SYNTHESIS AND PROCESSING OF MURINE LEUKEMIA VIRUS PROTEINS

by

Leonard H. Evans, B.S.

A THESIS

Presented to the Department of Biochemistry
and the Graduate Division of the University of Oregon Health
Sciences Center in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy
June 1977

APPROVED:

..........[redacted]........

(Professor in Charge of Thesis)

.......[redacted]....

(Chairman, Graduate Council)

ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. David Kabat for his interest and guidance, both as a friend and as my thesis advisor, throughout the course of these studies. I would also like to thank Dr. Kabat and other members of the laboratory for providing a warm and friendly atmosphere, as well as a stimulating environment to carry on research.

I am grateful to Dr. Charles Faust for many helpful discussions and for his interest in my research and career.

I would especially like to thank Dr. Howard Mason, who first introduced me to biochemical research. His warmth and concern, both on a personal and professional level, is deeply appreciated.

Finally, I would like to thank my wife, Pat, for typing this manuscript and more, for her unwavering encouragement and support of an oftentimes preoccupied graduate student.

TABLE OF CONTENTS

	Page
I. INTRODUCTION	1
I:1 Development of RNA Tumor Virology: The Viral Etiology of Spontaneous Neoplasms in Birds and Mammals	2
I:2 Morphology and Structure of RNA Tumor Viruses	8
I:3 Chemical Constituents of RNA Tumor Viruses: Structural Proteins and RNA	11
I:4 Reverse Transcriptase and the DNA Provirus	15
I:5 Genetic Markers of RNA Tumor Viruses	18
I:5.1 Host Range Restriction	18
I:5.2 Reverse Transcriptase Mutants	20
I:5.3 Transformation	21
I:5.4 Internal Group-Specific Antigens	23
I:6 Structure of the RNA Viral Genome	24
I:6.1 Avian Sarcoma and Leukosis Viruses	24
I:6.2 Murine Leukemia and Sarcoma Viruses and the Friend Virus Complex	26
I:7 The Synthesis of RNA Tumor Virus Proteins	27
I:8 Thesis Objective and Experimental Rationale	32
I:9 Organization of the Thesis	34
II. MATERIALS AND METHODS	35
II:1 Materials	35
II:2 Growth and Maintenance of Cells	35
II:3 Assays of Virus and Viral Components	36
II:3.1 MuLV	36
II:3.2 SFFV	37
II:3.3 Reverse Transcriptase Assay	37
II:3.4 Radioimmunoassay of p30	38
II:4 Cell Lysis for Detection of Cell-Associated Virus	40
II:5 Preparation of Virus	41
II:5.1 Purification of Virus from Eveline II Cell Culture Medium	41
II:5.2 Preparation of Stock Spleen Extract and Virus Preparation from Infected Mouse Serum	42
II:5.2.1 Preparation of Stock Spleen Extract	42
II:5.2.2 Preparation of Virus from Infected Mouse Serum	42
II:5.3 Preparation of Radioactive Virus	43
II:5.3.1 $[^{35}S]$-labeled F-MuLV	43
II:5.3.2 $[^{14}C]$-labeled MuLV	44

Page

II:6 Preparation of Rabbit Antiserum to F-MuLV.............. 44
II:7 Radioactive Labeling of Eveline Cells and Extraction of Cells for Immune Precipitation.......................... 46
II:8 Immune Precipitation Procedure.......................... 47
II:9 SDS-Polyacrylamide Gel Electrophoresis................. 48
II:10 Protein Determinations................................. 51
II:11 Scintillation Procedures............................... 52

III. ASSOCIATION OF FRIEND MURINE LEUKEMIA VIRUS WITH SUBCELLULAR MATERIALS.. 54

III:1 General.. 54
III:2 Results.. 55
III:2.1 The Distribution of Friend MuLV Infectivity on Isopycnic Sucrose Density Gradients............ 55
III:2.2 Electrophoresis of Proteins of the Dense and Light Virus Bands.............................. 56
III:2.3 Non-Virus Components in the 1.16 g/cm^3 and 1.24 g/cm^3 Virus Bands........................ 61
III:2.4 Rapidly Sedimenting MuLV Infectivity in Homogenates of Cultured Eveline Cells.......... 66
III:3 Discussion... 69

IV. THE SYNTHESIS OF FRIEND MURINE LEUKEMIA VIRUS PROTEINS IN CULTURED CELLS.. 71

IV:1 Purification and Characterization of Virus Produced by Eveline II Cells.. 71
IV:1.1 General.. 71
IV:1.2 Biological Nature of Virus Produced by Eveline II Cells.. 71
IV:1.3 Purification of Virus From Eveline Cells on Isopycnic Sucrose Gradients..................... 74
IV:1.4 Comparison of p30 Content of Purified F-MuLV from Eveline II Cells with Purified Rauscher Leukemia Virus (R-MuLV)...................... 74
IV:1.5 Electrophoretic Analysis of Viral Proteins on SDS/Polyacrylamide Gels......................... 77
IV:1.6 Electron-Microscopic Examination of F-MuLV from Eveline II Cells............................ 82
IV:1.7 Discussion... 83
IV:2 Detection of Murine Leukemia Virus Polyproteins in Cells Producing Friend Leukemia Virus......................... 86
IV:2.1 Immune Precipitation of F-MuLV Proteins by Antisera to F-MuLV............................ 86
IV:2.2 Immunoprecipitation of Intracellular Virus-Specific Proteins............................. 89
IV:2.3 Detection of Virus-Specific Polypeptides in Pulse-Chase Studies............................ 90
IV:2.4 Stability of Large Molecular Weight Polypeptides in SDS/Urea/Polyacrylamide Gels................ 97

Page

IV:2.5 Molecular Weight Determination of the Slowly Migrating Polypeptides 100
IV:2.6 Kinetics of Labeling of the Three Large Polyproteins 103
IV:2.7 Precipitation of Large Polypeptides with Antisera Directed Against p12, p30, and gp69/71 106
IV:2.8 Discussion 109
IV:2.8.1 The Detection and Properties of MuLV Polyproteins 109
IV:2.8.2 Implications Concerning Messenger RNAs. 113
IV:2.8.3 Role of MuLV Polyproteins 114
IV:3 Post-translational Processing of MuLV-Specific Proteins in Eveline II Cells 115
IV:3.1 General 115
IV:3.2 Pulse-Chase Analysis of F-MuLV Protein Synthesis in Eveline II Cells 116
IV:3.3 Identification of the Virus-Specific Proteins Using Monospecific Antisera to Virion Proteins... 121
IV:3.4 Pulse-Chase Analysis of gp69/71 Precursors 124
IV:3.5 Pulse-Chase Analysis of p30 Precursors 130
IV:3.6 Alternative Processing Pathways in MuLV Infected Cells 133
IV:3.7 Discussion 142

V. SUMMARY AND CONCLUSIONS 144

REFERENCES 149

LIST OF ILLUSTRATIONS

		Page
Figure III.1	MuLV, SFFV and Reverse Transcriptase Activities From Large Scale Preparation of Serum Virus........	58
Figure III.2	SDS/Polyacrylamide Gel Electrophoresis of Virus Bands at 1.24 and 1.16 g/cm^3......................	60
Figure III.3	Radioimmunoassay for p30 in Friend Leukemia Virus from Eveline II Cells and in the Serum Virus Bands at 1.24 g/cm^3 and 1.16 g/cm^3......................	63
Figure III.4	Detection of Bands at 1.24 g/cm^3 and 1.16 g/cm^3 from Uninfected Mouse Serum.......................	65
Figure III.5	Electron Microscopic Examination of Isopycnic Bands from Infected and Uninfected Mouse Serum.....	68
Figure IV.1	Isopycnic Sucrose Density Gradient of Virus from Eveline II Cells.................................	76
Figure IV.2	Competition Radioimmunoassay Comparing p30 Content of Rauscher MuLV and Friend MuLV from Eveline II Cells..	79
Figure IV.3	Electrophoretic Analysis of F-MuLV from Eveline II Cells: Molecular Weights of the Major Viral Proteins..	81
Figure IV.4	Electron Microscopic Examination of Purified F-MuLV from Eveline II Medium.....................	85
Figure IV.5	Precipitation of Disrupted [^{14}C]-labeled Virus with Anti-F-MuLV Serum............................	88
Figure IV.6	Pulse-Chase Analysis of Friend Leukemia Virus Proteins Synthesized in Eveline II Cells...........	93
Figure IV.7	Kinetics of Labeling and Degradation of Virus-Specific Polypeptides in Eveline II Cells..........	96
Figure IV.8	Labeling Kinetics of *Class (i)* Polypeptides........	99

Page

Figure IV.9	Stability of Proteins on SDS/Urea Gels; Kinetics of Labeling and Decay of Large Polyproteins; and Pulse-Chase Analysis of p30-Specific Proteins in Eveline II Cells................................	102
Figure IV.10	Molecular Weight Determination of Slowly Migrating Polypeptides....................................	105
Figure IV.11	Precipitation of Large Polyproteins with Antisera Directed Against the Viral Proteins p12, p30 and gp69/71..	108
Figure IV.12	Pulse-Chase Analysis of F-MuLV-Specific Proteins with Antiserum to Whole Disrupted F-MuLV..........	119
Figure IV.13	Precipitation of 10 Minute Pulse-labeled Cells with Monospecific Antisera.......................	123
Figure IV.14	Pulse-Chase Analysis of gp69/71-Specific Proteins in Eveline II Cells..............................	126
Figure IV.15	Kinetics of Labeling of gp69/71-Specific Proteins in Eveline II Cells..............................	129
Figure IV.16	Comparison of p30 and gp69/71-Specific Proteins in Pulse-Chased Eveline II Cells....................	132
Figure IV.17	Precipitation of gp69/71, p30 and p12 Specific Proteins from Late Log-Phase Eveline II Cells.....	135
Figure IV.18	Production of Infective and Radiolabeled Virus Particles During Cellular Growth.................	138
Figure IV.19	Pulse-Chase Analysis of F-MuLV-Specific Proteins in Stationary Phase Cells........................	141
Figure V.1	Processing Scheme for the MuLV-Specific Proteins..	146

I. INTRODUCTION

Tumor virology had its origins in the early part of this century and proceeded gradually for a period of forty to fifty years. Much of the research during this period was devoted to the description of malignant diseases and to establishing their viral etiology. The development of electron microscopy and improved cell culture techniques during the 1950's had a tremendous impact on the field. Electron microscopy enabled researchers to identify and study the detailed morphology of virus particles; and the development of cell culture techniques opened the field to molecular biological studies of the infective, replicative, and, in the case of sarcoma viruses, the transforming functions of the viruses. The following introduction is intended to provide a survey of many of the important developments in the field of RNA tumor virology. The early developments in tumor virology, including both RNA and DNA tumor viruses, have been exhaustively chronicled by Ludwik Gross in his book, Oncogenic Viruses (98). Much of the early historical introduction surveys portions of this book. Original references have been cited when directly consulted or when the original source was unavailable but represented a major advance. More recent developments in the field are surveyed in Tooze's book, The Molecular Biology of Tumour Viruses (207), as well as a recent Cold Springs Harbor symposium monograph (211); and in the proceedings of a recent ICN-UCLA symposium on animal viruses (17).

I:1 Development of RNA Tumor Virology: The Viral Etiology of Spontaneous Neoplasms in Birds and Mammals.

In 1908 Ellermann and Bang observed the transmission of an erythromyeloblastic leukemia by cell-free filtrates from leukemic cells of afflicted chickens (70). Leukemia was not designated at that early time as a neoplastic disorder however, and the significance of the observation was not immediately recognized. A short time later Peyton Rous, studying a transplantable solid sarcoma of chickens, successfully demonstrated the transmission of the tumor to healthy chickens by innoculation of cell-free filtrates derived from sarcoma cells (163,164). There was no doubt that the sarcoma studied by Rous was a cancer and the finding marked the true beginning of tumor virology.

The years that followed these early observations witnessed the gradual development of tumor virology. Following his initial discovery, Rous demonstrated the viral etiology of a number of spontaneously arising chicken tumors (165,166) and the work was independently confirmed in 1914 by Fujinami and Inamoto in Japan (84). However several years elapsed before the avian tumor viruses attracted the attention of other investigators, and it was not until the nineteen twenties and thirties that the avian tumor viruses and the diseases they cause were further characterized.

Ellerman and Bang noted in their early work that the manifestation of the disease transmitted by the cell-free filtrates from leukemic cells was variable in different birds under apparently

identical conditions. Many of the early studies were focused on the description of chicken leukemias and on establishing their viral etiology. The different manifestations of the disease were collectively termed the avian leukoses or the avian leukosis complex (100) which was divided into two general groups. The first group included true leukemias which were characterized by the appearance of large numbers of malignant cells in the circulation. Among these were the erythroleukemias and the myeloblastic leukemias. The second group termed the aleukemic group or lymphomatoses were characterized by tissue infiltration rather than gross circulatory involvement. There was no clear demarcation in the two forms of the disease. Often the same virus stock would induce different forms of the disease in different birds, and quite often the pathology would vary in the same chicken during the course of an infection. Whether these conditions are due to different viruses in the virus stocks or to variations in the host response to the same virus is still unclear in many instances; however it appears that certain of these diseases may be caused by unique viruses as evidenced by virus isolates which induce predominately only one manifestation of the disease. Examples of this are the myeloblastosis and erythroblastosis viruses studied extensively by Beard and his associates (23,25,26).

Other important early developments involved the host range of the sarcoma viruses. The original virus studied by Rous was restricted in its activity to just a few strains of chickens. However continued passage of the virus led to more virulent forms which could infect and induce tumors in a wide variety of chickens. In 1928 Fujinami and

Suzue demonstrated that a strain of chicken sarcoma virus could induce tumors in ducks (85) and it was later established that the virus could induce tumors in a number of other fowl. Many years later Svet-Moldavsky extended the host range of these viruses to mammalian species by the demonstration of sarcoma induction by the virus in rats (199).

About the same time that Rous demonstrated the transmission of chicken tumors by cell-free extracts, geneticists began to inbreed mice in efforts to obtain strains which were uniformly susceptible to chemical carcinogens. By the nineteen thirties a number of highly inbred strains of mice were available. The females of one such strain, the C3H strain developed by Strong, exhibited a very high incidence of mammary gland carcinomas (196). It was soon noted that the offspring of females of the high incidence strain crossed with males of low incidence strains developed a high incidence of the carcinoma, whereas when the parental strains were reversed, the offspring remained relatively free of breast cancer (186). In addition, offspring of C3H mice foster-nursed by females of low incidence strains did not exhibit a high incidence of the cancer (35,37). Bittner and others subsequently established that the agent responsible for the mammary gland carcinoma was in fact a milk-borne virus transmitted from mother to suckling offspring (2,36,37).

Two strains of mice, the Ak strain developed by Furth and the C58 strain developed by McDowell, exhibited extremely high occurrences of spontaneous leukemia with up to 90% of the mice dying from the disease during middle age (86,129). Repeated attempts in many

different laboratories failed to successfully transmit the disease to low incidence strains of mice by cell-free extracts (19,152,157). Although the age of the host had been shown to be an important factor in the transmission of the mammary gland carcinomas as well as in the transmission of the chicken leukemia and sarcoma viruses, most attempts to transmit leukemias were performed using adult mice. In 1951 Ludwik Gross reported the induction of leukemia in over 50% of innoculated mice of a low incidence strain when innoculated as neonates with cell-free extracts from Ak leukemia cells (95). These results were obtained with centrifuged extracts rather than filtrates, however, and subsequent experiments with filtrates yielded significant but less striking results (94). Gross' results met with much skepticism and initial attempts to repeat the work in other laboratories were unsuccessful (123,189). Retrospectively it appears that the early difficulties encountered in the transmission of the disease were due, in part, to the low level of infective particles in the spontaneously arising leukemias and the lack of knowledge concerning the restricted host ranges of murine leukemia viruses. Confirmation of the results did not come until five years later when a number of laboratories, including some which had previously been unable to repeat the observations, reported the cell-free transmission of mouse leukemia (66,87, 190,223). By repeated passage by filtrates Gross subsequently succeeded in deriving a highly potent strain of the virus which induced lymphatic leukemia in over 95% of the innoculated animals within two to three months (97).

Soon after the viral etiology of mouse leukemias was established,

a number of attempts were initiated in different laboratories to find viruses capable of transmitting other forms of cancer in mice. These studies were unsuccessful in transmitting the original tumors, however, some of them resulted in the isolation of viruses capable of inducing leukemias in innoculated mice (82,93,135,153). Among the better studied of these isolates are the Friend leukemia virus, the Moloney leukemia virus and the Rauscher leukemia virus, all named after their respective discoverers. Initial studies on the Moloney strain of murine leukemia virus indicated that it was very similar to the virus isolated from Ak mice by Gross. The Rauscher and Friend viruses, however, both induced a progressive erythroblastosis in susceptible mice. Subsequent studies revealed that these two isolates are in fact complexes of two distinct viral entities; a replication-defective erythroblastosis virus and a competent "helper" lymphatic leukemia virus which provides replicative functions for the former in coinfected cells (53). Furthermore, the lymphatic leukemia components of these viruses, as well as the lymphatic leukemia virus isolated by Moloney, induce surface changes on leukemic cells which are antigenically distinct from those induced by the lymphatic leukemia viruses obtained from spontaneous leukemia (3,143). This difference forms the basis for the two serological subgroups of murine leukemia viruses; the FMR (Friend-Moloney-Rauscher) subgroup and the G-AKR subgroup (referring to the leukemia virus isolated by Gross and to leukemia viruses isolated from Ak mice, Rockefeller strain).

Although attempts to transmit sarcomas in mice by innoculation of cell-free extracts from sarcoma tumors were unsuccessful, Harvey,

in 1964, obtained a virus stock which induced sarcomas in mice after passage of Moloney leukemia virus in rats (106). A number of sarcoma viruses have subsequently been isolated, all of which are associated with leukemia viruses. The Moloney sarcoma virus was obtained after the innoculation of high doses of Moloney leukemia virus in mice (136). Kirsten and Mayer isolated a sarcoma virus after passage of leukemia virus in rats (119), and the Abelson virus, which is apparently able to induce both sarcomas and erythroleukemia, has been isolated from prednisone treated mice infected with Moloney leukemia virus (1). All of the sarcoma viruses in mice are defective to various extents and require coinfection with leukemia viruses in order to replicate in infected cells. Leukemia virus and sarcoma viruses have subsequently been isolated from several mammalian species including the cat, the wooley monkey and the gibbon ape. In all cases, mammalian sarcoma viruses have been found to be defective in replication. The relationship between leukemia and sarcoma viruses in mammals is fundamentally different than in birds where either leukemia or sarcoma viruses can replicate in permissive cells (176).

Particles with the typical morphology of leukemia viruses (section I:2) have been observed in a wide variety of cultured cells including human (88,146). It appears very likely that most, if not all,mammalian cells harbor endogenous genes coding for viruses with the typical morphology of leukemia viruses. The extent to which these viruses participate in the etiology of human leukemias is a matter which is still unresolved.

I:2 Morphology and Structure of RNA Tumor Viruses.

Early studies on the properties of the infective agent in chicken sarcomas indicated that it was quite small and relatively labile. On the basis of ultracentrifugation studies and retention of infectivity on graded membranes, Elford and Andrewes concluded that the virus was somewhat less than 100 mμ in diameter (69). The sarcomagenic activity of the virus was found to be 3 orders of magnitude more resistant to X-irradiation than sarcoma cells, indicating a much smaller target size for the virus (120). In vitro incubation at physiological temperature inactivated the virus in less than 48 hours and heating at 55°C for 15 minutes destroyed all infectivity. The virus was also found to be inactivated by bile and ordinary antiseptic solutions. However, the virus survived freezing and lyophilization and could be stored in this manner for long periods of time (99).

Gross and others reported properties very similar to those of the chicken sarcoma viruses for the infective agent in murine leukemias (96,97). Graded membrane filtration indicated that the virus was about 70 to 100 mμ in diameter. The agent was highly resistant to irradiation by gamma rays. The virus was inactivated by heating at 50°C for 30 minutes and was sensitive to ether. Like the chicken sarcoma virus the mouse leukemia virus could be frozen and stored at -70°C and was relatively stable to lyophilization.

With the advent of electron microscopy it became possible to identify the tumor viruses and to study their morphology. The detection of particles in tumor cells by electron microscopy was first

reported by Claude, Porter and Pickels in 1947 (50) who detected small electron-dense particles in chicken sarcoma cells. The particles were uniformly spherical and had a diameter of about 70 to 80 mμ; however, the resolution was not sufficient to obtain a more detailed description of their morphology. With the development of thin sectioning techniques and later, freeze-etching, it became possible to obtain more detailed information about the structure of the virus. Initial studies on the structure of chicken sarcoma virus indicated that the particle contained a distinct external membrane with a centrally located electron-dense core or nucleoid. The diameter of the virus particle, including the membrane, is about 100 mμ. Particles with this characteristic morphology were found to be associated with most chicken sarcomas as well as with the chicken leukemias (29). Particles with virtually the same structure were soon identified as a common feature in mouse leukemia (32,57). Subsequent studies have shown that the particles are present in virtually all virus-induced as well as many spontaneous leukemias in the mouse (77,78). Studies by Epstein (71,72) established a quantitative correlation of the tumor inducing activity of extracts prepared from chicken sarcomas and the number of particles observed in tumor tissue, suggesting that the particles observed are in fact the agents responsible for the transmission of the tumor.

Electron microscopic examination of mouse mammary gland carcinomas also led to the identification of the virus responsible for the transmission of this tumor (31). Like the avian tumor viruses and the mouse leukemia virus, the particles are spherical, about 100 mμ in

diameter, contain an outer envelope and an electron-dense nucleoid. In contrast to the sarcoma and leukemia viruses, however, the mouse mammary tumor virus does not have a centrally located nucleoid but rather the nucleoid is elliptically located on or near the inner surface of the viral envelope (30).

RNA tumor virus particles have been classified into three groups, designated A-type, B-type and C-type, on the basis of the structure of the nucleoid or on its position in relation to the viral envelope (29,167). Except for the murine mammary tumor virus, all mature RNA viruses have C-type morphology. The nucleoid appears as a solid spherical structure that is centrally located within the viral envelope. The murine mammary tumor viruses are the only well characterized viruses exhibiting B-type morphology. As noted above, B-type particles are similar in size and structure to C-type particles but are distinguished by the eccentric location of the nucleoid with respect to the viral envelope. A-type particles are typically found intracellularly and are characterized by a toriod or doughnut-shaped nucleoid. These particles are found in cells producing either B-type or C-type particles and are thought to be progenitors of mature virus particles.

Freeze-etching studies have provided a more detailed description of C-type RNA tumor viruses (141). The virus is encapsulated in an outer envelope which appears as a unit membrane with knob-like projections or "spikes" on its surface. Immediately interior to the viral envelope is a core shell comprised of hexagonal subunits. The core shell surrounds a core membrane which in turn encloses the ribonucleoprotein nucleoid.

I:3 Chemical Constituents of RNA Tumor Viruses: Structural Proteins and RNA.

The overall chemical composition of C-type RNA tumor viruses is approximately 20-30% lipid, 60-70% protein, 2% carbohydrate and 1-2% RNA (42,151). Small amounts of DNA have been a frequently reported finding in virus preparations but are thought to be of host origin (124,159,160,214). The carbohydrate of the virus is contained in glycoprotein components and the lipid is the major constituent of the virus membranes.

When the RNA of the virus is extracted with phenol and sedimented on neutral sucrose gradients, it is resolved into a rapidly sedimenting 60-70S species and a slowly sedimenting 4-5S RNA species (161). Upon denaturation by heating or treatment with dimethylsulfoxide, the 60-70S component dissociates into at least two 30-35S subunits, which are single stranded and have a molecular weight of approximately 3,000,000 as determined by sedimentation and by direct measurements of their length by electron microscopy (13,55,58). This general description appears to be a universal feature of all C-type virus RNA as well as of the RNA of mouse mammary tumor viruses. Recent studies indicate that the subunits of the viral RNA are identical for a particular virus. Thus, the viruses are polyploid (28,63,220,222). The molecular and genetic structure of the 30-40S viral RNA will be discussed in more detail in a later section.

The 4-5S RNA components contain host cell tRNAs (27,47,73). Some of these may be contaminants encapsulated during the maturation of the

virus. However, one of the tRNAs has been shown to directly participate in the replication of the viral genome (67,170). When viral RNA is heated to 65°C almost all of the 4S RNA is dissociated from the 60-70S RNA. When the RNA is further heated to 95°C an additional amount of 4S RNA is separated from the rapidly sedimenting species (169). This tightly associated tRNA is homogeneous and corresponds to $tRNA^{tryp}$ in the avian viruses (170) and to $tRNA^{pro}$ in murine viruses (107). These tRNAs are apparently identical to the respective tRNAs found in the host cell. Recent studies indicate that the tRNAs function as primers for the transcription of the viral genome (169, 200). By base pairing to the viral genome as well as specifically binding the viral polymerase (section I:4), the tRNA confers a high degree of site specificity for the initiation of transcription (107). Interestingly, the initiation of transcription does not begin at the 3'-end of the 30-35S RNA template and proceed to the 5'-end as might be expected. Rather, initiation begins near the 5'-end, proceeds to the 5' terminus, and then continues transcription at the 3'-end (48, 107,201). The significance of this phenomenon and the precise mechanism by which it occurs is the subject of much current investigation.

The proteins of the avian and murine RNA tumor viruses exhibit general similarities and a number of homologies between avian and murine virus proteins have been established. There are six major proteins of the avian viruses and five major proteins in murine leukemia viruses which have been identified by gel filtration or gel electrophoresis (41,79,142). According to convention, the viral

proteins are designated with the letter p (protein) or gp (glycoprotein) followed by the apparent molecular weight of the protein times 10^{-3} (6); however, the use of these designations in discussions of both avian and murine viral proteins involves overlapping terminology. Thus in the following discussion only the murine leukemia virus proteins will be referred to by the conventional nomenclature.

The projections or "spikes" on the viral envelope contain the major glycoproteins of the viruses which are necessary for the adsorption of the virus to the cells during infection (104,158). The avian tumor virus glycoproteins have apparent molecular weights of approximately 85,000 and 40,000 daltons as estimated by sodium dodecyl sulfate (SDS) gel electrophoresis (41,61). It is not clear if these two glycoproteins represent distinct polypeptides or differences in only the carbohydrate moieties. The murine tumor virus glycoproteins migrate with apparent molecular weights of 70,000 daltons and 45,000 daltons in SDS gels and are designated gp70 and gp45 respectively (41, 61). However, recent evidence suggests that the 45,000 dalton glycoprotein differs from the larger glycoprotein only in the carbohydrate portion of the molecule (132). In the Rauscher and Friend strains of murine leukemia virus the 70,000 dalton glycoprotein is resolved into two closely migrating species which have been termed gp69/71 (193,194). These glycoproteins are believed to differ only in the carbohydrate portion of the molecule.

The virus core contains several lower molecular weight polypeptides. Avian viruses contain proteins of 10,000, 12,000, 15,000, 19,000 and 27,000 daltons as determined by gel filtration in the

presence of guanidine hydrochloride (41,79). The murine leukemia viruses contain proteins of 10,000 (p10), 12,000 (p12), 15,000 (p15) and 30,000 daltons (p30) but lack a 19,000 dalton component (41). The major protein of the core shell in murine viruses is p30 which constitutes the predominant protein of the virus. The analogous protein in the avian viruses is the 27,000 dalton component. The virus nucleoid consists of a ribonucleoprotein complex which contains the RNA genome in close association with the viral polymerase and at least one of the low molecular weight proteins. Fleissner and Tress (80) reported that a basic protein of low molecular weight is present in the ribonucleoprotein structures of both avian and murine viruses. This protein corresponds to p10 of the murine virus and the 12,000 dalton component of the avian virus. It has recently been shown that p10 preferentially binds single-stranded RNA and DNA (51). The binding was not specific for the viral RNA in that calf thymus single-stranded DNA was bound with equal affinity.

The topographical location and functions of the remaining lower molecular weight proteins found in the interior of the virus are poorly understood. Recent studies suggest that p12, which is the only phosphoprotein of the murine virus, may bind viral RNA in small amounts (178). The binding was reported to be highly specific in that complexes were formed only with p12 proteins incubated with viral RNAs from the save viruses. Further studies are required to establish the validity of these observations. The murine leukemia virus protein p15 is also associated with the virus core; however, little

is known about its specific location or function. This protein has been notoriously difficult to study because of its lack of solubility in normal buffers. There have been various reports of additional murine leukemia virus proteins in the 15,000 dalton molecular weight range. Ikeda et al. (112) have reported a component eluting in the void volume of guanidine hydrochloride gel filtration columns which migrates in SDS gels with an apparent molecular weight of approximately 16,000 daltons. Ihle et al. (111) have obtained evidence that a protein of approximately 15,000 daltons is associated with the viral envelope. The identity of this polypeptide is not clear at the present time, however, a recent report by Naso et al. (140) may have some bearing on the problem. These workers have identified two viral proteins which are termed p15E and p12E. p15E migrates in SDS gels very similarly to the component described above. p12E was shown to contain amino acid sequences present in p15E and it was therefore postulated that p12E was derived from p15E. However a note added in proof to this report states that both of these components share amino acid sequences with the envelope glycoprotein gp69/71. In light of this finding it appears probable that p15E and p12E are related to the proteins detected by Ihle et al. (111) and Ikeda et al. (112) and may correspond to breakdown products of the envelope glycoprotein. Further studies will be required to resolve this problem.

I:4 Reverse Transcriptase and the DNA Provirus.

Although virus production in explants of tumor tissue was observed by Furth as early as 1934, it was not until the development of refined

cell culture techniques in the 1950's that studies of in vitro infection, replication and transformation became possible. One of the early applications of cell culture was the development of a reproducible quantitative assay for avian sarcoma viruses by Temin and Rubin (205). When fibroblasts from chick embryos growing in monolayers were infected with the sarcoma virus discrete colonies of transformed cells were readily discernable by virtue of their changed morphology. The number of such colonies of transformed cells was found to be directly proportional to the dilution of infecting virus which indicated that a cell could be transformed by a single avian sarcoma virus.

The ability to productively infect cultured cells enabled researchers to study the effects of a wide variety of metabolic inhibitors on the infective and replicative processes of RNA tumor viruses. One of the early important observations was that when DNA synthesis was inhibited concommitant with virus infection the infection was blocked (9,10,11,204). This requirement for DNA synthesis was restricted to the first 8-12 hours after infection. Furthermore, treatment of infected cells with actinomycin D, which blocks the transcription of RNA from double-stranded DNA, resulted in a drastic decrease in virus production (202). These results differed from the results obtained from many non-oncogenic RNA viruses which did not require DNA synthesis, and whose replication was unaffected by actinomycin D. On the basis of these observations, Temin proposed a double-stranded DNA intermediate, termed the provirus, in the replication of RNA tumor viruses (203). According to Temin's hypothesis the

infecting virus RNA was transcribed by an RNA-dependent DNA polymerase to a double-stranded DNA provirus, which in turn served as a template for the synthesis of progeny viral RNA. Temin further postulated that the provirus could be stably integrated into the host genome, which would account for the heritability of the transforming functions of the virus. At the time this hypothesis was presented there was a dogma concerning the unidirectional flow of genetic information from DNA to RNA to protein and the proposal was met with considerable scepticism. This was overcome by the demonstration by Temin and Mizutani (206), and independently by Baltimore (15), that an enzyme which catalysed the precise reaction hypothesized by Temin was present in both murine and avian RNA tumor viruses. The enzyme was termed reverse transcriptase.

The actual demonstration of an intracellular provirus came from transfection experiments. It is well established that the addition of viral nucleic acid to cells can effect a productive infection, albeit, at a very low level as compared to intact virus. This phenomenon, which is termed transfection, has been observed with SV-40 DNA and poliovirus RNA as well as other viral nucleic acids. Shortly after the discovery of reverse transcriptase, Hill and Hillova demonstrated the existence of infectious proviral DNA in rat cells which had been infected with avian sarcoma virus (108). When chicken cells are treated with DNA from infected mammalian cells they become transformed and liberate virus identical to the original infecting virus (109). Infectious provirus has also been detected in cells infected with murine leukemia virus. Three forms of infectious pro-

virus have been detected in infected cells; a double-stranded linear unintegrated form; a double-stranded circular form and a stably integrated form (101,184). The first two are observed during early infection, while the last is observed later in infection and is stably established in infected cell lines. Gianni et al. (89) have recently succeeded in isolating the circular double-stranded form of the provirus from murine leukemia virus infected cells in a virtually pure form. The molecular weight of the double-stranded form has been calculated at very near two times that of the single stranded RNA subunits, i.e., 6×10^6 daltons. The observation that this DNA is infectious and gives rise to complete virus particle formation by infected cells provides further definitive evidence that all of the genetic information of the virus is contained in the 30-35S subunits.

I:5 Genetic Markers of RNA Tumor Viruses.

I:5.1 Host Range Restriction.

The elucidation of the genetic structure of the avian tumor viruses has proceeded more rapidly than of the murine viruses. The primary reason for this is the availability of suitable genetic markers in the avian viruses. As noted earlier, the avian sarcoma and leukosis viruses exhibit a high degree of host range specificity. The virus can infect and transform some strains of chickens but not others. When cell culture techniques became available the same patterns of restriction were found with cultured cells derived from the avian hosts. It was observed that primary infection by certain

isolates of avian leukosis and sarcoma viruses would block the super-infection of the cell by some, but not all other avian virus isolates and on the basis of host range and interference patterns, the avian tumor viruses have been grouped into 5 subgroups designated A-E (65, 208,216). Infection of susceptible cells by a particular subgroup inhibits further infection by other viruses of the same subgroup but not by other subgroups that the virgin cell is permissive to. The host range specificity of avian viruses was subsequently found to be a property of the envelope glycoprotein of the virus as well as of specific receptors for these glycoproteins on the cell surface. For a cell to be permissive for infection by a particular virus it must contain a specific receptor for that virus. Once a cell had been infected with a virus from a particular subgroup, the receptor sites are blocked. A particular cell may contain more than one type of receptor, however, and superinfection by another subgroup of virus for which a receptor is present results in the successful infection of the cell. This phenomenon has provided a convenient genetic marker for the envelope glycoproteins of the virus.

