluble in cold 5% trichloroacetic acid. Our results suggest that the carbohydrate side chain(s) which are heterogeneously sialylated are relatively rich in sialic acid and

deficient in N-acetyl glucosamine. Another carbohydrate side chain lacks sialic acid and has relatively more N-acetyl glucosamine.

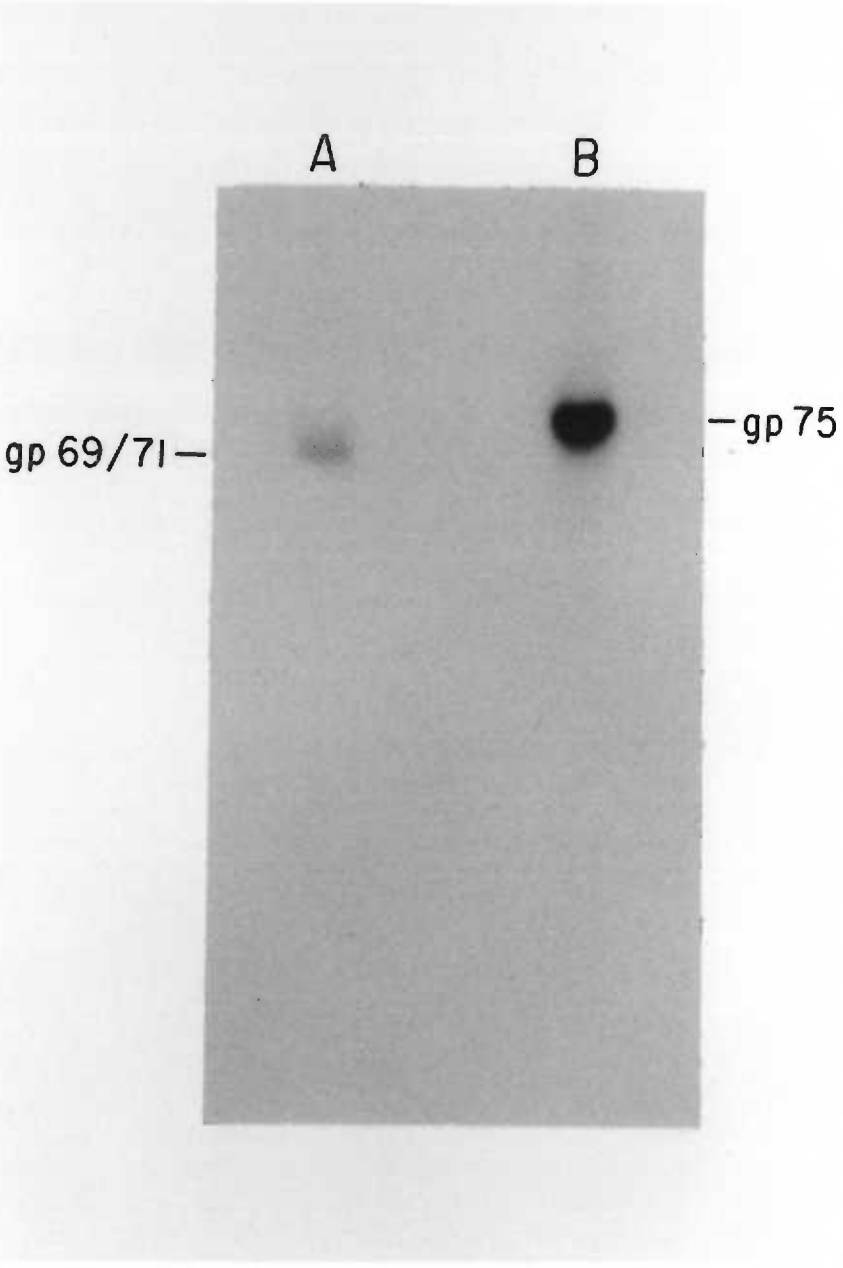
The experiments described in this section (i.e., in Figs. 11-13) were all repeated using the B4Sc-1 clone of Eveline II virus. Although the amount of radioactivity was lower due to the lower yield of virus and the inefficiency of labelling the infected Sc-1 cells with D-[^{3}H]glucosamine (section II,C4), the results were otherwise the same as with whole Eveline II virus. In particular, all of the glycopeptides observed in the whole Eveline II virus glycoproteins were also present in the gp71 of the B4Sc-1 virus.

C. Eveline MuLV Differs from the Original Strain of Friend MuLV.

The virus produced by Eveline II cells is generally considered to be Friend MuLV (125,155-157,164). However, during the course of this work and previous studies (41,192), we have found that Eveline II MuLV differs substantially from Friend MuLV. Figure 14 shows the results of an experiment in which Eveline II cells and Friend erythroleukemia cells (cell line F4-6/K) were radioiodinated on their plasma membranes using lactoperoxidase. The radioactive glycoproteins were precipitated from the cell extracts that contained detergents with antiserum to gp69/71 and the immunoprecipitates were analyzed by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate. The Friend cell virion glycoprotein (gp75) is larger than the glycoprotein on Eveline II cells (gp69/71). Furthermore, other experiments indicate more clearly that Eveline II cells contain both gp69 and gp71 on their surface, whereas cells infected

Figure 14. Cell Surface Glycoproteins of Eveline II Cells and Friend Erythroleukemia Cells, as Revealed by Lactoperoxidase-Catalyzed Iodination of Cells with [^{125}I]iodine.

The erythroleukemia cells are the F4-6/K cell line of Ostertag et al. (167-169). After removal of unreacted iodine, the cellular proteins were dissolved, immune precipitated with monospecific antibody to gp69/71, and analyzed by electrophoresis as described in Materials and Methods. The figure shows an autoradiogram of the polyacrylamide gel. A longer exposure of the film revealed the clear labelling of both gp69 and gp71 in the Eveline II cell sample.


A
B
gp 69/71
gp 75

with Friend MuLV contain only one surface glycoprotein. Similarly, the various p30 precursors of the two viruses have different molecular weights (41,192). In addition, the virus proteins produced by Friend erythroleukemia cells were found to be indistinguishable from those encoded by the original strain of Friend virus (obtained from the American Type Culture Collection). The distinctive properties of F-MuLV and Eveline II MuLV were also independent of the cell line in which the viruses were grown.

D. Discussion.

General Comments. The envelope glycoprotein of MuLVs and other mammalian retroviruses frequently separates during electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate either into two separate components or into a heterodisperse broad band which could contain several unresolved components (8,91, 92,126,160,161). Accordingly, the MuLV glycoproteins have been commonly termed "gp69/71" (160). Furthermore, these proteins are heterogeneous in their isoelectric point (106,124).

