Rockefeller University
Digital Commons @ RU

Student Theses and Dissertations

1982

# Strategies for Oncogenesis by Avian Leukosis Viruses

Benjamin G. Neel

Follow this and additional works at: https://digitalcommons.rockefeller.edu/student_theses_and_dissertations

Part of the Life Sciences Commons

PRO·BONO·HUMANI·GENERIS
The Rockefeller University
1901
THE LIBRARY

LD 4711.6 N378 1982 c.1 RES
Neel, Benjamin G.
Strategies for oncogenesis
by avian leukosis viruses

Rockefeller University Library
1230 York Avenue
New York, NY 10021-6399

# STRATEGIES FOR ONCOGENESIS BY AVIAN LEUKOSIS 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

Benjamin G. Neel, A.B.

1 April 1982

The Rockefeller University

New York, New York

© Copyright by Benjamin G. Neel, 1982

To my grandmother, Ida Dortch, who taught me to persevere.

TABLE OF CONTENTS

Summary.....i
Acknowledgements.....v
List Of Tables.....vii
List of Figures.....viii
List of Abbreviations.....x
Nomenclature for *onc* Genes.....xii

Introduction

A. The Biology of RNA Tumor Viruses.....1
Taxonomy.....1
The virus particle.....2
The virus life cycle.....3
Endogenous proviruses.....7
B. The Molecular Biology of RNA tumor viruses.....8
The genetic map.....8
The structure of the provirus.....11
Start and stop signals for transcription of viral genes.....14
Synthesis of viral RNA.....20
Posttranscriptional modifications of viral RNA...21
Translation of viral mRNA.....26
C. Mechanisms of Oncogenesis by RNA Tumor Viruses...26
Rapidly transforming viruses.....26
Slowly transforming viruses.....32
D. Etiology and Pathogenesis of ALV-induced Diseases.....33
Etiologic agents.....33
Differences in pathogenicity between exogenous and endogenous viruses: the role of *env* and *c* in ALV oncogenesis.....33
Spectrum of neoplastic disease induced by ALV....35
Pathogenesis of B-cell lymphomas induced by ALV..36
Other diseases induced by ALV.....38
E. A Model for Oncogenesis by ALV.....39
F. The Scope of This Thesis.....41

Materials and Methods

Animal cells and viruses.....44
Production of lymphoid leukosis.....45
RNA extraction.....46
Poly(A) selection of cellular RNA.....47
Glyoxal gel electrophoresis and blot hybridizations.47
Preparation of complementary DNA (cDNA) probes.....49
Analysis of tumor DNA.....50
Bacterial cells and viruses.....51
Restriction endonuclease digestions.....51
Molecular cloning of tumor junction fragments.....51
Isolation of c-myc clones.....53
Isolation of molecular clones of human DNA homologous to onc genes.....53
Preparation of $^{32}$P-labeled nick-translated probes...54
Analysis of cloned DNAs by electrophoresis and blot hybridization.....54
Restriction mapping.....55
In vitro transcription.....55
5'-end selection of 16L viral RNA.....56
RNase $T_1$ fingerprinting.....56
Analysis of virus-related proteins in 16L virus-infected cells.....56

Results

A. Oncogenesis by ALV; The Essentials of the Promoter Insertion Model.....58
Tumor incidence and characteristics.....58
Analysis of viral DNA in lymphoid tumors.....58
Analysis of viral RNA in lymphoid tumors.....61
Recovery of virus from ALV-induced tumors.....64
Identification of a gene activated by promoter insertion.....66
B. The Promoter Insertion Mechanism - Molecular Details.....70
Molecular cloning of tumor junction fragments...70
Isolation of a clone of the normal c-myc gene...71
Restriction mapping of tjf and c-myc clones.....73