Host range restriction is also observed in murine RNA tumor viruses and is expressed in cultured cells, however, in murine viruses the phenomenon is much more complex. Most murine RNA tumor viruses can be classified as either N or B tropic, depending upon their ability to replicate in cultured fibroblasts derived from mice (105). N-tropic virus replicate 100-1000 times more efficiently in NIH-Swiss mouse cells than in Balb/c mouse cells. Conversely, B-tropic virus replicate more efficiently in Balb/c cells. Different mouse strains

have been classified as either N-type or B-type depending upon their susceptibility to N-tropic and B-tropic virus. A third type of murine virus is the NB-tropic virus which is able to replicate in both N and B-type cells.

In contrast to the avian system, the host range of murine leukemia virus does not depend on the envelope glycoprotein, but rather on the genetic composition of the host. Current evidence indicates that a host gene product inhibits a function of the virus required for both replication and transformation (22). The target of this restriction is apparently a protein of the virus (156). This is evidenced by the observation that when infection conditions are manipulated to allow for the encapsulation of the RNA of a virus of one tropism by the proteins of the virus of another tropism, the resulting mixed virus exhibits the host restriction of the virus from which the proteins were derived. Other lines of evidence also support this contention. A likely protein of the virus which would serve as a target for both the inhibition of replication and transformation by the host cell is the viral polymerase; however, no direct evidence for this has been reported. Thus host range restriction cannot yet serve as a convenient genetic marker for murine virus in a manner analogous to the avian tumor viruses.

I:5.2 Reverse Transcriptase Mutants

Another valuable genetic marker in avian tumor viruses has been provided by the selection of a temperature sensitive reverse transcriptase mutant of the Rous sarcoma virus (126,133,215). These mutants

adsorb and penetrate cells at the non-permissive temperature but do not replicate in the cell or transform it. If infection occurs at the permissive temperature, however, and the culture is maintained at that temperature for only a short time (less than one day), replication and transformation will proceed even if the culture is returned to the non-permissive temperature. Thus, reverse transcriptase is necessary for the initiation of replication and transformation but not for their maintenance. Recently a temperature sensitive mutant in reverse transcriptase has been described for Rauscher murine leukemia virus and should provide an important genetic marker for murine viruses (210).

I:5.3 Transformation.

A third genetic marker for avian sarcoma viruses is provided by the transforming function of these viruses. As discussed earlier, when chicken fibroblasts are infected with the avian sarcoma viruses, discrete foci of malignantly transformed cells are distinguishable by virtue of their changed morphology. While leukosis viruses can infect and replicate in fibroblasts, they have little or no effect on the growth characteristics or morphology of these cells. The same relationship is observed with murine leukemia and sarcoma viruses. On the basis of their effect on cultured fibroblasts, C-type RNA tumor viruses have been generally categorized as transforming or non-transforming viruses. Transforming viruses are, by definition, the sarcoma viruses, while non-transforming viruses include the avian leukosis viruses and murine leukemia viruses as well as transformation-

defective avian sarcoma viruses.

The most common temperature sensitive mutants of avian sarcoma viruses are the transformation-defective mutants. These viruses are able to replicate at the non-permissive temperature but are unable to transform the cell. When the cells are shifted to the permissive temperature they reversibly switch to a transformed morphology, indicating that transformation is under continuous control by the viral genes (12,33). Thus, transformation is not a hit and run effect of the avian sarcoma virus but requires the persistent expression of a transforming "sarc" gene.

Although avian sarcoma viruses are competent in both replication and transformation, all known mammalian RNA tumor viruses capable of transforming fibroblasts in culture are replication-defective and require coinfection with "helper" leukemia viruses for the replication of infectious particles (176). They can, however, non-productively infect and transform fibroblasts. Transformation-defective mammalian sarcoma virus have not been isolated. However, one sarcoma virus, the S^+L^- (sarcoma$^+$, leukemia$^-$) isolate of Moloney sarcoma virus requires the coinfection of a leukemia virus to fully express its sarcomagenic potential (20). Cells infected with this sarcoma virus in the absence of helper leukemia virus (S^+L^- cells) do not exhibit typical transformed morphology during active growth. If cultures are allowed to grow to confluency and remain in the stationary phase for a period of time, however, they begin to exhibit transformed characteristics. Superinfection of these cells during the growing state with leukemia viruses causes them to exhibit fully transformed

characteristics. The mechanism of this phenomenon is unknown; however, it provides a relatively convenient method for the rapid assay of murine leukemia viruses (21). Superinfection with dilutions of leukemia viruses results in foci of transformed cells which can be enumerated. It is not yet clear if a specific gene or gene product is responsible for leukemogenesis by avian leukosis virus or murine leukemia virus. A recent hypothesis put forward by Peebles et al. (150) in which endogenous oncogenic information of either cellular or viral origin, such as the S^+L^- system above, is activated by the infecting leukemia or leukosis virus suggests a mechanism which does not require the postulation of transforming viral genes.

I:5.4 Internal Group-Specific Antigens.

As discussed above, for the avian sarcoma and leukosis viruses there are three genetic elements defined by specific mutations. A fourth gene which codes for all of the major internal proteins is defined by the observation that all of these viral proteins are synthesized as a single polypeptide precursor in infected cells (217, 218). Recently two temperature-sensitive mutants of Rauscher murine leukemia virus have been isolated which accumulate the precursor at the restrictive temperature (188). The genes coding for these proteins are necessarily tandom on the viral genome and can be considered as a unit. The genes of the avian tumor viruses have been designated: env, for the envelope glycoprotein determining host range; pol, for the viral polymerase; gag, for the internal proteins (group specific antigens) of the virus; and sarc, which

represents the sarcoma function of the avian sarcoma viruses (16). The leukosis viruses and certain transformation defective sarcoma viruses are thought to contain, only the gag, env and pol genes, while the sarcoma viruses contain in addition to these, the sarc gene. Direct chemical evidence for this is discussed below.

I:6 Structure of the RNA Viral Genome.

I:6.1 Avian Sarcoma and Leukosis Viruses.

The genetic information of the RNA tumor viruses is contained in a 70S molecule which dissociates into probably two 30-35S subunits upon treatment by heat or DMSO. Oligonucleotide mapping procedures have shown that the virus is diploid in that the two subunits from cloned virus preparations are very similar or identical (63,114). The 30-35S RNA is single stranded, contains poly(A) at the 3'-OH end and a 7-methyl guanosine-capped 5'-OH terminus (92,116). The molecule thus has many of the properties of eukaryotic messenger RNA.

Duesberg and Vogt reported that uncloned preparations of avian sarcoma viruses contain two sizes of electrophoretically separable 30-35S subunits, whereas certain transformation defective sarcoma viruses and avian leukosis viruses contain only the smaller subunit (60,62). When cloned preparations of sarcoma viruses were examined, it was revealed that the clones contained either the large or the small subunit. Furthermore, only those clones which carried the large subunit retained the capacity to transform fibroblasts. The presence of smaller subunits in uncloned sarcoma virus preparations was due

to spontaneously arising transformation-defective segregates. Subsequent studies revealed that non-transforming viruses, i.e., the leukosis viruses and transformation-defective sarcoma viruses, lacked 10 to 15% of the mass of the subunits of transforming viruses, suggesting that the transformation-defective sarcoma viruses are the result of deletion mutations of the sarc gene. An analysis of RNase T_1-resistant oligonucleotides of the viruses revealed that transformation defective mutants lack up to three large oligonucleotides which are present in the parent virus, indicating that these oligonucleotides are part of the sarc gene (122). By partially fragmenting the viral genome, isolating the 3'-OH poly(A) containing fragments by annealing to oligo-dT columns and subsequently distributing the poly(A)-containing fragments according to size by sedimentation, these investigators were able to obtain progressively larger fragments of the viral genome originating from the 3' end. By identifying T_1-resistant oligonucleotides contained in the progressively larger poly(A)-tagged fragments, a linear oligonucleotide map of the viral genome was obtained. The location of the sarcoma-specific oligonucleotides on this map was shown to be near the 3' end, thus chemically defining the location of this gene on the viral genome (221). Subsequent work correlating oligonucleotides with a deletion mutant of the envelope glycoprotein, as well as with recombinant viruses originating from crossovers, allowed the construction of a partial genetic map of the viral genome. This information coupled with genetic linkage studies allowed a nearly unambiguous genetic map to be assigned to the known viral genes (64). Weissmann's group, using the same approach in an

independent study, has derived the same gene order (115). The genetic map appears to be 5'-gag-pol-env-sarc-3' for the avian sarcoma viruses and 5'-gag-pol-env-3' for the leukosis viruses.

I:6.2 Murine Leukemia and Sarcoma Viruses and the Friend Virus Complex.

The relationship between mammalian leukemia and sarcoma viruses is more complex than in avian systems. Present evidence suggests that the murine sarcoma viruses arose by recombination of a leukemia virus with endogenous host cell sarcomagenic information (131,175, 177). Unlike avian sarcoma viruses, all murine sarcoma viruses are defective for replication. Also in contrast to the avian viruses, the RNA subunits of murine leukemia viruses are larger than the subunits of the corresponding sarcoma viruses, suggesting that the acquisition of the sarcoma virus-specific sequences by the infecting leukemia virus may occur with the loss of a portion of the murine leukemia virus genome (130). In support of this model, Dina et al. (56) have recently shown that the RNA of Harvey murine sarcoma virus, which was derived by the passage of Moloney leukemia virus in rats, contains about 40% of the Moloney leukemia virus genome as well as additional sequences of an endogenous rat C-type virus. The elucidation of the genetic relationship between murine leukemia and sarcoma viruses awaits further investigation.

The Friend leukemia virus complex is analogous to the murine leukemia virus/sarcoma virus complexes. This virus, which induces a progressive erythroleukemia in mice has been shown to consist of at

least two components. The virus responsible for the erythroleukemia is a defective virus which requires coinfection with a lymphatic leukemia virus in order to replicate in infected animals (53). Like the murine sarcoma viruses, the genome of the erythroleukemia component is smaller than the genome of the leukemia virus (130). Sequence relationships between this virus and the lymphatic leukemia virus with which it was originally isolated have not been established. The defective erythroleukemia virus component has been termed the spleen focus forming virus (SFFV) by virtue of the fact that the virus can be quantitated by enumerating discrete foci of proliferating reticular cells on the surface of the spleens of infected mice during early infection (8). The original helper murine leukemia virus (MuLV) isolated with the SFFV component is termed the Friend leukemia virus (F-MuLV) and is closely related to the Rauscher (R-MuLV) and Moloney murine leukemia viruses (M-MuLV).

I:7 The Synthesis of RNA Tumor Virus Proteins.

As discussed in the foregoing section, the genome of the RNA tumor viruses has the properties of a very large eukaryotic mRNA in that it is single-stranded, contains poly(A) at the 3' end and has a 7-methylguanosine-capped 5' end. Furthermore the viral RNAs isolated from a number of RNA tumor viruses have been shown to direct the synthesis of viral proteins (118,138,149,219). In addition, the predominant virus-specific RNA species associated with polyribosomes in infected cells is of the 30S to 35S size range, suggesting that this species serves as a messenger RNA in infected cells; however,

other prominent virus-specific RNAs of smaller size classes are also observed (76,90,91,180). Although eukaryotic mRNA is not known to possess more than one efficient initiation site per molecule, the information for at least five or six unique proteins is contained in 30-35S viral RNA. This suggests that there may be an unusual mechanism for the synthesis of the viral proteins.

The formation of viral proteins from larger precursor polypeptides by proteolytic cleavage is well documented for a number of animal viruses including members of the picornaviruses, alphaviruses and paramyxoviruses, as well as others (45,110,113,137,168,173,174,198, 217,218). In certain of these viruses, notably poliovirus and encephalomyocarditis virus of the picornavirus group, the entire genomic content of the virus, like the RNA tumor viruses, is represented in a single stranded RNA species which is able to serve as a messenger RNA in cell-free protein synthesizing systems. In vitro translation of the RNA from these viruses results in the synthesis of large polyproteins, some of which contain the amino acid sequences of all of the structural proteins of the virus (38,117,162). In infected cells poliovirus and encephalomyocarditis virus proteins are derived from a number of large precursors, which are in turn, derived by nascent cleavage of the translation product of a messenger RNA representing the entire genome (45,110,113,198). The above considerations suggest that the RNA tumor virus proteins may be derived in a manner analogous to the picornavirus proteins.

The avian tumor virus genome contains information sufficient to encode about 300,000 daltons of proteins (28); however, no evidence

of a viral specific protein approaching this size has been obtained. Vogt and Eisenman, studying viral protein synthesis in avian myeloblastosis virus-infected cells, have identified a 76,000 dalton polypeptide precursor which undergoes specific proteolytic cleavage to the major internal gag proteins of the virus (217). In subsequent detailed studies by this group, no larger virus-specific polypeptides were observed, even in cells unable to proteolytically process the 76,000 dalton precursor (218). In vitro translation of avian sarcoma virus RNA has yielded only a 76,000 dalton species which is apparently identical to the precursor detected in vivo (149,219). In addition to the 76,000 dalton precursor to the internal group-specific antigens, a 70,000 dalton precursor to the envelope glycoprotein has been identified in infected cells, and is apparently glycosylated to yield the 85,000 dalton envelope glycoprotein (103,125). Recently Smith has obtained evidence that a 20S mRNA, which is a prominent species in infected cells, is the primary site for the synthesis of the envelope glycoprotein, whereas the 76,000 dalton gag polypeptides are formed largely on the 30-35S mRNA.[1] This observation would indicate that the 35S viral mRNA contains an internal cyrptic initiation site which is activated either by direct transcription of the smaller mRNA from the DNA provirus, or by fragmentation of the larger mRNA species. Recent evidence indicates that a number of viral mRNAs possess cryptic internal initiation sites which are activated in this manner (46,183). There is no evidence concerning the synthesis of the viral polymerase

---

[1] A. Smith, personal communication, November, 1976.

in avian sarcoma virus-infected cells.

The analysis of viral protein synthesis by murine leukemia viruses has provided some evidence of high molecular weight viral protein precursors representing a major portion of the genome. Cell-free translation of 35S viral RNA from Moloney MuLV has resulted in the synthesis of three polypeptides of 60,000, 70,000 and 180,000 daltons, each of which contains the amino acid sequences of p30, the major structural protein of the virus (118). Likewise, in vitro translation of Rauscher leukemia virus RNA was reported to yield two classes of polypeptides of 50,000 to 70,000 daltons and 140,000 to 185,000 daltons (138). In vivo studies of MuLV protein synthesis have not yielded uniform results. Shanmugan et al. (179) could detect no viral protein precursors in cells producing Moloney murine sarcoma-leukemia virus. More recently, van Zaane et al. (213) have reported the presence of two precursor polypeptides of 82,000 and 65,000 daltons which were precipitated with antiserum made against whole disrupted virions, as well as an additional 72,000 dalton polypeptide which occurred only in cells treated with an arginine analog. No higher molecular weight virus-specific polypeptides were detected in these studies. In contrast to other in vivo studies, Naso, Arcement and Arlinghaus (139) have reported major viral-specific polypeptides of 180,000, 140,000, 110,000 and 80,000 and 60,000 daltons in cells infected with Rauscher MuLV. A number of these polypeptides were reportedly processed to the viral p30 during pulse chase studies. More recently, this group has reported only two relatively minor virus-specific polypeptides of approximately 200,000 daltons, in

addition to several major components less than 100,000 daltons (4,5, 140). The relationship of the minor high molecular weight components in their most recent studies to the prominent high molecular weight components in their previous work is unclear. It appears from their most recent studies that the 200,000 dalton proteins contain antigens of p30 and reverse transcriptase but not of the envelope glycoprotein gp69/71. The lower molecular weight precursors reported in these studies include two approximately 90,000 dalton precursors to the envelope glycoprotein, and proteins of 80,000 and 65,000 daltons which are reported to contain p30.

A model has recently been proposed in which the 200,000 dalton precursors to p30 and the 90,000 dalton precursors to the envelope glycoproteins are derived by nascent cleavage of a translation product of a large mRNA representing the entire viral genome (5). The 90,000 dalton proteins, which are presumably glycosylated nascently or very soon after nascent cleavage, are processed to the envelope glycoproteins, while the 200,000 dalton precursor is processed to the viral polymerase and to the lower molecular weight virion core proteins by a series of sequential proteolytic cleavages. The 80,000 and 65,000 dalton proteins are proposed intermediates in this cleavage pathway.

Additional studies also indicate that p30 is synthesized by way of larger polypeptide precursors in murine RNA tumor virus infected cells. Famulari et al. (75) have recently described p30 precursors of 65,000 and 75,000 daltons and two approximately 90,000 dalton precursors to the envelope glycoprotein in R-MuLV-infected cells. Feline cells, when infected with a feline pseudotype of Moloney

sarcoma virus (Moloney sarcoma virus encapsulated in proteins specified by feline leukemia virus), are unable to cleave a 60,000 dalton precursor to p30 specified by the sarcoma genome (144). The intact 60,000 dalton precursor is, in fact, detected in progeny virus of such cells. Recently Stephenson et al. (188) have described two temperature sensitive mutations of R-MuLV which result in the intracellular accumulation of a 60,000 dalton p30 precursor at the non-permissive temperature.

I:8 Thesis Objective and Experimental Rationale.

This study was undertaken to examine virus-specific protein synthesis in MuLV-infected cells in an effort to determine the post-translational events giving rise to mature virion proteins. In particular, it was of interest to determine if the MuLV proteins derive from a large precursor polypeptide representing the entire virus genome (as in picornavirus-infected cells) or by way of smaller precursors. It is noted that the studies reporting the detection of murine leukemia virus protein precursors described in the foregoing section are quite recent. At the onset of this study only one investigation of MuLV-specific protein synthesis had been reported (179), and no precursors to the murine leukemia virus proteins were detected. Nevertheless, at the present time there is still no consensus regarding the post-translational processes involved in MuLV protein synthesis in infected cells and virtually no information concerning the functional significance of these processes.

In contrast to poliovirus and other viruses in which proteolytic

processing of viral proteins has been extensively examined, infection of cells with RNA tumor viruses does not result in a significant reduction in host protein synthesis. Of the total cellular protein synthesis, less than 1% is devoted to viral proteins in RNA tumor virus infected cells (68). Thus the analysis of viral protein synthesis is complicated by an overwhelming background of host protein synthesis. The only feasible approach to this problem is to separate virus-specific proteins from host proteins by specific immunoprecipitation. In addition to highly specific immunological procedures, this approach requires a suitable source of virus for the production of antibodies, as well as biological and biochemical methods for the assay, purification, and characterization of the virus.

The initial choice of virus strain and cell lines to be utilized in the study was the Friend leukemia virus complex (MuLV and SFFV) propagated in erythroleukemia cells (83,145,197). A number of these cell lines are routinely maintained in the laboratory for other studies and were readily available. Although the analysis of viral protein synthesis necessitates the use of cell cultures, most murine leukemia virus-infected cultures liberate only small amounts of virus particles. Usually less than one mg of viral protein can be obtained from a liter of growth medium. Infected mouse serum, however, has been reported to contain very large amounts of virus and was expected to be an excellent source of virus for immunization (24,154). A number of interesting observations were made during the course of the purification and characterization of Friend leukemia virus from mouse serum which are presented in chapter III of this dissertation.

However, this source did not prove to be suitable for the production of virus for immunization purposes. Ultimately, the production of virus from cell culture medium became feasible because a line of mouse cells (Eveline II cells) was made available which produce very large amounts of the lymphatic leukemia component of Friend leukemia virus (40,141,171). The Eveline II cell line was also used exclusively for the analysis of MuLV protein synthesis in this study.

I:9 Organization of the Thesis.

For purposes of cohesiveness, the results and discussion of the properties of Friend leukemia virus isolated from mouse serum have been presented in a separate chapter (Chapter III). Furthermore, the results and discussion in Chapter IV, dealing with characterization of MuLV from Eveline cells and the synthesis of MuLV proteins has been presented in three sections: the first dealing with the characterization of the virus used for the preparation of antisera, the second dealing with the detection and characterization of very large polyproteins in MuLV-infected cells, and the third with the post-translational processing of virus-specific proteins. Although the latter two sections are closely related, and each contributes to the other to a degree, different aspects of MuLV protein synthesis are stressed and their separation contributes to the clarity of the presentation.

II. MATERIALS AND METHODS

II:1 Materials.

The source of the materials used in this study are indicated in the text of this chapter. All other materials were of the finest quality commercially available.

II:2 Growth and Maintenance of Cells.

Eveline II cells, which are chronically infected with the Friend leukemia virus, were kindly provided by D. Bolognesi, Duke University Medical Center. The cells were grown as suspension cultures in Dulbecco's modified Eagle medium (GIBCO, dry powdered medium) containing 0.62 grams $NaHCO_3$ per liter at 37°C. The medium was supplemented with 10% fetal calf serum (GIBCO) which had been heat inactivated at 56°C for 30 minutes, 100 units/ml of penicillin (GIBCO), 100 μg/ml of streptomycin (GIBCO), and 100 μg/ml of gentamycin (Schering Corp.). 60 ml cultures, growing in 250 ml disposable Erlenmeyer culture flasks (Corning) at 37°C, were passaged at 2 to 3 day intervals. The cells were kept in suspension by placing the cultures on a New Brunswick gyrotory shaker oscillating 90 rpm. The cell concentration was maintained between 6×10^5 and 3×10^6 cells/ml. Cells were counted in a hemocytometer after drawing into a Pasteur pipette to disaggregate clusters of cells.

S^+L^- cells were kindly provided by P. J. Fischinger, National

Institutes of Health. The cells were grown as monolayer cultures in $25cm^2$ tissue culture flasks (GIBCO) containing 5 mls of McCoy 5A medium (GIBCO) supplemented with 100 units/ml of penicillin (GIBCO), 100 μg/ml of streptomycin (GIBCO) and 0.25 μg/ml of fungizone (GIBCO). For transfers, the medium was decanted and the monolayer was rinsed 2 times with 0.7 mls of trypsin-EDTA (GIBCO, 0.5 g/l trypsin, 0.2 g/l EDTA). 0.7 mls of trypsin-EDTA was added a third time and incubated at 37°C for 10-12 minutes after which 2.8 mls of fresh growth medium was added. The cells were disaggregated by drawing into a Pasteur pipette and then counted in a hemocytometer. For normal transfers approximately $0.5 - 2 \times 10^5$ cells were plated onto 25 cm^2 flasks and adjusted to 5 ml with growth medium. The cultures were incubated in 5% CO_2 at 37°C for 24 hours after which the caps were tightly closed and incubated for an additional 3 days. Four days after plating of the cells, an additional 3 mls of medium was added and the cells incubated for 3 more days. The cultures were transferred in this manner once a week.

II:3 Assays of Virus and Viral Components.

II:3.1 MuLV

The titer of MuLV was measured using a variation of the S^+L^- method of Bassin et al. (21). 2×10^5 S^+L^- cells were plated onto $25cm^2$ tissue culture flasks and the culture volume adjusted to 5 ml with McCoy 5A medium supplemented as described in section II.2. After 24 hours of incubation in 5% CO_2 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 dextran solution was decanted, a 0.5 ml virus sample was added, and the flasks were incubated for another 30 minutes. Five milliliters of fresh medium was then added and the flasks were incubated, tightly closed, at 37°C. After 3 days an additional 3 ml of medium was added. Foci were counted 6-7 days after sample application.

II:3.2 SFFV.

The titer of spleen focus-forming virus (SFFV) was determined in 6 to 8-week-old DBA/2 male mice by the macroscopic spleen focus assay of Axelrad and Steeves (8). Mice were injected intravenously in the tail vein with a virus sample adjusted to 0.5 mls in Eagle's medium (GIBCO). Usually three dilutions of each virus sample were assayed in duplicate or triplicate (6-9 mice/sample). After 9 days the mice were sacrificed, and the spleens were removed and fixed in Bouin's solution (.015% picric acid, 9.25% formaldehyde, 5% acetic acid). The foci measuring greater than 0.5 mm in diameter were scored.

II:3.3 Reverse Transcriptase Assay.

Reverse transcriptase activity was determined by measuring the incorporation of [^{3}H]dGTP (New England Nuclear) into poly dG using a poly rC template (Collaborative Research, Waltham, Mass.) and an oligo dG_{12-18} primer (Collaborative Research). The virus sample, suspended in TSE buffer (0.1 M NaCl; 0.01 M Tris-HCl, pH 7.4; 0.001 M EDTA), was diluted with an equal volume of 0.2% Triton X-100 and

incubated at 37°C for 10 minutes. The solution was adjusted to a final volume of 100 μl containing 0.05 M Tris-HCl, pH 8.3; 0.006 M $MgCl_2$; 0.010 M dithiothreitol; 0.06 M NaCl; 0.00016 M [^3H] dGTP (~25-50 cpm/pmol); 10 μg/ml of poly rC, and 5 μg/ml of oligo dG_{12-18}. The reaction was allowed to proceed at 37°C for 15 minutes and was terminated by the addition of 0.5 ml of 0.1 M sodium pyrophosphate, 100 μg of *Escherichia coli* tRNA, and 0.5 ml of 25% trichloracetic acid (TCA). The samples were incubated on ice for 10 minutes, filtered on 24 mm glass fiber filters, and counted in toluene-based scintillation fluid as described in the scintillation procedures (section II:11).

Stock solutions were used to make up a reagent mixture to which a sample of disrupted virus was added to attain the final assay concentrations described above. Table II.1 shows the stock solutions and the volumes required to make up a reagent mixture for 10 assays. The amount of H_2O in the reagent mixture can be varied from 0 to 270 μl to accommodate sample volumes up to 27 μl. Other reagents (e.g., NaCl) can be varied to correct for salt concentrations in the sample.

II:3.4 Radioimmunoassay of p30.

p30, the major internal polypeptide of MuLV, was measured by a modification of the competition radioimmunoassay of Stephenson et al. (187). The virus sample was disrupted in PE buffer (10 mM potassium phosphate, pH 7.8; 10 mM EDTA; 0.6% bovine serum albumin) plus 0.5% Triton X-100 (Sigma). Serial dilutions of the sample were incubated for 1 hour at 37°C with a limiting amount of goat antiserum to p30 (enough to precipitate 50% of the [^{125}I]-labeled p30 added)

Table II.1 Stock Solutions and Composition of Reagent Mixture for Reverse Transcriptase Assay

Component	Concentration	Volume/10 Assays[a]
dGTP	15.85 mM (10mg/ml)	0.010 ml
[^{3}H] dGTP	.06 mM (15.6μCi/μg)	0.025 ml
poly rC	1 mg/ml	0.010 ml
oligo dG_{12-18}	1 mg/ml	0.005 ml
Tris-HCl, pH 8.3	100 mM	0.500 ml
NaCl	500 mM	0.110 ml
$MgCl_2$	100 mM	0.060 ml
dithiothreitol	1 M[b]	0.010 ml
H_2O	---	170 ml[c]
Total volume of reagent mixture		0.900 ml

[a]100μl assay volume.

[b]Store at -20°C.

[c]Volume of H_2O assuming sample volume of 0.01 ml.

in a reaction mixture adjusted to 0.160 ml with PE buffer containing 0.1% non-immune goat serum. [^{125}I]-labeled p30 (10,000 counts/min) in 0.020 ml was added, and the incubation was continued for an additional 3 hours at 37°C. 0.020 ml of undiluted rabbit anti-goat IgG (Pacific Biologicals) was added to each reaction mixture and the samples were incubated overnight at 4°C. The immunoprecipitates were pelleted by centrifugation at 2,000 x g for 30 minutes at 4°C. A 0.10 ml aliquot of the supernatant was taken and the radioactivity measured in a Beckman Biogamma counter. The precipitated radioactivity was calculated by determining the loss of radioactivity from the supernatant as compared to a control precipitation which contained only non-immune goat serum. For plotting competition curves, the precipitated radioactivity was expressed as a percentage of that obtained in the absence of competing antigen. Antiserum to p30 was generously donated by R. V. Gilden, Flow Laboratories. [^{125}I]-labeled p30 was kindly provided by S. R. Tronick, Viral Carcinogenesis Branch, National Cancer Institute.

II:4 Cell Lysis for Detection of Cell-Associated Virus.

Eveline II cells, growing in the logarithmic phase, were pelleted by low speed centrifugation and were washed 3 times in phosphate buffered saline (PBS) (0.137 M NaCl, 0.027 M KCl, 0.0015 M KH_2PO_4, 0.008 M Na_2HPO_4, pH 7.0) by resuspension and pelleting. The washed cells were resuspended to the original culture volume in 10 mM NaCl; 1.15 mM $MgCl_2$; 10 mM Tris-HCl, pH 7.5 and the cells were disrupted in a Dounce homogenizer with 50 strokes with a tight fitting pestle

and 30 strokes with a loose fitting pestle. Complete lysis was confirmed by phase contrast microscopic examination. Where indicated, the homogenate was cleared of cellular debris by centrifugation at 10,000 rpm for 10 minutes in a Sorvall SS-34 rotor.

II:5 Preparation of Virus.

II:5.1 Purification of Virus from Eveline II Cell Culture Medium.

MuLV was purified from the growth medium of logarithmically growing cultures of Eveline II cells by isopycnic banding in sucrose gradients (59). The cells were sedimented by centrifugation at 3000 rpm for 10 minutes at 4°C in a Sorvall GSA rotor, and the supernatant was further clarified by centrifugation at 10,000 rpm for 10 minutes. 25 ml of clarified medium was layered onto each of several discontinuous sucrose gradients consisting of 5 mls of 60% sucrose (Schwartz-Mann, RNase-free, ultrapure reagent) and 7 mls of 15% sucrose in TSE buffer and was centrifuged for 90 minutes at 25,000 rpm in a Beckman SW 27.1 rotor. At the conclusion of the centrifugation, the medium was removed, replaced with more clarified medium and centrifuged as above. This procedure was repeated up to four times until all the medium had been centrifuged. The virus bands at the surface of the 60% sucrose solution were collected, diluted with 3 volumes of TSE buffer and layered onto 35 ml 15-60% sucrose gradients constructed using a double chambered Buchler gradient maker. The virus was isopycnically banded by centrifugation for 3 hours at 25,000 rpm. The virus band at 1.15-1.17 g/cm^3 from each of the gradients was collected,

diluted in 7 volumes of TSE buffer and pelleted by sedimentation at 25,000 rpm for 90 minutes.

II:5.2 Preparation of Stock Spleen Extract and Virus Preparation from Infected Mouse Serum.

II:5.2.1 Preparation of Stock Spleen Extract.

DBA/2 mice were infected by intraperitoneal innoculation with 0.10 ml of Friend leukemia virus (American Type Culture Collection) obtained as a 20% wt/wt spleen extract. 14 days after infection the mice were sacrificed and the spleens were removed and homogenized in TSE buffer in a Dounce homogenizer. The amount of TSE buffer added was equal to 4 times the spleen weight. The homogenate was centrifuged at 3000 rpm for 10 minutes in a Sorvall SS-34 rotor to remove debris, and it was further clarified by centrifugation at 10,000 rpm for 10 minutes. The supernatant was then used for reinfection. After 6 passages in mice, a large preparation of the spleen extract was stored in serum bottles at -70°C. This preparation was employed for all subsequent innoculations.

II:5.2.2 Preparation of Virus from Infected Mouse Serum.

Blood from DBA/2 mice which had been infected with Friend leukemia virus (20% wt/wt spleen extract) was harvested 14 days post-infection by severing the axillary vessels. The blood, containing 10 units of heparin/ml, was cleared of cells by centrifugation at 3000 rpm for 10 minutes in a Sorvall SS-34 rotor. The serum was further clarified by centrifugation at 10,000 rpm for 10 minutes. Clarified

serum was layered onto a discontinuous gradient consisting of 5 ml of 60% sucrose and 10 mls of 15% sucrose in TSE buffer and was centrifuged at 25,000 rpm for 60 minutes in a Beckman SW 27.1 rotor. The material at the surface of the 60% sucrose cushion was collected and diluted with 3 volumes of TSE buffer, and then layered onto a 35 ml 15-60% continuous sucrose gradient in TSE buffer. The gradients were centrifuged at 25,000 rpm for 3 hours. The gradients were fractionated by pumping through a flow cell in a recording spectrophotometer (Gilford model 245) with continuous recording of the absorbance at 252 nm. Where indicated, the virus bands were diluted 1:8 in TSE buffer and were pelleted by sedimentation at 25,000 rpm for 90 minutes.

II:5.3 Preparation of Radioactive Virus.

II:5.3.1 [^{35}S]-labeled F-MuLV.

60 ml suspension cultures of Eveline II cells were sedimented by low speed centrifugation at 37°C. The pelleted cells were suspended in methionine-free MEM (minimum essential medium, GIBCO) and resedimented as above. The cells were then resuspended to the original culture volume in methionine-free MEM containing 10% dialysed fetal calf serum (GIBCO) and 80-100 μCi of [^{35}S]-L-methionine, and incubated at 37°C for one hour. At the conclusion of the incubation, the cells were sedimented as above and resuspended in normal growth medium. [^{35}S]-labeled virus was harvested from the growth medium as described previously (section II:5.1) after 16-24 hours of additional growth. The labeling medium was used to radiolabel subsequent cultures until

about 60% of the radioactivity was incorporated.

II:5.3.2 [^{14}C]- labeled MuLV.

MuLV were labeled with [^{14}C] by the addition of 300 μCi [^{14}C]-leucine to a 60 ml Eveline culture at the time of transfer. [^{14}C]-labeled virus was harvested from the culture medium as described in section II:5.1 after two days of growth.

II:6 Preparation of Rabbit Antiserum to F-MuLV.

Purified F-MuLV from Eveline II cells, which had been pelleted by high speed centrifugation, was resuspended in 2% Triton X-100 to a protein concentration of 1 mg/ml and vigorously shaken at 0°C for 20 minutes. An equal volume of diethyl ether was added and the mixture was shaken for an additional 30 minutes. The extraction mixture was centrifuged at 3000 rpm in a Sorvall SS-34 rotor at 4°C for 10 minutes and the aqueous layer was withdrawn. The remaining ether in the aqueous phase was removed by bubbling with nitrogen. For the primary immunization, a volume containing one mg of disrupted virus was mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories). The rabbit was injected intramuscularly in each thigh and subcutaneously in each shoulder with equal portions of the antigen. Booster injections were administered intravenously after six weeks with one mg of disrupted virus in the absence of adjuvant. Subsequent booster injections were administered in the same manner at two to three week intervals. Blood was collected by cardiac puncture 7-10 days after each booster injection and allowed

to stand at room temperature for 4 hours and overnight at 4°C. The contracted clot was removed by centrifugation at 10,000 rpm for 10 minutes in a Sorvall SS-34 rotor. The supernatant was then recentrifuged at 10,000 rpm as described above.