Our results show that the size heterogeneity of the MuLV envelope glycoprotein from Eveline II cells is caused by multiple viral genomes present in the cell culture and that the charge heterogeneity is caused primarily by heterogeneous sialylation of side chain carbohydrate. The carbohydrate on the glycoprotein can apparently accept many sialic acid residues, resulting in approximately eight discrete glycoprotein components (Figs. 8 and 10). Evidence based upon labelling with D-[^{3}H]glucosamine suggests that

the carbohydrate which is sialylated contains relatively little [^{3}H]N-acetylglucosamine because neuraminidase treatment released almost all of its radioactivity (Fig. 13). However another carbohydrate side chain on the glycoprotein apparently lacks sialic acids and contains more N-acetylglucosamine (Figs. 12 and 13).

We analyzed the envelope glycoproteins encoded by four different virus clones derived from the Eveline II virus. Two of these cloned viruses encode only for the synthesis of gp71, whereas the other two encode for the synthesis of both gp69 and gp71. The latter two clones conceivably could have been caused by infection with virus aggregates present in the preparation of Eveline II virus. However, we did not see virus aggregates in the electron microscope. Alternatively, such clones would be expected if the RNA genome which codes for gp69 can combine together with the genome encoding for gp71 to form heterozygous MuLV particles. Evidence for heterozygotes was previously obtained for Rous sarcoma virus (17) and for Moloney MuLV (193). Presuming that the MuLV genome is diploid and that the gp69 and gp71 genomes are equally prevalent, we would expect half of the virus clones to be heterozygotes and each type of homozygote to occur one-fourth of the time. Future experiments will include a search for homozygous gp69 virus by isolating a large number of clones from Eveline II virus particles purified by sucrose gradient sedimentation (193). Availability of these clonally purified homozygous MuLVs should also facilitate a more detailed analysis of the differences between gp69 and gp71.

Although charge heterogeneity of the envelope glycoproteins has previously been observed and ascribed to sialylation (106,124), the evidence obtained was inconclusive. Witte et al. (106) examined the gp69/71 of Moloney-sarcoma-leukemia virus by two-dimensional gels, similar to those used here. They observed an "acidic/heterogeneous" band which was not resolved into multiple components. This material was more homogeneous "near the limits of definition of our tube gel IEF system" (106) after treatment with neuraminidase. Their conclusion about the homogeneity of a protein in this region of the isoelectric focusing gel suffers from the same deficiencies as our gels. That is, there is very little resolution in this region of the system. The control gp69/71 focused in their experiment as a heterodisperse band that was approximately twice as broad as the neuraminidase treated material which focused at the top of the isoelectric focusing gel. Although the untreated glycoprotein appeared in the isoelectric focusing dimension as a broad band, other viral proteins also appeared to be similarly broad on the gel. It is therefore not clear to us whether they observed true heterogeneity or incomplete focusing. Marquardt et al. (124) electrophoresed isolated gp70 at two pH's (4.3 and 8.9) in nondenaturing buffer. They described a broadly migrating gp70 band which was made more homogenous after treatment with neuraminidase. However, the analysis of untreated control glycoprotein was not shown and the electrophoretic position of the broad band was not indicated. Furthermore, approximately half of the neuraminidase treated material

remained at the interface between the stacking and running gels. Our electrophoretic separations in highly denaturing conditions showed that the untreated glycoprotein migrates toward the anode in a relatively heterodisperse fashion.

Terminal sialic acid residues are typically added in the Golgi apparatus to the carbohydrate side chains of glycoproteins (93). Heterogeneity of sialylation has been reported for several glycoproteins (177,194-197). For example, mouse alpha-fetoprotein exhibits a changing pattern of electrophoretic mobility with development caused by changes in its sialic acid content; and yolk sac has an acceptor pool of the asialo-alpha-fetoprotein (195). Also, the carbohydrate side chains of the vesicular stomatitis virus glycoprotein are reported to contain either 0, 1, 2 or 3 sialic acid residues (177).

Generality of These Results. A major question raised by our cloning results concerns their generality. In other words, are all cell lines which produce two different sized MuLV glycoproteins simultaneously infected with at least two different viral genomes? Although a definitive answer to this question will obviously require cloning experiments with virus from other cell lines, there has been no published evidence that cells infected with a single MuLV genome produce more than one size class of glycoprotein. Even in cases where cloned virus may have been used to establish a cultured cell line, genetic divergence of multiple viral genomes within the culture cannot be excluded. Also, as mentioned above, cloned virus

particles may be heterozygotes since virions contain at least two 35S RNA subunits. Unless evidence is presented to the contrary, it therefore seems reasonable to believe that each haploid MuLV genome has only one *env* gene (5) which produces only one size class of envelope glycoprotein. Our results also imply that the type of cell does not significantly influence the size of the fully processed glycoprotein (Fig. 7). We have also obtained evidence supporting these conclusions using Rauscher MuLV. Although uncloned Rauscher virus contains the gp69/71 doublet (92,126,161), we have studied a cloned Rauscher virus which encodes only for gp71 (Fig. 9, lane F).

Origin of the Multiple Viral Genomes. Based on these considerations, it would appear that many of the established cell lines commonly used as sources of MuLV and other mammalian type C retroviruses may contain two or more viral genomes which encode for different size classes of envelope glycoprotein. It is therefore important to consider the origin of these multiple viral genomes.

Various studies suggest that the discrete size classes of glycoproteins which commonly occur in virus preparations are closely related variants. For example, peptide mapping studies and serological studies of gp69 and gp71 of Moloney and Rauscher MuLVs have indicated that the two glycoproteins are indistinguishable (114,161). Furthermore, the gp69/71 of Eveline II cells behaves as a single homogeneous material when analyzed by immunological methods (155-157). In addition, we did not detect any tryptic glycopeptides in the uncloned Eveline II virus which were absent in the cloned B4Sc-1 virus.

The simplest interpretation of these results is that the two glycoproteins are highly homologous and are encoded by closely related MuLV genomes. This latter idea is consistent with our two-dimensional separations of proteins from Eveline II virus and from the cloned B4Sc-1 virus; the only proteins of these virus preparations which differed significantly were the envelope glycoproteins. One possible explanation for these results is that a small deletion or modification in the gp71 gene produces a viral genome which encodes for gp69.

It would therefore appear that variant genomes with altered envelope glycoprotein genes may occur frequently in preparations of mammalian C-type retroviruses. For example, we have recently found that the original strain of Friend virus contains a defective MuLV with a partial deletion of its envelope glycoprotein gene (198). Conceivably, such glycoprotein variants may arise relatively frequently because of some special property of this region of the genome. Alternatively, such variants may frequently occur because they are selected for during virus replication in vivo. For example, such variants in the envelope glycoprotein might permit the virus and the tumor cells to escape the immune response of the host. Furthermore, glycoprotein heterogeneity might facilitate the infection of different tissues which could have different glycoprotein receptors.