Localization of putative coding domains in c-myc.....73
Identification of possible precursors to tumor-specific RNAs.....78
In vitro transcription of tumor junction fragments and c-myc.....80
C. Isolation of a Recombinant Rapidly Transforming Virus from an ALV-induced Fibrosarcoma.....89
Biological properties of 16L Virus.....89
Size and genetic content of 16L RNA.....90
Oligonucleotide fingerprinting of 16L RNA.....92
Analysis of virus-specific proteins in 16L-infected cells.....94
D. Isolation of Molecular Clones of Human DNA related to v-onc genes.....97
Molecular cloning of human myc-related DNA.....98
Molecular cloning of human src-related DNA.....104

Discussion

A. Lymphomagenesis by ALV.....108
Virus-related DNA and RNA in ALV-induced lymphomas.....108
Activation of c-myc by promoter insertion.....110
Defectiveness of the integrated provirus.....114
The two-gene model of ALV lymphomagenesis.....117
The latency of lymphoma induction by ALV.....119
Structure of the c-myc gene in normal cells and in ALV-induced lymphomas.....120
The gene product of c-myc.....127
Orientation of the provirus: the role of the LTR.....128
Comparison of oncogenesis by ALV and MC29.....131
B. The Generation of 16L Virus.....134
C. The Relevance of ALV-induced lymphomagenesis....139
Oncogenesis by slowly transforming viruses.....139
Oncogenesis by non-viral agents.....144

References.....147

Summary

The mechanism of oncogenesis by avian leukosis viruses (ALVs) was examined.

ALVs are slowly transforming RNA tumor viruses that do not transform cells in tissue culture and that cause neoplastic disease only after a latent period of 4-12 months. ALVs generally induce widely disseminating B cell lymphomas originating in the bursae of Fabricius of infected birds. Occasionally, they cause other neoplasms such as sarcomas, carcinomas and nephroblastomas, as well as a variety of nonneoplastic diseases. Unlike rapidly transforming RNA tumor viruses, which appear to cause neoplastic transformation as a result of high expression (or inappropriate expression) of a "transforming gene" (onc gene) present in the rapidly transforming virus genome, ALVs lack onc genes. Until recently, the mechanism of oncogenesis by these viruses was obscure.

Analysis of the virus-related DNA (performed by S. Astrin, Fox Chase Cancer Center) and RNA in ALV-induced lymphomas revealed that the tumors were clonal and thus presumably derived from a single infected cell. Tumor proviruses were often defective and tumors often failed to express normal ALV mRNAs. Thus viral gene expression is not required for maintenance of neoplastic transformation. Most importantly, tumors from different birds had integration sites in common. Tumor cells synthesized high levels of discrete new poly(A) RNAs consisting of viral 5' sequences covalently linked to cellular sequences. No virus capable of transforming tissue culture fibroblasts or of inducing

tumors in animals was isolated from ALV-induced lymphomas tested.

W.S. Hayward (Rockefeller University) found that most ALV-induced lymphomas expressed high levels of RNA that hybridizes to v-myc probe. v-myc is the transforming gene of the rapidly transforming virus MC29. The new poly(A) RNAs found in ALV-induced lymphomas contained myc information and ALV proviruses were found integrated adjacent to c-myc (c-myc is the cellular counterpart of v-myc). These results strongly suggest that in lymphoid tumors induced by ALV, an ALV provirus is integrated adjacent to a specific cellular gene, which is usually c-myc. Proviral integration adjacent to c-myc activates transcription of this gene, resulting in neoplasia.