The serum from immunized rabbits was adsorbed against a homogenate of Swiss mouse tissue which had been insolubilized according to a modification of the procedure of Avrameas and Ternyck (7). Five male and five female Swiss mice (Simonson Labs) were bled from the axillary vessels. The liver, spleen, heart, kidneys, lungs and much of the muscle and connective tissue were removed and combined with the blood. The tissue was suspended in 0.1 M sodium phosphate buffer, pH 7.4, and was homogenized in a Vertis homogenizer for 4 minutes at high speed with constant cooling in ice water. The final protein concentration of the homogenate was approximately 50 mg protein/ml. A 6% solution of glutaraldehyde was added dropwise with stirring until the homogenate became viscous (approximately 1 ml of glutaraldehyde per 10 mls of homogenate). The mixture was allowed to stand at room temperature for five hours. The suspension was adjusted to pH 5.0 by the addition of acetic acid and allowed to stand at room temperature for an additional 12 hours. The suspension was then diluted with PBS and centrifuged at 10,000 rpm for 10 minutes in a Sorvall SS-34 rotor. The precipitate was washed with PBS until the pH of the supernatant was neutral. Virtually all of the protein was insolubilized by this procedure. The insolubilized tissue was extensively washed in 0.2 M glycine-HCl, pH 2.8 at 4°C, and again with PBS until the supernatant was neutral. For the adsorption of immune serum, the insolubilized

tissue was centrifuged at 16,000 rpm for 30 minutes in a Sorvall SS-34 rotor. The supernatant was decanted, and the pellet was drained by inverting the tube. The pellet was suspended in immune serum by homogenization in a Dounce homogenizer, and stirred for 4 hours at room temperature. Each ml of immune serum was adsorbed with approximately 300 mg of insolubilized mouse protein. The adsorbant was regenerated by extensive washing with 0.2 M glycine-HCl, pH 2.8 at 4°C followed by washing with PBS. The regenerated adsorbant was stored at 4°C in 0.01% sodium azide and was equilibrated with PBS prior to use.

II:7 Radioactive Labeling of Eveline Cells and Extraction of Cells for Immune Precipitation.

Eveline cells were sedimented by low speed centrifugation (International Clinical Centrifuge, Setting = 3) at 37°C and were resuspended in methionine-free MEM containing 10% dialysed fetal calf serum with added [^{35}S]-L-methionine in the amounts indicated in the legends to the figures. Where indicated, pulse-labeled cells were chased by the addition of a 500-fold excess of unlabeled methionine to the labeling medium. In one experiment (Figure IV.6), the pulse was terminated by sedimenting the cells and resuspending them in normal growth medium. Samples of the cultures were taken at various times during the experiments, immediately cooled in ice water and pelleted by centrifugation at 350 rpm for five minutes at 4°C in an IEC 269 rotor. The cell pellets were extracted by the addition of immune buffer A (0.01 M NaH_2PO_4, pH 7.6; 0.001 M Na_2EDTA; 1% Triton

X-100; 0.5% sodium desoxycholate; 0.1% sodium dodecyl sulfate (SDS)). The volume of immune buffer A used to extract the cells was equal to 20% of the original sample volume taken from the culture. The cell extracts were centrifuged at 200,000 x g for 20 minutes in a Beckman 65 rotor prior to immune precipitation.

Antiserum directed against Rauscher leukemia virus p12 was kindly donated by S. Tronick, Viral Carcinogenesis Branch, National Cancer Institute. Antisera against Rauscher gp69/71 and p30 were generously supplied by Dr. J. Gruber, National Cancer Institute, National Institutes of Health.

II:8 Immune Precipitation Procedure.

Centrifuged cellular extracts were diluted with a volume of immune buffer B (immune buffer A containing 20 mg/ml of bovine serum albumin and 50 μg/ml of poly L-lysine) equal to 50% of the final immune incubation mixture. In some experiments unlabeled virus, disrupted in immune buffer A, was added as carrier for primary anti-serum precipitations. Immune serum adjusted to 1% Triton X-100, 0.5% sodium desoxycholate and 0.1% SDS was added, and the reaction mixture was incubated overnight at 4°C. The precipitates were collected by centrifugation through a 20% sucrose cushion in immune buffer C (immune buffer A containing 0.5 M NaCl) in an IEC 269 rotor at 2900 rpm for 20 minutes.

The precipitates were resuspended in 1 ml of immune buffer A with a Pasteur pipette (vortexing is not sufficient) and centrifuged at 2900 rpm for 10 minutes in an IEC 269 rotor. The precipitates

were washed 2 more times with 1 ml of immune buffer A, 3 times with 1 ml of immune buffer C, and centrifuged through a second 20% sucrose cushion in immune buffer C as described above. This precipitate was resuspended in 10 mM Na_2HPO_4, pH 7.6; 0.15 M NaCl and pelleted by centrifugation in an IEC rotor at 2900 rpm for 20 minutes. The pellet was drained of excess supernatant and was subsequently dissolved in electrophoresis sample buffer as described below. For secondary antiserum precipitations, antiserum was added to the cell extract after the addition of immune buffer B, as above, and incubated for one hour. The appropriate antiserum to IgG (rabbit anti-goat IgG or goat anti-rabbit IgG, Pacific Biologicals) was added in an amount determined to be sufficient to achieve maximum precipitation of radioactivity. The reaction mixture was incubated overnight and was then processed as described above.

II:9 SDS-Polyacrylamide Gel Electrophoresis.

Acrylamide and N,N'-methylenebisacrylamide (Eastman Organic Chemicals) were recrystallized before use. Acrylamide was dissolved in $CHCl_3$ at 50°C, filtered and crystallized at -20°C overnight. The crystals were collected by filtration and washed with heptane. Methylenebisacrylamide (BIS) was dissolved in water at 90°C and crystallized at 4°C. The crystals were dissolved in acetone at 50°C and recrystallized at -20°C.

Electrophoresis in 8% polyacrylamide cylindrical gels in the presence of 0.6% SDS was performed according to the procedures of Bitte and Kabat (34). 0.25 mls of freshly made 10% ammonium persulfate

was added to 10 mls of 20% acrylamide (wt/wt), containing 0.5% BIS (wt/wt), and diluted to 25 mls with electrophoresis buffer (0.036 M Tris; 0.03 M NaH_2PO_4; 0.001 M EDTA). 20 µl of N,N,N',N',-tetramethyl-ethylenediamine (TEMED) (Eastman Organic Chemicals) was added, and the solution was mixed and pipetted into plastic tubes to form gels 8 cm in length by 6 mm in diameter. The solutions were overlaid with 100 µl of H_2O and allowed to polymerize for at least 30 minutes at room temperature. The gels in the tubes were soaked overnight in electrophoresis buffer containing 0.6% SDS and then were pre-electrophoresed in electrophoresis buffer containing 0.6% SDS for one hour at 5 mA/gel prior to use. Samples to be electrophoresed were dissolved in electrophoresis buffer containing 0.6% SDS, 1% 2-mercaptoethanol, and 10% sucrose by heating at 60°C for 30 minutes. The samples were applied to gels and electrophoresed for approximately 3 hours at 5 mA/gel. After electrophoresis, the gels were removed from the tubes and were soaked in 12.5% TCA overnight at room temperature. The gels were stained for protein in a 0.2% coomassie brilliant blue solution in 10% acetic acid; 20% methanol for 10-12 hours, and the unbound strain was removed by immersion in 10% acetic acid; 20% methanol for 24 hours with several changes. For the detection of radioactive proteins the gels were sliced longitudinally as described (34) and were then processed for fluorography as described below.

Longitudinally sliced gels were processed for fluorography by the method of Bonner and Laskey (43). The gels were equilibrated with 20 volumes dimethylsulfoxide (DMSO) (Eastman Organic Chemicals) for 30 minutes and the equilibration was repeated with fresh DMSO.

The gels were then immersed in 4 volumes of a 20% (wt/wt) solution of 2,5-diphenyloxazole (PPO) (New England Nuclear) in DMSO and equilibrated for 3 hours. The gels were then placed in 20 volumes of water for at least one hour after which they were dried onto Whatman 3 mm paper in an apparatus described by Fairbanks (74). The dried gels were overlaid with Kodak X-Omat medical X-ray film and exposed at -70°C.

Slab gel electrophoresis was carried out by a modification of the procedure of Laemmli (121). 10% polyacrylamide slab gels were made in 0.1875 M Tris, pH 8.8 containing 0.1% SDS and 8 M urea (Mallinckrodt, analytical grade). 3.75 mls of 1.5 M Tris, pH 8.8 and 0.3 mls of 10% SDS were added to 10 mls of a 30% acrylamide (wt/wt) solution containing 0.8% BIS (wt/wt). 14.4 g of urea was added and the solution was adjusted to 30 mls with H_2O. The solution was gently warmed by immersion in a 37°C bath until the urea had dissolved. 0.15 ml of 10% ammonium persulfate was added followed by .01 mls of TEMED. The solution was mixed and pipetted into a gel mold consisting of two glass plates separated by lucite spacers as described by Reid et al. (155). The gel was overlaid with water and allowed to polymerize for at least 30 minutes. 5% stacking gels were made in 0.0625 M Tris, pH 6.8 containing 0.1% SDS and 6 M urea. 1.25 mls of 0.5 M Tris, pH 6.8 and 0.1 mls of 10% SDS were added to 1.67 mls of a 30% acrylamide (wt/wt) solution containing 0.8% BIS (wt/wt). 3.6 g of urea was added and the solution was adjusted to 10 mls with water. The solubilization of the urea was hastened as above. 0.1 mls of 10% ammonium persulfate was added followed by 0.005 mls of TEMED. The solution was mixed and pipetted onto the separating gel. A sample-well template

was inserted into the stacking gel solution and the solution was sprayed with a fine mist of water. The gel was allowed to polymerize for at least one hour. Samples were dissolved in sample buffer containing 0.05 M Tris-HCl, pH 6.8; 1% SDS; 1% 2-mercaptoethanol; 20% glycerol, and 6 M urea by heating at 100°C for 2 minutes. The samples were applied to the gel and the gel was electrophoresed in 0.05 M Tris, pH 8.3; 0.38 M glycine and 0.1% SDS at 30 mA. Unless otherwise indicated, electrophoresis was continued until the tracking dye (bromphenol blue added to the upper electrode chamber) had reached the bottom of the gel. The gels were fixed, stained and processed for fluorography as described above for longitudinal sections of cylindrical gels.

Where indicated, the films were exposed for varying times and were scanned with a Transidyne General integrating densitometer. Bands which were proportional to the exposure time were quantitated for each exposure, and curves constructed by comparing band intensities of adjacent samples on the gels.

II:10 Protein Determinations.

Protein was assayed either by the procedure of Lowry et al. (127) or by a modification of the procedure of Schaffner and Weissmann (172) as described below. Protein samples containing 20 to 100 μg of protein were adjusted to 0.25 mls with H_2O. 50 μl of 0.6% SDS in 0.6 M Tris-HCl, pH 8.0, was added to each sample and mixed. 100 μl of 50% TCA was added and the sample incubated on ice for at least 2 minutes. The sample was filtered on 0.22 μm filters (Millipore) and washed with 5%

TCA. The filter was placed in a scintillation vial and covered with a 0.2% solution of Amido-Schwartz 10B (Allied Chemical) in 45% methanol containing 10% acetic acid and allowed to stain for 10 minutes. The stain, which can be reused, was removed and the filter was rinsed with H_2O two times. The filters were then rinsed with approximately 5 mls of 90% methanol containing 2% acetic acid for approximately 2 minutes. The rinsing was repeated until the borders of the filter appeared colorless (usually 3 times). The filters were then rinsed twice with H_2O and were drained thoroughly. The remaining stain was eluted with 1 ml of 0.025 M NaOH, 0.00005 M EDTA in 50% ethanol for 10 minutes or until all of the stain had eluted. The elution can be hastened by incubating capped vials at 37°C. The eluted dye was quantitated by its absorbance at 630 nm and the protein was estimated from a standard curve using bovine serum albumin. With this standard, the slope of the curve is 0.02 absorbance units per μg of protein. The background with zero protein was typically from .01 to .02 absorbance units.

II:11 Scintillation Procedures.

[^{3}H]-dGTP, [^{35}S]-L-methionine and [^{14}C]-leucine, incorporated into TCA insoluble counts, were collected on glass fiber filters. The filters were dried, placed in scintillation vials and the radioactivity solubilized for 3 hours at room temperature or 1 hour at 37°C in 0.5 ml of toluene-protosol (New England Nuclear) (2:1). 5 mls of toluene containing 0.3% PPO, 0.03% dimethyl POPOP (1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene) and 0.1% acetic acid were added, and the vial was stored in the dark at 4° for 4 hours. Radioactivity was

counted in a Packard Tricarb liquid scintillation spectrophotometer.

[^{35}S]-labeled immune precipitates were drained and solubilized by the addition of 0.5 mls toluene-protosol (2:1) by incubation for 1 hour at 37°C. The solution was transferred to a scintillation vial, combined with 5 mls of the scintillation fluid described above and counted after storage in the dark for 4 hours at 4°C.

Where indicated, [^{14}C]-leucine incorporated into TCA insoluble material was filtered onto 0.45 μm Millipore filters and assayed for radioactivity in a Nuclear Chicago low background gas flow counter.

III. ASSOCIATION OF FRIEND MURINE LEUKEMIA VIRUS WITH SUBCELLULAR MATERIALS

III:1 General.

The most widespread and the only convenient method for purification of C-type RNA tumor viruses takes advantage of their characteristic buoyant density (160,212). The buoyant density of these viruses on isopycnic sucrose density gradients is typically reported to be 1.15 to 1.17 g/cm^3. When Friend leukemia virus from infected mice was prepared in this manner, however, two distinct bands of infectivity were observed. One band was found at the reported buoyant density of C-type viruses (approximately 1.16 g/cm^3) and the other at a density of approximately 1.24 g/cm^3. This observation was of considerable interest for a number of reasons. Virus cores, derived by removing the viral envelope by mild detergents, exhibit a buoyant density of 1.24 to 1.26 g/cm^3 (14,39,195). These structures are believed to be non-infectious; however, Bolognesi et al. (39) have reported that virus cores prepared from avian myeloblastosis virus are infectious for chick fibroblasts. Preparations of Friend leukemia virus have been reported to contain appreciable amounts of particles with A-type morphology (54). A biological activity of A-type particles has not been established and the behavior of these structures on isopycnic gradients has not been described. Conceivably the presence of two infective peaks might represent a separation of these

components. Finally, the infection of fibroblasts with Friend virus results in only a transient replication of appreciable levels of the SFFV component of the complex (F. Lilly and R. A. Steeves, cited in (191)). SFFV infectivity in the culture medium is detected only during the first few transfers after infection, whereas F-MuLV replicates efficiently in fibroblasts. Stewart and Maizel (191) have observed a component released from Friend virus-infected mouse fibroblasts which bands at a density of 1.22 to 1.24 g/cm^3 during the early passages after infection. The disappearance of this component during subsequent transfers suggested to the authors that it may be related to the SFFV component of the Friend virus complex.

The above reports suggested a number of possible explanations for the presence of two discrete bands of infectivity in mouse serum, and a more detailed examination of these components was undertaken. In addition, an unusual form of virus is described which is present in homogenates of infected cells.

III:2 Results.

III:2.1 The Distribution of Friend MuLV Infectivity on Isopycnic Sucrose Density Gradients.

Initial experiments indicated that the serum of DBA/2 mice 14 days after infection contained two types of infectious MuLV which band in sucrose density gradients at 1.15-1.17 g/cm^3 and 1.24-1.25 g/cm^3. The dense component was often up to 20% of the total infectivity.

In order to further study the two peaks, virus from mouse serum was banded in a sucrose density gradient and the fractions were

assayed for MuLV, SFFV, and reverse transcriptase. As shown in Figure III.1, the MuLV infectivity approximately paralleled the absorbance at 252 nm which was used as a measure of light scattering and presumably reflects the number of particles in the gradient fractions. SFFV activity extended to the dense region of the gradient but was not as pronounced as MuLV activity in this region. The reverse transcriptase activity was also relatively low in the dense region, although a shoulder of activity was present. In other experiments the reverse transcriptase activity in the dense peak was more pronounced.

III:2.2 Electrophoresis of Proteins of the Dense and Light Virus Bands.

The 1.16 g/cm^3 and 1.24 g/cm^3 virus-containing bands from isopycnic sucrose gradients were analyzed by electrophoresis in 8% polyacrylamide gels containing 0.6% sodium dodecyl sulfate. As seen in Figure III.2, the two bands had a simple polypeptide composition. In the dense peak, there are two major proteins of approximately 70,000 daltons and two partically resolved proteins with sizes of approximately 15,000 daltons. The material which bands at 1.16 g/cm^3 also contains proteins migrating with apparent molecular weights of approximately 70,000; however, the majority of the protein migrates as a single component at approximately 15,000 daltons. In addition, a small amount of a 30,000 dalton protein was observed. Although several of these polypeptides have apparent molecular weights similar to the MuLV proteins (41,81), approximately 60% of MuLV protein is

Figure III.1 MuLV, SFFV and Reverse Transcriptase Activities From Large Scale Preparation of Serum Virus.

Friend leukemia virus from the serum of 125 mice (approximately 50 mls) was prepared by isopycnic banding as described in the experimental procedures. The gradient was fractionated and aliquots were taken for assays. The density indicated by the arrows was read from a graph constructed by directly weighing 100 µls of selected fractions. A. Absorbance at 252 nm. B. MuLV infectivity (S^+L^- foci). C. SFFV infectivity. D. Reverse Transcriptase Activity.

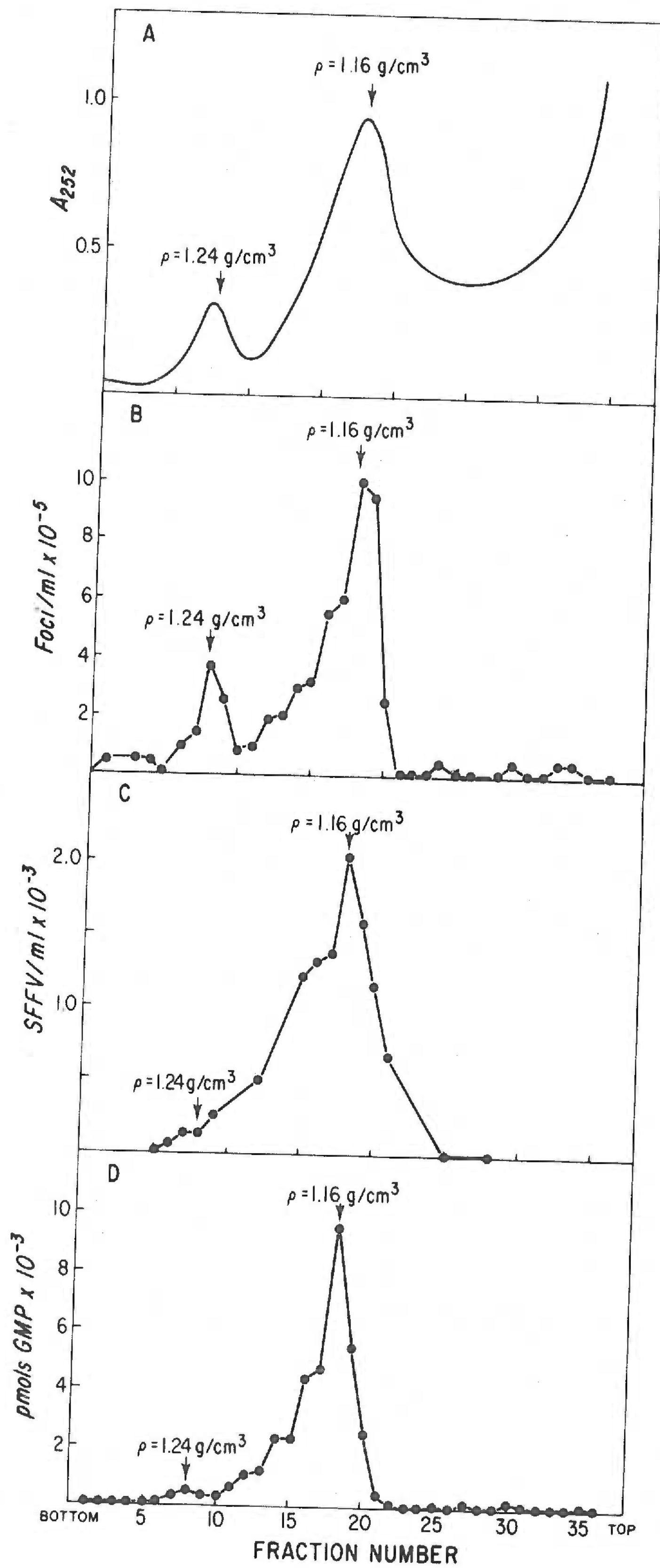
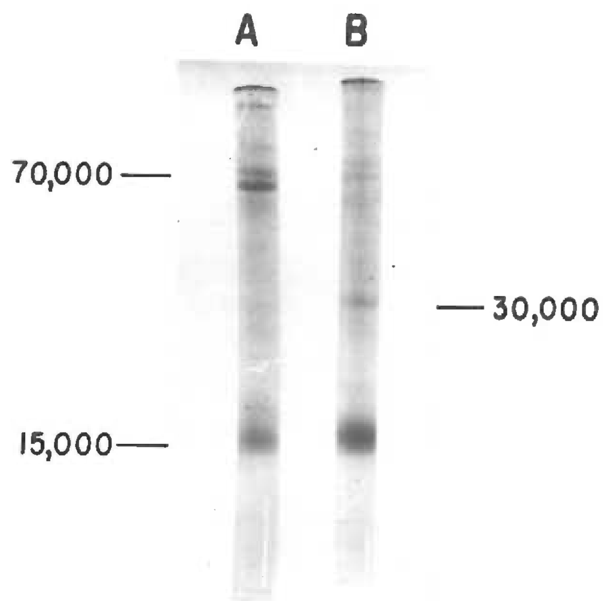

A
B
C
D
A_{252}
1.0
0.5
ρ = 1.16 g/cm³
ρ = 1.24 g/cm³
Foci/ml x 10⁻⁵
SFFV/ml x 10⁻³
pmols GMP x 10⁻³
2.0
1.0
10
8
6
4
2
BOTTOM
5
10
15
20
25
30
35
TOP
FRACTION NUMBER

Figure III.2 SDS/Polyacrylamide Gel Electrophoresis of Virus Bands at 1.24 and 1.16 g/cm^3.

The peak fractions from a gradient ran in parallel with that shown in Figure III.1 were diluted in TSE buffer and pelleted. The pellets were dissolved in electrophoresis sample buffer and electrophoresed on cylindrical 8% polyacrylamide gels containing 0.6% SDS as described in the Materials and Methods. The proteins were visualized by staining with coomassie blue. A. Band at 1.24 g/cm^3. B. Band at 1.16 g/cm^3.


A
B
70,000
30,000
15,000

p30, a 30,000 dalton protein; and MuLV also contain major proteins of 12,000 and 10,000 daltons (41,81).

III:2.3 Non-Virus Components in the 1.16 g/cm^3 and 1.24 g/cm^3 Virus Bands.

The simplest hypothesis which would explain these results is that the virions in the two isopycnic virus bands are heavily contaminated with non-virus materials. Since these materials might associate with virus and be involved in the virion density heterogeneity (Figure III.1) the constitution of these bands was further investigated.

One approach which was employed was to quantitatively analyze p30 by radioimmunoassay (187). Figure III.3 shows the results of this analysis. It is apparent that the p30 antigenicity per mg of protein of purified Friend MuLV isolated from cell culture medium is much greater than that of either of the serum virus peaks. If the p30 content per virion in the serum is similar to that of the purified virus from cell culture medium, these results would be consistent with approximately 10% and 3-4% purity of the virus in the 1.16 g/cm^3 and 1.24 g/cm^3 bands, respectively.

To determine if host material was banding in the region of the virus bands, the virus purification procedure was carried out with uninfected mouse serum. As shown in Figure III.4, two peaks of material banding at 1.16 g/cm^3 and at 1.24 g/cm^3 were readily apparent. Although the amount of protein recovered in these peaks was only about half of that obtained from an equivalent volume of infected mouse serum, the electrophoretic characteristics of these proteins were

Figure III.3 Radioimmunoassay for p30 in Friend Leukemia Virus from Eveline II Cells and in the Serum Virus Bands at 1.24 g/cm^3 and 1.16 g/cm^3.

Purified F-MuLV from Eveline cells and pellets of the peak fractions from the gradient employed in Figure III.2 were assayed for the ability to compete with 10,000 counts/min. of [^{125}I]-labeled viral p30 for binding a limited amount of antibody to p30. The reaction was carried out as described in the Materials and Methods. (Δ——Δ), Friend leukemia virus from Eveline II cells. (o——o), Serum virus banding at 1.16 g/cm^3. (●——●), Serum virus banding at 1.24 g/cm^3.

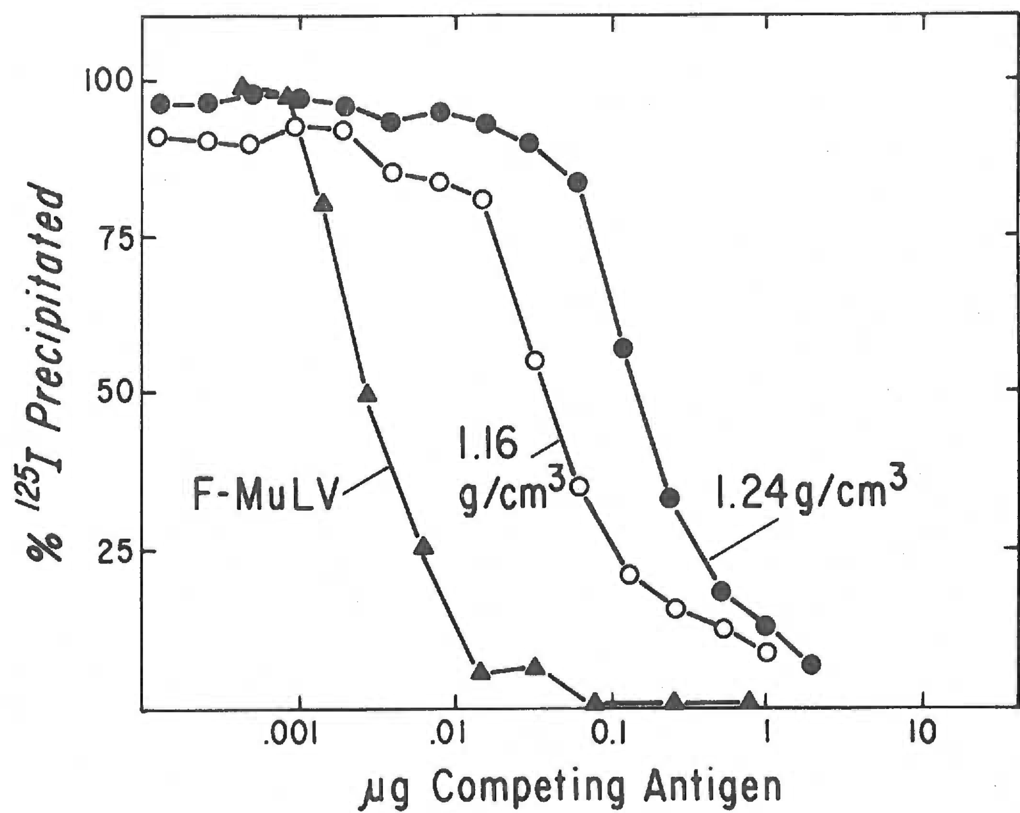
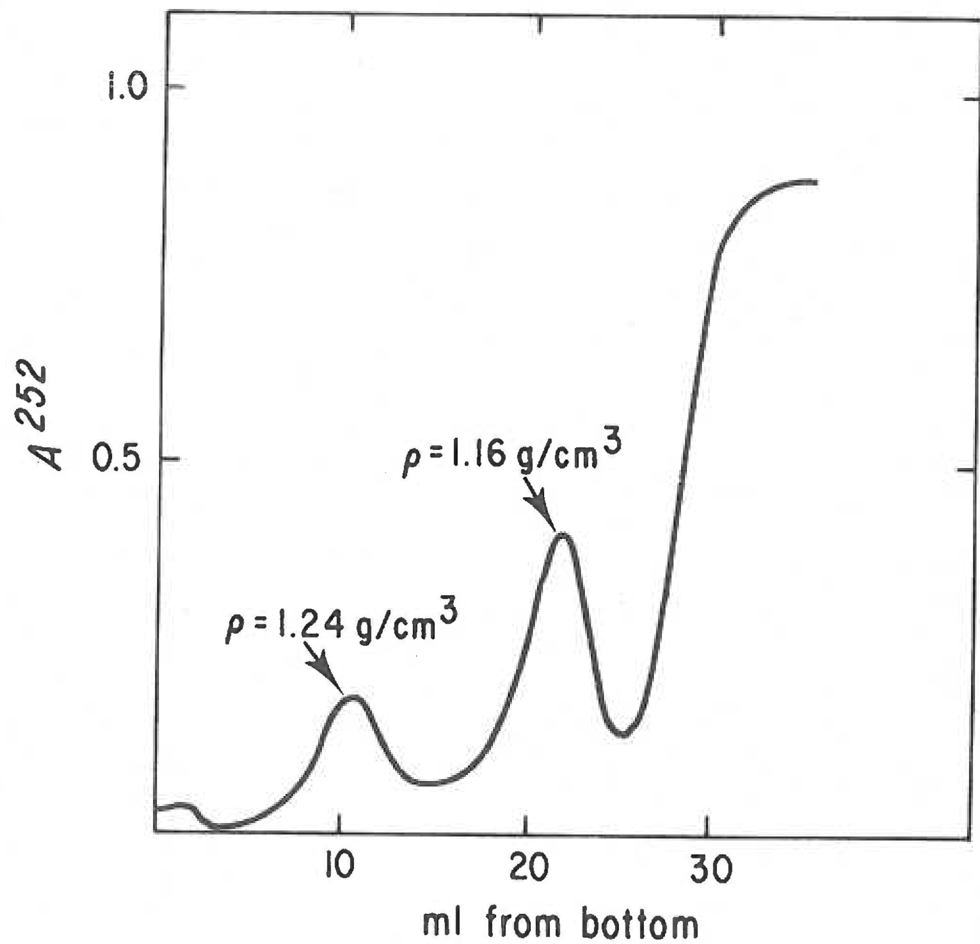

100
75
50
25
% ^{125}I Precipitated
F-MuLV
1.16
g/cm^3
1.24 g/cm^3
.001
.01
0.1
1
10
μg Competing Antigen

Figure III.4 Detection of Bands at 1.24 g/cm^3 and 1.16 g/cm^3 From Uninfected Mouse Serum.

The serum from 200 uninfected mice (approximately 100 mls) was collected and taken through the virus preparation as described in the Materials and Methods. Densities indicated by the arrows were measured as in Figure III.1.



1.0
0.5
A^{252}
ρ = 1.16 g/cm^3
ρ = 1.24 g/cm^3
10
20
30
ml from bottom

identical to those in Figure III.2 except that the minor 30,000 dalton protein was absent in the 1.16 g/cm^3 peak from uninfected mice.

An electron microscope analysis of the 1.16 g/cm^3 and 1.24 g/cm^3 bands from infected mice is shown in Figure III.5. Clearly, these isopycnic bands from sucrose gradients contain large amounts of subcellular particles which contaminate the virions. Furthermore the virus particles in the 1.24 g/cm^3 fraction appear to be attached to the subcellular materials. Such attachment would provide an explanation for the apparent higher density of this virus. Structures resembling virus cores were not observed.

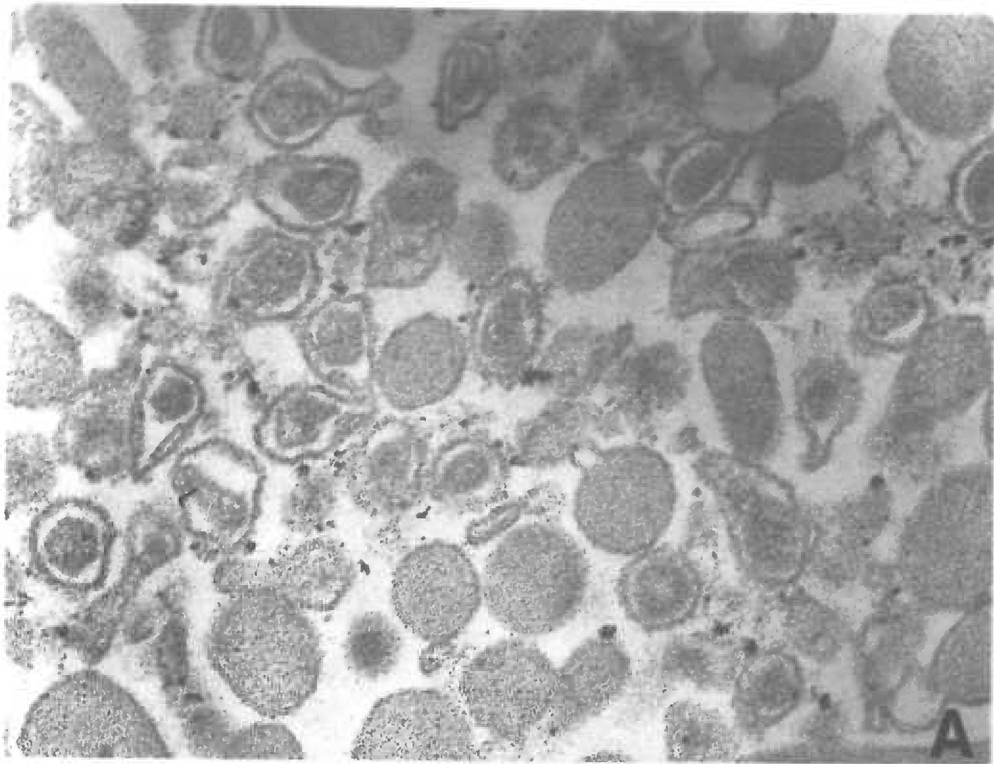
III:2.4 Rapidly Sedimenting MuLV Infectivity in Homogenates of Cultured Eveline Cells.