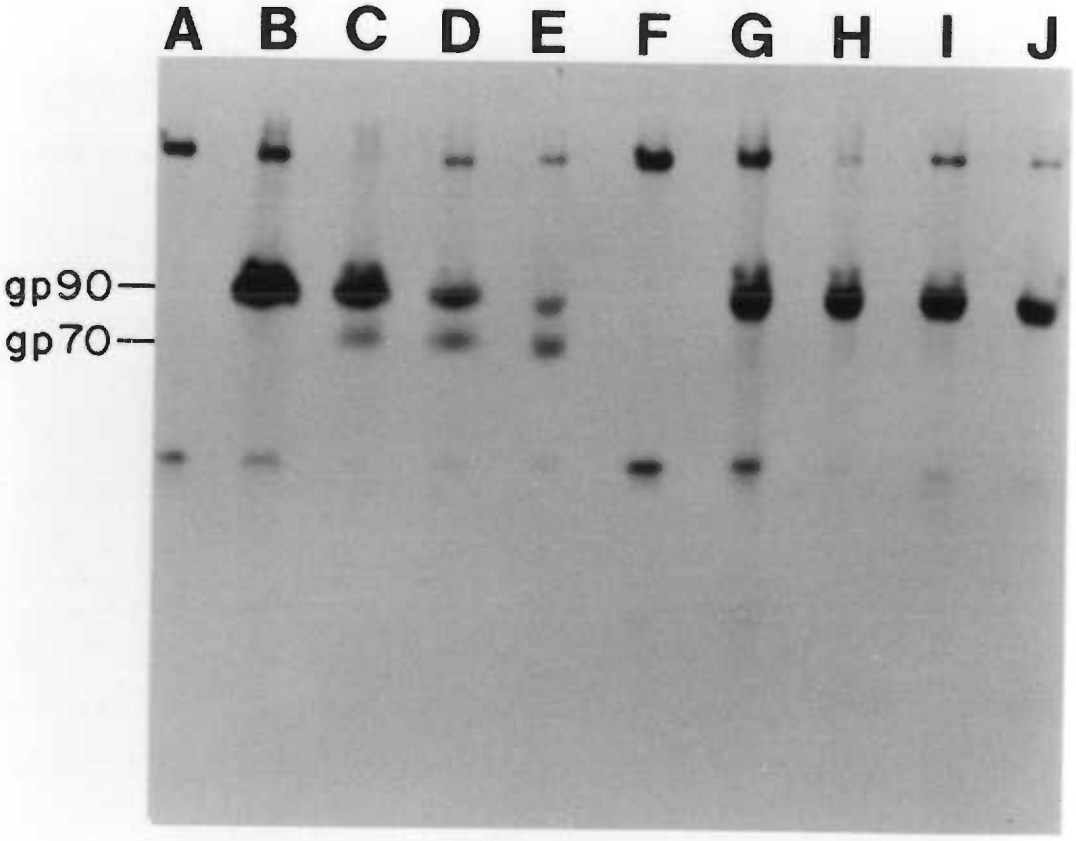
V. A MURINE LEUKEMIA VIRUS MUTANT WITH A TEMPERATURE-SENSITIVE DEFECT IN GLYCOPROTEIN SYNTHESIS

A. Previous Work with Rauscher MuLV ts26.

A group of temperature-sensitive mutants of Rauscher MuLV (R-MuLV) have been isolated and partially characterized by Stephenson and Aaronson and their co-workers (199-201, 50,51). These form infectious progeny when grown at 31° but not at 39°, whereas wild-type virus forms infectious progeny at either temperature. Several of these mutants were reported to be defective in the processing of $Pr65^{gag}$ to the mature virus core or *gag* proteins (50,51,201,202). We have studied the polypeptide processing of one of these mutants called ts26. After shifting from permissive (31°) to restrictive (39°) temperature, ts26 infected cells were pulse-labelled with L-[^{35}S]methionine and subsequently chased with excess unlabelled methionine. Radioactive proteins were immune precipitated from the labelled cell extracts and were analyzed on polyacrylamide gels in the presence of SDS and urea. Our results confirmed previous reports that cells infected with ts26 process $Pr65^{gag}$ approximately 1.5 to 3 times more slowly than do cells infected with wild-type R-MuLV (201). We also studied the synthesis and processing of gp70 related proteins in ts26 infected cells. As shown in Figure 15 we observed that while $gPr90^{env}$ is made at 39°, there is a nearly complete inhibition of its processing to form gp70. On the basis of these data and related studies, we have proposed that ts26 has a primary temperature-sensitive defect in gp70 synthesis and that the reduced

Figure 15. Pulse-Chase Analysis of gp70-Specific Proteins from Wild-Type and ts26 Rauscher Murine Leukemia Virus Infected Cells Grown at 39°.

Monolayer cultures (75 cm^2) of virus infected cells at 50-70% confluency were pulse-labelled at 39° for 30 min with 15 μCi/ml L-[^{35}S]methionine in 3 ml of methionine-free Minimal Essential Medium. At the conclusion of the pulse period the cells were chased by addition of unlabelled methionine (10 ml complete Minimal Essential Medium) for 1, 2 and 4 hr. Following the pulse and each of the chase periods, the cell monolayer was lysed and the cell extracts were immune precipitated by secondary antiserum precipitations using monospecific antiserum to gp70. The immune precipitates were analyzed by electrophoresis in SDS-urea gels (section II,F1). Lanes A-E are immune precipitates from wild-type R-MuLV infected cells grown at 39°: (A) normal goat serum control precipitation; (B) 30 min pulse; (C) 30 min pulse and 1 hr chase; (D) 30 min pulse and 2 hr chase; (E) 30 min pulse and 4 hr chase. Lanes F-J are immune precipitates from ts26 infected cells grown at 39°: (F) normal goat serum control precipitation; (G) 30 min pulse; (H) 30 min pulse and 1 hr chase; (I) 30 min pulse and 2 hr chase; (J) 30 min pulse and 4 hr chase. "gp90" is synonomous with the gPr90^{env} discussed in the text.


A
B
C
D
E
F
G
H
I
J
gp90
gp70

rate of $Pr65^{gag}$ processing is probably a secondary effect (107).