Molecular clones of the provirus-host cell junctions (tumor junction fragments) from two independent ALV-induced lymphomas were isolated. The structures of these clones were compared with a clone of the normal c-myc gene. Restriction mapping confirms that the ALV provirus is covalently linked to c-myc in both tumors. Normal proviral integration events had occurred adjacent to the c-myc locus, without gross structural alteration of c-myc. The long terminal repeat (LTR) of an ALV provirus is integrated upstream of the bulk of ALV coding sequences in both tumors, and is oriented such that transcription could initiate within the LTR and continue downstream into c-myc. Comparison of the restriction map of a tumor junction fragment clone with the map of the v-myc gene shows that, in c-myc, there are two regions of v-myc-related sequences (which are probably exons), separated by 1 kb of sequences unrelated to v-myc (intron). DNA sequence analysis (G. Gasic, Rockefeller University) suggests that integration has

occurred in c-myc exons adjacent to splice donor sites. Thus there may be at least 4 exons and 3 introns in c-myc. A model is proposed for the genesis of the tumor-specific RNAs containing viral 5' and c-myc information in ALV-induced lymphomas.

In vitro transcription experiments indicate the presence of two potential initiation sites for synthesis by RNA polymerase II in c-myc. Nearly all (>85%) proviral integrations adjacent to c-myc in ALV-induced lymphomas occur downstream from these two sites and upstream of the bulk of c-myc coding sequences. Experiments are in progress to define the initiation site(s) in vivo. The finding by other investigators (Payne et al., 1982) that other proviral orientations can also lead to activation of c-myc and lymphomagenesis is discussed.

One ALV-induced fibrosarcoma was obtained. This tumor produced a replication-defective rapidly transforming virus. The particular strain of ALV used was td107A, a transformation-defective mutant of subgroup A Schmidt-Ruppin Rous sarcoma virus. The virus isolated from the tumor (16L virus) transforms fibroblasts and causes fatal sarcomas in infected newborn chickens within two weeks. Its genomic RNA is 6.0 kb, and it contains fps sequences [fps is the transforming gene of Fujinami sarcoma virus (FSV) and UR1]. RNase $T_1$ fingerprint analysis shows that the 5' and 3' terminal sequences of 16L virus are identical to (and presumably derived from td107A RNA). The central part of 16L viral RNA consists of the fps-related sequences. These oligonucleotides fall into four classes: (i) oligonucleotides common to the putative transforming regions of FSV and UR1 virus; (ii) an oligonucleotide also present in FSV, but not in UR1; (iii) an

oligonucleotide also present in UR1, but not in FSV; and (iv) an oligonucleotide not present in either FSV, UR1 or td107A. Cells infected with 16L virus synthesize a protein of molecular weight 142,000 that is a gag fusion protein with protein kinase activity. These results suggest that 16L virus arose by recombination between td107A and the c-fps gene. A model for the generation of rapidly transforming viruses by recombination between slowly transforming viruses and cellular genes is presented.

Molecular clones of human DNA related to myc and src were obtained by screening a human recombinant bacteriophage library. Preliminary structural analysis by restriction mapping was performed. These studies suggest that coding sequences in both human genes are probably interrupted by introns. Also, the organization of these c-onc genes, especially the human myc-related gene appears to be different in chickens and humans. These clones should be useful for investigating non-viral oncogenesis in humans.

The finding that ALVs induce neoplasia by activating the expression of a normal cellular gene suggests that other oncogenic agents might also cause transformation by activating c-onc genes. The generality of oncogene activation in oncogenesis by other viral as well as non-viral agents is discussed.

Acknowledgements

Many people have contributed to my academic and emotional survival and growth during my time at Rockefeller. This work is partly theirs--for without them it could not have been accomplished.

I would like to thank all the members of the Hanafusa and Hayward labs for making the 11th Floor such a special place to work. It is indeed rare to find a lab in such a hotly competitive area as tumor virology that is as warm and friendly a place. In particular, I would like to thank my fellow students Carl Rettenmier, Roger Karess, and Steve Anderson, for teaching me the ropes and keeping me company late at night; Bernard Mathey-Prevot for his tolerance of my continual "protein chemist" jokes, and for late movie runs, and Tatsuo Takeya, Masa Shibuya, and Lu-Hai Wang for sharing with me their expertise in nucleic acid biochemistry and their good humor. Celeste Simon, Nancy Goldberg, Cheng-Kon Shih and Greg Gasic deserve special thanks for putting up with me in the lab, being there late at night, and for being good friends as well.