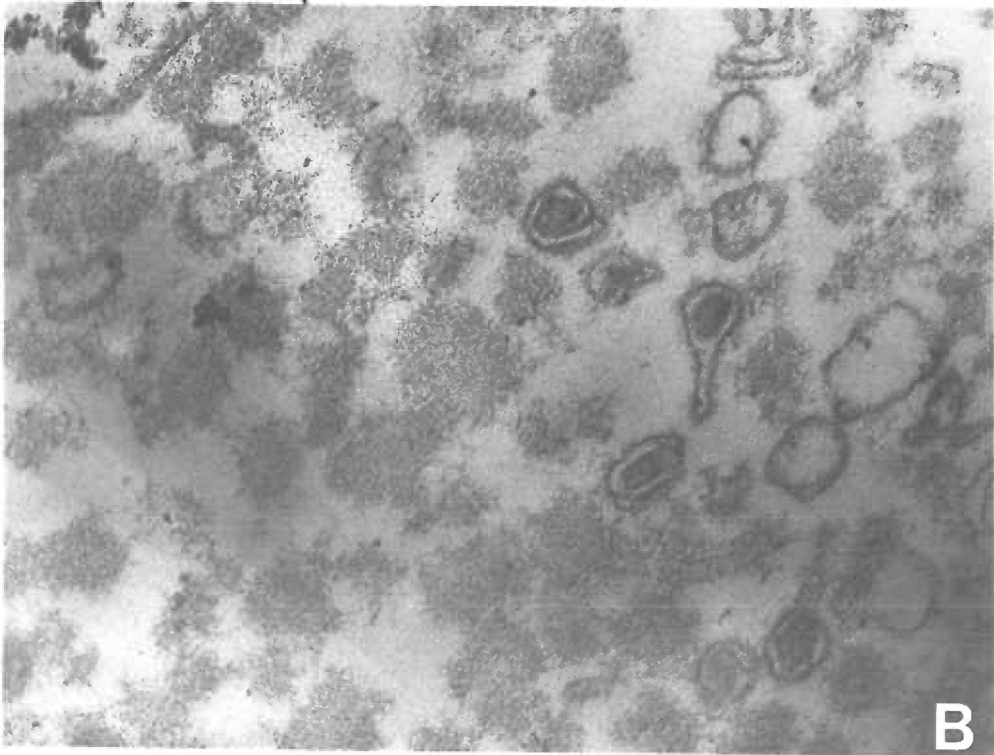
Cultured Eveline II cells are extremely active in production and release of infectious Friend MuLV (128). It was found that the cells also contain an unusual form of cell-associated virus.

When Eveline II cells growing in the logarithmic phase are washed extensively and lysed by homogenization in a low ionic strength buffer, the amount of infectivity in the cell homogenate is 20-50% of the infectivity found in the culture medium. If, however, the cell homogenate is first cleared of cellular debris by low speed centrifugation, only 10-20% of the infectivity in the homogenate remains free in the supernatant. Sonication of the cell homogenate prior to low speed centrifugation did not release the infectious virus from the rapidly sedimenting subcellular materials. Attempts to study the buoyant density of the cell-associated virus have been hindered by a loss of

Figure III.5 Electron Microscopic Examination of Isopycnic Bands from Infected and Uninfected Mouse Serum.

Glutaraldehyde-fixed pellets of the isopycnic bands from infected mouse serum were thin-sectioned and examined by electron microscopy. A. 1.16 g/cm^3 band from infected mouse serum. B. 1.24 g/cm^3 band from infected mouse serum. Magnification is approximately 100,000 X. Electron microscopy was performed by U. I. Heine, National Cancer Institute, National Institutes of Health.


A


B

infectivity of this population during sucrose density gradient centrifugation. On one occasion a significant proportion of the infectivity was recovered and over 95% was found at the top of the gradient at a density of less than 1.1 g/cm^3. In subsequent attempts to repeat this experiment, little or no infectivity was recovered from the gradients.

III:3 Discussion.

These results indicate that mouse serum contains subcellular materials which have the same buoyant densities as C-type virus (1.16 g/cm^3) and viral cores (1.24 g/cm^3). In the absence of productive infection with murine leukemia virus, the material banding in these regions exhibits no infectivity, polymerase activity or morphological relationship to virus particles. However, upon infection, infectious virus are found intermixed with the endogenous material in both regions of the gradient. The finding that the material banding at 1.24 g/cm^3 contains appreciable amounts of infectious C-type particles is unexpected and may reflect a degree of affinity of the virus for the endogenous material. The electron microscopic evidence is consistent with this possibility, since the virus particles appeared to be attached to particles with indistinct edges (Figure III.5B). These particles have not been further identified.

The observation that MuLV and SFFV infectivities are distributed relatively differently in the two buoyant density bands (Figure III.1) should not be considered to imply that these virus components are physically distinguishable. A more likely explanation is based on the

fact that SFFV is a dependent virus (53). Therefore, it will only infect cells coinfected with MuLV; and its titer is diluted out in an exponential, rather than a linear, manner.

This study may have some bearing on several observations previously reported in the literature. A number of studies have reported the presence of virus particles or viral components which distribute on isopycnic gradients at atypical buoyant densities (44,189,190,220). These reports as well as reports of the infectivity of virus cores (39) and variable levels of virion proteins in different virus preparations may, in some cases, be the result of contaminants similar to those described here. In addition, this study demonstrates that the usual methods used for virus purification are not always sufficient. It would also appear that the absence of virus at its typical buoyant density may not be sufficient to exclude the possibility that high levels of virus are present in a particular sample. The studies presented on cell-associated infectivity also suggest that this population of virus may not exist as free structures with the typical purification properties of C-type viruses. On the contrary, this virus is apparently also associated with subcellular components. These results could have particular relevance in screening procedures for virus contamination of cell cultures (102) and for the detection of tumor viruses in neoplastic tissues.

IV. THE SYNTHESIS OF FRIEND MURINE LEUKEMIA VIRUS PROTEINS IN CULTURED CELLS

IV:1 Purification and Characterization of Virus Produced by Eveline II Cells.

IV:1.1 General.

The studies of virus isolated from the serum of infected mice indicated that this source of virus would not be suitable for preparing large quantities of virus or for the preparation of immunological probes. Although most infected mouse cell lines produce only small quantities of virus particles, a line has been reported to produce very large quantities of the MuLV component of Friend leukemia virus (128). These cells, termed Eveline cells, were derived by W. Schafer in Germany. Recently a subline of Eveline cells (Eveline II cells) has been maintained at Duke University by D. Bolognesi for studies of purified viral proteins. The cells used in this study were kindly provided by his laboratory. These cells were derived by infection of secondary fibroblasts from an inbred line of Swiss mice with the original Friend leukemia virus and have since been adapted for growth in suspension cultures.

IV:1.2 Biological Nature of Virus Produced by Eveline II Cells.

The Friend leukemia virus is a complex of an independent lymphatic leukemia virus and an erythroleukemia virus which is dependent upon

the former for its replication (53). Previous studies have indicated that the erythroleukemia component (SFFV) replicates poorly or not at all in cells other than target erythropoietic stem cells, even in the presence of helper virus; however, the literature contains conflicting reports regarding this point (56,191).

The biological activities of the virus produced by Eveline cells are summarized in Table IV.1. For comparison, the biological activities of Friend virus obtained from infected mouse serum are also summarized. MuLV infectivity in the medium of logarithmically growing cultures was typically in the range of 30,000 to 100,000 foci per 10^6 cells, whereas SFFV infectivity, measured at 9 days post-infection by the procedure of Axelrad and Steeves (8), was uniformly zero at doses up to one ml of culture medium. When neonate mice were injected with one ml of culture medium from logarithmically growing cells, however, 70% of the innoculated mice developed typical macroscopic spleen foci at approximately three weeks of age, indicating that a low level of SFFV was replicating in Eveline cells. Neonate mice surviving innoculation had not developed enlarged lymph nodes or thymuses typical of lymphatic leukemia when examined at 9 months after injection. Many of these mice exhibited enlarged spleens, however, and cell-free extracts made from these spleens contained high titers of SFFV as well as MuLV. Thus, large amounts of MuLV are made by Eveline cells. However, the ability of this MuLV to induce lymphatic leukemia is uncertain, partly because the small numbers of SFFV alter the pathology of the disease. Although the values obtained from the SFFV assay and the S^+L^- assay are not strictly comparable in terms of the number of infective particles, it

Table IV.1 Comparison of Biological Activities of Virus Produced by Eveline Cells and Friend Virus from Infected Mouse Serum.

Virus Source	MuLV[a]	SFFV[b]	MuLV / SFFV	Lymphatic Leukemia Induction in Mice
Culture Medium of Eveline Cells Growing in Log Phase	~50,000/10^6 cells	<1/10^6 cells	>50,000	Unclear[c]
Serum of DBA/2 Mice Infected With Friend Virus	~1 x 10^6/ml	~2 x 10^4/ml	~50	Yes[d]

[a]MuLV as measured by the S^+L^- assay.

[b]As measured by the SFFV assay of Axelrad and Steeves (8).

[c]As measured in neonatally innoculated mice at 9 months of age.

[d]Reported by Dawson, Rose and Fieldsteel (52).

would appear that MuLV is present in a vast excess over SFFV in the virus produced by Eveline cells. The ratio of SFFV to MuLV is at least three orders of magnitude higher in serum from Friend virus-infected mice than in Eveline II culture medium.

IV:1.3 Purification of Virus From Eveline Cells on Isopycnic Sucrose Gradients.

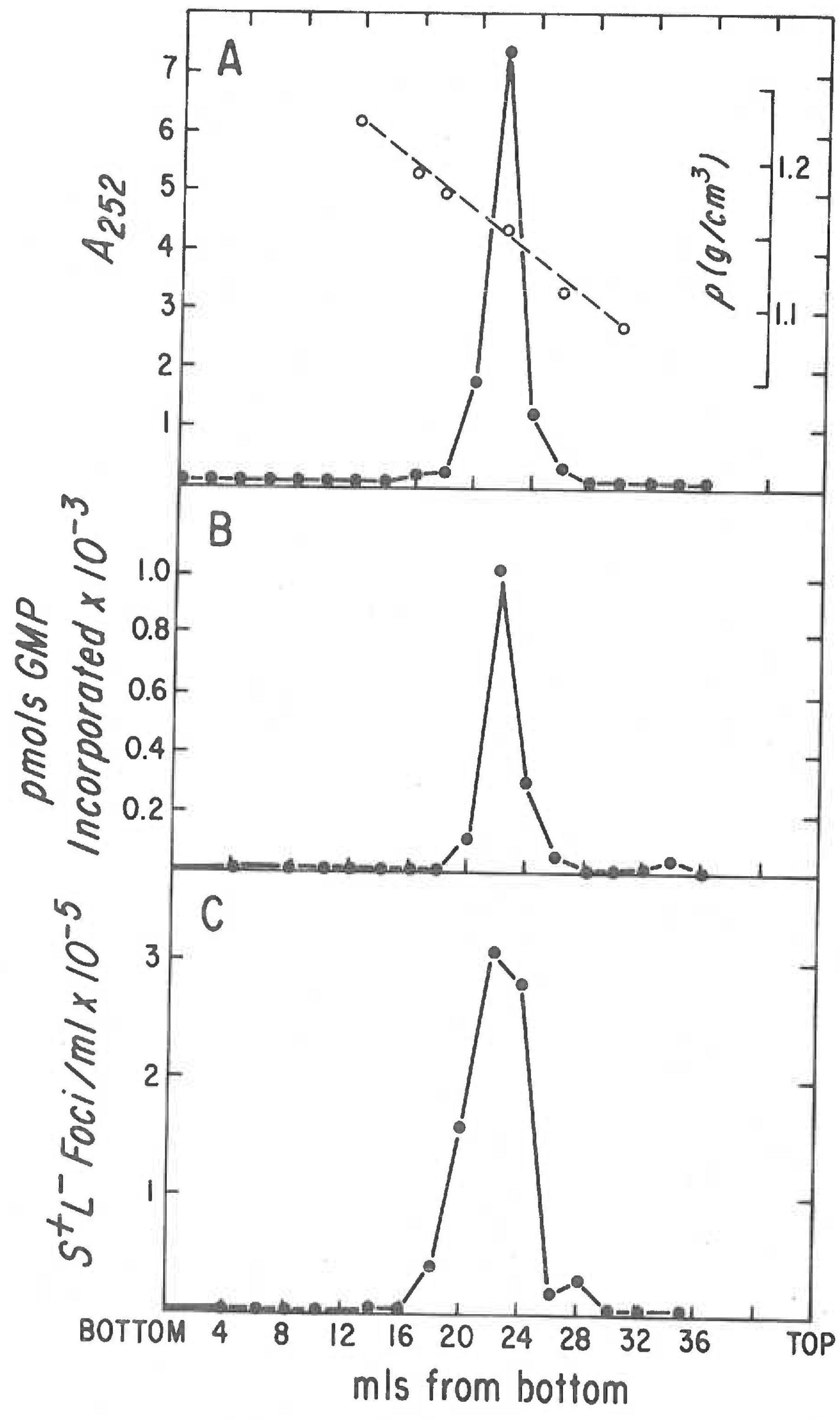
Figure IV.1 shows the results of an isopycnic sucrose density gradient preparation of virus from 250 mls of culture medium from Eveline II cells growing in the logarithmic phase. Fractions of the gradient were measured for MuLV infectivity, reverse transcriptase activity and absorbance at 252 nm. The maximum values obtained for all three parameters occurred in the same fraction which had a density of slightly over 1.15 g/cm^3. The small shoulder of MuLV infectivity is not a consistent finding and probably represents variation in the S^+L^- assay. The small amount of reverse transcriptase activity at the top of the gradient has been observed on a number of gradients and may represent small amounts of the enzyme released from disrupted virus. The peak fractions from this gradient were pelleted by sedimentation at 25,000 rpm for 90 minutes in a Beckmen SW 27.1 rotor and the resulting pellet contained 3.25 mg of protein. This is representative of the usual yield from the cells which is between 8 and 15 mg of viral protein per liter of culture medium.

IV:1.4 Comparison of p30 Content of Purified F-MuLV from Eveline II Cells with Purified Rauscher Leukemia Virus (R-MuLV).

The virus purified from Eveline II cells was compared to a

Figure IV.1 Isopycnic Sucrose Density Gradient of Virus From Eveline II Cells.

Virus from 250 mls of an Eveline II culture (1.93×10^6 cells/ml) was purified by isopycnic banding on a 15-60% sucrose gradient as described in the Materials and Methods. The gradient was fractionated into two ml fractions by pumping from the bottom of the gradient. 50 µl aliquots of the fractions were diluted to 1 ml for the determination of absorbance at 252 nm on a Varian Techtron (Model 635) spectrophotometer. 20 µl aliquots of the fractions were assayed for reverse transcriptase and serial dilutions of aliquots were assayed for MuLV infectivity by the S^+L^- assay as described in the Materials and Methods. Densities of selected fractions were determined by directly weighing 100 µl of the fractions. A. Absorbance at 252 nm corrected to 1 ml. B. Reverse transcriptase activity (pmol GMP incorporated/15 min/20 µl. C. MuLV infectivity per ml (S^+L^- foci).


A
B
C
A_{252}
ρ (g/cm^3)
pmols GMP Incorporated x 10^{-3}
S^+L^- Foci/ml x 10^{-5}
BOTTOM
TOP
mls from bottom

commercially prepared sample of Rauscher leukemia virus (Litton Bionetics, Inc.) by a competition radioimmunoassay for p30. Figure IV.2 shows the results of this assay. It is seen that the F-MuLV from Eveline cells competes slightly more efficiently for the antibody to p30 than does the R-MuLV. The difference observed may be underestimated in that the monospecific antiserum employed, as well as the radioiodinated p30, are specific for the R-MuLV protein. These results suggest that the F-MuLV preparation is of higher purity than the R-MuLV. This finding is not unexpected considering that the commercial procedure for the purification involves only a single rate zonal gradient procedure. Alternatively, the proportion of p30 in the virions from the Eveline II cells may be slightly greater than in the R-MuLV sample.

IV:1.5 Electrophoretic Analysis of Viral Proteins on SDS/Polyacrylamide Gels.

A sample of isopycnically purified virus from Eveline II cells was analyzed by electrophoresis on cylindrical 8% polyacrylamide gels containing 0.6% SDS as described in Materials and Methods. As seen in Figure IV.3, six major components were resolved in the gels. The two closely migrating bands at approximately 70,000 daltons correspond to the envelope glycoproteins gp69/71. The darkly stained protein migrating with a molecular weight of approximately 25,000 daltons in this gel corresponds to p30, the major structural protein of murine RNA tumor viruses. In similar molecular weight determinations, values of 25-28,000 daltons have been obtained for the molecular

Figure IV.2 Competition Radioimmunoassay Comparing p30 Content of Rauscher MuLV and Friend MuLV From Eveline II Cells.

Serial dilutions of F-MuLV from Eveline II cells and R-MuLV from a commercial source (Litton Bionetics, Inc.) were assayed for competition with approximately 10,000 rpm of [^{125}I]-p30 for binding to an anti-p30 antibody as described in the Materials and Methods. (●-----●), R-MuLV. (o——o), F-MuLV. The numbers in parentheses indicate the amount of protein required to achieve 50% inhibition of [^{125}I]-p30 precipitation.

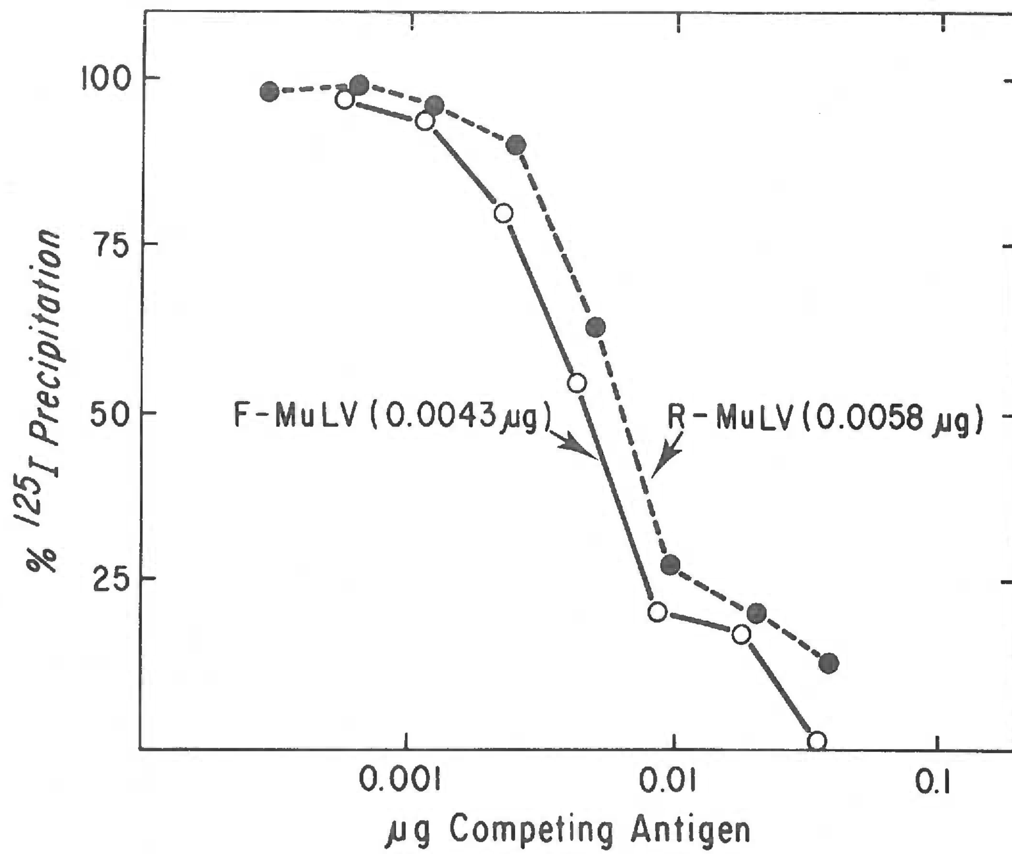
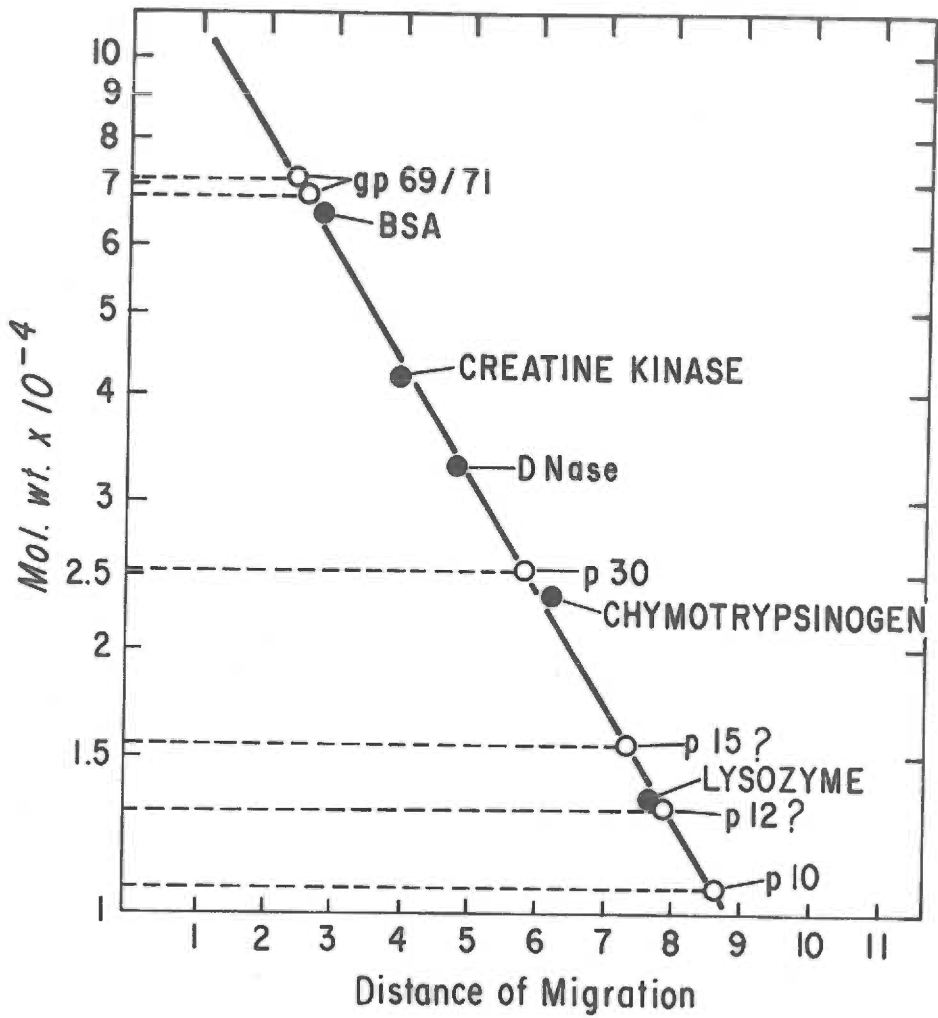
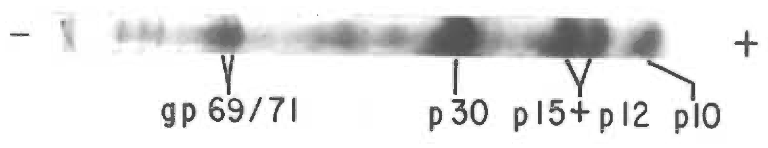

100
75
50
25
% ^{125}I Precipitation
F-MuLV (0.0043 μg)
R-MuLV (0.0058 μg)
0.001
0.01
0.1
μg Competing Antigen

Figure IV.3 Electrophoretic Analysis of F-MuLV from Eveline II Cells: Molecular Weights of the Major Viral Proteins.

Approximately 50 μg of F-MuLV protein was dissolved in sample buffer and electrophoresed on 8% polyacrylamide gels containing 0.6% SDS as described in the experimental procedures. Proteins of known molecular weight were electrophoresed in parallel gels. Electrophoresis was for 195 minutes at 5 mA/gel. Closed circles; standard proteins of known molecular weight. Open circles; major viral proteins. Because of confusion regarding the correspondence of the elution order of p15 and p12 on gel filtration columns with their migration on SDS-polyacrylamide gels, the identities of these proteins were not designated. However, it has recently been established that the migration of p15 and p12 in this gel system does, in fact, correspond to their elution order on gel filtration columns. Thus the postulated assignments for these proteins in the figure are correct.



10
9
8
7
6
5
4
3
2.5
2
1.5
1
Mol. wt. x 10^-4
gp 69/71
BSA
CREATINE KINASE
D Nase
p 30
CHYMOTRYPSINOGEN
p 15 ?
LYSOZYME
p 12 ?
p 10
1 2 3 4 5 6 7 8 9 10 11
Distance of Migration



−
+
gp 69/71
p 30
p 15 + p 12
p 10

weight of this protein. The three smaller virus proteins which migrate with apparent molecular weights of approximately 15,000, 13,000 and 10,000 daltons correspond to the viral proteins p15, p12 and p10, respectively.

Electrophoretic analysis of virus labeled with [^{35}S]-methionine and subsequent autoradiography revealed that the bands corresponding to gp69/71, p30 and p12 are labeled with this amino acid. The assignment of p12 as the major low molecular weight [^{35}S]-labeled protein has also been confirmed by its elution from gel filtration columns in the presence of guanidine-HCl.[2] The virion proteins p10 and p15 are not detected by autoradiography and are apparently devoid of methionine. A number of minor components are seen on stained gels (Figure IV.2). Some of these are not apparent on autoradiograms of [^{35}S]-labeled virus and may correspond to small amounts of contaminating proteins from the medium. Analysis of [^{35}S]-labeled virus on slab gels containing urea (see Figures IV.12 and IV.17) resolved some additional minor components. Among these is the viral glycoprotein gp45 which is reported to be a subglycosylated form of gp69/71 (132). This protein is not clearly resolved on cylindrical gels.

IV:1.6 Electron-Microscopic Examination of F-MuLV from Eveline II Cells.

A sample of purified F-MuLV from Eveline II cells was examined by

---

[2] S. Dressler and D. Kabat, unpublished observations.

electron microscopy to determine the amount of cellular debris which might be present. Figure IV.4 shows an electron micrograph of the preparation. It is seen that the sample is a nearly homogeneous population of C-type particles. The tail-like structures on a number of the particles are frequently observed in electron micrographs of RNA tumor viruses and are thought to be a result of the fixation procedure (54).

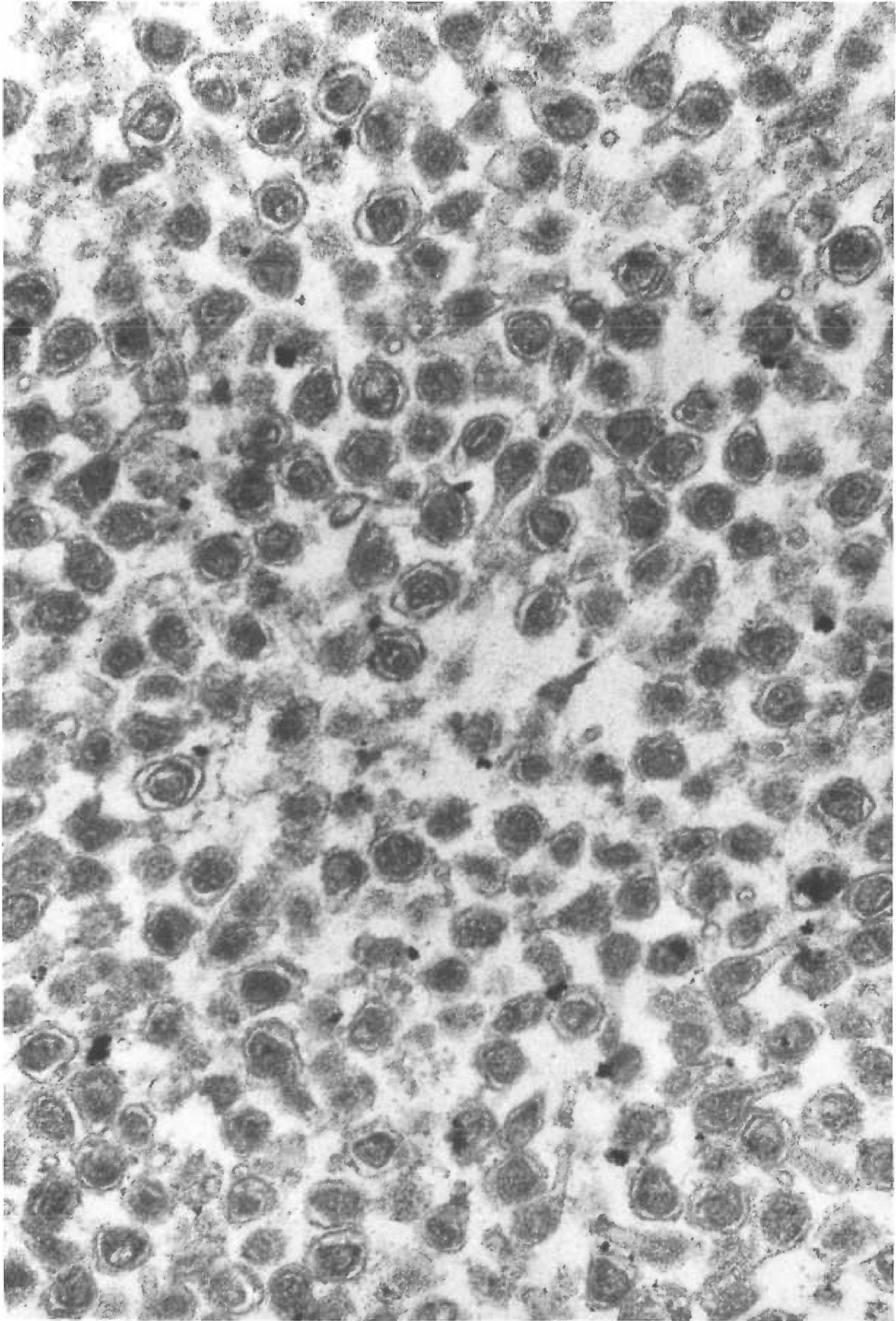
IV:1.7 Discussion.

The characterization of Friend virus produced by Eveline II cells largely conforms to the properties of virus produced by these cells reported in the literature (41). The cell line produces very large amounts of the MuLV component of the Friend leukemia virus complex. The replication of detectable levels of the SFFV component has not been previously reported; however, the infectious properties of the virus produced by these cells has not received more than a cursory examination in previous studies. Lymphatic leukemia was not observed in mice innoculated neonatally with large amounts of the virus; however, a larger number of animals and a longer incubation time would be required to fully establish its leukemogenic activity.

Virus prepared from the culture medium of logarithmically growing cells exhibits a high degree of purity by a number of criteria. The virus bands in a very narrow density range on continuous sucrose gradients, and the electrophoretic analysis of the virus proteins, revealed negligible contamination by non-viral proteins. That the proportion of p30 is significantly higher than the proportion of this

Figure IV.4 Electron Microscopic Examination of Purified F-MuLV From Eveline II Medium.

Section of a pellet of F-MuLV purified by isopycnic banding on sucrose density gradients from Eveline II cell culture medium. Magnification ~100,000 X. Electron microscopy was performed by U. I. Heine, National Cancer Institute, National Institutes of Health.



protein in a preparation of Rauscher leukemia virus is likely an indication of higher purity of the F-MuLV. Finally, direct examination of a pellet of purified F-MuLV revealed negligible contamination with cellular debris. The electrophoretic analysis, radioimmunoassay and electron-microscopic examination of the virus were performed on portions of a large preparation which was employed for the immunization of rabbits.

IV:2 Detection of Murine Leukemia Virus Polyproteins in Cells Producing Friend Leukemia Virus.

IV:2.1 Immune Precipitation of F-MuLV Proteins by Antisera to F-MuLV.

Rabbit antiserum directed against disrupted Friend MuLV and adsorbed against insolubilized mouse proteins, as described in the Materials and Methods,was tested for its ability to precipitate viral proteins using [^{14}C]-labeled virus purified on isopycnic sucrose gradients. As shown in Figure IV.5, approximately 70-75% of the total TCA precipitable counts were precipitated by the antiserum. A similar result was obtained using [^{35}S]-methionine-labeled virus. The maximum immune precipitable radioactivity was also approximately 70-75% using the secondary antiserum technique.

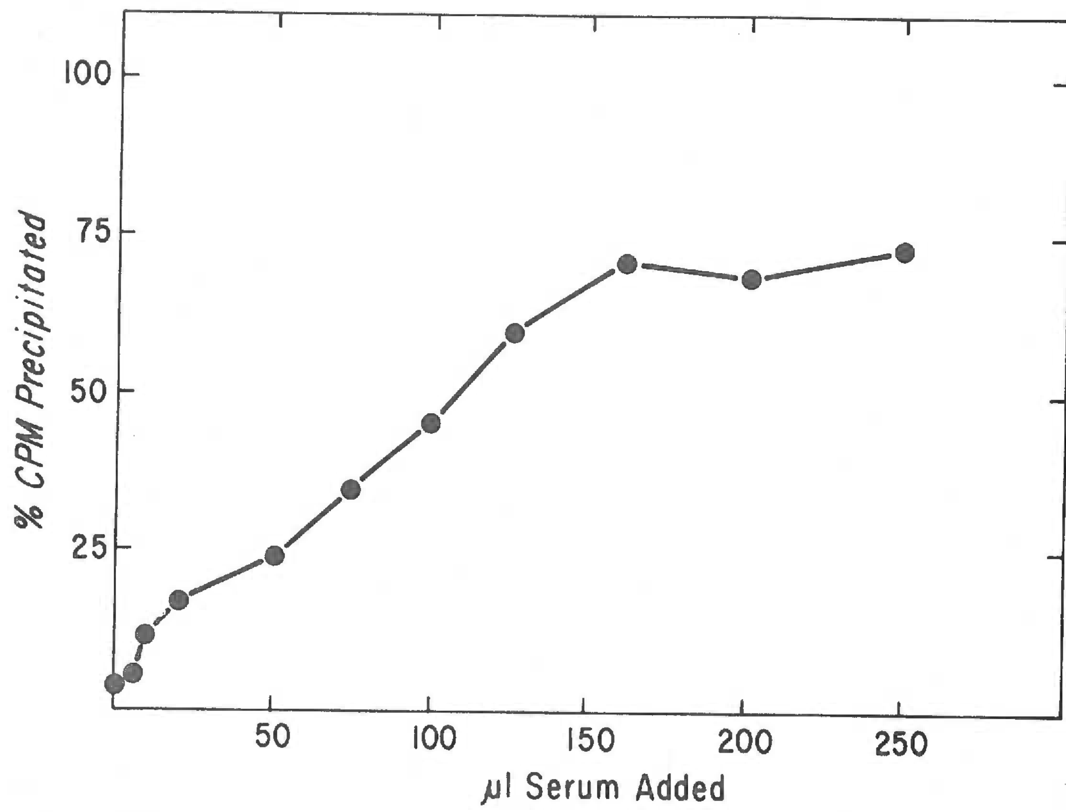
Electrophoretic analysis of [^{35}S] -labeled F-MuLV proteins on 8% polyacrylamide gels containing 0.6% SDS indicated that gp69/71, p30 and p12 were precipitated by the antiserum. The antiserum was subsequently shown to precipitate the viral proteins p10 and p15 as well, and has been employed in radioimmunoassays of these proteins.[3] The

---

[3] C. Sherton, unpublished observations.

