1981

# Transcriptional and Translational Products of Avian Erythroblastosis and Avian Myeloblastosis Viruses

Steven Mathias Anderson

# Transcriptional and Translational Products of Avian Erythroblastosis and Avian Myeloblastosis Viruses.

A thesis submitted to the Faculty of
the Rockefeller University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
by

Steven M. Anderson A.B.

1 April 1981

The Rockefeller University
New York, New York

## Summary

The transcription and translation products of two avian acute leukemia viruses, avian erythroblastosis virus (AEV) and avian myeloblastosis virus (AMV), were examined.

AEV is defective in virus replication and requires the presence of a helper virus to produce infectious progeny. AEV-transformed nonproducer cells were found to contain no proteins participating in virus replication. Instead, nonproducer cells were found to contain a 75,000 dalton gag-related polyprotein (p75) which contains the p19 region of the gag gene at its amino terminus. Two-dimensional tryptic peptide mapping indicated that p75 contained novel peptides in addition to those derived from p19. p75 was found to be a phosphoprotein containing a phosphoserine residue. Two-dimensional tryptic phosphopeptide maps of p19 and p75 were identical, indicating that all phosphorylation of AEV p75 occurs in the gag portion of the molecule. Unlike many other transforming proteins, AEV p75 was not found to be a protein kinase using the immune complex protein kinase assay.

AEV nonproducer cells were found to contain two viral-related RNA species of 28 S and 21 S. Both RNA species contain the AEV unique sequences and the "strong stop" region of the viral genome, as detected by cDNA hybridizatin. In addition to the 28 S RNA, which is the genomic RNA of AEV, the 21 S subgenomic RNA was incorporated into the virus particle. In vitro translation of specific size classes of AEV virion RNA demonstrated that the 28 S RNA encodes p75, and that the 21 S

RNA encodes two proteins of 46,000 and 48,000 daltons. These two proteins, p46 and p48, were not immunoprecipitated by antisera directed against the gag, pol or env gene products. Two-dimensional tryptic peptide mapping indicates these two proteins are closely related to each other.

An antiserum specific to the unique sequences in AEV p75 was prepared by immunizing chickens that had regressed from erythroblastic leukemia induced by a temperature-sensitive mutant of AEV, AEVts34. This antiserum did not cross-react with p46/p48 indicating that these proteins are not related to AEV p75. This antibody was used in immunofluorescent staining of AEV-transformed rat cells and staining was obeserved in the cellular membrane, cell ruffles, and in a perinuclear organelle.

The possible role of p75 and p46/p48 in oncogenic transformation by AEV is discussed.

AMV is a replication-defective acute leukemia virus. Nonproducer clones of AMV-transformed myeloblasts contain $Pr180^{gag-pol}$, $Pr76^{gag}$, and the individual gag proteins p19, p27, p12 and p15. The genome of AMV is 34 S and it produces a subgenomic mRNA of 21 S. This RNA contains the "strong stop" region, the AMV-specific sequences, and the 3' C region. As in the case of AEV, the genomic RNA and the subgenomic RNA of the virus both were incorporated into the virus particle. In vitro translation of AMV virion RNA identified three AMV-specific proteins of 56,000, 48,500, and 47,000 daltons. The 56,000 and the 48,500 dalton proteins were encoded by 20-22 S RNA, which is the same size as the AMV subgenomic RNA. The 47,000 dalton

protein was encoded by 22-24 S RNA. $^{7m}$GTP was found to block translation of the 56,000 and 48,500 dalton proteins but not the 47,000 dalton protein, indicating that the 56,000 and 48,500 dalton proteins are translated from capped RNAs but the 47,000 dalton protein is not. These three proteins are not immunoprecipitated by antisera directed against the gag, pol or env gene products. Two-dimensional tryptic peptide mapping had indicated that these three proteins were not related to Pr76$^{gag}$ or to the env gene product gPr92$^{env}$, the glycosylated envelope precursor to gp85 and gp37. And antiserum prepared by immunizing rabbits with purified AMV ATPase did not precipitate the 56,000, 48,500 or 47,000 in vitro translation products of AMV virion RNA. This antiserum did, however, immunoprecipitate AMV ATPase-related polypeptides from AMV-transformed myeloblasts.

# Acknowledgments

As I have approached this date, often with great anxiety, I have come to realize that no thesis project is the result of one person's efforts. There are many who have contributed to my thesis research and to my development as a scientist.

I would like to thank all the members of the Hanafusa laboratory for making it such an enjoyable place to work. Drs. Bill Hayward, Lu-Hai Wang, and Teruko Hanafusa have each shared with me their special techniques, talents and thoughts in each of their special areas of science. Special thanks go to fellow students Carl Rettenmier, Roger Karess, and Ben Neel for contributions to both science and society.

There are many members of the faculty to whom I am indebted for acting as teachers and for sharing their excitement of science with me. These include: Jay Valinsky, Warren Jelinek, Sid Strickland, Allen Goldberg, and Bob Lamb.

I should like to thank several fellow students for acting as friends beyond the call of duty: Jim Kreuger, Steve Aley, Warren Gallin, Wendi Neckameyer, Linda Hanley-Bowdoin, Wilma Friedman, and Celeste Simon.

I owe a great deal and gratitude to my collaborator on all the work with AMV, Dr. J.H. Chen of Life Sciences, Inc. Chen has not only been a great friend, but his infectious enthusiasm made working with him a great joy.

I owe my greatest debt to my thesis advisor, Saburo Hanafusa. He has given me the freedom to explore any area of interest to me and he has encouraged me in my every effort. He has always been available for help and consultation, and for that I am very grateful. He has set high standards for the type of experiments I did and for the type of results that were meaningful. His modesty, honesty, and thoughtfulness set him apart from all others, and endear him to those who know him.

Last of all, but not least, I thank my wife Margaret for her understanding and support throughout my time here at Rockefeller. She has helped me to survive here in New York. She has helped to make the rough times better, and the good times great. That this has reached the stage that it has, is a tribute to her.

"Think of how much more money--no, I mean how much more social position and power for doing good a successful doctor has than one of these scientists that just putter, and don't know what's going on in the world."

Arrowsmith

Sinclair Lewis

# Table of Contents

# List of Tables

# List of Figures

## Abbreviations

| | |
|---|---|
| AEV | avian erythroblastosis virus |
| ALV | avian leukosis virus |
| AMV | avian myeloblastosis virus |
| APT | amino phenyl thioether |
| c region | common region at 3' end of viral RNA |
| cDNA | complementary DNA |
| $CFU_C$ | colony forming unit-culture |
| chf | chick helper factor, glycoprotein of endogenous retrovirus |
| Crot | molecular concentration of RNA x time in seconds |
| CSA or CSF | colony stimulating Activity or Factor |
| DPT | diazophenyl thioether |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetraacetic acid |
| gs | group-specific antigen, gag gene product of endogenous retrovirus |
| h | hour |
| kb | Kilobase |
| LLV | Lymphatic Leukosis virus |
| LTR | long terminal repeat |
| NP | nonproducer |
| RAV | Rous-associated virus |
| RIPA | Radioimmunoprecipitation assay buffer |
| RSV | Rous sarcoma virus |
| RSV(1) | noninfectious particle released by nonproducer cells transformdd by Bryan RSV |
| SDS | sodium dodecyl sulfate |
| SR-RSV | Schmidt-Ruppin strain of RSV |
| TBR | tumor-bearing rabbit |
| td | transformation defective |
| ts | temperature-sensitive mutant |

## Nomenclature of Viral Genes and Transformation-Specific Sequences

The following names were agreed upon at the 1980 RNA Tumor Virus Meeting at Cold Spring Harbor:

| | |
|---|---|
| env | gene encoding gp85 and gp37 |
| erb | specific sequences of AEV |
| gag | gene encoding p19, p27, p12 and p15 |
| mac or myc | specific sequence of avian myelocytomatosis viruses |
| myb | specific sequence of avian myeloblastosis viruses |
| onc | general term for oncogenic sequence |
| pol | gene encoding reverse transcriptase |
| ras | specific sequence of rat-derived sarcoma virus |
| src | gene encoding $pp60^{src}$ |

Endogenous cellular homologue of a transforming gene is known as c-onc, e.g., c-erb, c-ras, c-src

# I. Introduction

## A. Review of Avian RNA Tumor Viruses

Taxonomy. The RNA tumor viruses or oncoviruses are members of the Retroviridae (Fenner, 1976; Mathews, 1979). The Retroviridae are enveloped RNA viruses which replicate via a DNA intermediate as a result of possessing the novel enzyme RNA-dependent DNA polymerase. There are three subfamilies of Retroviridae: Oncovirinae (the RNA tumor viruses), Lentivirinae (Maedi and visna viruses), and Spumavirinae (the foamy virus group). The RNA tumor viruses are the most extensively studied of these three groups, primarily as a result of the interesting neoplastic diseases they cause in their hosts. The virus particle is about 100 nm in diameter and consists of a helical nucleocapsid core enclosed in a core shell of viral proteins, which is in turn enclosed in a lipoprotein envelope. The envelope contains protruding spikes which are the envelope glycoproteins gp85 and gp37. The protein core and shell comprise the gag proteins p19, p27, p12, and p15. Two positive-sense, capped polyadenylated RNA molecules, attached in a head to head dimer (Kung et al., 1975), are present in the virion core along with a number of small RNAs (Erikson and Erikson, 1970; Erikson and Erikson, 1971) and 10-20 molecules of reverse transcriptase.

Genomic structure. The genomes of all RNA tumor viruses have three genes required for the production of infectious progeny virus: gag, pol, and env. The gag gene codes for the internal structural proteins p19, p27, p12 and p15. The pol gene codes for the enzyme reverse transcriptase and the env

gene codes for the envelope glycoproteins gp85 and gp37. The order of the genes has been determined by mapping the RNase $T_1$-resistant oligonucleotides from various strains of viruses and their mutants (for a review see Wang, 1978) and the order is 5'-gag-pol-env-3' (see Figure 1). Transforming genes, such as the src gene of Rous sarcoma virus (RSV), are not required for replication of the virus and are thus lost very easily if there is not a constant selection pressure to keep them (Vogt, 1971). There is a sequence at the 3' end of all avian RNA tumor viruses, known as c for common, which is a highly conserved sequence. This sequence contains termination codons in all reading frames (Czernilofsky et al., 1980a). This region, however, does carry important functions since it contains a promotor site, a poly(A) addition signal, and the initiation site for plus (+) strand DNA synthesis (see below).

The virus life cycle. The life cycle of the virus begins with the adsorption of the virus to the cell surface which is followed by penetration of the cell membrane. Adsorption is a nonspecific ionic interaction that does not involve the viral envelope glycoproteins. Penetration, however, is a very specific process involving interaction of the envelope glycoprotein and specific cellular receptors (Crittenden, 1968). Host range specificity of avian RNA tumor viruses is apparently controlled by penetration (Hanafusa, 1975; Vogt, 1977), and it is considered to be determined by the presence of the cellular receptor for the specific virus envelope subgroup. The virus subgroup is determined by the viral envelope

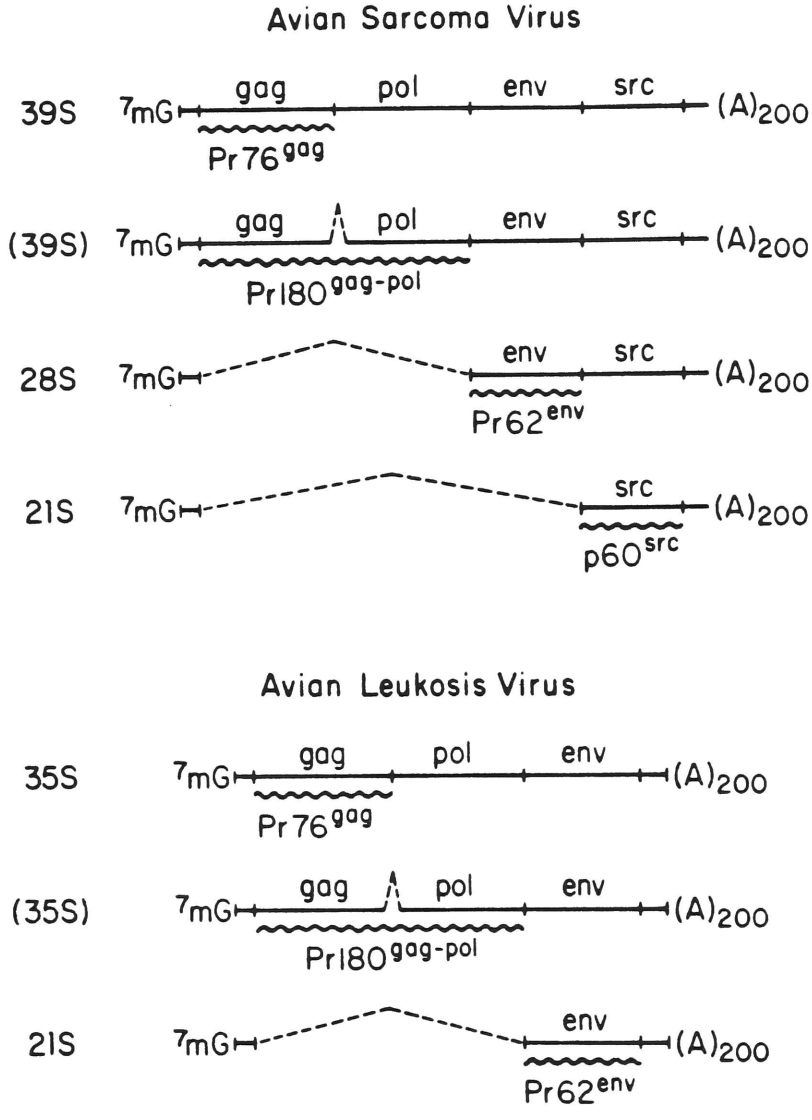


FIGURE 1. Genomic structure of avian sarcoma virus and avian leukosis virus. The structure of their mRNAs and their primary translation products. RNA sequences removed by splicing are indicated by broken lines. The putative mRNAs for $Pr180^{gag-pol}$ have not been directly identified. The indicated splice between the *gag* and *pol* genes is suggested by data indicating that there are multiple termination codons between the *gag* and *pol* genes, and by sequencing data indicating *gag* and *pol* are in different reading frames. (Figure courtesy of W.S. Hayward.)

glycoprotein, and all viruses can be divided into one of seven subgroups (A - G) based upon host range on genetically resistant cells, serum neutralization, and interference with focus-formation by RSV of specific subgroups (Hanafusa, 1975; Vogt, 1977).

Penetration is followed by uncoating, release of the viral RNAs and synthesis of the viral DNA intermediates. Viral DNA is synthesized by reverse transcriptase using $tRNA^{Trp}$, which binds near the 5' end of the viral RNA, as a primer for the reaction (Dahlberg et al., 1974; Taylor and Illmensee, 1975; Harada et al., 1975; Haseltine et al., 1977). Synthesis of the negative strand continues by jumping to the 3' end of either the same or another RNA subunit (Haseltine et al., 1976). Various models have been proposed to explain the mechanism involved in the jumping step (Bishop, 1978; Gilboa et at., 1979). This process is more complicated than just a simple jump because the linear double-stranded DNA product contains direct repeats at each termini (Hsu et al., 1978; Shank et al., 1978) which contain sequences from both the 5' and 3' ends of the viral genome. This direct terminal repeat has been named the large terminal repeat (LTR). Plus strand synthesis begins before complete synthesis of the negative strand and therefore it must be discontinuous (Varmus et al., 1978). Two double-stranded DNA intermediates are found in virus-infected cells: a closed circular form and a linear form (Varmus et al., 1978). There are two forms of the closed circular viral DNA containing either one or two copies of the LTR (Shank et al., 1978; Hughes et al., 1978).

Integration of the viral DNA into the host cell chromosomal DNA then takes place; however, the details of this step are not well understood at this time. The integrated provirus is colinear with the viral genome. Integration of the proviral DNA occurs at random sites in the host cell chromosomal DNA (Battula and Temin, 1977; Battula and Temin, 1978; Shank et al., 1978; Hughes et al., 1978; Keshet et al., 1979; Shimotommo and Temin, 1980). The integration of the provirus, however, does involve a specific mechanism in which a direct repeat of a short sequence of 4-6 bases is generated at each end of the provirus (Shimotohno et al., 1980, Shimotohono and Temin, 1980; Majors and Varmus, 1981; Ju and Skalka, 1980; Hishinuma et al., 1981). It has been suggested that the provirus integrates and functions in a manner analogous to insertion sequences and other transposable elements (Ju and Skalka, 1980; Temin, 1980; Hishinuma et al., 1981).

The structure of the integrated provirus has been extensively studied by restriction mapping and sequencing of molecularly cloned proviral DNA. The DNA contains a sequence of approximately 350 bases which is repeated at both ends of the provirus (Fig. 2). This long terminal repeat (LTR) consists of three definite segments: U3 (the 3' unique sequences), R (the short terminal repeat of the genome) and U5 (derived from the 5' end of the virus genome) (Ju and Skalka, 1980; Tarmamoto et al., 1980a; Yamamoto et al., 1980b).

DNA sequencing has revealed several interesting regions within the LTR including the presumed viral promoter (Yamamoto et al., 1980a; Yamamoto, et al., 1980b; Sutcliffe et al., 1980;

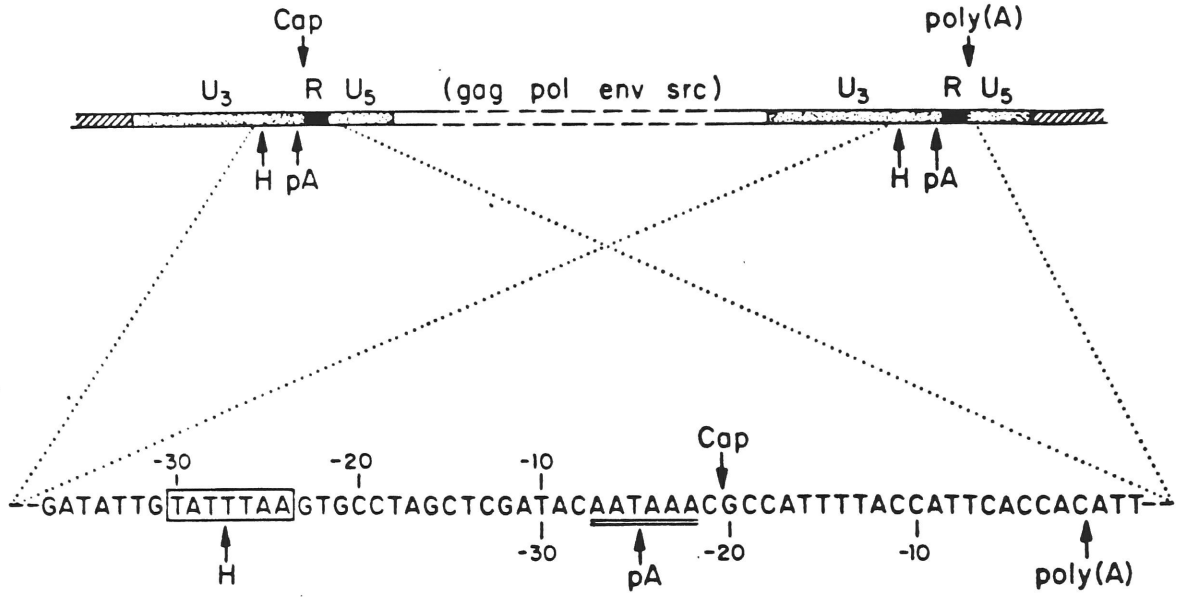


FIGURE 2. Nucleotide sequence of portions of RSV DNA involved in initiation of transcriptional and poly(A) addition. Positions corresponding to the capped and polyadenylated nucleotides of genomic RNA are indicated. Sequences apparently providing signals for capping and poly(A) addition are the Hogness box (H) and the AATAAA signal (pA). (Figure courtesy of W.S. Hayward.)

Shimotohuo et al., 1980; Van Beueren et al., 1980, Czernilofsky et al., 1980a; Dhar et al., 1980; Ju and Skalka, 1980; Benz et al., 1980; Majors and Varmus, 1981). The promotor is an AT-rich sequence approximately 30 nucleotides upstream from the cap site, and is located in relatively the same position as the Hogness box in a number of prokaryotic genes (see Ziff, 1980). Although several gene transcription units lack a Hogness box (Fiers et al., 1978; Reddy et al., Flavell et al., 1979; Baker et al., 1979), at least some systems show an absolute requirement for it (Wasylyk et al., 1980). A second interesting sequence, AATAAA, which is thought to code for cleavage and poly(A) addition (Proudfoot and Brownlee, 1976), is also in the LTR.

The integrated provirus serves as the template for the synthesis of all viral mRNAs and these transcripts undergo the usual posttranscriptional modifications including capping, splicing, poly(A) addition, and methylation. The synthesis and processing of viral mRNAs has recently been reviewed (Hayward and Neel, 1981). The size and genetic content of viral mRNAs has been determined by hybridization with gene-specific cDNA probes (Hayward, 1977; Weiss et al., 1977). The various mRNAs produced by several viruses are shown in Figure 1. RAV-2-infected cells contain mRNAs of 35 S and 28 S. The 35 S mRNA is translated to produce the gag and pol gene products, and the 21 S mRNA is translated to produce the env gene products. Cells infected with the Schmidt-Ruppin strain of Rous sarcoma virus contain mRNAs of 35 S, 28 S, and 21 S, which encode the gag and pol gene products, the env gene products, and the src

gene product, respectively. It is not clear whether there exist processing differences between those mRNAs which act as genomes for progeny virions and those which encode the gag and pol gene products. It is also not clear whether the mRNAs translated to produce $Pr76^{gag}$ and $Pr180^{gag-pol}$ are identical.

As noted above, the gag gene codes for the internal structural proteins of the virion. There are four gag proteins, p19, p27, p12 and p15 which are named by their molecular weights as determined by gel filtration (Fleissner, 1971). These four proteins are produced by cleavage of a precursor polyprotein $Pr76^{gag}$ (Vogt et al., 1975). The order of the four gag proteins in $Pr76^{gag}$ apparently is $NH_3$-p19-p27-p12-p15-COOH (Vogt et al., 1975; Shealy and Ruckert, 1978; Shealy et al., 1980). The gag protein p15 apparently contains a proteolytic activity capable of specifically processing $Pr76^{gag}$ to the individual gag proteins (Vogt et al., 1979). Protein p12 is phosphorylated (Lai, 1976) and is found in association with the virion RNA (Davis and Reuckert, 1972; Quigley et al., 1972; Fleissner and Tress, 1973). p19 is also a phosphoprotein (Lai, 1976; Erikson et al., 1977) and it is also apparently bound to the genomic RNA of the virion (Sen and Todaro, 1977). Recently, it has been suggested that p19 may play a key role in the processing of viral mRNAs by binding to double-stranded RNA regions and allowing specific cleavages by RNase III (Leis et al., 1978; Leis et al., 1980). The p27 protein is believed to comprise the core shell of the virion (Bolognesi et al., 1973; Stromberg et al., 1974). Nearest neighbor analysis of the proteins in intact virions with the reversible cross-linking agent

dithiobispropionimidate demonstrated that only homotypic multimers of the gag proteins could be detected (Pepinsky et al., 1980), indicating a highly ordered structure in the virion core and shell.

The primary product of the pol gene is thought to be $Pr180^{gag-pol}$ (Opperman et al., 1977; Hayman, 1978), but a precursor-product relationship between $Pr180^{gag-pol}$ and reverse transcriptase has not been firmly established. $Pr180^{gag-pol}$ is immunoprecipitated from labeled cell lysates with antisera directed against either gag or pol specific determinants (Opperman et al., 1977; Hayman, 1978). The mature form of reverse transcriptase found in the virion consists of two subunits of 95,000 and 65,000 daltons (Moelling et al., 1971; Gibson and Verman, 1974). All of the methionine- and cysteine-labeled tryptic peptides of the smaller α subunit of reverse transcriptase are present in the larger β subunit of reverse transcriptase. The β subunit, however, does contain two methionine-labeled tryptic peptides not present in the α subunit (Rettenmier, 1979). All of the β subunit peptides are present in $Pr180^{gag-pol}$ (Rettenmier, 1979). The α subunit is derived from the β subunit by proteolytic cleavage (Gibson and Verma, 1974). Cleavage of the β subunit with p15 yields the α subunit plus a 32,000 dalton protein (p32) that has a specific DNA endonuclease activity (Grandgenett et al., 1978; Schiff and Grandgenett, 1978; Vogt et al., 1979; Moelling et al., 1980). The α subunit, which is derived from the amino terminal portion of the β subunit, (Schiff and Grandgenett, 1978; Copeland et al., 1980; Eisenman et al., 1980), possesses both the polym-

erase and the RNase H activities (Grandgenett et al., 1973; Verma, 1975). $Pr180^{gag-pol}$ is processed by p15, but the products of this cleavage were not identifiable (Vogt et al., 1979; Moelling et al., 1980).

$Pr76^{gag}$ is produced by in vitro translation of genomic length viral RNA (Beemon and Hunter, 1977; Paterson et al., 1977; Pawson et al., 1976; Purchio et al., 1977; von der Helm and Duesberg, 1975). In vitro translation of approximately full length viral RNA also directs the synthesis of a 180,000 dalton gag-pol fusion product (Weiss et al., 1978; Philipson et al., 1978; Murphy et al., 1979), which can also be identified by direct immunoprecipitation of labeled cell extracts (Opperman et al., 1977). As stated above, the mRNAs coding for $Pr76^{gag}$ and $Pr180^{gag-pol}$ may or may not be identical. Weiss et al. (1978) found at least two termination codons between the gag and pol gene sequences. Recent sequencing data of D. Schwartz and W. Gilbert (quoted in Hayward and Neel, 1981) indicates that the gag and pol genes are separated by a region containing multiple termination codons between the two genes and that these two genes are in different reading frames. Thus one would expect that there must be different processing mechanisms involved in the production of the mRNAs which code for $Pr76^{gag}$ and $Pr180^{gag-pol}$.

The env gene products gp85 and gp37 are translated from a subgenomic mRNA (21 S in the case of RAV-2 and 28 S in the case of nondefective RSV). Immunoprecipitation of lysates from virus-infected cells labeled with either $^{35}S$-methionine or radiolabeled-N-acetylglucosamine demonstrates the major env

precursor is $gPr92^{env}$ (England et al., 1977; Buchhagen and Hanafusa, 1978; Moelling and Hayami, 1977). Immunoprecipitation with anti-gp85 antiserum of virus-infected cells treated with either 2-deoxyglucose or tunicamycin identified a protein of 56,000-58,000 daltons which is the unglycosylated precursor to gPr92 (Diggelman, 1979; Stohrer and Hunter, 1979). The precursor, $gPr92^{env}$ is then cleaved to yield the viral glycoprotein complex (VGP) which is composed of one molecule of both gp85 and gp37 linked by disulfide bonds (Leamson and Halpern, 1976; Klemenz and Diggelman, 1978; Shealy and Ruckert, 1978). In vitro translation of 21 S RAV-2 env mRNA or 28 S SR-RSV env mRNA yields a protein of 62,000-64,000 daltons which is immunoprecipitable with anti-gp85 antiserum (Pawson et al., 1980; Anderson et al., 1980). The larger size of the in vitro translation product versus the in vivo labeled unglycosylated precursor presumably reflects the presence of a signal sequence which has not yet been removed from the in vitro translation product.

The 21 S src mRNA of Rous sarcoma virus has been translated in vitro to yield $p60^{src}$ (Purchio et al., 1977; Beemon and Hunter, 1978; Purchio et al., 1978). There is currently no evidence for the processing of $pp60^{src}$ and it does not appear that there is a "signal sequence" at the amino-terminus of the src protein based upon the putative amino acid sequence of $pp60^{src}$ as determined by DNA sequencing (Czernilofsky et al., 1980b).

A virus which lacks one or more of these three genes, gag, pol, and env, is said to be defective because it is not able to

produce infectious virus particles. Examples of defective viruses include the Bryan strain of RSV (Hanafusa, 1963) and Fujinami sarcoma virus (T. Hanafusa et al., 1980). The Byran strain of RSV does not produce the env gene products gp85 and gp37, and Fujinami sarcoma virus does not produce any of the normal gag, pol, or env gene products (T. Hanafusa et al., 1980). Defective viruses require the presence of a replication-competent helper virus to complement their genetic defect(s).

Assembly of the virus particle takes place at the cell membrane, possibly with the gp85-gp37 complex acting as an organization site for virus assembly. Reverse transcriptase binds to the RNA dimers at a site within 100-400 bases from the 5' end of the RNA (Shank and Linial, 1980) and this may be an important step in the packaging of the viral RNAs. After the immature virus particle buds through the membrane, several maturational steps take place after budding, including formation of the 70 S RNA dimers and processing of the gag proteins (Witte and Baltimore, 1978). The env and pol gene products, however, are not required for virus assembly since noninfectious virus particles can be formed as in the case of RSV, which is defective in both pol and env (Hanafusa and Hanafusa, 1971). In summary, the life cycle of the RNA tumor viruses represents a very effective manner by which a host cell can be infected and programmed to produce progeny virus for the life of that cell, without causing the death of that host cell.

B. Early History of Avian Leukemia: 1850 to 1970, the Age of Pathology.

The first description of human leukemia in 1845 by Craigie (1845), Bennett (1845), and Vichow (1845) gave birth to a new field in medical science whose challenges have not diminished in 135 years since its birth. It was Wilhelm Virchow who introduced the term leukamie to describe a disease condition "welche sich durch eine progressive und bis zu ganz ungewohnlichen Masse anwachsenda Vermehrung der farblosen Korperchen im Blute charakerisiert," (which is characterized by a progressive increase, to a very large amount, of the colorless corpuscles of the blood). The origin and significance of these cells in the blood of patients was widely discussed. The validity of their observations is quite amazing in light of the fact that no cytochemical stains existed at this time. Although the origin of the leukemic cells was widely discussed, the problem remained unresolved. By 1856 Virchow had come to believe that the problem laid in the organs that produced these cells--presumably the spleen, liver, and lymph nodes since they were enlarged in leukemic patients. Virchow proposed that there were two types of leukemias based upon pathological findings; lymphatic leukemia and splenic leukemia. The latter term was modified to myeoid leukemia in recognition of the fact that the bone marrow is very important in the production of leukocytes (Neumann, 1870). The development of staining technology by Erlich in the 1880's resulted in an accurate description of the leukocytes present in normal blood and led to the realization

that there were multiple forms of leukemia.

With a knowledge of human leukemia in the background, it was not long before cases of leukemia were described in animals. Horses, pigs, dogs, cats, mice, and birds were all reported to have diseases analogous to human leukemia (for a review see: Ellerman, 1921; Olson, 1931; Engelbreth-Holm, 1942). The first reported case of leukemia in birds was described by Roloff in 1868. In 1896 Caparoni described histological changes in three enlarged fowl livers which he diagnosed as leukemia. Butterfield (1905) and Warthin (1907) both accurately described leukosis in their works and Warthin notes in his conclusion; "aleukemic and leukemic forms of lymphocytoma exist not infrequently in the common fowl, in all respects analogous to the similar conditions existing in man" (Warthin, 1907). The pathologies described consisted primarily of the presence of enlarged livers and spleens, and of a tremendous increase in the number of leukocytes in the blood. These pathologies were clearly different from the "infectious leucemia" described by Moore (1897) which later became known as fowl typhoid fever. This disease occurs both spontaneously and following experimental infection with Bacterium sanguinarium and results in a leukocyte count of 100,000 to 200,000 per $mm^3$ (normal is 30,000). The leukocytes in this disease are polymorphonuclear leukocytes and there is no infiltration of these cells into any organs of the body, a common observation in the other leukemias described.

The first transmission study was reported by Mosler in 1872. In this study, a leukemic patient's blood was injected

into dogs and rabbits. This study, and the many that followed (for reviews see: Ellerman, 1921; Opie, 1931; Engelbreth-Holm, 1942), were not successful in demonstrating either homologous or heterologous transmission of leukemia.

In their epoch studies of 1908, Ellerman and Bang (1908, 1909) demonstrated not only the transmission of leukemia from one chicken to another by injection of leukemic cells, but also more incredibly, that the leukemia could be transmitted by cell-free extracts! These results were met with a wave of objections that have been detailed by both Ellerman (1921) and Engelbreth-Holm (1942) in their reviews. Schriddle (1909) thought that he could produce a leukemic blood picture in birds following innoculation of chickens with organs from normal hens, to which Ellerman responded that this was at variance with all previous work. In addition, Ellerman noted that leukosis was more than just a change in the blood picture, and that these cells were also present in other organs, such as the liver. Skiba (1909) objected that the hematopoietic system of the bird is quite fragile and thus could easily react to a variety of conditions with a "leukemic" blood picture. He therefore questioned the leukemic nature of the disease and whether it was identical with the spontaneous disease observed on farms. Burckhardt (1912) pointed out there is a very large increase in the number of leukocytes in birds with tuberculosis and questioned the whether Ellerman's fowl leukosis was not really fowl tuberculosis. Ellerman (1921) demonstrated that he could separate tubercle bacilli from the leukosis agent by filtration and obtain clean cases of leukosis following infec-

tion with the filtrate. Finally, Kasarinoff (1910) had shown that a "leukemic" blood picture could be produced by various blood poisons and Henschen (1917) thought that leukosis might represent an anemia of toxic origin with subsequent involvement of the myeloid system.

The confirmation of Ellerman and Bang's work by Hirschfeld and Jacoby (1909) and Schmeisser (1915) clearly substantiated the infectious, transmissable nature of leukosis and appeared to rule out objections to this work. The isolation of the Rous sarcoma virus (1910) and the demonstration that sarcomas could be produced by a filterable agent (Rous, 1911), provided further support for Ellerman's research. At this time, however, leukemia was not considered to be a neoplastic growth and so this was not taken as supporting evidence by all scientists. In fact, Ellerman does not even cite the work of Rous in his book of 1921.

Ellerman was able to isolate at least eight strains of avian leukosis viruses and his observations, plus those of several other investigators, lead him to propose that there were three groups of leukosis viruses (Ellerman, 1921, Engelbreth-Holm, 1942):

1) Lymphogenous leukemia (Lymphoid leukosis). A primarily "aleukemic" or extravascular disease in which death generally occurs without clinical manifestations, but upon autopsy the liver is greatly enlarged (up to eight times its normal weight). The spleen must also be considerably enlarged. Fine or coarse whitish-grey nodules corresponding to the lymphocyte infiltration can

be seen on the surface of these two organs. Infiltration is also observed, in either the diffuse or the nodular type, in the bone marrow, ovaries, intestine, heart, lungs, thymus and kidneys. It was later realized that the bursa, which usually had atrophied in adults, was large and often contained numerous tumor nodules.

2) Myelogenous leukemia. Both aleukemic and leukemic forms of this disease were recognized and the possibility that both myelocytic and myeloblastic forms existed was raised by Ellerman (1921). In this disease the peripheral blood contains up to 600,000 leukocytes per $mm^3$ (normal is 30,000 per $mm^3$). There is some enlargement of the liver (twofold) and the spleen, but the general picture is one of anemia and somewhat pronounced emaciation. The differences between the leukemic and aleukemic forms has been stressed by Mathews (1929), Feldman (1932), Feldman and Olsen (1933) and Olsen (1940); they believed that the leukemic form was a myeloblastic or granuloblastic leukemia and the aleukemic form was a myelocytic form, perhaps analogous to chloroma in man. More recently, the same opinions have been expressed by Beard and his coworkers (Beard, 1968; Beard et al., 1970).

3) Erythroleucosis (Erythroblastosis). This group corresponds to the "intravascular lymphoid form" in Ellerman's first classification and includes a leukemia characterized by immature erythroblasts and what Eller-

> man called leukanaemia--a disease which seemed to occur in alternative passages with erythroblastic leukemia and which was characterized by the presence of "lymphoidcytes". The lymphoidcyte was later characterized as representing an early stage in erythroid development and renamed "erythrogomes" (Ellerman, 1921). Erythroblastosis is quite acute and death may occur before clinical manifestations, such as anemia, become evident. The liver and spleen are enlarged (fourfold) with a deep reddish-violet surface without any demarcations. The bone marrow, rather than being brown as in myeloid leukemia, is a uniform vivid red and often semi-liquid.

This classification has remained largely intact, even to the current day; the only modification being that of Beard and his coworkers (Beard, 1968) who have stressed the uniqueness of myelocytomatosis. As noted above, this disease may have been included in the myelogenous leukemia group by Ellerman who might have missed some of its unique properties. Ellerman did note that many of his strains caused a variety of leukemias upon passage and it appeared that "different pictures are thus produced by one and the same virus" (Ellerman, 1921). The point will be argued later that clonal virus isolates are capable of inducing a restricted pathological response. The conversion of myelogenous or erythroblastic leukemia virus to a lymphatic leukosis virus, however, can now be understood as a loss of the replication-defective acute leukemia virus and the growth of only the replication-competent lymphatic leukosis

virus.

In the preceding discussion I have not mentioned a disease first described by Marek (1907), which was evidently not observed by Ellerman. This disease known alternatively as Marek's disease, neurolymphomatosis, and fowl or range paralysis, has often been confused with lymphatic leukosis. Marek's disease can be clearly distinguished from lymphoid leukosis because the disease is characterized by paralysis of the extremities, caused by infiltration of nerves with lymphoid cells. Work in the 1960's and 1970's identified a herpes virus as the causative agent of Marek's disease thus confirming the different pathological pictures seen in these diseases (for a review see: Nazarian, 1980).

Following Ellerman's work, very little research on avian leukosis was carried out until the 1930's. During this time, apparently all of Ellerman's virus isolates were unfortunately lost. In the 1930's work began in several different laboratories. Much of the research up to 1942 is summarized by Engelbreth-Holm (1942). In 1935 Engelbreth-Holm and Rothe-Meyer (1935) published their work on five various virus isolates. One of these strains produced a erythroblastic leukemia (strain R), whereas a second, strain ES, produced both erythroblastosis and sarcomas. Both of these strains exist today known as strain R and strain ES4. The three other strains, $T_1$, AA, and ø, have all been lost. Strain $T_1$ produced both erythro- and myeloblastic leukemia whereas both strains AA and ø produced erythroblastic leukemia but were difficult to maintain.

It was these authors who first noted the multiple oncogenic potential of what appeared to be a single virus strain, strain ES. This strain was able to produce both sarcomas and erythroblastosis, a trait it carried throughout 84 passages in vivo (Engelbreth-Holm and Rothe-Meyer, 1935). They noted its ability to cause one pathology over another varied with the route of innoculation--sarcomas were produced following intramuscular or subcutaneous innoculation, whereas erythroblastic leukemia was primarily produced following intravenous innoculation. They hypothesized that the target cell of ES4 is a mesenchymal cell at a relatively early stage of differentiation which could differentiate along either of two different pathways, and that the pure erythroblastosis isolates such as strain R or pure sarcoma viruses such as RSV infect mesenchymal cells at a later stage of differentiation (Engelbreth-Holm and Rothe-Meyer, 1935). Thus they accounted for the multiple oncogenic potential of a virus in terms of the exact target cells which the virus is able to infect. To this day this hypothesis has not been clearly tested.

Similar observations on the multiple oncogenic potential of a strain to produce both erythroblastosis and sarcomas were described by Stubbs and Furth (1935) with strain 13, and by Oberling and Guerin (1933) with another independent isolate. Furth (1931) had noted this type of tumor previously, but did not associate the tumors with the causative agents of transmissible erythro- or myeloid leukemia.

In 1927 Begg described the isolation of a virus from a hen with a large ovarian tumor which was determined to be an en-

dothelioma. This isolate, named MH2, was found to produce endotheliomas following injection into chickens (Begg, 1927, Murray and Begg, 1930) and more recently has been found to cause kidney carcinomas and liver carcinomas (Moscovici et al., 1977). One strain of MH2 was also found capable of producing a high incidence of what appeared to be monocytic leukemia (Moscovici et al., 1977).

Furth later described an isolate, strain 2, which was capable of producing primarily lymphomatosis, myelocytomatosis and endothelioma (Furth 1933, 1934). The production of endothelioma and myelocytomatosis is similar to that observed by Begg (1927) and Murry and Begg (1930). The description of transmission of lymphomatosis (lymphoid leukosis) was a new finding, however. Although Ellerman (1921) felt that he had quite clearly demonstrated the transmissibility of lymphomatosis, the transmissibility of this disease was generally not as well accepted as the transmission of myeloblastosis or erythroblastosis (Furth, 1931, Stubbs and Furth, 1931). In fact, Furth stated in 1931 "the transmissibility of lymphoid leukosis is thus far unproven and that it occurs as commonly among uninnoculated as among innoculated fowls." Furth's statement is truer that it appears, since it was some time before researchers realized that many flocks were congenitally infected with lymphoid leukosis virus and thus bound to develop the disease without innoculation.

The study of lymphoid leukosis in this country began in earnest following the establishment of the Regional Poultry Research Laboratory at East Lansing, Michigan in 1939. In ad-

dition to beginning a complete study of lymphoid leukosis, the laboratory began to develop inbred lines of chickens which have become the basis of current research, just as the development of inbred strains of mice has been important to other fields of medical research. A wide variety of pathological lesions are associated with lymphoid leukosis including: visceral lymphomatosis ("big liver disease"--the hallmark of lymphoid leukosis), ocular lymphomatosis ("gray eye disease"), osteopetrosis ("big bone disease"), fibrosarcoma, endothelioma, nephroblastoma, erythroblastosis and hemorrhaging (for a review see Gross, 1970). A number of field isolates have been studied, but the most extensively characterized is RPL12 (Burmester, Prickett and Belding, 1946). Burmester and his coworkers have studied the effects of the route of innoculation, the volume of innoculum, virus titer, age of the host at innoculation and the genetic background of the chicken (Gross et al., 1959; Burmester et al., 1959; Burmester et al., 1960; Gross et al., 1962; Burmester and Purchase, 1969). Nearly every factor examined had an effect on the spectrum of tumors observed. Although it is difficult to draw any specific correlations, it is clear that visceral lymphomatosis was seen most often following injection with high dilution of virus (Fredrickson et al., 1964). Other pathologies were generally only seen when high virus titers were used for infection. In a study of field isolates from nineteen flocks across the country, Burmester and Fredrickson (1964) found that there was no correlation between the incidence of disease and the pathology observed in experimentally infected animals and the breed, age or size of the

chickens, the type of flock or the pathology seen in the original diseased chicken from which the virus was isolated. The appearance of lymphoid leukosis in "normal" uninfected birds was later correlated with the "vertical" transmission of virus from hen to chick via the egg (Cottral et al., 1954, Burmester et al., 1955).

Between 1940 and 1965 several very interesting avian leukemia viruses were isolated. The most important isolate was avian myeloblastosis virus BAI strain A (AMV) which was described by Hall et al. in 1941. AMV was isolated from a chicken with neurolymphomatosis (Marek's disease?); however, following passage it produced "erythroblastosis" at high incidence and with a decreasing latency (Hall et al., 1941). Beard's group adopted the name "erythromyeloblastosis" virus, later changing the name to myeloblastosis virus. AMV BAI strain A causes myeloblastosis, osteopetrosis, lymphoid leukosis, and nephroblastomas (Beard, 1963). The virus has been extensively studied because: (i) the virus caused a high incidence of disease with a short latency, (ii) the virus has a high titer (often $10^{10}$ - $10^{11}$ infectious units/ml plasma and $10^{10}$ - $10^{13}$ particles/ml plasma), and (iii) because leukemic cells could easily be grown in vitro. Much of the research with AMV has been reviewed by Beard (1963) and Moscovici (1975).

Three interesting isolates were found in Bulgaria by Todorov and his coworkers: MC29, MC31 and E26. MC29 and MC31 are both reported to have been isolated from chickens with myelocytomatosis and induce myelocytomatosis, liver carcinoma, kid-

ney carcinoma, as well as endotheliomas and mesotheliomas (Langlois et al., 1969a; Mladenov et al., 1967; Chabot et al., 1970; Beard et al., 1975; Beard et al., 1976; Beard et al., 1970). Recent studies of MC29 indicate that it induces hepato-carcinomas, adenocarcinomas and "soft tissue" sarcomas in addition to myelocytomatosis (Moscovici et al., 1977). MC31 has not been extensively studied. E26 was reported to cause an acute "erythroleukemia" within two to four weeks after innoculation of newborn chicks, quail and turkey (Ivanov et al., 1962). It may also cause myelocytomatosis in guinea fowl poults (Nedyalkov et al., 1975).

Another strain of virus capable of inducing myelocytomatosis was isolated by Loliger (1964a) in Germany. Following injection into newborn chicks this isolate was shown to cause myelocytomatosis in 10% of the birds in 4-12 weeks (Loliger, 1964b, Loliger and Schubert, 1966).

The most recent addition to this collection of viruses is OK10, which was isolated from the egg of a leukotic hen during a field test to determine the effects of 1-adamantanamine (Amantadine) on chickens naturally infected with leukosis (Oker-Blom et al., 1975). Three weeks after intraperitoneal injection of newborn chicks, tumors were observed in the mesentery, whereas intravenous injection resulted in tumors in the liver, kidneys and testes (Hortling, 1978).

What can perhaps be best said about these "early" observations on avian leukemia was that they established the following thoughts:

1) Virus capable of transmitting a disease could be isolated from leukemic birds.

2) Such isolates often produced a variety of different pathologies upon passage, but generally one specific pathology or group of pathologies can be reproducibly produced by such isolates following innoculation into new hosts.

3) The latency of disease often decreased with passage.

4) Transmission of the disease was affected by many factors including age, route of innoculation and genetic background of the bird.

The age of pathology in the field of avian leukemia viruses ended in the early 1960s. At this time it was not clear whether the virus isolates which had been studied for some thirty years were pure strains or mixtures of several viruses. Thus it could not be stated whether the multiple oncogenic potentials of some virus isolates were the result of one or several different viruses. The finding that the Bryan strain of RSV was a mixture of viruses, one of which was able to induce resistance to transformation by RSV (Rubin, 1960; Rubin and Vogt, 1962; Hanafusa et al., 1964), clearly indicated that some virus stocks were not as simple as had been envisioned. This result had clear implications for virologists who had assumed they were studying pure strains of avian leukemia viruses--in order for their studies to become meaningful, they had to use clonal stocks of viruses of a defined nature. This had been accomplished with RSV by working with an in vitro transformation system utilizing CEF. At this time, however, no one had developed biological systems for the

analysis of these viruses.

C. Review of Current Research: The Age of Cellular Biology and Molecular Biology The study of avian leukemia viruses entered the modern age with the development of in vitro systems in which specific target cells could be transformed by these viruses. This ultimately allowed the preparation of cloned virus stocks and the analysis of the cells transformed by these viruses. In addition, in vitro systems allowed each new biochemical technique to be applied to the study of the genetics and molecular biology of these viruses.

In a review entitled "Cell transformation by RNA tumor viruses," Hanafusa (1977) pointed out that there were clear differences among those viruses which had been referred to as the avian leukemia viruses. Hanafusa pointed out that the avian RNA tumor viruses could be divided into three groups: the sarcoma viruses, the acute leukemia viruses, and the lymphoid leukemia viruses (Table 1). The sarcoma viruses included the various strains of the Rous sarcoma viruses and several independent viruses including: Fujinami sarcoma virus (Fujinami and Inamoto, 1914), Y73 (Kawai et al., 1980), and PRCII (Carr and Campbell, 1958). These viruses all transform chicken embryo fibroblasts in vitro in 4-7 days and induce sarcoma formation in vivo in 5-12 days.

The lymphoid leukosis viruses (LLV) are replication-competent viruses which are not able to transform any known target cell in vitro, but in vivo cause lymphoid leukosis as described above. LLVs include a wide variety of field isolates of lymphatic leukemia viruses as well as the "helper" viruses of defective strains of transforming viruses (Vogt and Rubin,

TABLE 1

AVIAN AND MURINE LEUKEMIA VIRUSES

| | SARCOMA VIRUSES | ACUTE LEUKEMIA VIRUSES | LYMPHATIC LEUKEMIA VIRUSES |
|---|---|---|---|
| AVIAN | Rous Sarcoma Virus<br>Bryan High-Titer Strain<br>Prague Strain<br>Schmidt-Ruppin Strain<br>Harris Strain<br>Carr-Zilber Strain<br>Avian Sarcoma Virus B77<br>Fujinami Sarcoma Virus<br>Avian Sarcoma Virus PRCII<br>Avian Sarcoma Virus Y73<br>Avian Sarcoma Virus UR1<br>Avian Sarcoma Virus UR2 | Avian Erythroblastosis Virus<br>Strain E54<br>Strain R<br>Avian Myeloblastosis Virus<br>E26 Avian Leukemia Virus<br>Avain Myelocytomatosis Virus<br>Strain MC29<br>Strain OK10<br>Strain CMII<br>Strain MH2 | Avian Lymphomatosis Viruses<br>Strain RPL12<br>Resistance-Inducing Factors<br>Rous-Associated Viruses<br>Another Rous Contaminant (ARL) |
| MURINE | Moloney Sarcoma Virus<br>Harvey Sarcoma Virus<br>Kirsten Sarcoma Virus<br>Finkel-Biskis-Jinkins Sarcoma Virus<br>Gazdar Sarcoma Virus | Friend Spleen Focus-Forming Virus<br>Rauscher Spleen Focus-Forming Virus<br>Abelson Leukemia Virus | Murine Leukemia Viruses<br>Gross<br>Graffi<br>Moloney<br>Kirsten<br>Tewnant<br>Radiation-Induced Leukemia Virus<br>LLV Associated with Acute Leukemia viruses and Sarcoma viruses<br>AKR LLV |

1962) and transformation-defective mutants of nondefective Rous sarcoma viruses (Biggs et al., 1973; Purchase et al., 1977a). The latency of the disease is quite long, four to twelve months. Clone-purified stocks of LLV are still able to induce a variety of neoplasms in addition to lymphatic leukemia including osteopetrosis, nephroblastoma, and erythroblastosis (Purchase and Gilmour, 1975; Biggs et al., 1973; Purchase et al., 1977a; Smith and Moscovici, 1969) and in fact it is not clear whether there is a cloned LLV strain which is capable of causing only lymphatic leukemia. The earliest detectable pathological change induced by LLV are observed in the bursa, 4 to 8 weeks after the innoculation of newborn chicks (Purchase, 1976). The target for LLV is apparently a pre-B lymphocyte in the bursa, since bursectomy of newborn chicks renders them resistant to LLV (Peterson et al., 1964; Peterson et al., 1966; Purchase et al., 1977b). In addition, bursectomized chicks can be made susceptible again by transplanting new bursal cells into the host (Purchase et al., 1977b). LLV-induced lymphatic leukemia cells contain IgM on their surface but not IgG or IgA (Cooper et al., 1974), suggesting that there is a block in the switch from IgM to IgG or IgA. The results of Neel et al. (1981) and Payne et al. (1981) suggest that LLV may mediate lymphocyte transformation via insertion of a viral sequence which serves as an active promoter resulting in the increased expression of a normal cellular gene. The elevated expression of this gene is directly responsible for the transformation of the cell. In about 85% of birds with lymphoid leukosis, the sequence which is elevated is related to the virus MC29 (Hay-

ward et al., 1981).

The acute leukemia viruses differ from LLV in that they do transform specific bone marrow target cells in vitro and in addition they cause an acute disease with a latency of 2-4 weeks. Based upon RNA sequence homology and upon cell markers present in transformed bone marrow cells, the known acute leukemia virus isolates have been divided into three groups (see Table 2). The three groups are: the avian erythroblastosis virus group which contain the erb specific sequences; the avian myeloblastosis virus group which contain the myb specific sequences, and the avian myelocytomatosis virus group which contain the mac specific sequences. cDNA probes specific for these specific sequences are referred to as cDNAerb, cDNAmyb and cDNAmac. These properties of the acute leukemia viruses have been reviewed recently by Graf and Beug (1978) and by Hayman (1981). Since it is these viruses that are the subject of my thesis work, I shall describe what is known about the prototype member of each virus subgroup and point out differences where they are known to exist among the other members of that subgroup.