B. Absence of Plasma Membrane gp70 in ts26 Infected Cells at Restrictive Temperature.

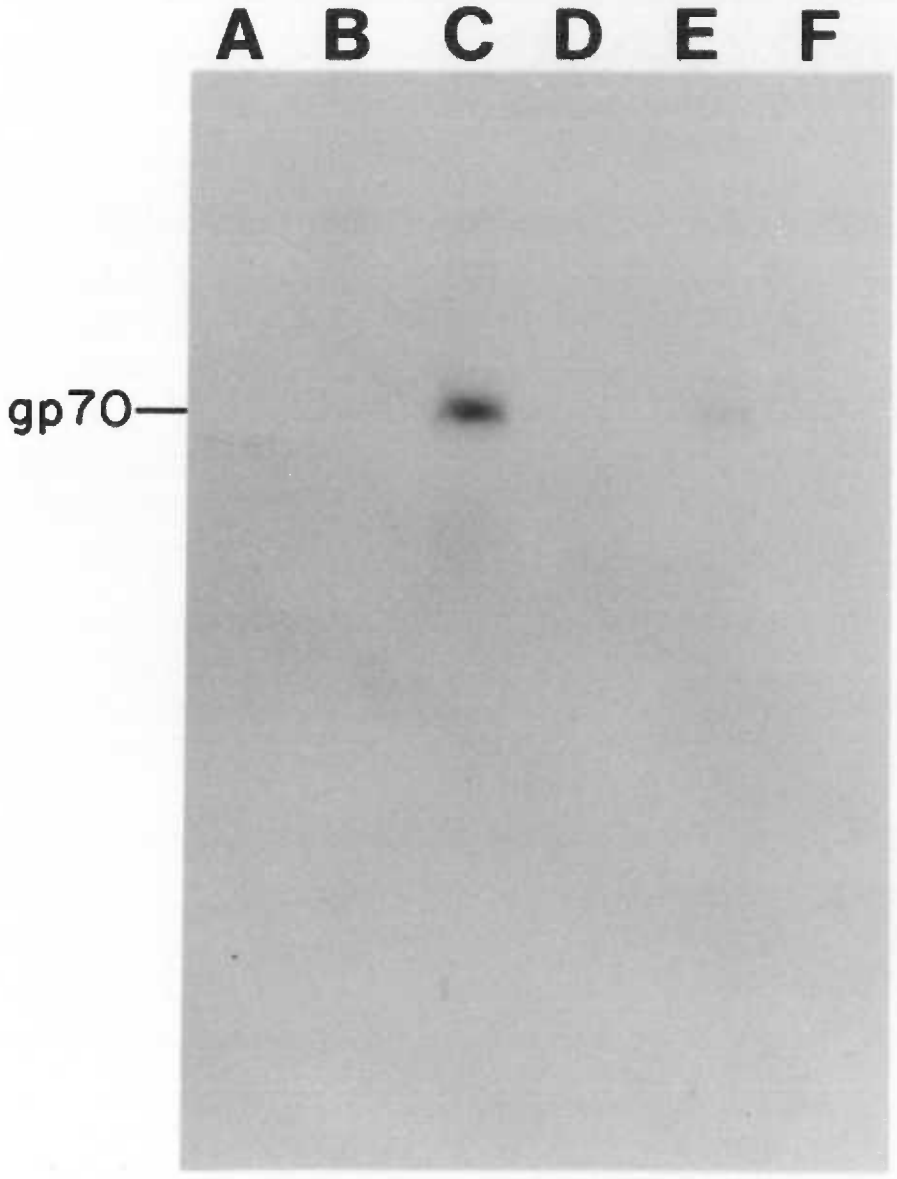
Normally, MuLV infected cells contain the *env* related proteins gp70 and p15E on their surface plasma membrane (section I,G). In the case of ts26, cleavage of $gPr90^{env}$ to mature envelope proteins is severely reduced (Fig. 15). Therefore, we were interested to determine whether any *env* related proteins occur on the plasma membrane of cells infected with ts26. The infected cells were labelled by lactoperoxidase catalyzed surface iodination with [^{125}I]iodine (133,174). Radioactive viral antigens were immune precipitated from cell extracts with monospecific antiserum to gp70 or p30 and were analyzed by polyacrylamide gel electrophoresis in the presence of SDS and urea. Figure 16 shows the results of this analysis. The plasma membrane of ts26 infected cells contains much less gp70 at 39° (lane E) than at 31° (lane C). This supports the conclusion that $gPr90^{env}$ processing is impaired at 39°. On the contrary, equal amounts of gp70 occur on the plasma membrane of cells infected with wild-type R-MuLV at both temperatures (data not shown). Monospecific antiserum to p30 did not precipitate any labelled components from the surface of these cells at either temperature (lanes B and D).

C. Release of Virus Particles from ts26 Infected Cells at the Restrictive Temperature.

Although they process the $gPr90^{env}$ molecule negligibly and

Figure 16. Cell Surface Glycoprotein of ts26 Infected Cells, as Revealed by Lactoperoxidase-Catalyzed Iodination of Cells with [^{125}I]iodine.

Cell surface labelling with [^{125}I]iodine was performed as previously described (section II,E and references 133 and 174). Cells infected with ts26 R-MuLV were grown at 31° or 39° prior to surface labelling. After removal of unreacted iodine, the cellular proteins were dissolved, immune precipitated with monospecific antiserum to gp70 and p30, and analyzed by electrophoresis in SDS-urea gels (section II,F1). Lanes A-C are immune precipitates from labelled cells grown at 31° while lanes D-F are immune precipitates from labelled cells grown at 39°. Lanes A and F are normal goat serum control precipitations. Lanes B and D are anti-p30 immune precipitates and lanes C and E are anti-gp70 immune precipitates.


A B C D E F
gp70

contain only a trace amount of plasma membrane gp70, cells infected with ts26 release defective MuLV particles when incubated at 39°. Cells infected with ts26 or with wild-type virus were labelled for 24 hrs with L-[^{35}S]methionine at 39° and the culture medium was then centrifuged at high speed to sediment particulate material. The pelleted proteins were efficiently redissolved in immune buffer A (0.01 M NaH_2PO_4, pH 7.6; 0.001 M EDTA; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS) and were then immunoprecipitated with monospecific antisera against viral proteins. The results in Table 3 indicate that cells infected with ts26 release particles at the restrictive temperature containing p30 but lacking gp70. Furthermore, as judged by their p30 content, these cells produce 40-50% as many MuLV particles as cells infected with wild-type virus.

Figure 17 shows an electrophoretic analysis of the radioactive virion proteins released from cells at 39° and immune precipitated by the gp70 antiserum. The antiserum specifically precipitated mature gp70 from the preparation of wild-type virus (lane A) and ts25 mutant which has normal gp70 synthesis (lane B). However, the particles released from ts26 infected cells lacked this glycoprotein (lane C).

The above results were unexpected because it had been reported previously that at 39° ts26 infected cells do not release particles containing reverse transcriptase (201). However, as shown in Table 4, we found reverse transcriptase in the defective ts26 particles released at 39° as measured either with endogenous

Table 3. Release of [^{35}S]Met-Labelled Virion by Infected Cells at 39°

Virus	Antiserum Used	Radioactivity Precipitated (cpm)
wild-type	MuLV-R	860
	p30	1240
	gp70	360
	Normal Goat Serum	35
ts26	MuLV-R	400
	p30	620
	gp70	30
	Normal Goat Serum	26

Figure 17. Immune Precipitation of gp70 from Particles Released at 39° by Cells Infected with Rauscher Murine Leukemia Virus Wild-Type and Temperature-Sensitive Mutants.