Several laboratories have collaborated with ours on this work. Foremost among these was Sue Astrin's lab. Sue deserves immense credit for her painstaking Southern analyses that demonstrated the existence of discrete classes of tumor junction fragments, which was the first strong evidence in favor of the promoter insertion model. I thank her and her coworkers for all that they have taught me as well. Ann Skalka, Grace Ju, and Fumio Hishinuma of the Roche Institute taught me everything I know about cloning. Thanks also to Takis Papas for supplying the valuable v-*myc* probe. Bernard Mathey-Prevot, Teruko Hanafusa, Saburo Hanafusa and especially Lu-Hai Wang were invaluable collaborators on the 16L virus work. Bill Okazaki supplied the useful lymphoma cell line RP9, and Jim Walberg performed numerous histopathological analyses on our chickens tirelessly and expertly. Nancy Goldberg and Susan Braverman provided excellent technical assistance on various phases of the work. I would also like to thank Warren Jelinek, Ed Ziff and Joe Nevins of the Darnell lab for useful discussions, and Sue Haynes and Joe Nevins for help with the in vitro transcription experiments.

Three special friends, Al Hillman, Bruce Charash, and Evie Placek were always there for support and encouragement, as well as good times. My parents and my brothers deserve thanks not only for their support, but also for diversions. Thanks alone are not nearly enough for my fiance Phyllis Koton, who has supplied understanding, love, and perspective, all in copious amounts through these last few hectic months.

Special thanks go to Lauren O'Connor for helping to type the manuscript, truly service beyond the call of duty.

Finally, my academic growth here at Rockefeller is largely due to the efforts of two very special scientists, but more important, two very special men.

Saburo Hanafusa promoted an atmosphere in the lab that emphasized cooperation and friendliness, but also made all of us work to the limits of our ability. I learned much from his encyclopedic knowledge of virology, and from his constant willingness to discuss anything interesting in biology. He is truly that rare creature, the gentleman and the scholar. I thank him as well as Teruko for making my laboratory experience so pleasant.

Last, but certainly most, is my adviser, Bill Hayward. It is hard to know where to begin in thanking him for his guidance, patience, and friendship over the past five years. He has taught me not only techniques, but how to think about problems and design approaches to them. He allowed me to work on a fascinating problem and then shared (at times to near-embarassing excess) credit that was mainly due to him. As a role model, one could not hope for better. I'd also like to thank Linda Hayward for her tolerance of my near-daily calls reporting new, "exciting" results, which often became artifacts the next morning.

# LIST OF TABLES


| Table No. | | Page No. |
|---|---|---|
| 1. | In vitro transcription of the c-myc locus | 87 |
| 2. | *fps*-related large RNase $T_1$-resistant oligonucleotides present in 16L viral RNA | 96 |