1. Avian Erythroblastosis Virus (AEV).

The two members of this group are AEV strain ES4 and AEV strain R, both isolated by Engelbreth-Holm and his coworkers in the 1930's (Engelbreth-Holm and Rothe-Meyer, 1935). The ES4 strain has been the most widely studied, and recently the possibility that strain R and strain ES4 are now the same viruses has been raised (Hayman et al., 1979). Infection of CEF with

TABLE 2

AVIAN ACUTE LEUKEMIA VIRUSES

| SPECIFIC SEQUENCE | VIRUS STRAIN | TYPE OF NEOPLASM IN CHICKEN OF ORIGIN | PREDOMINANT TYPE OF NEOPLASM PRODUCED BY VIRUS INFECTION | COUNTRY OF ORIGIN |
|---|---|---|---|---|
| erb | AEV-ES4 | Sarcoma and Myelocytomatosis | Erythroblastosis and Sarcoma | Denmark (1933) |
| | AEV-R | Erythroblastosis | Erythroblastosis | Denmark (1931) |
| myb | AMV-BAI-strain A | Neurolymphomatosis | Myeloblastosis | USA (1941) |
| | E26 | Not Known | Myeloblastosis, Erythroblastosis? | Bulgaria (1962) |
| mac | MC29 | Myelocytomatosis | Myelocytomasis, liver and Kidney Carcinoma | Bulgaria (1964) |
| | CMII | Myelocytomatosis | Myelocytomatosis | Germany (1964) |
| | MH2 | Endothelioma | Liver and Kidney Carcinoma, Sarcomas | England (1927) |
| | OK10 | Endothelioma | Monocytic leukemia (?) Endothelioma (?) | Finland (1975) |

AEV gives rise to discrete foci of transformed cells (Ishizaki and Shimizu, 1970; Graf et al., 1976a; Graf et al., 1976b) and plating AEV-infected bone marrow cells in soft agar gives rise to colonies of erythroblasts (Graf, 1975; Graf et al., 1976a; Graf et al., 1976b). Analysis of clones isolated in this manner demonstrated that both strains ES4 and R are replication-defective and require the presence of a helper virus to produce infectious virus (Graf et al., 1976b). Studies utilizing cloned stocks of AEV produced by superinfection of NP clones with helper virus confirmed early observations of Engelbreth-Holm (Engelbreth-Holm and Rothe-Meyer, 1935) that strain ES was capable of causing both sarcomas or erythroblastosis, depending on the route of innoculation used (Graf et al., 1976a; Graf et al., 1977a). These experiments clearly demonstrated that a cloned stock of AEV had a multiple oncogenic potential, something not ruled out by the Engelbreth-Holm studies since it was possible they were studying the effects of a mixture of viruses with different tropisms.

The RNA of AEV has been extensively analyzed and the genomic RNA found to be 28 S or 5.5 to 5.7 kb (Bister and Duesberg, 1979; Kamahora et al., 1979; Lai et al., 1979, see Figure 3). Mapping of RNase $T_1$ resistant oligonucleotides (Bister and Duesberg, 1979; Kamahora et al., 1979) has shown there is 1 kb at the 5' and 1.5 kb at the 3' end of the virus in common with the helper virus of AEV, and approximately 3 kb of unique sequence RNA in the middle of the molecule. Heteroduplex mapping (Lai et al., 1979) supports these data. The unique region of AEV is not related to the unique sequences present in MC29 or

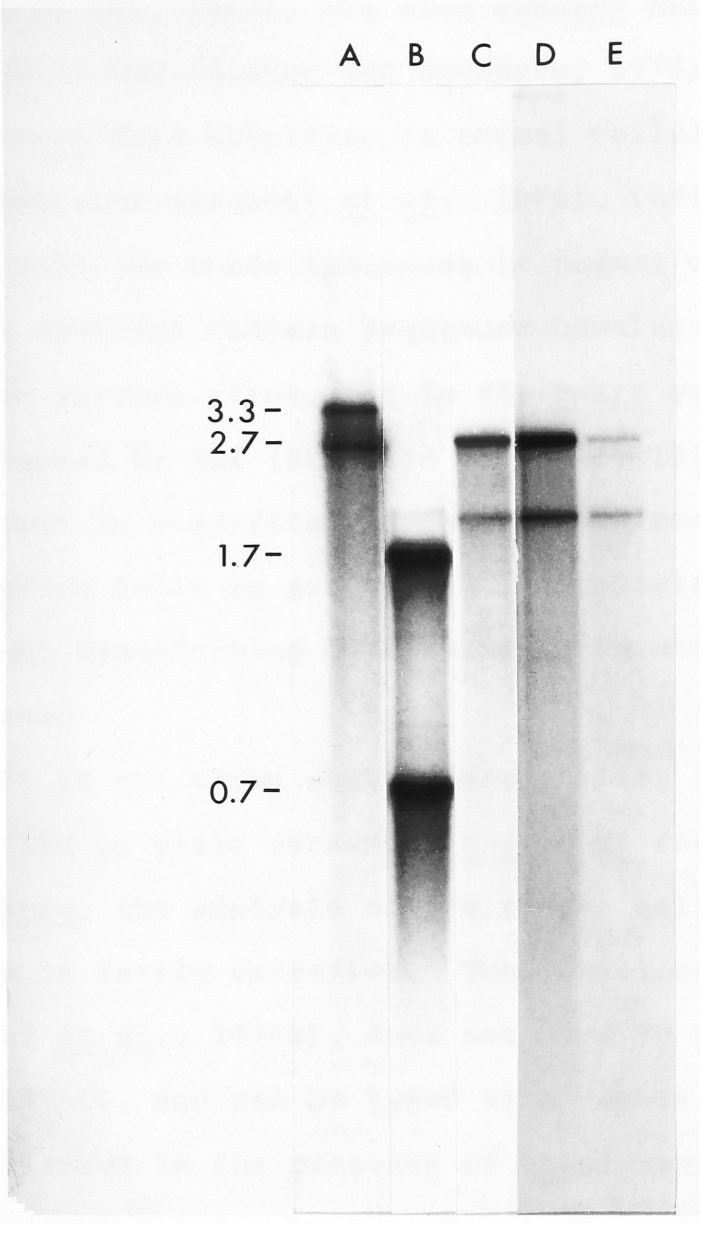


FIGURE 3. Sizing of AEV viral RNA by glyoxal gel electrophoresis. The viral RNA from Schmidt-Ruppin RSV-infected cells and three independently isolated AEV-transformed producer clones was labeled with $^{32}P$ and the poly(A)-containing RNA isolated. The viral RNAs and marker ribosomal RNAs were denatured with glyoxal and separated by electrophoresis in a 1% agarose gel. Lane A, Schmidt-Ruppin RSV RNA; lane B, ribosomal RNA marker RNA; and lanes C-E, viral RNA from three AEV producer clones. Molecular weight x $10^{-6}$ daltons is indicated on the left.

AMV by $T_1$ oligonucleotide fingerprinting (Bister and Duesberg, 1979a; Duesberg et al., 1980), nor does cDNAerb hybridize to either AMV, MC29 or RSV (Bister and Duesberg, 1979; Roussel et al., 1979). cDNAerb does hybridize to normal cellular DNA of all higher vertebrates (Roussel et al., 1979), indicating that there may be a role for these sequences in normal cell processes. AEV does not contain sequences homologous to the src gene of Rous sarcoma virus, nor is the c-src gene activated in cells transformed by AEV (Stehelin and Graf, 1978). The latter observation is significant because it is possible that AEV could transform cells by activating the cellular homologue of another virus' transforming gene rather than utilize its own transforming gene.

Although it is not known what target cell(s) is transformed by AEV to yield sarcomas in vivo or transformed fibroblasts in vitro, the analysis of the target cell present in the bone marrow is fairly extensive. The target cell is non-phagocytic (Graf et al., 1976b), does not have Fc receptors (Graf et al., 1976b), and can be lysed with rabbit anti-chicken erythrocyte antiserum in the presence of complement (Graf et al., 1977b). Recent studies by Gazzolo et al. (1980) indicate that the bone marrow target cells have a light density (1.060 - 1.065 $g/cm^3$), sediment at a rate of 4.0 - 6.0 mm/h at unit gravity (a measure of cell size), express an "immature antigen" at a low level, and express a brain-related antigen at a high level. These studies suggest the target cell for AEV is a subpopulation of the $BFU_E$ (Gazzolo et al., 1980), an early stage of erythrocyte maturation (Samarut and Bouabdelli, 1980).

Chickens treated with exogenous "erythropoietin" before infection with AEV are less susceptible to induction of erythroblastosis and show a decreased incidence of erythroblastosis (Rusov et al., 1975), further substantiating the claim that AEV's target cell is an immature erythroid precursor. Further discussion of this virus' transcription and translation products will be given in the Results section of this thesis.

2. The avian myelocytomatosis virus group.

This group of viruses includes four independent isolates: MC29, MH2, CMII and OK10. These four isolates share sequence homology as detected by RNase $T_1$ oligonucleotide fingerprinting (Duesberg and Vogt, 1979; Bister et al., 1980) and by hybridization with a cDNA probe specific to the unique sequence of MC29 (Roussel et al., 1979). The MC29-specific sequences have been named mac and these sequences are highly conserved among all higher vertebrates (Roussel et al., 1979 and Sheiness et al., 1980). The prototype of this group is MC29 and the various pathologies caused by the virus have been extensively studied (Mladenov et al., 1967; Langlois et al., 1969a; Chabot et al., 1970; Beard et al., 1975; Beard et al., 1976) and reviewed (Langlois et al., 1969a; Beard et al., 1970). As stated above MC29 induces myelocytomatosis, liver and kidney carcinomas, endotheliomas, mesotheliomas, adenocarcinomas and soft tissue sarcomas (Beard et al., 1970; Beard et al., 1975, Moscovici et al., 1977). MC29 is able to transform CEF (Langlois and Beard, 1967; Bolognesi et al., 1968) as well as bone marrow cells (Todorov and Yakimov, 1967). Graf (1973) noted that two types of

colonies were produced by MC29-infected CEF plated in soft agar; one corresponding to a fibroblast target cell and other to a bone marrow target cell. Further studies by Graf *et al.* (1976b) have revealed that MC29 transforms both macrophage and bone marrow cells and that these target cells in both macrophage and bone marrow cells, can be removed by allowing phagocytic cells to ingest iron filings and separating them with a magnet.

MC29-transformed bone marrow cells have fc receptors, are phagocytic, and express a macrophage specific antigen(s) on their cell surface (Graf *et al.*, 1977b). A *mac* specific cell surface marker, detectable by immunofluorescence is expressed by cells transformed by any virus of this group (Beug *et al.*, 1979). Gazzolo *et al.* (1979) have further characterized the bone marrow target for MC29 and MH2. The target cell has a light density (1.0550 to 1.0760 $g/m^3$), expresses both fc and complement receptors, and it is phagocytic. They were able to separate the target cells for AMV and MC29 by sedimentation at unit gravity (Gazzolo *et al.*, 1979).

The defectiveness of MC29 was demonstrated by Bister *et al.* (1977) and Bister and Vogt (1978). NP clones of MC29-transformed quail cells were isolated. The only viral protein detectable in these NP cell lines, by immunoprecipitation with antisera directed against the viral structural proteins, was a 110,000 dalton protein immunoprecipitated by anti-*gag* antiserum (Bister *et al.*, 1977). The protein contains p19 and part of p27 from the *gag* gene (Bister *et al.*, 1977; Rettenmier *et al.*, 1979). The virus is defective in *gag*, *pol*, and *env* (Bister and

Vogt, 1978).

The viral RNA of MC29 is 28 S or 5.7 kb (Duesberg et al., 1977; Mellon et al., 1978; Duesberg et al., 1979) with 5' and 3' common sequences flanking a contiguous stretch of unique sequences (Duesberg et al., 1977; Mellon et al., 1978; Duesberg et al., 1979). The virus produces only one mRNA of 28 S (Sheiniss et al., 1981) which presumably codes for the 110,000 gag-related polyprotein (Mellon et al., 1978). There are no sequences in MC29 homologous to the src gene of RSV and the expression of c-src is not enhanced in MC29 transformed cells (Stehelin and Graf, 1978).

Like MC29, CMII transforms both CEF and chicken bone marrow cells (Graf et al., 1977c). CMII-transformed bone marrow cells are phagocytic and require the presence of colony stimulating activity (CSA) to grow in soft agar (Graf et al., 1977c; Gazzolo et al., 1979). In addition, the target cells can be separated from the normal $CFU_c$ by cell separation experiments (Graf et al., 1977c). Normal granulocyte-macrophage progenitor cells are able to grow in vitro and produce colonies called $CFU_c$ (colony forming unit-culture). The growth of $CFU_c$, as well as many leukemic cells, is strictly dependent upon a source of growth factors referred to as colony stimulating factors (CSF) or colony stimulating activity (CSA) (Metcalf, 1977; Till and McCulloch, 1980). CMII-transformed quail NP clones contain a 90,000 dalton gag-related polyprotein but no $Pr76^{gag}$, $Pr180^{gag-pol}$ or gp85 (Hayman et al., 1979). Thus CMII, like MC29, is defective in gag, pol and env. Comparison of methionine tryptic peptide maps of the CMII 90,000 dalton pro-

tein and the MC29 110,000 dalton protein demonstrated that they share two non-gag-related peptides (Kitchener and Hayman, 1980). The RNA of CMII is 6 kb and RNase $T_1$ oligonucleotide fingerprinting indicates that CMII shares sequence homology with both MC29 and MH2 (Bister et al., 1979) which coincides with sequence homology observed by hybridization of CMII RNA with a MC29-specific cDNA probe (Roussel et al., 1979).

MH2 is able to transform both CEF and chick yolk sac macrophage in a fashion similar to MC29 (Hu et al., 1978). Nonproducer cells contain only a single gag-related polyprotein of 100,000 daltons, and do not contain $Pr76^{gag}$, $Pr180^{gag-pol}$ or gp85 (Hu et al., 1978). The virus is defective in gag, pol and env (Hu and Vogt, 1979). The RNA of MH2 is 28 S or 5.7 kb and RNase $T_1$ fingerprinting indicates it contains a unique sequence closely related to that of MC29 (Duesberg and Vogt, 1979; Bister et al., 1979).

OK10 is able to transform chicken bone marrow cells and quail embryo fibroblasts (Graf et al., 1979) and OK10-transformed bone marrow cells express an antigen which is specific for cells transformed by avian myeloblastosis viruses (Beug et al., 1979). NP clones of OK10-transformed quail fibroblasts can be isolated (Graf et al., 1979). The RNA of OK10 is approximately 6 kb (Bister et al., 1980). OK10 RNA hybridizes to a MC29-specific cDNA probe (Roussel et al., 1979), and fingerprinting of RNase $T_1$ resistant oligonucleotides confirms this sequence homology (Bister et al., 1980). These four viruses have common 5' gag sequences and 3' specific sequences but vary in the amount of gag and pol sequences they contain in

the 5' end of their specific sequences (Bister et al., 1980). OK10-transformed NP quail embryo fibroblasts contain $Pr76^{gag}$ and a 200,000 dalton gag-related polyprotein (p200) (Ramsey and Hayman, 1980). OK10 p200 contains all but one of the methionine-labeled tryptic peptides present in $Pr180^{gag-pol}$ and also contains four OK10-specific peptides, one of which is common to MC29 and CMII (Ramsey and Hayman, 1980). The cells also produce a noninfectious virus particle that lacks reverse transciptase activity (Ramsey and Hayman, 1980).

3. The avian myeloblastosis virus group.

This group of viruses contains one very well characterized member, AMV, and a second member, E26. This grouping is based upon sequence homology with a cDNA probe specific for AMV (cDNA myb) (Roussel et al., 1979) and upon the presence of the myb specific cell marker on both AMV- and E26-transformed bone marrow cells (Beug et al., 1979).

AMV has been the subject of two very comprehensive reviews (Beard, 1963; Moscovici, 1975). The BAI strain A of AMV was thought to be a pure strain of leukemia virus, however, two viruses of subgroups A and B were isolated by endpoint dilution or by growth on CEF which are resistant to certain envelope subgroups (Vogt 1965; Vogt and Ishizaki, 1966). These two isolates proved not to cause myeloblastosis, but did cause osteopetrosis and were named MAV-1 and MAV-2 (Smith and Moscovici, 1969). MAV-1 and MAV-2 are typical replication competent helper viruses with the exception of their ability to cause osteopetrosis with high incidence (Smith and Moscovici, 1969).

*In vitro* studies of bone marrow transformation with AMV began very early: the first successful attempt was by Doljanski and Pikovski (1942) and this work was confirmed by Beaudreau *et al.* (1960) and Lagelof (1960). Further studies on transformation of avian hematopoietic cells were reported by Baluda and Goetz (1961), Baluda *et al.* (1964), and Moscovici and Vogt (1968). Moscovici and his coworkers have emphasized transformation of chicken yolk sac cells utilizing a technique recently described (Moscovici and Moscovici, 1973). NP clones of AMV-transformed yolk sac cells were first isolated by Moscovici and Zanetti (1970) and further analysis by Moscovici and coworkers (Moscovici *et al.*, 1975; Moscovici, 1975), indicated that AMV was defective in at least the *env* gene. AMV could be rescued from NP yolk sac clones by helper viruses from subgroups B, C, and D (Moscovici, 1975) indicating there may be a resistance to specific subgroups of helper viruses in yolk sac cells which is not expressed in CEF (Gazzolo *et al.*, 1975).

The growth and differentiation of hematopoietic stem cells can be followed by a variety of *in vitro* assays (Metcalf, 1977; Till and McCulloch, 1980). Cells committed to differentiation along the myeloid/granulocyte pathway are called colony forming units (CFUc) and their growth is strictly dependent upon the presence of an inducer called colony stimulating factor (CSF) (Metcalf, 1977; Till and McCulloch, 1980). Dodge and Moscovici (1973) developed a CFUc assay for avian hematopoietic stem cells and compared the growth of normal bone marrow cells and leukemic myeloblasts. Normal hematopoietic cells produce a large diffuse colony of cells (Type I colonies) which appear to

be phagocytic cells (macrophages) and granulocytes (Dodge and Moscovici, 1973). Apparently normal colonies (Type I) can be produced by both primitive hematopoietic stem cells and by differentiated macrophages (Dodge et al., 1975). Leukemic cells form smaller, dense colonies of cells (Type II colonies) which are less dependent upon the presence of CSF, but which have a lower plating efficiency (Dodge and Moscovici, 1973). In a study of CSF levels in the sera of normal, leukemic and regressor chickens, leukemic birds had lower levels of CSF capable of supporting normal colony formation but higher levels of CSF capable of supporting colony formation by leukemic myeloblasts, when compared to either normal birds or birds that had regressed from AMV induced leukemia (Silva et al., 1974). A recent study (Bryant et al., 1980) has shown that the CSA of normal and leukemic plasma were the same when tested undiluted, but dilution showed there was more CSA in leukemic plasma. Gel filtration clearly showed 5-10 times as much CSA in leukemic plasma as compared to normal chicken plasma. Leukemic plasma also contained a very potent inhibitor of colony formation not present in normal plasma (Bryant et al., 1980).

AMV viral RNA has been sized and found to be 33-34 S or 7.5 kb (Chen et al., 1981; Gonda et al., 1981). Analysis of unintegrated proviral DNA (Bergman et al., 1980), integrated proviral DNA (Souza et al., 1980c), and of a molecularly cloned proviral AMV DNA supports this size (Souza et al., 1980a; Souza, et al., 1980b). AMV does not contain sequences homologous to the src gene (Stehelin et al., 1976, Roussel et al., 1979, Chen et al., 1980), but instead a unique sequence called

myb which presumably represents the transforming sequence of this virus (Roussel et al., 1979; Chen et al., 1980). The myb specific sequence has been analyzed by RNase $T_1$-fingerprinting (Duesberg et al., 1980) and restriction mapping (Souza et al., 1980b), and heteroduplex mapping (Souza et al., 1980d), and it maps at the 3' end of the viral genome. The myb sequence is highly conserved in all higher vertebrates (Roussel et al., 1979). In the chicken it is expressed in all the tissues examined (Chen, 1980), but its expression varies with both the age of the chicken and the tissue examined (Chen, 1980). The myb gene is transcribed to produce a subgenomic mRNA of 2.1-2.3 kb (Chen et al., 1981; Gonda et al., 1981) which contains only the viral leader and 3' c region in addition to the myb sequences. All of the gag gene and all or part of the pol gene, but none of the env gene, are present in the viral genome (Chen et al., 1981; Gonda et al., 1981). Thus AMV is defective in at least the env gene and perhaps also pol. A noninfectious virus particle containing the viral RNA is produced by AMV NP cell indicating that the gag gene is functional, but these particles either lack reverse transcriptase or contain very low amounts of it (Duesberg et al., 1980; Chen et al., 1981).

The other member of the avian myeloblastosis virus group, E26, is very poorly characterized. As stated above, E26 was reported to cause an acute "erythroleukemia" in newborn chicks, quail and turkey (Ivanov et al., 1962). It may also cause myelocytomatosis in guinea fowl poults (Nedyalkov et al., 1975). A characterization of the genome of E26 has not been published. E26 transforms chicken bone marrow cells and quail

embryo fibroblasts (Graf *et al.*, 1979) and NP clones of cells can be isolated indicating E26 is defective. E26-transformed bone marrow cells appear similar to AMV-transformed myeloblasts and contain a *myb* specific cell surface antigen (Beug *et al.*, 1979). NP clones of E26-transformed bone marrow cells contain a *gag*-related polyprotein of 125,000 daltons but no $Pr76^{gag}$, $Pr180^{gag-pol}$, or gp85 (see Figure 24) (S.M. Anderson and C. Moscovici, unpublished observations).

Scope of this thesis.

In my research I have sought to identify the transforming protein of two acute leukemia viruses, avian erythroblastosis virus (AEV) and avian myeloblastosis virus (AMV). The transciptional and translational products of AEV were identified and characterized. AEV-specific proteins were identified by immunoprecipitation of labeled cell extracts and by in vitro translation of AEV virion RNA. Two translational products were identified and characterized. An antibody specific for one of these two proteins was prepared and used in further analysis of this protein. The possible role of each protein in transformation by AEV is discussed.

The translation products of AMV were also analyzed by immunoprecipitatin of labeled cell extracts and by in vitro translation of AMV virion RNA. AMV-specific translation products were identified.

# II. Materials and Methods

## Materials and Methods

Primary cells and cell lines. Chicken embryo fibroblasts (CEF) were prepared from eleven day-old embryos as described (Hanafusa, 1969). All embryos used in these studies were negative for both group-specific (gs) antigen and chick helper factor (chf). gs antigen is the gag gene product of endogenous viruses and is detected by a complement-fixation assay using visceral extracts from chick embryos as antigen and antisera from tumor-bearing hamsters. chf is the env gene product of endogenous avian viruses. The presence of chf is a determined by assaying for the production of subgroup E pseudotyped virus, the subgroup of chf, following infection of CEF with a RSV(RAV-2) (H. Hanafusa et al., 1970; T. Hanafusa et al., 1970). Both assays are routinely performed in the Hanafusa laboratory (on all chicken embryos). Fertile chicken eggs obtained from either SPAFAS (Norwich, Conn) or Life Sciences, Inc (St. Petersburg, Florida). CEF were grown in either Scherer's media with 5% calf serum and 10% tryptose phosphate broth or Ham's F10 with 5% calf serum and 10% tryptose phosphate broth. Both media contained 10 units/ml penicillin and 0.5 mg/ml streptomycin.

Primary quail embryo cells were prepared from thirteen day-old embryos obtained from Life Sciences, Inc (St. Petersburg, FL) and were grown in Ham's F10 with 5% calf serum, 2% chick serum, 10% tryptose phosphate broth, and 0.5% dimethyl sufloxide with penicillin and streptomycin as above.

Yolk sac cells were prepared as described by Moscovici and Moscovici (1973) and were grown in BT-88 (Moscovici and Moscov-

ici, 1973), or in RPMI 1640 (GIBCO) with 10% tryptose phosphate broth, 5% calf serum, 5% heat-inactivated chick serum, 1% 100 X BME vitamins (Flow), 0.008 mg/ml folic acid, 0.5% dimethyl sulfoxide, 10 units/ml penicillin and 0.5 mg/ml streptomycin.

A variety of cell lines were obtained from many different sources. Table 3 lists these cell lines, the media used for their propagation, the properties of the cell lines and the donors of these cell lines.

Viruses. AEV, Strain ES4 was obtained from R. Ishizaki, Duke University (currently at the Cancer Resesarch Laboratory, Nippon Medical School, Tokyo, Japan). This virus was a subgroup B virus and I isolated a B subgroup nontransforming helper virus (EAV), by endpoint dilution. AEV ts34 (RAV-2) was the gift of Thomas Graf and it was isolated following mutagenisis with 5-azacytidine (Graf et. al, 1978).

The AMV-A and AMV-B was obtained from Dr. Joseph Beard, Life Sciences, Inc. The group A and B helper viruses were isolated by endpoint dilution (Smith and Moscovici, 1969). MAV-1 and MAV-2, the subgroup A and B helper viruses of AMV, respectively, are nontransforming viruses which cause lymphoid leukosis, nephroblastoma and osteopetrosis (Smith and Moscovici, 1969). AMV(RAV-1) AMV(RAV-2) and AMV(tdB77) stocks were provided by Dr. Carlo Moscovici (Veterans Administration Hospital, Gainesville, Florida). E26 was the gift of Dr. Carlo Moscovici.

MC29 subgroups A and B were obtained from Dr. Langlois, Duke University. The subgroup A and B nontransforming helper

TABLE 3

CELL LINES USED IN THESE STUDIES

| CELL LINE | VIRUS | MEDIA | TYPE OF CELL | SOURCE |
|---|---|---|---|---|
| *ts*34 A6L1 [LSEC(AE/EB2-1] | AEV *ts*34 | RPMI 1640 as described | chicken bone marrow | T. Graf |
| BM2 | AMV NP | RPMI 1640 as described | chick bone marrow | C. Moscovici |
| AC3b | AEV | DME, 10% calf | rat fibroblast | K. Quade |
| AC4a | AEV | DME, 10% calf | rat fibroblast | K. Quade |
| MC2 | AEV | DME, 10% calf | rat fibroblast | K. Quade |
| MC1a | AEV | DME, 10% calf | rat fibroblast | K. Quade |
| 208F | -- | DME, 10% calf | normal rat fibroblast | K. Quade |
| MC29Q8 | MC29 NP | F10, 5% calf, 2% chick, 10% TPB | quail fibroblast | K. Bister |
| 297-C43 | human adenovirus type 5 | DME, 10% calf | hamster cell line | J. Nevins |
| SV40-transformed muntjac | SV40 | DME, 10% calf | muntjac cell line | E. Gershey |
| SFFV/NRK | Friend SFFV | DME, 10% calf | NRK cells | E. Scolnick |
| HSV/NIH | Harvey sarcoma virus | DME, 10% calf | NIH mouse cell line | E. Scolnick |
| ST-FeSV/mink | Snyder Theilen FeSV | DME, 10% calf | mink cell line | E. Scolnick |
| C127D | -- | DME, 10% calf | normal NIH cell line | E. Scolnick |

viruses, MCAV-A and MCAV-B, were isolated by endpoint dilution from these virus stocks.

RAV-1, RAV-2, RAV-7, RAV-50, RAV-61, td109, the Schmidt-Ruppin strain of Rous sarcoma virus, and the Bryan high titer strain of Rous sarcoma virus were from the stocks of the Hanafusa laboratory.

Isolation of nonproducer (NP) clones. Chicken embryo fibroblasts were infected with tenfold dilutions of virus 4-8 h after plating. DEAE-dextran was added to each infection to enhance adsorption of non-A subgroup virus stocks to the cells. The cells were trypsinized 8-12 h later, washed with media, and plated in 10 ml soft agar over a 15 ml hard agar base. Soft agar contained 20% (vol/vol) 2X F10 (GIBCO Labortories), 6% (vol/vol) tryptose phosphate broth (Difco Laboratories), 2.9% (vol/vol) of a 2.8% solution (wt/vol) $NaHCO_3$, 4% (vol/vol) calf serum (GIBCO), 2% (vol/vol) heat-inactivated chick serum (GIBCO), 1% (vol/vol) 100X MEM vitamins (Flow laboratories), 0.008mg/ml folic acid, 0.5% (vol/vol) dimethyl sulfoxide, 10 u/ml penicillin, 0.5 mg/ml streptomycin, 40% (vol/vol) conditioned media from primary culture of chicken embryo fibroblasts, 20% (vol/vol) agar (1.8% wt/vol solution, Difco), and 1% (vol/vol) chicken neutralizing antiserum directed against the helper virus present in the virus stock. Hard agar subbase consisted of 40% (vol/vol) 2X F10, 10% (vol/vol) tryptose phosphate broth, 6.25% (vol/vol) of a 2.8% solution (wt/vol) $NaHCO_3$, 6% (vol/vol) calf serum (GIBCO) 2% (vol/vol) heat-inactivated chick serum, 1% (vol/vol) 100X MEM vitamins (Flow),

0.008 mg/ml folic acid, 0.5% (vol/vol) dimethyl sulfoxide, 10 u/ml penicillin, 0.5 mg/ml streptomycin, 33% (vol/vol) Agar (1.8% wt/vol Difco).

After one week the cultures were fed with an additional 10 ml soft agar. Two weeks after infection, discrete colonies of cells were picked from the soft agar with 1 ml syringes and grown as clones in either 35 mm tissue culture plates or in Linbro tissue culture multiple-well plates ($2cm^2$/well, Flow). Nonproducer clones were identified by screening for polymerase positive virus particles in the supernatant fluids of infected cells; nonproducer clones of acute leukemia virus-transformed cells do not release polymerase positive particles.

Polymerase assay. Five to ten ml of tissue culture supernatant fluids were clarified by a low speed spin (2000 rpm for 10 minutes in a Damon/International PR-J centrifuge). The clarified sups were spun at 36,000 rpm in a Beckman Type 40 Rotor for 1 h. The supernatant fluids were poured off, the tubes inverted in a test tube rack and placed at $4^{\circ}$ C to drain. The pellets were solubilized in 50 ul of 0.083 M Tris [pH 8.3], 0.01 M magnesium acetate, 0.1 M NaCl, 0.16% NP-40, 0.32 M dithiothreitol. 50 ul of the solubilized virus was mixed with 50 ul of template-primer mixture; 3.75 ug (rC)n: (dG)12-18 (Collaborative Research) and 250pmol $^{3}$H-dGTP 500cpm/pmol (New England Nuclear). The reaction was incubated at $37^{\circ}$ C for 1 h. The reaction was terminated by adding 0.2 ml of 0.025% (wt/vol) bovine serum albumin and 0.7 ml 20% (wt/vol) trichloracetic acid, 0.014 M sodium pyrophosphate. The samples were incubated

on ice for 1 h and the acid precipitates collected on GFC/A (Millipore) filters, washed 3 times with 5% TCA, then dried and counted in a nonaqueous scintillation cocktail such as Betafloar (National Diagnostics). Negative samples contained 200-500 cpm/filter whereas positive samples contained 10,000 to 120,000 cpm/filter.

Isotopic labeling of cells and viruses. Cells were labeled in one of the following manners: (i) with 100-500 uCi $^{35}$S-methionine (Amersham or New England Nuclear) in 2 ml methionine-free Eagle Miminal Essential Media with 2% calf serum for 4-12 h; (ii) with 100-500 uCi of $^{35}$S-Cystine (New England Nuclear) in 2 ml cystine-free minimal essential media with 25% methionine and 2% calf serum for 4 h; (iii) for 12 h with 1-10 mCi carrier-free $^{32}$P-ortho-phosphate (Amersham or New England Nuclear) in 10 ml phosphate-free M199 (GIBCO) with 2% dialyzed calf serum.

Virus for protein analysis was labeled with either $^{35}$S-methionine or $^{35}$S-cystine for two successive 12 h pulses with 250 uCi in 5 ml of the appropriate amino acid deficient media, followed by a 12 h chase in complete media. The culture fluids were clarified by centrifugation at 8,000 rpm in Sorval SS-34 rotor for 10 minutes. The supernatant was then centrifuged through a layer of 20% (wt/vol) sucrose in TEN (10 mm Tris [pH 7.4], 1 mM EDTA, 150 mM NaCl) onto a cushion of 60% (wt/vol) sucrose in TEN at 24,000 RPM for 3 h in a Beckman SW27 rotor. The virus banding at the interface between the 20% and 60% sucrose was collected, diluted three-fold with TEN, layered

over a linear 20-50% sucrose gradient, and spun overnight at 22,000 rpm in a Beckman SW27.1 rotor. The material banding at 35 to 40% sucrose was pelleted in a Beckman type 40 rotor at 40,000 rpm for 1.5 h.

$^{32}$P-labeled virus for protein analysis, was prepared by labeling cultures for 12 h with 1 mCi carrier free $^{32}$P-orthophosphate in 10 ml phosphate-free M199 with 2% dialyzed calf serum and then chasing for 12 h in complete medium. The culture fluids were clarified by spinning at 8,000 rpm for 15 minutes in a Sorval SS-34 rotor. The virus was then pelleted by spinning for 14 h at 12,000 rpm in a Sovral SS-34 rotor.

Virus pellets were either solubilized directly in SDS sample buffer (62.5 mM Tris [pH 6.8], 3% SDS, 50 mM dithiothreitol, 10% glycerol with 0.001% bromophenol blue) or disrupted in RIPA buffer (0.05 M Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% (wt/vol) Triton-X100, 1% sodium deoxycholate, 0.5% SDS) for immunoprecipitation of specific virion proteins.

Immunoprecipitation. Cells were lysed in RIPA buffer with 1% Trasylol (a general protease inhibitor, FBA Pharmaceuticals, New York, New York). 1.0 ml RIPA was used per 100 mm plate, 0.4 ml RIPA per 60 mm plate, and 1.0 ml RIPA per $10^7$-$10^8$ cells grown in suspension culture. The cell lysates were disrupted with a vortex mixer for 30 sec and then spun at 36,000 rpm in a Beckman type 40 rotor for 15 minutes. The supernatant was either used immediately for immunoprecipitation or frozen at -70° C. Immunoprecipitation was performed utilizing Protein A from Staph. aureus as an immunoadsorbent (Kessler, 1975). Clarified

cell lysates were immunoprecipitated by adding 5 ul of antibody to 100 ul of cell lysate and incubating on ice for 1 h. 15 ul of a 50% suspension of Protein A-Sepharose (Sigma or Pharmica) in RIPA buffer was added for 1 h with gentle mixing on a rotating platform at 4° C. The immune complexes were washed five times with RIPA buffer, solubilized in 50 ul of SDS gel sample buffer, heated at 100° C for 4 minutes, and the proteins analyzed in SDS polyacrylamide gels.

Immunoprecipitation with chicken antiserum was carried out by a modification of this method. 5 ul of chicken antiserum was added to 100 ul of cell lysate and incubated on ice for 1 h. The IgG fraction of rabbit anti-chicken IgG was obtained from Miles Laboratories and was bound to Protein A-Sepharose by adding 220 ug of the rabbit IgG to 150 ul of a 50% suspension of Protein A-Sepharose and mixing gently for 1 h on a rotating platform. The chicken IgG immune complexes were collected by adding 15 ul of a 50% suspension of the (rabbit anti-chicken IgG)-Protein A-Sepharose complex to each immunoprecipitate and mixing on a rotating platform for 2 h at 4° C.The immune complexes were then processed as above.

Antisera. Antisera specific for each of the individual gag proteins were the kind gift of Dr. Volker Vogt, Cornell University. Anti-RAV-60 gp85 was prepared in this laboratory and was the gift of Dr. J. H. Chen and reacts with the env gene products of subgroups A-F (personal communication, C. Rettenmier).

Anti-AMV reverse transcriptase was obtained from two sources: Dr. J. H. Chen, Life Sciences, Inc., St. Petersburg,

Florida and Dr. Robert Nowinsky, University of Washington, Seattle, Washington. The anti-reverse transcriptase antisera from Dr. Chen reacted with gag proteins. To block antibodies directed against gag proteins, 10 ul was incubated with 120 ug of disrupted RAV-2 virions for 60 minutes on ice. The appropriate volume of absorbed antisera was used directly for immunoprecipitation. Anit-SV40 "T" serum was the gift of Dr. M.T. Hsu, Rockefeller University and antiserum specific for adenovirus Ela proteins was the gift of Dr. A.J. Levine, SUNY at Stonybrook. Antisera specific for the transforming proteins of Friend spleen focus-forming virus and the McDonough strain of Feline sarcoma virus was the gift of Dr. S. Ruscetti, National Cancer Institute. Rat anti-Harvey p21 serum was the gift of Drs. T. Shih and E.M. Scolnick, National Cancer Institute.

Anti-virion antibody was prepared by immunizing rabbits with 50 ug of disrupted purified RAV-2. Various anti-virion antisera showed different patterns of cross-reactivity with the viral proteins. Tumor-bearing rabbit sera, which precipitated $pp60^{src}$ of RSV, were the gift of Roger Karess and were prepared as described (Brugge and Erikson, 1977).

Chicken anti-p75 antiserum was prepared as follows: one-week old $gs^{-}chf^{-}$ chicks (SPAFAS, Norwich, Conn) were injected with $10^5$ FFU AEV ts34(RAV-2). In the next four weeks 10 of 19 chicks died of erythroblastosis. Eight weeks after innoculation the remaining chicks were immunized weekly with $10^7$ AEV ts34-transformed erythroblasts. 1-2 ml blood was collected weekly prior to immunization. Antisera were screened by immunoprecipitation of $^{35}S$-methionine labeled AEV-transformed

cells. Antisera capable of immunoprecipitating p75 were then screened with and without absorption with disrupted virion protein for 1 h on ice.

SDS polyacrylamide gels. Polyacrylamide slab gels (30:0.8, acrylamide:bis-acrylamide) containing 0.1% SDS were prepared using the discontinuous buffer system of Laemmli (Laemmli, 1970). The gels were stained with 0.05% Coomassie brilliant blue in 25% (vol/vol) 2-propanol, 7% (vol/vol) acetic acid and destained in methanol-acetic acid-water (5:1:5 [vol/vol]). Some gels were impregnated with PPO (2,5-diphenyloxazole) using either the PPO-DMSO method of Laskey and Mills (Laskey and Mills, 1975) or En$^3$Hance (New England Nuclear) according to manufacture's instructions. The processed gels were dried on Whatman 3MM paper and exposed to DuPont Cronex 2DC film.

Two-dimensional gel electrophoresis of proteins. Two-dimensional separation of proteins, using isoelectric focusing and SDS slab gel electrophoresis, was performed by the method of O'Farrell (1975). Isoelectric focusing in the first dimension was performed at constant power for a total of 6,000 to 8,000 volt-hours in a 4% polyacrylamide tube gels containing 9 M urea, 2% NP-40 and 2% ampholines (pH 3.5-10, LKB Instruments). Samples were loaded at the anode of the isoelectric focusing gel. After separation in the first dimension, gels were equilibrated in 6.25 mM Tris (pH 6.8), 2.3% SDS, 5% 2-mercaptoethanol for 30 minutes at room temperature. The second dimension was a 5 to 15% gradient slab gel. The gels were

fixed, stained and dried as described above.

Tryptic peptide analysis of radiolabeled proteins. Protein bands were located by autoradiography, excised from the gel, the paper scraped off, and washed four times for 15-30 minutes with 10% methanol in silconized 10 x 75 mm test tubes. Protein bands from gels which had been treated with the fluorographic agent En$^3$Hance, were first swelled in 1 ml 10% methanol for 30 minutes, washed four times for 30 minutes per wash in 50% methanol--50% acetic acid, to remove the precipitated PPO, and then washed four times with 10% methanol to remove the acetic acid. The washed gel slices were lyophilized to dryness and then oxidized with 0.3-0.5 ml performic acid (30% hydrogen peroxide and 88-90% formic acid, 1:19 [vol/vol], preincubated 1 h at room temperature) for 1 h on ice. The gel slices were lyophilized to dryness, rehydrated with 1 ml water, dried again, rehydrated a second time with 1 ml water, and then dried again to remove the remaining performic acid. 0.3 to 0.5 ml of 50 ug/ml L-(tosylamido 2-phenyl)ethyl chromethyl ketone-treated bovine trypsin (252 U/mg Worthington Biochemical Corp., Freehold, N.J.), in 0.05 M ammonium bicarbonate (pH 8) was added at approximately 8 h intervals. Total digestion time was 20-24 hours in a total of 1-1.5 ml of the trypsin solution (50-75 ug trypsin). The gel bands were removed, and the residue was lyophilized 2-3 times to remove ammonium bicarbonate. The final wash was in 100 ul of water to concentrate the sample. Samples were then dissolved in 10 ul of the appropriate electrophoresis buffer and applied to 10 x 20 cm or 20 x 20 cm cellulose coated

glass plates (E. Merck).

High voltage electrophoresis was carried out in the first dimension at either a pH 4.7 or pH 1.9. The pH 4.7 buffer consisted of n-Butanol-pyridine-acetic acid-water (2:1:1:18) and the pH 1.9 buffer consisted of acetic acid-formic acid-water (15:5:80). Electrophoresis at pH 4.7 was for 130 minutes at 600 volts on 20 x 20 cm plates and electrophoresis at pH 1.9 was for 20 minutes at 1000 volts on 10 x 20cm plates. Progress was monitored by a mixture of 2% orange G and 1% acid fuchsin dissolved in electrophoresis buffer. Ascending chromatography was then performed in the second dimension in n-butanol-pyridine-acetic acid-water (65:50:10:40). The dried plates were processed for fluorography by either (i) dipping into melted 0.4% (wt/wt) diphenyloxazole in 2-methylnaphthalene (Bonner and Stedman 1978) or (ii) sprayed with $En^3Hance$ spray (New England Nuclear). The treated plates were exposed to pre-flashed DuPont Cronex 2DC with DuPont Lightning Plus Intensifying Screens.

Assay for presence of Ac-Met-Glu-dipeptide. The presence of the acetylated Met-Glu dipeptide, the characteristic amino-terminal dipeptide of p19 and $Pr76^{gag}$ was determined as previously described (Rettenmier et al., 1979). The gel bands were processed as described above for tryptic peptides, except that they were digested with 50 ug of S. aureus V-8 protease (approximately 500 u/mg; Miles Laboratories, Elkhart, Ind.) in 1.0 ml 0.05 M sodium bicarbonate buffer pH 8.0. The peptides were spotted on 10 x 20 cm thin layer cellulose plates 1.5 cm from

the bottom in the center of the plate. Electrophoresis was for 15 minutes at 1000 V at pH 6.5 in pyridine-acetic acid-water (100:3:897). Ascending chromatography in the second dimension was in butanol:pyridine:acetic acid:water (65:50:10:40). The plates were processed for radioautography as described above.

Phosphoamino acid analysis. Tryptic peptides were prepared as above, lyophilized to concentrate the peptides, dissolved in 10 ul 6N HCl, and hydrolyzed in a sealed capillary tube for 90 minutes in a boiling water bath. The hydrolysates were lyophilized several times to remove HCl, and finally dissolved in 5-10 ul of pH 3.5 buffer (pyridine-acetic acid-water, 1:10:189). The samples were mixed with standards of phosphoserine, phosphothreonine and phosphotyrosine. Phosphoserine and phosphothreonine were from Sigma; phosphotyrosine was prepared by the method of Rothberg (et. al 1978) and was the gift of Roger Karess. Samples were spotted onto cellulose coated glass plates and electrophoresed in pH 3.5 buffer for 90 minutes at 500 volts. Marker peptides were located by spraying with ninhydrin and the $^{32}P$ label located by autoradiography.

In some experiments two-dimensional separation of phosphoamino acids was carried out as described by Hunter and Sefton (Hunter and Sefton, 1980). Electrophoresis was performed in the first dimension at pH 1.9 (acetic acid:formic acid:water, 15:5:80) at 1000 V for 1 h, followed by electrophoresis in the second dimension at pH 3.5 (acetic acid:pyridine:water, 50:5:945) for 1 h at 1000 volts. The plates were sprayed with ninhydrin and processed as above.

Isolation and preparation of RNAs.

Viral RNA. Virus was purified from clarified tissue culture fluids by pelleting the virus in a Beckman type 19 rotor at 17,000 rpm for 3 h. The pellet was dissolved in TEN and the virus banded in a 15-50% sucrose (TEN) gradient. The material banding between 35 and 40% sucrose was pooled, diluted 3-fold, and concentrated by spinning through 20% sucrose onto a 50% sucrose cushion. The purified virus was diluted two-fold with TEN and an equal volume of preincubated (1 h, 37° C) Proteinase K, (EM Laboratories, Inc., Elmsford, N.Y.) 1 mg/ml in 0.2% SDS, 10 mM EDTA, 10 mM Tris (pH 7.4), was added. The extract was incubated for 1 h at 37° C. After incubation, an equal volume of 10 mM Tris (pH 7.4) was added and the lysate extracted 2-3 times with phenol:chloroform:isoamyl alcohol (1:1:0.01 [vol/vol]) containing 0.1% 8-hydroxyquinoline and once with chloroform containing 1% isoamyl alcohol. The aqueous phase was collected, made 0.2 M sodium acetate, and precipitated by the addition of two volumes of ethanol.

RNA from tissue culture cells. RNA was isolated from tissue culture cells as described (Hayward, 1977). Cell monolayers were washed with isotonic buffer (4 g Trizma base [Sigma], 8 g NaCl, 0.38 g KCl, 0.1 g $Na_2HPO_4$, and 1 g glucose per liter, pH 7.4) and lysed in 0.5% SDS, 10 mM Tris (pH 7.4), 10 mM EDTA with 1 mg/ml Proteinase K (preincubated for 1 h at 37° C). The lysate was allowed to stand at room temperature for 1 h and during the first 20 minutes the DNA was sheared by 3-5 passages through a 20 guage needle. The lysate was then extracted 3-5 times with phenol:chloroform:isoamyl alcohol and once with

chloroform containing 1% isoamyl alcohol and precipitated with ethanol as above.

Selection of Poly(A)-containing RNA. Poly(A)-containing RNA was selected by either Poly U-Sepharose column chromatography or oligo dT cellulose chromatography. Poly U-Sepharose (Pharmacia) chromatography was as described (Hayward, 1977). RNA was dissolved in 0.2 NETS (0.2 M NaCl, 10 mM EDTA, 10 mM Tris [pH 7.4], 0.2% SDS) to an RNA concentration of less than 2 mg/ml and applied to a Poly(U)-Sepharose column such that there was approximately 1 ml of packed Sepharose per 1 mg of cell nucleic acid. The column was washed with 5-10 column volumes of ETS (10 mM EDTA, 10 mM TRIS [pH 7.4], 0.2% SDS), 2 volumes of 50% [vol/vol] formamide in ETS and 1 volume of 90% formamide in ETS. DNA and nonpoly(A)-containing RNA appear in the flow through, whereas poly(A)-containing RNA is eluted with 50% formamide. The poly(A)-containing RNA was pooled, the salt was adjusted to 0.2 M NaCl, and precipitated with two volumes of ethanol. In some cases yeast tRNA was added as a carrier.

Oligo dT cellulose (Collaborative Research) column chromatography was as described (Wang et al., 1975). Nucleic acid was loaded in 0.5 M LiCl, 10 mM EDTA, 10 mM Tris [pH 7.4], 0.2% SDS. The column was washed with 0.1 M LiCl, 10 mM EDTA, 10 mM Tris [pH 7.4]-0.2% SDS, eluted with 1 x ETS (10 mM EDTA, 10 mM Tris [pH 7.4], 0.2% SDS) and then eluted with sterile double-distilled water. Poly(A)-containing RNA elutes with 1 x ETS and with water. The desired fractions were pooled, heated to $90^{\circ}$ C for 90 sec to denature the RNA, made 0.5 M LiCl, and re-

chromatographed on a oligo dT column equilibrated with 0.5 LETS. Between different samples, the column was washed with 0.2 N NaOH and immediately reequilibrated with 0.5 LETS. The poly(A)-containing RNA was pooled, sodium acetate concentration brought to 0.25 M, and the RNA precipitated with two volumes of ethanol.

Poly(A)-containing RNA was sized by sucrose density gradient sedimentation. RNA was dissolved in 0.2-0.4 ml ETS, heated for 90 sec at 90° C to denature the RNA, and layered onto a 15 to 30% (wt/vol) sucrose gradient in 50 mM NaCl, 10 mM Tris (pH 7.4) 10 mM EDTA, 0.2% SDS. Centrifugation was for 6-7 h at 40,000 rpm in a Beckman SW40 Rotor. Marker RNAs of 35 S (RAV-2), 28 S and 18 S (chick ribosomal RNAs) were sedimented in parallel gradients.

Preparation of cDNA probes. cDNA strong stop (cDNAss) was synthesized from RAV-2 or RAV-0 by a modification of Haseltine et al. (1977), as described previously (Hayward et al., 1979). The strong stop DNA was purified twice on polyacrylamide gels; the first gel was run with the primer intact and the second was run following treatment with alkali to remove the primer. The resulting product is a single-stranded DNA 101 nucleotides long, which hybridizes to only the 5' of all viral mRNAs under the conditions used here.

cDNA specific for the unique region of AEV (cDNAaev) was synthesized using 28-30 S poly(A)-containing RNA purified from AEV(RAV-2) virions as the template, and random DNA primers generated by digestion of calf thymus DNA with DNase I. Condi-

tions for cDNA synthesis and primer preparation were as described by Taylor et al. (1976), except that the primer was treated with 0.2 N NaOH (30 minutes, 100° C) as a final step in the preparation, to hydrolyze contaminating cellular RNA present in commercial calf-thymus DNA. The cDNA was precipitated once with ethanol dissolved in 1 ml 0.2 M NaOH, and then incubated at 100° C for 30 minutes to hydrolyze the RNA. Unincorporated triphosphates were removed by column chromatography on Sephadex G-75 0.2 M NaCl, 10 mM EDTA, 10 mM Tris (pH 7.4), and 0.1% SDS. The void volume containing the cDNA was precipitated by the addition of two volumes of ethanol. cDNA sequences common to the helper viruses RAV-2 and EAV were removed by two cycles of hybridization and hydroxylapatite column chromatography as described (Hayward, 1977). The nonhybridized cDNA pool from the negative selection was then subjected to a positive selection against 28-30 S AEV(RAV-2) viral RNA. The cDNAaev-AEV RNA hybrids were separated from the unhybridized probe by sucrose density sedimentation in a 15-30% sucrose gradient spun for 6 hours at 40,000 rpm in a Beckman SW40 rotor. The cDNAaev-AEV 28 S RNA hybrid fraction was collected, the RNA hydrolized, and then cDNA precipitated. 98% of the resulting cDNA probe hybridized to AEV viral RNA at a Crt of 2.

Nucleic acid hybridization. Hybridizations were carried out under conditions of RNA excess at moderate stringency (Hayward, 1977). Hybridization mixtures were 30% formamide, 0.45 M NaCl, 0.045 M sodium citrate, 0.005 M EDTA, 0.20% SDS. 3 ul of each gradient fraction and 3 ul of reaction mixture, with 200-300

cpm of the cDNA probe and components at necessary concentration to give the desired final concentration were sealed in capillary tubes and incubated at 50° C for the indicated times. The extent of hybridization was monitored with S1 nuclease as described (Hayward, 1977).