Figure IV.5 Precipitation of Disrupted [^{14}C]-labeled Virus With Anti-F-MuLV Serum.

[^{14}C]-labeled F-MuLV was immune precipitated with increasing amounts of rabbit anti-F-MuLV by the primary antiserum procedure described in Materials and Methods. 10^3 TCA-insoluble cpm (8.8 μg virus protein) were added per assay. Immune precipitates were solubilized and counted as described in Materials and Methods.


100
75
50
25
% CPM Precipitated
50
100
150
200
250
µl Serum Added

anti-virus serum was thoroughly adsorbed with insolubilized uninfected mouse proteins (see Materials and Methods) and it did not precipitate any proteins from uninfected mouse fibroblasts which had been labeled with [^{35}S]- L-methionine.

IV:2.2 Immunoprecipitation of Intracellular Virus-Specific Proteins.

The immunoprecipitation procedures employed in this study incorporate features of a number of immunoprecipitation schemes reported by others. The use of detergents for the preparation of cellular extracts is necessary to insure that one is analyzing a representative sample of cellular protein and also greatly reduces non-specific precipitation of proteins. The levels of detergents employed (0.5% DOC, 1% Triton-X-100 and 0.1% SDS) in these procedures were taken from van Zaane et al. (213). Even with these levels of detergents, a significant level of radioactivity is observed in precipitations with non-immune serum. This problem can be partially avoided by the use of high levels of bovine serum albumin and the inclusion of poly-L-lysine[4] in the reaction mixture. The use of sucrose cushions during washing procedures further reduces the non-specific precipitation (49) as does the inclusion of high salt in solutions used to wash the immunoprecipitate.

Even with these precautions a residual level of non-specific radioactivity is present when the cellular extract is precipitated with control serum or even in the absence of added serum. This

[4] E. Herbert, personal communication.

residual level can be reduced to background radioactivity in primary antiserum precipitations and to 1-2% of the total immune precipitated radioactivity in secondary antiserum precipitations by centrifugation of the cellular extract at 200,000 x g for twenty minutes prior to immune precipitation. Electrophoretic analysis of immunoprecipitates from extracts of cells which had not been previously centrifuged did not result in an increased level of any of the proteins detected in centrifuged extracts, indicating that the centrifugation did not result in the loss of appreciable amounts of virus-specific proteins. As shown in Table IV.2, approximately 96-99% of the incorporated methionine was recovered in the soluble extract. Approximately 1-2% of this soluble radioactivity was precipitated by antiserum directed against whole disrupted virus, whereas only slight amounts of radioactivity were precipitated by control rabbit serum either directly or as a coprecipitate in an antigen-antibody complex. Electrophoretic analysis of the control serum precipitates revealed no discrete radioactive protein bands (see Figures IV.16 and IV.17).

IV:2.3 Detection of Virus-Specific Polypeptides in Pulse-Chase Studies.

In the initial experiments, immunoprecipitated proteins were analyzed by electrophoresis in polyacrylamide gels in the presence of 0.6% sodium dodecyl sulfate. Figure IV.6 shows an autoradiogram from an experiment in which the cells had been pulse-labeled for 35 minutes with [^{35}S]-L-methionine and subsequently chased for 120 minutes with a 500-fold excess of unlabeled methionine. Only three radioactive

Table IV.2 Efficiency of Cell Extraction and Specificity of Immunoprecipitation

	A (20 minute pulse)	B (30 minute pulse)
Cell extract	320,000[a]	473,000[a]
Cell extract after centrifugation at 200,000 x g	314,000[a]	472,000[a]
Immune serum precipitates:		
Primary antiserum precipitation	4,620	7,410
Secondary antiserum precipitation	5,880	8,820
Control serum precipitates:		
Primary antiserum precipitation	11	17
Secondary antiserum precipitation	97	104

[a]Trichloracetic acid precipitable cpm per 50 µl of extract.

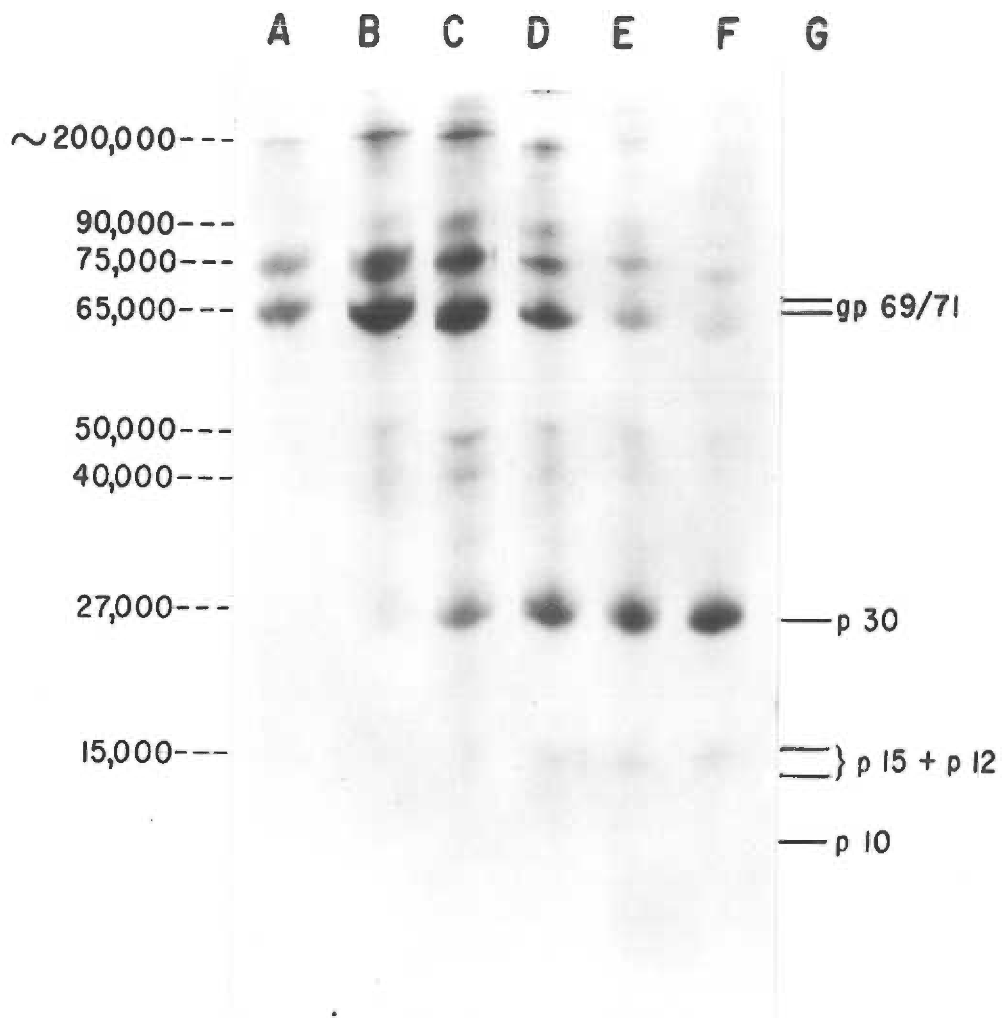
4 ml samples of an Eveline cell culture (1.4×10^6 cells/ml) were taken during a pulse-chase experiment in which the culture was pulse-labeled with 20 µCi/ml of [^{35}S]-L-methionine. Sample A was taken after a 20 minute pulse and sample B after a 30 minute pulse. The cells were pelleted by low speed centrifugation and extracted with 0.8 mls of immune buffer A (see Materials and Methods)and centrifuged at 200,000 x g for 20 minutes. Immune precipitations were performed using 50 µl of the extracts and rabbit antiserum to F-MuLV or control serum by both the primary and secondary antiserum techniques described in the Materials and Methods.

Figure IV.6 Pulse-chase Analysis of Friend Leukemia Virus Proteins Synthesized in Eveline II Cells.

A 50 ml suspension culture of Eveline cells growing at 1.7×10^6 cells/ml was pulse-labeled with 300 μCi of [^{35}S]-L-methionine (100 μCi/mmol, New England Nuclear) in methionine-free medium for 35 minutes. At the end of the pulse period the culture was chased in medium containing an approximately 500-fold excess of unlabeled methionine for two hours. Samples taken at various times during the experiment were analyzed in cylindrical 8% polyacrylamide gels as described. Each sample represents an equivalent volume of the culture. The samples applied to the gels were primary antiserum immune precipitates from:

(A) 10 minute pulse-labeled cells; (B) 30 minute pulse-labeled cells;
(C) 35 minute pulse-labeled cells followed by a 10 minute chase period;
(D) 35 minute pulse-labeled cells followed by a 30 minute chase period;
(E) 35 minute pulse-labeled cells followed by a 60 minute chase period;
(F) 35 minute pulse-labeled cells followed by a 120 minute chase period;
(G) Migration of F-MuLV proteins.

Standard proteins of known molecular weight and purified F-MuLV were run on parallel gels and visualized with coomassie blue. Approximate molecular weights of the viral-specific polypeptides are indicated by the dashed lines on the left of the autoradiogram. The region of migration of p15 and p12, which are not always well resolved on cylindrical gels, is designated by the bracketed area. p12 is the only viral protein in this region of the gel which is highly labeled with [^{35}S]-methionine.

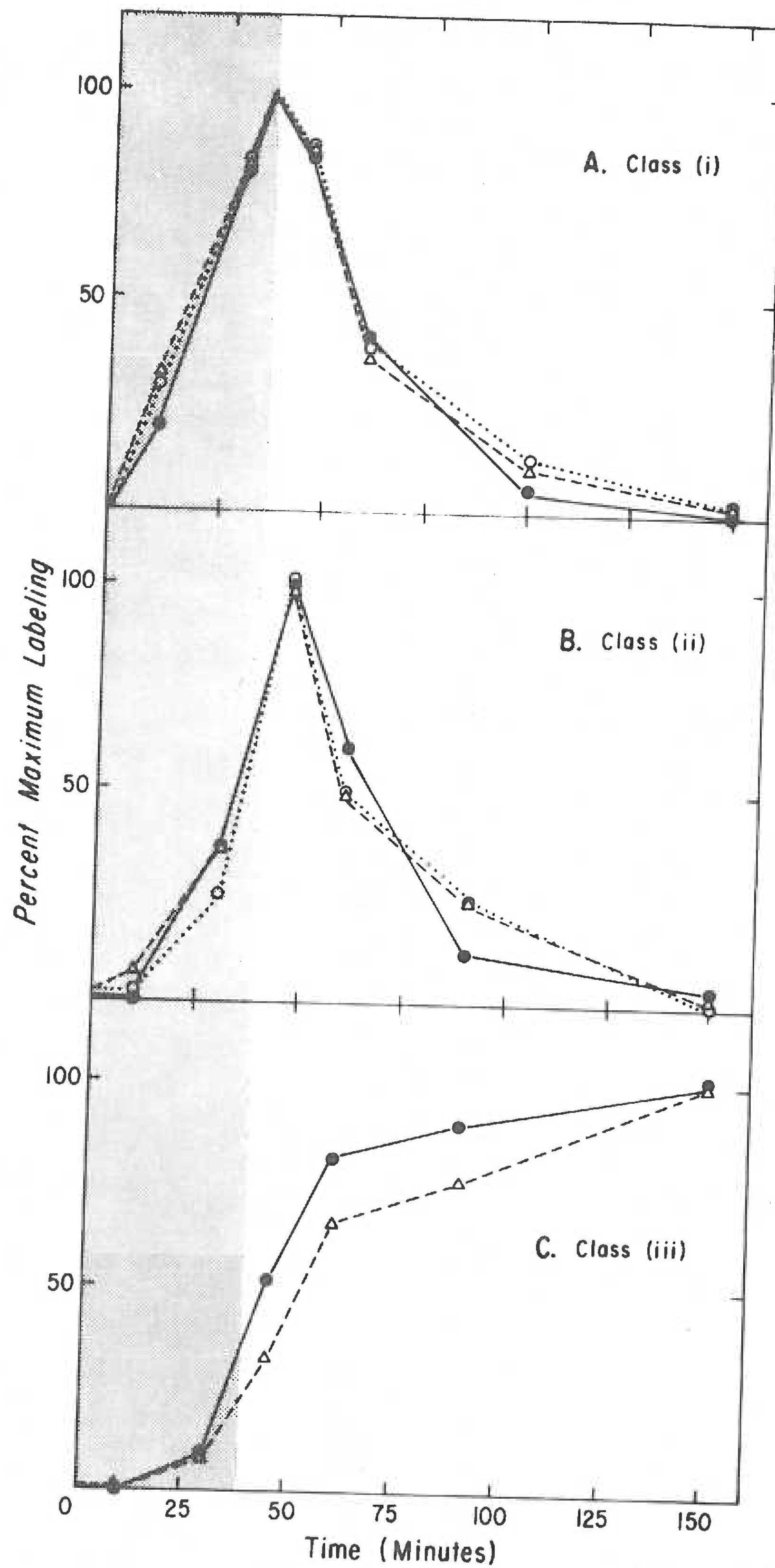

A B C D E F G
~200,000---
90,000---
75,000---
65,000---
50,000---
40,000---
27,000---
15,000---
gp 69/71
p 30
} p 15 + p 12
p 10

proteins were detectable after 10 minutes of labeling - a minor slowly migrating protein and two major components with estimated molecular weights of 65,000 and 75,000. After 30 minutes, the initially labeled bands were more intense and several minor components were detectable. During the chase period the proteins apparent at 10 minutes as well as a number of the subsequently labeled proteins dramatically decreased in intensity, while bands co-migrating with the viral proteins p30 and p12 increased in intensity throughout the chase period. Essentially the same results were obtained when the samples were analyzed by the two-step antibody precipitation method or when the samples were exhaustively precipitated by the addition of carrier virus and additional antiserum.

As seen in Figure IV.6, dramatic changes in the radioactive protein pattern occurred during the pulse-chase experiment. In order to obtain a more quantitative description of these changes, the gels were subjected to fluorography for varying periods of time and the films were scanned with an integrating densitometer. Figure IV.7 depicts integrals of the band densities normalized to the percent of maximum labeling attained during the experiment. It can be seen that this analysis has revealed three kinetically distinct classes of virus specific polypeptides: *Class (i)*. These proteins are labeled in an approximately linear fashion during the pulse period and decline in labeling throughout the chase period. These may represent primary translation products or, alternatively, rapidly derived intermediates in viral protein processing. *Class (ii)*. These proteins are labeled only after a lag in the pulse period, continue to increase in labeling

Figure IV.7 Kinetics of Labeling and Degradation of Virus-Specific Polypeptides in Eveline II Cells.

Fluorographs from the gels shown in Figure IV.6 were scanned with a Transidyne General integrating densitometer (see Materials and Methods). The integrals of the various bands were normalized to the percent of the maximum intensity attained during the experiment and plotted as a function of the sampling time. A. *Class (i)* polypeptides (see text); (●——●) high molecular weight polyproteins; (o-----o) polypeptide of approximately 65,000 daltons; (Δ-----Δ) polypeptide of approximately 75,000 daltons. B. *Class (ii)* polypeptides; (●——●) polypeptide of approximately 90,000 daltons; (Δ-----Δ) polypeptide of approximately 50,000 daltons; (o-----o) polypeptide of approximately 40,000 daltons. C. *Class (iii)* polypeptides; (●——●) polypeptide of approximately 30,000 daltons; (Δ-----Δ) polypeptide of approximately 15,000 daltons. The shaded area in each graph represents the pulse period. Maximum labeling for *class (i)* polypeptides was estimated by extrapolation to the end of the pulse period.



100
50
A. Class (i)
100
50
B. Class (ii)
Percent Maximum Labeling
100
50
C. Class (iii)
0
25
50
75
100
125
150
Time (Minutes)

during the early chase period, and thereafter decline. This class consists of presumed intermediates in processing pathways and includes the 90,000, 50,000 and 40,000 dalton polypeptides. *Class (iii)*. These proteins are labeled late in the pulse period and continue to increase in labeling throughout the chase period. This class contains the end products of intracellular processing and includes proteins which co-migrate with virion p30 and p12.

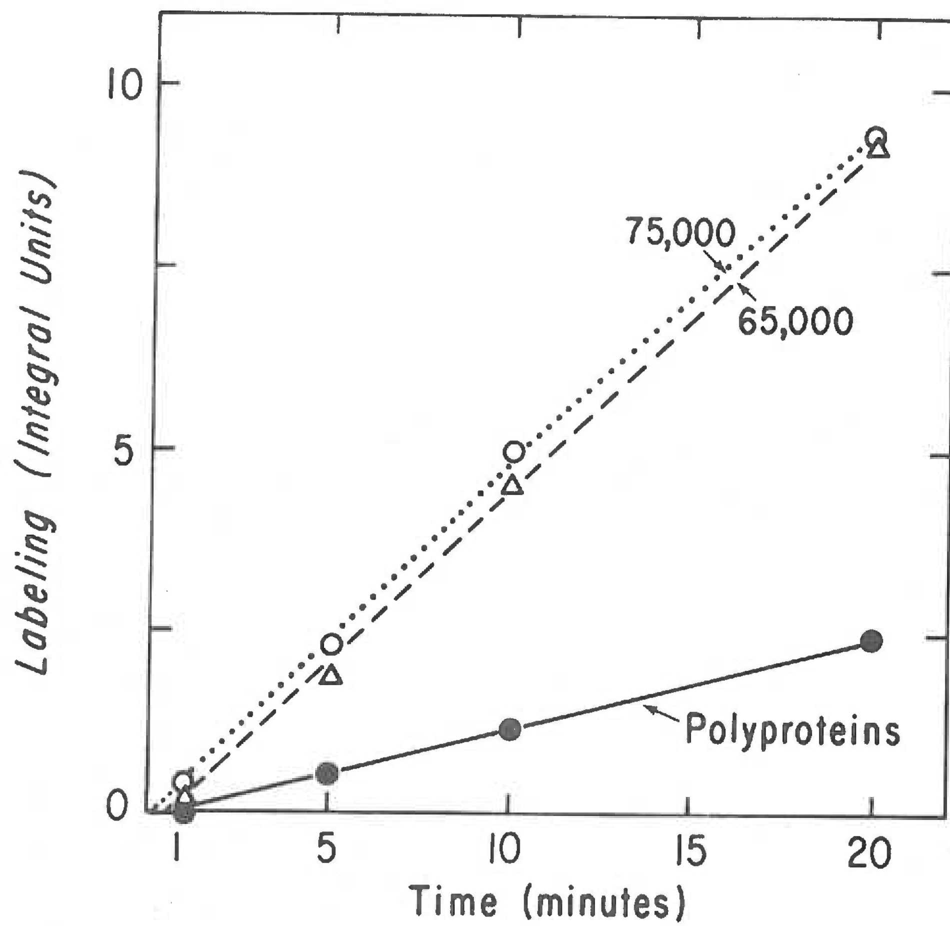
It should be noted that the actual labeling of the high molecular weight polyprotein is only about 20% as high as the lower molecular weight *class (i)* precursors with sizes of 75,000 and 65,000 daltons (see Figure IV.6), and that the rate of its disappearance during the chase is not more rapid (Figure IV.7A). This result would not be expected if the large protein was an obligatory precursor of the 75,000 and 65,000 dalton proteins. Furthermore, the linear labeling kinetics exhibited by all of these *class (i)* proteins does not suggest a precursor-product relationship. In subsequent pulse-chase experiments (see Figure IV.12) the linear labeling kinetics of the 65,000 and 75,000 dalton proteins and the large polyprotein were more rigorously studied. Figure IV.8 shows the labeling of these proteins during a twenty minute pulse period. As seen in Figure IV.8, the 65,000 and 75,000 dalton polypeptides, as well as the large polyprotein, were detected after labeling for only 1 minute and they each accumulated radioactivity without any lag phase.

IV:2.4 Stability of Large Molecular Weight Polypeptides in SDS/Urea/Polyacrylamide Gels.

Many protein aggregates are stable during electrophoresis in SDS/

Figure IV.8 Labeling Kinetics of *Class (i)* Polypeptides.

Eveline II cells (1.4×10^6 cells/ml) were pulse-labeled with 50 μCi [^{35}S]-L-methionine/ml for 20 minutes. Samples were taken at 1, 5, 10, and 20 minutes and immune precipitated with rabbit antiserum to FLV by direct precipitation as described in the Materials and Methods. The precipitates were prepared for electrophoresis and electrophoresed on 10% polyacrylamide slab gels containing 8 M urea as described. Electrophoresis was continued until the dye front reached the end of the gel. The labeling was quantitated as described in Figure IV.7 and the intensities of the bands were plotted as a function of labeling time. The plots represent: (●———●) the high molecular weight polyproteins; (o-----o) the polypeptide of approximately 75,000 daltons and (Δ-----Δ) the polypeptide of approximately 65,000 daltons.



Labeling (Integral Units)
10
5
0
1
5
10
15
20
Time (minutes)
75,000
65,000
Polyproteins

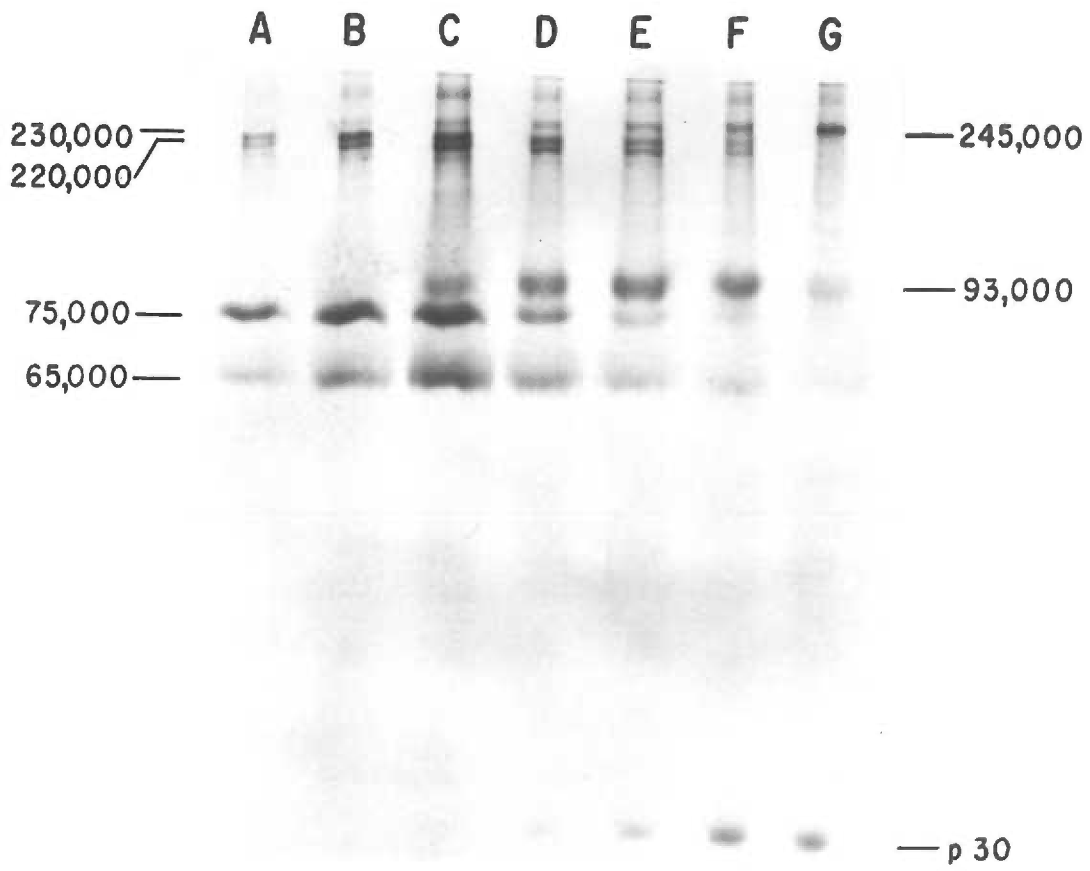
polyacrylamide gels; indeed the immunoprecipitation is performed in this detergent. In order to determine if the large radioactive virus protein (Figure IV.6) is an aggregate of smaller polypeptides, immune precipitates from a pulse-chase experiment were dissolved in sample buffer containing 6 M urea, 1% sodium dodecyl sulfate and 1% B-mercaptoethanol; the samples were heated to 100°C for 2 minutes and were then electrophoresed in 10% polyacrylamide slab gels containing 8 M urea and 0.1% sodium dodecyl sulfate. As seen in Figure IV.9, in these highly denaturing conditions the large [^{35}S]-labeled protein was better resolved and three closely migrating large components can be seen. No disaggregation of any radioactive viral polypeptide into smaller constituents was observed. This gel system was found to also substantially increase the resolution of the lower molecular weight proteins and has been used routinely in the subsequent experiments.

IV:2.5 Molecular Weight Determination of the Slowly Migrating Polypeptides.

The proteins known to be encoded by the C-type RNA tumor viruses include the major internal antigens of the virus, the envelope glycoprotein, and the viral polymerase (16) and represent a combined molecular weight of approximately 200,000 to 230,000 daltons in murine systems (41,132,134). It was of interest, therefore, to obtain an accurate estimate of the molecular weights of the slowly migrating proteins detected in the pulse-chase experiments to determine if they are sufficiently large to contain all of the viral

Figure IV.9 Stability of Proteins on SDS/Urea Gels; Kinetics of Labeling and Decay of Large Polyproteins; and Pulse-Chase Analysis of p30-Specific Proteins in Eveline II Cells.

Eveline II cells (~1.5 x 10^6 cells/ml) were pulse-labeled with 50 µCi/ml of [^{35}S]-L-methionine for 30 minutes and chased by the addition of unlabeled methionine for 2 hours. Samples taken during the experiment were precipitated with antisera to p30 by secondary antiserum precipitation and analyzed in 10% polyacrylamide gels containing 8 M urea as described in Materials and Methods. The samples correspond to immune precipitates obtained from cells after a: (A) 10 minute pulse; (B) 20 minute pulse; (C) 30 minute pulse; (D) 30 minute pulse and 15 minute chase; (E) 30 minute pulse and 30 minute chase; (F) 30 minute pulse and 60 minute chase; (G) 30 minute pulse and 120 minute chase. The approximate molecular weight of the virus-specific proteins are indicated at the sides of the gel. The smaller [^{35}S]-labeled proteins are described in more detail in section IV:3.5.


A
B
C
D
E
F
G
230,000
220,000
75,000
65,000
245,000
93,000
p 30

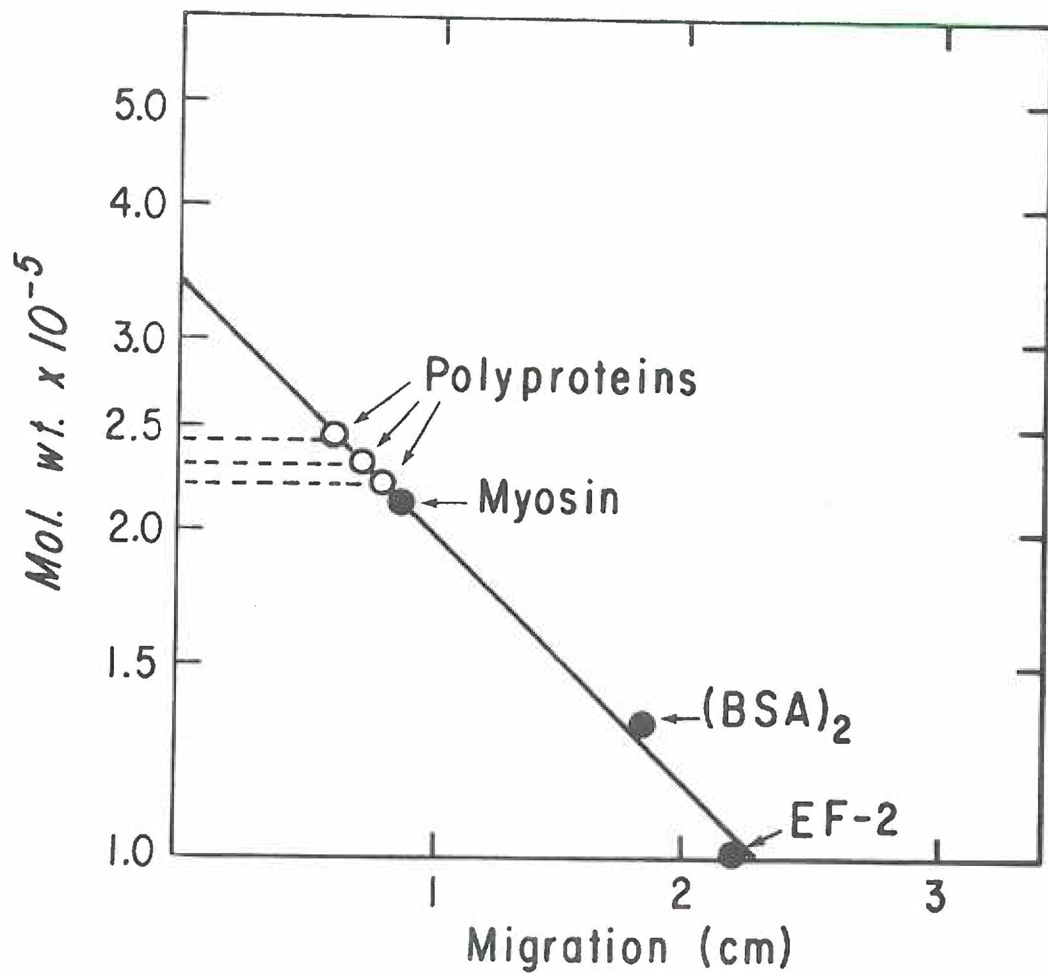
encoded proteins. For this purpose, 10% polyacrylamide slab gels (121) were utilized in order to obtain a close comparison of the viral specific proteins with standard proteins of known molecular weight. Cells were pulse-labeled for 30 minutes and the immunoprecipitated viral proteins were electrophoresed for a time sufficient for accurate molecular weight determinations of the large-sized proteins. All three of the large proteins migrate slightly slower than rabbit myosin (molecular weight 212,000). As shown in Figure IV.10, their molecular weights were estimated to be 220,000, 230,000 and 245,000 and are sufficiently large to contain all of the known MuLV proteins. Although the three proteins were less well resolved, the same molecular weight estimates were obtained in gels lacking urea.

IV:2.6 Kinetics of Labeling of the Three Large Polyproteins.

The pulse-chase labeling experiments described earlier (Figures IV.6 and IV.8) did not resolve the three large polyproteins sufficiently to permit analysis of their individual labeling kinetics. Figure IV.9 shows the results of a pulse-chase experiment in which the samples were electrophoresed in an SDS/urea gel for a time sufficient to separate the three large polyproteins. Unlike the 220,000 and 230,000 dalton proteins, the 245,000 dalton polypeptide is labeled with a lag in the pulse period and it continues to be labeled during the chase period, indicating that it is not a primary translation product. It is conceivable that this protein is derived from a larger precursor; however, only insufficient trace amounts of a larger

Figure IV.10 Molecular Weight Determination of Slowly Migrating Polypeptides.

Eveline II cells (1.6 x 10^6 cells/ml) were pulse-labeled for 30 minutes with 50 µCi [^{35}S]-L-methionine/ml in methionine-free medium and immune precipitated with rabbit antiserum to FLV using the secondary antiserum technique described in Materials and Methods. The precipitate was analyzed in 10% polyacrylamide slab gels containing 8 M urea as described. The gels were electrophoresed for the time required for the dye front to migrate twice the length of the gel. Molecular weight standards were myosin (molecular weight 212,000), non-reduced bovine serum albumin $(BSA)_2$ (molecular weight 135,000) and [^{14}C]-ADP-ribosylated elongation factor II (EF-2) (molecular weight 100,000).


Mol. wt. x 10⁻⁵
5.0
4.0
3.0
2.5
2.0
1.5
1.0
Polyproteins
Myosin
(BSA)2
EF-2
1
2
3
Migration (cm)

protein was present in the immunoprecipitates. Therefore, and for other reasons described below, it is suggested that the 245,000 dalton protein is formed by a modification of one of the smaller polyproteins. As shown in Figure IV.9 none of the polyproteins is degraded more rapidly than the 75,000 and 65,000 dalton proteins.

IV:2.7 Precipitation of Large Polypeptides with Antisera Directed Against p12, p30, and gp69/71.