Cells infected with wild-type, ts25 and ts26 R-MuLV were labelled with L-[^{35}S]methionine at 39°. Medium from these labelled cell cultures was centrifuged at high speed to pellet particulate material. The pelleted material was solubilized in immune buffer A (section II,E) and immune precipitated with monospecific antiserum to gp70. The immune precipitates were electrophoresed in SDS-urea gels (section II,F1). Lane A, wild-type; Lane B, ts25 (a mutant with temperature-sensitive Pr65^{gag} processing); Lane C, ts26.

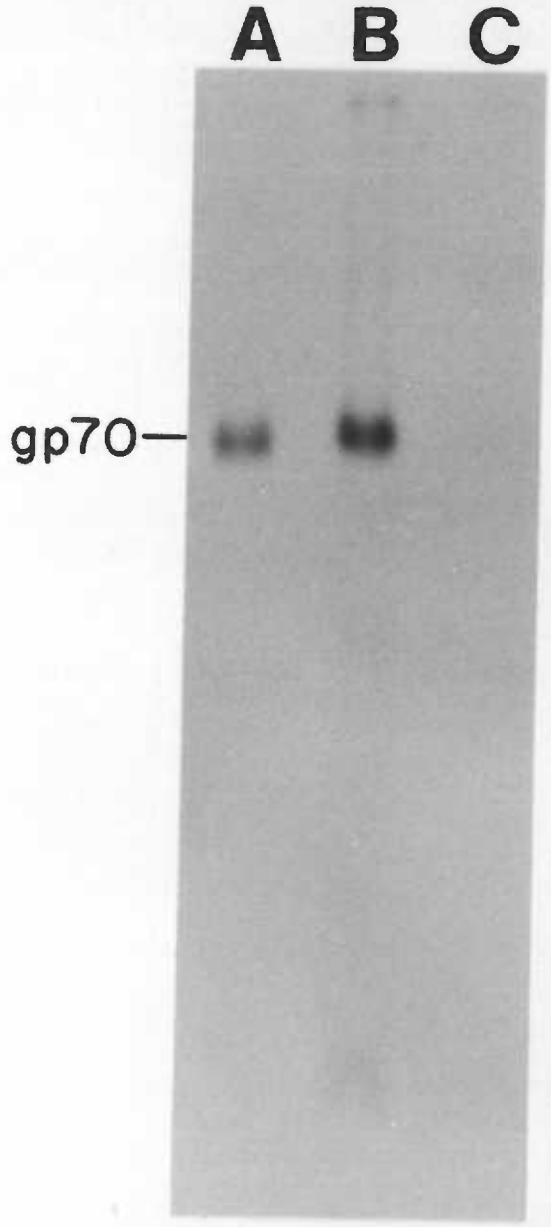

A
B
C
gp70

Table 4. Reverse Transcriptase in Particles Released from Cells Infected with ts26

Virus	Temperature	poly(rc) template	cpm
wild-type	31	+	1494
		-	316
	39	+	1122
		-	205
ts26	31	+	1222
		-	204
	39	+	740
		-	296

template or with an added poly(rC)-oligo(dG) template.

We therefore performed a more thorough analysis of the R-MuLV particles. The L-[^{35}S]methionine labelled particles were purified by isopycnic centrifugation in sucrose gradients and the virion proteins were analyzed by two-dimensional separation in polyacrylamide gels using isoelectric focusing in one dimension and sodium dodecyl sulfate electrophoresis in the second dimension (162,163). The particles released at 39° from cells infected with ts26 banded at a density very similar to wild-type virus (1.14-1.16 g/cc) and they contained all of the labelled virion proteins except gp70, p15E, and p12E. Figure 18 is a two-dimensional map of wild-type R-MuLV propagated at 39°. Figure 19 is the two-dimensional map of ts26 virus formed at 39°. The absence of gp70, p15E and p12E is indicated by the bracketed arrows. As mentioned above p15E and gp70 both derive from the same precursor (90-92). It is believed that p12E is a proteolytic fragment of p15E (92).

Additionally, the p30 components in the defective ts26 particles had different isoelectric points from the major p30 species present in wild-type virus (Figs. 18 and 19 asterisk). This was probably caused by a processing alteration at 39°, because the p30 in ts26 virus grown at 31° had the same isoelectric point components as wild-type p30 (data not shown). In addition, ts26 virus grown at 31° contained a normal amount of gp70.

An electron microscopic examination of the R-MuLV particles grown at 39° is shown in Figure 20. At this level of resolution the

Figure 18. Two-Dimensional Polyacrylamide Gel Electrophoresis of Wild-Type Rauscher Murine Leukemia Virus Formed at 39°.

Virion proteins were radioactively labelled at 39° with L-[^{35}S]methionine as described in Materials and Methods (section II,C4). Purified wild-type R-MuLV was solubilized in lysis buffer (section II,F2) containing 0.5% SDS. The solubilized virion proteins were electrophoresed in two-dimensions essentially as described by O'Farrell (162,163) with the modifications described in Materials and Methods (section II,F2). Following electrophoresis the second-dimension slab gel was processed for fluorography (176). The figure is oriented with the high pH end to the left and the low pH end to the right.