Glyoxal gel electrophoresis and RNA blotting. Poly(A)-containing RNA was selected by oligo dT cellulose column chromatography, and precipitated twice, the second time in a silconized 0.5 ml microfuge tube (Eppendorf). The RNA was dissolved in 5 ul of glyoxal buffer [100 ul deionized glyoxal, 350 ul DMSO, 70 ul 100 mM sodium phosphate (pH 6.8), 180 ul water] and denatured at 50° C for 30 minutes as described (McMaster and Carmichael, 1977). 3 ul of tracking dyes (0.05% xylene cyanol, 0.05% Bromophenol Blue, 20% glycerol) was added to the sample and the RNA subjected to electrophoresis in a 1% agarose gel (Seakem LE, Marine Colloids Div., FMC Corp) in a horizontal apparatus (Bio-Rad). Electrophoresis was for 30 minutes at 75 volts and 2.5 h at 150 volts. Transfer of RNA was carried by the method of Alwine et al. (Alwine et al., 1977). The gel was soaked in 250 ml 50 mM NaOH for 1 h at room temperature. It was then washed twice for 20 minutes with 200 ml 200 mM sodium acetate, pH 4.0. Aminophenylthioether (APT) paper was prepared by a modification of Seed (B. Seed, personal communication) and was the gift of Ben Neel. The paper was activated by treatment with 1% $NaNO_2$ in ice cold 1.2 N HCl for 30 minutes to yield diazophenylthioether (DPT) paper, then washed three times for 3 minutes with ice cold double-distilled water and twice for two

minutes with 200 mM sodium acetate, pH 4.0. The transfer was then carried out as described (Alwine et al., 1977). 800-1000 ml of 200 mM sodium acetate buffer (pH 4.0) was blotted through the filter. The filter was then washed with prehybrid mix [50% formamide, 5 x SSC, 5 x Denhardts, 1% glycine, 250 ug/ml sonicated, denatured salmon sperm DNA, 50 mM sodium phosphate (pH 6.5), 0.1% SDS] for four hours. 5 x Denhardts is composed of 0.1% bovine serum albumin, 0.1% polyvinylpyrollidine, and 0.1% Ficoll and 5 x SSC is 0.45 M NaCl, 0.45 M sodium citrate. Hybridization was carried out in 50% formamide, 5 x SSC, 1 x Denhardts, 100 ug/ml sonicated, denatured salmon sperm DNA, 20 mM sodium phosphate (pH 6.5), 0.1% SDS. The probe was dissolved in 1 x ETS and added to the reaction mixture. Approximately 25-50 ul of reaction mixture per $cm^2$ of filter was used in the hybridization. Hybridization was carried out in a sealed plastic bag for 3 days at $37^{\circ}$ C. Following hybridization, the filter was washed three times with 250 ml 2 x SSC containing 0.1% SDS for 5 minutes per wash at room temperature with rocking. It was then washed (with rocking) twice with 250 ml prewarmed 0.1 x SSC containing 0.1% SDS for 15 minutes at $37^{\circ}$ C and twice with 100 ml per wash of 50% formamide, 5 x SSC, 0.1% SDS. Finally, the filter was blotted dry with Whatman 3MM paper and exposed to Kodak XR-5 film with a DuPont Cronex Lighting Plus intensifying screen.

In vitro translation of viral RNAs. RNA to be translated was precipitated three times to remove residual SDS from the sucrose gradient. In vitro translation was performed using the

mRNA-dependent reticulocyte lysate system of Pelham and Jackson (1976). 7 ul of the prepared lysate, 4 ul of $^{35}$S-methionine (New England Nuclear) and 0.25-0.5 ug of RNA dissolved in 2 ul sterile double distilled water were mixed and incubated at 30° C for 60 minutes. A 40 ul amount of SDS sample buffer was added to stop the reaction. Translation products were analyzed on 8.5% SDS polyacrylamide gels. The gels were processed for fluorography with En$^3$Hance (New England Nuclear) and autoradiography was for 12-48 h with DuPont Cronex 2DC film and DuPont Lightning Plus.

The labeled products of *in vitro* translation were characterized by a sequential immunoprecipitation procedure. 85 ul of RIPA with 1% Trasylol was added to the 13 ul reaction mixture, 5 ul of anti-p19 antisera or anti-*gag* antisera was added, and the mixture incubated on ice for one hour. Protein A-Sepharose was added and mixed as described above. The anti-*gag* immune complexes were pelleted by centrifugation in a Beckman Microfuge, the supernatant fluid removed, and immunoprecipitated with rat anti-gp85. This procedure was repeated once more with rabbit anti-reverse transcriptase following precipitation with the anti-gp85 antiserum. The immunoprecipitates were analyzed on 8.5% SDS polyacrylamide gels.

*Protein kinase assay*. Cell extracts were immunoprecipitated as described above and 25 ul of reaction mixture (50 mM Hepes [pH 7.0], 0.15 M KCl, 5 mM dithiothreitol, 10 mM magnesium acetate, 0.1 uM g$^{32}$P-ATP (3000 Ci/mmol, Amersham) was added to the washed immune complexes. The tube was mixed briefly with a

# III. Transcriptional and Translational Products of Avian Erythroblastosis Virus

vortex mixer and incubated for 10 minutes at 30° C. The reaction was terminated by the addition of 40 ul of SDS gel sample buffer and the products analyzed on 7.5% SDS polyacrylamide gels.

Immunofluorescent staining of cells. Immunofluorescent staining was performed as described (Wang and Goldberg, 1978; Krueger et al., 1980a). Covers slips (No. 1, Corning) were washed overnight in the 1% detergent 7x, rinsed several times in double-distilled water, rinsed in 70% ethanol and flamed before use. Cells plated at low density in 100 mm plates containing coverslips. The cells were fixed in 3.7% formaldehyde in phosphate buffered saline, pH 7.2 (PBS). The coverslips were washed 3 times with PBS (10 minutes per wash). The cells were lysed for 5 minutes in 0.1% Triton X-100 in PBS, then washed three times for 20 minutes in PBS. Coverslips were placed in a humidified atmosphere and 50 ul of a ten-fold dilution of antibody in PBS was added to each coverslip. Incubation was for 20 minutes at room temperature. The coverslips were drained and washed in three changes of PBS, 20 minutes per wash. The secondary antibody, FITC conjugated-rabbit anti-chicken IgG (Miles) or FITC conjugated-goat anti-rabbit IgG was then added for 20 minutes at room temperature. The coverslips were drained and then washed 3 times for 20 minutes per wash as above and mounted in 50% glycerol, 50% double strength PBS salts. Photographs were made with Kodak TriX ASA400 push processed to ASA1600, FITC conjugated-goat anti-rabbit IgG was the gift of Dr. Eugenia Wang, Rockefeller University.

I chose to study AEV for several reasons. (i) AEV transforms both CEF and bone marrow cells. This means that the in vitro analysis of the virus would be simplified because of th relative ease with which these cells can be grown. (ii) The differentiation of erythroblasts can easily be monitored because there are many available. (iii) The fact that AEV has a multiple oncogenic potential indicates that there might be either multiple target cells, multiple gene products involved in transformation, or perhaps multiple pathways via which a transforming protein may function.

Before I began my research, Graf and his coworkers had established the defectiveness of AEV (Graf et al., 1976a) and the multiple oncogenic potential of cloned stocks of AEV had been confirmed (Graf et al., 1976a; Graf et al., 1976b; Graf et al., 1977a). My early efforts were directed toward developing an in vitro focus assay for AEV and confirming the defectiveness of AEV.

Colonies of AEV-transformed cells were isolated from soft agar and grown as independent clones. The supernatant fluids of such clones were assayed for the presence of infectious virus by colony formation in soft agar or by focus assay under hard agar, and for the presence of polymerase positive particles. When colonies were isolated from soft agar in the absence of neutralizing antibody directed against the helper virus, less that 5% of the colonies were nonproducers when assayed for the presence of infectious virus. When neutralizing antibody was added to the soft agar, however, 80 to 90% of the colonies isolated were nonproducers. None of the nonproducer

clones analyzed produced polymerase positive particles, and so the polymerase assay was used to screen isolated clones for nonproducers since it is much faster than biological assays such as focus formation.

Infectious AEV could be rescued from AEV NP cells with the following viruses: RAV-1, RAV-2, RAV-7, RAV-50 and RAV-61 (Table 4). No infectious virus could be rescued from AEV NP cells superinfected with Golden pheasant virus or Amherst pheasant virus (Table 4). Similar data was obtained upon the rescue of MC29 from NP clones of MC29-transformed CEF (Table 4). These experiments were performed by superinfecting AEV- or MC29- NP clones with the indicated helper viruses and transferring the cultures once. Supernatant fluids from all helper virus-infected cells contained polymerase positive virus particles. The presence of infectious MC29 was determined by focus assay on SPAFAS gs-chf- CEF and the presence of infectious AEV by colony formation of infected gs-chf- CEF in soft agar. The results (Table 4) clearly demonstrated that only RAV-1, RAV-2, RAV-7, RAV-50 and RAV-61 can function as helper viruses. Although Golden pheasant virus (GPV) and Amherst pheasant virus (APV) replicated in these cells, they were not able to effectively rescue AEV or MC29.

These data are somewhat surprising in light of the fact that both GPV and APV are able to function as helper viruses for the Bryan strain of RSV (T. Hanafusa et al., 1976). The Bryan strain of RSV does not produce the envelope glycoproteins, however, it does form a noninfectious virus particle (Hanafusa and Hanafusa, 1968; Robinson, 1967; Dougherty and Di

TABLE 4

Effectiveness of various helper viruses in rescueing AEV and MC29 from nonproducer cells.

| helper virus used to infect NP cell | MC29 NP Clone 1 | | AEV NP Clone 30 | |
|---|---|---|---|---|
| | Polymerase Assay[a] | FFU/ml Focus Assay[b] | Polymerase Assay[a] | CFU/ml Colony Assay[c] |
| RAV-1 | + | $2.8 \times 10^3$ | + | $>10^5$ |
| RAV-2 | + | $1.25 \times 10^4$ | + | $>10^5$ |
| RAV-7 | + | $2.8 \times 10^3$ | + | $>10^5$ |
| RAV-50 | + | $1.7 \times 10^4$ | + | $>10^5$ |
| RAV-61 | + | $8.3 \times 10^4$ | + | $>10^5$ |
| GPV | + | <10 | + | <10 |
| APV | ND[d] | ND | + | <10 |
| Mock | - | <10 | - | <10 |

a polymerase assay was performed 6 days post-infection on 10ml of a 24 hr tissue culture fluid. Positive samples were 18,000 to 56,600 cpm, negative samples were >500 cpm.

b $1.0 \times 10^6$ cells infected with 0.1 ml diluted virus in presence of 1mg/ml polybrene, overlaid 12 hr later with FLO w/ 2.5% Calf 0.5% chick serum 10% TPB and agar. Innoculation was at 37°C. Focci counted at 8 days.

c $1.0 \times 10^6$ cells infected with 0.1 ml diluted virus in presence of 1mg/ml polybrene. 12 hr later cells planted in soft agar as described. Colonies counted at 14 days.

d not determined.

Stephano, 1965). Thus all that need be provided for RSV is an envelope glycoprotein; AEV and MC29 require more than just the *env* function of a helper virus (Bister and Vogt, 1978; see below). Apparently, the mechanism by which the RNA of GPV and APV is packaged into their respective virions, does not recognize the genomic RNAs of either AEV or MC29, and thus no infectious AEV or MC29 virus particles are produced. APV and GPV are not capable of acting as helper viruses for RSVα, a *pol*-*env*- mutant of the Bryan strain of RSV, indicating that the reverse transcriptases of pheasant viruses and chicken viruses are not interchangeable. Thus, it was also possible that AEV is defective in the *pol* gene.

Infectious AEV was not produced by co-cultivating AEV NP cells with either uninfected gs+chf-CEF cells or gs- $h_E$ cells (Table 5). Co-cultivation of Bryan RSV(-) cells, with AEV NP cells, did not result in the production of either infectious RSV or AEV (Table 4).

The fact that AEV could not complement the genetic defect of the Bryan strain of RSV, which is *env*-, meant that AEV did not produce the envelope glycoproteins gp85 and gp37. Since AEV was not complemented by gs- $h_E$ cells, which produce the envelope glycoproteins of an endogenous oncovirus, the defectiveness of AEV must be more extensive than just the *env* gene. Since Bryan RSVα, a mutant of RSV which is both *env*- and *pol*-, produces a noninfectious virus particle, we assumed that AEV was also defective in the *gag* gene. No conclusions can be drawn about the presence of the *pol* gene in AEV since the polymerase assay is dependent upon the production of a virus par-

TABLE 5

RESCUE OF AEV FROM NONPRODUCER CELLS BY CO-CULTIVATION WITH VARIOUS TYPES OF CELLS

| AEV NP cells co-cultivated with: | Colony of AEV/ml culture fluid |
|---|---|
| $gs^-chf^-$CEF | <10 |
| $gs^+chf^-$CEF | <10 |
| $gs^-$hE CEF | <10 |
| RSV($^-$) CEF | <10 |
| RAV-2-infected CEF | $5 \times 10^4$ |

$6 \times 10^5$ cells of each type were mixed and plated in 60 mm petri dishes. Supernatant fluids collected 7 days later were assayed for infectious AEV by colony formation in soft agar. Colonies were counted at 14 days. Co-cultivation of RSV($^-$) cells with RAV-2-infected CEF yielded $2 \times 10^6$ FFU/ml RSV.

ticle. This was examined, however, by analyzing proteins present in AEV-transformed cells as described in the following section.

Proteins present in AEV nonproducer cells. In order to determine whether AEV contained any functional genes, AEV NP clones were labeled with $^{35}$S-methionine and immunoprecipitated with antisera directed against the individual viral structural proteins. NP clones were used for this analysis because they should contain only the AEV genome and thus the analysis would not be confused by the presence of helper virus gene products. The results (Figure 4) demonstrate that AEV contains none of the normal viral structural proteins or protein precursors. Immunoprecipitation of RAV-2 infected cells (Figure 4, left panel) shows each of the individual gag proteins (p27, p19, p15 and p12), $Pr76^{gag}$, $Pr180^{gag-pol}$, $gPr92^{env}$, gp85 and gp37. AEV nonproducer cells immunoprecipitated with the same antisera demonstrates the presence of only one protein of 75,000 daltons which is precipitated by anti-p19 antiserum (Figure 4, right panel). This protein is not $Pr76^{gag}$ since it is not precipitated by anti-p27 or anti-p12 antiserum. There are no pol or env gene products detectable in AEV nonproducer cells. These data confirm the prediction, based upon biological data, that AEV is defective in gag and env. Since no polymerase protein or precursor protein of any size is seen, AEV is probably also defective in the pol gene.

Do all AEV-transformed cells contain p75? Twelve independently isolated clones were labeled with $^{35}$S-methionine and im-

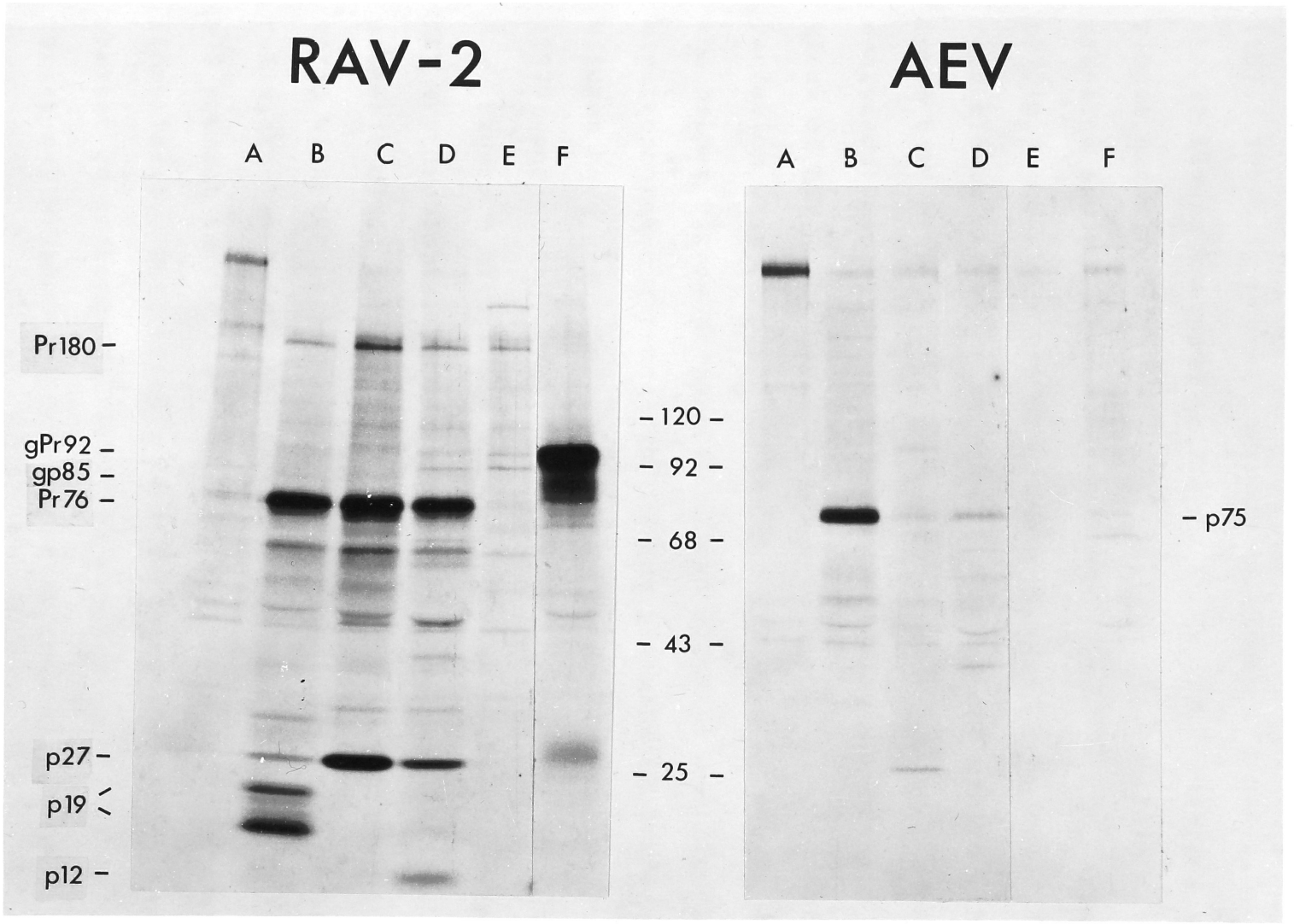


FIGURE 4. Viral-related proteins present in $^{35}$S-methionine-labeled lysates of RAV-2-infected CEF (left) and a nonproducer clone of AEV-transformed CEF (right). Polypeptides were separated on 5-15% gradient SDS polyacrylamide gels. Antisera used were: nonimmune serum, lane A; anti-p19 serum, lane B; anti-p27 serum, lane C; anti-p12 serum, lane D; anti-reverse transcriptase serum, lane E; and anti-gp85 serum, lane F. Molecular weight x $10^{-3}$ daltons is indicated in the center and viral proteins of interest are indicated on the right and left.

munoprecipitated with rabbit anti-gag antisera. Figure 5 shows six of these twelve clones, two of which are NP clones (110 and 115). The only protein present in clones 110 and 115, as in the NP clones analyzed in Figure 4, is p75. The four producer clones shown, clones 101, 108, 109 and 114, contain all the gag proteins and precursors, including: $Pr180^{gag-pol}$, $Pr76^{gag}$, p27, p19 and p12. p75 is clearly resolved from $Pr76^{gag}$ in all four clones. $Pr76^{gag}$ of RAV-2 and RAV-7 comigrates with the upper band at 76,000 daltons seen in each producer clone analyzed. Clone 108 is infected with EAV, the natural helper virus of AEV. The gag proteins of EAV show electrophoretic variation when compared to the gag proteins of RAV-2, which is the helper viruse present in clones 101, 109 and 114. Such electrophoretic variance in the gag proteins of different viruses have been characterized by Rettenmier and Hanafusa (1977) and Shaikh et al. (1978,1979).

Figure 5 also shows the gag-related proteins present in AEV erythroblasts. AEV-erythroblasts were isolated from a leukemic bird, labeled with $^{35}$S-methionine, and immunoprecipitated with the anti-gag antiserum. In these erythroblast cells, the amount of the gag proteins relative to the amount of p75 present is clearly less than that seen in AEV-transformed fibroblasts. Whether this is a fortuitous observation, or whether it reflects differential expression of p75 in various target cells cannot be determined at this time. Since p75 is the only viral-related protein present in all AEV-transformed cells, it is a candidate for the transforming protein of AEV. That such a protein could be identified with antiserum directed

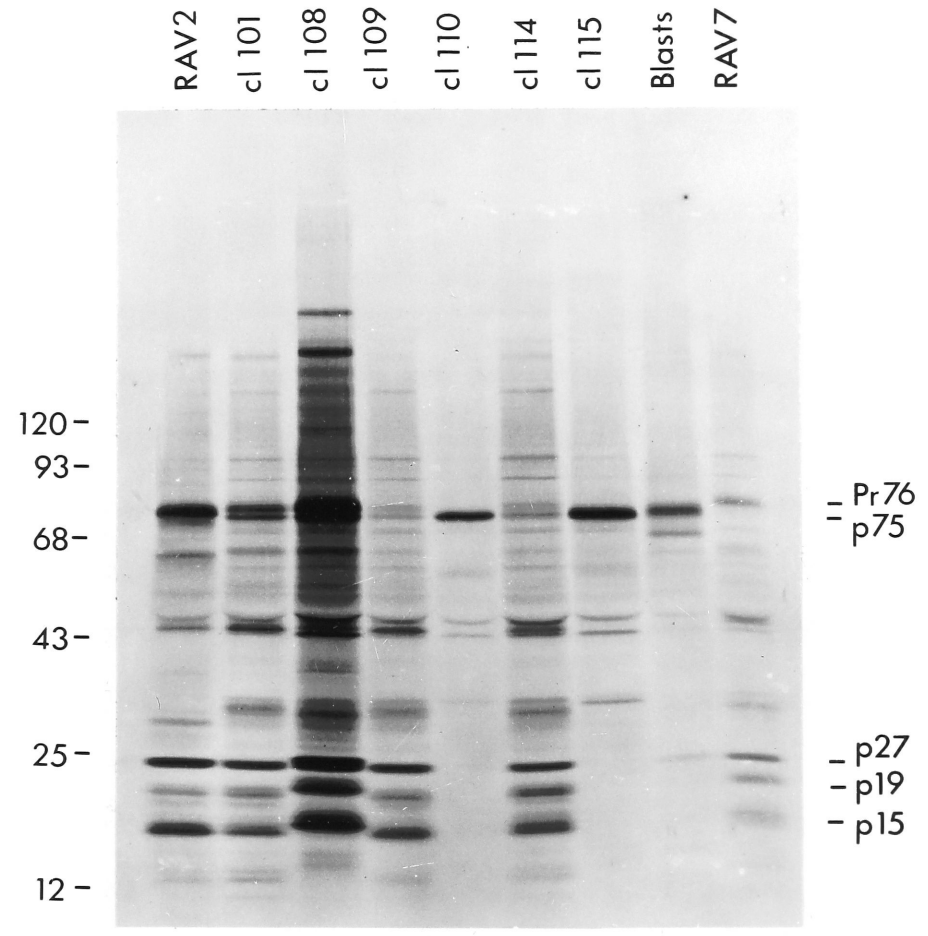


FIGURE 5. Presence of AEV p75 in producer and nonproducer clones of AEV-transformed cells. Six independent clones of AEV-transformed CEF were isolated and labeled with $^{35}$S-methionine. In addition, RAV-2-infected CEF, RAV-7-infected CEF, and AEV erythroblasts from a leukemic chicken were also labeled with $^{35}$S-methionine. Lysates were immunoprecipitated with rabbit anti-*gag* antiserum and analyzed on a 5-15% gradient SDS polyacrylamide gel. Molecular weight x $10^{-3}$ is indicated on the left and viral proteins of interest are indicated on the right.

against the viral structural proteins is quite fortunate. The src gene product pp60$^{src}$ shares no determinants with viral structural proteins and would not have been found by these techniques.

Two dimensional gel electrophoresis of AEV p75. A method for the two-dimensional separation of proteins has been described by O'Farrell (1975) in which proteins are first separated by isoelectric focusing and then SDS slab gel electrophoresis. I analyzed the proteins present in an AEV producer clone which could be immunoprecipitated with rabbit anti-gag antiserum by this method (Figure 6). Unfortunately, there was some breakdown of the pH gradient at higher pHs, however, all the proteins of interest are resolved. The pI of p15 was 7.2-7.3. Two p19 spots are present with pIs of 7.4-7.6 and 6.0-6.2. Pr76$^{gag}$ appeared in a streak from pI 6.6 to 7.4 with major spots at 7.0 and 7.35. AEV p75 streaked from 7.35 to 7.6 with a major spot with a pI of 7.5. These data are consistent with the finding of Rettenmier and Hanafusa (1977) on the gag proteins present in the virions of various Rous-associated viruses. These results further substantiate the claim that p75 is distinct from Pr76$^{gag}$.

Tryptic peptide mapping of AEV p75. Since p75 contains only the p19 region of the gag gene, the majority of this protein should represent the translational product of the unique sequences present in AEV. The presence of novel peptides in p75 was determined by preparing two-dimensional tryptic peptide

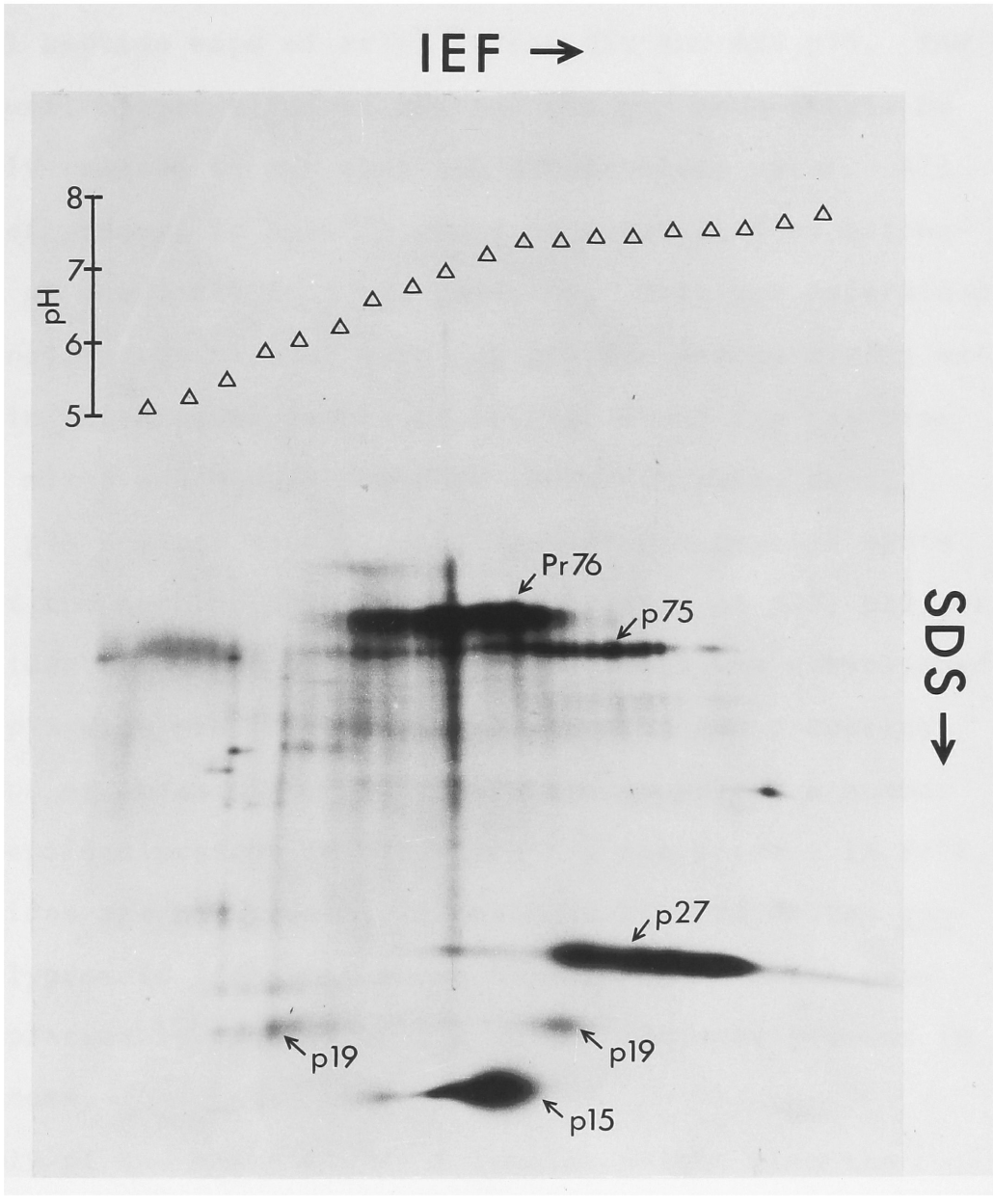


FIGURE 6. Two-dimensional electrophoresis of proteins from an AEV producer clone. An AEV-transformed producer clone was labeled with $^{35}$S-methionine and immunoprecipitated with rabbit anti-*gag* antiserum. The precipitates were analyzed by two-dimensional electrophoresis. The pH of a parallel tube gel is indicated across the top of the gel. Proteins of interest are indicated.

maps of $^{35}$S-methionine-labeled p75. Figure 7 shows two-dimensional peptide maps of Pr76$^{gag}$ from EAV and AEV p75. EAV is the natural helper virus of AEV and its gag gene should be more closely related to AEV than any other helper virus. All the peptides present in Pr76$^{gag}$ could be identified as belonging to one of the individual gag proteins. This was determined by preparing peptide maps of each gag protein and by mixing experiments in which equal counts of both Pr76 and the gag proteins were mixed and mapped together (data not shown here). The map of p75 (center panel) contains four p19 peptide spots but none of the methionine spots characteristic of p27, p12, or p15. The identification of these peptide spots was established by mixing p75 with the individual gag proteins and preparing peptide maps of these mixtures. There are, however, a number of novel peptides present in p75 which are not present in Pr76. These peptides are not present in the MC29 110,000 dalton gag-related polyprotein (data not shown here). These novel peptides are presumably encoded by the unique sequence present in the AEV genome.

The p19 of EAV has a higher molecular weight than the p19's of many other RNA tumor viruses (see above). In addition, EAV p19 contains several novel methionine-labeled tryptic peptides. Two of these peptides are present in p75 and they are marked with arrows in Figure 7.

AEV p75 contains N-terminal Ac-Met-Glu dipeptide. The N-termini of p19 and Pr76$^{gag}$ are blocked (Herman et al., 1975; Palmiter et al., 1978), probably as the result of N-terminal

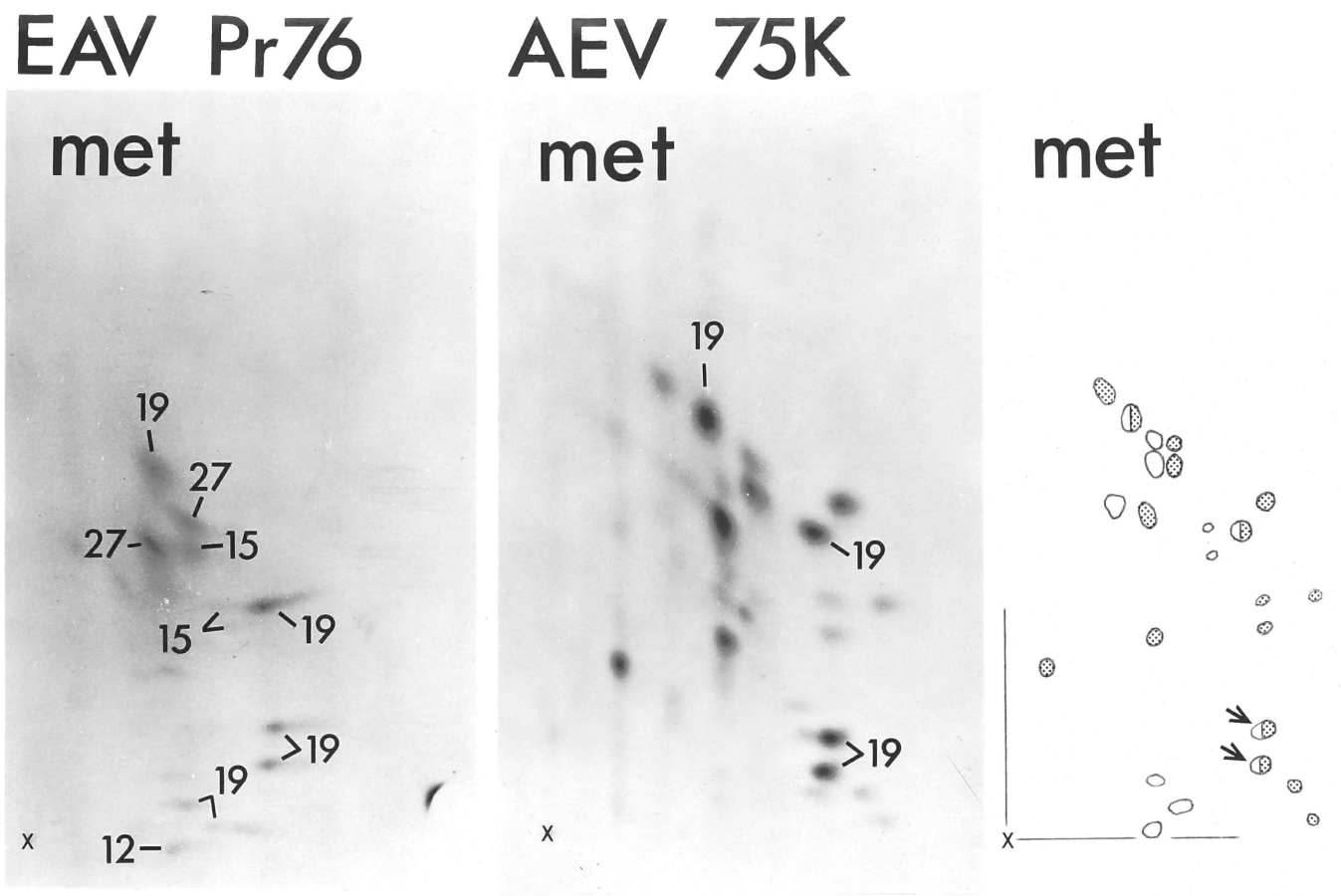


FIGURE 7. Comparison of $^{35}$S-methionine-labeled tryptic peptides of EAV Pr76$^{gag}$ (left) and AEV p75 (center) by two dimensional fingerprinting. Peptides were spotted on cellulose-coated thin-layer plates in the lower left corner (x) of each panel. Electrophoresis at pH 1.9 was performed from left to right in the horizontal direction with the anode at the left, followed by ascending chromatography from bottom to top. At the right is a schematic diagram showing the tryptic peptides found in EAV Pr76$^{gag}$ (indicated by the open circles ○), AEV p75 (indicated by the dark circles ●), and those *gag*-related peptides present in both (indicated by the half-filled circles ◐). Two type-specific tryptic peptides unique to the EAV p19 are marked with the arrows (↙) in the schematic diagram.

acetylation (Palmiter et al., 1978). Under conditions that prevent N-terminal acetylation, the amino terminal amino acid sequence of the Prague strain of RSV was found to be Met-Glu-Ala-Val-Ile-Lys..., and the N-terminal methionine could be labeled with $^{35}$S-Met-tRNA$_f^{Met}$ (Palmiter et al., 1978). Pactamycin mapping studies (Vogt et al., 1975; Shealy and Ruckert, 1978; Rettenmier et al., 1979; Shealy and Ruckert, 1980) locates p19 at the N-terminal end of Pr76$^{gag}$, and analysis of methionine-labeled p19 tryptic peptides has identified a peptide with the same amino acid composition as the N-terminus of Pr76$^{gag}$ (Palmiter et al., 1978).

S. aureus V8 protease, which cleaves at the carboxy side of glutamyl acid residues (Houmard and Drapeau, 1972), should release the dipeptide acetylmethionylsulfoneglutamic acid from both p19 and Pr76$^{gag}$. This blocked dipeptide, which we call the p19* peptide, can be labeled with $^{35}$S-methionine and should be negatively charged at pH 6.5, but neutral at pH 1.9. RAV-2 Pr76$^{gag}$ was digested with V8 protease and the 19* peptide was identified (Figure 8). The 19* dipeptide comigrated with synthetic Ac-Met-Glu prepared by Carl Rettenmier and Mark Rieman. This dipeptide was also found in AEV p75 (Figure 8, right panel). This indicates that the translation of p75 begins at the gag initiation codon and contains at least the first two amino acids of the gag gene. The presence of the 19* dipeptide is thus diagnostic for a viral protein whose translation begins at the gag gene initiation codon and includes the first two amino acids of the gag gene.

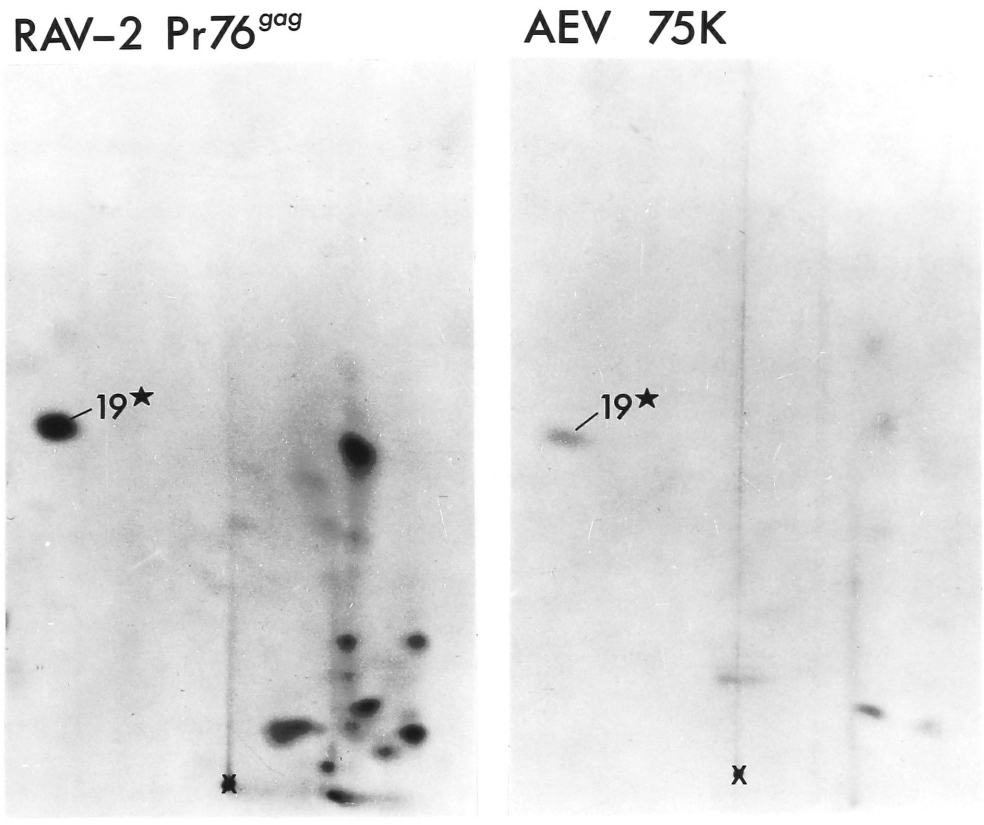


FIGURE 8. Autoradiograms showing two-dimensional analysis of V8 protease digestion products of $^{35}$S-methionine-labeled RAV-2 Pr76$^{gag}$ (left) and AEV p75 protein (right). Samples were spotted at the origin (x), and electrophoresis at pH 6.5 was performed in the horizontal direction with the anode at the left. Ascending chromatography was from bottom to top. The negatively charged 19* peptide is indicated in each panel.

Modification of AEV p75. The transforming proteins of a variety of different RNA tumor viruses are modified in various ways. The 50,000-55,000 dalton transforming protein of the Friend Spleen Focus-forming virus is a glycoprotein (Dresler et al., 1979,; Ruscetti et al., 1980; MacDonald et al., 1980). The transforming proteins of RSV (Collet et al., 1979; Karess et al., 1979; Hunter and Sefton, 1980), Fujinami sarcoma virus (Feldman et al., 1980), Y73 (Kawai et al., 1980), PRCII (Neil et al., 1981), Harvey sarcoma virus (Shih et al., 1979), Abelson murine leukemia virus (Van der Ven et al., 1980b), and the Gardner-Arnstein and Snyder Theilen strains of feline sarcoma virus (Van der Ven et al., 1980a) are all phosphoproteins. Since AEV p75 represents a candidate for the transforming protein of AEV, I sought to determine whether it might be glycosylated or phosphorylated.

The glycosylation of p75 was examined by labeling RAV-2 infected and NP cells with either $^{35}$S-methionine or $^{3}$H-N-acetylglucosamine (Figure 9, left panel). RAV-2 infected cells labeled with $^{35}$S-methionine show the presence of all viral proteins: p12/p15, p19, p27, Pr76$^{gag}$, gp85, gp37, and gPr92$^{env}$ (Figure 9, lane C). $^{3}$H-N-acetylglucosamine was incorporated into gPr92$^{env}$, gp85 and gp37 (Figure 9, lane D). There was no glucosamine label incorporated into material comigrating with $^{35}$S-methionine labeled p75 (Figure 9, lanes E and F respectively). Therefore, it appears that p75 is not a glycoprotein.

AEV p75 does appear to be phosphorylated, however, as $^{32}$P-labeled AEV NP cells immunoprecipitated with anti-virion antiserum contain a protein which comigrates with $^{35}$S-

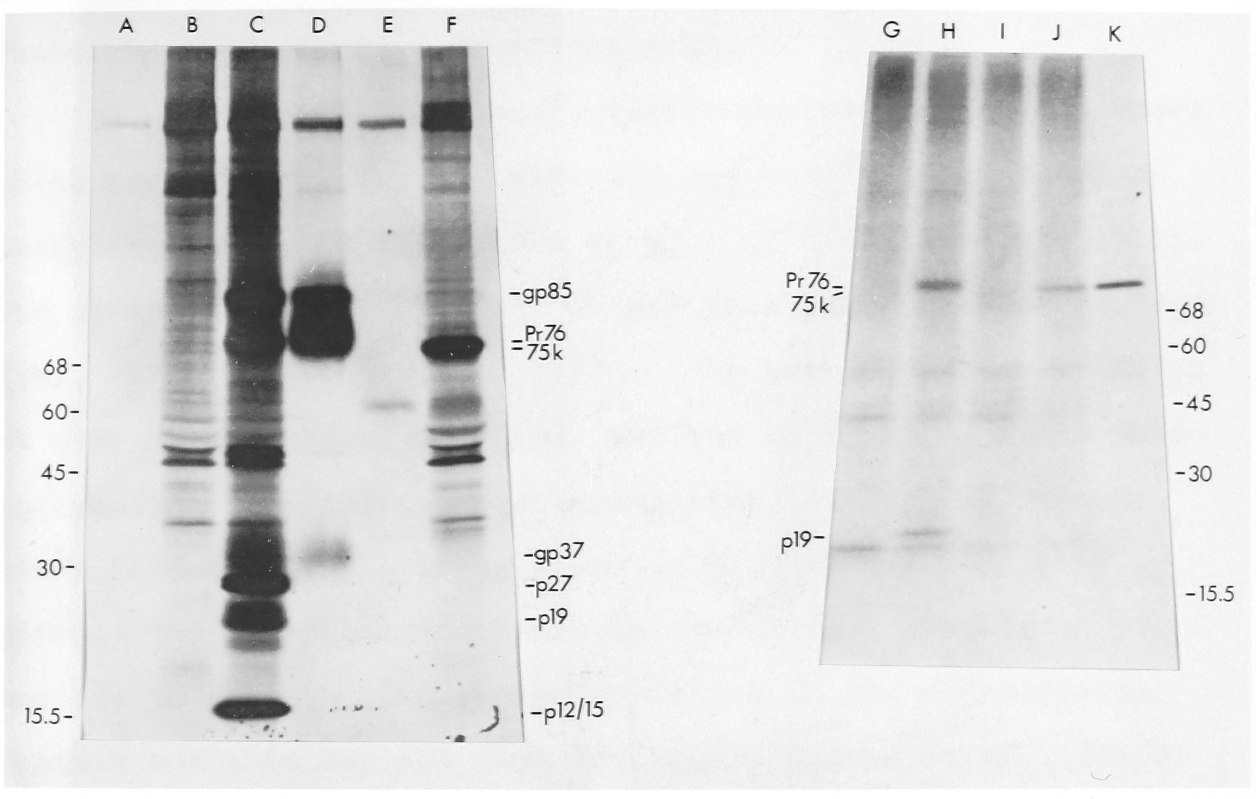


FIGURE 9. Modification of AEV p75. The glycosylation and phosphorylation of AVE p75 were investigated. In the left panel, *td*109-infected cells were labeled with $^{35}$S-methionine (lanes B and C) or $^{3}$H N-acetylglucosamine (lanes A and D), and AEV NP Cells with either $^{35}$S-methionine (lane F) or $^{3}$H N-acetylglucosamine (lane E). Immunoprecipitation was with the following antisera: nonimmune serum, lanes A and B; rabbit anti-virion serum, lanes C-F. In the right panel, phosphorylation of AEV p75 is examined. EAV-infected cells (lanes G and H) were labeled with $^{32}$P and AEV NP cells were labeled with $^{32}$P (lanes I and J) or with $^{35}$S-methionine (lane K). Immunoprecipitation was with the following antisera: nonimmune serum, lanes G and I; anti-virion serum, lanes H, J, and K. Precipitates were analyzed on 5-15% polyacrylamide gels. Molecular weight x $10^{-3}$ is indicated on the outside and viral proteins of interest are indicated in the center.

methionine labeled p75 (Figure 9, lanes J and K respectively). The amount of $^{32}P$ incorporated into p75 is comparable to the amount of $^{32}P$ incorporated into $Pr76^{gag}$ (Figure 9, lane H). Therefore, AEV p75 is a phosphoprotein.

In addition to the transformation proteins mentioned above which are phosphoproteins, p19, p12 and $Pr76^{gag}$ are all phosphorylated (Lai, 1976; Erikson et al., 1977; Rettenmier, 1981). The phosphoamino acid present in p19 is a phosphoserine residue (Lai, 1976; Erikson et al., 1977). The transformation proteins of RSV, Fujinami sarcoma virus, Y73 and PRCII all contain phosphotyrosine in addition to phosphoserine (Hunter and Sefton, 1980; Feldman et al., 1980; Kawai et al., 1980; Neil et al., 1980). The phosphoserine residues of Fujinami, PRCII and Y73 may lie in the gag gene region contained in the transforming protein but this has not been rigorously demonstrated. Therefore it was of interest to determine the phosphoamino acid present in AEV p75 and compare it with those present in the p19 or $Pr76^{gag}$ since AEV p75 contains the p19 region of the gag gene.

The products of a mild acid hydrolysis were analyzed by electrophoresis at pH 3.5, conditions which separate phosphothreonine and phosphotyrosine, a problem which confused earlier work with $pp60^{src}$. The phosphoamino acid present in p19, p75 and Pr76 was phosphoserine (Figure 10). There is no evidence for either phosphotyrosine or phosphothreonine in any of these three proteins. Lai (1976) noted the presence of phosphothreonine in p19, however, neither the data present here, nor the the data of Erikson et al. (1977) confirms this obser-

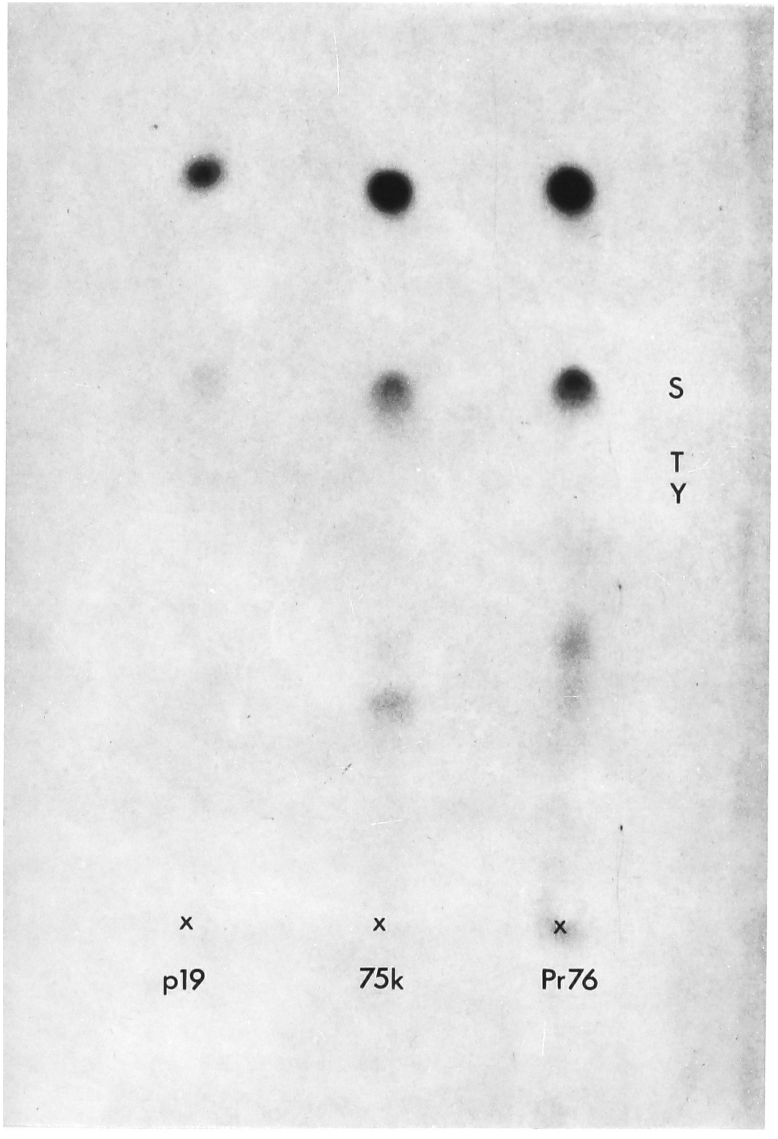


FIGURE 10. Phosphoamino acid analysis of p19, p75 and $Pr76^{gag}$. Total acid hydrolysis of $^{32}P$-labeled p19, p75 and $Pr76^{gag}$ was performed as described and the hydrolysate was spotted at the bottom of the plate (x). Electrophoresis at pH 3.5 was performed from bottom to top. The positions of marker phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) are indicated.

vation.

These results suggest that AEV p75 might be phosphorylated at a serine residue in the gag-related portion of p75 which contains p19 sequences. Alternatively, p75 might be phosphorylated at multiple sites in both the p19 region as well as the unique region of p75. In order to determine which of these possibilities might be correct, two-dimensional peptide maps of $^{32}$P-labeled p19 and p12 from EAV, the natural helper virus of AEV, were prepared and compared to that of AEV p75. The results (Figure 11) indicate that there are a number of phosphopeptides present in all three proteins, and that the tryptic phosphopeptides of EAV p19 and AEV p75 appear closely related. The tryptic phosphopeptide map of p12 is clearly different from that of p19 and p75 (Figure 11). Pr76$^{gag}$ contains both p19 and p12 phosphopeptides, although it appears that the p19 peptides are more prominent than those of p12 (data not shown here). Two dimensional peptide maps prepared at pH 1.9 instead of pH 4.7 as shown in Figure 10, confirm the data presented here. Thus, AEV p75 is phosphorylated at a serine residue present in the p19 region of the gag gene contained in p75, and that there are apparently no other major phosphopeptides present in p75 not contained in p19.