# LIST OF FIGURES


| Fig. No. | | Page No. |
|---|---|---|
| 1. | The virus life cycle | 5 |
| 2. | Genetic maps of some representative viruses | 9 |
| 3. | Structure of retroviral RNA and DNA | 12 |
| 4. | Nucleotide sequence of portions of RSV DNA involved in initiation of transcription and poly(A) addition | 18 |
| 5. | Avian sarcoma virus and avian leukosis virus mRNAs and their primary tranlation products | 25 |
| 6. | The promoter insertion model of oncogenesis | 40 |
| 7. | New virus-related RNAs in lymphomas | 63 |
| 8. | New virus-related lymphoma RNAs contain non-viral information | 65 |
| 9. | myc-specific and viral 5'-specific RNAs in ALV-induced lymphomas | 68 |
| 10. | Molecular cloning of a tumor junction fragment and the normal c-myc gene | 72 |
| 11. | Restriction maps of tumor junction fragments | 74 |
| 12. | Identification of putative coding and non-coding domains in c-myc | 76 |
| 13. | Identification of putative tumor-specific precursor RNAs in ALV-induced lymphomas | 79 |
| 14. | Dependence of in vitro transcription reactions on DNA concentration | 81 |
| 15. | In vitro transcription of a tumor junction fragment | 83 |
| 16. | In vitro transcription of c-myc | 85 |
| 17. | Map of transcripts initiated in vitro from c-myc | 88 |
| 18. | Morphologies of cells infected with tumor supernatants | 91 |
| 19. | Fingerprint patterns of RNase $T_1$-resistant oligonucleotides of td107A and 16L viral RNAs | 93 |
| 20. | Oligonucleotide maps of 16L virus and related viruses | 95 |
| 21. | Localization of myc-specific and human repeat sequences in λHm-10 | 99 |
| 22. | Restriction maps of λHm-10 EcoR1 inserts | 101 |
| 23. | The extent of myc-related information in λHm-10 | 103 |

| ig. No. | | Page No. |
|---|---|---|
| 24. | Localization of *src*- and repeat-related sequences in λHs-9 | 105 |
| 25. | Restriction maps of λHs-9 Eco Rl inserts | 107 |
| 26. | Activation of c-*myc* by promoter insertion ALV-induced lymphomagenesis | 111 |
| 27. | Model of the c-*myc* locus and its putative transcript following integration of an ALV provirus upstream from c-*myc* | 123 |
| 28. | Comparison of oncogenesis by ALV and the rapidly transforming virus MC29 | 133 |

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ABM | aminobenzylmethoxy (paper) |
| AEV | avian erythroblastosis virus |
| ALV | avian leukosis virus |
| AMV | avian myeloblastosis virus |
| APT | aminophenylthioether (paper) |
| ATP | adenosine triphosphate |
| bp | base pairs (nucleotide pairs) |
| cDNA | complementary DNA |
| CEF | chicken embryo fibroblast |
| chf | chick helper factor (glycoprotein of endgenous virus) |
| CTP | cytidine triphosphate |
| dATP | deoxyadenosine triphosphate |
| dCTP | deoxycytidine triphosphate |
| DEAE | diethylaminoethyl |
| dGTP | deoxyguanosine triphosphate |
| DNase | deoxyribonuclease |
| dNTP | deoxynucleoside triphosphate |
| EAV | erythroblastosis virus-associated virus (helper virus of AEV) |
| EDTA | ethylenediamine tetraacetic acid |
| FeLV | Feline leukemia virus |
| FeSV | feline sarcoma virus |
| FFU | focus-forming units |
| FSV | Fujinami sarcoma virus |
| gs | group-specific antigen (<u>gag</u> gene product of endogenous virus) |
| GTP | guanosine triphosphate |
| kb | kilobase |
| LTR | long terminal repeat |
| mA | milliamperes |
| MAV | myeloblastosis virus-associated virus (helper virus of AMV) |
| MCAV | myelocytomatosis virus-associated virus (helper virus of MC29) |

| | |
|---|---|
| MC29 | myelocytomatosis virus 29 |
| MCF | mink cell focus-forming (virus) [dual-tropic murine recombinant virus] |
| MMTV | murine mammary tumor virus |
| moMSV | Moloney murine sarcoma virus |
| MSV | murine sarcoma virus |
| MoLV | murine leukemia virus |
| NNS | nonneoplastic syndrome (see text) |
| PB | primer binding site |
| R | short terminal redundancy in retroviral genome |
| rASV | recovered avian sarcoma virus |
| RAV | Rous-associated virus |
| REV | reticuloendotheliosis virus |
| RNase | ribonuclease |
| RPL | Regional poultry lab (virus) |
| RSV | Rous sarcoma virus |
| SDS | sodium dodecyl sulfate |
| SSC | standard saline-citrate (see text) |
| td | transformation defective |
| tjf | tumor junction fragment |
| TTP | thymidine triphosphate |
| U3 | sequences unique to the 3' end of the retroviral genome |
| U5 | sequences unique to the 5' end of the retroviral genome |
| YAV | yet another virus |