The viral protein content of the polyproteins were investigated by immune precipitation with monospecific antisera directed against the virion proteins p12, p30 and gp69/71. The specificities of these antisera have been extensively studied and are reported to lack cross-reactivity with other viral proteins (4,193,209). Furthermore these sera have been studied in this laboratory using radioimmune assay techniques and purified virion proteins and were found to lack cross-reactivity.[5]

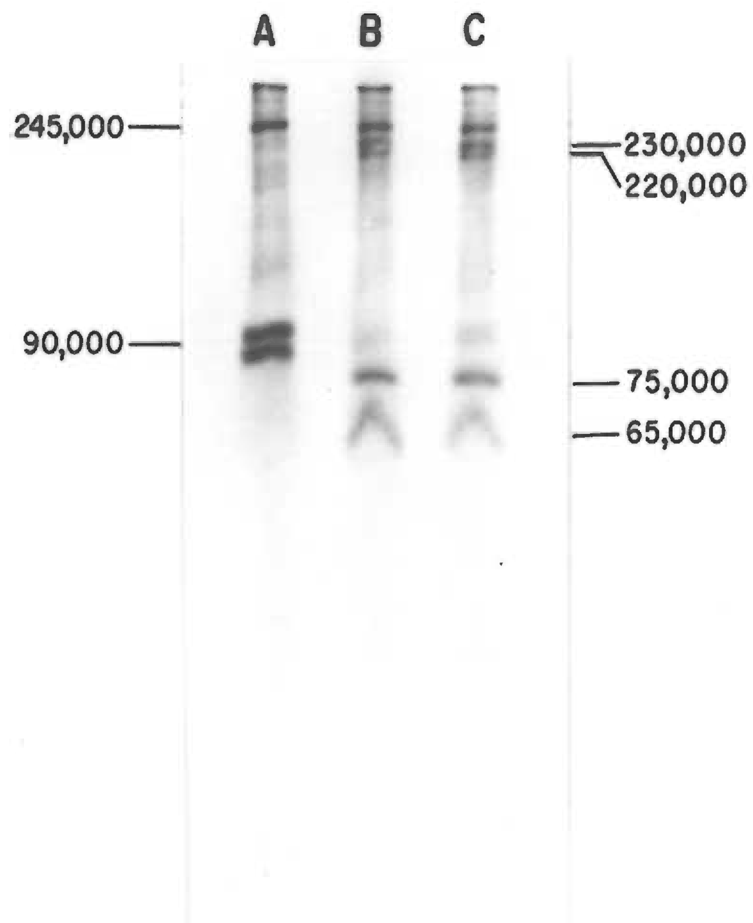
Figure IV.11 shows the results of an experiment in which the cells were pulse-labeled for 30 minutes with [^{35}S]-L-methionine. It is seen that anti-gp69/71 precipitates only the 245,000 dalton polyprotein whereas both anti-p12 and anti-p30 precipitate all three of the polyproteins. Anti-gp69/71 also precipitates two proteins of approximately 90,000 daltons whereas anti-p30 and anti-p12 precipitate the 65,000 and 75,000 dalton proteins.

---

[5] C. Sherton and D. Kabat, in preparation.

Figure IV.11 Precipitation of Large Polyproteins with Antisera Directed Against the Viral Proteins p12, p30 and gp69/71.

Eveline II cells (1.6 x 10^6 cells/ml) were pulse-labeled with 20 μCi [^{35}S]-L-methionine/ml in methionine-free medium for 30 minutes. The culture was aliquoted and precipitated with goat antisera directed against purified viral proteins using the secondary antiserum technique as described in Materials and Methods. The immune precipitates were electrophoresed in 10% polyacrylamide slab gels containing 8 M urea for two times the time required for the dye front to reach the end of the gel. The sample wells contained immune precipitates obtained with: (A) antiserum to gp69/71; (B) antiserum to p12; (C) antiserum to p30.


A
B
C
245,000
90,000
230,000
220,000
75,000
65,000

IV:2.8 Discussion.

IV:2.8.1 The Detection and Properties of MuLV Polyproteins.

The results of the pulse-chase kinetic studies with [^{35}S]-methionine revealed three clearly distinct classes of virus-specific polypeptides in infected cells. *Class (i)* consists of polypeptides which are labeled without a lag during the pulse period and decline in labeling throughout the chase period. Polypeptides exhibiting these labeling kinetics presumably represent primary translation products released directly from ribosomes or, alternatively, very rapidly derived products of processing. *Class (ii)* virus-specific polypeptides are only labeled after a lag, continue to be labeled early into the chase period, and thereafter decline. These labeling characteristics would be expected for intermediates in processing pathways. *Class (iii)* proteins are labeled late in the pulse period and continue to increase in labeling throughout the chase period. The two proteins in this class co-migrated with the viral proteins p30 and p12 which are end products of protein processing.

Included in the first kinetic class of polypeptides described above are two very large polypeptides which were determined to have molecular weights of approximately 220,000 and 230,000. A third larger protein (approximately 245,000 daltons) was shown to be labeled only after a significant lag period (Figure IV.9). These high molecular weight polypeptides are larger than any RNA tumor virus-specific polypeptides previously reported and are sufficiently large to contain all of the proteins which have been proven to be

encoded by the MuLV genome. To investigate this possiblity, monospecific antisera to three unique virus proteins were employed. Antisera to p12 and p30, both of which are products of the gag gene coding for the internal group specific antigens of the virus (16), precipitated all three large polypeptides as well as a number of the smaller proteins. Antiserum against the envelope glycoprotein gp69/71 precipitated the largest of the polypeptides (245,000 daltons) and two smaller polypeptides.

The significance of the existence of three polyproteins of similar size and the failure of anti-gp69/71 serum to precipitate the polyproteins of 220,000 and 230,000 daltons is not clear. The differences in estimated size of the polyproteins are insufficient to allow for the presence of gp69/71 in only the 245,000 dalton protein; furthermore, the labeling studies imply that the 245,000 dalton polyprotein probably derives from the smaller proteins by a post-synthetic modification (Figure IV.9). A more likely explanation, which is consistent with a number of otherwise anomalous observations, is that the anti-gp69/71 serum is directed primarily against the carbohydrate portion of the glycoprotein and the 220,000 and 230,000 dalton proteins lack the carbohydrate residues required for antigenicity. If this is the case, the lag in the labeling of the largest polyprotein would be the result of a relatively slow glycosylation of the smaller polyproteins which confers gp69/71 antigenicity on the polyprotein. Strong evidence supporting this interpretation is in fact available. Naso et al. (140) have described two 90,000 dalton precursors to gp69/71 in Rauscher leukemia virus-infected cells. These proteins

are similar to the gp69/71 precursors described in this study (see Figure IV.11A) and are known to be glycosylated. In experiments to be presented in the next section, it is shown that these proteins are labeled with a lag phase characteristic of intermediates in viral protein processing (see Figure IV.7). However, the anti-gp69/71 serum does not precipitate the more rapidly labeled unglycosylated precursors of the 90,000 dalton glycoproteins which must exist in the cell in appreciable amounts. For these reasons it is concluded that the 220,000-245,000 dalton proteins are all very likely related and contain the polypeptide sequences of the *gag* gene proteins p30 and p12 as well as the envelope glycoprotein gp69/71. This latter conclusion is most clearly established for the 245,000 dalton polyprotein.

During the course of this work, there was no evidence for any high molecular weight polypeptides in murine leukemia virus-infected cells which contained viral antigens in addition to p30. However, a very recent report indicates that the approximately 200,000 dalton protein ("Pr la+b") of Arlinghaus and co-workers can be precipitated by antiserum to both p30 and reverse transcriptase (5). Although no data was obtained concerning the presence of the viral polymerase in the large polyproteins described here, recent studies elucidating the genetic structure of avian leukosis virus indicate that the viral genes are in the order 5'-*gag*-*pol*-*env*-3' (64,115) where the *gag* gene codes for the major internal proteins of the virion, the *pol* gene codes for the viral DNA polymerase and the *env* gene codes for the envelope glycoprotein (16). If this arrangement holds true for MuLV, then the precipitation of the 245,000 dalton polyprotein

with antisera directed against gag gene products p12 and p30 as well as env gene product gp69/71 would indicate the presence of the viral polymerase in the polyprotein. Indeed, careful comparison of these results with those of Arlinghaus and co-workers (5) suggests that their Pr 1a+b are probably identical to the 220,000 and 230,000 dalton polyproteins. Their failure to precipitate Pr 1a+b with anti-gp69/71 serum (140) may be explained by a carbohydrate specificity of this antiserum as described above; these workers apparently used the same antiserum from the same source as that employed in these studies. However, the possibility cannot be excluded that Pr 1a+b contains only the gag and pol polypeptide sequences, whereas the largest polyprotein contains gag, pol and env.

Naso et al. (139) have reported the detection of a number of major MuLV-specific polypeptides of high molecular weight which were apparently converted to the viral p30 in Rauscher leukemia virus infected cells. These workers postulated a sequential cleavage mechanism leading to p30 in which a large precursor polypeptide reported to be 140,000 daltons was cleaved into intermediate sized molecules which were subsequently cleaved to the viral protein. However, many of the highly labeled proteins seen in these early studies were not detected in their later papers (4,5,140) and therefore the earlier kinetic analysis can no longer be accepted. Indeed, the results presented here do not support the hypothesis that the cellular p30 primarily derives from precursors larger than 100,000 daltons. The 220,000-245,000 dalton polyproteins, which are the only proteins larger than 100,000 daltons seen in the immunoprecipitates, contain only a small

proportion of the incorporated [^{35}S]-methionine (Figures IV.6, IV.8 and IV.9) compared with the 65,000 and 75,000 dalton proteins, which, as will be described in a later section, also contain p30 and p12 (also see Figure IV.11). If the polyproteins were obligatory precursors to the lower molecular weight virus-specific proteins they would be expected to turn over relatively rapidly in order to maintain a low steady state level relative to the smaller, but more highly labeled 75,000 and 65,000 dalton proteins. However, the rate of disappearance of the large polyproteins during chase periods is not more rapid than the disappearance of lower molecular weight precursors (Figure IV.7 and IV.9). Furthermore, the labeled 65,000 and 75,000 dalton precursors to p12 and p30 were detected within one minute after the addition of [^{35}S]-L-methionine, and they were labeled at an approximately constant rate between 0-20 minutes (Figure IV.8). It is concluded that the smaller virion proteins are not derived entirely from the large polyproteins.

IV:2.8.2 Implications Concerning Messenger RNAs.

In C-type leukovirus infected cells the major viral-specific RNA associated with polyribosomes is approximately 30-35S; however, substantial amounts of smaller viral mRNAs are detected, the most prominent being a 20S species (76,90,91,180). The synthesis of a 220,000 to 245,000 dalton polypeptide would require a messenger RNA of approximately 2.0-2.5 x 10^6 daltons which would be expected to have a sedimentation coefficient of 30-35S (185). Therefore the large polyproteins described here must be synthesized on the large size class

of viral mRNA; presumably this is the intact 30-35S subunit of the viral genome.

Recent evidence indicates that a number of viral mRNAs possess cryptic internal initiation sites (46,183). In these cases, the large mRNA is fragmented (or else selective transcription occurs) to yield a smaller mRNA derived from the 3' end in which the formerly cryptic initiation site is now able to actively function. Gielkens et al. (166) have recently reported that the 20S viral mRNA in MuLV infected cells synthesizes different proteins than the 30-35S mRNA. Furthermore, a recent study indicates that the 20S viral mRNA in avian sarcoma virus infected cells is the primary site for synthesizing the envelope glycoprotein whereas the gag polypeptides are formed mainly on the 30-35S mRNA.[6]

IV:2.8.3 Role of MuLV Polyproteins.

The role of the large 220,000-245,000 dalton polyproteins in infected cells is not known. Although it is possible that they may represent translation products which have escaped cleavage normally occurring in the nascent state, there is considerable evidence based on cell-free protein synthesis that the gag gene at the 5' end of the 35S viral mRNA is followed by a polypeptide chain termination codon (118, 149,219). Furthermore, infected cells produce much less reverse transcriptase than p30 (147). For these reasons it seems more likely that the polyproteins result from the misreading of a normally recognized termination codon on a large virus-specific mRNA. Conceivably,

---

[6]A. Smith, personal communication.

they may provide a mechanism for unequal synthesis of proteins such as the viral polymerase whose representation in cells and virions is much less than its genetic representation in the viral genome. Furthermore, the basic process of polyprotein formation might also be involved in cell transformation by avian sarcoma virus. Since the transforming _sarc_ gene lies at the 3' end of the avian sarcoma virus RNA (114,221), unless a small sarcoma-specific mRNA is produced from the 3' end of the viral RNA, the formation of the _sarc_ encoded polypeptide would very likely require cleavage from a precursor polyprotein. In this context it is important to note that small quantities of an approximately 300,000 dalton polypeptide which precipitates specifically with anti-F-MuLV antiserum and with the monospecific antiserum to purified viral proteins (see Figure IV.9) has been detected in some experiments. Whether the additional polypeptides in this protein are involved in leukemogenesis or in other viral functions (e.g., proteolysis or glycosylation) remains unknown.

IV:3 Post-translational Processing of MuLV-specific Proteins in Eveline II Cells.

IV:3.1 General.

According to the sequential model of MuLV protein synthesis discussed earlier a 200,000 dalton and 90,000 MuLV precursor are derived by nascent cleavage of a translation product of 30-35S viral mRNA (5). The 90,000 dalton protein, which is glycosylated nascently or very soon after nascent cleavage, is processed to the envelope glycoproteins while the 200,000 dalton protein is processed to the viral polymerase

and to the lower molecular weight virion core proteins. Intermediate molecular weight proteins are thought to represent sequential cleavage products in the processing pathway. From the experiments described in the foregoing section, it was concluded that the three large polyproteins detected in Eveline cells apparently contain all of the proteins known to be encoded by the viral genome. However, the results indicated that these large polyproteins are not on a major pathway leading to the virion structural proteins.

This section describes an analysis of the synthesis and degradation of the major lower molecular weight virus-specific proteins in F-MuLV-infected cells. The results indicate that the post-translational processing of MuLV-specific proteins is much more complex than previously envisioned. In particular, the major precursors to the viral core proteins are involved in different processing pathways rather than as sequential intermediates in a single pathway.

IV:3.2 Pulse-Chase Analysis of F-MuLV Protein Synthesis in Eveline II Cells.

Previous studies of murine leukemia virus protein synthesis have reported only limited kinetic data. This is, in part, due to the use of monolayer cultures of infected cells in earlier studies (4,75,213). Detailed pulse-chase experiments on intact cells growing in monolayers require the use of several replicate cultures and it is necessary to carefully control for variations in cellular density. In addition, when high specific activity radioactive amino acids are employed, variations in the amounts of endogenous amino acids place a serious limitation on

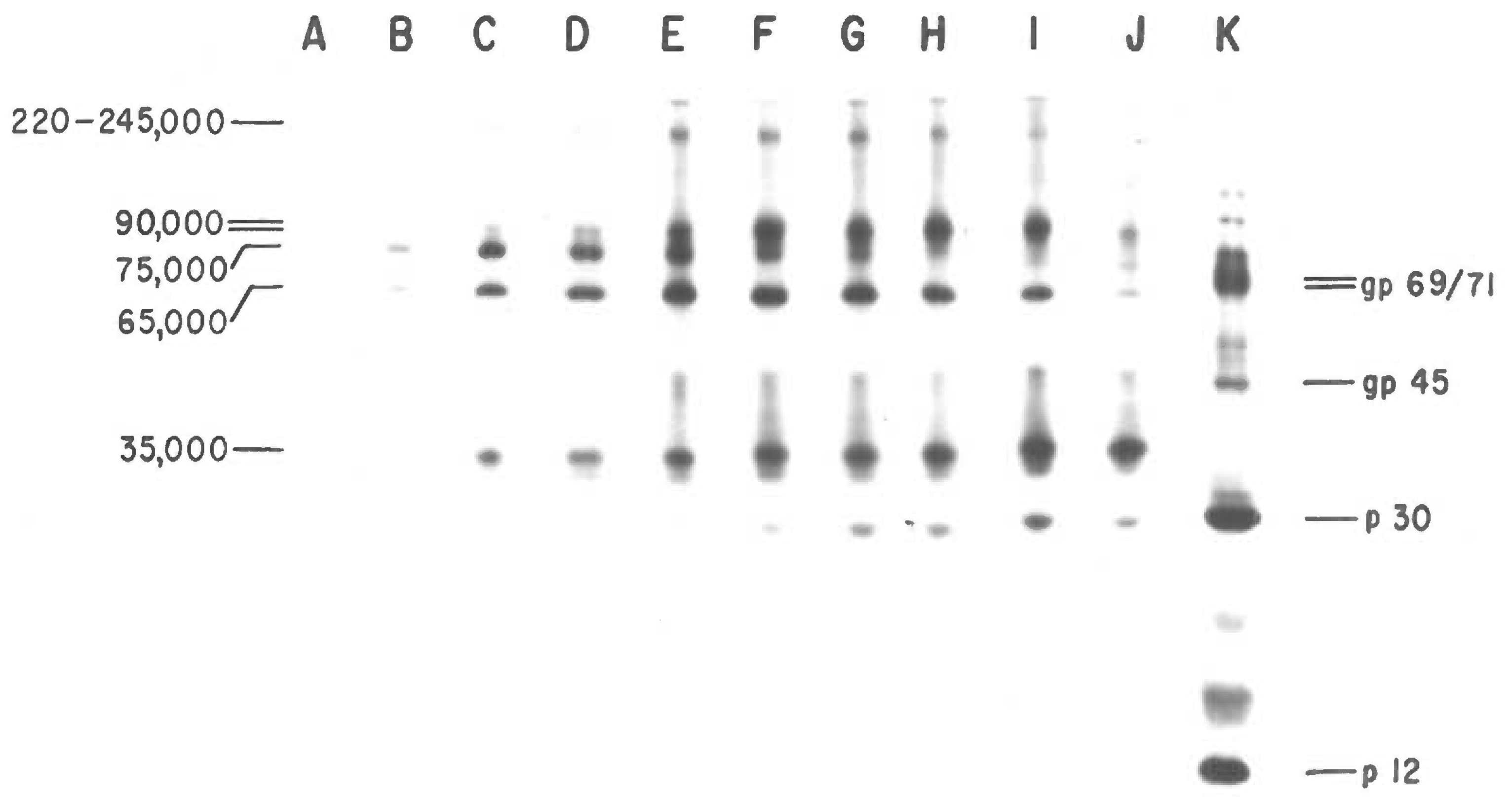
the quantitative information that can be obtained. In some studies this problem has been approached by studying MuLV protein synthesis in cell lysates (75,213), however, this represents a serious perturbation of the system and the results obtained may not accurately reflect the events occurring in intact cells.

The above problems have been largely overcome in the present study by the use of suspension cultures of F-MuLV-infected cells. The use of suspension cultures enables one to obtain repeated, representative samples of the same culture during the course of an experiment. Figure IV.12 shows the results of a pulse-chase experiment in which five samples were taken during a 30 minute pulse period and an additional five samples taken during a two hour chase period. The samples were immune precipitated with antiserum to F-MuLV and the precipitates were analyzed on polyacrylamide slab gels as described in Materials and Methods. At early times in this experiment three major proteins are apparent. These include the 65,000 and 75,000 dalton proteins observed in the pulse-chase experiment described earlier and an approximately 35,000 dalton protein which was not previously observed. Also evident are the minor polyproteins described in the preceding section. The radioactive proteins immune precipitated from the earliest sample (slot A), taken one minute after the addition of radiolabel, are only apparent after longer exposure times and correspond in positions and relative intensities to the radioactive proteins precipitated from the sample taken at 5 minutes (slot B).

In a previous section it was shown that the 65,000 and 75,000 dalton proteins as well as the 220,000 and 230,000 dalton polyproteins

Figure IV.12 Pulse-chase Analysis of F-MuLV-specific Proteins with Antiserum to Whole Disrupted F-MuLV.

Eveline II cells (1.4 x 10^6 cells/ml) were pulse-labeled with 50 μCi/ml of [^{35}S]-L-methionine for 30 minutes. At the conclusion of the pulse period the cells were chased by the addition of unlabeled methionine for two hours. Samples taken at various times were immune precipitated by primary antiserum precipitations and analyzed in 10% polyacrylamide slab gels containing 8 M urea as described in Materials and Methods. Each sample represents an equivalent volume of the culture taken after a: (A) 1 minute pulse; (B) 5 minute pulse; (C) 10 minute pulse; (D) 20 minute pulse; (E) 30 minute pulse; (F) 30 minute pulse and 15 minute chase; (G) 30 minute pulse and 22 minute chase; (H) 30 minute pulse and 30 minute chase; (I) 30 minute pulse and 60 minute chase; (J) 30 minute pulse and 120 minute chase. [^{35}S]-labeled F-MuLV was electrophoresed in slot (K). Approximate molecular weights of the MuLV-specific proteins precipitated by the antiserum are indicated at the left of the autoradiogram.



A
B
C
D
E
F
G
H
I
J
K
220-245,000
90,000
75,000
65,000
35,000
gp 69/71
gp 45
p 30
p 12

are labeled in an approximately linear fashion through 30 minute labeling periods. The detection of these proteins after only one minute of labeling and their linear labeling kinetics suggest that they are primary translation products or very rapidly derived intermediates in viral protein processing, and are not related as a precursor to its products. The 35,000 dalton protein is also detectable after only one minute and continues to increase in labeling during the pulse period and much of the subsequent chase period, suggesting that it may be derived both as a primary translation product and as a processing intermediate. As in the earlier pulse-chase experiment (Figure IV.6), proteins migrating with molecular weights of approximately 90,000 daltons are observed at later pulse times (slots D and E). It is noted that none of the proteins detected during the pulse period correspond to major virion proteins (slot K).

During the chase period (slots F-J) a number of striking changes occur in the virus-specific proteins. The 65,000 and 75,000 dalton proteins begin to decrease in labeling at the beginning of the chase period. The disappearance of the 75,000 dalton protein is more rapid in this experiment than the disappearance of the 65,000 dalton component and is nearly absent 30 minutes into the chase period (slot H), whereas the 65,000 dalton protein is still prominent after a 60 minute chase (slot I). In the previous experiment (Figure IV.6) the disappearance of the 65,000 and 75,000 dalton proteins occurred at approximately the same rate. The 90,000 dalton proteins, which are labeled with a lag, and the early labeled 35,000 dalton component continue to increase in labeling well into the chase period and after a two hour chase the

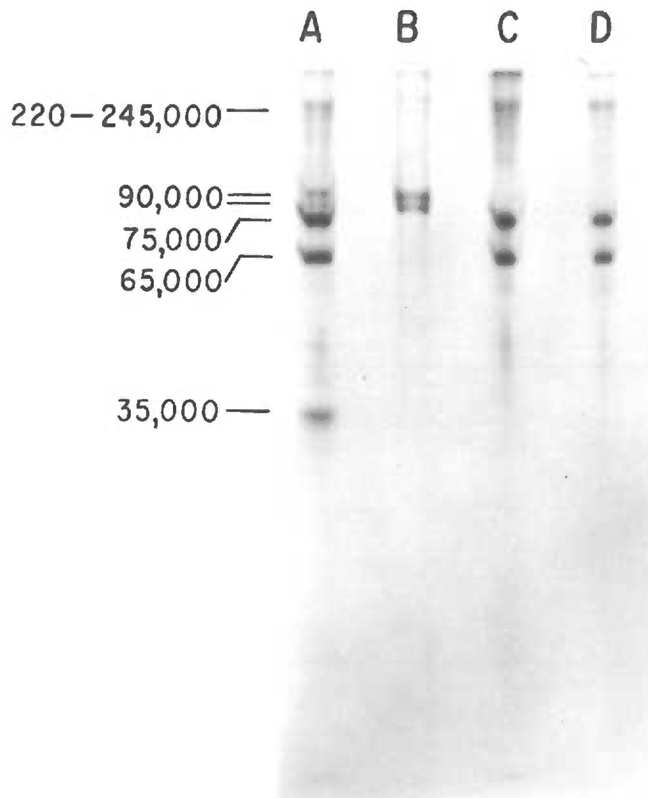
35,000 dalton protein is the predominant protein in the immune precipitate. A lag in labeling during pulse periods and an increase in labeling during the chase period indicates that the 90,000 dalton components are intermediates in viral protein processing. Early in the chase period a protein which co-migrates with the virion protein p30 is detected and it continues to be labeled for at least one hour into the chase period. The decrease in this and other components after two hours of chase presumably represents the export of proteins from the cells as released virions. The failure to detect other [^{35}S]-labeled virion proteins may indicate that they are derived by relatively late processes shortly before release from the cell.

IV:3.3 Identification of the Virus-Specific Proteins Using Monospecific Antisera to Virion Proteins.

Aliquots of the 10 minute pulse-labeled sample in Figure IV.12 (slot C) were precipitated with antisera made to purified gp69/71, p30 and p12 and compared to the precipitate obtained with antiserum to F-MuLV. As shown in Figure IV.13, anti-F-MuLV precipitated two approximately 90,000 dalton proteins and the 75,000, 65,000 and 35,000 dalton proteins as expected (see Figure IV.12, slot C). Antiserum to gp69/71 precipitated the two 90,000 dalton proteins, and antisera to p30 and p12 precipitated the 65,000 and 75,000 dalton proteins. These results are in close agreement with those of Arcement et al. (4) and Famulari et al. (75) who have described similar precursors to p30 and gp69/71 in Rauscher leukemia virus-infected cells. The 35,000 dalton component, which is precipitated by antiserum to F-MuLV, was not precipitated by any of the monospecific antisera. No direct evidence regarding the

Figure IV.13 Precipitation of 10 Minute Pulse-labeled Cells with Monospecific Antisera.

Aliquots of the sample taken at 10 minutes in the experiment shown in Figure IV.12 were precipitated with antisera to whole disrupted virus, gp69/71, p30, and p12 by primary antiserum precipitation. The precipitates were analyzed as in Figure IV.12. The samples applied to the gel were cell extracts precipitated with: (A) antisera to whole disrupted F-MuLV; (B) antiserum to gp69/71; (C) antiserum to p30; (D) antiserum to p12. Approximate molecular weights of the virus-specific proteins are indicated at the left of the autoradiogram.


A
B
C
D
220—245,000
90,000
75,000
65,000
35,000

identity of this component has been obtained. In agreement with the earlier studies, antiserum to gp69/71 precipitated the largest of the polyproteins whereas antisera to p30 and p12 precipitated all three polyproteins (Figure IV.11).

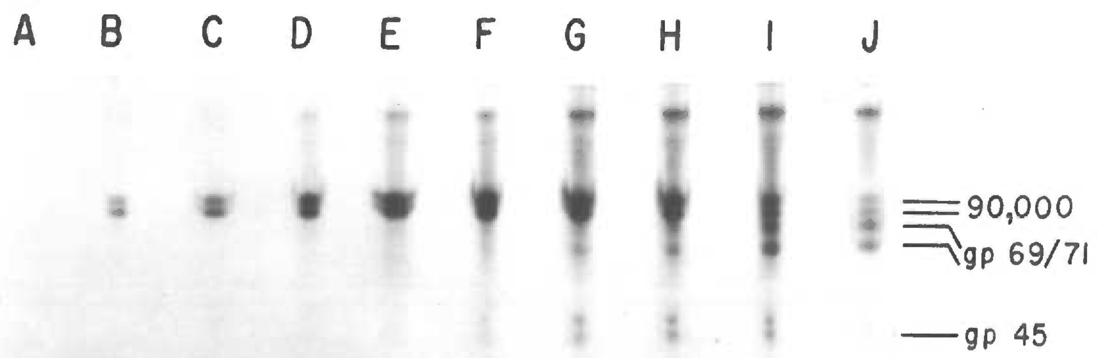
IV:3.4 Pulse-Chase Analysis of gp69/71 Precursors.

In agreement with Figure IV.13 Naso et al. (140) have described two 90,000 dalton glycoprotein precursors to gp69/71 in Rauscher leukemia virus infected cells. However, the unglycosylated forms of these proteins were not detected, and it was, therefore, proposed that the primary precursors to gp69/71 are very rapidly glycosylated either nascently or very soon after release from ribosomes (187).

In order to obtain further information concerning this possibility, aliquots of samples from the experiment in Figure IV.12 were immune precipitated with monospecific antiserum to gp69/71 and analyzed on polyacrylamide slab gels. Figure IV.14 shows the results of this experiment. It is seen that no gp69/71 precursors in addition to the two 90,000 dalton proteins are detected even at the earliest sampling times. The gp69/71 precursors are resolved throughout the experiment and continue to increase in intensity well into the chase period. At late times in the chase period the precursors are processed to the virion proteins gp69/71. This is in contrast to results obtained with Rauscher leukemia virus in which the gp69/71 proteins have been reported to be devoid of methionine (5,140). Clearly this is not the case for F-MuLV envelope glycoproteins. Also apparent in the later chase periods are small amounts of an approximately 45,000-50,000 dalton protein

Figure IV.14 Pulse-Chase Analysis of gp69/71-Specific Proteins in Eveline II Cells.

Aliquots of the samples taken during the experiment shown in Figure IV.12 were precipitated with antiserum to gp69/71 by primary antiserum precipitation. The precipitates were analyzed as in Figure IV.12. The samples represent immune precipitates obtained after a: (A) 1 minute pulse; (B) 5 minute pulse; (C) 10 minute pulse; (D) 20 minute pulse; (E) 30 minute pulse; (F) 30 minute pulse and 15 minute chase; (G) 30 minute pulse and 22 minute chase; (H) 30 minute pulse and 30 minute chase; (I) 30 minute pulse and 60 minute chase; (J) 30 minute pulse and 120 minute chase. The virus-specific proteins are indicated at the right of the gel. $[^{35}S]$-F-MuLV was electrophoresed in another well (not shown) to determine the positions of migration of gp69/71 and gp45.

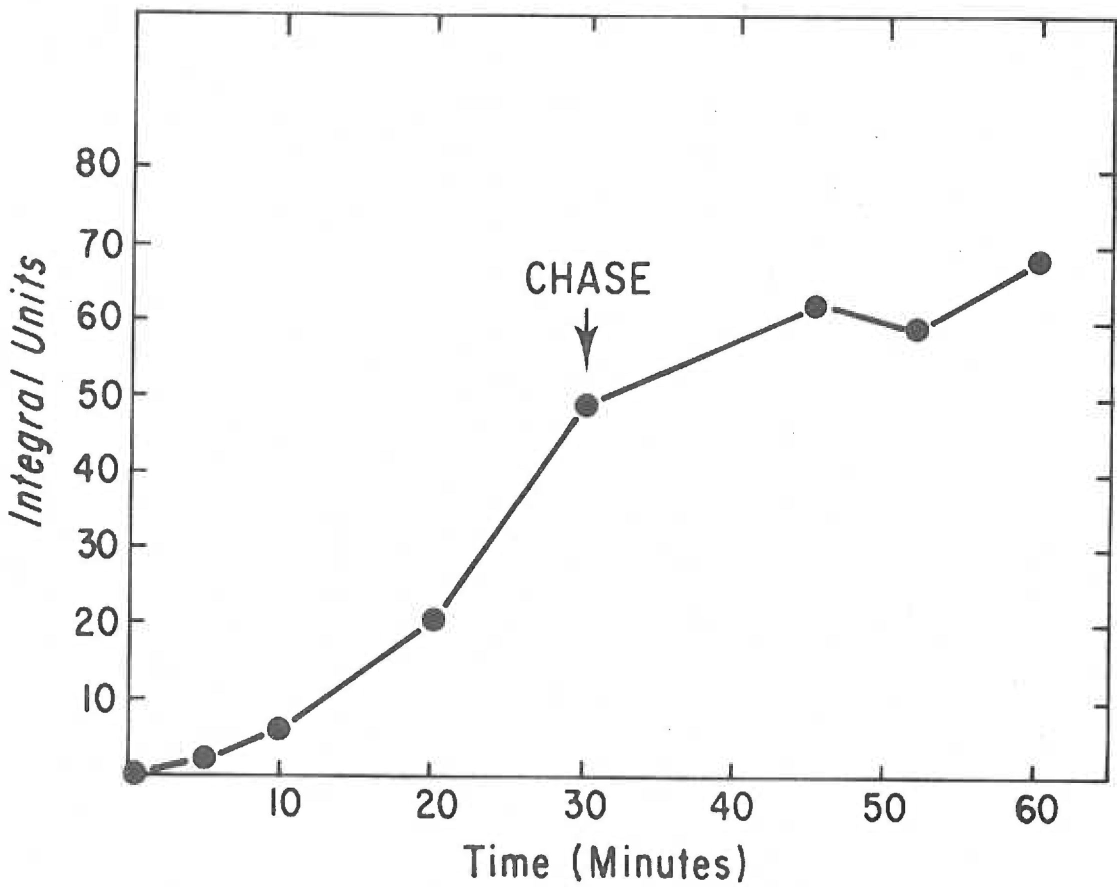

A
B
C
D
E
F
G
H
I
J
90,000
gp 69/71
gp 45

which co-migrates with gp45 present in the virus preparation. This protein has been reported to be a subglycosylated derivative of gp69/71 (132). These results suggest that this glycoprotein is derived, at least in part, during the processing of the glycoprotein precursors rather than by a modification of the envelope glycoprotein in assembled extracellular virions.

To obtain a more quantitative description of the results in Figure IV.14, the gels were subjected to fluorography for varying periods of time and the 90,000 dalton bands were quantitated with an integrating densitometer as described in Materials and Methods. Figure IV.15 shows a plot of the relative integrals of the 90,000 bands up to 30 minutes into the chase period. No attempt was made to separately quantitate the two bands; however, their intensities appear to be very nearly identical throughout the experiment (Figure IV.14). It appears from this data that the gp69/71 precursors are labeled with a lag and increase in labeling in the early chase period. Their labeling kinetics suggest that the 90,000 dalton proteins are intermediates in MuLV processing and imply that their precursor(s), which must exist in the cell in appreciable amounts, are not detected by the antiserum employed in this study. In agreement with the kinetic study on the large polyproteins presented earlier, anti-gp69/71 precipitates small amounts of a single large polyprotein which exhibits a lag in its labeling (see Figure IV.9). As discussed earlier, a possible explanation for these results is that the anti-gp69/71 serum is directed primarily against the carbohydrate portion of the envelope glycoproteins. According to this proposal, the unidentified precursors to the 90,000 dalton

Figure IV.15 Kinetics of Labeling of gp69/71-Specific Proteins in Eveline II Cells.

Fluorographs of the gel shown in Figure IV.14 were quantitated with an integrating densitometer as described in Materials and Methods. The intensity of the 90,000 bands was plotted as a function of time for the first 60 minutes of the experiment. The arrow indicates the beginning of the chase period.


CHASE
Integral Units
80
70
60
50
40
30
20
10
10
20
30
40
50
60
Time (Minutes)

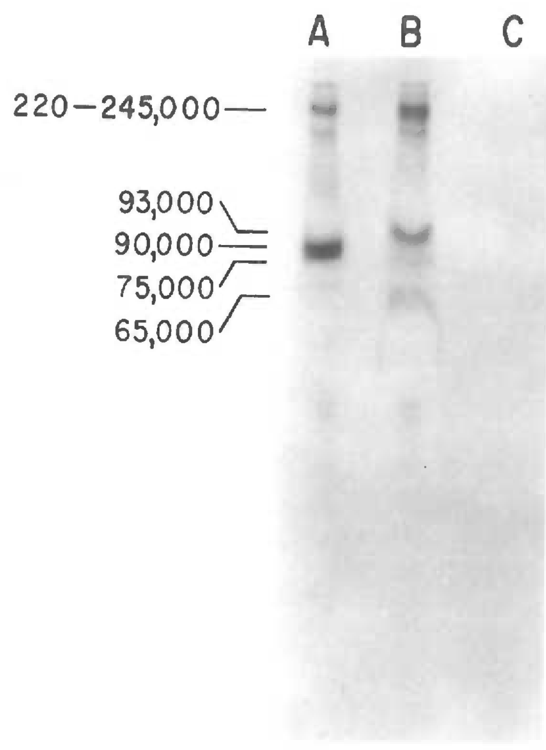
glycoproteins lack the carbohydrate antigens required for immunoprecipitation. A similar phenomenon could account for the lag in the labeling of the large polyprotein.