The maps of all the proteins analyzed here clearly show the presence of more than one phosphopeptide. Previous analysis of the phosphopeptides of p19 by Erikson et al. (1977) and Rettenmier (1981) have shown the presence of only one large peptide spot. Although it is possible that some of the peptides I see are incomplete digestion products, the data

presented here have been reproducible in several experiments with electrophoresis carried out at both pH 4.7 and pH 1.9. In addition, the same results were obtained with two different lots of commercially prepared TPCK-trypsin. Digestion for up to 30 hours did not alter the patterns observed. The fact that p75 and p19 are isolated from widely separated regions of the gel makes it unlikely that there has been extensive contamination of these preparations with a common protein(s). The common spots observed in both p12 and p19 could be derived from p19 since it has been demonstrated that some p12 preparations contain p19 fragments (Shealy et al., 1980).

Enzymatic activities associated with p75. Protein kinase activities are associated with the transforming proteins of a number of different transforming viruses including: RSV (Collet and Erikson, 1978; Levinson et al., 1978) Fujinami sarcoma virus (Feldman et al., 1980), PRCII (Neil et al., 1981), Y73 (Kawai et al., 1980), Gardner-Arnstein strain of feline sarcoma virus (Van der Ven et al., 1980b), Abelson murine leukemia virus (Witte et al., 1979), and murine sarcoma virus (Sen et al., 1979). I was very interested in determining whether the gag-related polyproteins of several avian acute leukemia viruses had protein kinase activities associated with them. The assay used in these experiments was the immune complex protein kinase assay in which $\gamma$-$^{32}$P-ATP is added to the immune complexes isolated with Protein A-Sepharose. In this assay either the heavy chain of IgG, the transforming protein itself, or both are phosphorylated. Cell extracts of AEV, MC29 and AMV

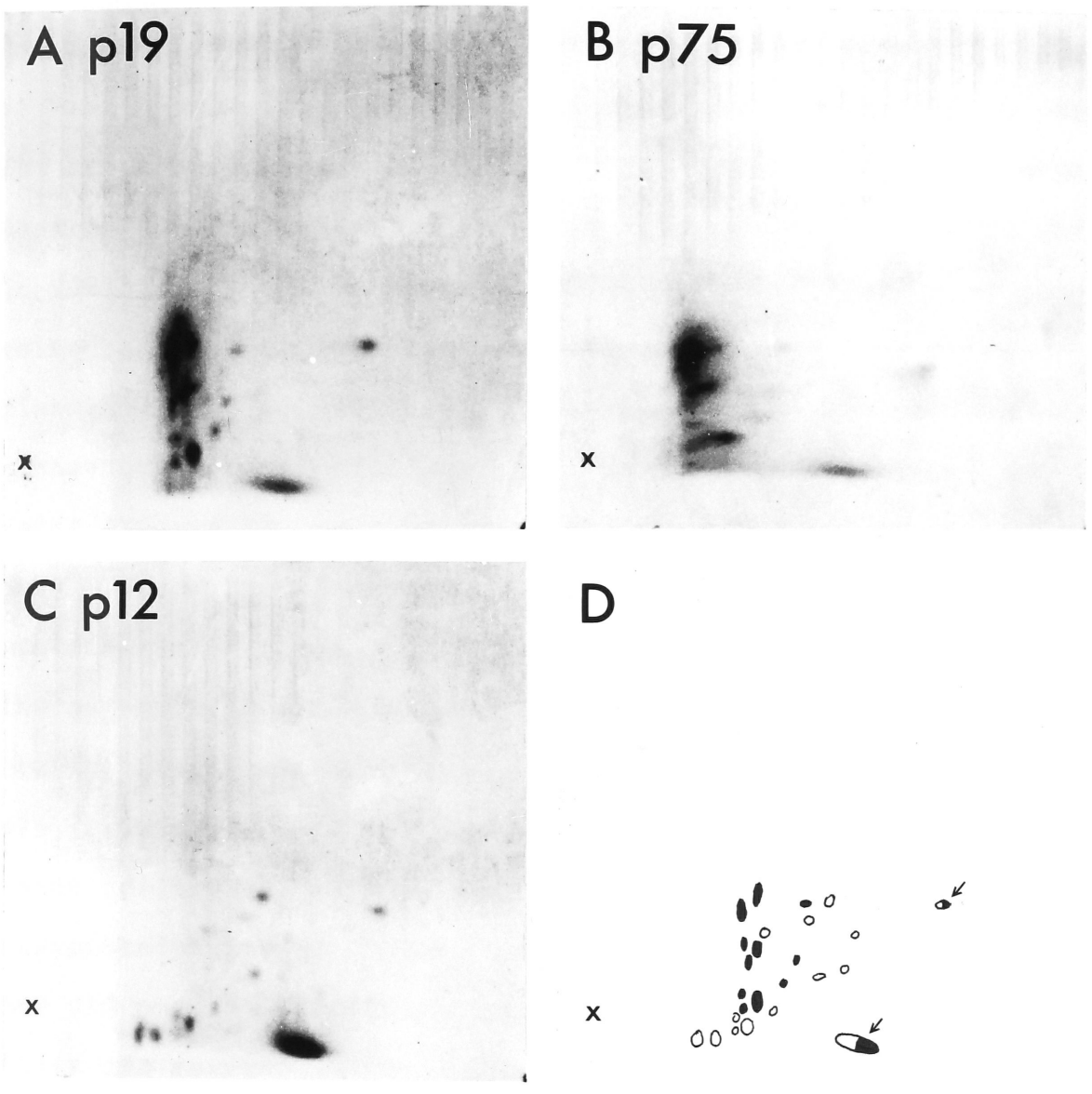


FIGURE 11. Two-dimensional tryptic phosphopeptide maps of p12, p19, and AEV p75. $^{32}$P-labeled EAV p12 and p19, and AEV p75 were worked up as from peptide mapping as described in Methods. Peptides were spotted at the bottom left corner (x) and electrophoresis at pH 1.9 was performed in the horizontal direction with the anode at the left. Ascending chromatography in the second dimension was from bottom to top. Panel A, p19; panel B, p75; panel C, p12; panel D, schematic drawing. Peptides common to p19 and p75 are indicated by dark circles (●), p12 peptides by the open circles (o), and those common to both by the half-filled circles (◐) marked by arrows.

NP clones were screened for protein kinase activity (Figure 12, left panel) and parallel cultures were labeled with $^{35}S$-methionine and immunoprecipitated with the same antiserum used for the kinase assay (Figure 12, right panel). The antiserum used in these experiments was from a tumor-bearing rabbit and in addition to containing antibodies to pp60$^{src}$, it also contained antibodies directed against gag and env gene determinants. None of the acute leukemia viruses have protein kinase activities associated with their gag-related polyproteins (Figure 12, left panel, lanes F, G and H), although pp60$^{src}$ from Schmidt-Ruppin RSV transformed cells did show a protein kinase activity (Figure 12, left panel, lanes A and I). The gag-related proteins of these viruses were observed in the precipitates from $^{35}S$-methionine labeled parallel cultures: AEV p75 (Figure 12, lane F), AMV Pr76$^{gag}$, the individual gag proteins (Figure 12, lane G) and the 110,000 dalton gag-related polyprotein of MC29 (Figure 12, lane H). Thus AEV, MC29, and AMV did not show protein kinase activities associated with their gag-related polyproteins using this assay, and with ATP as a phosphate donor. The addition of calf thymus histones, casein or bovine serum albumin to the immune complexes in the presence of $\gamma$-$^{32}P$-ATP did not result in phosphorylation of any of these potential substrates (data not shown here). Thus none of these putative transforming proteins contain protein kinases using a variety of substrates using this assay.

Harvey and Kirsten sarcoma viruses are two independently isolated rat-derived sarcoma viruses which can transform a variety of cells in vitro. In addition to causing sarcomas in

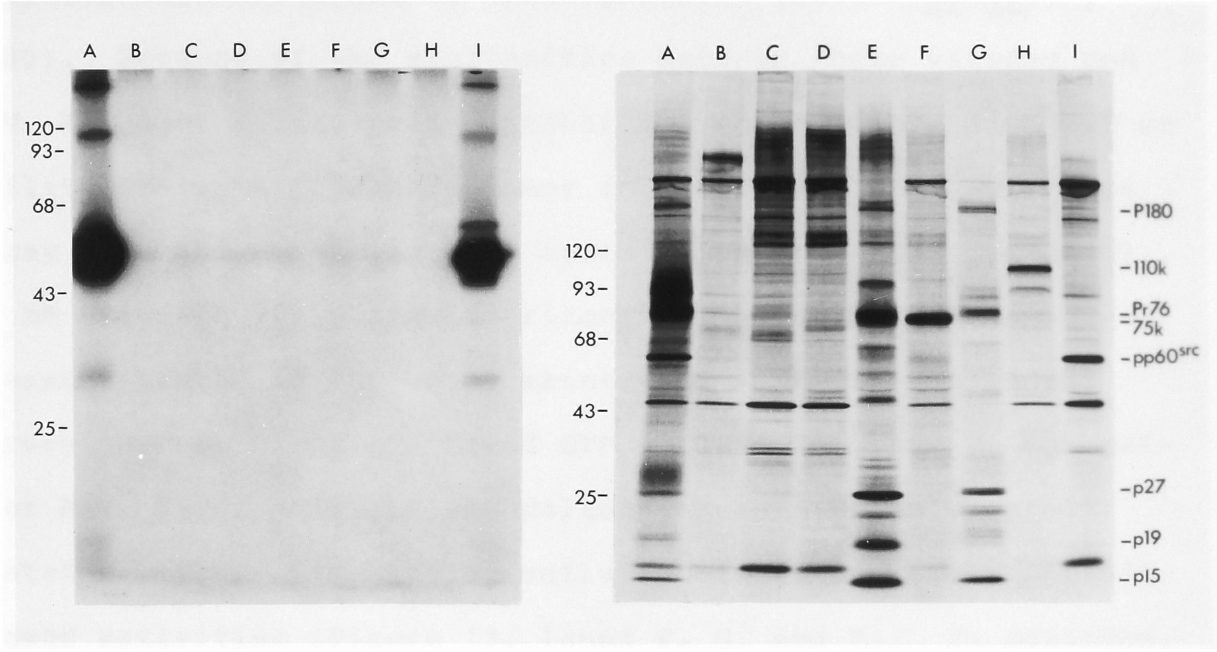


FIGURE 12. Kinase activity of *gag*-related polyproteins of avian leukemia viruses. The immune complex protein kinase assay was performed as described in the Methods with $\gamma$-$^{32}$P-ATP as a phosphate donor (left panel). Parallel cultures were labeled with $^{35}$S-methionine (right panel) and immunoprecipitated with the same antisera. SR-RSV-B-transformed CEF, lanes A and I; normal CEF, lanes B and C; normal quail embryo fibroblasts, lane D; RAV-2-infected CEF, lane E; AEV NP clone of transformed CEF, lane F; and MC29-transformed NP quail cell line Q8, lane H. Immunoprecipitation was with the following antisera: nonimmune serum, lane B; unabsorbed tumor-bearing rabbit serum with anti-bodies to pp60$^{src}$, the *gag* proteins and the *env* gene products, lanes A and C-I. Kinase reaction products were analyzed on a 5-15% gradient SDS polyacrylamide gel. Molecular weight x $10^{-3}$ daltons is indicated on the left of each panel and viral proteins of interest are indicated on the right.

adult mice, Harvey and Kirsten sarcoma viruses cause erythroleukemia in newborn mice (Scher et al., 1975). Both viruses encode a 21,000 dalton transformation protein which binds GDP and GTP (Scolnick et al., 1979). No other di- or triphosphates are bound by these proteins (Shih et al., 1980). Because of the similarities between these viruses and AEV, I sought to determine whether p75 could either bind GTP or utilize GTP as a phosphate donor in the immune complex kinase assay used above. Cells were lysed in 100 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris (pH 7.4), and 1% Triton X-100 and the immune complexes isolated in the usual manner and $\gamma^{32}$P-GTP was added. Harvey sarcoma virus p21 bound GTP (Figure 13, lane D) but neither AEV p75 or MC29 110,000 dalton gag-related polyprotein contained either GTP binding activity or GTP-dependent protein kinase activities (Figure 13, lanes F, G, and H). In addition, rat anti-Harvey p21 antiserum did not immunoprecipitate p75, although it did precipitate a protein of about 20,000 daltons from chicken cells which could represent avian c-ras, the endogenous cellular homologue of the Harvey sarcoma virus p21 (data not shown here).

The gag-related polyproteins of AEV and MC29, as well as $pp60^{src}$ of RSV, are apparently not ATPases. This was determined by performing the standard immune complex protein kinase assay, stopping the reaction with SDS gel sample buffer, spinning out the Sepharose beads, and spotting the supernatant fluid on DEAE-52 paper. ATP and free phosphate were separated by high voltage paper electrophoresis (Figure 14). The amount of $P_1$ released from $\gamma^{32}$P-ATP by AEV p75, MC29 p110 or RSV $pp60^{src}$

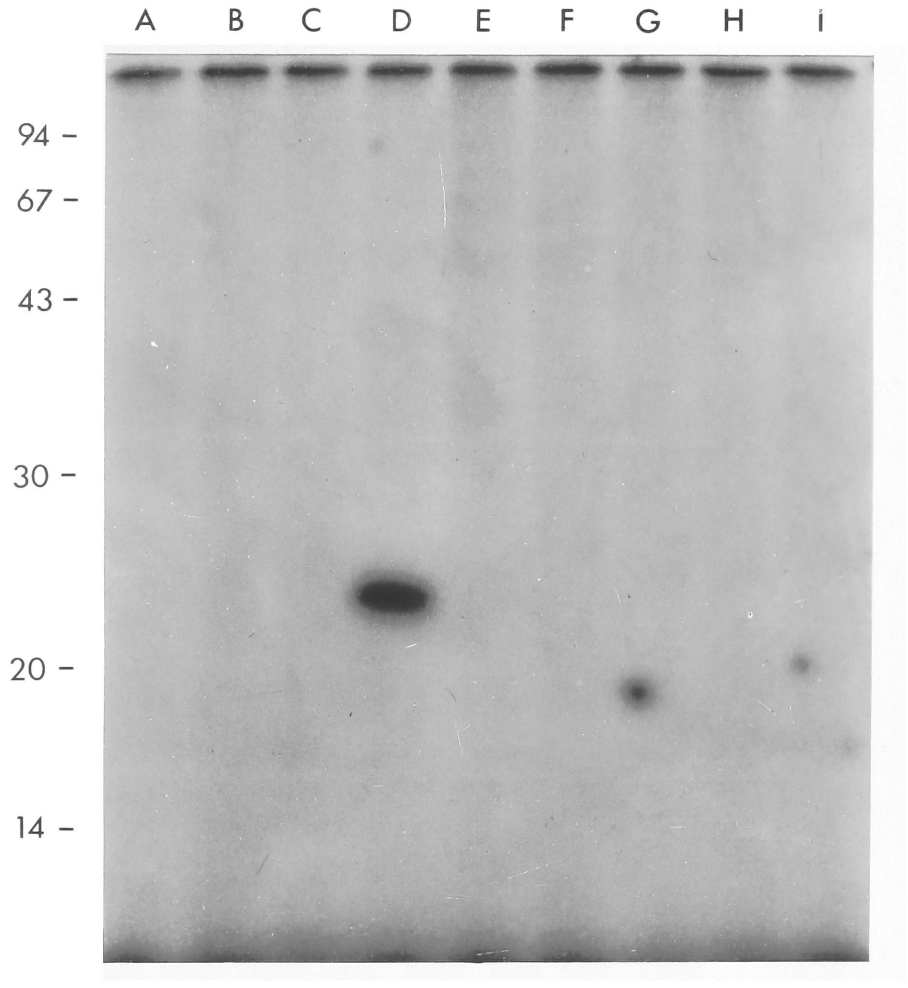


FIGURE 13. GTP binding and kinase activity of AEV p75 and MC29 110,000 dalton *gag*-related polyproteins. Cells were lysed in 100 mM NaCl, 5 mM $MgCl_2$, 20 mM Tris (pH 7.4), 1% Triton X-100, disrupted by mixing on a vortex mixer, and then spun at 36,000 rpm in a Beckman Type 40 rotor for 15 minutes. Lysates were then immunoprecipitated as described and the immune complexes isolated on Protein A-Sepharose beads. $10^{-7}$M $\gamma$-$^{32}$P-GTP (New England Nuclear) was added to the immune complexes for 10 minutes at 32° C. The reaction was terminated by adding SDS gel sample buffer and the reaction products were analyzed on a 12% SDS polyacrylamide gel. Lanes A and B, normal mouse cell line C127D; lanes C and D, Harvey sarcoma virus-transformed NIH 3T3 cells; lane E, normal rat cell line 208F; lane F, AEV-transformed rat cell line AC3b; lane G, MC29-transformed rat cell line MC2; and lanes H and I, AEV-transformed CEF NP clone. Immunoprecipitation was with the following antisera: normal rat serum, lanes A and C; normal rabbit serum, lane I; rat anti-Harvey p21 serum, lanes B and D; rabbit anti-p19 serum, lanes E-H. Molecular weight x $10^{-3}$ daltons is indicated on the left.

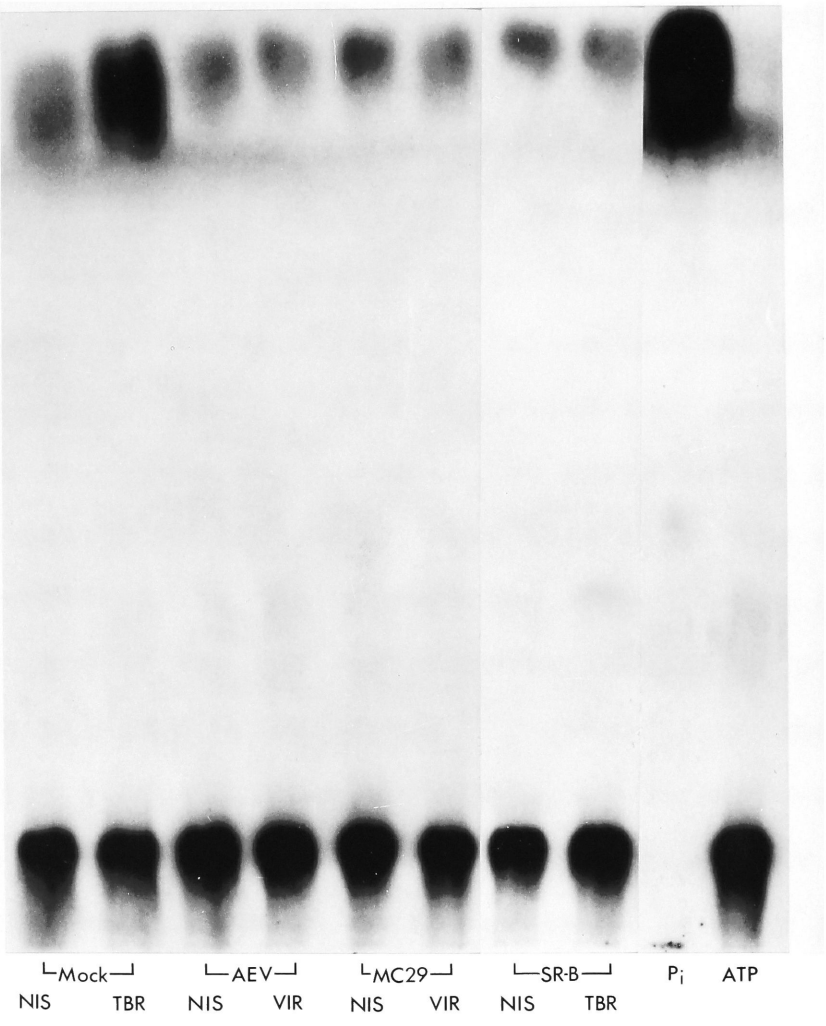


FIGURE 14. Are the *gag*-related polyproteins of AEV and MC29 ATPases? The immune complex protein kinase assay reaction with $\gamma$-$^{32}$P-ATP as substrate was performed as described in Methods. The reaction was for 10 minutes at 30$^{o}$ C and the immune complexes were spun out. The supernatant fluids were removed and 5 ul spotted on Whatman DEAE-52 paper and high voltage electrophoresis was performed to separate ATP from free phosphate. Antisera used were nonimmune rabbit serum (NIS) and tumor-bearing rabbit serum (TBR) used in Figure 11.

was less than that released by immunoprecipitates from uninfected cells (Mock). In addition, AEV p75 did not contain deacetylase activity using $^{3}H$ acetate-labeled duck histones as a substrate (data not shown here).

Is p75 incorporated into the virion? The gag-related polyproteins of Abelson murine leukemia virus (Reynolds et al., 1978) and of the Snyder-Theilen strain of feline sarcoma virus (Stephenson et al., 1977) are incorporated into pseudotyped virus particles. This has aided in the purification of these putative transforming proteins. Were this to be the case with AEV p75 it would aid in the preparation of antiserum against this protein and in the further characterization of p75. This question was examined by purifying $^{35}S$-methionine labeled virus particles from a AEV-NP clone superinfected with RAV-2. Such a preparation includes both RAV-2 and AEV virions. AEV p75 was not seen in either the total virion proteins (Figure 15, lane A) or in proteins precipitated with either anti-gag antiserum or anti-p19 antiserum (Figure 15, lanes C and D respectively). Since AEV p75 contains p19 sequences, immunoprecipitation with anti-p19 antiserum should enrich for p19 and AEV p75 if it was present in the virion. Thus, p75 is apparently not incorporated into the virion like the gag-related transformation proteins of the Snyder-Theilen strain of feline sarcoma virus and Abelson murine leukemia virus.

Presence of a second gene in AEV. The genomic RNA of AEV is 28 S as determined by gel electrophoresis or sedimentation in

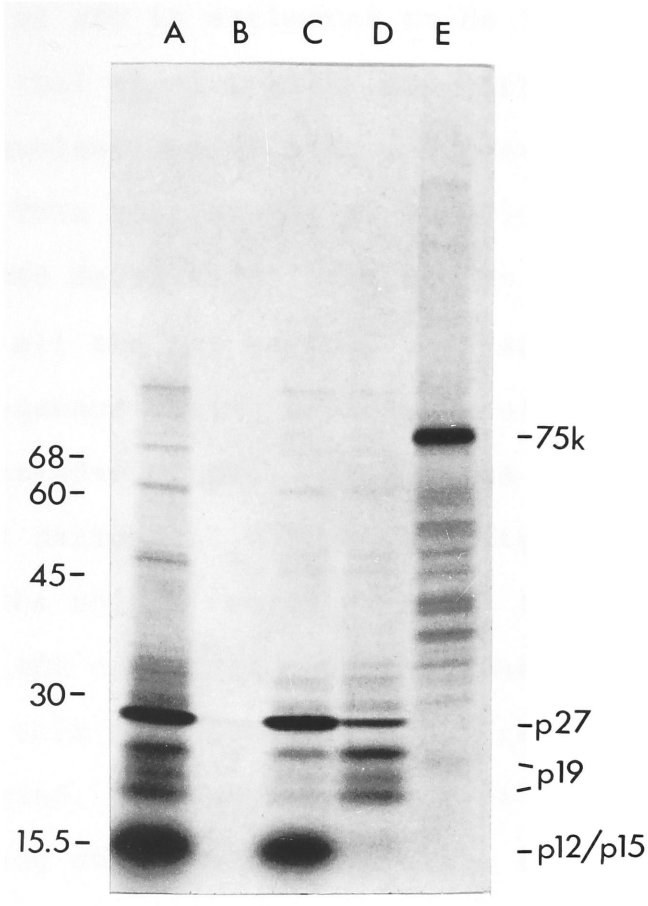


FIGURE 15. Presence of AEV p75 in the virion. $^{35}$S-methionine-labeled AEV(RAV-2) virions were purified as described in the Methods and analyzed on a 5-15% gradient SDS polyacrylamide gel. Lane A, total virion proteins; Lane B, virion proteins immunoprecipitated with nonimmune serum; lane C, virion proteins immunoprecipitated with anti-*gag* serum; lane D, virion proteins immunoprecipitated with anti-p19 serum; lane E, AEV NP clones immunoprecipitated with anti-p19 serum. Molecular weight x $10^{-3}$daltons is indicated on the left and viral proteins of interest are noted on the right.

sucrose density gradients (Bister and Duesberg, 1979; Kamahora et al., 1979) and heteroduplex mapping (Lai et al., 1979). The unique sequence of AEV is estimated to be 3 kb based upon heteroduplexing (Lai et al., 1979) and mapping of RNase $T_1$ resistant oligonucleotides (Bister and Duesberg, 1979; Kamahora et al., 1979). This corresponds to a coding capacity of 100,000 to 120,000 daltons for this region of the genome alone. If p75 contains all the p19 region, then approximately one half of the unique sequence coding capacity would be required to code for the remainder of p75. This means that there are 45,000 to 55,000 daltons of coding capacity remaining unaccounted for in the unique region of AEV. It seemed possible, therefore, that AEV contained a second gene.

To examine this question, the virus-related RNAs in AEV NP cells were analyzed. Two cDNA probes were used in these studies, "cDNA strong stop" and a cDNA probe specific for the unique region of AEV ($cDNA_{aev}$). cDNA strong stop is complementary to the first 101 nucleotides present at the 5' end of all viral RNAs. The $cDNA_{aev}$ probe hybridized to 98% with AEV viral RNA at a Crt of 2. Poly(A)-containing RNA from AEV NP cells was isolated, sized in sucrose gradients and the RNA in each fraction hybridized to both probes. Two peaks of hybridization were detected with both probes (Figure 16). One peak of hybridization co-sedimented with 28 S chicken ribosomal RNA which is the same size as the viral genomic RNA. The second peak of hybridization is approximately 21 S in size. The homology between either RAV-2 or RAV-0 cDNA strong stop and AEV RNA was not as extensive as that between $cDNA_{aev}$ and AEV RNA. However,

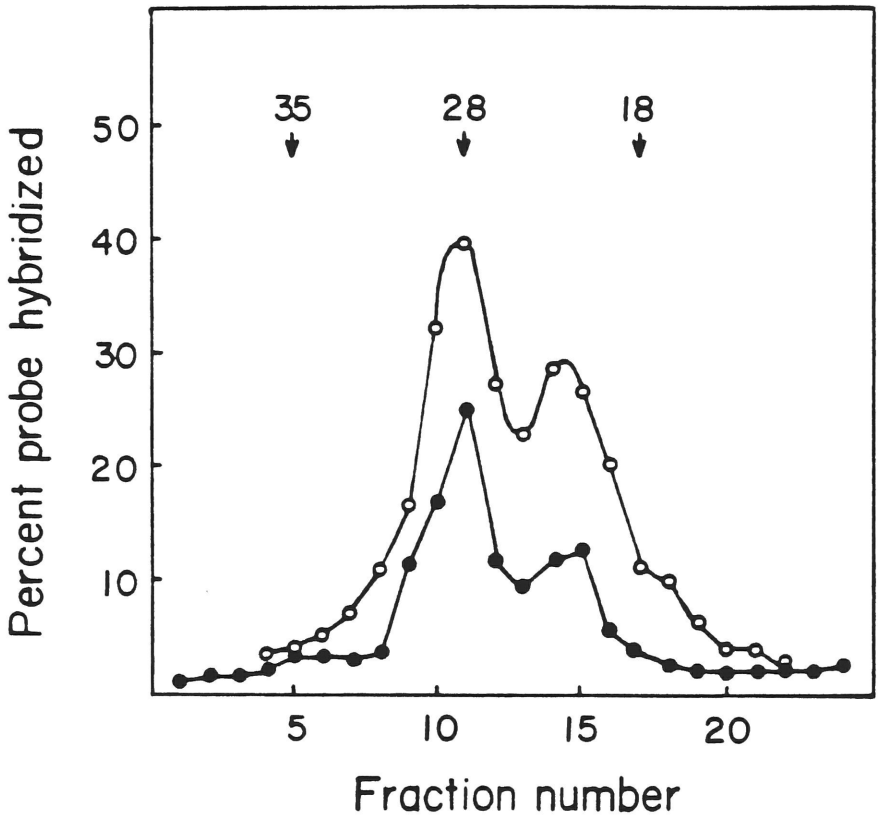


FIGURE 16. Viral RNAs present in AEV NP clone 49. Poly(A)-containing RNAs were sized by sedimentation in a 15-30% sucrose gradient as described in the Methods. Hybridization was for 20 h with either cDNA strong stop (●) or cDNAaev (o). The positions of RAV-2 35 S viral RNA, 28 S rRNA and 18 S rRNA are indicated.

the same ratio of 28 S to 21 S mRNA species was observed following hybridization with cDNA strong stop, as was observed with the $cDNA_{aev}$ probe. Based upon the kinetics of hybridization with $cDNA_{aev}$ we estimated that there are 400-500 copies per cell of the 28 S AEV RNA species and 200-250 copies per cell of the subgenomic 21 S AEV mRNA.

Poly(A)-containing RNA from an AEV NP cell clone was separated by gel electrophoresis, transferred to DPT paper by blotting and hybridized with $^{32}P\text{-}cDNA_{aev}$. The blot shows the presence of two AEV specific RNA species (Figure 17, lane C), confirming the results of solution hybridization experiments.

The $^{32}PcDNA_{aev}$ probe was shown to be specific for AEV viral RNA by analyzing AEV(RAV-2) viral RNA. AEV(RAV-2) viral RNA, which is a mixture of 35 S RAV-2 RNA and 28 S AEV RNA, was electrophoresed in a 1% agarose gel, the RNA transferred to DPT paper and the blot hybridized with either $^{32}P$-cDNA strong stop or $^{32}P\text{-}cDNA_{aev}$. cDNA strong stop hybridized to RNAs of 35 S, 28 S and 21 S (Figure 17, lane A). The 35 S and 28 S RNAs presumably represent the genomes of RAV-2 and AEV, respectively. The 21 S strong stop-containing RNA could be the 21 S RAV-2 *env* RNA which is has been shown to be incorporated into the virion particle (Stacey *et al*,, 1976). When the same blot was hybridized with $cDNA_{aev}$, the probe reacted with two RNA species: one RNA species of 28 S and a second of approximately 21 S (see Figure 17, lane B). These results show that AEV NP cells produce two AEV-specific RNA species of 28 S and 21 S. AEV virus particles also contain both of these two RNA species. Although NP cells appear to contain two molecules of the 28 S

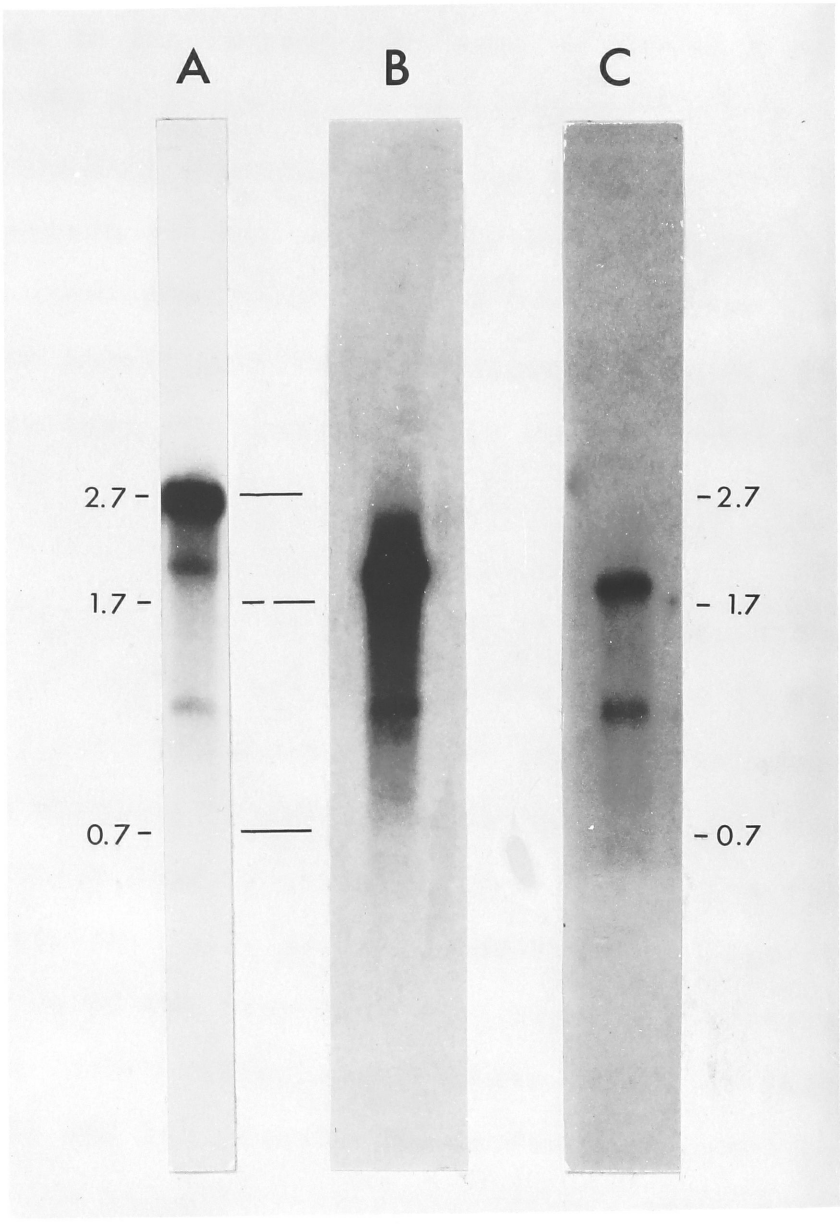


FIGURE 17. Analysis of viral RNAs present in purified virions and in an AEV-transformed fibroblast NP clone. Poly(A)-containing RNA was isolated from AEV(RAV-2) virions (lanes A and B) and AEV NP cell clone 830 (lane C). RNA was separated by electrophoresis in a 1% agarose gel, and was transferred to DPT paper. Hybridizations were with cDNA strong stop (lane A) and with cDNAaev (lanes B and C). The positions of 35 S RAV-2 and 28 S and 18 S rRNA markers are indicated (molecular weight x $10^6$ daltons).

RNA species for each molecule of the 21 S RNA, the viral particles seem to incorporate much more of the 28 S genomic AEV RNA than of the 21 S subgenomic RNA of AEV.

These data provided the first evidence that transformed cells contain two AEV-specific transcripts, one of genome-size and the other subgenomic. Since both of these RNAs hybridized with cDNA specific to the AEV-unique sequences, it is possible that more than one transformation gene is present in the AEV genome.

In vitro translation of AEV viral RNA. Because the two size classes of AEV RNAs are found in the virion, in vitro translation of virion RNA would serve to identify the gene products of both RNA species. Poly(A)-containing viral RNA was fractionated in sucrose density gradients and RNAs of the following size classes pooled: 35 S, 28-30 S, and 20-24 S. Approximately 0.25-0.5 ug of RNA from each size class was translated in vitro using the rabbit reticulocyte lysate system of Pelham and Jackson (1975) and the translation products analyzed by SDS polyacrylamide gel electrophoresis (Figure 18). Translation of 35 S RNA yielded a major translation product of 76,000 daltons and translation of 28-30 S RNA primarily programmed the synthesis of a 74,000-75,000 dalton protein. Several products are produced by the translation of the 20-24 S RNA pool. Proteins of 64,000, 48,000 and 46,000 stand out as major translation products. Several proteins of below 45,000 daltons are seen in translations of virion RNA especially of this size class, although they can also be observed in translations of 35 S

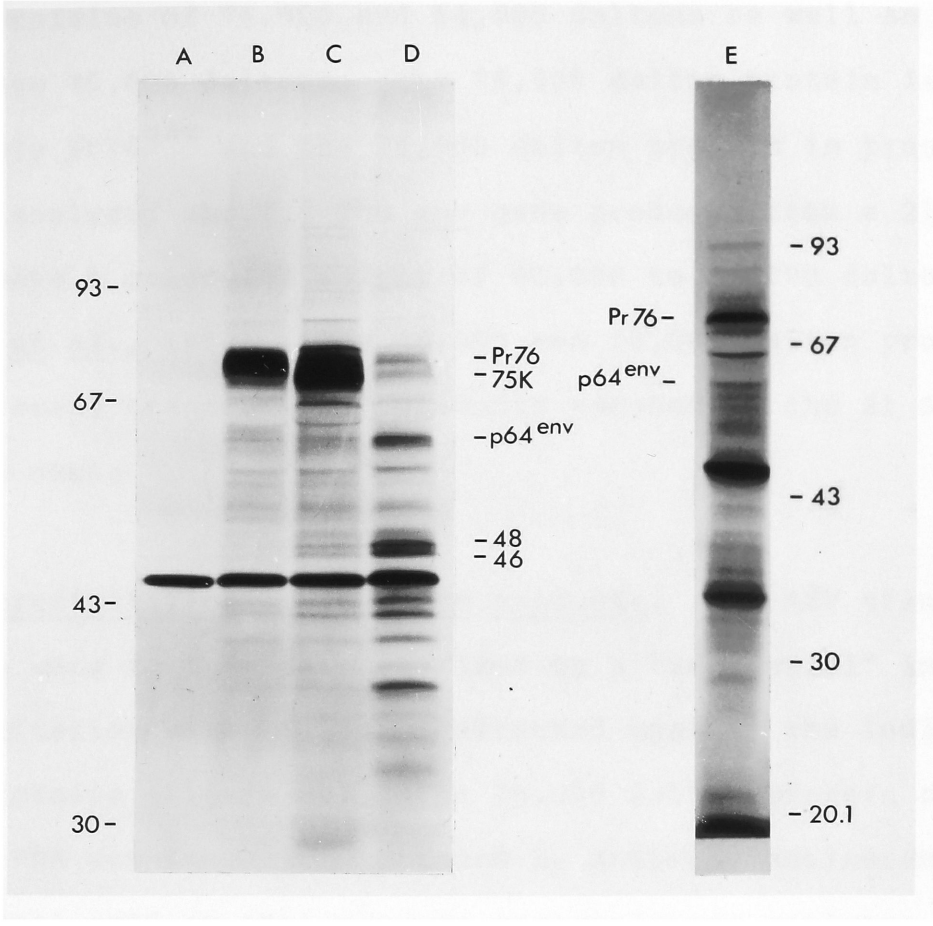


FIGURE 18. Analysis of the translational products of AEV (RAV-2) virion RNA. Poly(A)-containing virion RNAs were sized by sedimentation in a 15-30% sucrose gradient. The following RNA size classes were pooled: 35 S, 28 to 30 S, and 20 to 24 S. *In vitro* translation of 0.25 to 0.5 ug of virion RNA was performed using the rabbit reticulocyte lysate system. The translation products were analyzed in a 8.5% SDS polyacrylamide gel. Lane A, no added RNA; lane B, 35 S RNA; lane C, 28-30 S RNA; lane D, 20-24 S RNA. Lane E contained the translational products of total virion RNA of EAV, the natural helper virus of AEV. Molecular weight x $10^{-3}$ daltons is indicated on the left and right and virus-specific translation products are indicated in the center.

viral RNA. In vitro translation of unsized EAV virion RNA yields proteins of 76,000 and 64,000 daltons as well as those seen below 45,000 daltons. The 76,000 dalton protein is presumably $Pr76^{gag}$ and the 75,000 dalton protein is presumably AEV p75 analyzed above. The env gene produced from a 21 S RNA, should have a molecular weight of 60,000 to 64,000 daltons (Pawson et al., 1980). The 46,000 and 48,000 dalton proteins may represent translational products encoded by the 21 S AEV specific mRNA.

Immunoprecipitation of in vitro products. The AEV translation products were further characterized by a "sequential" immunoprecipitation with antiserum directed against the individual viral proteins (Figure 19). The 76,000 dalton protein produced by 35 S RNA was immunoprecipitated by anti-p19 antiserum, but not by anti-gp85 or anti-reverse transcriptase antisera (Figure 19, lanes B,C,D), and it is presumably $Pr76^{gag}$. There is some carry-over of $Pr76^{gag}$ into the immunoprecipitation with anti-gp85. The major product of 28-30 S RNA is also precipitated by anti-p19 antiserum (Figure 19, lane F). It is not precipitated by anti-reverse transcriptase or anti-gp85 and it is the AEV gag-related polyprotein, p75. The 64,000 dalton translational product of 20-24 S RNA is precipitated by anti-gp85 serum and represents the initial translation product of the env gene (Figure 19, lane K). The 46,000 (p46) and 48,000 (p48) dalton proteins are not precipitated by any of the antisera used (Figure 19, lanes J-L). The material seen in Lane L at about 46,000 daltons appears to have been excluded from the region

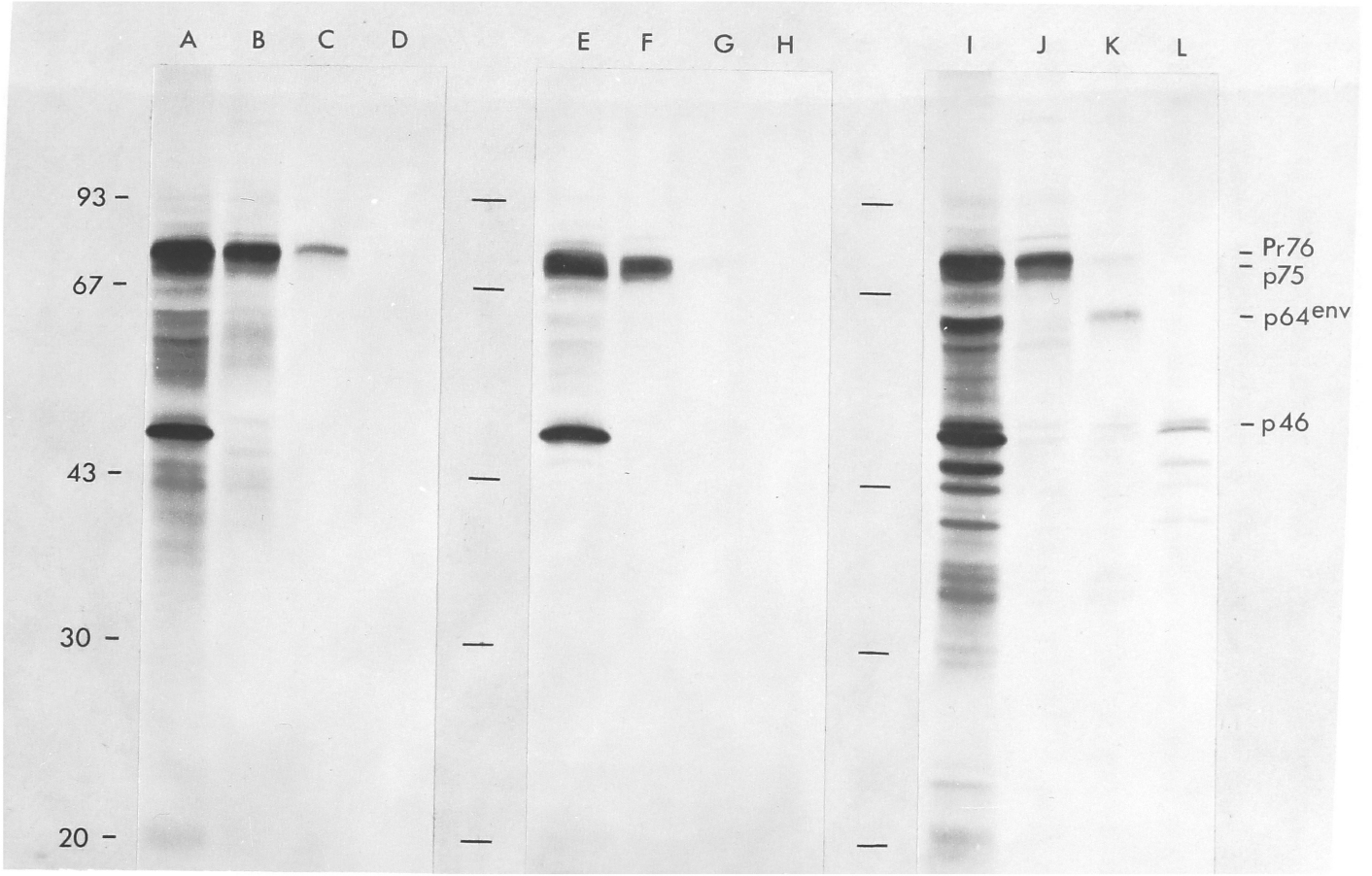


FIGURE 19. Immunoprecipitation of the translation products of AEV(RAV-2) virion RNA. AEV(RAV-2) viral RNA was purified as described in Figure 17 and translated *in vitro*. The translation products were immunoprecipitated sequentially with anti-p19 serum, anti-gp85 serum, and anti-reverse transcriptase serum. The precipitates were analyzed in a 8.5% SDS polyacrylamide gel. Lanes A-D, 35 S RNA; lanes E-H, 28-30 S RNA; and lanes I-L, 20-22 S RNA. Lanes A, E and I are the total translation products (unimmunoprecipitated); lanes B, F and J were precipitated with anti-p19 serum, lanes C, G and K were precipitated with anti-gp85 serum, and lanes D, H and L were precipitated with anti-reverse transcriptase serum. Molecular weight x $10^{-3}$ is indicated on the left and translation products of interest are indicated on the right.

above it by a protein present in the reticulocyte lysate. It was not seen in repeats of the experiment. There is some p75 produced by translation of the 20-24 S RNA and this presumably reflects translation of RNA fragments present in this fraction.

These data indicate that the 35 S RNA is likely to be that of RAV-2 and its major translational product is $Pr76^{gag}$. The 28-30 S RNA pool is a mixture of 35 S RAV-2 RNA and 28 S AEV RNA which code for a mixture of $Pr76^{gag}$ and the AEV p75 protein. The RNAs of the 20-24 S pool include the 21 S RAV-2 *env* mRNA and the AEV subgenomic RNA. The 21 S *env* mRNA codes for $p64^{env}$, whereas the 46,000 and 48,000 dalton proteins appear to be the translational products of the subgenomic AEV RNA and they are not related to the *gag*, *pol*, or *env* gene product of replication-competent RNA tumor viruses.

*Inhibition of in vitro translation by* $^{7m}$*GTP*. $^{7m}$GTP has the property of blocking the translation of capped mRNAs in both the wheat germ system (Hicken *et al*, 1976; Weber *et al*, 1976; Hickey *et al*, 1977) and the rabbit reticulocyte system (Suzuki, 1976). In order to determine whether the translation products of AEV virion RNA were produced by capped poly(A)-containing mRNAs or by translation of RNA fragments, translations were performed in the presence and absence of $^{7m}$GTP. As shown in Figure 20, the synthesis of $Pr76^{gag}$, p75, $p64^{env}$ and the p46/p48 band was inhibited by the presence of $^{7m}$GTP. Thus it appears that p46 and p48 are translated from capped poly(A)-containing mRNAs and not from fragments of viral RNAs.

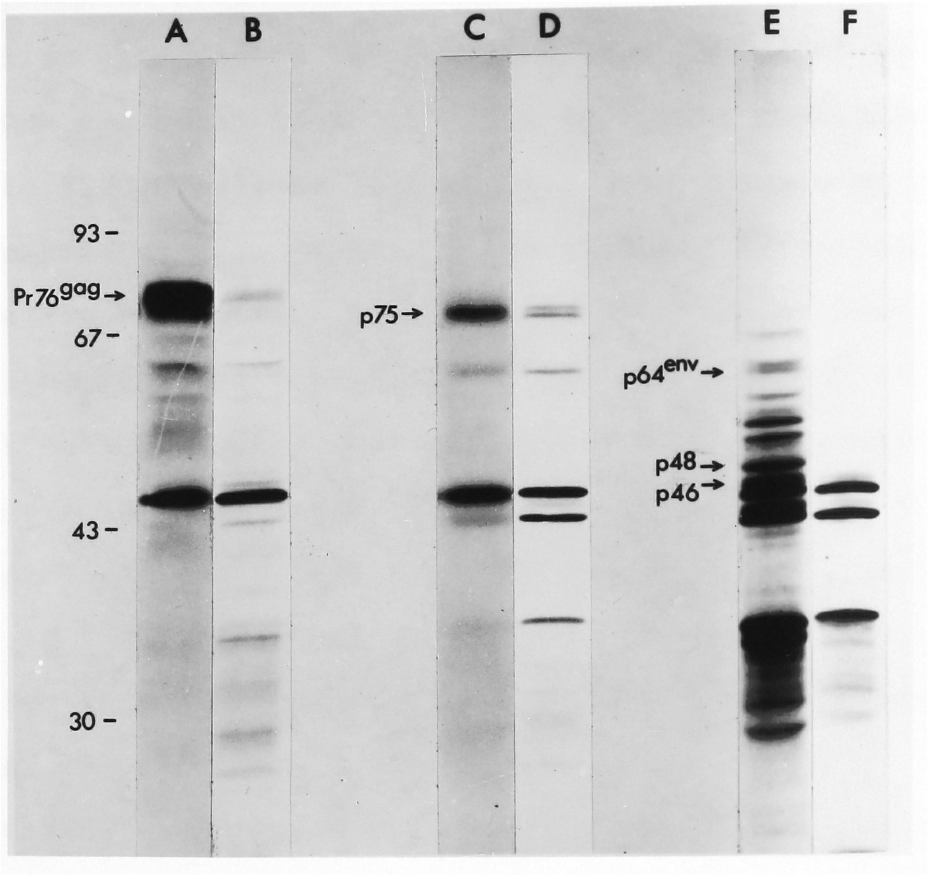


FIGURE 20. The effect of $^{7m}$GTP on *in vitro* translation. Viral RNA isolated as in Figure 17 was translated *in vitro* in the presence and absence of 1 μM $^{7m}$GTP. Translation products were analyzed in a 8.5% SDS polyacrylamide gel. Lanes A, B, 35S RNA; lanes C, D, 28-30 S RNA; and lanes E, F, 20-24 S RNA. Lanes A, C, and E, no $^{7m}$GTp; lanes B, D and F, with $^{7m}$GTP. Molecular weight x $10^{-3}$ daltons is indicated on the left.

Further characterization of p46 and p48. While these investigations were in progress, several other investigators carried out similar experiments in which the translation of 20-24 S AEV virion RNA was noted to program the synthesis a protein of 40,000 to 45,000 daltons (Lai et al., 1980; Pawson et al., 1980; Yoshida et al., 1980). Although these investigators did not directly demonstrate the presence of a second mRNA species produced by AEV, the size class of the RNA encoding this new translational product suggested to them that there might be a second gene in AEV. These authors, however, only observed one novel translational product encoded by 20-22 S AEV instead of the two, p46 and p48, that I observed. Thus, these two polypeptides were analyzed by two-dimensional tryptic fingerprinting.

The results shown in Figure 21, clearly indicate that p46 and p48 are very closely related to each other; their fingerprints differ in only one peptide which is present in p48 (marked by the vertical arrow) which is not present in p46. Concomitant with the presence of this new peptide, there has been a decrease in the amount of a second peptide spot in p48 (marked by the horizontal arrow), compared to the amount of this peptide present in p46. The new peptide in p48 is more negatively charged than the peptide from which it appears to have been derived.