## NOMENCLATURE FOR *onc* GENES

At the 1980 RNA tumor virus meeting at Cold Spring Harbor, New York, the following names were agreed upon (summarized in Coffin et al., 1981)

| *onc* Gene | Virus Strain |
|---|---|
| *abl* | Abelson MuLV |
| *erb-A* | AEV |
| *erb-B* | AEV |
| *fes* | Snyder-Theilen & Gardner-Arnstein strains of FeSV |
| *fms* | McDonough strain of FeSV |
| *fps* | FSV and related viruses |
| *mos* | Moloney MSV |
| *myb* | AMV and E26 |
| *myc* | MC29 and other viruses |
| $ras_H$ | Harvey sarcoma virus |
| $ras_K$ | Kirsten sarcoma virus |
| *rel* | reticuloendotheliosis virus-T |
| *ros* | UR2 |
| *sis* | simian sarcoma virus |
| *src* | RSV |
| *yes* | Y73 and Esh |

When present in a viral genome, an *onc* gene is referred to as v-*onc*; its cellular counterpart is c-*onc*

## A. The Biology of RNA Tumor Viruses

Taxonomy. RNA tumor viruses (Oncovirinae), also known as oncoviruses (or the earlier "oncornaviruses"), are members of the family Retroviridae (Mathews, 1979). Retroviridae are enveloped, positive strand RNA viruses that replicate via a DNA intermediate (Temin, 1964a,b; Bader, 1964), utilizing the enzyme reverse transcriptase (DNA-dependent RNA polymerase) (Baltimore, 1970; Temin and Mizutani, 1970). During the past 10-20 years, RNA tumor viruses have been studied extensively by virologists and molecular biologists both because of their novel mode of replication (RNA->DNA->RNA) and because of their ability to transform cells in tissue culture and induce neoplastic disease in animals. The other members of the Retroviridae, the foamy viruses (Spumavirinae) and the Maedi/visna virus group (Lentivirinae) have been less extensively studied because they fail to induce neoplastic pathology and are difficult to grow in vitro; these viruses will not be discussed here. For the rest of this thesis, where the term "retrovirus" is used, it will refer only to the RNA tumor virus subfamily.

Various schemes have been utilized to subdivide the RNA tumor virus group. The classic taxonomic approach is to divide the subfamily into two genera, the Type C oncovirus group and the Type B oncovirus group, and a proposed genus, the Type D oncovirus group, based on virus particle morphology. Type C viruses are then further classified as mammalian, avian, or reptilian,

depending on the normal animal host (Mathews, 1979). Hanafusa (1977) divided RNA tumor viruses into sarcoma viruses, acute leukemia viruses, and lymphatic leukemia viruses, based on the usual pathology induced by the virus. An alternative classificaton scheme, based on the structures and disease-producing properties of different viruses (Hayward and Neel, 1981) will be used in this thesis. Rapidly transforming viruses, which include replication competent (Rous sarcoma virus) and replication defective (avian defective sarcoma viruses, avian acute leukemia viruses, murine and feline sarcoma viruses, Abelson leukemia virus) induce neoplastic disease in host animals rapidly (2-4 weeks), and with high efficiency (approximately 100% of infected animals). They also transform appropriate target cells in tissue culture. Studies in many laboratories have established that the transforming ability of these viruses resides in a specific gene or genes (oncogenes or onc genes) present in the viral genome (see below). Slowly transforming viruses, which include the avian leukosis viruses (ALVs), the murine (MuLVs) and feline (FeLVs) leukemia viruses and the murine mammary tumor virus (MMTV), induce neoplasia only after a latent period of 4-12 months, and with lower efficiency than the rapidly transforming viruses. Slowly transforming viruses do not cause morphologic transformation of any target cell thus far tested in tissue culture. Their genomes lack oncogenes. In addition to the rapidly and slowly transforming viruses, which are exogenous to the host, there are also endogenous retroviruses (see below).