IV:3.5 Pulse-Chase Analysis of p30 Precursors.

A number of observations suggest that the 90,000 dalton proteins detected in the pulse-chase experiment shown in Figure IV.12 may contain proteins in addition to the gp69/71 precursors. The lag in the labeling of the 90,000 dalton components in the pulse-chase experiment appeared more pronounced in the samples precipitated by anti-F-MuLV serum (Figure IV.12) than in the samples precipitated by anti-gp69/71 (Figure IV.14). In addition the 90,000 dalton bands appear to broaden and migrate more slowly during the chase period suggesting that another late-labeling component may be present. Furthermore, in some monospecific precipitations with antiserum to p30 and p12, small amounts of a protein migrating slightly behind the gp69/71 precursors were detected. To examine the processing of p30 precursors in more detail, a pulse-chase experiment was analyzed using monospecific antiserum to purified p30. As shown in Figure IV.9, presented earlier, a late-labeling protein of approximately 90,000 daltons is precipitated by the anti-p30 serum. In contrast to the gp69/71 precursors, which are detectable after a 10 minute pulse, the approximately 90,000 dalton component precipitated by antiserum to p30 is not detected at this time (also see Figure IV.13). Figure IV.16 shows a comparison of the immune precipitates obtained from a pulse-chased sample using antiserum to gp69/71 and antiserum to p30. Although the two 90,000 dalton precursors to gp69/71 were not clearly resolved

Figure IV.16 Comparison of p30 and gp69/71-Specific Proteins in Pulse-Chased Eveline II Cells.

Eveline II cells which had been pulse-labeled with [^{35}S]-L-methionine for 30 minutes and chased with unlabeled methionine for 22 minutes as described in Figure IV.12 (slot G) were extracted and precipitated with antiserum to gp69/71 and to p30 by secondary antiserum precipitation. The precipitates were analyzed on polyacrylamide slab gels in the presence of 8 M urea as described in Figure IV.12. The gels were electrophoresed for approximately 2 times that required for the dye front to reach the end of the gel. The wells contained immune precipitates obtained with: (A) anti-gp69/71; (B) anti-p30; (C) non-immune goat serum. The approximate molecular weights of the virus-specific proteins are indicated at the left of the autoradiogram.


A
B
C
220—245,000
93,000
90,000
75,000
65,000

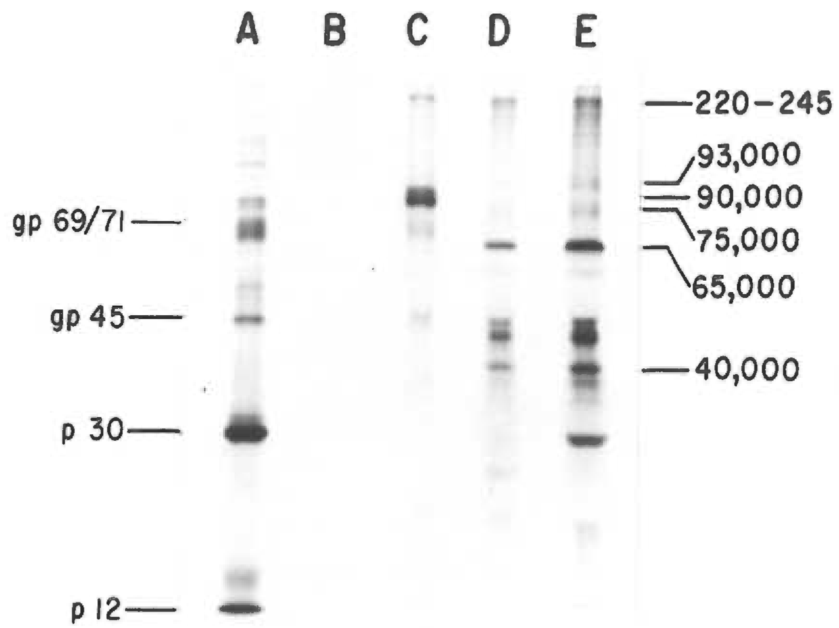
from one another on this gel, it is apparent that the approximately 90,000 dalton p30-specific precursor migrates slightly slower than the gp69/71 precursors. On this basis an approximate molecular weight of 93,000 daltons has been assigned for this protein. Also shown in Figure IV.17 is a secondary antiserum precipitation using non-immune serum illustrating the lack of detectable radioactive proteins in control precipitates. Although the precise precursor-product relationships between the various p30 precursors cannot be unambiguously assigned from the experiment in Figure IV.9, the 93,000 dalton late-labeling component appears to arise at the expense of the 75,000 dalton protein. The results of experiments presented below are consistent with this proposal.

IV:3.6 Alternative Processing Pathways in MuLV Infected Cells.

The results of the above kinetic studies are consistent with the possibility that different pathways of viral protein processing exist in MuLV-infected cells. That these pathways may be functionally distinct is suggested by the observation that different pathways appear to be favored in different growth conditions. Figure IV.17 shows the results of an experiment in which cells growing in the late logarithmic phase were pulse-labeled for one hour and immune precipitated with antisera to gp69/71, p30 and p12. Antiserum to gp69/71 precipitates a high molecular weight protein and the two closely migrating 90,000 dalton gp69/71 precursors. Small amounts of the virion proteins gp69/71 are also observed. Antisera directed against p30 and p12 precipitate the 65,000 dalton protein and two additional proteins of

Figure IV.17 Precipitation of gp69/71, p30 and p12 Specific Proteins from Late Log-Phase Eveline II Cells.

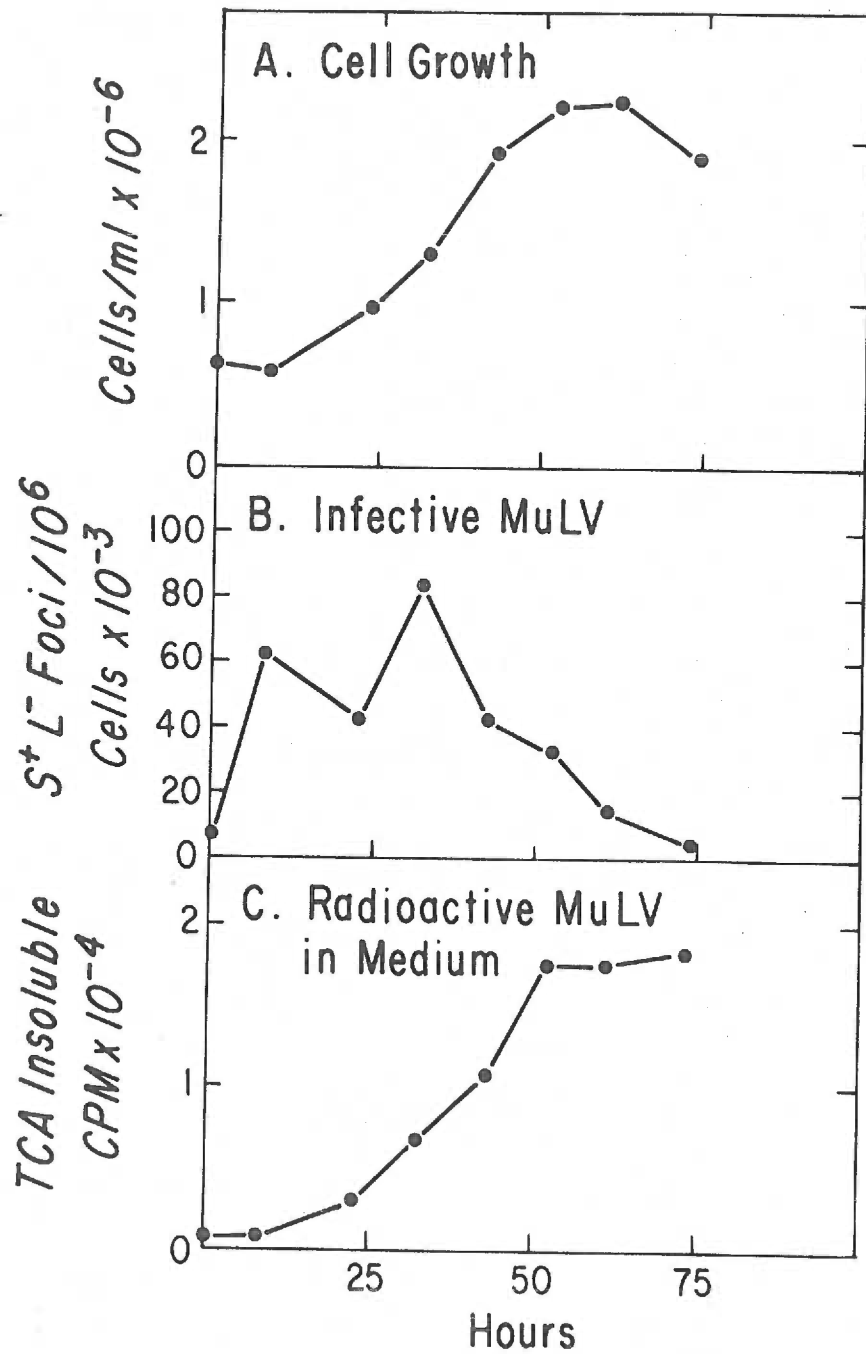
Eveline II cells growing in the late-logarithmic phase (3 x 10^6 cells/ml) were pulse-labeled with 10 μCi/ml of [^{35}S]-L-methionine for one hour. The culture was divided into aliquots, extracted and immune precipitated by primary antisera precipitation with non-immune goat serum and antiserum made to gp69/71, p30 and p12. The immune precipitates were analyzed as in Figure IV.12. The sample wells contained: (A) [^{35}S]-L-methionine-labeled F-MuLV; (B) precipitate obtained with non-immune serum; (C) precipitate obtained with goat antiserum to gp69/71; (D) precipitate obtained with goat antiserum to p12; and (E) precipitate obtained with goat antiserum to p30. The approximate molecular weights of virus-specific proteins are indicated at the right of the autoradiogram.



A
B
C
D
E
gp 69/71
gp 45
p 30
p 12
220-245
93,000
90,000
75,000
65,000
40,000

approximately 50,000 and 40,000 daltons. The 40,000 dalton precursor to both p12 and p30 is of particular importance in that it indicates that the genes encoding these two proteins are adjacent on the viral genome. The 50,000 and 40,000 dalton proteins were not detected in any samples of the pulse-chase analysis using antiserum to p30 (Figure IV.9). Furthermore, the proteins of 75,000 and 93,000 daltons which are precipitated by monospecific antisera to both p30 and p12 are only slightly labeled in this experiment. This result lends further support to the suggestion that the 93,000 dalton protein is derived by a modification of the 75,000 dalton protein. The low amounts of the 75,000 and 93,000 dalton proteins likely reflect a low level of synthesis of the 75,000 dalton protein and consequently a low amount of the 93,000 dalton protein derived from it. It is conceivable that the levels of both the 75,000 and 93,000 dalton components reflect a very rapid turnover of these proteins; however, this seems unlikely considering the stability of the 93,000 dalton component in pulse chase studies (see Figure IV.9). In any event, the viral protein processing in this experiment is clearly different than that observed in the earlier experiments in which the cells were in the mid-logarithmic phase of growth.

Recent studies have indicated that cultured cells infected with MuLV release infectious virions only during periods of active growth and cease virus production in the G_o state of proliferative arrest (148,181). The data in Figure IV.18 suggests that this is also true of Eveline II cells. As shown in Figure IV.18, the release of virus ceases when the cells enter the stationary phase of the cellular growth

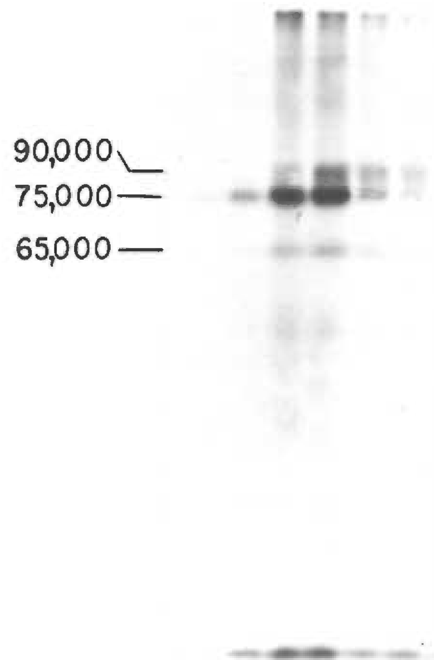


A. Cell Growth
Cells/ml x 10^-6
2
1
0
B. Infective MuLV
S+L- Foci/10^6
Cells x 10^-3
100
80
60
40
20
0
C. Radioactive MuLV
in Medium
TCA Insoluble
CPM x 10^-4
2
1
0
25
50
75
Hours

cycle. Interestingly, the dilution of stationary cells into fresh culture medium is followed within 8 hours by a burst of infectious virus release into the medium. However, this burst of infectivity is not paralleled by a similar burst of radioactive virus release. These results suggest that stationary phase cells, which are not releasing virus, may nevertheless contain appreciable amounts of virus proteins that are released only when the cells are provided fresh medium. It is conceivable that alternate pathways of viral protein processing could provide a mechanism by which the cellular control of virus production is mediated. Figure IV.19 shows the results of a preliminary pulse-chase experiment using stationary phase cells. Clearly, virus-specific proteins continue to be synthesized in stationary phase cells; however, the pattern of protein processing is strikingly different than that observed in logarithmically growing cells (see Figure IV.12). The predominant radioactive components are the 75,000 dalton protein and a late labeling 90,000 dalton protein. The 65,000 dalton protein, which is a major component in logarithmically growing cells, was only slightly labeled in this experiment. The appearance of the prominant late-labeling 90,000 dalton protein is consistent with the proposal that it is derived from the 75,000 dalton protein; however, the contribution of the 90,000 dalton gp69/71 precursors to the late-labeling component is not known. The large polyproteins, which have been detected in all previous experiments, were not apparent in the stationary phase culture suggesting that the formation of these proteins may be of functional significance.

Figure IV.19 Pulse-chase Analysis of F-MuLV-Specific Proteins in Stationary Phase Cells.

An Eveline II cell culture in the stationary phase was pulse labeled for 30 minutes with 50 μCi/ml of [^{35}S]-L-methionine. The culture was chased for an additional hour by the addition of unlabeled methionine. Samples taken at various times were immune precipitated and analyzed on slab gels as described in Figure IV.12. The samples applied to the gels were precipitated from samples taken after a: (A) five minute pulse; (B) 10 minute pulse; (C) 20 minute pulse; (D) 30 minute pulse; (E) 30 minute pulse and 30 minute chase; (F) 30 minute pulse and 60 minute chase. The approximate molecular weights of the MuLV-specific proteins are indicated at the left of the autoradiogram.


A B C D E F
90,000
75,000
65,000

IV:3.7 Discussion.

The results of the studies on MuLV protein processing indicate that the post-translational processing of MuLV-specific proteins is far more complex than has been previously suggested (5). The major envelope glycoprotein precursors detected in these studies are two approximately 90,000 dalton proteins which are probably glycosylated (140). The labeling kinetics of these proteins suggests that an additional non-glycosylated precursor exists in infected cells, but was not detected by the antisera employed in this study.

Major p30 and p12-containing proteins of approximately 93,000, 75,000, 65,000, 50,000 and 40,000 daltons were described. The 75,000 and 65,000 dalton proteins appear to be directly released from the ribosomes and are not intermediate processing products as previously suggested (4,5). It is not known if these proteins derive from alternative cleavage sites on nascent polypeptide chains or from separate virus-specific messenger RNAs in the cell (76,90). The 93,000 dalton protein, which has not been previously described, appears to be derived by a modification (probably glycosylation) of the 75,000 dalton protein and is detectable only after 30 minutes of labeling in mid-logarithmically growing cells. The detection of a 40,000 dalton protein which is precipitated with antisera to p30 and p12 indicates that the genes encoding these two proteins are adjacent on the viral genome. An apparently similar 40,000 dalton precursor of p30 and p12 has recently been described by Barbacid et al. (18).

The experiments using cells in different states of growth provide

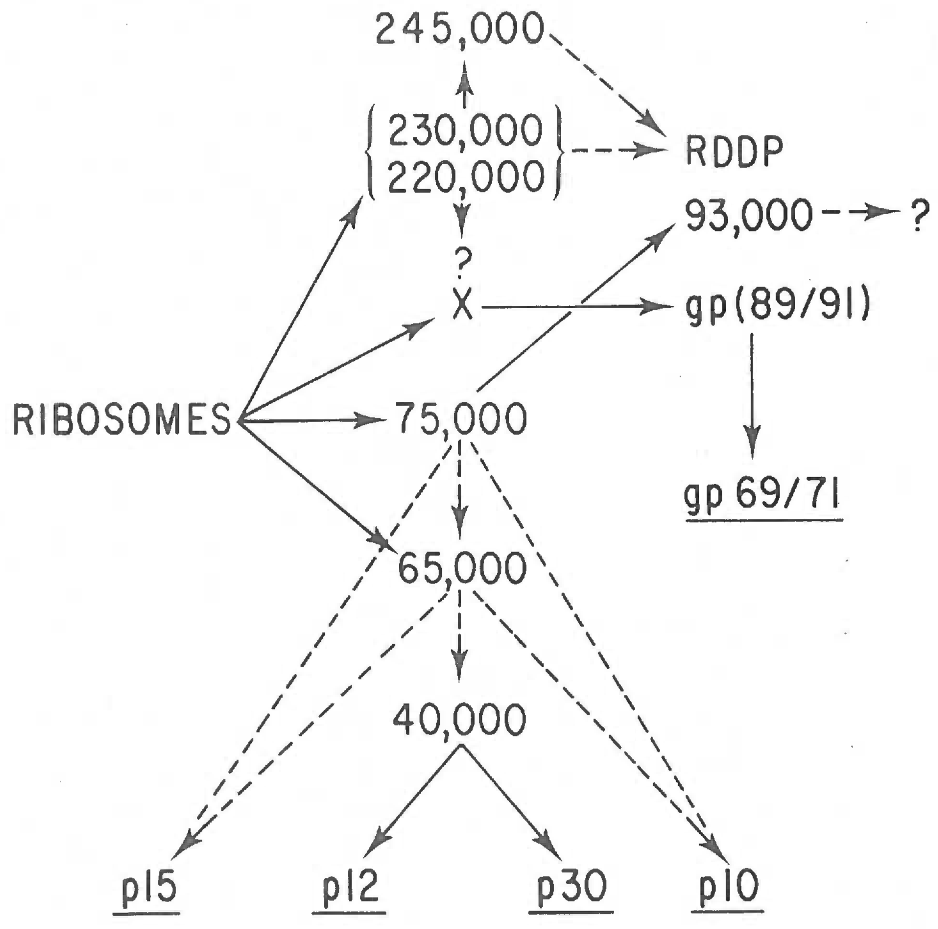
additional evidence that there are different pathways for the formation and processing of MuLV-specific proteins and, furthermore, that different pathways may be favored in different phases of cellular growth. The preliminary evidence on stationary phase cells suggests that the majority of the gag-gene products are shunted towards the 75,000 dalton protein which is subsequently processed to the 93,000 dalton protein. In contrast to all experiments on logarithmically growing cultures, no large polyproteins were detected in this experiment, suggesting a possible function for the polyproteins. One possibility is that these polyproteins provide a source of the reverse transcriptase which is present in low amounts in cells and virions compared to the virion structural proteins (147). These results are quite intriguing considering the absence of infective or radioactive virus release from stationary phase cells (Figure IV.18). The detection of appreciable amounts of the 40,000 and 50,000 dalton proteins and the lack of the 75,000 and 93,000 dalton proteins in late logarithmically growing cells further suggests that processing pathways differ in different phases of cellular growth. Additional studies will be required to more clearly define the cellular growth conditions which favor the various processing pathways of MuLV-specific proteins.

V. SUMMARY AND CONCLUSIONS

A MuLV protein processing map based on the results of this study and those of other workers is shown in Figure V.1. It includes most of the virus-specific proteins which were detected. The main features which require explanation are as follows: (i) It is proposed that ribosomes release several different virus-specific proteins including the 220,000 and 230,000 dalton polyproteins, the 75,000 and 65,000 dalton proteins, and a protein designated X which is a precursor to the two 90,000 dalton glycoprotein intermediates (designated gp(89/91) in Figure V.1) in gp69/71 synthesis. This proposal concerning ribosome release is suggested by the results since the kinetics of labeling data is inconsistent with a simple precursor-product relationship among any of these proteins. (ii) Following their formation, the 220,000 and 230,000 dalton proteins are glycosylated to form the 245,000 dalton polyprotein, but the subsequent fate of these large proteins is unclear. Presumably, they represent a source of the virion reverse transcriptase (RDDP) (5). (iii) The 75,000 dalton protein which precipitates with anti-p12 and anti-p30 sera is likely a precursor of the 93,000 dalton protein; presumably the modification involves glycosylation. (iv) The ultimate fate of the 93,000, 75,000 and 65,000 dalton proteins is unclear. However, they all very likely contain p10, p12, p15 and p30 (18,188); and it is likely that at least the 65,000 dalton protein is a direct precursor to these virion proteins (18). (v) The 40,000 dalton protein which precipitates with antisera against p12 and p30 is

Figure V.1 Processing Scheme for the MuLV-Specific Proteins.

Major precursors and intermediates detected in these experiments and their inter-relationships. The solid arrows denote reactions for which evidence is presented. The dashed arrows represent possible but uncertain pathways. Additional proteins detected in this study are not indicated (e.g., 35,000 daltons, Figure IV.12 and IV.13) because their place and role in this scheme is not clarified. Mature virion proteins are underlined.


245,000
230,000
220,000
RDDP
93,000
?
?
X
gp(89/91)
RIBOSOMES
75,000
gp 69/71
65,000
40,000
p15
p12
p30
p10

seen in late logarithmic phase cells but large amounts of this protein were not detected in other conditions of cellular growth. Although included in the processing map, it may not be an obligatory intermediate in p30 or p12 formation. The same argument applies to an approximately 50,000 dalton protein detected in late logarithmic phase cells which has not been included in the processing map. Small amounts of 40,000 and 50,000 dalton proteins were labeled as intermediates in one pulse-chase experiment (Figure IV.6), however, their identities were not determined.

Although the results of this study suggest that there are probably 4-5 different virus-specific proteins which are released directly from ribosomes (Figure V.1), the data does not indicate whether they are formed on separate mRNAs or whether they are all produced from a single large mRNA. In the latter case, they could be released by proteolytic cleavage at different positions on nascent polypeptide chains or, alternatively, by partial read-through of inefficient termination sites on large mRNAs. Present information, however, suggests that the gag precursors of 75,000 and 65,000 daltons are formed on a 30-35S viral mRNA (91,118) and that the envelope glycoprotein precursors are probably synthesized on a 20S viral mRNA[7] (91). Furthermore, the 220,000-245,000 dalton proteins must be synthesized on the 30-35S viral mRNA. Other observations suggest that the gag gene lies at the 5' end of the 30-35S mRNA and that it is followed by a termination codon (118, 219). This termination codon must be partially suppressed to allow for

---

[7]A. Smith, personal communication.

the production of the large polyproteins containing *gag* antigens.

It seems clear that there is no single pathway for genesis of virus-specific proteins. On the contrary, several proteins released from ribosomes contain p30 and p12 antigens (the 65,000, 75,000, 220,000 and 230,000 dalton proteins) and several probably also contain gp69/71 polypeptides (X in Figure V.1, and the 220,000 and 230,000 dalton proteins). It is not known if these virus-specific proteins are all ultimately processed to virion proteins. Alternatively, they may be involved in regulatory functions or inserted into membranes (182) or simply degraded.

Additional evidence that there are different pathways for formation and processing of MuLV-specific proteins derives from experiments using cells in different states of growth. Preliminary evidence implies that the relative abundance of different virus-specific proteins differs in mid-log-phase cells, late log-phase cells, and stationary phase cells. Apparently, the different pathways of viral gene expression are favored by cells in different stages of the cellular growth cycle.

REFERENCES

1. Abelson, H.T. & Rabstein, L.S. Lymphosarcoma: Virus-induced thymus-independent disease in mice. Cancer Res., 1970. 30, 2213-2222.

2. Andervont, H.B. & Bryan, W.R. Properties of mouse mammary-tumor agent. J. Nat. Cancer Inst., 1944. 5, 143-149.

3. Aoki, T., Old. L.J. & Boyse, E.A. Serological analysis of leukemia antigens of the mouse. Nat. Cancer Inst. Monogr., 1966. 22, 449-457.

4. Arcement, L.J., Karshin, W.L., Naso, R.B., Jamjoom, G. & Arlinghaus, R.B. Biosynthesis of Rauscher leukemia viral proteins: presence of p30 and envelope p15 sequences in precursor polyproteins. Virology, 1976. 69, 763-794.

5. Arlinghaus, R.B., Naso, R.B., Jamjoom, G.A., Arcement, L.J. & Karshin, W.L. Biosynthesis and processing of Rauscher leukemia viral precursor polyproteins. In D. Baltimore, A.S. Huang, and C.F. Fox (Eds.) Animal Virology. ICN-UCLA Symp. Mol. and Cell. Biol., 1976. 4, 689-716.

6. August, J.T., Bolognesi, D.P., Fleissner, E., Gilden R.V. & Nowinski, R.C. A proposed nomenclature for the virion proteins on oncogenic RNA viruses. Virology, 1974. 60, 595-600.

7. Avrameas, S. & Ternynck, T. The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbants. Immunochem., 1969. 6, 53-66.

8. Axelrad, A.A. & Steeves, R.A. Assay for Friend leukemia virus: rapid quantitative method based on enumeration of macroscopic spleen foci in mice. Virology, 1964. 24, 513-518.

9. Bader, J.P. The role of DNA in the synthesis of Rous sarcoma virus. Virology, 1964. 22, 462-468.

10. Bader, J.P. The requirement for DNA synthesis in the growth of Rous-associated virions. Virology, 1965. 26, 253-261.

11. Bader, J.P. Metabolic requirements for infection by Rous sarcoma virus. I. Transient requirement for DNA synthesis. Virology, 1966. 29, 444-451.

12. Bader, J.P. Temperature-dependent transformation of cells infected with a mutant of Bryan Rous sarcoma virus. J. Virol., 1972. 10, 267-276.

13. Bader, J.P. & Steck, T.L. Analysis of the ribonucleic acid of murine leukemia virus. J. Virol., 1969. 4, 454-459.

14. Bader, J.P., Brown, N.R. & Bader, A.V. Characteristics of cores of avian leuko-sarcoma viruses. Virology, 1970. 41, 718-728.

15. Baltimore, D. RNA-dependent DNA polymerase in virions of RNA tumor viruses. Nature, 1970. 226, 1209-1211.

16. Baltimore, D. Tumor viruses: 1974. Cold Spring Harbor Symp. Quant. Biol., 1975. 39, 1187-1200.

17. Baltimore, D., Huang, A.S. & Fox, C.F. (Eds.) Animal Virology, ICN-UCLA Symp. Mol. and Cell. Biol., 1976. 4, 1-787.

18. Barbacid, M., Stephenson, J.R. & Aaronson, S.A. gag Gene of mammalian type-C RNA tumor viruses. Nature (London), 1976. 262, 554-559.

19. Barnes, W.A. & Furth, J. A transmissible leukemia in mice with atypical cells. Am. J. Cancer, 1937. 30, 75-94.

20. Bassin, R.H., Tuttle, N. & Fischinger, P. Isolation of murine sarcoma virus transformed mouse cells which are negative for leukemia virus from agar suspension culture. Int. J. Cancer, 1970. 6, 95-107.

21. Bassin, R.H., Tuttle, N. & Fischinger, P.J. Rapid cell culture assay technique for murine leukaemic viruses. Nature (London), 1971. 229, 564-566.

22. Bassin, R.H., Duran-Troise, G., Gerwin, B.I., Gisselbrecht, S. & Rein, A. Murine sarcoma virus pseudotypes aquire a determinant specifying N or B tropism from leukaemia virus during rescue. Nature, 1975. 256, 223-225.

23. Beard, J.W. Virus of avian myeloblastic leukosis. Poultry Sci., 1956. 35, 203-223.

24. Beard, J.W., Sharp, D.G. & Eckert, E.A. Tumor viruses. Adv. Virus Res., 1955. 3, 149-197.

25. Beard, D., Beaudreau, G.S., Bonar, R.A., Sharp, D. & Beard, J.W. Virus of avian erythroblastosis. III. Antigenic constitution and relation to the agent of avian myeloblastosis. J. Nat. Cancer Inst., 1957. 18, 231-259.

26. Beard, D. & Beard, J.W. Virus of avian erythroblastosis. VI. Further studies on neutralization by antiserum to normal chicken protein. J. Nat. Cancer Inst., 1957. 19, 923-939.

27. Beaudreau, G.S., Sverak, L., Zischka, R. & Beard, J.W. Attachment of ^{14}C-amino acids to BAI strain A (myeloblastosis) avian tumor virus RNA. Natl. Cancer Inst. Monograph, 1964. 17, 791-803.

28. Beemon, K.L., Faras, A.J., Haase, A.T., Duesberg, P.H. & Maisel, J.E. Genomic complexities of murine leukemia and sarcoma, reticuloendotheliosis, and visna viruses. J. Virol., 1976. 17, 525-537.

29. Bernhard, W. Electron microscopy of tumor cells and tumor viruses. A review. Cancer Res., 1958. 18, 491-509.

30. Bernhard, W. The detection and study of tumor viruses with the electron microscope. Cancer Res., 1960. 20, 712-727.

31. Bernhard, W. & Bauer, A. Mise en évidence de corpuscules d'aspect virusal dans des tumeurs mammaires de la souris. Étude au microscope électronique. Compt. Rend. Acad. Sci. (Paris), 1955. 240, 1380-1382.

32. Bernhard, W. & Guérin, M. Présence de particles d'aspect virusal dans les tissus tumor aux de souris atteintes de leucémie spontanée. Compt. Rend. Acad. Sci. (Paris), 1958. 247, 1802-1805.

33. Biquard, J.-M. & Vigier, P. Characteristics of a conditional mutant of Rous sarcoma virus defective in ability to transform cells at high temperature. Virology, 1972. 47, 444-455.

34. Bitte, L. & Kabat, D. Isotopic labeling and analysis of phosphoproteins from mammalian ribosomes. Meth. Enzymol., 1974. 30, 563-590.

35. Bittner, J.J. Some possible effects of nursing on the mammary gland tumor incidence in mice. Science, 1936. 84, 162.

36. Bittner, J.J. Milk-influence of breast tumors in mice. Science, 1942. 95, 462-463.

37. Bittner, J.J. Mammary cancer in fostered and unfostered C3H breeding females and their hybrids. Cancer Res., 1943. 3, 441-447.

38. Boime, I. & Leder, P. Protein synthesis directed by encephalomyocarditis virus mRNA. III. Discrete polypeptides translated from a monocistronic messenger in vitro. Arch. Biochem. Biophys., 1972. 153, 706-713.

39. Bolognesi, D.P., Gelderblom, H., Bauer, H., Mölling, K. & Hüper, G. Polypeptides of avian RNA tumor viruses. V. Analysis of the virus core. Virology, 1972. 47, 567-578.

40. Bolognesi, D., Luftig, R. & Shaper, J.H. Localization of RNA tumor virus polypeptides. I. Isolation of further virus substructures. Virology, 1973. 56, 549-564.

41. Bolognesi, D.P., Hüper, G., Green, R.W. & Graf, T. Biochemical properties of oncornavirus polypeptides. Biochim. Biophys. Acta, 1974. 355, 220-235.

42. Bonar, R.A. & Beard, J.W. Virus of avian myeloblastosis. XII. Chemical constitution. J. Natl. Cancer Inst., 1959. 23, 183-195.

43. Bonner, W.M. & Laskey, R.A. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem., 1974. 46, 83-88.

44. Bukrinskaya, A.G., Miller, G.G., Lebedeva, E.N. & Zhdanov, W.M. Intracellular virus-specific structures and RNAs in oncornavirus-producing human cells. J. Virol., 1974. 13, 478-487.

45. Butterworth, B.E., Hall, L., Stoltzfus, C.M. & Rueckert, R.R. Virus-specific proteins synthesized in encephalomyocarditis virus-infected Hela cells. Proc. Natl. Acad. Sci. USA, 1971. 68, 3083-3087.

46. Cancedda, R., Villa-Komaroff, L., Lodish, H.F. & Schlesinger, M. Initiation sites for translation of Sindbis virus 42S and 26S messenger RNA. Cell, 1971. 6, 215-222.

47. Carnegie, J.W., Deeney, A.O.C., Olson, K.C. & Beaudreau, G.S. An RNA fraction from myeloblastosis virus having properties similar to transfer RNA. Biochim. Biophys. Acta, 1969. 190, 274-284.

48. Cashion, L.M., Joho, R.H., Planitz, M.A., Billeter, M.A. & Weissmann, C. The initiation sites of Rous sarcoma virus RNA-directed DNA synthesis in vitro. In D. Baltimore, A.S. Huang, and C.F. Fox (Eds.) Animal Virology. ICN-UCLA Symp. Mol. and Cell. Biol., 1976. 4, 147-159.

49. Cashman, D. & Pitot, H.C. Characterization of radioactive immunochemically precipitated protein antigens using SDS-polyacrylamide gels. Anal. Biochem., 1971. 44, 559-569.

50. Claude, A., Porter, K.R. & Pickels, E.G. Electron microscope study of chicken tumor cells. Cancer Res., 1947. 7, 421-430.

51. Davis, J., Scherer, M., Tsai, W.P. & Long C. Low-molecular-weight Rauscher leukemia virus protein with preferential binding for single-stranded RNA and DNA. J. Virol., 1976. 18, 709-718.

52. Dawson, P.J., Rose, W.M. & Fieldsteel, A.H. Lymphatic leukaemia in rats and mice innoculated with Friend virus. Brit. J. Cancer, 1966. 20, 114-121.

53. Dawson, P.J. Tacke, R.B. & Fieldsteel, A.H. Relationship between Friend virus and an associated lymphatic leukaemia virus. Brit. J. Cancer, 1968. 22, 569-576.