It is tempting to speculate that p48 is derived from p46 by some post-translational modification such as phosphorylation. Attempts to phosphorylate either p46 or p48 have not been successful. A translation of 20-24 S AEV RNA was per-

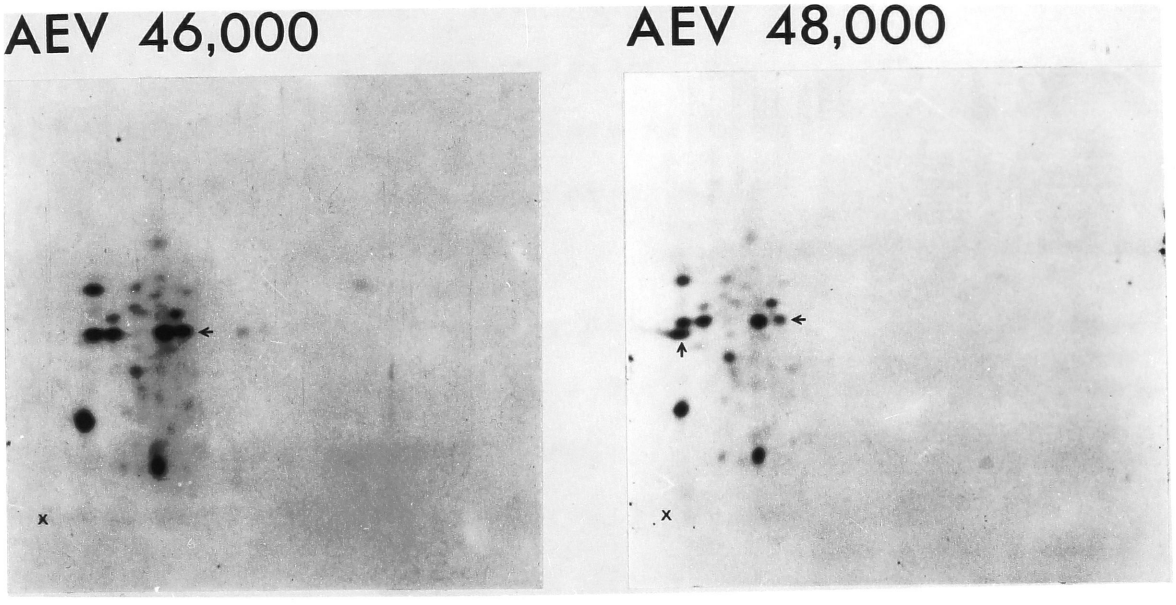


FIGURE 21. Two-dimensional tryptic peptide analysis of the 46,000 dalton (left) and 48,000 dalton (right) *in vitro* translation products of 20-22 S AEV virion RNA. Virion RNA was translated *in vitro* with $^{35}$S-methionine as the label, the bands of interest excised, and worked up as described in Methods. Peptides were spotted in the lower left corner (x) of each panel. Electrophoresis was in the horizontal direction with the anode at the left, at pH 4.7. Ascending chromatography was from bottom to top. The peptide unique to the 48,000 dalton protein is marked with a vertical arrow (↑) and the peptide which is diminished in the 48,000 dalton protein is marked with a horizontal arrow (←).

formed with unlabeled amino acids and after 1 h, $\gamma^{32}$P-ATP was added to the translation products to determine if p46 or p48 could be phosphorylated. The high background level of proteins that were phosphorylated in the rabbit reticulocyte system did not allow a clear answer to the question of whether p46 or p48 could be phosphorylated. When casein was added to the lysate system, it was readily phosphorylated above background levels (data not shown here). Thus there may merely have not been enough p46 or p48 available to observe such a phosphorylation event.

p46 and p48 are apparently not synthesized with the same efficiency by all reticulocyte lysate preparations used, indicating that the enzymes which modify p46, if they do exist, may not be as stable as those involved in translation of RNA. Neither p46 or p48 contain the 19* dipeptide present in AEV p75 (see above), indicating that translation of p46 and p48 does not begin at the initiation codon for the gag gene (data not shown here). An alternative to the protein modification model proposed above, is that p48 is the product of reading through one stop codon and that translation has continued until a second stop codon has been reached. If this is the case, the new peptide present in p48 should be located at the carboxy terminus of p48 and the amount of the peptide noted by the horizontal arrow present in p48 might reflect the extent of contamination of p46 with p48.

The characterization of p46 and p48 has been limited by the fact that this protein was isolated as a translation product of 21 S AEV mRNA. At this time there is no antibody which

is able to cross react with this protein molecule. Further research on p46 and p48 will be hindered until such an antiserum becomes available.

Preparation of an antibody specific for AEV p75. Using a modification of the technique of Beug et al. (1981), I have recently produced an antiserum from chickens that have regressed from a AEV ts34-induced erythroblastosis, that has antibodies directed against the AEV specific determinants in p75. One-week old chicks were inoculated with approximately $10^5$ FFU of AEV ts34. During the next four weeks ten of the nineteen birds died of erythroblastosis. Eight weeks after the original inoculation the survivors were immunized with $10^7$ AEV ts34-transformed bone marrow cells, and the antisera were screened by immunoprecipitation of AEV-transformed cells. Promising antisera were then screened following absorption with disrupted virus protein to demonstrate that the antisera contained antibodies directed against the unique sequences present in AEV and not just against the gag portion of p75. Immunoprecipitation of AEV ts34-transformed bone marrow cells and EAV-infected CEF indicated that the major protein recognized by anti-p75 in transformed bone marrow cells is p75 (Figure 22, lanes B and C). Neither the absorbed or unabsorbed anti-p75 antiserum precipitated the individual gag proteins p12/p15, p19 or p27 from the AEV ts34 cell extract although these proteins were clearly present in the cell lysate as seen in precipitations with the anti-virion antiserum (Figure 22, lanes B and C versus lane D). The gag proteins were also not precipitated from EAV-infected

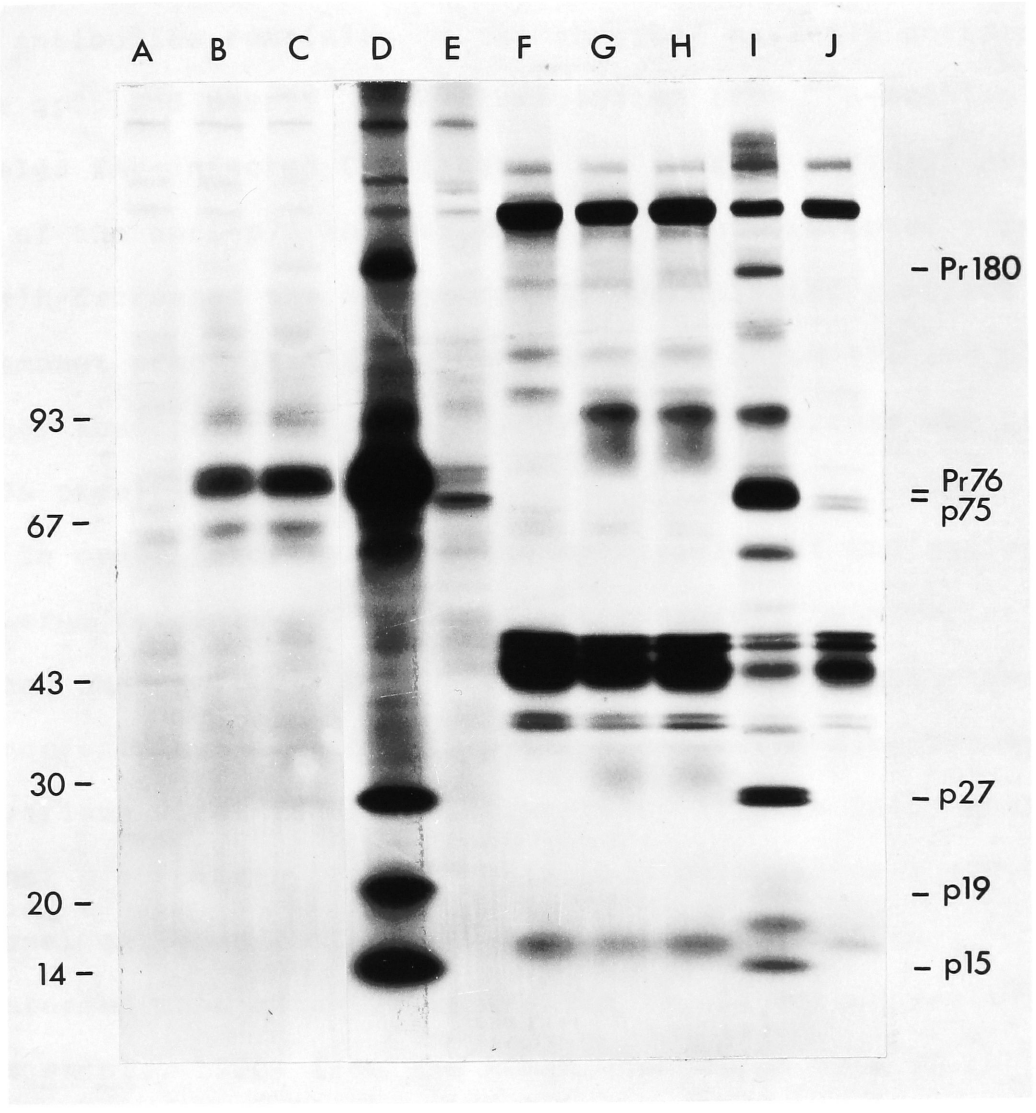


FIGURE 22. Immunoprecipitation of AEV*ts*34-transformed erythroblasts and EAV-infected CEF with anti-p75 serum. Lanes A-E are $^{35}$S-methionine labeled AEV*ts*34-transformed erythroblasts and lanes F-J are labeled EAV-infected CEF. Immunoprecipitation is with the following antisera: lanes A and F, normal chick serum; lanes B and G, anti-p75 serum; lanes C and H, absorbed anti-p75 serum; lanes D and I, anti-*gag* serum; and lanes E and J, normal rabbit serum. Molecular weight x $10^{-3}$ daltons is indicated on the left and viral proteins of interest are indicated on the right.

CEF (Figure 22, lanes G and H). There is some residue anti-gp85 antibodies remaining in the absorbed anti-p75 antiserum since gp85 and $pgPr95^{env}$ is precipitated from $^{35}S$-methionine labeeled EAV-infected CEF (Figure 22, lanes G and H). Absorption of the anti-p75 antiserum with 35ug of disrupted virus protein decreased the amount of p75 precipitated compared to the amount precipitated by the unabsorbed serum (Figure 23). Further absorption of the antibody did not decrease the amount of p75 precipitated by the antiserum (Figure 23).

In order to demonstrate the specificity of the anti-g75 antiserum for only AEV p75, cells transformed by a variety of RNA and DNA tumor viruses were labeled with $^{35}S$-methionine and immunoprecipitated with anti-p75 and antiserum directed against the various viral transforming proteins (Figure 24). Anti-p75 did not precipitate the SV40 T (93,000 daltons) or t (20,000 daltons) antigens (McCormick et al., 1979) from SV40-transformed muntjac cells or the Ela 53,000 dalton protein(s) (Ross et al., 1980) from the human adenovirus type 5-transformed hamster cell line 297-C43 (Figure 24). In addition, the transforming proteins of the following RNA tumor viruses were not immunoprecipitated by anti-p75 (Figure 24): Harvey sarcoma virus (p21, Shih et al., 1979), Friend spleen focus-forming virus (gp52, Ruscetti et al., 1980), the Snyder-Theilen strain of feline sarcoma virus (Barbacid et al., 1980), the McDonough strain of feline sarcoma virus (Barbacid et al., 1980), Schmidt-Ruppin strain of RSV ($p60^{src}$), Fujinami sarcoma virus (T. Hanafusa et al., 1979), MC29, AMV, and E26.

It would be interesting if the anti-p75 antiserum could

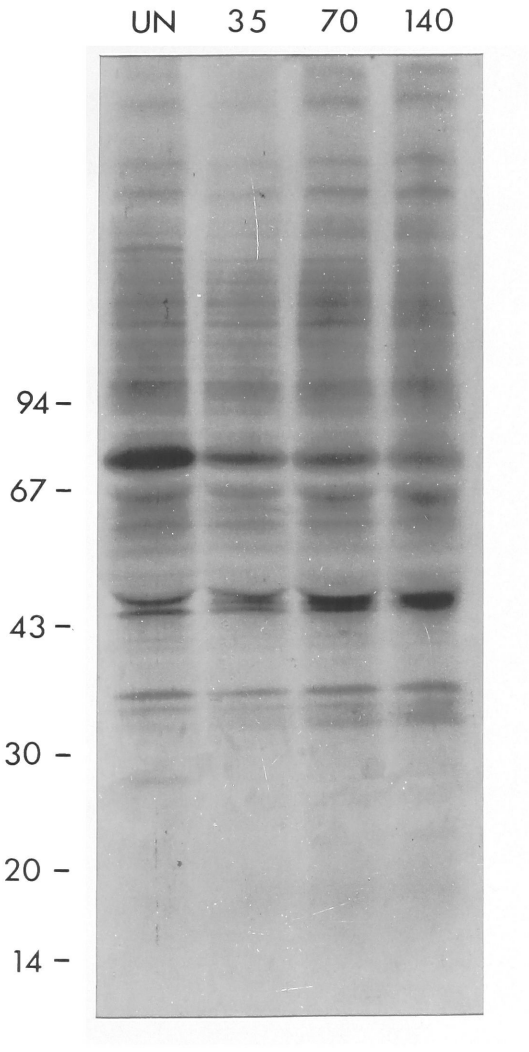


FIGURE 23. Absorption of anti-p75 antiserum with disrupted RAV-2 virions. Unlabeled virions were purified as described in Methods. Chicken anti-p75 was absorbed with varying amounts of RAV-2 virion proteins disrupted in RIPA for 1 h on ice. $^{35}$S-methionine-labeled AEV *ts*34 (RAV-2)-infected bone marrow cells were immunoprecipitated with the absorbed antibody. Lane A, unabsorbed antiserum; lane B, absorbed with 35 ug/5 ul serum; lane C, absorbed with 70 ug/5 ul serum; lane D, absorbed with 140 ug/5 ul serum. The precipitates were analyzed in a 5-15% gradient SDS polyacrylamide gel and molecular weight x $10^{-3}$ daltons is indicated on the left.

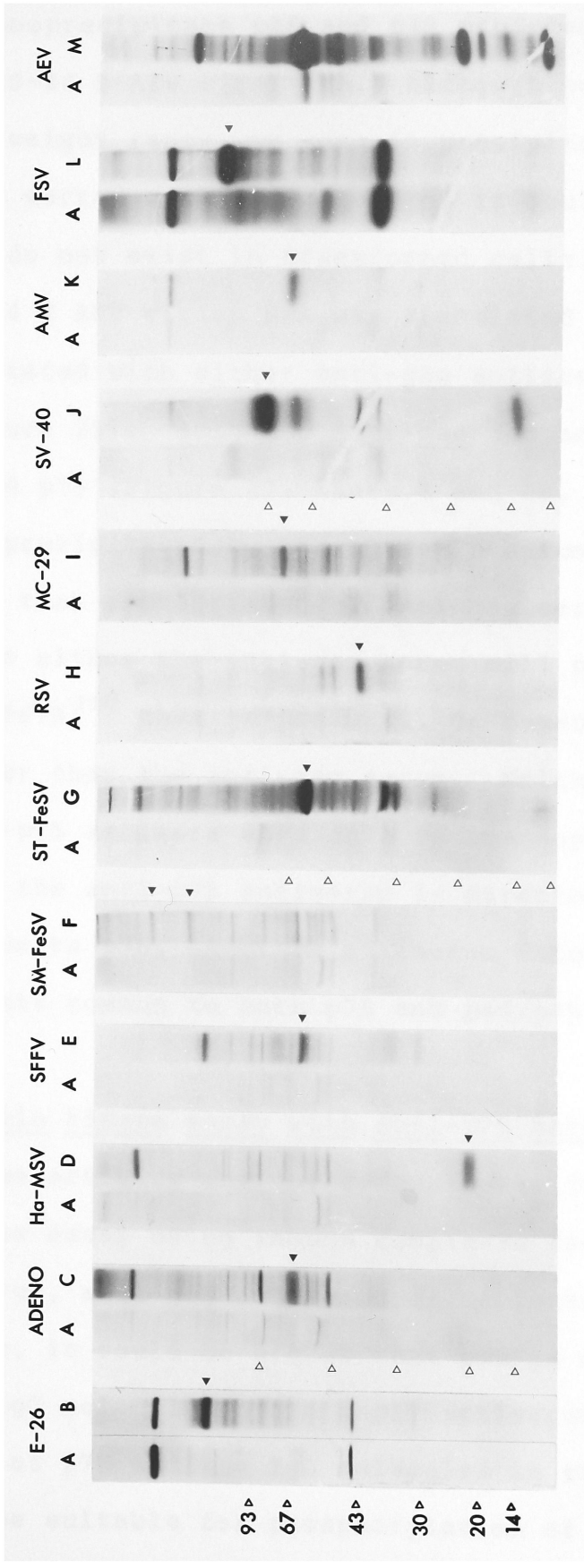


FIGURE 24. Immunoprecipitation of virus-transformed cell lines with anti-;75 serum. The indicated cell lines were immunoprecipitated with either anti-p75 serum (lanes labeled A) or antiserum directed against their specific transforming proteins. Lanes B, I, K, L and M, rabbit anti-*gag* serum; lane C, hamster anti-ND4 serum; lane D, rat anti-Harvey p21 serum lane E, rat anti-gp52 serum ; lane F, anti-FeLV *gag* protein serum; Lane G, anti SF-FeSV transformed mink cell serum; lane H, tumor-bearing rabbit antiserum (anti-p60); lane J, SV40 tumor-bearing hamster antiserum. The specific transforming proteins of interest are indicated by the dark arrows. Molecular weight x $10^{-3}$ daltons is indicated by the open arrows for each set of panels.

immunoprecipitate p46 and p48 produced by *in vitro* translation of 20-24 S AEV viral RNA. Although no protein of this molecular weight range was seen in precipitations of AEV-transformed bone marrow cells (Figure 21), it could be argued that p46 and p48 do not exist in transformed cells. Therefore, 28-30 S and 20-24 S AEV virion RNA was translated *in vitro* and immunoprecipitated with either anti-*gag* antiserum or anti-p75 antiserum (Figure 25). Both the anti-*gag* and anti-p75 antisera precipitated p75 (Figure 22, lanes B and C), although the amount of p75 precipitated by the anti-p75 serum was considerably less than that precipitated by anti-*gag* serum. This could be because either the anti-*gag* serum will precipitate both p75 and any $Pr76^{gag}$ that is produced, or because the anti-p75 serum is weaker than the anti-*gag* serum. Neither the anti-*gag* nor the anti-p75 antisera were able to immunoprecipitate p46 or p48. Thus the anti-p75 antiserum is directed against determinants unique to p75, and this antiserum detected no antigenic determinants common to both p75 and p46/p48.

*Protein kinase assay with anti-p75 serum.* Although a protein kinase activity was not demonstrable in AEV p75 by the protein kinase assay using immune complexes formed with anti-p19 antiserum, anti-*gag* antiserum or unblocked tumor-bearing rabbit serum, it could be argued that p75 is not able to phosphorylate the IgG molecules of anti-p19 antiserum because the conformation of p75 and the IgG molecules in the immune complexes may not be suitable for phosphorylation of the latter. The protein kinase activity of Fujinami sarcoma virus p140 shows a prefer-

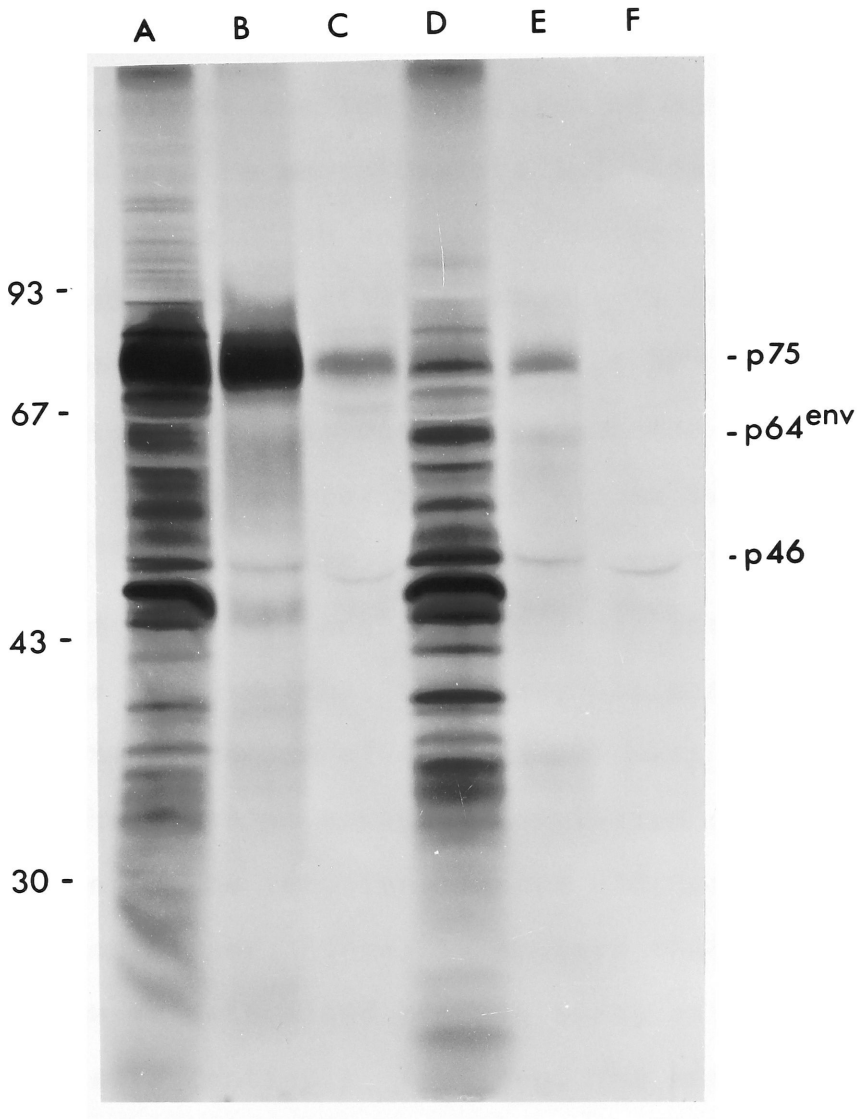


FIGURE 25. Immunoprecipitation of AEV(RAV-2) virion RNA translation products with anti-p75 serum. The 28-30 S viral RNA fraction (lanes A-C) and the 20-24 S viral RNA fraction (lanes D-F) of AEV(RAV-2), prepared as in Figure 17, were translated *in vitro* with the rabbit reticulocyte system. The total translation products of 28-30 S and 20-24 S AEV(RAV-2) virion RNA are shown in lanes A and D, respectively. Translation products were immunoprecipitated with either rabbit anti-p19 serum (lanes B and E) or with absorbed chicken anti-p75 serum (lanes C and F). Translation products were analyzed on a 8.5% SDS polyacrylamide gel. Molecular weight x $10^{-3}$ daltons is indicated on the left and translation products of interest are indicated on the right.

ence for the antibodies which it phosphorylates; p140 only weakly phosphorylates the IgG molecules of anti-p19 antiserum although it is able to phosphorylate IgG molecules in tumor-bearing rabbit serum which apparently recognizes the gag determinants of p140 (Feldman et al., 1980). To determine whether such a problem existed in the analysis of AEV p75, p75 was immunoprecipitated by rabbit anti-p19 serum and chicken anti-p75 serum and the immune complex protein kinase reaction performed. Although $pp60^{src}$ was active in a control reaction, p75 immunoprecipitated by either antiserum did not act as a protein kinase (Figure 26). Neither the heavy chain of rabbit IgG nor the light or heavy chains of chicken IgG were phosphorylated by AEV p75 and there was no autophosphorylation of p75. The addition of casein to the reaction mixture did not result in its phosphorylation either. Thus, it appears that AEV p75 is not a protein kinase as determined by this assay using four different types of antisera (anti-p19, anti-gag, TBR and anti-p75).

Immunofluorescent studies with anti-p75 antiserum. The availability of an antiserum specific for the unique region of AEV p75 makes it possible to carry out experiments to determine the localization of p75 in a variety of transformed cells. Preliminary results of immunofluorescence studies with chicken anti-p75 antiserum indicates that, in AEV-transformed rat cells, p75 is located in the cellular membrane, in cell ruffles and in a perinuclear region (Figure 27). Panels B and D in Figure 27 clearly show localization of immunofluorenscent labeling in cell ruffles. The membrane staining is observed in Panels F

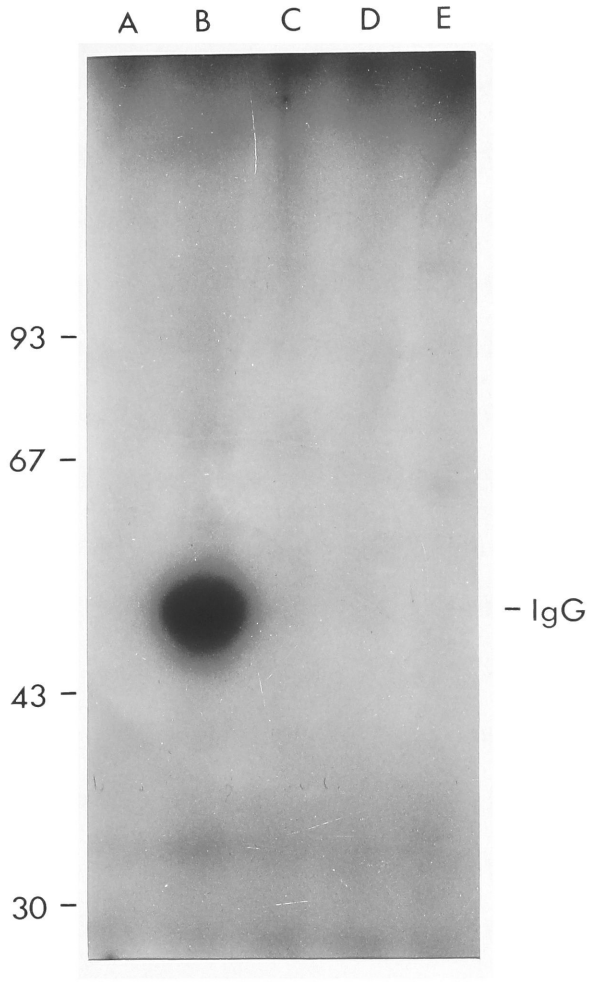


FIGURE 26. Kinase assay of AEV p75. Schmidt Ruppin RSV-infected cells and AEV-transformed rat cell line AC3b were immunoprecipitated and the immune complex protein kinase assay performed as described in the Methods. Lanes A and B, SR-RSV-B-transformed CEF and lanes C-E, AEV-transformed rat cell line AC3b. Immunoprecipitation was with the following antisera: normal rabbit serum, lane A; tumor-bearing rabbit serum which precipitates $pp60^{src}$, lane B; normal chicken serum, lane C; rabbit anti-p19 serum, lane D; absorbed chicken anti-p75 serum, lane E. Kinase products were analyzed on a 7.5% SDS polyacrylamide gel. Molecular weight x $10^{-3}$ daltons is indicated on the left and the position of rabbit IgG is indicated on the right.

and H. Perinuclear staining is seen in all panels (B,D,F and H). There is clearly no staining of either the nucleus or the nuclear envelope. The perinuclear region that is stained by anti-p75 has not been further characterized at this time. Normal rat fibroblasts do not show any of these patterns of fluorescence. There is a slight staining of a cytoplasmic granule, but this is not a very intense staining. Studies with AEV-transformed CEF and AEV-transformed chicken erythroblasts are in progress. Cell fraction studies, in which the amount of AEV p75 present in different cell fractions is determined by immunoprecipitation, are also in progress. The transforming proteins of several other RNA tumor viruses, including RSV (Rohrschneider, 1979; Willingham et al., 1979; Courtneidge et al., 1980; Krueger et al., 1980a,b) Fujinami sarcoma virus (R. Feldman, personal communication) and Harvey sarcoma virus (Willingham et al., 1980) are membrane-associated. Therefore, it would be interesting if this membrane of transforming proteins has anything to do with either the sarcomagenicity of these viruses or with the pathways by which these viruses transform cells.

Attempts to identify a cellular homologue of AEV p75. Attempts to identify the cellular homologue of p75 in tissues of normal chickens have not been successful at this time. Direct immunoprecipitation of $^{35}$S-methionine labeled normal spleen, liver, bursal, lymphocytes and blood cells with absorbed anti-p75 antiserum, has not identified a candidate for the endogenous p75 homologue (data not shown here). Experiments using the tech-

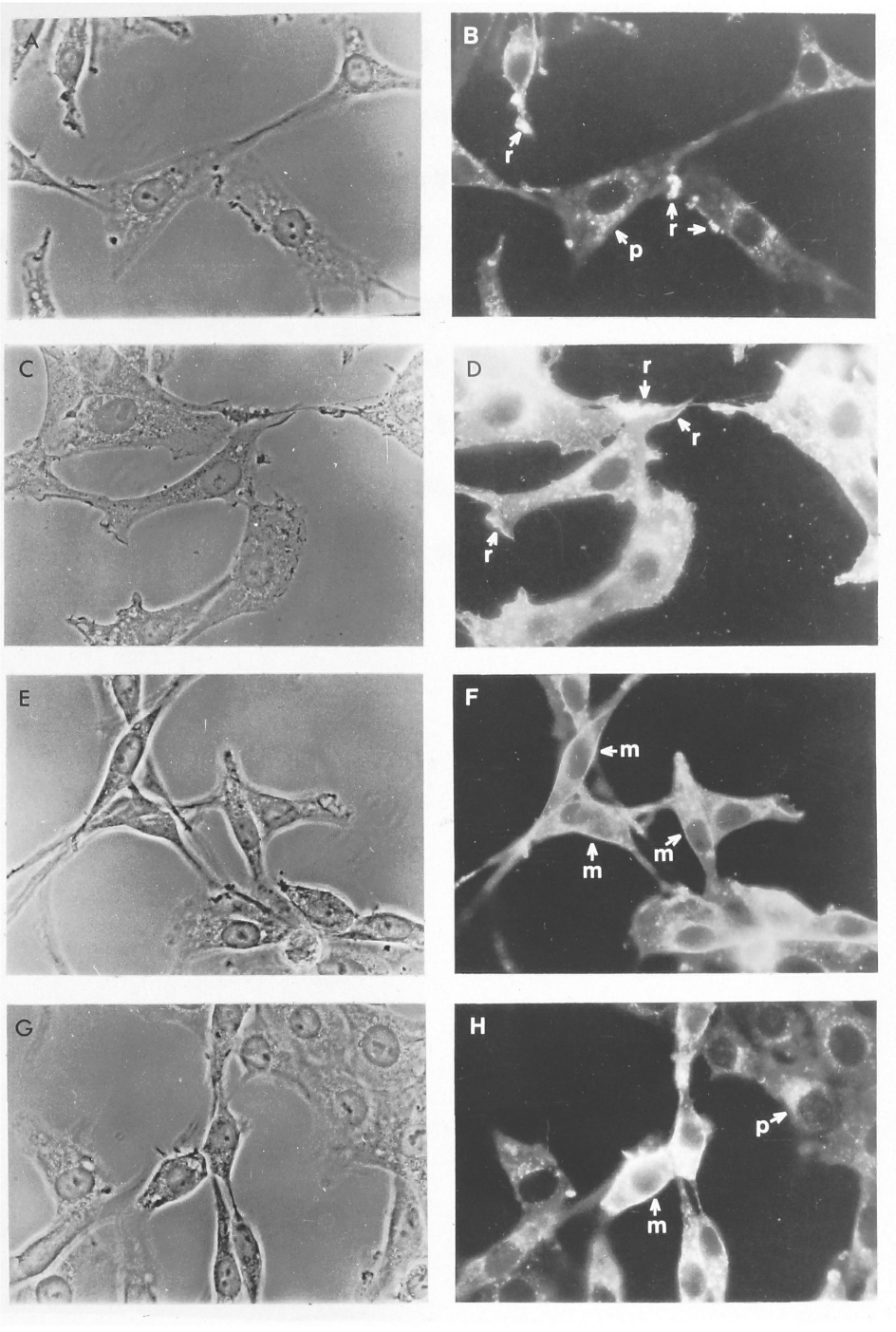


FIGURE 27. Immunofluorescent staining of AEV-transformed rat cell line AC3b. All cells were stained with chicken anti-p75 serum that had been absorbed with normal rat cells and with 35 ug/5 ul antibody disrupted virion protein. Panels A, C, E and G are phase contrast photographs of the same field shown in panels B, D, F and H, respectively, which show the fluorescent staining patterns seen with anti-p75. Arrows indicate points of interest. Membrane staining (m), stained cell ruffles (r), and perinuclear staining (p) is noted.

nique of transferring unlabeled proteins from SDS polyacrylamide gels to nitrocellulose filters and probing these filters with anti-p75 antiserum have also not met with success. Identification of viral proteins in infected cells and of the 120,000 dalton gag-pol fusion protein present in $gs^+$ cells, using a rabbit anti-virion antiserum has been successful using the method of protein transfer to nitrocellulose filter with rabbit antibodies as probes. There apparently are some technical difficulties involving the use of chicken antiserum which I have not yet solved.

# IV. Discussion of Research on Avian Erythroblastosis Virus

## Discussion

In the preceding chapter I have described my work with AEV. Analysis of nonproducer clones of AEV-transformed CEF isolated from soft agar, confirmed the defectiveness of AEV as described by Graf et al. (1976b). The transforming activity of AEV could only be rescued by replication-competent helper viruses including RAV-1, RAV-2, RAV-7, RAV-50 and RAV-61. Golden pheasant virus and Amherst pheasant virus were not capable of rescuing AEV from NP cells, although both these viruses replicated in AEV NP cells. The inability of these two viruses to rescue AEV from NP cells is presumably due to sequence differences that exist between AEV and these viruses in the region of the genome required for correct packaging of the virion RNA (Shank and Linial, 1980). AEV can not be rescued from NP cells following co-cultivation with gs+chf-, gs-hE, and Bryan RSV(-) cells, nor can AEV NP cells co-cultivated with RSV(-) cells rescue infectious RSV.

Immunoprecipitation of AEV NP cells with antisera specific for the viral structural proteins demonstrated that AEV coded for a protein of 75,000 daltons (p75). This protein was precipitated only by anti-p19 serum. No other structural proteins or protein precursors were observed in these cells. This protein was clearly distinguishable from $Pr76^{gag}$ based upon precipitation with antisera directed against each of the gag proteins and by two-dimensional gel electrophoresis. Two-dimensional tryptic peptide mapping demonstrated that AEV p75 contained p19 peptides in addition to unique peptides. The

cross-reaction of p75 with specific antisera and the tryptic peptide mapping data have also been described by Hayman et al. (1979) and their data are in full agreement with the data presented here.

Further analysis of AEV p75 demonstrated that it is phosphorylated and that it contains a phosphoserine residue, the same phospho-amino acid present in p19 and p12 (Lai, 1976; Erikson et al., 1977). Phospho-tryptic peptides maps of p12, p19 and p75 were prepared and the map of p75 was the same as that of p19. No phosphopeptides unique to p75 were identified. These results suggest that p75 is phosphorylated at a serine residue in the p19 gag region it contains. In addition, AEV p75 contains the 19* dipeptide indicating that the translation of p75 begins at the gag gene initiation codon and that p75 contains at least the first two amino acids of the gag gene. Bister et al. (1980b) have recently confirmed my observation on the phosphorylation of AEV p75, however, these authors did not examine the phosphoamino acid(s) present in p75 nor did they analyze the phosphopeptides of p75.

AEV p75 is not capable of phosphorylating either itself or IgG molecules in the immune complex protein kinase assay. This was true when either ATP or GTP are used as phosphate donors and with the following antisera: rabbit anti-p19 serum, rabbit anti-gag serum, unblocked tumor-bearing rabbit serum, and chicken anti-p75 serum. This protein also does not contain associated ATPase or histone deacetylase activities.

AEV NP cells contain two viral mRNAs that can be detected by hybridization with both a cDNA probe specific for the unique

sequences present in AEV or with "cDNA strong stop." The RNAs present in AEV NP cells are 28 S, the size of the AEV genome, and 21 S. Both of these RNA species can be detected in the virions of pseudotyped AEV and sufficient amounts can be purified to allow *in vitro* translation of both RNAs. As determined by *in vitro* translation experiments, the 28 S RNA encodes the 75,000 dalton *gag*-related polyprotein and the 21 S RNA encodes two AEV-specific proteins of 46,000 and 48,000 daltons (p46 and p48). Two-dimensional peptide mapping of p46 and p48 indicates that they are closely related. The 46,000 dalton protein may undergo post-translational modification, such as phosphorylation, to produce the 48,000 dalton protein. The presence of a second AEV-specific mRNA has also been recently described by Sheiness *et al*. (1981). Using probes derived from a molecularly cloned preparation of AEV, they have shown that this second RNA species is derived from the 3' end of the AEV-specific sequences. Several investigators have also described *in vitro* translation products of AEV virion RNA (Lai *et al*., 1980; Pawson *et al*., 1980; Yoshida and Toyoshima, 1980). All of these investigators have identified a protein of 40,000 to 45,000 daltons which is produced by translation of 20-24 S AEV virion RNA.

The role of p46/p48 in transformation by AEV has not been further analyzed because there are no antibodies currently available which recognize this protein(s). This protein is translated from the 20-22 S mRNA isolated from either AEV *td*359 or AEV *ts*34 virion RNA (Beug, *et al*., 1980). AEV *td*359 and AEV *ts*34 are the two known transformation mutants of AEV.

An antibody directed against the AEV-specific sequences present in p75 was prepared using a modification of a protocol described by Beug et al. (1981) in which chickens that had regressed from AEV ts34-induced erythroblastosis were immunized with AEV ts34-transformed erythroblasts. The transforming proteins of a variety of DNA and other RNA tumor viruses are not immunoprecipitated by anti-p75 antiserum.

A model for the structure of the AEV genome and its subgenomic mRNA is shown in Figure 28. For the purpose of comparison, I show the structures of the 35 S and 21 S RAV-2 mRNAs from virus-infected cells (Hayward, 1977; Weiss et al., 1977). AEV 28 S RNA has of leader of 0.35 to 0.45 Kb based upon sequencing of the RAV-2 leader sequence (G.P. Gasic and W.S. Hayward, personal communication) and approximately 0.6 kb of the 5' end of the gag gene. This is followed by a contiguous stretch of approximately 3 Kb of sequences unique to AEV and approximately 1.5 Kb of sequences at the 3' end of the viral genome which are shared with the 3' end of RAV-2. This model is consistent with data available for $T_1$ oligonucleotide fingerprinting (Bister and Duesberg, 1979; Kamahora et al., 1979) and with heteroduplex mapping (Lai et al, 1979) and restriction mapping of molecularly cloned AEV (Venstrom et al., 1980). AEV p75 is produced by translation beginning at the gag gene initiation codon and terminating approximately half-way through the unique sequence of AEV. The model predicts there is an AUG codon and the appropriate sequence for a splice acceptor site present in the middle of the AEV specific sequence. The splicing of the 5' leader sequence to this acceptor site

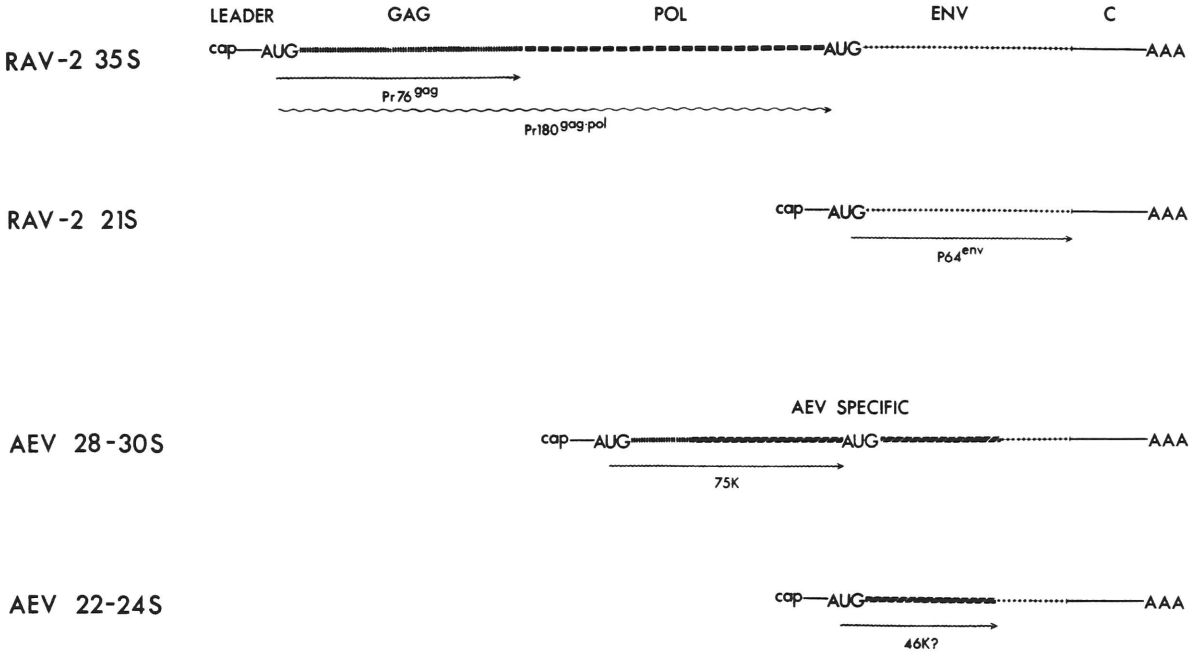


FIGURE 28. Model for the genomic structure of AEV and its subgenomic mRNA. Structures of the RAV-2 35 S mRNA codes for $Pr76^{gag}$ and $Pr180^{gag-pol}$, and the 21 S mRNA codes for teh unglycosylated *env* gene product, which is then glycosylated to yield $gPr95^{env}$, the precursor of gp85 and gp37. The positions of the *gag*, *pol* and *env* genes, as well as those of the leader and c regions, are indicated.

results in the production of a 21 S subgenomic mRNA containing primarily AEV-specific sequences. This subgenomic mRNA apparently encodes the 46,000 dalton protein seen in *in vitro* translations of AEV 20-24 S viral RNA. That this second gene contains its own AUG codon is suggested by the fact that these proteins do not contain the 19* peptide diagnostic for proteins whose synthesis begins at the *gag* gene initiation codon and contain the first two amino acids of the *gag* gene. Alternatively, the splicing mechanism producing the 21 S mRNA must be very precise in locating the *gag* gene AUG in the proper position. Sequence analysis of the middle of the AEV unique region in molecularly cloned AEV DNA, should help to further evaluate these predictions.

The finding that there are two genes present in AEV raises the question of whether both are involved in the transformation of cells by AEV. There are four models predicting the ways in which these hypothetical *onc* genes could interact to result in transformation (Figure 29). The first model, model A, assumes that there is one gene which is responsible for the transformation of both target cells. Model B states that there are two genes, each required for the transformation of only one target cell. Model C hypothesizes that there are two genes, one required for the transformation of one target cell, and one required for the transformation of both target cells. The most complex model, model D, assumes that there are two transformation genes, which are both required for the transformation of both target cells.

There are two known mutants of AEV; AEV *ts*34 (Graf *et al.*,

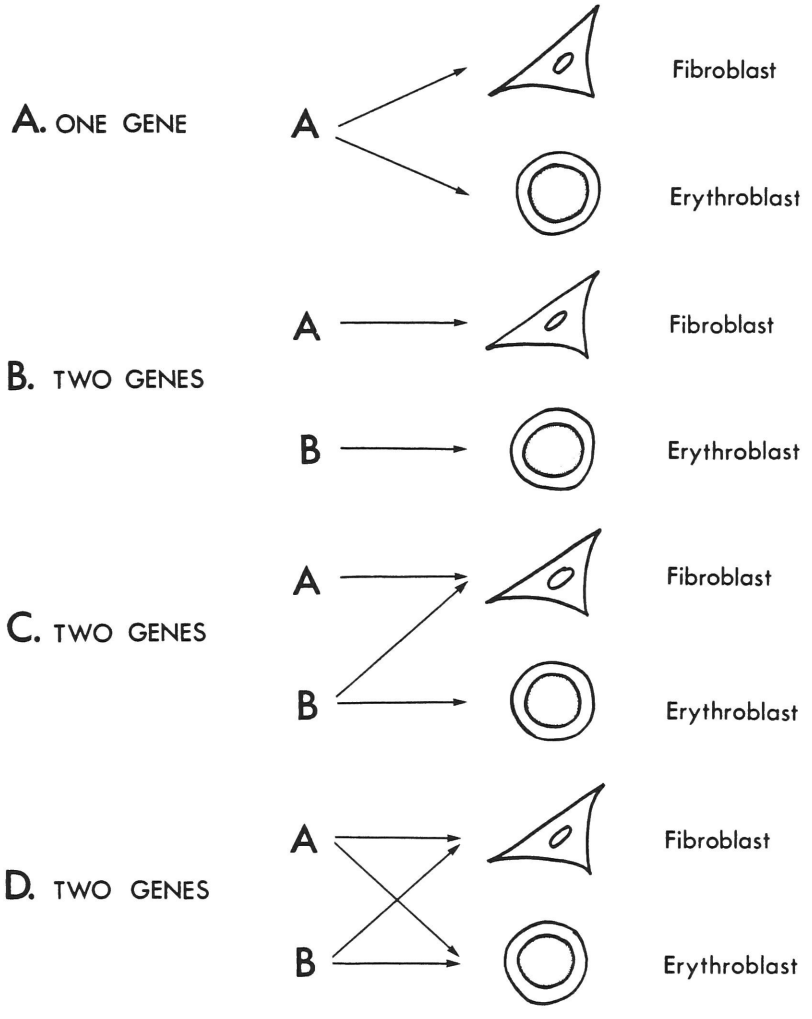


FIGURE 29. Possible models for transformation of two targets by AEV involving one or two gene products. See text for discussion.

1978; Beug and Graf, 1980) and AEV td359 (Royer-Pokora et al., 1979). Transformation of both erythroblasts (Graf et al., 1978) and fibroblasts (Beug and Graf, 1980) by AEV ts34 is temperature-sensitive. AEV td359 is a nonconditional mutant for the transformation of bone marrow cells and the induction of leukemia, but it is still able to transform fibroblast cells in vitro and induce sarcomas in vivo (Graf et al., 1978). AEV td359 contains a deletion of some sequences at the carboxy-terminus of p75 (Beug et al., 1980), and thus produces what is known as △p75. Subgenomic RNA from both AEV ts34 and td359 is able to produce a protein of about 45,000 daltons by in vitro translation (Beug et al., 1980).

These data may be of help in evaluating the four models presented above. The fact that the lesion in AEV td359 results in both the inability to transform bone marrow cells and in the synthesis of △p75, clearly implicates this gene in the transformation of erythroblasts. It does not mean that p46 is not involved in the transformation of erythroblasts, it merely means that at least p75 is required.

The fact that AEV p75 is required for erythroblast transformation does not rule out a role in fibroblast transformation. It is possible that different domains of the same protein are required for the transformation of different target cells and that a lesion in one part of the molecule might not effect the action of the other domain. It has been hypothesized that different regions of $pp60^{src}$ are responsible for the pleiotropic effects of src observed in different cell systems (Friis et al., 1977; Friis and Weber, 1977). The work

of Calothy et al. (1978) in the chick neuroretinal system with the Rous sarcoma virus and its mutants also supports this model. These examples show that AEV p75 could have multiple functions and thus be responsible for the transformation of both target cells.

The pleiotropism of AEV p75 is strongly supported by the observation that the lesion of AEV ts34 affects transformation of both fibroblasts and erythroblasts, if one assumes the lesion of ts34 is in p75. Alternatively, the results with AEV ts34 could be taken to indicate that there exists a protein which is required for the transformation of both target cells, and since p75 may only be required for the transformation of bone marrow cells (data from td359), one might be led to believe that the lesion in ts34 actually lies in the second gene product, p46. The fact that AEV ts34 p75 does not have a temperative-sensitive antigenicity and that it does not turn over at a faster rate at nonpermissive temperature, compared to permissive temperature, could also be taken to indicate that the ts lesion does not lie in p75. The 20-24 S viral RNA of both ts34 and td35 can be translated in vitro to produce p46 (Beug et al., 1980; data not shown). A temperature-sensitive lesion, however, would not necessarily cause a change in the molecular weight of a protein, thus p46 could be ts in AEV ts34. Unfortunately, the data available can not really settle the argument of whether there are one or two transforming genes in AEV. The preparation of an antiserum directed against p46 would be of great help in determining the role of this protein in cell transformation by AEV.

Another very interesting question is whether the transformation process utilized by AEV shares any common steps or pathways with that of RSV or any other transforming virus. The fact that AEV-transformed CEF share certain properties with other transformed cells, such as elevated glucose transport, loss of LETS, and disappearance of actin cables (Royer-Pokora et al., 1978), indicates that at least some of the pleiomorphic effects of the "transformation cascade" expressed in AEV-transformed CEF may be the same as those expressed in cells transformed by other viruses such as RSV. At least one of the cellular proteins which is altered following transformation by RSV, is also altered following transformation of fibroblasts by AEV (Radke and Martin, 1979). Whether other cellular proteins affected by RSV transformation are also altered in AEV-transformed cells has yet to be determined.

Recent work of Racker and his coworkers has demonstrated the cellular src homologue, c-src, may be the same as $PK_f$, an enzyme involved in the control of the $Na^+K^+$-ATPase (M. Spector, R.B. Pepinsky, V. Vogt and E. Racker, personal communication). The pathway (Spector et al., 1981) for this process is:

$$PK_f \rightarrow PK_L \rightarrow PK_S \rightarrow PK_M \rightarrow Na^+K^+\text{-ATPase}$$

In addition, it has recently been found that the 20,000 dalton subunit of $PK_M$ is the endogenous Harvey sarcoma virus p21 (E.M. Scolnick, M. Spector and E. Racker, personal communication). Since the endogenous counterparts of two viral transforming proteins have been localized along this pathway, it is tempting to speculate that others may also interact with this pathway. The endogenous Harvey sarcoma p21 is found at very high levels

in a hematopoietic precursor cell line (Scolnick et al., 1981) and thus it appears as though the control of cell differentiation may also intersect with this pathway at some point. In this light it is also very interesting that the differentiation of Friend erythroleukemic cell lines can be stimulated by treatment with oubain (Bernstein et al., 1976), an inhibitor of the $Na^+K^+$-ATPase. Obviously, it would be of interest to determine whether either AEV p75 or p46 interact with any of the enzymes along this pathway or whether these cellular homologues are part of this pathway. $PK_M$ has a 40,000 dalton subunit and $PK_L$ has subunits of 44,000 and 48,000 daltons (Spector et al., 1981) and p46 could be related to one of these proteins.

While the question of how a virus transforms a cell is certainly of importance, it is also of great interest to determine how a virus is able to apparently block the differentiation of a cell, as AEV does in the case of transformed bone marrow cells. One important question is whether the process involved in transforming a cell, such as a fibroblast, is the same that involved in blocking the differentiation of a stem cell or differentiating cell. The question of whether a cell whose differentiation is blocked is actually transformed, i.e. malignant, is also very important. It is also essential to distinguish between cell transformation by a specific gene product and mechanisms by which the growth of a cell is affected merely by the presence of the virus or by virus growth (Gross et al., 1979).

In the case of AEV, the expression of viral genes has clear effects upon the differentiation of erythroid cells.