The virus particle. The virus particle (virion) consists of

an internal core of structural proteins, an outer membrane derived from the host cell membrane, and surface glycoproteins embedded in the viral membrane (Nermut et al., 1972), which give the virion a spiked appearance. The viral genome is single stranded RNA with a sedimentation value ranging from about 34-39S (7.5-9.5 kb) for nondefective viruses. Two chemically and genetically identical RNA subunits are joined by hydrogen bonding at or near their 5' ends to form a 60-70S complex (Kung et al., 1975). A number of low molecular weight (4-7S) cellular RNAs are found in the particle, either free or associated with the 60-70S complex (Erikson and Erikson, 1970, 1971; Bishop et al., 1970; Sawyer and Dahlberg, 1973; Faras et al., 1973; Sawyer et al., 1974; Waters et al., 1974; Waters, 1975; for review, see Waters and Mullin, 1977). These RNAs include a selected population of tRNAs, plus 5S and 7S ribosomal RNAs (Erikson and Erikson, 1976). One of the tRNAs [$tRNA^{trp}$ for avian viruses (Harada et al., 1975), $tRNA^{pro}$ for murine viruses (Peters et al., 1977)] serves as a primer for synthesis of negative strand viral DNA (Dahlberg et al., 1974). The function, if any, of the other low molecular weight RNAs is unknown, but they may serve a role in viral RNA processing or packaging.

The virus life cycle. Many of the steps in the replication cycle have been deduced from studies of conditional (temperature-sensitive) or nonconditional mutants (Vogt, 1977). These studies have demonstrated that three viral genes are required for virus replication: gag (coding for the virion structural proteins or group-specific antigens), pol (reverse

transcriptase), and *env* (envelope glycoprotein). All of these gene products are present in virus particles. Other functions (replication of the integrated provirus, RNA synthesis and protein synthesis) are supplied by the host cell.

The infectious cycle begins with the adsorption of virus particles to the cell surface followed by penetration of the cell membrane. Adsorption is nonspecific, involving ionic interactions, but penetration of the cell membrane appears to require a highly specific interaction between cellular receptors and the envelope glycoproteins of the virus. Host range specificity of the virus is generally determined at the penetration step (Hanafusa, 1975; Vogt, 1977).

The intracellular events involved in virus replication are shown schematically in Fig. 1. In the cytoplasm, single-stranded viral RNA is transcribed by reverse transcriptase into a double-stranded linear DNA copy. The DNA is then transported to the nucleus, where some of the linear molecules are converted to closed circular forms. The DNA is integrated into host cell chromosomes via a mechanism that is not well understood. Circular forms of viral DNA may be intermediates in integration (by analogy with bacterial episomes), but there is as yet no direct evidence for this. Integration involves a specific site on the viral DNA, but integration occurs at many sites within the host genome.

The integrated viral DNA (provirus) functions as a stable host cell genetic element. Viral genetic information encoded in the provirus can be transmitted in two ways: (1) by replication of the viral DNA as part of the host cell DNA, and transmission