54. de Harven, E. & Friend, C. Structure of virus particles partially purified from the blood of leukemic mice. Virology, 1964. 23, 119-124.

55. Delius, H., Duesberg, P.H. & Mangel, W.F. Electron microscope measurements of Rous sarcoma virus RNA. Cold Spring Harbor Symp. Quant. Biol., 1975. 39, 835-843.

56. Dina, D., Beemon, K. & Duesberg, P. The 30S Moloney sarcoma virus RNA contains leukemia virus nucleotide sequences. Cell, 1976. 9, 299-309.

57. Dmochowski, L. & Grey, C.E. Subcellular structures of possible viral origin in some mammalian tumors. Ann. N.Y. Acad. Sci., 1957. 68, 559-615.

58. Duesberg, P.H. Physical properties of Rous sarcoma virus RNA. Proc. Natl. Acad. Sci. USA, 1968. 60,1511-1518.

59. Duesberg, P.H. & Robinson, W.S. Nucleic acids and proteins isolated from the Rauscher mouse leukemia virus (MLV). Proc. Natl. Acad. Sci. USA, 1966. 55, 219-227.

60. Duesberg, P.H. & Vogt, P.K. Differences between the ribonucleic acids of transforming and nontransforming avian tumor viruses. Proc. Natl. Acad. Sci. USA, 1970. 67, 1673-1680.

61. Duesberg, P.H., Martin, G.S. & Vogt, P.K. Glycoprotein components of avian and murine RNA tumor viruses. Virology, 1970. 41, 631-646.

62. Duesberg, P.H. & Vogt, P.K. RNA species obtained from clonal lines of avian sarcoma and from avian leukosis virus. Virology, 1973. 54, 207-218.

63. Duesberg, P.H., Kawai, H.S., Wang, L.H., Vogt, P.K., Murphy, H.M. & Hanafusa, H. RNA of replication-defective strains of Rous sarcoma virus. Proc. Natl. Acad. Sci. USA, 1975. 72, 1569-1573.

64. Duesberg, P.H., Wang, L.H., Mellon, P., Mason, W.S. & Vogt, P.K. Towards a complete genetic map of Rous sarcoma virus. In D. Baltimore, A.S. Huang and C.F. Fox (Eds.) Animal Virology. ICN-UCLA Symp. Mol. and Cell. Biol., 1976. 4, 107-125.

65. Duff, R.G. & Vogt, P.K. Characteristics of two new avian tumor virus subgroups. Virology, 1969. 39, 18-30.

66. Dulaney, A.D., Maxey, M., Schillig, M.G. & Goss, M.F. Neoplasms in C3H mice which received AK-leukemic extracts when newborn. Cancer Res., 1957. 17, 809-814.

67. Eiden, J.J., Bolognesi, D.P., Langlois, A.J. & Nichols, J.L. The initial nucleotide sequence of DNA transcribed from avian myeloblastosis virus 70S RNA by RNA-dependent DNA polymerase. Virology, 1975. 65, 163-172.

68. Eisenman, R., Vogt, V.M. & Diggelmann, H. Synthesis of avian RNA tumor virus structural proteins. Cold Spring Harbor Symp. Quant. Biol., 1975. 39, 1067-1075.

69. Elford, W.J. & Andrewes, C.H. Estimation of the size of a fowl tumour virus by filtration through graded membranes. Brit J. Exp. Path., 1935. 16, 61-66.

70. Ellermann, V. & Bang. O. Experimentelle leukämie bei hühnern. Centrabl. f. Bakt., 1908. 46, 595-609.

71. Epstein, M.A. Intracellular identification of the Rous virus. Nature, 1956. 178, 45-46.

72. Epstein, M.A. The identification of the Rous virus; a morphological and biological study. Brit. J. Cancer, 1956. 10, 33-48.

73. Erikson, E. & Erikson, R.L. Isolation of amino acid acceptor RNA from purified avian myeloblastosis virus. J. Mol. Biol., 1970. 52, 387-390.

74. Fairbanks, G., Jr., Levinthal, C. & Reeder, R.H. Analysis of ^{14}C-labeled proteins by disc electrophoresis. Biochem. Biophys. Res. Commun., 1965. 20, 393-399.

75. Famulari, N.G., Buchhagen, D.L., Klenk, H.D. & Fleissner, E. Presence of murine leukemia virus envelope proteins gp70 and p15(E) in a common polyprotein of infected cells. J. Virol., 1976. 20, 501-508.

76. Fan, H. & Baltimore, D. RNA metabolism of murine leukemia virus: detection of virus-specific RNA sequences in infected and uninfected cells and identification of virus-specific messenger RNA. J. Mol. Biol., 1973. 80, 93-117.

77. Feldman, D.G. & Gross, L. Electron-microscopic study of the mouse leukemia virus (Gross) and of tissues from mice with virus-induced leukemia. Cancer Res., 1964. 24, 1760-1783.

78. Feldman, D.G. & Gross, L. Electron microscopic study of the distribution of the mouse leukemia virus (Gross) in genital organs of virus-infected C3Hf mice and of AK mice. Cancer Res., 1967. 27, 1513-1527.

79. Fleissner, E. Chromatographic separation and antigenic analysis of proteins of the oncornaviruses. I. Avian leukemia-sarcoma viruses. J. Virol., 1971. 8, 778-785.

80. Fleissner, E. & Tress. E. Isolation of a ribonucleoprotein structure from oncornaviruses. J. Virol., 1973. 12, 1612-1615.

81. Fleissner, E., Ikeda, H., Tung, J-S., Vitella, E.S., Tress, E., Hardy, W., Stockert, E., Boyse, E.A., Pincus, T. & O'Donnell, P. Characterization of murine leukemia virus-specific proteins. Cold Spring Harbor Symp. Quant. Biol., 1975. 39, 1057-1066.

82. Friend, C. Cell-free transmission in adult Swiss mice of a disease having the character of a leukemia. J. Exp. Med., 1957. 105, 307-318.

83. Friend, C., Scher, W., Holland, J.G. & Sato, T. Hemoglobin synthesis in murine virus-induced leukemic cells in vitro: stimulation of erythroid differentiation by dimethyl sulfoxide. Proc. Natl. Acad. Sci. USA, 1971. 68, 378-382.

84. Fujinami, A. & Inamoto, K. Ueber geschwülste bei japanischen haushühnern insbesondere über einen transplantableu tumor. Zeitschr. f. Krebsforsch., 1914. 14, 94-119.

85. Fujinami, A. & Suzue, K. Contribution to the pathology of tumor growth. Experiments on the growth of chicken sarcoma in the case of heterotransplantation. Trans. Japan. Path. Soc., 1928. 18, 616-622.

86. Furth, J., Siebold, H.R. & Rathbone, R.R. Experimental studies on lymphomatosis of mice. Am. J. Cancer, 1933. 19, 521-604.

87. Furth, J., Buffett, R.F., Banasiewicz-Rodriguez, M. & Upton, A.C. Character of agent inducing leukemia in newborn mice. Proc. Soc. Exp. Biol. and Med., 1956. 93, 165-172.

88. Gallo, R.C., Wong-Staal, F., Reitz, M., Gallagher, R.E., Miller, N. & Gillespie, D.H. Some evidence for infectious type-C virus in humans. In D. Baltimore, A. Huang and C. F. Fox (Eds.) Animal Virology, ICN-UCLA Symp. Mol. and Cell. Biol., 1976. 4, 385-407.

89. Gianni, A.M., Hutton, J.R., Smotkin, D. & Weinberg, R.A. Proviral DNA of Moloney leukemia virus: purification and visualization. Science, 1976. 191, 569-571.

90. Gielkins, A.L.J., Salden, M. & Bloemendal, H. Virus-specific messenger RNA on free and membrane-bound polyribosomes from cells infected with Rauscher leukemia virus. Proc. Natl. Acad. Sci. USA, 1974. 71, 1093-1097.

91. Gielkins, A.L.J., van Zaane, D., Bloemers, H.P.J. & Bloemendal, H. Synthesis of Rauscher murine leukemia virus specific polypeptides in vitro. Proc. Natl. Acad. Sci. USA, 1976. 73, 356-360.

92. Gillespie, D., Marshall, S. & Gallo, R.C. RNA or RNA tumour viruses contains poly A. Nature New Biol., 1972. 236, 227-231.

93. Graffi, A. Chloroleukemia of mice. Ann. N.Y. Acad. Sci., 1957. 68, 540-558.

94. Gross, L. Pathogenic properties and "vertical" transmission of the mouse leukemia agent. Proc. Soc. Exp. Biol. and Med., 1951. 78, 342-348.

95. Gross, L. "Spontaneous" leukemia developing in C3H mice following innoculation in infancy, with AK-leukemia extracts, or AK-embryos. Proc. Soc. Exp. Biol. and Med., 1951. 76, 27-32.

96. Gross, L. Studies on the nature and biological properties of a transmissible agent causing leukemia following innoculation into newborn mice. Ann. N.Y. Acad. Sci., 1957. 68, 501-521.

97. Gross, L. Biological and pathogenic properties of a mouse leukemia virus. Acta Haemat., 1960. 23, 259-275.

98. Gross, L. Oncogenic viruses. London: Pergamon Press, 1970.

99. Gross, L. Oncogenic viruses. London: Pergamon Press, 1970. (pages 105-106).

100. Gross, L. Oncogenic viruses. London: Pergamon Press, 1970. (pages 206-216).

101. Guntaka, R.W., Mahy, B.W.J., Bishop, J.M. & Varmus, H.E. Ethidium-bromide inhibits appearance of closed circular viral DNA and integration of virus-specific DNA in duck cells infected by avian sarcoma virus. Nature, 1975. 253,507-509.

102. Hackett, A. Monitoring for presence of oncogenic virus in tissue culture. In P.F. Kruse, Jr. and M. K. Patterson, Jr. (Eds.) Tissue culture methods and applications. New York: Academic Press, Inc., 1973. (pages 539-548).

103. Halpern, M.S., Bolognesi, D.P. & Lewandowski, L.J. Isolation of the major viral glycoprotein and putative precursor from cells transformed by avian sarcoma viruses. Proc. Natl. Acad. Sci. USA, 1974. 71, 2342-2346.

104. Hanafusa, H. Analysis of the defectiveness of Rous sarcoma virus. III. Determining influence of a new helper virus on the host range and susceptibility to interference of RSV. Virology, 1965. 25, 248-255.

105. Hartley, J.W., Rowe, W.P. & Huebner, R.J. Host range restrictions of murine leukemia viruses in mouse embryo cell cultures. J. Virol., 1970. 5, 221-225.

106. Harvey, J.J. An unidentified virus which causes the rapid production of tumours in mice. Nature, 1964. 204, 1104-1105.

107. Haseltine, W.A. & Baltimore, D. In vitro replication of RNA tumor viruses. In D. Baltimore, A. S. Huang and C. F. Fox (Eds.) Animal Virology. ICN-UCLA Symp. Mol. and Cell. Biol., 1976. 4, 175-213.

108. Hill, M. & Hillova, J. Production virale dans les fibroblasts de poule traités par l'acide desoxyribonucléique de cellules XC de rat transformées par le virus de Rous. Compt. Rend. Acad. Sci., 1971. 272, 3094-3097.

109. Hill, M. & Hillova, J. Recovery of the temperature-sensitive mutant of Rous sarcoma virus from chicken cells exposed to DNA extracted from hamster cells transformed by the mutant. Virology, 1972. 49, 309-313.

110. Holland, J.J. & Kiehn, E.D. Specific cleavage of viral proteins as steps in the synthesis and maturation of enteroviruses. Proc. Natl. Acad. Sci. USA, 1968. 60, 1015-1022.

111. Ihle, J., Hanna, M., Schafer, W., Hunsmann, G., Bolognesi, D. & Huper, G. Polypeptides of mammalian oncornaviruses. III. Localization of p15 and reactivity with natural antibody. Virology, 1975. 63, 60-67.

112. Ikeda, H., Hardy, W., Tress, E. & Fleissner, E. Chromatographic separation and antigenic analysis of proteins of the oncornaviruses. V. Identification of a new viral protein, p15(E). J. Virol., 1975. 16, 53-61.

113. Jacobson, M.F., Asso, J. & Baltimore, D. Further evidence on the formation of poliovirus proteins. J. Mol. Biol., 1970. 49, 657-669.

114. Joho, R.H., Billeter, M.A. & Weissmann, C. Mapping of biological functions on RNA of avian tumor viruses: location of regions required for transformation and determination of host range. Proc. Natl. Acad. Sci. USA, 1975. 72, 4772-4776.

115. Joho, R.H., Stoll, E., Friis, R.R., Billeter, M.A. & Weissmann, C. A partial genetic map of Rous sarcoma virus RNA: location of polymerase, envelope and transformation markers. In D. Baltimore, A. S. Huang, and C. F. Fox (Eds.) Animal Virology. ICN-UCLA Symp. Mol. and Cell. Biol., 1976. 4, 127-145.

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

117. Kerr, I.M., Brown, R.E. & Tovell, D.R. Characterization of the polypeptides formed in response to encephalomyocarditis virus ribonucleic acid in a cell-free system from mouse ascites tumor cells. J. Virol., 1972. 10, 73-81.

118. Kerr, I.M., Olshevsky, V., Lodish, H.F. & Baltimore, D. Translation of murine leukemia virus RNA in cell-free systems from animal cells. J. Virol., 1976. 18, 627-635.

119. Kirsten, W.H. & Mayer, L.A. Morphological responses to a murine erythroblastosis virus. J. Nat. Cancer Inst., 1967. 39, 311-336.

120. Lacassagne, A. & Nyka, W. Sur les conditions de sterilisation des virus par les rayous X: le virus vaccinal. Compt. Rend. Soc. Biol., 1938. 128, 1038-1040.

121. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), 1970. 227, 680-685.

122. Lai, M. M-C., Duesberg, P.H., Horst, J. & Vogt, P.K. Avian tumor virus RNA: a comparison of three sarcoma viruses and their transformation-defective derivatives by oligonucleotide fingerprinting and DNA-RNA hybridization. Proc. Natl. Acad. Sci. USA, 1973. 70, 2266-2270.

123. Law, L.W., Dunn, T.B. & Boyle, P.J. Neoplasms in the C3H strain and in F_1 hybrid mice of two crosses following introduction of extracts and filtrates of leukemic tissues. J. Nat. Cancer Inst., 1955. 16, 495-539.

124. Levinson, W., Bishop, J.M., Quintrell, N. & Jackson, J. Presence of DNA in Rous sarcoma virus. Nature (London), 1970. 227, 1023-1025.

125. Lewandowski, L.J., Smith, R.E., Bolognesi, D.P. & Halpern, M.S. Viral glycoprotein synthesis under conditions of glucosamine blocks in cells transformed by avian sarcoma viruses. Virology, 1975. 66, 347-355.

126. Linial, M. & Mason, W.S. Characterization of two conditional early mutants of Rous sarcoma virus. Virology, 1973. 53, 258-273.

127. Lowry, O.H., Rosenbrough, J.T., Farr, A.L. & Randall, R.J. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 1951. 193, 265-275.

128. Luftig, R.B., McMillan, P.M. & Bolognesi, D.P. An ultrastructural study of C-type virion assembly in mouse cells. Cancer Res., 1974. 34, 3303-3310.

129. MacDowell, E.C. & Richter, M.N. Mouse leukemia. IX. The role of heredity in spontaneous cases. Arch. Path., 1935. 20, 709-724.

130. Maisel, J., Klement, V., Lai, M. M-C, Ostertag, W. & Duesberg, P. Ribonucleic acid components of murine sarcoma and leukemia viruses. Proc. Natl. Acad. Sci. USA, 1973. 70, 3536-3540.

131. Maisel, J., Scolnick, E.M. & Duesberg, P.H. Base sequence differences between the RNA components of Harvey sarcoma virus. J. Virol., 1975. 16, 749-753.

132. Marquardt, H. & Oroszlan, S. The major viral envelope glycoproteins of Rauscher murine leukemia virus: isolation and chemical characterization. Fed. Proc., 1976. 35, 1610 (Abstract).

133. Mason, W.S., Friis, R.R., Linial, M. & Vogt. P.K. Determination of the defective function in two mutants of Rous sarcoma virus. Virology, 1974. 61, 559-574.

134. Moelling, K. Further characterization of the Friend mouse leukemia virus reverse transcriptase-RNase H complex. J. Virol., 1976. 18, 418-425.

135. Moloney, J.B. Biological studies on a lymphoid leukemia virus extracted from sarcoma S.37. I. Origin and introductory investigations. J. Nat. Cancer Inst., 1960. 24, 933-951.

136. Moloney, J.B. A virus-induced rhabdomyosarcoma of mice. Nat. Cancer Inst. Monogr., 1966. 22, 139-142.

137. Morser, M.J., Kennedy, S.I.T., & Burke, D.C. Virus-specified polypeptides in cells infected with Semiliki forest virus. J. Gen. Virol., 1973. 21, 19-29.

138. Naso, R.B., Arcement, L.J., Wood, T.G., Saunders, T.E. & Arlinghaus, R.B. The cell-free translation of Rauscher leukemia virus RNA into high molecular weight polypeptides. Biochim. Biophys. Acta, 1975. 383, 195-206.

139. Naso, R.B., Arcement, L.J. & Arlinghaus, R.B. Biosynthesis of Rauscher leukemia viral proteins. Cell, 1975. 4, 31-36.

140. Naso, R.B., Arcement, L.J., Karshin, W.L., Jamjoom, G.A. & Arlinghaus, R.B. A fucose-deficient glycoprotein precursor to Rauscher leukemia virus gp69/71. Proc. Natl. Acad. Sci. USA, 1976. 73, 2326-2330.

141. Nermut, M.V., Hermann, F. & Schafer, W. Properties of mouse leukemia, viruses. III. Electron microscopic appearance as revealed after conventional preparation techniques as well as freeze-drying and freeze-etching. Virology, 1972. 49, 345-358.

142. Nowinski, R.C., Fleissner, E., Sarkar, N.H. & Aoki, T. Chromatographic separation and antigenic analysis of proteins of the oncornaviruses. II. Mammalian leukemia-sarcoma viruses. J. Virol., 1972. 9, 359-366.

143. Old, L.J., Boyse, E.A. & Stockert, E. Typing of mouse leukemias by serological methods. Nature, 1964. 201, 777-779.

144. Oskarsson, M.K., Robey, W.G., Harris, C.L., Fischinger, P.J., Haapala, D.K. & Vande Wonde, G.F. A p60 polypeptide in the feline leukemia virus pseudotype of Moloney sarcoma virus with murine leukemia virus p30 antigenic determinants. Proc. Natl. Acad. Sci. USA, 1975. 72, 2380-2384.

145. Ostertag, W., Melderis, H., Steinheider, G., Kluge, N. & Dube, S. Synthesis of mouse haemoglobin and globin mRNA in leukemic cell cultures. Nature (London), 1972. 239, 231-234.

146. Panem, S. Spontaneous type C virus expression in human embryonic lung cells (HEL-12): Comparison of cloned and mixed cell populations. In D. Baltimore, A. Huang and C. F. Fox (Eds.) Animal Virology, ICN-UCLA Symp. Mol. and Cell. Biol., 1976. 4, 409-417.

147. Panet, A., Baltimore, D. & Hanafusa, T. Quantitation of avian RNA tumor virus reverse transcriptase by radioimmunoassay. J. Virol., 1975. 16, 146-152.

148. Paskind, M.P., Weinberg, R.A. & Baltimore, D. Dependence of Moloney murine leukemia virus production on cell growth. Virology, 1975. 67, 242-248.

149. Pawson, T., Martin, G.S. & Smith, A.E. Cell-free translation of virion RNA from nondefective and transformation-defective Rous sarcoma viruses. J. Virol., 1976. 19, 950-967.

150. Peebles, P.T., Scolnick, E.M. & Howk, R.S. Increased sarcoma virus RNA in cells transformed by leukemia viruses: model for leukemogenesis. Science, 1976. 192, 1143-1145.

151. Quigley, J.P., Rifkin, D.B. & Reich, E. Phospholipid composition of Rous sarcoma virus host cell membranes and other enveloped RNA viruses. Virology, 1971. 46, 106-116.

152. Rask-Nielsen, H.C. & Rask-Nielson, R. Further studies on a transmissible myeloid leukosis in white mice. II. Acta path. and Microbiol. Scand., 1938. 15, 169-175.

153. Rauscher, F.J. A virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. J. Nat. Cancer Inst., 1962. 29, 515-543.

154. Rauscher, F.J. & Allen, B.V. Growth curve of a murine leukemia virus in mice. J. Natl. Cancer Inst., 1964. 32, 269-275.

155. Reid, M.S. & Bieleski, R.L. A simple apparatus for vertical flat-sheet polyacrylamide gel electrophoresis. Anal. Biochem., 1968. 22, 374-381.

156. Rein, A., Kashmiri, S.V.S., Bassin, R.H., Gerwin, B.I. & Duran-Troise, G. Studies on the mechanism of FV-1 restriction: properties of determinants specifying viral tropism. In D. Baltimore, A. S. Huang and C. F. Fox (Eds.) Animal Virology. ICN-UCLA Symp. Mol. and Cell. Biol., 1976. 4, 269-282.

157. Richter, M.N. & MacDowell, E.C. Studies on mouse leukemia. VII. The relation of cell death to the potency of innoculated cell suspensions. J. Exp. Med., 1933. 57, 1-20.

158. Rifkin, D.B. & Compans, R.W. Identification of the spike proteins of Rous sarcoma virus. Virology, 1971. 46, 485-489.

159. Riman, J. & Beaudreau, G.S. Viral DNA-dependent DNA polymerase and the properties of thymidine-labeled material in virions of an oncogenic virus. Nature, 1970. 228, 427-430.

160. Robinson, W.S., Pitkanen, A. & Rubin, H. The nucleic acid of the Bryan strain of Rous sarcoma virus: purification of the virus and isolation of the nucleic acid. Proc. Natl. Acad. Sci. USA, 1965. 54, 137-144.

161. Robinson, W.S., Robinson, H.L. & Duesberg, P.H. Tumor virus RNAs. Proc. Natl. Acad. Sci. USA, 1967. 58, 825-834.

162. Rouminantzeff, M., Summers, D.F. & Maizel, J.V., Jr. Comparison of polysomal structures of uninfected and poliovirus infected HeLa cells. Virology, 1971. 44, 239-248.

163. Rous, P. A transmissible avian neoplasm (sarcoma of the common fowl). J. Exp. Med., 1910. 12, 696-705.

164. Rous, P. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. J. Exp. Med., 1911. 13, 397-411.

165. Rous, P. On certain spontaneous chicken tumors as manifestations of a single disease. I. Spindle-celled sarcomata rifted with blood sinuses. J. Exp. Med., 1914. 19, 570-576.

166. Rous, P. & Lange, L.B. The characters of a third transplantable chicken tumor due to a filterable cause. A source of intra-canicular pattern. J. Exp. Med., 1913. 18, 651-664.

167. Sarkar, N.H., Moore, D.H. & Nowinski, R.C. Symmetry of the nucleocapsid of the oncornaviruses. In P. Emmelot and P. Bentvelzen (Eds.) RNA tumor viruses and host genome in oncogenesis. Amsterdam: North Holland, 1972. pp. 71-79.

168. Sampson, A.C.R. & Fox, C.F. Precursor protein for Newcastle disease virus. J. Virol., 1973. 12, 579-587.

169. Sawyer, R.C. & Dahlberg, J.E. Small RNAs of Rous sarcoma virus: characterization by two-dimensional polyacrylamide gel electrophoresis and fingerprint analysis. J. Virol., 1973. 12, 1226-1237.

170. Sawyer, R.C., Harada, F. & Dahlberg, J.E. Virion-associated RNA primer for Rous sarcoma virus DNA synthesis: Isolation from uninfected cells. J. Virol., 1974. 13, 1302-1311.

171. Schafer, W. & Seifert, E. Production of a potent complement-fixing murine leukemia virus antiserum from the rabbit and its reactions with various types of tissue culture cells. Virology, 1968. 35, 323-328.

172. Schaffner, W. & Weissmann, C. A rapid, sensitive and specific method for the determination of protein in dilute solution. Anal. Biochem., 1973. 56, 502-514.

173. Scheid, A. & Choppin, P.W. Identification of biological activities of paramyxovirus glycoproteins. Activation of cell fusion, hemolysis, and infectivity by proteolytic cleavage of an inactive precursor protein of Sendai virus. Virology, 1974. 57, 475-490.

174. Schlesinger, M.J. & Schlesinger, S. Large-molecular-weight precursors of Sindbis virus proteins. J. Virol., 1973. 11, 1013-1016.

175. Scolnick, E.M. & Parks, W.P. Harvey sarcoma virus: a second murine type C sarcoma virus with rat genetic information. J. Virol., 1974. 13, 1211-1219.

176. Scolnick, E.M., Goldberg, R.J. & Parks, W.P. A biochemical and genetic analysis of mammalian RNA-containing sarcoma viruses. Cold Spring Harbor Symp. Quant. Biol., 1975. 39, 885-895.

177. Scolnick, E.M., Howk, R.S., Anisowicz, A., Peebles, P., Scher, C. & Parks, W. Separation of sarcoma virus-specific and leukemia virus-specific genetic sequences of Moloney sarcoma virus. Proc. Natl. Acad. Sci. USA, 1975. 72, 4650-4654.

178. Sen, A., Sherr, C.J. & Todaro, G.J. Specific binding of the type C viral core protein p12 with purified viral RNA. Cell, 1976. 7, 21-32.

179. Shanmugan, G., Vecchio, G., Attardi, D. & Green, M. Immunological studies on viral polypeptide synthesis in cells replicating murine sarcoma-leukemia virus. J. Virol., 1972. 10, 447-455.

180. Shanmugan, G., Bhaduri, S. & Green, M. The virus-specific RNA species in free and membrane-bound polyribosomes of transformed cells replicating murine sarcoma-leukemia viruses. Biochem. Biophys. Res. Commun., 1974. 56, 697-702.

181. Sherton, C.C., Evans, L.H., Polonoff, E. & Kabat. D. Relationship of Friend murine leukemia virus production to growth and hemoglobin synthesis in cultured erythroleukemia cells. J. Virol., 1976. 19, 118-125.

182. Smart, J.E. & Hogg, N. Alteration in membrane glycoproteins after type-C virus infection of murine fibroblasts. Nature, 1976. 261, 314-316.

183. Smith, A.E., Bayley, S.T., Mangel, W.F., Shure, H., Wheeler, T. & Kamen, K.I. Characterization and translation of polyoma virus messenger RNAs. In Proceedings of the 10th FEBS meeting Amsterdam: North Holland Publishing Co., 1975. pp. 151-164.

184. Smotkin, D., Gianni, A.M., Yoshimura, F. & Weinberg, R.A. Transfection of Moloney MuLV DNA. In D. Baltimore, A. S. Huang and C. F. Fox (Eds.) Animal Virology. ICN-UCLA Symp. Mol. and Cell. Biol., 1976. 4, 237-242.

185. Spirin, A.S. Some problems concerning macromolecular structure of RNA. Progr. Nucl. Acid. Res., 1963. 1, 301-345.

186. Staff of Roscoe B. Jackson Memorial Laboratory, per C. C. Little, Director. The existence of non-chromosomal influence in the incidence of mammary tumors in mice. Science, 1933. 78, 465-466.

187. Stephenson, J.R., Vilsnack, R.E. & Aaronson, S.A. Radioimmunoassay for avian C-type virus group specific antigen: detection in normal and virus-transformed cells. J. Virol., 1973. 11, 893-899.

188. Stephenson, J.R., Tronick, S.R. & Aaronson, S.A. Murine leukemia virus mutants with temperature-sensitive defects in precursor polypeptide cleavage. Cell, 1975. 6, 543-548.

189. Stewart, S.E. Neoplasms in mice innoculated with cell-free extracts or filtrates of leukemic mouse tissues. I. Neoplasms of the parotid and adrenal glands. J. Nat. Cancer Inst., 1955. 15, 1391-1415.

190. Stewart, S.E. Neoplasms in mice innoculated with cell-free extracts or filtrates of leukemic mouse tissues. II. Leukemia in hybrid mice produced by cell-free filtrates. J. Nat. Cancer Inst., 1955. 16, 41-53.

191. Stewart, M.L. & Maizel, J.V. Friend leukemia and endogenous viruses. I. Production of Friend and endogenous C-type particles by mouse cells cultured in vitro. Virology, 1975. 65, 55-66.

192. Stewart, M.L. & Maizel, J.V. Friend leukemia and mouse endogenous viruses. II. Proteins of Friend leukemia and mouse endogenous C-type particles produced by Balb/c mouse cell cultures. Virology, 1975. 65, 67-76.

193. Strand, M. & August, J.T. Structural proteins of oncogenic ribonucleic acid viruses. Interspec II, a new interspecies antigen. j. Biol. Chem., 1973. 248, 5627-5633.

194. Strand, M. & August, J.T. Structural proteins of mammalian oncogenic RNA viruses: multiple antigenic determinants of the major internal protein and envelope glycoprotein. J. Virol., 1974. 13, 171-180.

195. Stromberg, K. Surface-active agents for isolation of the core component of avian myeloblastosis virus. J. Virol., 1972. 9, 684-697.

196. Strong, L.C. The establishment of the C3H inbred strain of mice for the study of spontaneous carcinoma of the mammary gland. Genetics, 1935. 20, 586-591.

197. Sugano, H., Furasawa, M., Kawaguchi, T. & Ikawa, Y. Enhancement of erythrocytic maturation of Friend virus-induced luekemia cells. Bibl. Haematol., 1973. 39, 943-954.

198. Summers, D.F. & Maizel, J.V., Jr. Evidence for large precursor proteins in poliovirus synthesis. Proc. Natl. Acad. Sci. USA, 1968. 59, 966-971.

199. Svet-Moldavsky, G.J. Sarcoma in albino rats treated during the embryonic stage with Rous virus. Nature, 1958. 182, 1452-1453.

200. Taylor, J.M., Garfin, D.E., Levinson, W.E., Bishop. K.M. & Goodman, H.M. Tumor virus ribonucleic acid directed deoxyribonucleic acid synthesis: Nucleotide sequence at the 5' terminus of nascent deoxyribonucleic acid. Biochemistry, 1974. 13, 3159-3163.

201. Taylor, J.M., Illmensee, R., Trusal, L.R. & Summers, J. Transcription of avian sarcoma virus RNA. In D. Baltimore, A. S. Huang and C. F. Fox (Eds.) Animal Virology. ICN-UCLA Symp. Mol. and Cell. Biol., 1976. 4, 161-173.

202. Temin, H. The effects of actinomycin D on the growth of Rous sarcoma virus in vitro. Virology, 1963. 20, 577-582.

203. Temin, H.M. Nature of the provirus of Rous sarcoma. Natl. Cancer Inst. Monogr., 1974. 17, 557-570.

204. Temin, H.M. The participation of DNA in Rous sarcoma virus production. Virology, 1974. 23, 486-494.

205. Temin, H.M. & Rubin, H. Characteristics of an assay for Rous sarcoma virus and Rous sarcoma cells in tissue culture. Virology, 1958. 6, 669-688.

206. Temin, H.M. & Mizutani, S. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature, 1970. 226, 1211-1213.

207. Tooze, J. (Ed.) The molecular biology of tumor viruses. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1973.

208. Tooze, J. (Ed.) The molecular biology of tumor viruses. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1973. pp. 528-536.

209. Tronick, S.R., Stephenson, J.R. & Aaronson, S.A. Immunological characterization of a low molecular weight polypeptide of murine leukemia virus. Virology, 1973. 54, 199-206.

210. Tronick, S.R., Stephenson, J.R., Verma, I.M. & Aaronson, S.A. A thermolabile reverse transcriptase of a mammalian leukemia virus mutant temperature sensitive in its replication and sarcoma virus helper functions. J. Virol., 1975. 16, 1476-1482.

211. Tumor Viruses, Cold Spring Harbor Symp. Quant. Biol., 1975. 39, 1-1187.

212. Valentine, A.F. & Bader, J.P. Production of virus by mammalian cells transformed by Rous sarcoma and murine sarcoma viruses. J. Virol., 1968. 2, 224-237.

213. van Zaane, D., Gielkens, A.L.J., Dekker-Michielson, M.J.A. & Bloemers, H.P.J. Virus-specific precursor polypeptides in cells infected with Rauscher leukemia virus. Virology, 1975. 67, 544-552.

214. Varmus, H.E., Levinson, W.E. & Bishop, J.M. Extent of transcription by the RNA-dependent DNA polymerase of Rous sarcoma virus. Nature New Biol., 1971. 233, 19-21.

215. Verma, I.M. Studies on reverse transcriptase of RNA tumor virus. I. localization of thermolabile DNA polymerase and RNase H activities on one polypeptide. J. Virol., 1975. 15, 121-126.

216. Vogt, P.K. & Ishizaki, R. Patterns of viral interference in the avian leukosis and sarcoma complex. Virology, 1966. 30, 368-374.

217. Vogt, V.M. & Eisenman, R. Identification of a large polypeptide precursor of avian oncornavirus proteins. Proc. Natl. Acad. Sci. USA, 1973. 70, 1734-1738.

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

219. von der Helm, K. & Duesberg, P.H. Translation of Rous sarcoma virus RNA in cell-free systems from ascites Krebs II cells. Proc. Natl. Acad. Sci. USA, 1975. 72, 614-618.

220. Vosika, G.J., Krivit, W., Gerrard, J.M., Coccia, P.F., Nesbit, M.E., Coalson, J.J. & Kennedy, B.J. Oncornavirus-like particles from cultured bone marrow cells preceding leukemia and malignant histiocytosis. Proc. Natl. Acad. Sci. USA, 1975. 72, 2804-2808.

221. Wang, L.H., Duesberg, P., Beemon, K. & Vogt, P.K. Mapping RNase T_1-resistant oligonucleotides of avian tumor virus RNAs: sarcoma-specific oligonucleotides are near the poly(A) end and oligonucleotides common to sarcoma and transformation-defective viruses are at the poly(A) end. J. Virol., 1975. 16, 1051-1970.

222. Weissmann, C., Parsons, J.T., Coffin, J.W., Rymo, L., Billeter, M.A. & Hofsteffer, H. Studies on the structure and synthesis of Rous sarcoma virus RNA. Cold Spring Harbor Symp. Quant. Biol., 1975. 39, 1043-1056.

223. Wooley, G.W. & Small, M.C. Experiments on cell-free transmission of mouse leukemia. Cancer, 1956. 9, 1102-1106.