AEV-transformed erythroblasts express H5, the erythrocyte specific histone (Graf et al., 1977bc), but they do not synthesize hemaglobin as determined by benzidine staining and radio-immune assay (Graf et al., 1977c). AEV leukemic cells rapidly take up $^{59}Fe$, but instead of the iron being incorporated into heme, it is apparently converted to ferritin (Bather et al., 1963). In addition, although the globin genes are transcribed to produce pre-mRNA or hnRNA, this RNA is apparently not processed to yield mRNA which is translated to produce globin (Therwath and Scherer, 1978). Thus the differentiation of the erythroblast has been interrupted at a very precise point in the differentiation process. Whether the virus accomplishes this via modification of a general cell processes, such as the $Na^+K^+$-ATPase cascade, or by specifically inhibiting processing of a differentiation pathway-specific gene product which might stimulate or allow further differentiation of that cell (e.g. globin), is not known. Clearly the answers to these questions will have great implications for both developmental biologists and oncologists.

# V. Translational Products of Avian Myeloblastosis Virus

Although AMV has been a favorite virus of many molecular biologists because it is a rich source of reverse transcriptase, the biochemical analysis of AMV has lagged behind that of many other RNA tumor viruses. This has been primarily because AMV-transformed myeloblasts are not the most convenient cells to grow *in vitro*. The discovery that AMV was defective (Moscovici *et al.*, 1975; Moscovici, 1975) and the analysis of other acute leukemia viruses such as MC29 and AEV, set the conceptual framework for understanding the structure of AMV. Preparation of a cDNA probe specific for AMV has demonstrated that the genome of AMV is 34 S or 7.5 kb (Chen *et al.*, 1981; Gonda, 1981). The AMV specific *myb* sequences are located at the 3' end of the viral genome and are transcibed to produce a 21 S subgenomic mRNA. I have sought to analyze the intracellular proteins of AMV and compare them with those observed following *in vitro* translation of AMV virion RNA. The purpose has been to identify the putative *myb* gene product(s). The apparent absolute specificity of AMV for one target cell, makes the comparison of its transformation protein(s) to those with multiple target cells such as AEV-ES4 and RSV very interesting.

*Viral Proteins Present in AMV Transformed Myeloblasts*. To determine what viral proteins could be detected in AMV NP myeloblast clones, myeloblasts were labeled with $^{35}$S-methionine and immunoprecipitated with anti-reverse transcriptase, anti-gp85, and antisera specific for each of the individual *gag* proteins (See Figure 30A). NP cells contained Pr180$^{gag\text{-}pol}$, the read-through product of the *gag* and *pol* genes (Opperman *et al.*,

1977; Hayman, 1978), and the gag gene products $Pr76^{gag}$, p19, p27, p12 and p15. Since the individual gag proteins were found in addition to $Pr76^{gag}$, $Pr76^{gag}$ must be processed in NP cells. The env gene product gp85 was not observed in AMV NP cells, consistent with observations that AMV does not contain env sequences detectable by cDNA hybridization (Chen et al., 1981; Gonda et al., 1981). Immunoprecipitation of AMV producer myeloblasts clones demonstrated the presence of all normal viral proteins and protein precursors including $Pr180^{gag-pol}$, $Pr76^{gag}$, p19, p27, p12, p15, gp37, gp85 and $gPr92^{env}$ (data not shown here).

Figure 30B shows a second independently isolated AMV NP myeloblast clone that produces a protein of 110,000 daltons which is precipitated by anti-gag antiserum. This protein is seen in about 50% of the AMV clones examined (J. H. Chen, personal communication), thus this protein is probably not a translation product of the AMV genome. The low amount of this protein present in cells has prevented its further analysis. On a gross level, there does not appear to be any biological differences between between those clones which contain this 110,000 dalton protein and those that do not contain this protein.

Presence of AMV specific subgenomic mRNA within the virion. An AMV-specific subgenomic mRNA of 21 S has recently been described (Chen et al., 1981; Gonda et al., 1981). This RNA contains the first 101 nucleotides present at the 5' end of the viral genome, which is known as the strong stop region, the AMV

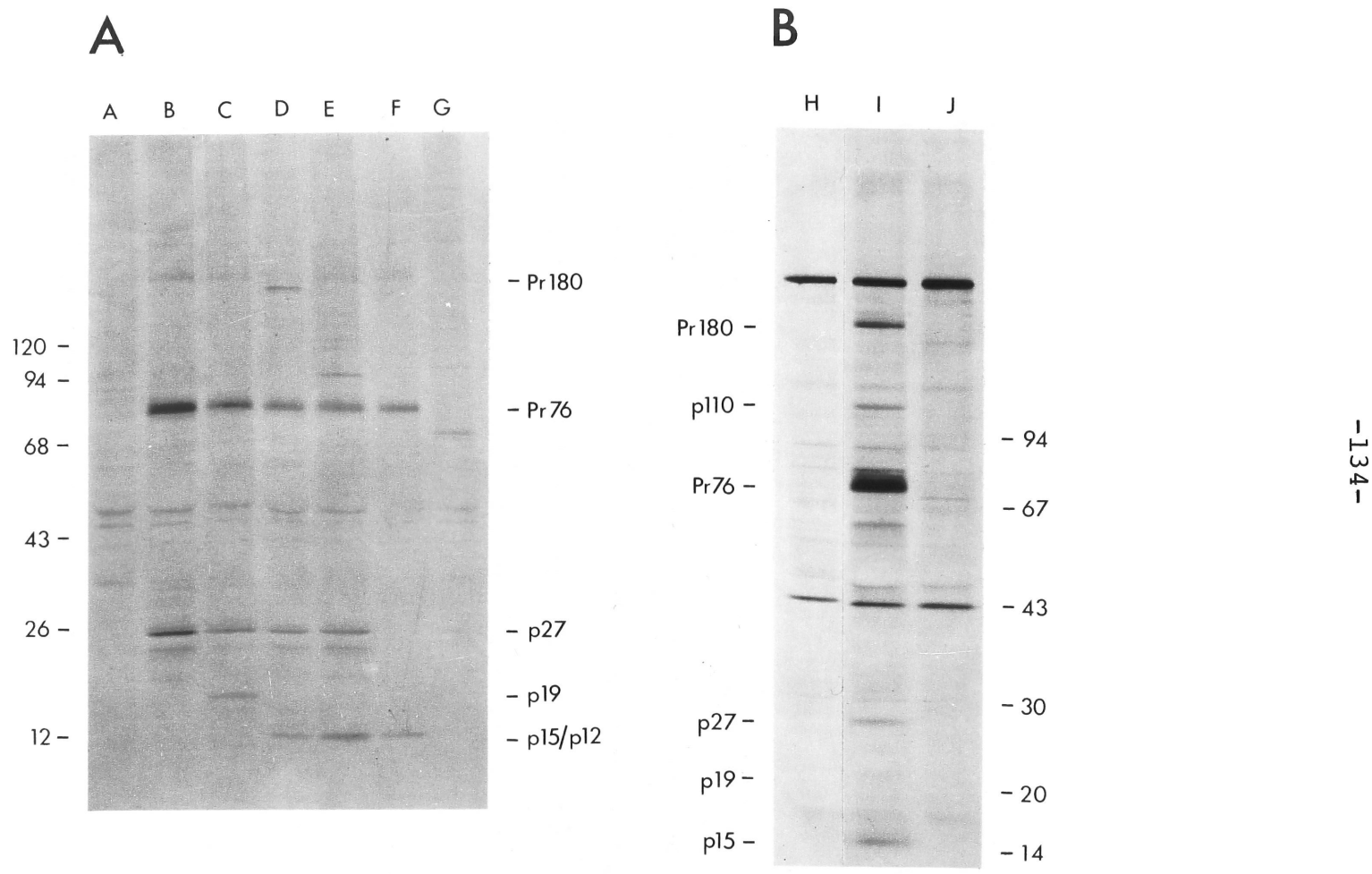


FIGURE 30. Immunoprecipitation of two independently isolated AMV-transformed nonproducer myeloblast clones with specific antisera. Two independently isolated AMV NP clones (panels A and B, respectively) were labeled with $^{35}$S-methionine and the lysates immunoprecipitated with the following antisera: nonimmune rabbit serum, lanes A and H; anti-p19 serum, lane B; anti-p27 serum, lane C; anti-p12 serum, lane D; anti-p15 serum, lane E; anti-reverse transcriptase serum, lane F; anti-gp85 serum, lanes G and J; and anti-*gag* serum which recognizes p19, p27, p12, and p15, lane I. The immunoprecipitates were analyzed in 5-15% SDS polyacrylamide gels. Molecular weight x $10^{-3}$ is indicated on the outside and proteins of interest are indicated in the center.

unique sequences called myb, and the "c" region (Chen et al., 1981; Gonda et al., 1981). Previous work with the RAV-2 21 S env mRNA (Stacey et al., 1977) and the 21 S AEV-specific subgenomic RNA (see above), demonstrated that these RNAs could be incorporated into the virion and could be efficiently translated both in vivo and in vitro . To see if the AMV subgenomic RNA was also packaged, AMV and its helper virus, MAV-1, virion RNAs were denatured with glyoxal, electrophoresed in a 1.0% agarose gel, and the RNA transferred to DBM paper by the technique of Alwine et al. (1977). The blot was then hybridized with either $cDNA_{AMV}$ or $cDNA_{MAV-1}$. The results indicate that a small amount of the 20-21 S AMV specific subgenomic RNA is incorporated into the AMV virion (Figure 31, Lane 2). Very little 21 S env mRNA is observed in these preparations of virion RNA (Figure 31, lanes 4 and 5) apparently reflecting the low efficiency of packaging subgenomic mRNAs into the virion. The AMV virion associated 20-22 S RNA was utilized to identify the translational products of this AMV-specific RNA by in vitro translation experiments.

In vitro translation of AMV virion RNA. AMV virion RNA was purified from virus obtained from plasma of leukemic chickens and sized in sucrose density gradients. RNA from each sucrose gradient fraction was translated in vitro using the rabbit reticulocyte lysate system. Major AMV translational products (Figure 32A) were seen at 76,000, 56,000, 48,500, 47,000, 43,000, 41,000, 37,000, 35,000, 34,000 and 31,000 daltons. Figure 32B shows the translational products of MAV-1 virion RNA

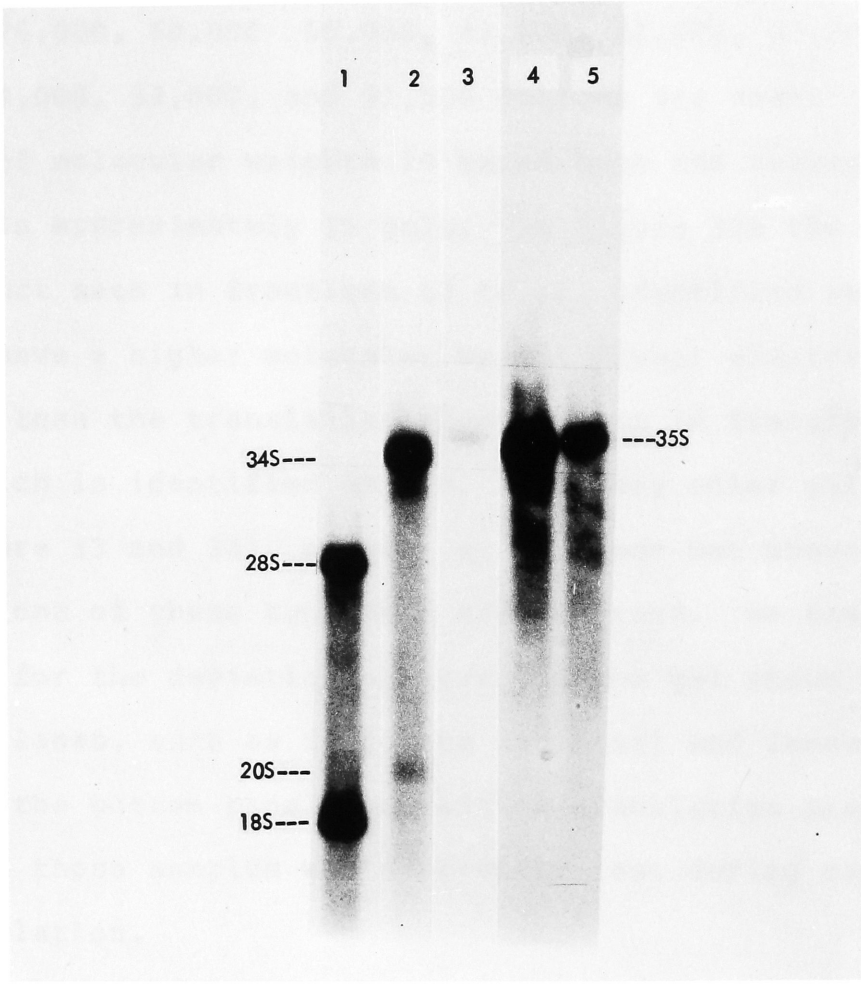


FIGURE 31. Size of AMV-specific RNA. 1.5 ug of poly(A)-containing 70 S AMV-A and MAV-1 RNA were denatured in 1 M glyoxal in 10 mM phosphate (pH 7.0) at 50° C for 30 minutes, sized by electrophoresis in a 1% agarose gel, and transferred to DBM paper. Blots were hybridized with either $^{32}$P-cDNA$_{AMV}$ (lanes 2 and 3) or $^{32}$P-cDNA$_{MAV-1}$ (lanes 4 and 5). Approximately 10,000 cpm probe/cm$^2$ filter paper was used in the hybridizations. Lane 1, $^{32}$P ribosomal RNA; lanes 2 and 4, AMV-A 70 S RNA; and lanes 3 and 5, MAV-1 70 S RNA.

prepared and sized by sucrose gradient sedimentation in the same manner as the AMV RNA was prepared. Translational products of 76,000, 60,000 56,000, 43,000, 41,000, 37,000, 35,000, 34,000, 32,000, and 31,000 daltons are seen. The assignment of molecular weights is based upon the average of that observed in approximately 15 gels. In Figure 32A the translation product seen in fractions 13 to 18, identified as p47, appears to have a higher molecular weight (lower electrophoretic mobility) than the translation product seen in fractions of 16 to 20, which is identified as p48. In every other gel shown here (Figure 33 and 34), as well as in those not shown here, the positions of these two bands are reversed. We have no explanation for the deviation observed in the gel shown in Figure 32. Some lanes, such as 12 in the top panel and lanes 3, 15 and 24 in the bottom panel, contain no translation products. The RNA in these samples was apparently lost during experimental manipulation.

The two proteins of 41,000 and 43,000 daltons are minor bands that appear to be specific to the lysate system. The bands at 31,000, 34,000, 35,000 and 37,000 daltons appear to be virus-specific translational products produced by various strains of Rous sarcoma viruses-and Rous associated viruses (R. E. Karess and S. M. Anderson, unpublished data). The 76,000 dalton protein seen in translations of AMV and MAV-1 is $Pr76^{gag}$; its identity has been confirmed by immunoprecipitation and by the presence of the 19* dipeptide (see below). The 60,000 dalton protein seen in MAV-1 translation is the unglycosylated env gene product (see below). Proteins of 56,000

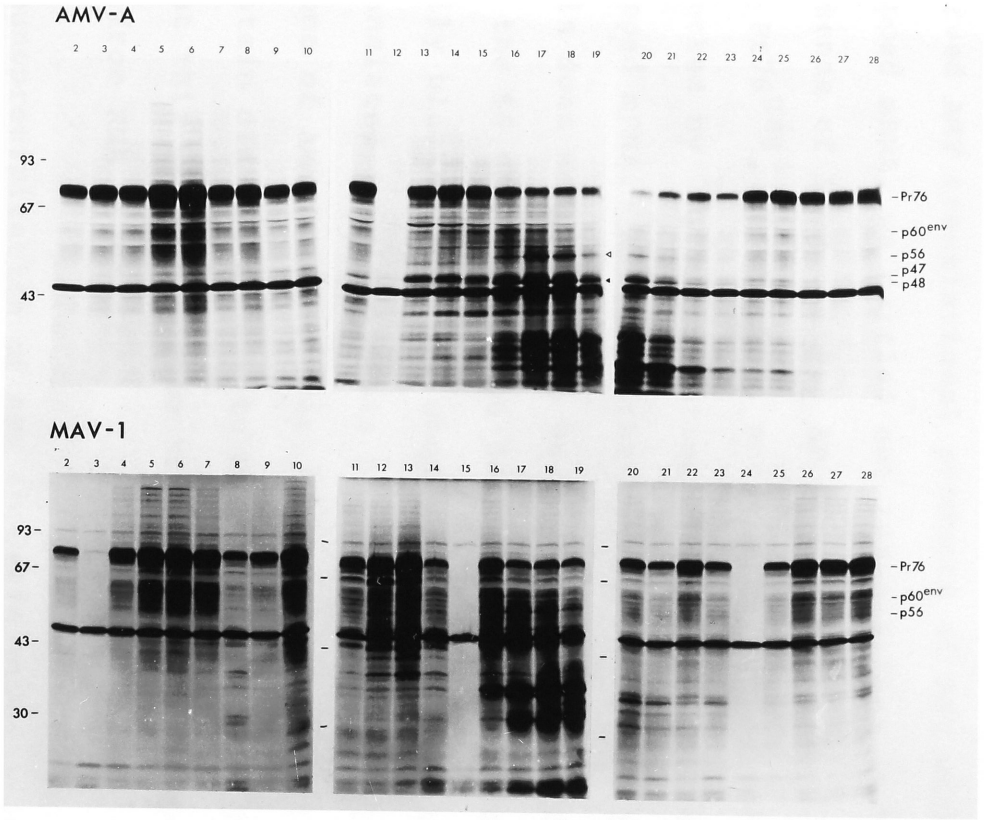


FIGURE 32. *In vitro* translation of AMV-A (top panel) and MAV-1 (bottom panel) viral RNA. Poly(A)-containing viral RNA was sized in a sucrose gradient and the RNA in the fractions concentrated by precipitation. 0.25-0.5 ug RNA from each of the fractions was translated *in vitro* with the rabbit reticulocyte lysate system and the products analyzed in 8.5% SDS polyacrylamide. Fraction 6 contains 35 S viral RNA and fraction 17 contains 20-22 S viral RNA. The AMV-specific 48,500 and 56,000 dalton proteins are indicated by the closed (◀) and open (◁) triangles, respectively. Sucrose gradient fraction number is indicated across the top. Molecular weight x $10^{-3}$ daltons is indicated on the left and translation products of interest are indicated on the right.

daltons are seen in translations of both MAV-1 and AMV RNA and they will be referred to as $p56_{MAV}$ and $p56_{AMV}$, respectively. The proteins of 47,000, 48,500 and 32,000 daltons seen in translations of AMV virion RNA are not seen in translations of MAV-1 virion RNA.

$^{7m}$GTP block of translation. To determine whether all the observed AMV translational products of AMV were translated from capped mRNA, we carried out translations in the presence and absence of 1 uM $^{7m}$GTP. As shown in Figure 33, the translation of $Pr76^{gag}$, the 56,000, and the 48,500 dalton proteins were blocked by $^{7m}$GTP indicating that they are translated from capped mRNA. Although the synthesis of the 32,000 dalton protein does not appear to be blocked by $^{7m}$GTP to the same extent as the 56,000 and 48,500 dalton proteins, its synthesis was totally blocked by $^{7m}$GTP when a second preparation of AMV RNA was translated in vitro (data not shown here). In both preparations of AMV RNA, however, the synthesis of the 47,500 dalton protein did not appear to be inhibited by $^{7m}$GTP, indicating that this protein may be translated from noncapped RNA species or from RNA fragments.

Immunoprecipitation of AMV in vitro translational products. To identify those translational products which might be related to known viral structural proteins, sequential immunoprecipitation of the translation products with anti-gag, anti-gp85, and anti-reverse transcriptase antisera was performed. The 76,000 dalton protein was immunoprecipitated by anti-gag antiserum

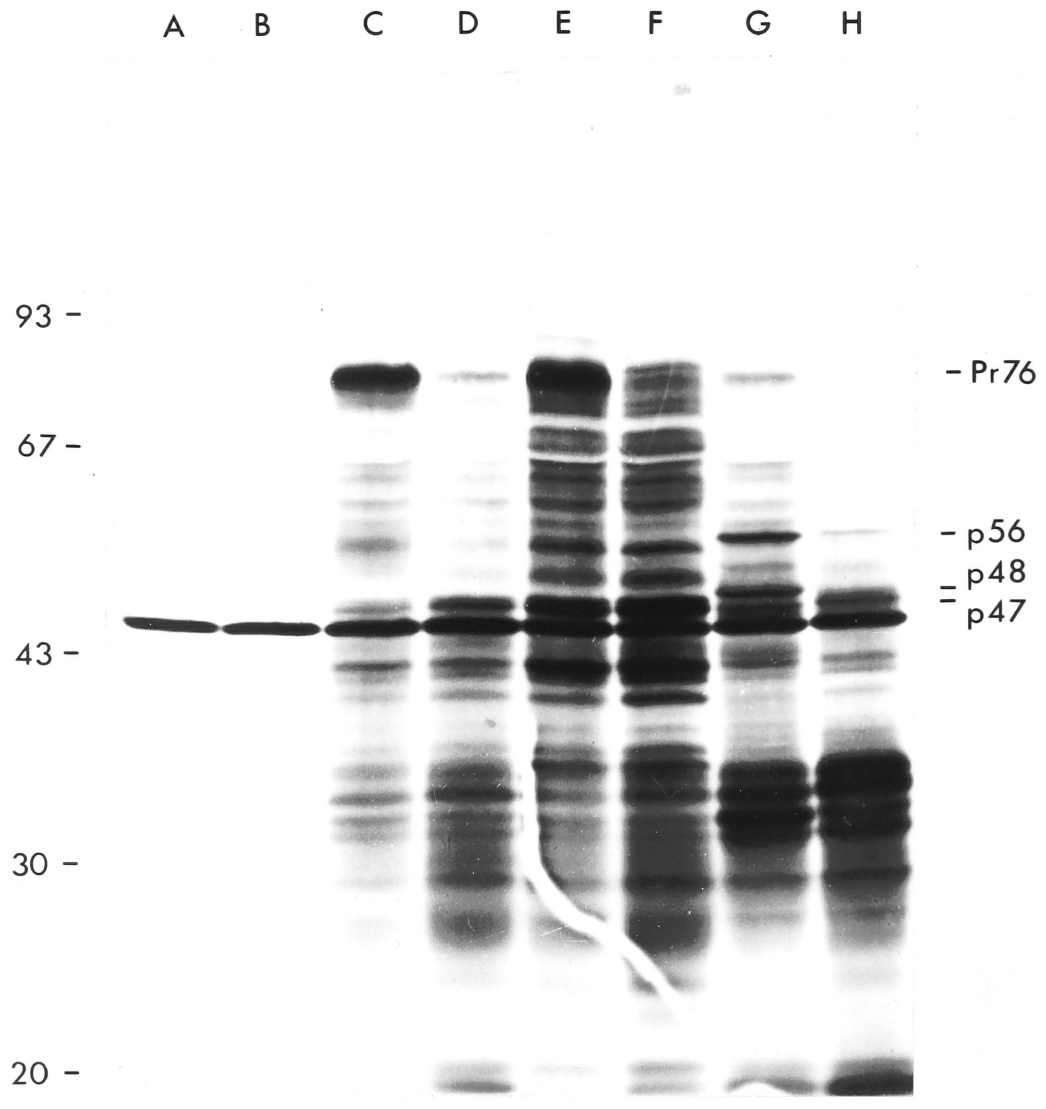


FIGURE 33. Inhibition of *in vitro* translation by $^{7m}$GTP. Parallel translations of virion RNA were performed in the absence (lanes A, C, E and G) and presence (lanes B, D, F and H) of 1 uM $^{7m}$GTP. Lanes A and B, no added RNA; lanes C and D, 35 S AMV RNA (fraction 5); lanes E and F, 24 S AMV RNA (fraction 15); lanes G and H, 21 S AMV RNA (fraction 18). The translation products were displayed on a 8.5% SDS polyacrylamide gel. Molecular weight x $10^{-3}$ daltons is indicated on the left and the position of viral translation products is noted on the right.

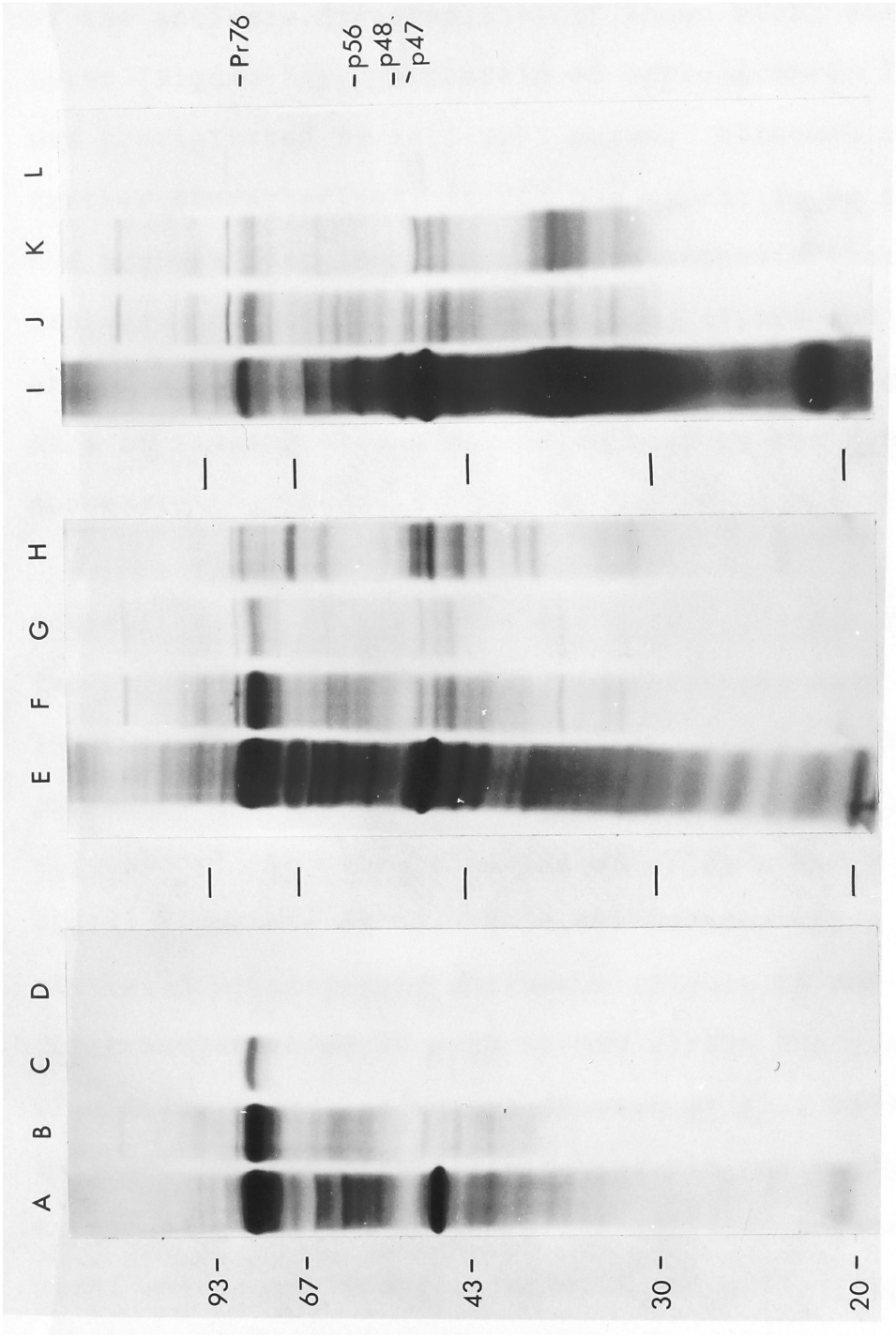


FIGURE 34. Immunoprecipitation of AMV-specific translation products. Parallel translations were performed and the products of one translation were sequentially immunoprecipitated with anti-*gag*, anti-gp85 and anti-reverse transcriptase sera. Lanes A-D, 35 S AMV RNA (fraction 5); lanes E-H, 24 S AMV RNA (fraction 15); and lanes I-L, 21 S AMV RNA (fraction 18). Lanes A, E and I show total translation products of each size RNA. Immunoprecipitation was with the following antisera: anti-*gag* serum, lanes B, F and J; anti-gp85 serum, lanes C, G and K; anti-reverse transcriptase serum, lanes D, H and L. Peptides were analyzed on 8.5% SDS polyacrylamide gels. Molecular weight x $10^{-3}$ daltons is indicated on the left and AMV-specific products are indicated on the right.

(Figure 34, Lane 2) indicating that it is $Pr76^{gag}$. None of the other AMV translational products were immunoprecipitated by any of the antisera directed against known viral structural proteins (Figure 34). A protein of approximately 35,000 daltons was precipitated by anti-gp85 serum. Although it was not further characterized, it did not appear to be the 32,000 dalton protein described above. This suggests that the AMV translational products of 56,000, 48,500, 47,000 and 32,000 daltons are not related to any of the viral structural genes. One or more of these proteins may be encoded by the myb specific sequences.

Identification of the MAV-1 env gene product. The intracellular env gene products of RNA tumor viruses have been extensively studied. The env gene of helper viruses is transcribed and processed to produce a 21 S env mRNA (Hayward, 1977; Weiss et al., 1977). In vitro translation of 21 S RAV-2 virion RNA yields a protein of 62,000-64,000 daltons which is immunoprecipitated by anti-gp85 antiserum (Figure 16 and 17) and in vitro translation of 28 S td SR-RSV virion RNA yields a protein of similar molecular weight (Pawson et al., 1980). This translational product presumably represents the unglycosylated precursor of $gPr92^{env}$, which is the cellular precursor to the viral envelope glycoproteins gp85 and gp37, (Diggelman, 1979; Storher and Hunter, 1979). Immunoprecipitation of tunicamycin treated virus-infected cells with anti-gp85 antiserum has identified an unglycosylated env gene product of 60,000 daltons (Diggelman, 1979; Storher and Hunter, 1979). The intracellular

unglycosylated env gene product of 60,000 daltons is smaller than the unglycosylated env gene product observed by in vitro translation (62,000 to 64,000 daltons). This difference in size presumably reflects the presence of a signal sequence on the in vitro translation product which has been cleaved from the protein observed in tunicamycin-treated cells.

We sought to identify the env gene products of MAV-1 for comparison with the AMV gene products. 21 S MAV-1 virion RNA was translated in vitro and the translational products analyzed in a 8.5% SDS polyacrylamide gel (Figure 35). The products of a parallel translation reaction were sequentially immunoprecipitated with anti-gp85 antiserum, then with anti-p27 antiserum and the precipitates were analyzed by gel electrophoresis. A polypeptide of 60,000 daltons is immunoprecipitated by anti-gp85 serum, but not with anti-gag antiserum. The gag gene product $Pr76^{gag}$, presumably translated from fragmented RNA, is observed after immunoprecipitation with anti-p27 antiserum (Figure 35, lane C). The 56,000 dalton protein encoded for by MAV-1 is not immunoprecipitated by anti-p27 or anti-gp85 antiserum.

The intracellular protein products of the MAV-1 env gene were identified by the following experiment. Parallel cultures of MAV-1 infected CEF with or without tunicamycin treatment, were labeled, immunoprecipitated, and the precipitates analyzed by gel electrophoresis (Figure 36). Immunoprecipitation of untreated MAV-1 infected cells with anti-gp85 demonstrated the presence of the normal envelope precurser $gPr92^{env}$ (Diggelman, 1979; Storher and Hunter, 1979) (Figure 36, Lane C). The

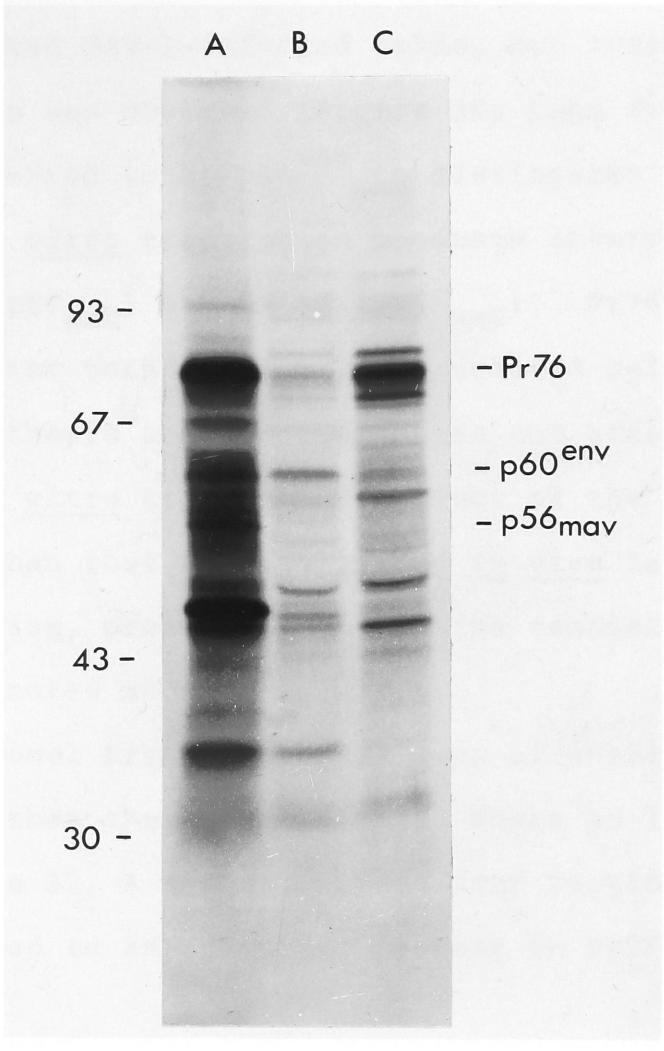


FIGURE 35. Translation products of MAV-1 21 S virion mRNA. Parallel translations of MAV-1 21 S RNA were carried out. The products of one translation were analyzed directly (lane A). The translation products of a parallel reaction were sequentially immunoprecipitated with anti-gp85 serum (lane B) and then with anti-p19 serum (lane C). The translation products were analyzed on a 8.5% SDS polyacrylamide gel. Molecular weight x $10^{-3}$ daltons is indicated on the left and selected MAV-1 translation products are indicated on the right.

anti-gp85 antiserum did not precipitate $gPr92^{env}$ from tunicamycin-treated MAV-1-infected cells, but instead a protein of 56,000 daltons was observed (Figure 36, Lane F). This protein will be referred to as $p56^{env}$ to distinguish it from the 56,000 dalton *in vitro* translation products observed with either MAV-1 RNA ($p56_{AMV}$) or AMV RNA ($p56_{AMV}$). $Pr76^{gag}$ was immunoprecipitated from both treated and untreated cells, indicating that its synthesis and processing was not affected by tunicamycin. The *in vitro* translation product of the MAV-1 *env* gene is larger than that seen following *in vivo* labeling and immunoprecipitation, presumably due to the removal of the signal sequence as noted above.

Two-dimensional tryptic peptide maps of $gPr92^{env}$ and of the $p56^{env}$ show that these two proteins share at least four major spots (Figure 37, A and B). These four peptides do not appear to be related to any peptides present in $Pr76^{gag}$ (Figure 37C).

*Peptide maps of AMV translational products*. Two dimensional tryptic peptide maps of the major *in vitro* translation products of AMV were prepared (Figure 38). A peptide map of $p56_{MAV}$ was also prepared (Figure 38C). Comparison of the peptide maps of the $p56_{MAV}$ and $p56_{AMV}$ indicates that these translational products are not identical. Neither protein appear to be related to either $Pr76^{gag}$ (Figure 38) or the *env* gene products $p56^{env}$ and $gPr92^{env}$ shown in Figure 37. The 48,500 translational product is also not related to $Pr76^{gag}$ or the *env* gene products $gPr92^{env}$ or $p56^{env}$. In addition, these translational products do not appear to be related to the 46,000 and 48,000 dalton

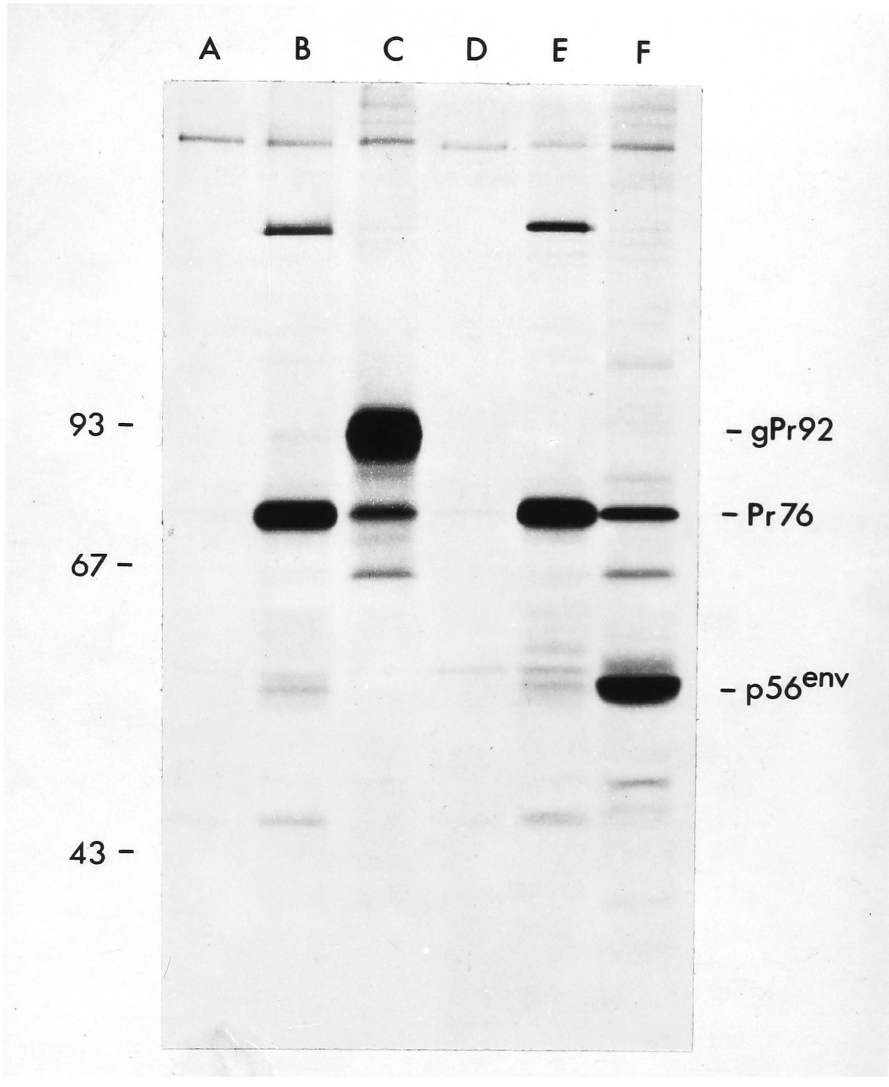


FIGURE 36. The effect of tunicamycin on MAV-1 gene products. MAV-1-infected CEF were treated for 2 h with or without 0.5 ug/ml tunicamycin, starved for 2 h in methionine-free media, and then labeled for 2 h with 200 uCi/ml $^{35}$S-methionine in methionine-free media. Tunicamycin treatment continued during the methionine starvation and the radiolabeling of the cells. The cells were lysed in RIPA buffer and immunoprecipitated with the following antisera: nonimmune serum, lanes A and D; anti-*gag* serum, lanes B and E; and with anti-gp85 serum, lanes C and F. Control cells are in lanes A-C and tunicmycin-treated cells in lanes D-F. The immunoprecipitates were analyzed on a 7.5% polyacrylamide gel. Molecular weight x $10^{-3}$ daltons is indicated on the left and key viral proteins are noted on the right.

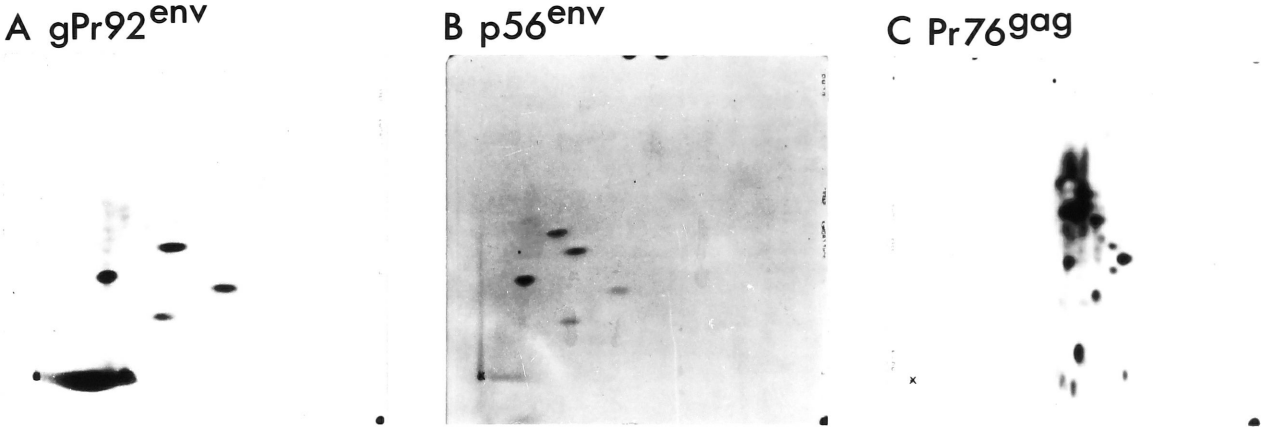


FIGURE 37. Two-dimensional peptide maps of MAV-1 *env* gene products. Selected gel bands from the gel shown in Figure 7 were excised from the gel and processed for tryptic fingerprinting as described in the Methods. The samples were spotted in the lower left (x) and electrophoresis was towards the cathode at pH 4.7, followed by ascending chromatography. MAV-1 $gPr92^{env}$, lane A; MAV-1 $p56^{env}$, lane B; MAV-1 $Pr76^{gag}$, lane C.

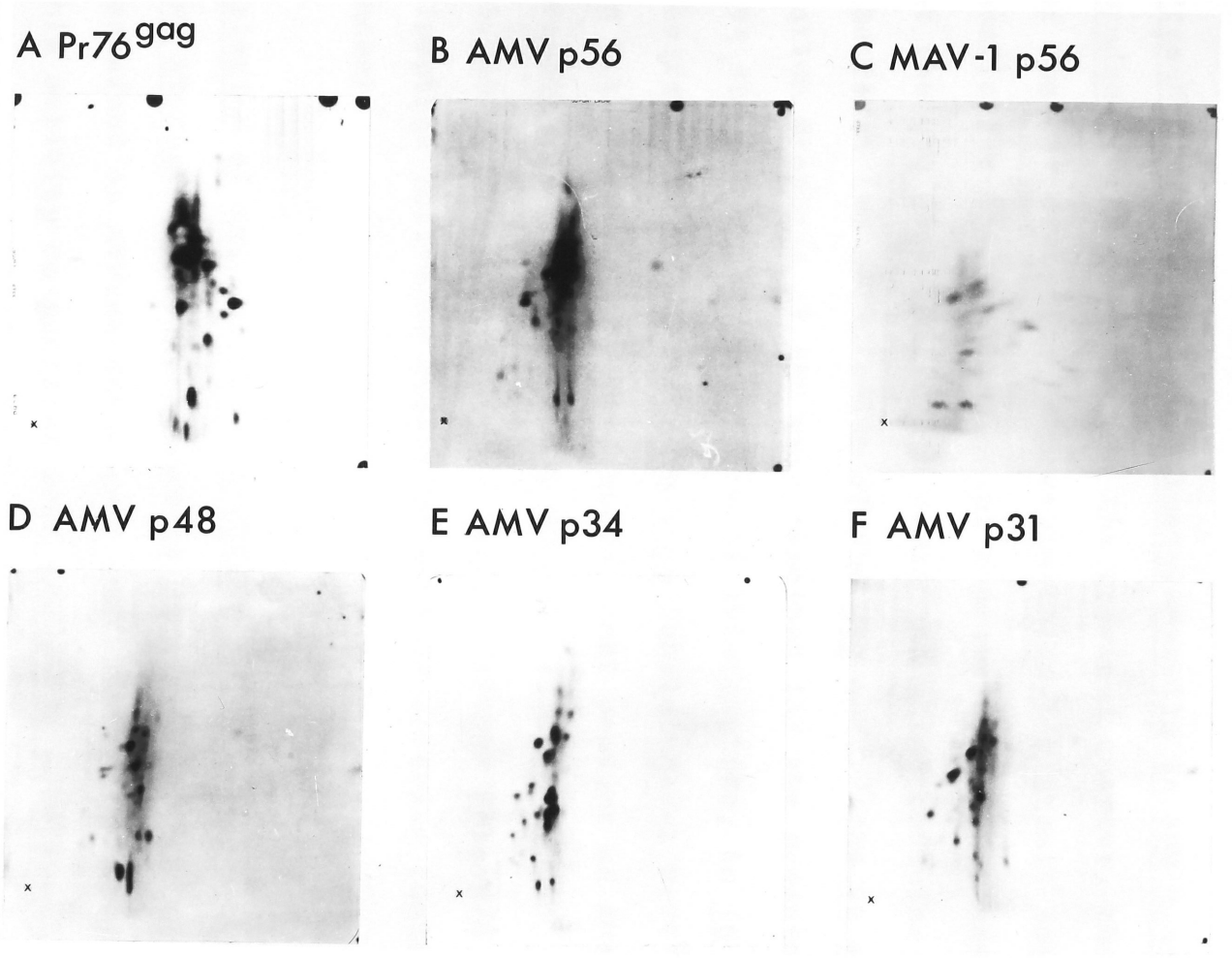


FIGURE 38. Two-dimensional tryptic fingerprints of AMV-A and MAV-1 *in vitro* translation products. Translation products were separated on 8.5% gels and the bands of interest located, excised, and processed as described. The samples were spotted in the lower left corner (x) and electrophoresis in the horizontal direction was toward the cathode at pH 4.7, followed by ascending chromatography from bottom to top. $Pr76^{gag}$, panel A; AMV 56,000 dalton peptide, panel B; MAV-1 56,000 dalton peptide, panel C; AMV 48,500 dalton peptide, panel D; AMV 34,000 dalton peptide, panel E; AMV 31,000 dalton peptide, panel F.

proteins observed in translations of AEV 20-24 S virion RNA (Figure 21).

Presence of the dipeptide Acetyl-Met-Glu in AMV translational products. As described in the previous chapter, the presence of the 19* dipeptide, N-acetylmethionyl-glutamic acid, is diagnostic for the translation of a protein whose synthesis begins at the gag gene initiation codon and which contains at least the first two amino acids of the gag gene. The in vitro translation products of AMV were examined for the presence of the 19* dipeptide. The presence of this peptide, in in vitro synthesized AMV $Pr76^{gag}$ and in vivo labeled MAV-1 $Pr76^{gag}$ is shown in Figure 39. No other translational product of AMV (including proteins of 56,000, 49,500, 47,000, 43,000, 41,000, 37,000, 35,000, 34,000, 32,000, and 31,000 daltons released the 19* dipeptide following digestion with V8 protease (data not shown).

Relation of AMV-specific in vitro translation products to the AMV ATPase. In 1952, Mommaerts et al. noted the AMV plasma contained an ATPase activity. Further studies demonstrated this activity co-purified with AMV virus particles and that its concentration was directly proportional to the number of virus particles determined by direct electron microscopy (Mommaerts et al., 1954; Green and Sharp, 1955; Green and Beard, 1955). Normal plasma, plasma from AEV strain R-infected birds and plasma from RPL12-infected birds do not have an ATPase activity (Haddah et al., 1960; Beard, 1963). The enzymatic activity can

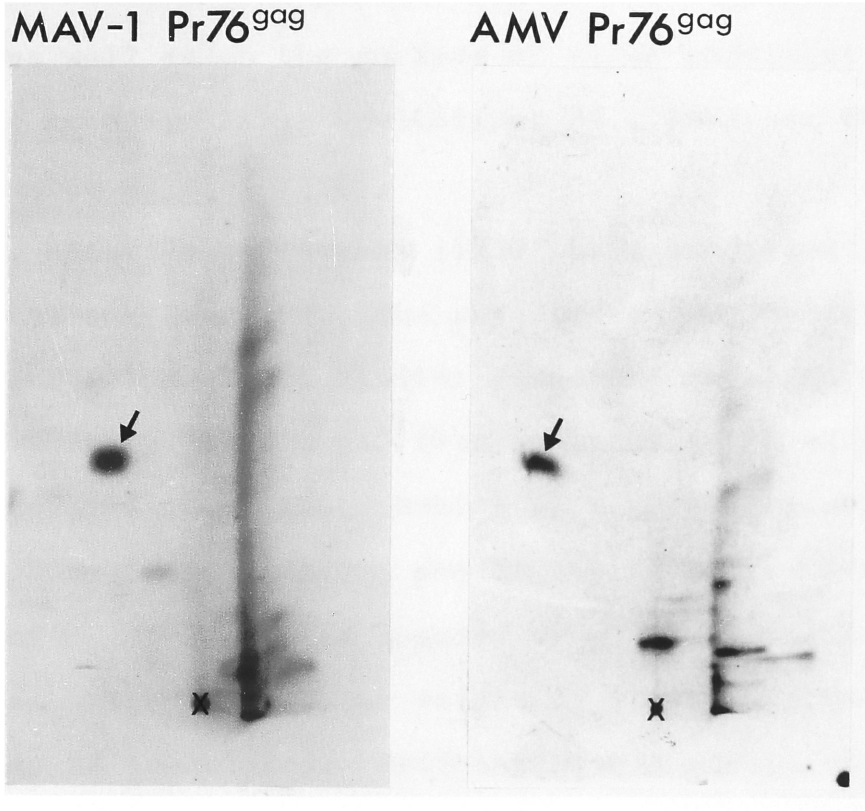


FIGURE 39. Presence of the 19* dipeptide in *in vivo*-labeled $Pr76^{gag}$ and *in vitro* synthesized AMV $Pr76^{gag}$. $^{35}S$-methionine-labeled MAV-1 $Pr76^{gag}$ and AMV $Pr76^{gag}$ were digested with V8 protease and the digestion products spotted in the center of the plate (x). Electrophoresis was at pH 6.5 in the horizontal direction with the anode at the left. Ascending chromatography was from bottom to top. The negatively charged 19* peptide is indicated by the arrow in each panel.

be localized, by cytochemical techniques, on the surface of AMV-transformed cells in the plasma, thymus and in nephroblastoma tissue, as well as on the surface of virus particles themselves (de The et al., 1963a; Novikoff et al., 1962; de The et al., 1963b; de The et al., 1964).

Recently, Banerjee and Racker (1977) have succeeded in purifying the ATPase from AMV. The purified enzyme hydrolyzed ATP, GTP, CTP, UTP and ITP at similar rates and required Ca++ and Mg++ for activity. By SDS gel electrophoresis the enzyme can be disassociated into five subunits with molecular weights of 62,000, 60,000, 28,000, 24,000 and 18,000 daltons. A subunit structure of $\alpha_2 \beta_2 \gamma \delta \epsilon$ was assumed based upon staining intensities, from which a molecular weight of 314,000 daltons for the complex can be calculated. Sedimentation analysis of the enzyme complex yields an estimated molecular weight of 650,000 daltons, so the enzyme may exist as a dimer.

Since AMV apparently acquires the ATPase from the cell in which it grows, it could be argued that this enzyme is characteristic of the target cell AMV infects, or that this enzyme is expressed in cells at the stage AMV "blocks" their differentiation. Alternatively, the ATPase, or part of it, could be encoded by AMV. Therefore, the relation between the in vitro translation products of AMV virion RNA and the AMV ATPase was investigated. A rabbit antiserum prepared against the purified AMV ATPase was prepared by Dr. J.H. Chen of Life Sciences, Inc., and it was used in the following studies.