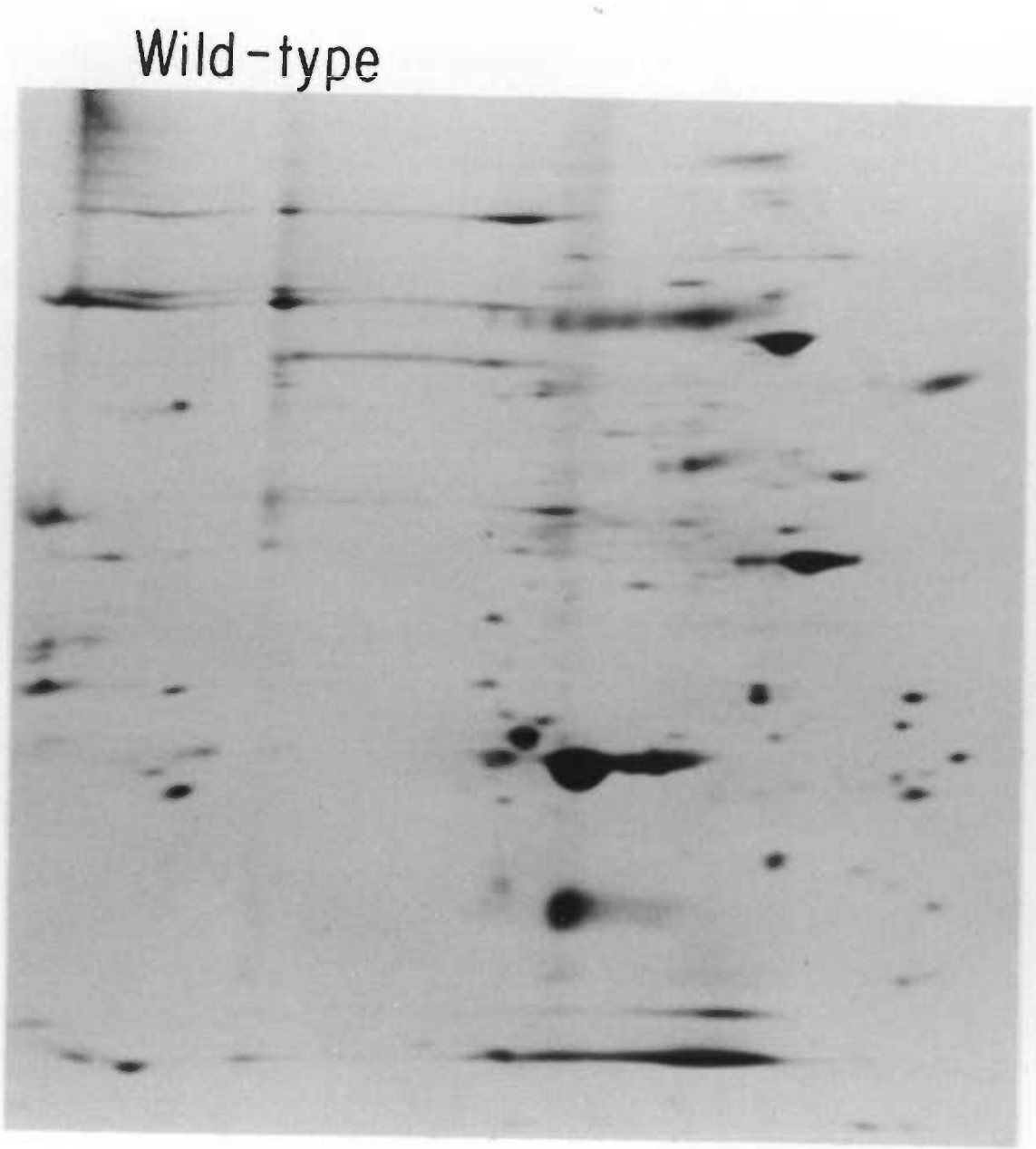

Wild-type

Figure 19. Two-Dimensional Polyacrylamide Gel Electrophoresis of the ts26 Mutant of Rauscher Murine Leukemia Virus Formed at 39°.

MuLV proteins were labelled, purified and electrophoresed as in Figure 18. Bracketed arrows indicate the missing virus proteins: upper brackets, gp70; lower brackets, p15E and p12E. The asterisk indicates the major p30 component seen in the wild-type and ts26 at 31°.

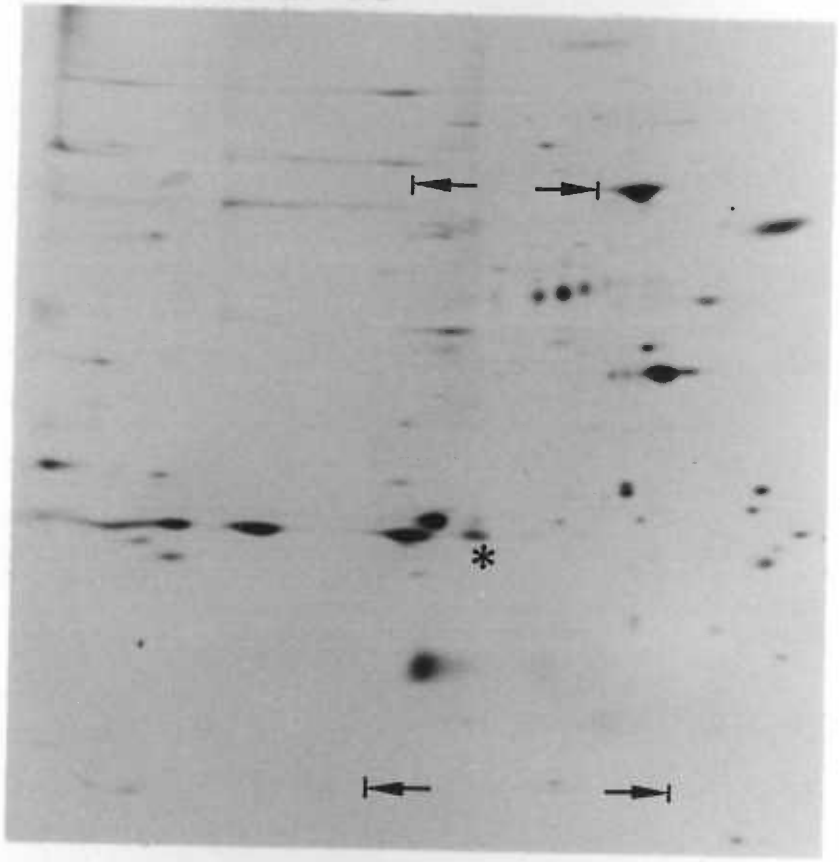
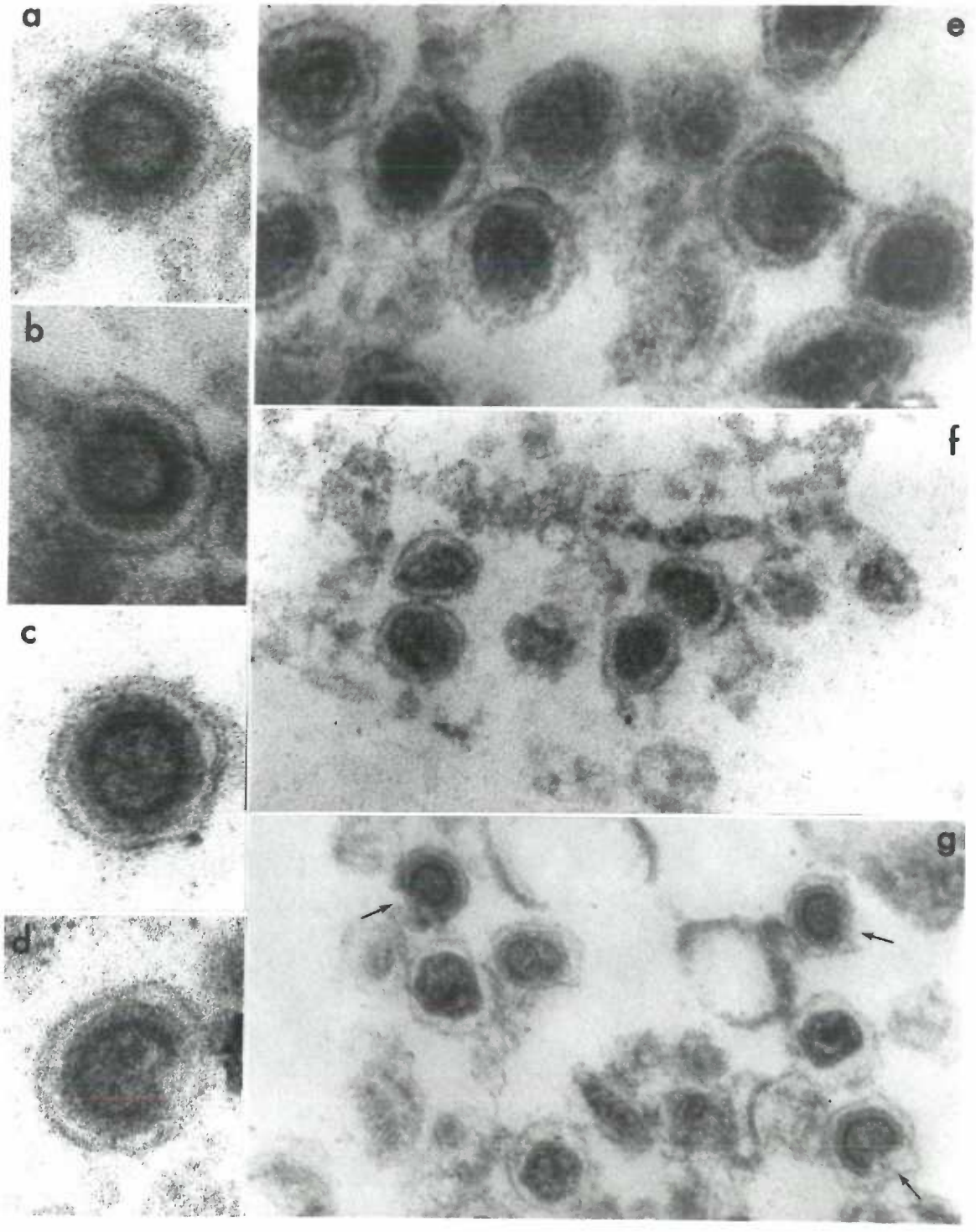

Mutant at 39°
*

Figure 20. Electron Microscopic Examination of Wild-Type and ts26 Rauscher Murine Leukemia Virus Particles Produced at 39°C.

The virus produced by wild-type and ts26 R-MuLV infected cells grown at 39° was harvested and purified by isopycnic banding in sucrose gradients. Purified virus was glutaraldehyde-fixed and thin-sectioned for electron microscopy. Panels (a)-(d) show single R-MuLV particles magnified 110,000 times: (a) and (b) are wild-type particles, (c) and (d) are ts26 particles. Panel (e) is a typical field of wild-type particles magnified 91,000 times while panels (f) and (g) are fields from ts26 preparations magnified 57,000 times. In panels (d) and (g, arrows) particles with incomplete crescent-shaped nucleoids can be seen. These are not seen in the wild-type virus preparations. Electron microscopy was performed by M. Webb, Dept. Anatomy, U.O.H.S.C.


a
b
c
d
e
f
g

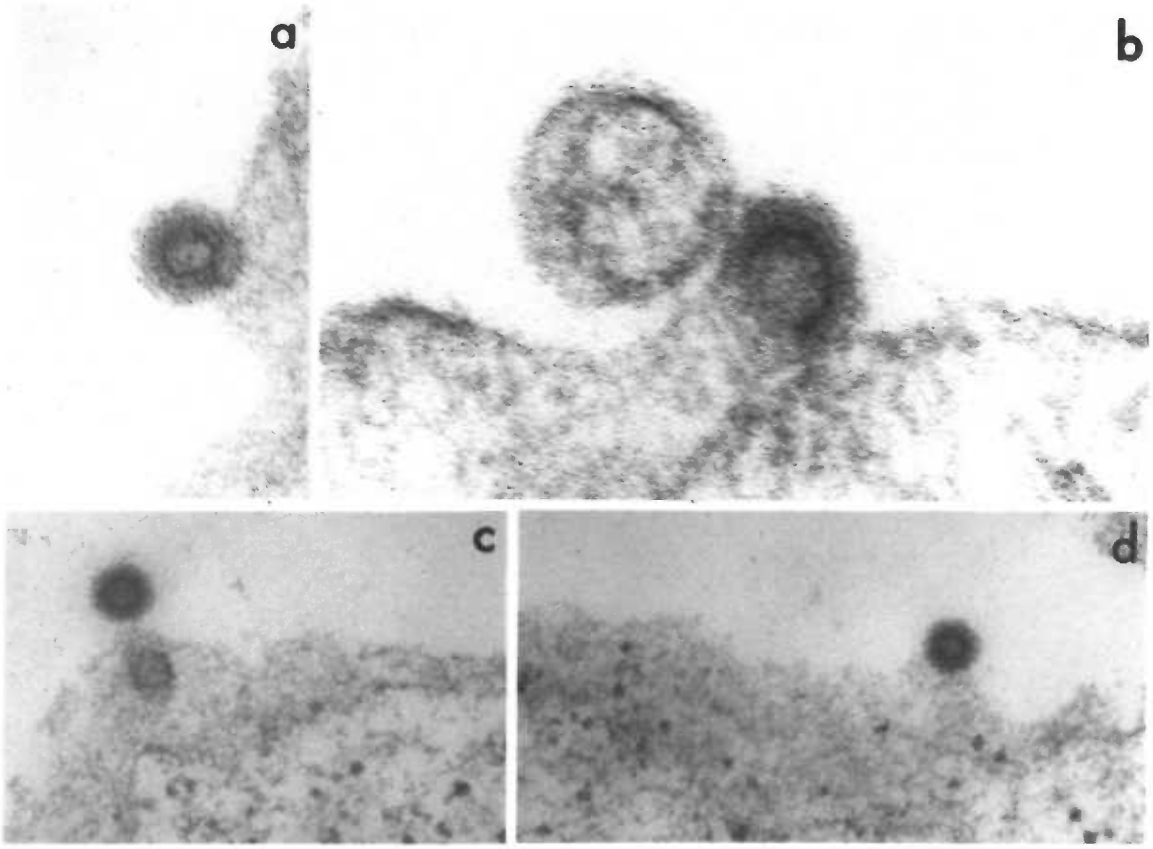
defective ts26 MuLV appeared indistinguishable from wild-type virus. It is noteworthy that the defective ts26 MuLV possess an outer membrane, even though they lack the membrane proteins gp70 and p15E. Presumably, this outer membrane must be acquired when the virion cores are released from the cells, very likely by a process of budding through the cell plasma membrane. Figure 21 shows wild-type R-MuLV budding from infected cells. This budding from wild-type R-MuLV infected cells was observed when the cells were grown at either 31° or 39°. However, a preliminary electron microscopic analysis of cells infected with ts26 and incubated at 39° showed no evidence for virus budding. Specifically, no budding virus was seen on the plasma membranes or on the intracellular membranes of 20 cell sections which were carefully examined. By way of contrast, a similar analysis of cells infected with wild-type virus revealed 12 virus buds. These results confirm an earlier electron microscopic study of cells infected with ts26 (203) which indicated that budding is severely reduced in cells infected with the mutant R-MuLV. These results create a dilemma concerning the mechanism of defective MuLV release.