The anti-AMV ATPase antiserum was first screened against AMV-transformed myeloblasts. In Figure 40, AMV-transformed

myeloblasts were immunoprecipitated with anti-gag serum (lane A), anti-AMV ATPase serum (lane B), and anti-AMV ATPase serum which had been absorbed with 35 ug of disrupted virus protein (lane C). The anti-ATPase serum clearly has antibodies directed against some gag determinants since $Pr76^{gag}$, p27 and p19 were precipitated by the antiserum. Some, but not all, of these antibodies were removed by absorption with disrupted virus. The amount of $Pr76^{gag}$, p27 and p19 precipitated by the anti-ATPase antiserum was reduced when absorbed serum was used (lane B vs lane C). The anti-ATPase serum precipitates many proteins which are not related to viral structural proteins. Of special interest are two proteins of 64,000 and 60,000 daltons which probably correspond to the 62,000 and 60,000 dalton subunits of AMV ATPase reported by Banerjee and Racker (1977). The antiserum clearly precipitates $Pr76^{gag}$ and absorption of the serum with disrupted virus reduces that amount of $Pr76^{gag}$ precipitated (Figure 40, lanes B and C). The three smaller subunits of 28,000, 24,000 and 18,000 daltons have not been clearly identified in immunoprecipitates of labeled cell at this time. An intriguing possibility is that some of these smaller proteins, such as the 28,000 and 18,000 dalton species, could be the AMV gag proteins p27 and p19 respectively. This possibility has not been explored and may merit further investigation. Alternatively, these proteins may either be poorly labeled with methionine or present in very low amounts.

This anti-ATPase antiserum has been used to screen the AMV-specific translation products. The results, shown in Figure 41, demonstrated that $p56_{AMV}$, p48 and p47 are not precipi-

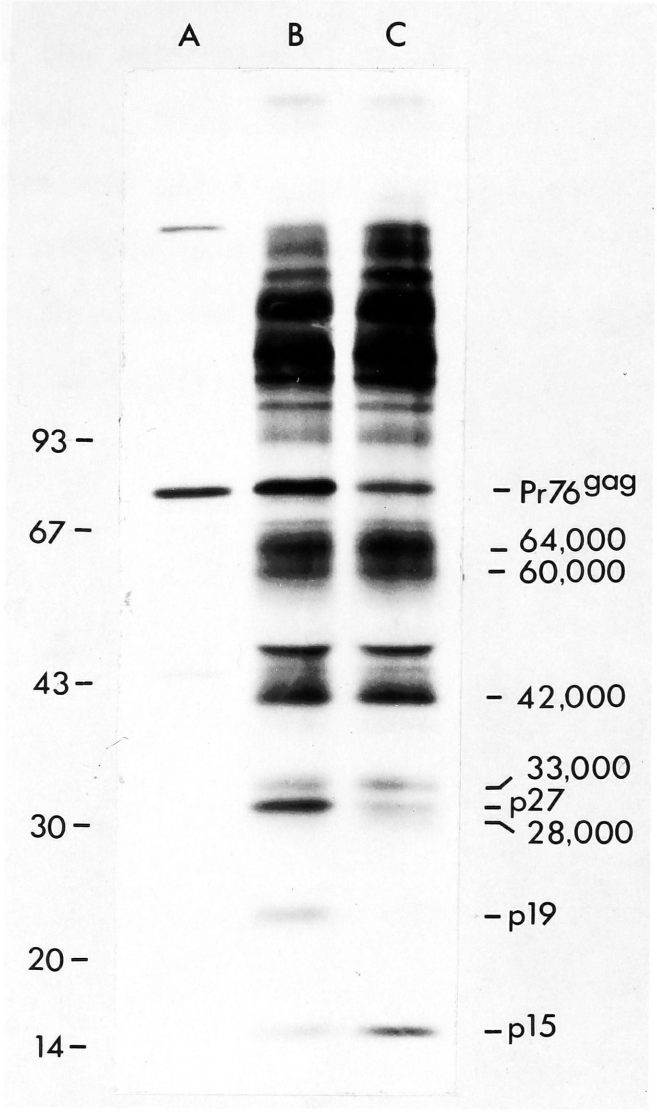


FIGURE 40. Immunoprecipitation of AMV NP myeloblasts with anti-AMV ATPase serum. AMV NP clone was labeled with $^{35}S$-methionine and immunoprecipitated with the following antisera: rabbit anti-*gag* protein serum, lane A; Rabbit anti-AMV ATPase serum, lane B; anti-AMV ATPase that had been absorbed with 35 ug disrupted virion proteins, lane C. Molecular weight x $10^{-3}$ daltons is indicated on the left and proteins of interest are indicated on the right.

tated by this antiserum. A small amount of $Pr76^{gag}$ is apparently precipitated (lanes B and D), however, which is not unexpected since the anti-ATPase serum clearly contains gag antibodies (Figure 40). Thus, the AMV-specific translation products, $p56_{AMV}$, p48 and p47 are not related to the AMV-ATPase. Presumably, this ATPase is a host cell enzyme, although it is still possible that some subunits of the enzymes are host-derived and other subunits are viral structural proteins, such as p27 and p19.

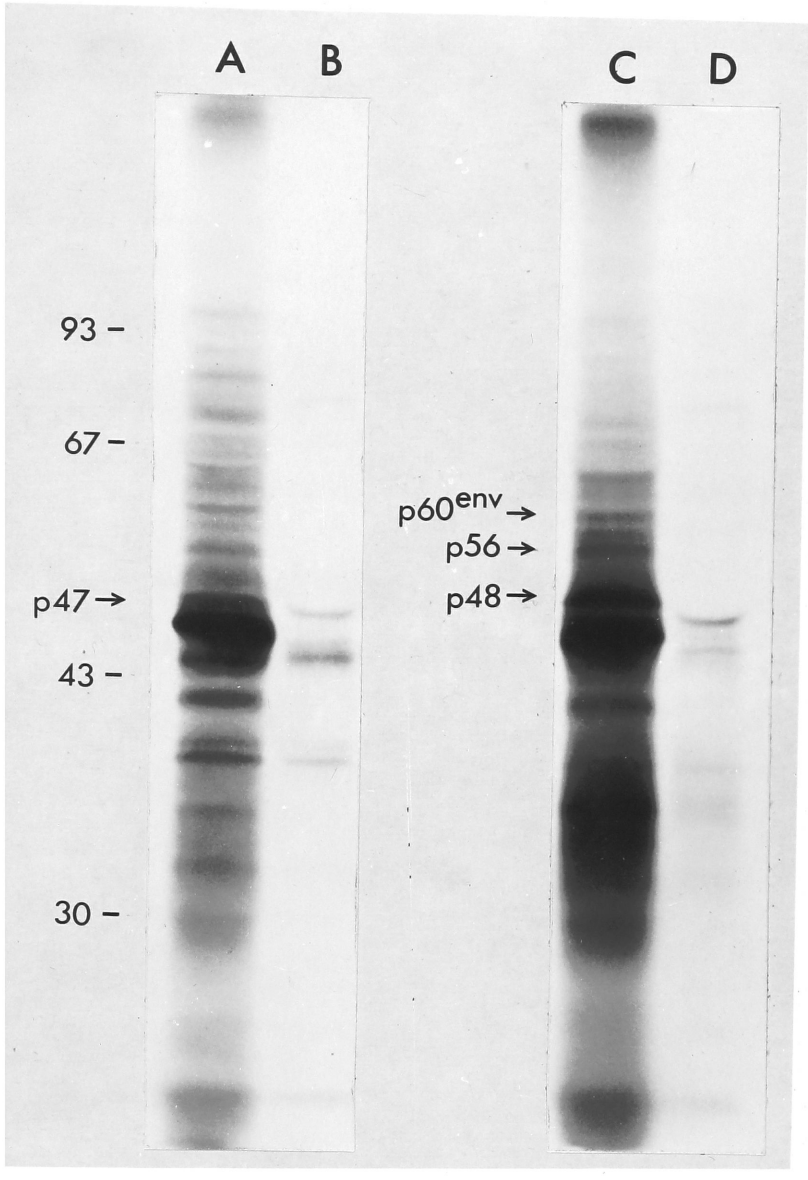


FIGURE 41. Immunoprecipitation of the *in vitro* translation products of AMV virion RNA with the anti-AMV ATPase serum. Parallel translations of RNA from fraction 15 (lanes A and B) and fraction 19 (lanes C and D) of the AMV virion RNA translated in Figure 32. Lanes A and C are the total translation products of these RNA fractions and lanes B and D show those proteins which were immunoprecipitated by the anti-AMV ATPase serum. Molecular weight x $10^{-3}$ is indicated on the left and translation products of interest are indicated by the arrows.

# VI. Discussion of Research on Avian Myeloblastosis Virus

## Discussion

We have studied the translational products of AMV *in vivo* and *in vitro*. The major protein in AMV NP cells immunoprecipitated by antisera directed the viral structural proteins is $Pr76^{gag}$. This *gag* protein precursor is evidently processed to the individual *gag* proteins, because each of the individual *gag* proteins is present in NP cells and because noninfectious virus particles are released by NP cells (Duesberg *et al.*, 1980; Chen *et al.*, 1981). Virus particle formation apparently requires only the viral RNA and the products of the *gag* gene since RSV α, which contains only an active *gag* gene, is able to produce virus particles (Hanafusa and Hanafusa, 1971). $Pr180^{gag-pol}$, the readthrough product of the *gag* and *pol* genes, is present in NP cells, indicating that most, if not all, of the *pol* gene is transcriptionally active. The defective virus particles released by NP cells, however, do not have detectable levels of reverse transcriptase (Duesberg *et al.*, 1980). The envelope glycoprotein gp85 was not detectable in NP cells. This is consistent with hybridization studies showing that AMV lacks sequences homologous to cDNAenv (Chen *et al.*, 1981; Gonda *et al.*, 1981). AMV producer clones contain all of the normal structural proteins and protein precursors. A protein of 110,000 daltons is immunoprecipitated by anti-*gag* antiserum, however, this does not appear to be an AMV specific translational product since it is present in only approximately 50% of the AMV clones analyzed (J.H. Chen, personal communication). We assume that this protein results from either the enhanced expression of a

cellular protein, or from modification of a cellular protein following transformation by AMV.

Recently, several investigators have reported upon the viral proteins present in AMV-transformed cells as detected by immunoprecipitation (Duesberg *et al.*, 1980; Silva and Baluda, 1980). The analysis of viral proteins in AMV nonproducer and producer clones by Duesberg *et al.* (1980) are in agreement with the data presented here. Silva and Baluda (1980) described two glycoproteins immunoprecipitated by anti-gp85 antiserum (gp120 and h27) and one protein (p100) immunoprecipitated by antisera prepared against the individual *gag* proteins. The glycoproteins gp120 and h27 were also found in normal yolk sac and spleen cells. The p100 protein was not found in yolk sac cells infected with AMV. We have not detected any of these three proteins in the AMV myeloblasts we have examined and thus assume that these proteins are unique to the cell lines or antisera used by these authors. The 110,000 dalton protein seen by Silva and Baluda (1980) in SPAFAS yolk sac cells transformed *in vitro* by AMV may be the same as a protein of the same molecular weight that was observed in some AMV myeloblast clones, such as that seen in Figure 29.

AMV produces two mRNAs, 34 S full length AMV virion RNA, and a subgenomic mRNA of 21 S which contains AMV-specific sequences. This 21 S mRNA is packaged within the AMV virion in addition to the 34 S genomic RNA. We therefore used virion RNA for *in vitro* translation experiments. Four AMV specific translational products were found which are not related to any of the known viral structural proteins by immunoprecipitation or

peptide mapping. These proteins have molecular weights of 56,000, 48,500, 47,000 and 32,000 daltons. Based upon two-dimensional peptide mapping, the 56,000 and the 48,500 dalton proteins do not appear to be related to $Pr76^{gag}$, the *env* genes products $p56^{env}$ or $gPr92^{env}$, or the proteins produced by translation of the AEV subgenomic 21 S mRNA. None of these four proteins contained the 19* dipeptide diagnostic for the N-terminus of *gag* gene products (Rettenmier *et al.*, 1979b) and thus they are not translated from a mRNA containing the *gag* gene initiation codon and the first two codons of the *gag* gene. Based upon immunoprecipitation with specific antisera, none of these four proteins are related to the *gag*, *pol* or *env* gene products.

Antiserum prepared against the ATPase present in the AMV virion and in AMV-transformed cells did not precipitate any of the AMV-specific translation products. Immunoprecipitation of AMV-transformed NP myeloblast clones with the same antisera demonstrated it precipitates two proteins of 64,000 and 60,000 daltons which may correspond to the 62,000 and 60,000 dalton subunits observed by Banerjee and Racker (1977). The three smaller subunits of 28,000, 24,000 and 18,000 daltons were not clearly identified in immunoprecipitates made with this antiserum. The possibility was raised that one or more of these small subunits might be related to either of the *gag* proteins p27 or p19, however, no data was presented in favor of such a view. It appears, however, that none of the AMV-specific translation products described here are related to the AMV ATPase.

The 47,000 dalton protein is produced by 22-24 S RNA, whereas the 56,000, 48,500 and 32,000 dalton proteins are produced by 20-22 S RNA, which is the size of the AMV subgenomic mRNA species. The translation of the 32,000, 48,500 and the 56,000 dalton proteins, but not the 47,000 dalton protein, is inhibited by the presence of $^{7m}$GTP, a cap analogue known to block translation of capped mRNAs (Hickey et al., 1976; Suzuki, 1976; Weber et al., 1976; Hickey et al., 1977). This may indicate that the 47,000 dalton protein is not translated from a capped mRNA species but instead from an uncapped RNA species or RNA fragments. These facts taken together indicate that one or more of the 32,000, 48,500 and 56,000 dalton proteins, represent the translational product(s) of the myb gene and thus the transforming protein of AMV.

A question must be raised concerning whether one or both of the 56,000 dalton proteins could be related to the ability of AMV and certain MAV isolates to cause osteopetrosis (Smith and Moscovici, 1969). Competition hybridization experiments between MAV-2(O), an isolate which induces osteopetrosis in approximately 100% of infected birds (Smith and Moscovici, 1969), and an avian lymphoid leukosis isolate, has demonstrated that approximately 15-20% of their sequences are unrelated (Smith et al., 1976). It is therefore possible that there are sequences responsible for the induction of osteopetrosis. This question cannot be resolved by the data presented here, but it poses several interesting questions for future research.

I have analyzed the in vitro translational products of 20-22 S poly(A)-containing mRNA from AMV NP cells. Translation

of AMV cellular RNA yields peptides of 56,000 and 48,500 daltons which comigrate with the translational products of the 20-22 S AMV virion RNA. Preliminary data indicates the peptide maps of the cellular and the viral translational products are related (Chen, Anderson, Moscovici, unpublished data). This suggests that these two proteins are also present in AMV transformed cells.

I can not readily explain why there are multiple products of the myb gene of AMV; the 32,000, 48,500 and the 56,000 dalton proteins are all apparently encoded by 21 S myb mRNA. The size of the AMV unique mRNA is about 2.5 kb (Chen et al., 1981; Gonda et al., 1981) which includes a poly(A) tract of about 0.2 kb, the common region of about 0.7 kb, and a leader of about 0.3-0.4 kb. The actual coding region of this RNA would thus be 1.2-1.5 kb which corresponds to a coding capacity of 47,000 to 55,000 daltons. Heteroduplex mapping of AMV (Souza et al., 1980b) estimates that the AMV specific region is 0.9±0.16 kb which would correspond to a much smaller coding capacity of 33,000 to 38,000 daltons. This assumes that there is no other 3' viral sequences, such as part of the env gene, which are translated as part of the myb gene. This would indicate that the 56,000 and the 48,500 dalton protein can not be read in the same reading frame. It is possible, however, that both proteins are coded for by the same region of proviral DNA in two reading frames produced by different splicing patterns. Multiple splicing patterns account for the production of several proteins by the same region of DNA in the SV-40 early gene (Berk and Sharp, 1978; Reddy et al, 1979), the polyoma early

gene (Friedmann et al., 1979; Soeda et al., 1979; Kamen et al., 1979), and gene 8 of influenza virus (Lamb and Lai, 1980). If this is the case, then it is possible that both the 56,000 and the 48,500 dalton proteins are myb gene products.

Resolution of the question of what protein is the myb gene product of AMV requires the preparation of anti-myb antisera which might recognize one or more of these proteins. Such antisera do not exist at this time, although it is known that chickens do regress from AMV-induced leukemia (Silva and Moscovici, 1973). Immunization of such regressor chickens with AMV myeloblasts may yield antiserum specific for the transforming protein(s) of AMV, as described earlier for AEV.

Another possibility is to compare the peptide map of the 125,000 dalton gag-related polyprotein of E26 with that of the proteins described here. E26 is an avian acute leukemia virus which contains myb sequences (Roussel et al., 1979). The peptide map of its putative transforming protein may have peptides related to the transforming protein of AMV.

Ultimately the question of which protein is the transforming protein of AMV may be solved through the use of the molecularly cloned AMV genome (Souza et al., 1980c). The DNA sequence of the myb gene region should tell us if there is one or more open reading frames in addition to predicting an amino acid sequence for the protein(s). In addition, it may be possible to clone this sequence into a plasmid vector in such a fashion as to allow its expression (Guarente et al., 1980) and thus purify enough protein to immunize animals in the standard fashion. Alternatively, it may be possible to raise antibodies

against synthetic peptides whose sequences have been predicted from the nucleic acid sequence of a molecularly cloned DNA. This method has been used in the analysis of the R gene of Moloney murine leukemia virus (Sutcliffe et al., 1980; Lerner et al., 1981) and SV40 T antigen (Walter et al., 1980).

The identification of the AMV myb gene product will be the first step in understanding how this fascinating virus causes the transformation of hematopoeitic cells with such great specificity.

## General Discussion

The origin of RNA tumor viruses has been widely discussed. The finding that the onc genes of many RNA tumor viruses are present in the DNA of normal cells (Stehelin et al., 1976; Roussel et al., 1979) supports the belief that these viruses are derived from cellular sequences. Huebner and Todaro (1969) proposed the oncogene hypothesis in which they stated that the genetic information for leukemia viruses preexists in all cells, and the appearance of these viruses results from derepression of these sequences. Such a derepression could be the result of either a genetic change in the controlling sequences of the oncogene or from a temporary derepression caused by the presence of an "inducer" molecule. This theory is supported by research demonstrating the release of leukemia viruses from cells following treatment with x-rays or chemical substances such as mutagens (Weiss et al., 1971). This hypothesis does not deal with how or why oncogenes were formed in the cellular DNA and so one is still left with a "chicken and the egg" type of question.

In 1970, Temin (1970) advanced the protovirus hypothesis in which he proposed that leukemia viruses do not preexist in cellular DNA, but rather that they arise from other elements, called protoviruses, by genetic change in these elements. These protoviruses would be capable of transmitting genetic information from DNA through an RNA intermediate back to DNA, and would be capable of transmitting genetic information from cell to cell in an organism, either as free RNA or as a ribonu-

cleoprotein. Temin further proposed that protoviruses would contain genes for their own replication and for the control of cell multiplication, and they would be capable of genetic evolution (in a predetermined manner) as a result of alterations occurring in successive movements from one cell to another. If the free DNA form of a protovirus happened to integrate into a region of the genome controlling membrane proteins, the genome of an RNA leukemia could be formed.

Recently, Temin (1980) has modified his original model by suggesting that cellular movable genetic elements may be closely tied to the origin of retroviruses. This thought is based primarily upon the fact that retroviruses integrate into cellular DNA in a fashion that is analogous to that used by transposable elements in bacterial cells (Dhar *et al.*, 1980; Ju and Skalka, 1980; Sutcliffe *et al.*, 1980). Temin (1980) proposes that two insertion sequences were able to transpose around a DNA polymerase gene and that this combination might have become the ancestor of the reverse transcriptase currently present in RNA tumor viruses. Recombination of this element with other cellular genes or with other transposable elements carrying cellular genes, could give rise to an element carrying the ancestral *gag*, *pol*, and *env* genes. Further acquisition of cellular control sequences, such as the gene called *c-src*, could give rise to transforming viruses.

It is also possible that the genes controlling the normal differentiation of stem cells are carried on moveable elements. The control of mating type in yeast appears to be carried on moveable genetic elements (Strathern *et al.*, 1980). Recombina-

tion between moveable elements carrying these sequences and an element carrying the ancestral gag gene could give rise to an acute leukemia virus.

Do such recombination events occur? The generation of the recovered avian sarcoma viruses (Hanafusa et al., 1977) argues that this is possible. Hanafusa et al. injected newborn chicks with partial td mutants of RSV. These mutants contain a small portion of the src gene (Kawai et al., 1977). After 8-9 weeks, tumors appeared at sites distal to the site of injection and transforming virus could be isolated from the tumor material. These viruses encode a $pp60^{src}$ protein with an associated protein kinase activity (Karess et al., 1979). Fingerprinting of RNase $T_1$-resistant oligonucleotides and two-dimensional peptide mapping of the src proteins indicates that these viruses have acquired new sequences which are closely related to, but different from, the parental src gene (Wang et al., 1978; Karess and Hanafusa, 1981).

Recently, Neel and Hayward (personal communication) have isolated a defective transforming virus from a leukosis virus-infected chicken. Newborn chickens were injected with td107A, a deletion mutant which lacks all of the src gene. Four to five months later a bird was found to have a fibrosarcoma present in the breast. A defective transforming virus could be isolated from the tumor tissue and the transforming sequences of this virus are related to, but unique from, the transforming gene of Fujinami sarcoma virus.

Transforming viruses have also been isolated from in vitro systems in which nononcogenic viruses have apparently acquired

cellular sequences resulting in the production of a sarcoma virus. Rasheed et al. (1978) isolated a rat sarcoma virus, apparently related to Harvey and Kirsten sarcoma viruses, by cocultivating rat embryo fibroblasts that released low amounts of endogenous ecotropic type C virus with different chemically-transformed rat cell lines. These viruses can rapidly transform cells in vitro. Rapp and Todaro (1978,1979) have also isolated transforming viruses following infection of a variety of mouse cells with a mousetropic C3H murine leukemia virus. These viruses are capable of transforming cells in vitro or inducing a variety of sarcomas in vivo.

These investigations clearly show that retroviruses can acquire transforming sequences from cellular DNA. In the case of recovered avian sarcoma viruses, this process is quite efficient since the virus contains part of the src gene. The other process, in which there are no common sequences, is evidently not very efficient.

What is transformation and what is its relation to oncogenesis? In a strict sense, transformation is any inheritable change in a cell's properties, and with respect to oncogenesis the inheritable change results in tumorous growth or leukemia. Thus, oncogenic transformation is a inheritable change which results in the loss of the control of cell growth and/or cell differentiation. Oncogenic viruses are merely very efficient promotors of this change in the control of cell processes. They are very efficient because they carry genes coding for gene products which directly interfere with these normal cell processes. A clear example of this is the recovered

avian sarcoma viruses in which perhaps 80% of $pp60^{src}$ is derived from cellular sequences. Presumably these viruses transform cells by over-producing $pp60^{src}$ in infected cells (Karess et al, 1979). As you will recall, $pp60^{src}$ and $PK_f$, an enzyme involved in the control of the $Na^+K^+$-ATPase, may be identical, and thus the point at which one transforming protein may act has been localized.

How many onc genes (transforming genes) are there? Currently there are some 10-20 transforming proteins that have been identified. In theory, there should be at least one transforming gene for each existing major control point involved in the regulation of cell growth and/or differentiation. I say at least one because it may be possible for different transforming proteins to act upon the same control point via different mechanisms. The fact that the endogenous forms of two transforming proteins (RSV and Harvey sarcoma virus) have been localized in the control of the Na+K+-ATPase, indicates that there may be a few common pathways that are involved in cell transformation.

There is one virus, however, the Friend spleen focus-forming virus, which does not appear to utilize this same type of process in transforming cells. The onc protein of this virus is considered to be a recombinant glycoprotein, gp52, which contains both ecotropic and xenotropic murine leukemia virus envelope sequences (Troxler et al., 1980). It is possible that gp52, by nature of being a glycoprotein, may alter a cell surface receptor for a differentiation hormone such as erythropoietin, and thus prevent its binding to the receptor.

Alternatively, the molecule may look like a growth hormone molecule and directly interfere with its action.

I think that these thoughts on viral-induced cell transformation have clear implications of the concept of what is the target cell of a virus. In the simplest sense, a target cell is one in which the virus is able to grow, express its gene products, and thus cause transformation. In the case of the DNA tumor viruses SV40 and adenoviruses, it is actually those cells which are nonpermissive for virus replication which become transformed (Levine, 1976). Graf et al. (1980) demonstrated that the avian acute leukemia viruses are capable of infecting and replicating in cells which they are not able to transform. For example, AEV can infect, produce infectious progeny and synthesize p75 in macrophage cells, although AEV is not able to transform these cells. In the case of the avian RNA tumor viruses, it appears that a target cell should be defined as a cell which can be infected, allows the expression of the viral genome, and which has the specific pathway which the virus is able to alter to produce oncogenic transformation. Viruses which are capable of transforming or altering the growth of many different cell types, such as RSV, must be able to interfere with a reaction that is common to many different cell types. Conversely, viruses with a very restricted target cell population, such as AMV, must be very finely tuned so as to interact with a process present in only a very few cell types.

The preceding discussion suggests a rather interesting experiment. Cloning the sequence which codes for a molecule in-

volved in the control of cell growth and/or cell differentiation and ligating it into a nontransforming retrovirus, should result in the creation of a new transforming virus. Conversely, the introduction of a sequence coding for a molecule which can counteract the effects of a transforming protein (for example, a phosphatase for a protein kinase), might nullify the effects of the transforming protein. There types of experiments could allow one to further dissect the process of oncogenic transformation and the control of cell growth or differentiation in vivo with very specific molecular tools.

References.

1. Alwine, J.C., D.J. Kemp, and G.R. Stark. 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc. Natl. Acad. Sci. U.S.A. 74:5350-5354.

2. Anderson, S.M., W.S. Hayward, B.G. Neel, and H. Hanafusa. 1980. Avian erythroblastosis virus produces two mRNA's. J. Virology 36:676-683.

3. Baluda, M.A. and I.E. Goetz. 1961. Morphological conversion of cell cultures by avian myeloblastosis virus. Virology 15:185-199.

4. Baluda, M.A., C. Moscovici and I.E. Goetz. 1964. Specificity of the in vitro inductive effect of avian myeloblastosis virus. Nat. Cancer. Inst. Mono. 17:449-458.

5. Baker, C.C., J. Herisse, G. Courtois, F. Galibert, and E. Ziff. 1979. Messenger RNA for the Ad2 DNA binding protein: DNA sequences encoding the first leader and heterogeneity at the mRNA 5' end. Cell 18:569-580.

6. Banerjee, R.K., and E. Racker. 1977. Solubilization, purification, and characterization of a nucleoside triphosphatase from avian myeloblastosis virus. J. Biol. Chem. 252: 6700-6706.

7. Barbacid, M., A.V. Lauver, and S.G. Devare. 1980. Biochemical and immunological characterization of polyproteins coded for by the McDonough, Gardner-Arnstein, and Snyder-Theilen strains of feline sarcoma virus. J. Virology 33:196-207.

8. Bather, R., S. Dzuibalo, and C.le Q. Darcel. 1963. In vitro uptake of $^{59}Fe$ by blood cells from normal birds and birds with erythroblastosis. Canad. J. Biochem. Physiol. 41:2057-2064.

9. Battula, N., and H.M. Temin. 1977. Infectious DNA of spleen necrosis virus is integrated at a single site in the DNA of chronically infected chicken fibroblasts. Proc. Natl. Acad. Sci. U.S.A. 74:281-285.

10. Battula, N., and H.M. Temin. 1978. Sites of integration of infectious DNA of avian reticuloendotheliosis viruses in different avian cellular DNAs. Cell 13:387-398.

11. Beard, J.W. 1963. Avian virus growths and their etiologic agents. In: A. Haddow and S. Weinhouse, Advances in Cancer Research, vol. 7, pp. 1-127, Academic Press, New York.

12. Beard, J.W. 1968. Introduction to avian leukemia. In: M.A. Rich, Experimental leukemia, p 205-232. Appleton Century-Crofts, New York.

13. Beard, J.W., J.F. Chabot, D. Beard, U. Heine and G.E. Houts. 1976. Renal neoplastic response to leukosis virus strains BAI A (avian myeloblastosis virus) and MC29. Cancer Res. 36: 339:353.

14. Beard, D., J.F. Chabot, A.J. Langlois, E.A. Hillman, and J.W. Beard. 1970. Singularity of oncogenic activity of strain MC29 avian leukosis viruses. Arch. Geschwulstforsch. 35:315-325.

15. Beard, J.W., E.A. Hillman, D. Beard, K. Lapis, and U. Heine. 1975. Neoplastic response of the avian liver to host infection with strain MC29 leukosis virus. Cancer Res. 35:1603-1627.

16. Beaudreau, G.S., C. Becker, R.A. Bonar, A.M. Wallbank, D. Beard, and J.W. Beard. 1960. Virus of avian myeloblastosis. XIV. Neoplastic response of normal chicken bone marrow treated with the virus in tissue culture. J. Nat. Cancer Inst. 24:395-415.

17. Beemon, K., and T. Hunter. 1977. *in vitro* translation yields a possible Rous sarcoma virus *src* gene product. Proc. Natl. Acad. Sci. U.S.A. 74:3302-3306.

18. Begg, A.M. 1927. A filterable endothelioma of the fowl. Lancet 2: 912-915.

19. Bennett, J.H. 1845. Two cases of disease and enlargement of the spleen in which death took place from the presence of purulent matter in the blood. Edinburgh Med Surg J. 64:413-423.

20. Bergmann, D.G., L.M. Souza, and M.A. Baluda. 980. Characterization of avian myeloblastosis-associated virus DNA intermediates. J. Virology 34:366-372.

21. Berk, A.J., and P.A. Sharp. 1978. Spliced early mRNAs of simian virus 40. Proc. Nat. Acad. Sci. U.S.A. 75:1274-1278.

22. Bernstein, A., D.M. Hunt, V. Crichley, T.W. Mak. 1976. Induction by ouabain of hemoglobin synthesis in cultured Friend erythroleukemic cells. Cell 9:375-381.

23. Beug, H., and T. Graf. 1980. Transformation parameters

of chicken embryo fibroblasts infected with the ts34 mutant of avian erythroblastosis virus. Virology 100:348-356.

24. Beug, H., T. Graf and M.J. Hayman. 1981. Production and characterization of antisera specific for the erb-portion of the presumptive transforming protein of avian erythroblastosis virus. Virology 111: 201-210.

25. Beug, H., A. von Kirchbach, G. Doderlein, J.-F. Conscience and T. Graf. 1979. Chicken hematopoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. Cell 18:375-390.

26. Beug, H., G. Kitchener, G. Doederlein, T. Graf and M.J. Hayman. 1980. Mutant of avian erythroblastosis virus detective for erythroblast transformation: deletion in the erb portion of p75 suggests function of the protein in leukemogenesis. Proc. Natl. Acad. Sci. U.S.A. 77:6683-6686.

27. Biggs, P.M., B.S. Milne, T. Graf, and H. Bauer. 1973. Oncogenicity of non-transforming mutants of avian sarcoma viruses. J. Gen. Virol. 18:399-403.

28. Bishop, J.M. 1978. Retroviruses. Ann. Rev. Biochem. 47:35-88.

29. Bister, K. and P.H. Duesberg. 1979. Structure and specific sequences of avian erythroblastosis virus RNA: Evidence for multiple classes of transforming genes among avian tumor viruses. Proc. Natl. Acad. Sci. U.S.A. 76:5023-5027.

30. Bister, K., M.J. Hayman and P.K. Vogt. 1977. Defectiveness of avian myelocytomatosis virus MC29: Isolation of long-term nonproducer cultures and analysis of virus-specific polypeptide synthesis. Virology 82:431-448.

31. Bister, K., W.-H. Lee, and P.H. Duesberg. 1980a. Phosphorylation of the nonstructural proteins encoded by three avian acute leukemia viruses and by avian Fujinami sarcoma virus. J. Virology 36:617-621.

32. Bister, K., H.-C. Loliger, and P.H. Duesberg. 1979. Oligonucleotide map and protein of CMII: detection of conserved and nonconserved genetic elements in avian acute leukemia viruses CMII, MC29, and MH2. J. Virology 32:208-219.

33. Bister, K., G. Ramsay, M.J. Hayman and P.H. Duesberg. 1980b. OK10, an avian acute leukemia virus of the

MC29 subgroup with a unique genetic structure. Proc. Natl. Acad. Sci. U.S.A. 77:7142-7146.

34. Bister, K. and P.K. Vogt. 1978. Genetic analysis of the defectiveness in strain MC29 avian leukosis virus. Virology 88:213-221.

35. Bolognesi, D.P., A.J. Langlois, L. Sverak, R.A. Bonar, and J.W. Beard. 1968. In vitro chick embryo cell response to strain MC29 avian leukosis virus. J. Virology 2:576-586.

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

37. Bonner, W.M. and J.D. Stedman. 1978. Efficient fluorography of $^{3}H$ and $^{14}C$ on thin layers. Anal. Biochem 89:247-256.

38. Brugge, J.S. and R.L. Erikson. 1977. Identification of a transformation-specific antigen induced by an avian sarcoma virus. Nature (London) 269:346-348.

39. Bryant, D., R. Smith, S. Sharma and W. Dodge. 1980. Some circulating factors which influence granulocyte-monocyte production in the chick with myeloblastic leukemia. Cancer Res. 40:4031-4036.

40. Buchagen, D.L., and H. Hanafusa. 1978. Intracellular precursors to the major glycoprotein of avian oncoviruses in chicken embryo fibroblasts. J. Virology 25:845-851.

41. Burckhardt, J.L. 1912. Ueber das Blutbild bei Huhnertuberkalose und dessen Beziehunges zur sogenanntenH Huhnerleukamie nebst Bemerkungen uber das normale Huhnerblut. Z. Immunitatsforsch. 14:544.

42. Burmester, B.R., A.K. Fontes, and W.G. Walter. 1960. Pathogenicity of a viral strain (RPL12) causing avian visceral lymphomatosis and related neoplasms. III. Influence of host age and route of innoculation. J. Nat. Cancer Inst. 24:1423-1442.

43. Burmester, B.R. and T.N. Fredrickson. 1964. Transmission of virus from field cases of avian lymphomatosis. I. Isolation of virus in line 15I chickens. J. Nat. Cancer Inst. 32:37-63.

44. Burmester, B.R., R.F. Gentry, and N.F. Waters. 1955. The presence of the virus of visceral lymphomatosis in embryonated eggs of normal appearing hens. Poultry Sci. 34:609-617.

45. Burmester, B.R., M.A. Gross, W.G. Walter, and A.K. Fontes. 1959. Pathogenicity of a viral strain (RPL12) causing avian visceral lymphomatosis and related neoplasms. II. Host-virus interactions affecting response. J. Nat. Cancer Inst. 22:103-127.

46. Burmester, B.R., C.O. Prickett, T.C. Belding. 1946. A filterable agent producing lymphoid tumors and osteopetrosis in chickens. Cancer Res. 6:189-196.

47. Burmester, B.R., and H.G. Purchase. 1969. Occurrance, transmission and oncogenic spectrum of the avian leu-.osis viruses. Comparative Leukemia Research, 1969. Bibl. Laemat. 36:83-95. Karger, New York.

48. Butterfield, E.E. 1905. Aleukemic lymphadenoid tumors of the hen. Folia haem. 2:649-659.

49. Calothy, G., F. Poirier, P. Crisanti-Combes, and B. Pessac. 1978. Induction of cell proliferation in chick embryo neuroretinal cells infected with avian oncoviruses: a genetic study, p 122-133. In: S. Balati and C. de Giuli-Morghen (ed.), Avian tumor viruses. Piccin Editor, Padova, Italy.

50. Caparin, U. 1896. Fegati leucemici nei polli. Clin. vet. 19:433, quoted in Engelhreth-Holm (1942).

51. Carr, J.G. 1960. Kidney carcinoma of the fowl induced by the MH2 reticuloendothelioma virus. Brit. J. Cancer 14:77-82.

52. Carr, J.G., and J.G. Campbell. 1958. Three new virus-induced fowl sarcomata. Brit. J. Cancer 12:631-635.

53. Chabot, J.F., D. Beard, A.J. Langlois, and J.W. Beard. 1970. Mesotheliomas of peritoneum, epicardium, and pericardium induced by strain MC29 avian leukosis virus. Cancer Res. 30:1287-1308.

54. Chen, J.H. 1980. Expression of endogenous avian myeloblastosis virus information in different chicken cells. J. Virology 36:162-170.

55. Chen, J.H., W.S. Hayward, and C. Moscovici. 1981. Size and genetic content of virus-specific RNA in myeloblasts transformed by avian myeloblastosis virus (AMV). Virology 110:128-136.

56. Chen, J.H., M.G. Moscovici and C. Moscovici. 1980. Isolation of complementary DNA unique to the genome of avian myeloblastosis virus (AMV). Virology 103:112-122.

57. Collet, M.S., E. Erikson and R.L. Erikson. 1979.

Structural analysis of the avian sarcoma virus transforming protein: sites of phosphorylation. J. Virology 29:770-781.

58. Cooper, M.D., H.G. Purchase, D.E. Bockman, and W.E. Gathings. 1974. Studies on the nature of the abnormality of B cell differentiation in avian lymphoid leukosis: production of heterogenous IgM by tumor cells. J. Immunol. 113:1210-1222.

59. Copeland, T.D., D.P. Grandgenett, and S. Oroszlan. 1980. Amino acid sequence analysis of reverse transcriptase subunits from avian myeloblastosis virus. J. Virology 36:115-119.

60. Cottral, G.E., B.R. Burmester, and N.F. Waters. 1954. Egg transmission of avian lymphomatosis. Poultry Sci. 33:1174-1184.

61. Courtneidge, S.A., A.D. Levinson, and J.M. Bishop. 1980. The protein encoded by the transforming gene of avian sarcoma virus (pp60src) and a homologous protein in normal cells (pp60proto-src) are associated with the plasma membrane. Proc. Natl. Acad. Sci. U.S.A. 77:3783-3787.

62. Craigie, D. 1845. Case of disease of the spleen in which death took place in consequence of the presence of purulent matter in the blood. Edinburgh Med Surg J. 64:400-412.

63. Crittenden, L.B. 1968. Observation on the nature of a genetic cellular resistance to avian tumor viruses. J. Natl. Cancer Inst. 41:145-153.

64. Czernilofsky, A.P., W. DeLorbe, R. Swanstrom, H.E. Varmus, J.M. Bishop, E. Tischer, and H.M. Goodman. 1980a. The nucleotide sequence of an untranslated but conserved domain at the 3' end of the avian sarcoma virus genome. Nucl. Acid. Res. 8:2967-2984.

65. Czernilofsky, A.P., A.D. Levinson, H.E. Varmus, J.M. Bishop, E. Tischer, H.M. Goodman. 1980b. Nucleotide sequence of an avian sarcoma virus oncogene (src) and proposed amino acid sequence for gene product. Nature 287:198-203.

66. Dahlberg, J.E., R.C. Sawyer, J.M. Taylor, A.J. Faras, W.E. Levinson, H.M. Goodman, and J.M. Bishop. 1974. Transcription of DNA from the 70 S RNA of Rous sarcoma virus. I. Identification of a specific 4 S RNA which serves as primer. J. Virology 13:1126-1133.

67. Davis, N.L., and R.R. Rueckert. 1972. Properties of a ribonucleoprotein particle isolated from Nonidet P-

40-treated Rous sarcoma virus. J. Virology 10:1010-1020.

68. de The, G., C. Becker, J.W. Beard. 1964. Virus of avian myeloblastosis (BAI strain A). XXV. Ultracytochemical study of virus and myeloblast phosphatase activity. J. Nat. Cancer Inst. 32:201-236.

69. de The, G., U. Heine, J.R. Sommer, L. Arvy, D. Beard, and J.W. Beard. 1963a. Multiplicity of cell response to the BAI strain A (myeloblastosis) avian tumor virus. IV. Ultrastructural characteristics of the thymus in myeloblastosis and of the adenosinetriphosphatase activity of thymic cells and associated virus. J.N.Cl. 30:415-455.

70. de The, G., H. Ishiguro, U. Heine, D. Beard. 1963b. Multiplicity of cell response to the BAI strain A (myeloblastosis) avian tumor virus. VI. Ultrastructural aspects of adenosinetriphosphotase activity of nephroblastoma cells and virus. J. N. Cl. 30:1267-1301.

71. Dhar, R., W.L. McClements, L.W. Enquist, and G.F. Vande Woude. 1980. Nucleotide sequences of integrated Moloney sarcoma provirus long terminal repeats and their host and viral junctions. Proc. Natl. Acad. Sci. U.S.A. 77:3937-3941.

72. Diggelman, M. 1979. Biosynthesis of an unglycosylated envelope glycoprotein of Rous sarcoma virus in the presence of tunicamycin. J. Virology 30:799-804.

73. Dodge, W.H., and C. Moscovici. 1973. Colony formation by chicken hematopoietic cell and virus-induced myeloblasts. J. Cell. Physiol. 81: 371-386.

74. Dodge, W.H., R.F. Silva, and C. Moscovici. 1975. The origin of chicken hematopoietic colonies as assayed in semisolid agar. J. Cell. Physiol. 85:25-29.

75. Doljanski, L. and M. Pikovski. 1942. Agent of fowl leukosis in tissue cultures. Cancer Res. 2:626-631.

76. Dougherty, R.M., and H.S. DiStephano. 1965. Virus particles associated with "nonproducer" Rous sarcoma cells. Virology 27:351.

77. Dresler, S., M. Ruta, M.J. Murray and D. Kabat. 1979. Glycoprotein encoded by the Friend spleen focus-forming virus. J. Virology 30:564-575.

78. Duesberg, P.H., K. Bister, and C. Moscovici. 1979. Avian acute leukemia virus MC29: Conserved and variable RNA sequences and recombination with helper

virus.
Virology 99:121-134.

79. Duesberg, P.H., K. Bister, and C. Moscovici. 1980. Genetic structure of avian myeloblastosis virus, released from transformed myeloblasts as a defective virus particle. Proc. Natl. Acad. Sci. U.S.A. 77:5120-5124.

80. Duesberg, P.H., K. Bister, and P.K. Vogt. 1977. The RNA.f avian acute leukemia virus MC29. Proc. Natl. Acad. Sci. U.S.A. 74:4320-4324.

81. Duesberg, P.H., and P.K. Vogt, 1979. Avian acute leukemia viruses MC29 and MH2 share specific RNA sequences: evidence for a second class of transforming gene. Proc. Natl. Acad. Sci. U.S.A. 76:1633-1637.

82. Eisenman, R.N., W.S. Mason, and M. Linial. 1980. Synthesis and processing of polymerase proteins of wild-type and mutant avian retroviruses. J. Virology 36:62-78.

83. Ellerman, V. 1921. The leucosis of fowls and leucemia problems. Gyldendal, London.

84. Ellerman, V., and O. Bang. 1908. Experimentelle leukamie bei Huhnern. Cbl. Bakt. XLVI:595.

85. Ellerman, V., and O. Bang. 1909. Experimentelle leukamie bei Huhnern. Z. Hyg. LXIII:231.

86. Engelbreth-Holm, J., 1942. Spontaneous and experimental leukemia in animals. Oliver and Boyd. Edinburgh.

87. Engelbreth-Holm, J. and A. Rothe-Meyer. 1935. On the connection between erythroblastosis (haemoytoblastosis), myelosis and sarcoma in chicken. Acta Path. Microbiol. Scand. 12:352-365.

88. England, J.E., D.P. Bolognesi, B. Dietzschold, and M.S. Halpern. 1977. Evidence that a precursor glycoprotein is cleaved to yield the major glycoprotein of avian tumor virus. J. Virology 21:810-814.

89. Erikson, E., J.S. Brugge, and R.L. Erikson. 1977. Phosphorylated and nonphosphorylated forms of avian sarcoma virus polypeptide p19. Virology 80:177-185.

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

91. Erikson, E., and R.L. Erikson. 1971. Association of 4

S ribonucleic acid with oncornavirus ribonucleic acids. J. Virology 8:254-256.

92. Feldman, R.A., T. Hanafusa, and H. Hanafusa. 1980. Characterization of protein kinase activity associated with the transforming gene product of Fujinami sarcoma virus. Cell 22:757-765.

93. Feldman, W.H. 1932. Neoplasms of domesticated animals. W.B. Saunders Company. Philadelphia, Penn.

94. Feldman, W.H., and C. Olsen. 1933. The pathology of spontaneous leukosis of chickens. Am. Vet. Med. Assoc. Jour. 82:875-900.

95. Fenner, F. 1976. Classification and nomenclature of viruses. Intervirology 7:1-102.

96. Fiers, W., R. Contrearas, G. Hageman, R. Rogiers, A. Van de Voorde, H. Van Heoverswyn, J. Van Herrewghe, G. Volckaert, and M. Ysebaert. 1978. Complete nucleotide sequence of SV40 DNA. Nature (London) 273:113-120.

97. Flavell, A.J., A. Cowie, S. Legon, and R. Kamen. 1979. Multiple 5' terminal cap structures in late polyoma virus RNA. Cell 16:357-371.

98. Fleissner, E. 1971. Chromatographic separation and antigenic analysis of proteins of oncornaviruses. I. Avian leukemia-sarcoma viruses. J. Virology 8:778-785.

99. Fleissner, E., and E. Tress. 1973. Isolation of a ribonucleoprotein structure from Oncornaviruses. J. Virology 12:1612-1615.

100. Fredrickson, T.N., H.G. Purchase, and B.R. Burmester. 1964. Transmission of virus from field cases of avian lymphomatosis. III. Variation in the oncogenic spectra of passaged virus isolates. National Cancer Intitutes Mono. 17, p. 1-30.

101. Friedmann, T., A. Esty, P. LaPorte, and P. Deininger. 1979. The nucleotide sequence and genome organization of the polyoma early region: extensive nucleotide and amino acid homology with SV40. Cell 17:715-724.

102. Friis, R.R., R.T. Schwarz, and M.F.G. Schmidt. 1977. Phenotypes of Rous sarcoma virus-transformed fibroblasts: an argument for a mutlifunctional src gene product. Med. Microbiol. Immunol. 164:155-165.

103. Friis, R.R., and M.J. Weber. 1977. Properties and

speculations about the Rous sarcoma virus src gene product, p296-304. In: S. Barlati and C. de Giuli-Morghen (ed.), Avian RNA tumor viruses. Piccin Editore, Padova, Italy.

104. Fujinami, A., and K. Inamoto. 1914. Uber Geschwulste bei japanischene Haushuhnern iusbesondere uber einen transplantable Tumor. Z. Krebsforsch. 14:94-119.

105. Furth, J. 1931. Observations with a new transmissible strain of the leucosis (leucemia) of fowls. J. Exp. Med. 53:243-267.

106. Furth, J. 1933. Lymphomatosis, myelomatosis, and endothelioma of chickens caused by a filterable agent. I. Transmission experiments. J. Exp. Med. 58:253-275.

107. Furth, J. 1934. Lymphomatosis, myelomatosis, and endothelioma of chickens caused by a filterable agent. II. Morphological characteristics of the endotheliomata caused by this agent. J. Exp. Med. 59:501-517.

108. Gazzolo, L., C. Moscovici, and M.G. Moscovici. 1979. Response of hemopoietic cells to avian acute leukemia viruses: effects on the differentiation of the target cells. Cell 16:627-638.

109. Gazzolo, L., M.G. Moscovici, C. Moscovici, and P.K. Vogt. 1975. Susceptibility and resistance of chicken macrophage to avian RNA tumor viruses. Virology 67: 553-565.

110. Gazzolo, L., J. Samarut, M. Bouabdelli, and J.P. Blachet. 1980. Early precursors in the erythroid lineage are the specific target cells of avian erythroblastosis virus in vitro. Cell 22:683-691.

111. Gibson, W., and I.M. Verma. 1974. Studies on the reverse transcriptase of RNA tumor viruses. Structural relatedness of two subunits of avian RNA tumor viruses. Proc. Natl. Acad. Sci. U.S.A. 71:4991-4994.

112. Gilboa, E., S. Goft, A. Shields, F. Yoshimara, S. Mitra, and D. Baltimore. 1979. In vitro synthesis of a 9 kb terminally redundant DNA carrying the infectivity of Moloney murine leukemia virus. Cell 16:863-874.

113. Gonda, T.J., D.K. Sheiness, L. Fanshier, J.M. Bishop, C. Moscovici and M.G. Moscovici. 1981. The genome and the intracellular RNAs of avian myeloblastosis virus. Cell 23:279-290.

114. Graf, T. 1973. Two types of target cells for transfor-

mation with avian myelocytomatosis virus. Virology 54:398-413.

115. Graf, T. 1975. In vitro transformation of chicken bone marrow cells with avian erythroblastosis virus. Z. fur Naturforschung 30c:847-849.

116. Graf, T., N. Ade, and H. Beug. 1978. Temperature-sensitive mutant of avian erythroblastosis virus suggests a block of differentiation as a mechanism of leukaemogenesis. Nature 275:496-501.

117. Graf, T. and H. Beug. 1978. Avian leukemia viruses. Interaction with their target cells in vivo and in vitro. Biochimica Biophysica Acta 516:269-299.

118. Graf, T., H. Beug, and M.J. Hayman. 1980. Target cell specificity of defective avian leukemia viruses: Hematopoietic target cells for a given virus type can be infected but not transformed by strains of a different type. Proc. Natl. Acad. Sci. U.S.A. 77:389-393.

119. Graf, T., H. Beug, B. Royer-Pokaro, and W. Meyer-Glauner. 1977b. In vitro transformation of hematopoietic cells by avian erythroid and myeloid leukemia viruses: A model for the differentiation of normal and neoplastic cells. In: Differentiation of normal and neoplastic hematopoietuc cells., ed by B. Clarkson, P.A. Marks, and J.E. Till. Cold Spring Harbor Laboratory. p625-639.

120. Graf, T., D. Fink, H. Beug, and B. Royer-Pokora. 1977a. Oncornavirus-induced sarcoma formation obscured by rapid development of lethal leukemia. Cancer Res. 37:59-63.

121. Graf, T., N. Oker-Pokova, T.G. Todorov, and H. Beug. 1979. Transforming capacities and defectiveness of avian leukemia viruses OK10 and E26. Virology 99:431-436.

122. Graf, T., B. Royer-Pokora and H. Beug. 1976b. In vitro translation of specific target cells by avian leukemia viruses. In: Animal Virology. ICN-UCLA Symposia on Molecular and Cellular Biology Vol IV. D. Baltimore, A.S. Huang, and C.F. Fox, eds. Academic Press. pp. 321-337.

123. Graf, T., B. Royer-Pokora, W. Meyer-Glaener, M. Claviez, E. Gotz, and H. Beug. 1977. In vitro transformation with avian myelocytomatosis virus strain CMII: Characterization of the virus and its target cells. Virology 83:96-109.

124. Graf, T., B. Royer-Pokora, C.E. Schubert, and H. Beug. 1976a. Evidence for the multiple oncogenic potential of cloned leukemia virus: in vitro and in vivo studies with avian erythroblastosis virus. Virology 71:423-433.

125. Grandgenett, D.P., G.F. Gerard, and M. Green. 1973. A single subunit from avian myeloblastosis virus with both RNA-directed DNA polymerase and ribonuclease H activity. Proc. Natl. Acad. Sci. U.S.A. 70:230-234.

126. Grandgenett, D.P., A.C. Vora, and R.D. Schiff. 1978. A 32,000 dalton nucleic acid-binding protein from avian retrovirus cores possesses DNA endonuclease activity. Virology 89:119-132.