D. Discussion.

Our results show that the ts26 mutant of Rauscher MuLV has a temperature-sensitive lesion in the synthesis of gp70 and p15E (107). When incubated at 39°, ts26 infected cells accumulate $gPr90^{env}$ and their plasma membranes become depleted of gp70 (Fig. 16). We do not yet know whether the $gPr90^{env}$ molecules accumulate in the endoplasmic reticulum or whether there is any processing of

Figure 21. Electron Microscopic Examination of Wild-Type Rauscher Murine Leukemia Virus Particles Budding from Infected Cells.

Cells infected with wild-type virus were grown at 39° in 75 cm^2 T-flasks. When the cells were 50-70% confluent they were dispersed with trypsin-EDTA solution and gently pelleted. The cell pellets were glutaraldehyde-fixed and thin-sectioned for electron microscopy. The magnifications are: (a) 112,500X; (b) 177,500X; (c) and (d) 25,000X. Electron microscopy was performed by M. Webb, Dept. Anatomy, U.O.H.S.C.


a
b
c
d

their carbohydrates. However the uncleaved gPr90^{env} molecules do not become incorporated into the plasma membrane.

In addition to the evidence suggesting that the temperature-sensitive lesion in ts26 virus is in its *env* gene, the results presented here have important implications for the mechanism of virus budding. Consistent with a previous report (203), the appearance of budding virus from ts26 infected cells is severely reduced at the non-permissive temperature. This lack of visible budding could be interpreted to mean either that membrane gp70 is required for virus budding, in agreement with a recent model (23), or else that plasma membrane gp70 retards the rate of bud growth and release so that budding virions accumulate on the membrane when gp70 is present. If this were true, then in the absence of gp70 release would be so rapid that buds would seldom be observed. We currently favor the latter explanation because the cells infected with ts26 and incubated at 39° release substantial amounts of defective MuLV particles which contain an outer membrane and the virion proteins p30 and reverse transcriptase but lack gp70 and p15E. However, the p30 in defective particles has a different isoelectric point than the p30 found in wild-type particles (Fig. 18 and 19 asterisk) or in ts26 virus grown at 31°. Based on this evidence, we conclude that the *gag* proteins of ts26 are processed differently at 31°and 39°. This is in accord with previous studies (201,202) and with our finding that Pr65^{gag} processing is inhibited in ts26 infected cells at 39° (107).

These basic properties of the ts26 mutant agree closely with available evidence concerning mutants of Rous sarcoma virus (RSV) which are defective in membrane glycoprotein synthesis (204-208). For example, cells infected with the Bryan high titer strain of RSV, which has a deletion of its *env* gene, nevertheless release substantial amounts of defective type-C virus (204-206). Furthermore, the infected cells appear to contain very few virus buds (207). Similarly, defective particles are released by cells infected with an RSV mutant that has a temperature-sensitive defect in envelope glycoprotein synthesis (208). Furthermore, cells infected with certain strains of Moloney murine sarcoma virus lack envelope glycoprotein but release defective virus particles that contain p30 (209). These results provide precedence for our conclusion that defective type-C retrovirus can be released from cells that lack virion envelope glycoproteins and are not consistent with a recent molecular model (23) for MuLV budding.

VI. SUMMARY AND CONCLUSIONS

Two-dimensional isoelectric focusing/sodium dodecyl sulfate electrophoresis has revealed that murine leukemia virus particles contain a complex population of proteins. In addition to virus encoded proteins, numerous host cell proteins were found in the virion. Some of the host proteins in the virus were quite abundant, indicating that they may be selectively included in virus particles. These host proteins might play some functional role in the virus. The virus encoded proteins gp70 and p30 were found to be isoelectrically heterogeneous. This charge heterogeneity was not affected when the MuLV was cloned or propagated in different host cell types, indicating that it is caused by post-translational modifications. The nature of the modification to p30 is not known.

Further analysis of the gp70 molecule revealed that its isoelectric heterogeneity is due to heterogeneous sialylation. More specifically, gp70 contains a carbohydrate side chain which lacks sialic acid and at least one other carbohydrate side chain which is heterogeneously sialylated.

The characteristic positions of proteins in the two-dimensional virus maps has enabled us to use the MuLV proteins as probes for viral gene function. Examination of uncloned gp70 revealed that it consisted of two components of different size (gp69/71) which were separable by cloning. The indication is that MuLV cultures often contain two *env* genes, each on a different 35S RNA subunit. Furthermore, these subunits may combine to form

heterozygous MuLVs which are capable of expressing both *env* genes. Examination of MuLV clones differing in their tropism revealed that all the virion proteins, among these clones, were identical except p30. Three independent B-tropic clones, when adapted to NB-tropic, experienced the same alteration in their p30 molecules. This indicates that the p30 region of the *gag* gene, and its product, are responsible for determining the tropism of the ecotropic MuLVs.

The results of studies with a temperature-sensitive mutant (ts26) of Rauscher MuLV demonstrated that cleavage of the gPr90^{env} precursor to mature glycoprotein is required for the appearance of gp70 on the cell surface. Cells infected with the ts26 mutant produce virus-like particles at 39° even though they lack surface gp70. This indicates that gp70 is not required for virus exit from the cell. However it may be required for virus "budding". Examination of ts26 particles produced at 39° revealed that the envelope proteins gp70 and p15E were absent. In addition, the p30 in these particles had an altered isoelectric character. Pulse-labelling experiments indicate that Pr65^{gag} processing is slightly inhibited in ts26. The altered p30 species we observed may be processing intermediates which accumulate in the absence of gp70.

Appendix

In section IV-A we described an analysis of the envelope glycoproteins of four clones derived from the Eveline II virus (Figure 9). Two of these clones make both gp69 and gp71 (lanes D,E). We argued that these clones probably arose by infection of a single cell by a heterozygous MuLV particle. The heterozygote would possess two *env* genes, one encoding gp69 and the other encoding gp71. It is also possible that a given microtiter well received two infected cells, one infected with a homozygous gp69 virus and the other infected with a homozygous gp71 virus. We believe this is an unlikely explanation and in this section we have calculated the probability that this might occur.

Problems of this kind can be handled by the Poisson probability law (210-212). The probability that two infected cells would occur in any given microtiter well is given by the following equation:

$$P_2 = \frac{\lambda^2}{2} e^{-\lambda}$$

where λ is the mean number of virus producing microtiter wells. In this case, 200 wells were examined for MuLV and five were found. Therefore, $\lambda = 5/200 = 0.025$. Substituting this value for λ in the above equation gives a probability of 0.0003. This confirms our assertion that the probability of two infected cells in a given well is very low.

Two of the four clones we analyzed produced gp69 and gp71. The probability that both these "heterozygotes" resulted from wells receiving two infected cells is vanishingly small.

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