127. Green, I., and J.W. Beard. 1955. Virus of avain erythromyeloblastic leukosis. VI. Properties of the enzyme associated with the virus in dephosphorylating adenosine triphosphate. J. Nat. Cancer Inst. 15:1217-1229.

128. Green, I., and D.G. Sharp. 1955. The ATPase activity of avian erythromyeloblastic leukosis virus. I. Introductory kinetics. Biochemica Biophysica Acta 18:36-42.

129. Gross, L. 1970. Oncogenic Viruses, 2nd edition. Pargamon Press, New York, N.Y.

130. Gross, M.A., B.R. Burmester and N. Mantel. 1962. Pathogenicity of a viral strain (RPL12) causing avian visceral lymphomatosis and related neoplasms. IV. Virus bioassay based on a log-exponential distribution of host mortality times. J. Nat. Cancer Inst. 28:1111-1124.

131. Gross, M.A., B.R. Burmester, and W.G. Walter. 1959. Pathogenicity of a viral strain (RPL12) causing avian visceral lymphomatosis and related neoplasms. I. Nature of lesions. J. Nat. Cancer Inst. 22:83-101.

132. Guarente, L., T.M. Roberts and M. Ptashne. 1980. A technique for expressing eukaryotic genes in bacteria. Science 209:1428-1430.

133. Haddad, M.N., D. Weinstein, R.A. Bonar, G.S. Beaudreau, C. Becker, D. Beard, and J.W. Beard. 1960. Virus of avian myeloblastosis. XV. Structural loci of virus synthesis and adenosinetriphosphatase activity by electron and light microscopy of myeloblasts from tissue culture. J. Nat. Cancer Inst. 24:971-993.

134. Hall, W.J., C.W. Bean, and M. Pollard. 1941. Transmission of fowl leucosis through chick embryos and young

chicks.
Am. J. Vet. Res. 2:272-279.

135. Hanafusa, H. 1969. Rapid transformation of cells by Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 63:318-325.

136. Hanafusa, H. 1975. Avian RNA tumor viruses. In: F.F. Becker, Cancer: A Comprehensive Treatise, Vol. 2, p. 401-483. Plenum Press, New York.

137. Hanafusa, H. 1977. Cell transformation by RNA tumor viruses. In: H. Fraenkel-Conrat and R.R. Wagner, Comprehensive Virology, Vol 10, p. 49-90. Plenum Publishing Corporation.

138. Hanafusa, H., C.C. Halpern, D.L. Buchhagen, and S. Kawai. 1977. Recovery of avian sarcoma virus from tumors induced by transformation-defective mutants. J. Exp. Med. 146:1735-1747.

139. Hanafusa, H., and T. Hanafusa. 1968. Further studies on RSV production from transformed cells. Virology 34:630-636.

140. Hanafusa, H., and T. Hanafusa. 1971. Noninfectious RSV deficient in DNA polymerase. Virology 43:313-316.

141. Hanafusa, H., T. Hanafusa, and H. Rubin. 1963. The defectiveness of Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 49:572-580.

142. Hanafusa, H., T. Hanafusa, and H. Rubin. 1964. Analysis of the defectiveness of Rouse sarcoma virus. II. Specification of RSV antigenicity by helper viruses. Proc. Natl. Acad. Sci. U.S.A. 51:41-48.

143. Hanafusa, H., T. Miyamoto, T. Hanafusa. 1970. A cell-associated factor essential for formation of an infectious form of Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 66:314.

144. Hanafusa, T., H. Hanafusa, C.E. Metroka, W.S. Hayward, C.W. Rettenmier, R.C. Sawyer, R.M. Dougherty, and H.S. DiStefano. 1976. Pheasant virus: new class of ribodeoxyvirus. Proc. Natl. Acad. Sci. U.S.A. 73:1333-1337.

145. Hanafusa, T., H. Hanafusa, T. Miyamoto. 1970. Recovery of a new virus from apparently normal chick cells by infection with avian tumor viruses. Proc. Natl. Acad. Sci. U.S.A. 67:1797.

146. Hanafusa, T., L.-H. Wang, S.M. Anderson, R.E. Karess, W.S. Hayward, and H. Hanafusa. 1980. Characteris-

tics of the transforming gene of Fujinami sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 77:3009-3013.

147. Harada, F., R.C. Sawyer, and J.E. Dahlberg. 1975. A primer ribonucleic acid for initiation of in vitro Rous sarcoma virus deoxyribonucleic acid synthesis. Nucleotide sequence and amino acid acceptor activity. J. Biol. Chem. 250:3487-97.

148. Haseltine, W.A., D.G. Kleid, A. Panet, E. Rothenberg, and D. Baltimore. 1976. Ordered transcription of RNA tumor virus genomes. J. Mol. Biol. 106:109-131.

149. Haseltine, W.A., A.M. Maxim, and W. Gilbert. 1977. Rous sarcoma virus genome is terminally redundant: The 5' sequence. Proc. Natl. Acad. Sci. U.S.A. 74:989-993.

150. Hayman, M.J. 1978. Viral polyproteins in chick embryo fibroblasts infected with avian sarcoma leukosis viruses. Virology 85:241-252.

151. Hayman, M.J. 1981. Transforming proteins of avian retroviruses. J. Gen. Virology, J. Gen. Viology 52:1-14

152. Hayman, M.J., G. Kitchner, and T. Graf. 1979. Cells transformed by avian myelocytomatosis virus strain CMII contains a 90K gag-related protein. Virology 98:191-199.

153. Hayman, M.J., B. Royer-Pokora, and T. Graf. 1979. Defectiveness of avian erythroblastosis virus: synthesis of a 75K gag-related protein. Virology 92:31-45.

154. Hayward, W.S. 1977. Size and genetic content of viral RNAs in avian oncovirus-infected cells. J. Virology 24:47-63.

155. Hayward, W.S., S.B. Braverman, and S.M. Astrin. 1979. Transcriptional products and DNA structure of endogenous avian proviruses. Cold Spring Harbor Symp. Quant. Biol. 44:1111-1121.

156. Hayward, W.S., and B.G. Neel. 1981. Retroviral gene expression. Curr. Topics Microbiol. Immun., in press.

157. Hayward, W.S., B.G. Neel, and S.M. Astrin. 1981. Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. Nature (London), in press.

158. Henschen, F. 1917. Zur Frage der Huhner leukamie.

Arch. Tierheilk. 43:203.

159. Herman, A.C., R.W. Green, D.P. Bolognesi, and T.C. Vanaman. 1975. Comparative chemical properties of avian oncornavirus polypeptides. Virology 64:339-348.

160. Hickey, E.D., L.A. Weber, and C. Baglioni. 1976. Inhibition of initiation of protein synthesis by 7-methylguanosine-5'-monophosphate. Proc. Natl. Acad. Sci. U.S.A. 73:19-23.

161. Hickey, E.D., L.A. Weber, C. Baglioni, C.H. Kim, and R.H. Sarma. 1977. A relation between inhibition of protein synthesis and conformation of 5'-phophorylated 7-methylguanosine. J. Mol. Biol. 109:173-183.

162. Hirschfeld, H., and M. Jacoby. 1909. Zur Kenntnis der ubertragbaren Huhnerleukamie. Berl. Klin. Wschr. 40:159.

163. Hishinuma, F., P.J. DeBona, S. Astrin and A.M. Skalka. 1981. Nucleotide sequence of acceptor site and termini of integrated avian endogenous provirus f2ev1: integration creates a 6 bp repeat of host DNA. Cell 23:155-164.

164. Hortling, L. 1978. Rapid oncogenesis in vivo by chicken retrovirus OK10. Acta path. microbiol. scand. Sect. B 86:185-192.

165. Houmard, J., and G.R. Drapeau. 1972. Staphylococcal protease: a proteolytic enzyme specific for glutamoyl bonds. Proc. Natl. Acad. Sci. U.S.A. 69:3506-3509.

166. Hsu, T.W., J.C. Sabran, G.E. Mark, R.C. Guntaka, and J.M. Taylor. 1978. Analysis of unintegrated avian RNA tumor virus double-stranded DNA intermediates. J. Virology 28:810-818.

167. Hu, S.S.F., C. Moscovici, and P.K. Vogt. 1978. The defectiveness of Mill Hill 2, a carcinoma-inducing avian oncovirus. Virology 89:162-178.

168. Hu, S.S.F., and P.K. Vogt. 1979. Avian oncovirus MH2 is defective in gag, pol, and env. Virology 92:278-284.

169. Huebner, R.J., and G.J. Todaro. 1969. Oncogenes of RNA tumor viruses as determinants of cancer. Proc. Natl. Acad. Sci. U.S.A. 64:1087-1094.

170. Hughes, S.H., P.R. Shank, D.H. Spector, H.-J. Kung, J.M. Bishop, H.E. Varmus, P.K. Vogt, and M.L. Breitman. 1978. Proviruses of avian sarcoma virus are termi-

nally redundant, co-extensive with unintegrated linear DNA and integrated at many sites. Cell 15:1397-1410.

171. Hunter, T., and B.W. Sefton. 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. U.S.A. 77:1311-1315.

172. Ishizaki, R. and T. Shimizu. 1970. Heterogenicity of strain R avian (erythroblastosis) virus. Cancer Res. 30:2827-2831.

173. Ivanov, X., Z. Mladenov, S. Nedyalkov, and T.G. Todorov. 1962. Bull. Inst. Path. Comp. Anim. Domest. 9:5-36.

174. Ju, G., and A.M. Skalka. 1980. Nucleotide sequence analysis of the long terminal repeat (LTR) of avian retroviruses: Structural similarities with transposable elements. Cell 22:379-386.

175. Kamahora, T., H. Sugiyama, A. Nomoto, M. Yoshida, and K. Toyoshima. 1979. RNA specific for the transforming component of avian erythroblastosis virus strain R. Virology 96:291-294.

176. Kamen, R., J. Favaloro, J. Parker, R. Treisman, L. Lania, M. Fried, and A. Mellor. 1979. Comparison of polyoma virus transcription in productively infected mouse cells and transformed rodent cell lines. Cold Spring Harbor Symp. Quant. Biol. 44:63-75.

177. Karess, R.E., and H. Hanafusa. 1981. Viral and cellular src genes contribute to the structure of recovered avian sarcoma virus transforming protein. Cell, 24:155-164.

178. Karess, R.E., W.S. Hayward, and H. Hanafusa. 1979. Cellular information in the genome of recovered avian sarcoma virus directs the synthesis of transforming protein. Proc. Natl. Acad. Sci. U.S.A. 76:3154-3158.

179. Kasarinoff. 1910. Experimentelle Blutuntersuchungen bei Vogeln. Fol. haem. 10:391-413.

180. Kawai, S., P.H. Duesberg, and H. Hanafusa. 1977. Transformation-defective mutants of Rous sarcoma virus with src gene deletions of varying lenght. J. Virology 24:910-914.

181. Kawai, S., M. Yoshida, K. Segawa, H. Sugiyama, R. Ishizaki, and K. Toyoshima. 1980. Characterization of Y73, an avian sarcoma virus: a unique transforming gene and its product, a phosphopolyprotein with protein kinase activity. Proc. Natl. Acad. Sci. U.S.A.

77:6199-6203.

182. Keshet, E., J.J. O'Rear, and H.M. Temin. 1979. DNA of noninfectious and infectious integrated spleen necrosis virus (SNV) is colinear with unintegrated SNV DNA and not grossly abnormal. Cell 16:51-61.

183. Kessler, S.W. 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. 115:1617-1624.

184. Kitchener, G., and M.J. Hayman. 1980. Comparative tryptic peptide mapping studies suggest a role in cell transformation for the gag-related protein of avian erythroblastosis virus and avian myelocytomatosis virus strains CMII and MC29. Proc. Natl. Acad. Sci. U.S.A. 77:1637-1641.

185. Klemenz, R., and H. Diggelmann. 1978. The generation of the two envelope glycoproteins of Rous sarcoma virus from a common precursor polypeptide. Virology 85:63-74.

186. Krueger, J.G., E. Wang, E. Garber, and A.R. Goldberg. 1980a. Differences in intracellular location of $pp60^{src}$ in rat and chicken cells transformed by Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 77:4142-4146.

187. Krueger, J.G., E. Wang, and A.R. Goldberg. 1980b. Evidence that the src gene product of Rous sarcoma virus is membrane associated. Virology 101:25-40.

188. Kung, H.-J., J.M. Bailey, N. Davidson, M.O. Nicolson and R.M. McAllister. 1975. Structure, subunit composition, and molecular weight of RD-114 RNA. J. Virology 16:397-411.

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

190. Lagerlof, B. 1960. In vitro and in vivo investigations of fowl erythroleukemia. Acta. Path. Microb. Scand. 49. Supplement No. 138.

191. Lai, M.M.C. 1976. Phosphoproteins of Rous sarcoma viruses. Virology 74:287-301.

192. Lai, M.M.C., S.S.F. Hu, and P.K. Vogt. 1979. Avian erythroblastosis virus: transformation-specific sequences form a contiguous segment of 3.25 kb located in the middle of the 6-kb genome. Virology 97:366-

377.

193. Lai, M.M., J.C. Neil, and P.K. Vogt. 1980. Cell-free translation of avian erythroblastosis virus RNA yields two specific and distinct proteins with molecular weights of 75,000 adn 40,000. Virology 100:475-483.

194. Lamb, R.A., and C.J. Lai. 1980. Sequence of interrupted and uninterrupted mRNAs and cloned DNA coding for the two overlapping nonstructural proteins of influenza virus. Cell 21:475-485.

195. Langlois, A.J., D. Beard, and J.W. Beard. 1969a. Strain MC29, an avian leukosis virus of unique properties. /Comp. Leuk. Res. 36:96-105.

196. Langlois, A.J., and J.W. Beard. 1967. Converted-cell focus formation in culture by strain MC29 avian leukosis virus. Proc. Soc. Exp. Bio. Med. 126:718-722.

197. Langlois, A.J., R.B. Fritz, U. Heine, D. Beard, D.P. Bolognesi and J.W. Beard. 1969b. Response of the bone marrow to MC29 avian leukosis virus *in vitro*. Cancer Res. 29:2056-2074.

198. Langlois, A.J., L. Veprek, D. Beard, R.B. Fritz, and J.W. Beard. 1971. Isolation of a non-focus-forming agent from strain MC29 avian leukosis virus. Cancer Res. 31:1010-1018.

199. Laskey, R.A., and A.D. Mills, 1975. Quantitative film detection of $^{3}H$ and $^{14}C$ in polyacrylamide gels by fluorography. Eur. J. Biochem. 56:335-341.

200. Leamson, R.N., and M.S. Halpern. 1976. Subunit structure of the glycoprotein complex of avian tumor virus. J. Virology 18:956-968.

201. Leis, J.P., J. McGinnis, and R.W. Green. 1978. Rous sarcoma virus p19 binds to specific double-stranded regions of viral RNA: effect of p19 on cleavage of viral RNA by RNase III. Virology 84:87-98.

202. Leis, J.P., P. Scheible, and R.E. Smith. 1980. Correlation of RNA binding affinity of avian oncornavirus p19 proteins with the extent of processing of virus genome RNA in cells. J. Virology 35:722-731.

203. Lerner, R.A., J.G. Sutcliffe, and T.M. Shinnick. 1981. Antibodies to chemically synthesized peptides predicted from DNA sequences as probes of gene expression Cell 23:309-310.

204. Levine, A.J. 1976. SV40 and adenovirus early functions

involved in DNA replication and transformation. Biochimica Biophysica Acta 458:213-241.

205. Loliger, H.-Ch. 1964a. Experimentelle Untersachungen zur Ubertragung der Geflugelleukose. II. Serienubertragungen met einem myelosestamm. III. Vergleichende Untersuchangin zwischen zwei aus Spontanfallen isolierten myelosestammen und einem Lymphomatosestamm. Dtsch. Tierarztl. Wschr. 71:207-212.

206. Loliger, H.-Ch. 1964b. Histogenetic correlations between the reticular tissue and the different types of avian leukosis and related neoplasms. Natl. Cancer Inst. Mono. 17, p 37-61.

207. Loliger, H.-Ch., and H.J. Schubert. 1966. Ubertragungsversuche mit aviaren Myelozytomem (tumorformige Chloroleukose, s. Chlorome). Pathol. Vet 3:492-505.

208. MacDonald, M.E., F.H. Reynolds, W.J.M. Van der Ven, J.R. Stephenson, T.W. Mak, and A. Berstein. 1980. Anemia- and polycythemia-inducing isolates of Friend spleen focus-forming virus. Biological and molecular evidence for two distinct viral genomes. J. Exp. Med. 151:1477-1492.

209. Majors, J.E. and H.E. Varmus. 1981. Nucleotide sequence at host-proviral junction for mouse mammary tumor virus. Nature (London) 289:253-257.

210. Marek, J. 1907. Multiple Nervenentzundung (Polyneuritis). bei Huhnern. Deutsche tierarz H. Wochenschr. 15:417-421.

211. Mathews, F.P. 1929. Leukochloroma in the common fowl: Its relation to myelogenic leukemia and its analogies to chloroma in man. Arch. Path. 7:442-457.

212. Mathews, R.E.F. 1979. Classification and nomenclature of viruses. Intervirology 12:234-238.

213. McCormick, F., F. Chaudry, R. Havery, R. Smith, P.W.J. Rigby, and A.E. Smith. 1979. T antigens of SV40-transformed cells. Cold Spring Harbor Symp. Quant. Biol. 44:171-178.

214. McMaster, G.K., and G.G. Carmichael. 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. U.S.A. 74:4835-4838.

215. Mellon, P., A. Pawson, K. Bister, G.S. Martin, and P.H. Duesberg. 1978. Specific RNA sequence and gene pro-

ducts of MC29 avian acute leukemia virus. Proc. Natl. Acad. Sci. U.S.A. 75:5874-5878.

216. Metcalf, D. 1977. Hemopoietic cells. In vitro cloning of normal and leukemic cells. Springer-Verlag, New York, N.Y.

217. Mladenov, Z., U. Heine, D. Beard, and J.W. Beard. 1967. Strain MC29 avian leukosis virus. Myelocytoma, endothelioma, and renal growths: pathomorphological and ultrastructural aspects. J. Nat. Cancer Inst. 38:251-285.

218. Moelling, K., D.P. Bolognesi, H. Bauer, W. Busen, H.W. Plassman and P. Hausen. 1971. Association of viral reverse transcriptase with an enzyme degrading the RNA moiety of RNA-DNA hybrids. Nature New Biol. 234:240-243.

219. Moelling, K., and M. Hayami. 1977. Analysis of precursors to the envelope glycoproteins of avian RNA tumor viruses in chicken and quail cells. J. Virology 22:598-607.

220. Moelling, K., A. Scott, K.E.J. Dittmar and M. Owada. 1980. Effect of p15-associated protease from an avian RNA tumor virus on virus-specific polyprotein precursors. J. Virology 33:680-688.

221. Mommaerts, E.B., E.A. Eckert, D. Beard, D.G. Shard, and J.W. Beard. 1952. Dephosphorylation of adenosine triphosphate by concentrates of the virus of avian erythromyeloblastic leukosis. Proc. Soc. Exp. Biol. Med. 79:450-455.

222. Moore, V.A. 1897. Infectious leukemia in fowls: a bacterial disease frequently mistaken for fowl cholera. 12th and 13th Ann. Rep. U.S. Bureau of Animal Industry 1895-1896. p. 185.

223. Moscovici, C. 1975. Leukemic transformation with avian myeloblastosis virus: present status. Curr. Topics Microbiol. Immun. 71:79-101.

224. Moscovici, C., R.W. Alexander, M.G. Moscovici, and P.K. Vogt. 1977. Transforming and oncogenic effects of MH-2 virus. In: S. Barlati and C de. Giuli-Morghen, Avian RNA tumor viruses, p. 45-56. Piccin Medical Books, Padua. pp. 45-56.

225. Moscovici, C., L. Gazzolo, and M.G. Moscovici. 1975. Focus assay and defectiveness of avian myeloblastosis virus. Virology 68:173-181.

226. Moscovici, C. and M.G. Moscovici. 1973. Tissue culture

of avian hematopoietic cells. In: D.M. Prescott, Methods in Cell Biology, Vol. 7, pp. 313-328. Academic Press, New York, N.Y.

227. Moscovici, C. and P.K. Vogt. 1968. Effects of genetic cellular resistance on cell transformation and virus replication in chicken hematopoietic cell cultures infected with avian myeloblastosis virus (BAI-A). Virology 42:61-67.

228. Moscovici, C., and M. Zanetti. 1970. Studies on single foci of hematopoietic cells transformed by avian myeloblastosis virus. Virology 42:61-67.

229. Moscovici, M.G., and C. Moscovici. 1980. AMV-induced transformation of hematopoietic cells: growth patterns of producers and nonproducers. In: G.B. Rossi, In vivo and in vitro erythropoiesis: The Friend system, p. 503-514. Elsevier North-Holland, Amsterdam.

230. Mosler, F. 1872. Die Pathologie und Therapie der Leukamie. Hirschwald, Berlin.

231. Murphy, E.C., J.J. Kopchick, K.F. Watson, and R.B. Arlinghaus. 1978. Cell-free synthesis of a precursor polyprotein containing both gag and pol gene products by Rauscher murine leukemia virus 35S RNA. Cell 13:359-369.

232. Murray, J.A., and A.M. Begg. 1930. Histolog and histogenesis of a filterable endothelioma. In: 9th Sci. Report of the Imperial Research Fund, London. p. 1-13.

233. Nazerian, K. 1979. Marek's disease lymphoma of chicken and its causative herpesvirus. Biochimica Biophysica Acta 560:375-395.

234. Nedyalkov, S., S. Bozhkov, and G. Todorov. 1975. Experimental erythroblastosis in Japanese quail (Coturnix coturnix japonica) induced by the E-26 leukosis strain. Acta Vet. Brno. 44:75-78.

235. Nedyalkov, S., and Z. Mlandenov. 1967. Susceptibility of Turkey-Poults to avian leukosis strains Mc-29 anc Mc-31 and pathomorphological spectrum of neoplastic growths. Neoplasma 14:619-627.

236. Neel, B.G., W.S. Hayward, H.L. Robinson, J. Fang, and S.M. Astrin. 1981. Avian leukosis virus-induced tumors have common proviral integration sites and synthesize discrete new RNAs: Oncogenesis by promoter insertion. Cell 23:323-334.

237. Neil, J.C., J. Ghysdael, and P.K. Vogt. 1981.

Tyrosine-specific protein kinase activity associated with p105 of avian sarcoma virus PRCII. Virology 109: 223-228.

238. Neumann, E. 1870. Ein Fall von Leukamie mit Erkrankung des Knochenmarkes. Arch. Heilk. 11:1-14.

239. Novikoff, A.B., G. de The, D. Beard, and J.W. Beard. 1962. Electron microscopic study of the ATPase activity of the BAI strain A (myeloblastosis) avian tumor virus. J. Cell. Biol. 15:451-462.

240. Oberling, C., and M. Guerin. 1933. Nouvelles recherches sur la production de tumeurs malignes avec le virus de la leucemie transmissible des poules. Bull. Assoc. Franc. Cancer 22:326.

241. O'Farrell, P.H. 1975. High resolution two-dimentsional electrophoresis of proteins. J. Biol. Chem. 250:4007-4021.

242. Oker-Blom, N., L. Hortling, A. Kallio, E.-L. Nurmiaho, and H. Westermarck. 1978. OK10 virus, an avian retrovirus resembling the acute leukemia viruses. J. Gen. Virol. 40:623-633.

243. Oker-Blom, N., A. Kallio, and L. Hortling. 1975. Morphological changes in chick embryo cell cultures induced by avian leukosis viruses. Intervirology 5:342-353.

244. Olson, C. 1940. Transmissible fowl leukosis. A review of the literature. Mass. Agri. Expt. Station Bull. 370:1-48.

245. Opie, E.L. 1931. Experimental study of the leucemias and lymphomata. Medicine 7:31-63.

246. Opperman, H., J.M. Bishop, H.E. Varmus and L. Levintow. 1977. A joint product of the genes gag and pol of avian sarcoma virus: a possible precursor or reverse transcriptase. Cell 12:993-1005.

247. Palmiter, R.D., J. Gagnon, V.M. Vogt, S. Ripley, and R.N. Eisenman. 1978. The $NH_2$-terminal sequence of the avian oncovirus gag-precursor polyprotein ($Pr76^{gag}$). Virology 91:423-433.

248. Pawson, T., and G.S. Martin. 1980. Cell-free translation of avian erythroblastosis virus RNA. J. Virology 34:280-284.

249. Pawson, T., G.S. Martin, and A.E. Smith. 1976. Cell-free translation of virion RNA from nondefective and transformation-defective Rous sarcoma viruses. J.

Virology
19:950-967.

250. Pawson, T., P. Mellon, P.H. Duesberg, and G.S. Martin. 1980. env gene of Rous sarcoma virus: Identification of the gene product by cell-free translation. J. Virology 33:993-1003.

251. Payne, G.S., S.A. Courtneidge, L.B. Crittenden, A.M. Fadly, J.M. Bishop and H.E. Varmus. 1981. Analysis of avian leukosis virus DNA and RNA in bursal tumors: viral gene expression is not required for maintenance of the tumor state. Cell 23:311-322.

252. Pelham, H.R.B., and R.J. Jackson. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. 67:247-256.

253. Pepinsky, R.B., D. Cappiello, C. Wilkowski, and V.M. Vogt. 1980. Chemical crosslinking of proteins in avian sarcoma and leukemia viruses. Virology 102:205-210.

254. Peterson, R.D.A., B.R. Burmester, T.N. Fredrickson, H.G. Purchase, and R.A. Good. 1964. Effects of bursectomy and thymectomy on the development of visceral lymphomatosis in the chicken. J. Nat. Cancer Inst. 32:1343-1354.

255. Peterson, R.D.A., H.G. Purchase, B.R. Burmester, M.D. Cooper, and R.A. Good. 1966. Relationships among visceral lymphomatosis, bursa of Fabricius, and bursa-dependent lymphoid tissue of the chicken. J. Nat. Cancer Inst. 36: 585-598.

256. Philipson, L., P. Anderson, U. Olshevsky, R. Weinberg, D. Baltimore, and R. Gesteland. 1978. Translation of MuLV and MSV RNAs in nuclease-treated reticulocyte extracts: Enhancement of the gag-pol polypeptide with yeast suppressor tRNA. Cell 13:189-199.

257. Proudfoot, N.J. and G.G. Brownlee. 1976. 3' noncoding region sequences in eukaryotic messenger RNA. Nature (London) 263:211-214.

258. Purchase, H.G. 1976. Differentiation and diagnosis of avian lymphoid leukosis and Marek's disease. C.E.C. Publication EUR 549e, Luxembourg.

259. Purchase, H.G. and D.G. Gilmour. 1975. Lymphoid leukosis in chickens chemically bursectomized and subsequently inoculated (sic) with bursa cells. J. Nat. Cancer Inst. 55:851-855.

260. Purchase, H.G., D.G. Gilmour, C.H. Romero, and W. Oka-

zaki.
1977b. Post-infection genetic resistance to avian lymphoid leukosis resides in B target cell. Nature 270:61-62.

261. Purchase, H.G., W. Okazaki, P.K. Vogt, H. Hanafusa, B.R. Burmester, and L.B. Crittenden. 1977a. Oncogenicity of avian leukosis viruses of different subgroups and mutants of sarcoma viruses. Infect. Immun. 15:423-428.

262. Purchio, A.E., E. Erikson, J.S. Brugge, and R.L. Erikson. 1978. Identification of a polypeptide encoded by the avian sarcoma virus src gene. Proc. Natl. Acad. Sci. U.S.A. 75:1567-1571.

263. Purchio, A.E., E. Erikson, and R.L. Erikson. 1977. Translation of 35S and of subgenomic regions of avian sarcoma virus RNA. Proc. Natl. Acad. U.S.A. 74:4661-4665.

264. Quade, K. 1979. Transformation of mammalian cells by avian myelocytomatosis virus and avian erythroblastosis virus. Virology 98:461-465.

265. Quigley, J.P., D.B. Rifkin, and R.W. Compans. 1972. Isolation and characterization of ribonucleoprotein substructures from Rous sarcoma virus. Virology 50:65-75.

266. Radke, K., and G.S. Martin. 1979. Transformation by Rous sarcoma virus: effects of src gene expression on the synthesis and phosphorylation of cellular polypeptides. Proc. Natl. Acad. Sci. U.S.A. 76:5212-5216.

267. Ramsay, G., and M.J. Hayman. 1980. Analysis of cells transformed by defective leukemia virus OK10: Production of noninfectious particles and synthesis of Pr76gag and an additional 200,000-dalton protein. Virology 106:71-81.

268. Rapp, U.R., and G.J. Todaro. 1978. Generation of new mouse sarcoma viruses in cell culture. Science 201:821-824.

269. Rapp, U.R., and G.J. Todaro. 1979. Evolution of oncogenic mouse type-C viruses in cell culture. Cold Spring Harbor Symp. Quant. Biol. 44:1289-1295.

270. Rasheed, S., M.B. Gardner, and R.J. Huebner. 1978. In vitro isolation of stable rat sarcoma viruses. Proc. Natl. Acad. Sci. U.S.A. 75:2972-2976.

271. Reddy, V.B., P.K. Ghosh, P. Lebowitz, M. Piatak, and

S.M. Weissman. 1979. Simian virus 40 early mRNA's. I. Genomic localization of the 3' and 5' termini and two major splices in mRNA from transformed and lytically infected cells. J. Virology 30:279-296.

272. Reddy, V., B. Thimmapraya, R. Dhar, K. Subrumanian, S. Zain, J. Pan, P. Ghosh, M. Celma, and S. Weissman. 1978. The genome of Simian Virus 40. Science 200:494-500.

273. Rettenmier, C.W. 1979. Studies on the avian oncoviral gag gene products: structural protein markers and gag-related precursor polypeptides. Doctoral thesis. The Rockefeller University.

274. Rettenmier, C.W. 1981. Comparative peptide analysis of avian oncoviral structural protein markers. Virology 108:435-444.

275. Rettenmier, C.W., S.M. Anderson, M.W. Rieman and H. Hanafusa. 1979. gag-related polypeptides encoded by replication-defective avian oncoviruses. J. Virology 32:749-761.

276. Rettenmier, C.W., and H. Hanafusa. 1977. Structural protein markers in the avian oncoviruses. J. Virology 24:850-864.

277. Rettenmier, C.W., R.E. Karess, S.M. Anderson and H. Hanafusa. 1979. Tryptic peptide analysis of avian oncovirus gag and pol gene products. J. Virology 32:102-113.

278. Reynolds, F.H., T.L. Sacks, D.N. Deobagkar, and J.R. Stephenson. 1978. Cells nonproductively transformed by Abelson murine leukemia virus express a high molecular weight polyprotein containing structural and nonstructural components. Proc. Natl. Acad. Sci. U.S.A. 75:3974-3978.

279. Robinson, H.L. 1967. Isolation of noninfectious particles containing Rous sarcoma virus RNA from the medium of Rous sarcoma virus-transformed nonproducer cells. Proc. Natl. Acad. Sci. U.S.A. 57: 1655-1662.

280. Rohrschneider, L. 1979. Immunofluorence on avian sarcoma virus-transformed cells: Localization of the src gene product. Cell 16:11-24.

281. Roloff, F. 1868. Multiple lymphosarcome bie Huhne. Magaz. f. d. ges. Thierheilk. 34:190. Quoted by Engelbreth-Holm (1942).

282. Ross, S.R., S.J. Flint, and A.J. Levine. 1980. Identification of the adenovirus early proteins and

genomic map positions. Virology 100:419-432.

283. Rothberg, P.G., T. Harris, A. Nomoto, and E. Wimmer. 1978. $O^4$-(5'-uridylyl)tyrosine is the bond between the genome-linked protein and the RNA of poliovirus. Proc. Natl. Acad. Sci. U.S.A. 75:4868-4872.

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

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

286. Roussel, M., S. Saule, C. Lagrou, C. Rommens, H. Beug, T. Graf, and D. Stehelin. 1979. Three new types of viral oncogene of cellular origin specific for haematopoietic cell transformation. Nature (London) 281:452-455.

287. Royer-Pokora, B., H. Beug, M. Claviez, H.-J. Winkhardt, R.R. Friis, and T. Graf. 1978. Transformation parameters in chicken fibroblasts transformed by AEV and MC29 avian leukemia viruses. Cell 13:751-760.

288. Royer-Pokora, B.H., S. Grieseer, H. Beug, and T. Graf. 1979. Mutant avian erythroblastosis virus with restricted target cell specificity. Nature (London) 282:750-752.

289. Rubin, H. 1960. A virus in chick embryos which induces resistance in vitro to infection with Rous sarcoma virus. Proc. Natl. Acad. Sci. 46:1105-1119.

290. Rubin, H., and P.K. Vogt. 1962. An avian leukosis virus associated with stocks of Rous sarcoma virus. Virology 17:184-194.

291. Ruscetti, S., D. Troxler, D. Linenmeyer, and E. Scolnick. 1980. Three laboratory strains of spleen focus-forming virus: comparison of their genomes and translational products. J. Virology 33:140-151.

292. Rusov, C., B. Marinkovic,and B. Djukic. 1975. Effect of erythropoietin on the susceptibility of chicks to erythroblastosis virus. Avian Pathology 4:53-58.

293. Samarut, J., and M. Bouabdelli. 1980. In vitro development of CFU-E and BFU-E in cultures of embryonic and post-embryonic chicken hematopoietic cells. J. Cell. Physiol. 105:553-563.

294. Scher, C.D., E.M. Scolnck, and R. Siegler. 1975. Induction of erythroid leukaemia by Harvey and Kirsten sarcoma viruses. Nature (London) 256:225-226.

295. Schiff, R.D., and D.P. Grandgenett. 1978. Virus-coded origin of a 32,000 dalton protein from avian retrovirus cores: structural relatedness of p32 and the beta-polypeptide of the avian retrovirus DNA polymerase. J. Virology 28:279-291.

296. Schmeisser, H.C. 1915. Spontaneous and experimental leukemia of the fowl. J. Exp. Med. 22:820-838.

297. Schriddle, A. 1909. Giebt es eine infektiose Aetiologie der leukamie. Deutshe Med. Wochenschrift Nr. 6.

298. Scolnick, E.M., A.G. Papageorge and T.Y. Shih. 1979. Guanine nucleotide-binding activity as an assay for src protein of rat-derived murine sarcoma viruses. Proc. Natl. Acad. Sci. U.S.A. 76:5355-5359.

299. Scolnick, E.M., M.O. Weeks, T.Y. Shih, S.K. Ruscetti and T.M. Dexter. 1981. Markedly elevated levels of an endogenous sarc protein in a hemopoietic precursor cell line. Mol. Cell. Biology 1:66-74.

300. Sen, A., and G. Todaro. 1977. The genome-associated, specific RNA binding proteins of avian and mammalian type C viruses. Cell 10:91-99.

301. Sen, A., G.J. Todaro, D.G. Blair, and W.G. Robey. 1979. Thermolabile protein kinase molecules in a temperature-sensitive murine sarcoma virus pseudotype. Proc. Natl. Acad. Sci. U.S.A. 76:3617-3621.

302. Shaikh, R., M. Linial, S. Brown, A. Sen, and R. Eisenman. 1979. Recombinant avian oncoviruses. II. Alterations in the gag proteins and evidence for intragenic recombination. Virology 92:463-481.

303. Shaikh, R., M. Linial, J. Coffin, and R. Eisenman. 1978. Recombinant avian oncoviruses. I. Alterations in the precursor to the internal structural proteins. Virology 87:326-338.

304. Shank, P.R., S.H. Hughes, H.-J. Kung, J.E. Majors, N. Quintrell, R.V. Guntaka, J.M. Bishop, and H.E. Varmus. 1978. Mapping unintegrated avian sarcoma virus DNA: termini of linear DNA bear a 300 nucleotides present once or twice in two species of circular DNA. Cell 15:1383-1395.

305. Shank, P.R., and M. Linial. 1980. Avian oncovirus mutant (SE21Q1b) deficient in genomic RNA: characterization of a deletion in the provirus. J. Virology 36:450-456.

306. Shealy, D.J., A.G. Mosser, and R.R. Ruckert. 1980. Novel p19-related protein in Rous-associated virus

type 61: implications for avian gag gene order. J. Virology 34:431-437.

307. Shealy, D., and R.P. Rueckert. 1978. Proteins of Rous-associated virus 61, and avian retrovirus: Common precursor for glycoproteins gp85 and gp35 and use of pactomycin to map translational order of proteins in the gag, pol, and env genes. J. Virology 26:38-388.

308. Sheiness, D., L. Fanshier, and J.M. Bishop. 1978. Identification of nucleotide sequences which may encode the oncogenic capicity of avian retrovirus MC29. J. Virology 28:600-610.

309. Sheiness, D.K., S.H. Hughes, H.E. Varmus, E. Stubblefield, and J.M. Bishop. 1980. The vertebrate homolog of the putative transforming gene of avian myelocytomatosis virus: characteristics of the DNA locus and its RNA transcript. Virology 105:415-524.

310. Sheiness, D., B. Venstrom, and J.M. Bishop. 1981. Virus-specific RNAs in cells infected by avian myelocytomatosis virus and avian erythroblastosis virus: modes of oncogene expression. Cell 23:291-300.

311. Sherr, C.J., A. Sen, G.J. Todaro, A. Sliski, and M. Essex. 1978. Pseudotypes of feline sarcoma virus contain an 85,000-dalton protein with feline oncornavirus-associated cell membrane antigen (FOCMA) activity. Proc. Natl. Acad. Sci. U.S.A. 75:1505-1509.

312. Shih, T.Y., M.O. Weeks, H.A. Young, and E.M. Scolnick. 1979. Identification of a sarcoma virus-coded phosphoprotein in nonproducer cells transformed by Kirsten or Harvey murine sarcoma virus. Virology 96:64-79.

313. Shih, T.Y., A.G. Papageorge, P.E. Stokes, M.O. Weeks, and E.M. Scolnick. 1980. Guanine nucleotide binding and autophosphorylating activities associated with the purified p21 src protein of Harvey sarcoma virus. Nature (London) 287:686-691.

314. Shimotohno, K., S. Mizutani, H.M. Temin. 1980. Sequence of retrovirus provirus resembles that of bacterial trasposable elements. Nature (London) 185:550-554.

315. Shimotohno, K., and H.M. Temin. 1980. No apparent nucleotide sequence specificity in cellular DNA juxtaposed to retrovirus proviruses. Proc. Natl. Acad. Sci. U.S.A. 77:7357-7361.

316. Silva, R.F. and M.A. Baluda. 1980. Avian myeloblastosis virus proteins in leukemic chicken myeloblasts. J. Virology 35:766-774.

317. Silva, R.F., W.H. Dodge, and C. Moscovici. 1974. The role of humoral factors in the regression of leukemia in chickens as measured by in vitro colony formation. J. Cell. Physiol. 83:187-192.

318. Silva, R.F., and C. Moscovici. 1973. Spontaneous regression of leukemia in chickens infected with avian myeloblastosis virus. Proc. Soc. Exp. Biol. Med. 143:604-611.

319. Skiba, A. 1909. Beitrag zu Kenntnis der Leukamien mit besonderer Berucksichtigung dieser Krankheit beim Geflugel. Deutsch. tieraztl. Wschr. 17:405.

320. Smith, R.E., L.J. Davids and P.E. Neiman. 1976. Comparison of avian osteopetrosis virus with an avian lymphomatosis virus by RNA-DNA hybridization. J. Virology 17:160-167.

321. Smith, R.E., and C. Moscovici. 1969. The oncogenic effects of nontransforming viruses from avian myeloblastosis virus. Cancer Res. 29:1356-1366.

322. Soeda, E., J.R. Arrand, N. Smolar, and B.E. Griffin. 1979. Sequence from early region of polyoma virus DNA containing viral replication origin and encoding small, middle and (part of) large T antigens. Cell 17:357-370.

323. Sommer, J.R., D. Weinstein, C. Becker, G.S. Beaudreau, D. Beard and J.W. Beard. 1962. Virus of avian myeloblastosis. XIX. Protein, polysaccharide, lipide, and nucleic acid of myeloblasts and cytidine uptake *in vitro*. J. Nat. Can. Inst. 28:75-97.

324. Souza, L.M., and M.A. Baluda. 1980a. Identification of the avian myeloblastosis virus genome. I. Identification of restriction endonuclease fragments associated with acute myeloblastic leukemia. J. Virology 36:317-324.

325. Souza, L.M., M.J. Briskin, R.L. Hillyard, and M.A. Baluda. 1980b. Identification of the avian myeloblastosis virus genome. II. Restriction endonuclease analysis of DNA from proviral recombinants and leukemic myeloblast clones. J. Virology 36:325-336.

326. Souza, L.M., M.C. Komaromy, and M.A. Baluda. 1980c. Identification of a proviral genome associated with avian myeloblastic leukemia. Proc. Natl. Acad. Sci. U.S.A. 77:3004-3008.

327. Souza, L.M., J.N. Strommer, R.L. Hillyard, M.C. Komaromy, and M.A. Baluda. 1980d. Cellular sequences are present in the presumptive avian myeloblastosis virus genome. Proc. Natl. Acad. Sci. U.S.A. 77:5177-5181.

328. Spector, M., S. O'Neal, and E. Racker. 1981. Regulation of Phosphorylation of the β-subunit of the Ehrlich Ascites tumor Na+/K+ -ATPase by a protein kinase cascade. J. Biol. Chem. 256:4219-4227.

329. Stacey, D.W., V.G. Allfrey, and H. Hanafusa. 1977. Microinjection analysis of envelope-glycoprotein messenger activities of avian leukosis viral RNAs. Proc. Natl. Acad. Sci. U.S.A. 74:1614-1618.

330. Stehelin, D., and T. Graf. 1978. Avian myelocytomatosis and erythroblastosis viruses lack the transforming gene src of avian sarcoma viruses. Cell 13:745-750.

331. Stehelin, D., R.V. Guntaka, H.E. Varmus, and J.M. Bishop. 1976. Purification of DNA complementary to nucleotide sequences required for neoplastic transformation of fibroblasts by avian sarcoma viruses. J. Mol. Biol. 101:349-365.

332. Stehelin, D., H.E. Varmus, J.M. Bishop, and P.K. Vogt. 1976b. DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. Nature (London) 260:170-172.

333. Stohrer, R., and E. Hunter. 1979. Inhibition of Rous sarcoma virus replication by 2-deoxyglucose and tunicamycin: Identifiation of an unglycoslated env gene product. J. Virology 32:412-419.

334. Strathern, J.N., E. Spatola, C. McGill, and J.B. Hicks. 1980. Structure and organization of transposable mating type cassettes in Saccharomyces yeasts. Proc. Natl. Acad. Sci. 77:2839-2843.

335. Stromberg, K., N.E. Hurley, N.L. Davis, R.R. Ruckert, and E. Fleisssner. 1974. Structural studies of avian myeloblastosis virus: comparison of polypeptides in virion and core component by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Virology 13:513-528.

336. Stubbs, E.L., and J. Furth. 1931. Transmission experiments with leucosis of fowls. J. Exp. Med. 53:269-276.

337. Stubbs, E.L., and J. Furth. 1935. The relation of leukosis to sarcoma of chickens. I. Sarcoma and erythroleukosis (strain 13). J. Exp. Med. 61:593-

615.

338. Sutcliffe, J.G., T.M. Shinnick, N. Green, G.-T. Liu, H.L. Niman and R.A. Lerner. 1980b. Chemical synthesis of a polypeptide predicted from nucleotide sequence allows detection of a new retroviral gene product. Nature (London) 287:801-805.

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

340. Suzuki, H. 1976. Effect of 7-methylguanosine-5'-phosphate on rabbit globin synthesis. FEBS Letters 72:309-313.

341. Taylor, J.M. and R. Illmensee. 1975. Site on the RNA of an avian sarcoma virus at which primer is bound. J. Virology 16:553-558.

342. Taylor, J.M., R. Illemensee, and J. Summers. 1976. Efficient transcription of RNA into DNA by avian sarcoma virus polymerase. Biochim. Biophys. Acta 442:324-330.

343. Temin, H.M. 1970. Malignant transformation of cells by viruses. Persp. Biol. Med. 14:11-26.

344. Temin, H.M. 1980. Origin of retroviruses from cellular moveable genetic elements. Cell 21:599-600.

345. Therwath, A., and K. Scherrer. 1978. Post-transcriptional supprssion of globin gene expression in cells transformed by avian erythroblastosis virus. Proc. Natl. Acad. Sci. U.S.A. 75:3776-3780.

346. Till, J.E., and E.A. McCulloch. 1980. Hemopoietic stem cell differentiation. Biochimica Biophysica Acta 605:431-459.

347. Todorov, T.G., and M. Yakimov. 1967. Changes in the cultures of chicken bone marrow cells infected with the virus of chicken myelocytomatosis (strains Mc-29 and Mc-31). Neoplasma 14:613-617.

348. Troxler, D.H., S.K. Ruscetti, and E.M. Scolnick. 1980. The molecular biology of Friend virus. Biochimica Biophysica Acta 605:305-324.

349. Van Beveren, C., J.G. Goddard, A. Berns, and I.M. Verma. 1980. Structure of Moloney murine leukemia viral DNA: Nucleotide sequence of the 5' long terminal re-

peat and adjacent cellular sequences. Proc. Natl. Acad. Sci. 77:3307-3311.

350. Van der Ven, W.J.M., A.S. Khan, F.H. Reynolds, K.T. Mason, and J.R. Stephenson. 1980a. Translation products encoded by newly acquired sequences of indepen-delntly derived feline sarcoma virus isolates are structurally related. J. Virology 33:1034-1045.

351. Van der Ven, W.J.M., F.H. Reynolds, and J.R. Stephenson. 1980b. The nonstructural components of polyproteins encoded by replication-defective mammalian transforming retroviruses are phosphorylated and have associated protein kinase activity. Virology 101:185-197.

352. Varmus, H.E., S. Heasley, H.-J. Kung, H. Opperman, V.C. Smith, J.M. Bishop, and P.R. Shank. 1978. Kinetics of synthesis, structure and purification of avian sarcoma virus-specific DNA made in the cytoplasm of acutely infected cells. J. Mol. Biol. 120:55-82.

353. Verma, I.M. 1975. Studies on reverse transcriptase of RNA tumor viruses. I. Localization of thermolabile DNA polymerase and RNAse H activities on one polypeptide. J. Virology 15:121-126.

354. Virchow, R. 1845. Weisses Blut. Froriep's Notizen 36:151-156.

355. Vogt, V.M., A. Wight, and R. Eisenman. 1979. In vitro cleavage of avian retrovirus gag proteins by viral protease p15. Virology 98:154-167.

356. Vogt, P.K. 1965. A heterogeneity of Rous sarcoma virus revealed by selectively resistant chick embryo cells. Virology 25:237-267.

357. Vogt, P.K. 1971. Spontaneous segregation of non-transforming viruses from cloned sarcoma viruses. Virology 46:939-946.

358. Vogt, P.K. 1977. Genetics of RNA tumor viruses. In: H. Fraenkel-Conrat and R.R. Wagner, Comprehensive Virology, Volume 9. Plenum Press, New York, N.Y. p. 341-455.

359. Vogt, P.K., and R. Ishizaki. 1966. paterns of viral interference in the avian leukosis and sarcoma complex. Virology 30:368-374.

360. von der Helm, K., and P.H. Duesberg. 1975. Translation of Rous sarcoma virus RNA in a cell-free system from ascites Krebs II cells. Proc. Natl. Acad. Sci. U.S.A. 72:614-618.

361. Wahl, G.M., M. Stern, and G.R. Stark. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Nat. Acad. Sci. U.S.A. 76:3683-3687.

362. Walter, G., K.-H. Scheidtmann, A. Carbone, A.P. Laudano, and R.F. Doolittle. 1980. Antibodies specific for the carboxy- and amino-terminal regions of simian virus 40 large tumor antigen. Proc. Natl. Acad. Sci. U.S.A. 77:5197-5200.

363. Wang, E., and A.R. Goldberg. 1978. Binding of deoxyribonuclease I to actin: A new way to visualize microfilament bundles in nonmuscle cells. J. Histochem. Cytochem. 26:745-749.

364. Wang, L.-H. 1978. The gene order of avian RNA tumor viruses derived from biochemical analyses of deletion mutants and viral recombinants. Ann. Rev. Microbiol. 32:561-592.

365. Wang, L.-H., P. Duesberg, K. Beemm, and P.K. Vogt. 1975. Mapping of RNase $T_1$-resistant oligonucleodatides 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. Virology 16:1051-1070.

366. Wang, L.-H., C.C. Halpern, M. Nadel, and H. Hanafusa. 1978. Recombination between viral and cellular sequences generates transforming sarcoma virus. Proc. natl. Acad. Sci. 75:5812-5816.

367. Warthin, A.S. 1907. Leukemia of the common fowl. J. Inf. Dis. 4:369-381.

368. Wasylyk, B., C. Kedinger, J. Corden, O. Brison, and P. Chambon. 1980. Specific *in vitro* initiation of transcription on conalbumin and ovalbumin genes and comparison with adenoviruses-2 early and late genes. Nature (London) 285:367-373.

369. Weber, L.A., E.R. Feman, E.D. Hickey, M.C. Williams, and C. Baglioni. 1976. Inhibition of HeLa cell messenger RNA translation by 7-methylguanosine-5'-monophosphate. J.B.C. 251:5657-5662.

370. Weiss, R.A., R.R. Friis, E. Katz, and P.K. Vogt. 1971. Induction of avian tumor viruses in normal cells by physical and chemical carcinogens. Virology 46:9920-938.

371. Weiss, S.R., P.B. Hackett, H. Opperman, A. Ullrich, L.

Levintow, and J.M. Bishop. 19978. Cell-free translation of avian sarcoma virus RNA: suppression of the gag termination codon does not augment synthesis of the joint gag/pol product. Cell 15:607-614.

372. Weiss, S.R., H.E. Varmus and J.M. Bishop. 1977. The size and genetic composition of virus-specific RNAs in the cytoplasm of cells producing avian sarcoma-leukosis viruses. Cell 12:983-992.

373. Willingham, M.C., G. Jay, and I. Pastan. 1979. Localization of the ASV src gene product to the plasma membrane of transformed cells by electron microscopic immunocytochemistry. Cell 18:115-134.

374. Willingham, M.C., I. Pastan, T.Y. Shih, and E.M. Scolnick. 1980. Localization of the src gene product of the Harvey strain of MSV to plasma membrane of transformed cells my electron microscopic immunocytochemistry. Cell 19:1005-1014.

375. Witte, O.N., and D. Baltimore. 1978. Relationship of retrovirus polyprotein cleavages to virion maturation studied with temperature-sensitive murine leukemia virus mutants. J. Virology 26:750-761.

376. Witte, O.N., L. Sun, N. Rosenberg, and D. Baltimore. 1979. A trans-acting protein kinase identified in cells transformed by Abelson murine leukmia virus. Cold Spring Harbor Symp. Quant. Biol. 44:855-857.

377. Yamamoto, T., B. de Crombrugghe, and I. Pastan. 1980. Identification of a functional promoter in the long terminal repeat of Rous sarcoma virus. Cell 22:787-797.

378. Yamamoto, T., G. Jay, and I. Pastan. 1980b. Unusual features in the nucleotide sequence of a cDNA clone derived from the common region of avian sarcoma virus messenger RNA. Proc. Natl. Acad. Sci. U.S.A. 77:176-180.

379. Ziff, E.B. 1980. Transcription and RNA processing by the DNA tumor viruses. Nature (London) 287:491-499.

End