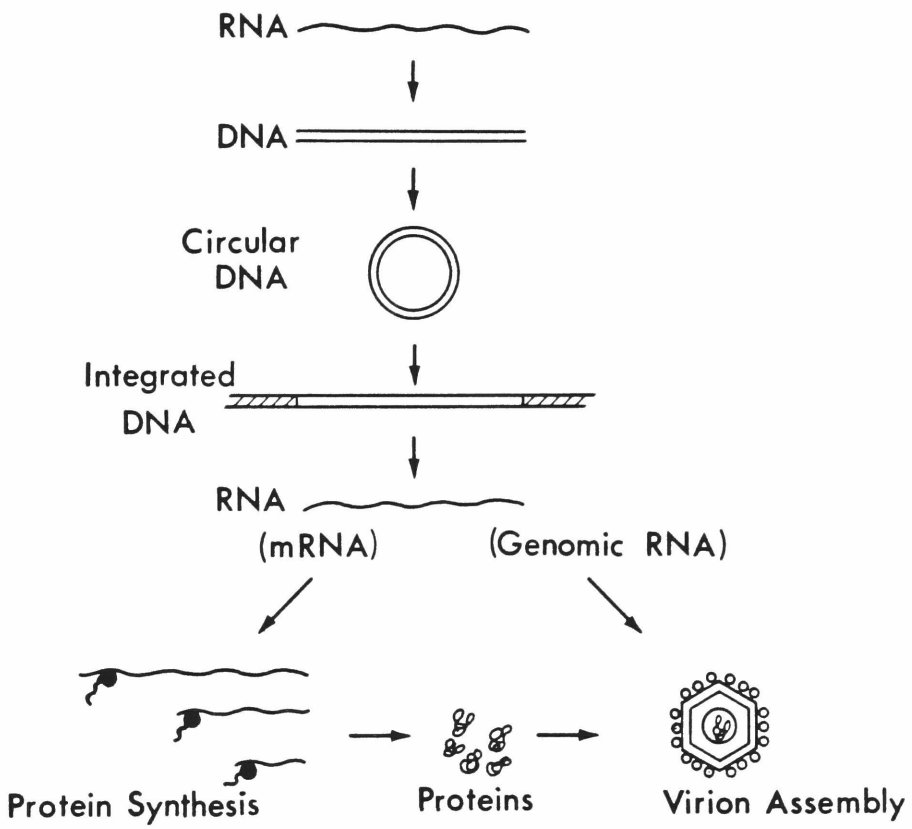



Figure 1. The virus life cycle

to daughter cells at each cell division (vertical transmission), and (2) by transcription of the proviral template into RNA, packaging into virus particles and infection of neighboring cells (horizontal transmission).

The provirus is the major template for synthesis of viral RNA. The primary transcript undergoes a series of posttranscriptional modifications (capping, internal methylations, polyadenylation, and splicing). Viral genomic RNA and mRNA have the same polarity (positive strand), and may be derived from the same pool of primary transcripts. The mechanism of discrimination between genomic RNA and mRNA and the precise step at which discrimination occurs, are unknown. The mRNA population consists of genome-length RNA plus one or more subgenomic RNAs, which are generated from the primary transcript by splicing.

Genes located near the 5' end of the viral genome are translated from mRNA similar in length to genomic RNA. The internal genes are translated from subgenomic mRNAs. The proteins involved in replication (the gag, pol, and env gene products) are synthesized as large precursor polypeptides, which are subsequently cleaved to generate the active proteins. Several of the proteins are modified, either by phosphorylation or glycosylation. Details of the pathways of protein synthesis and maturation have been described in a review by Eisenman and Vogt (1978).

The virus particles are assembled on the cell membrane, and bud off into the surrounding environment. Virus particles continue to be released throughout the life of the cell and its progeny. Condensation of viral RNA to form the 70S complex apparently occurs after virus release (Cheung et al., 1972;

Duesberg et al., 1973).

Endogenous proviruses. Most normal vertebrates contain viral genetic information closely related to that of retroviruses from the same animal species (for reviews, see Hanafusa, 1975; Vogt, 1977; Aaronson and Stephenson, 1977; Robinson 1978; Jaensich, 1980). These so-called "endogenous proviruses" are transmitted from parent to offspring through the germ line of the animal. Recent experiments in the avian (Astrin, 1978; Astrin et al., 1980) and murine (Cohen and Varmus, 1979) systems have shown that individual proviruses segregate in Mendelian fashion in genetic crosses.

The endogenous proviral genes are generally expressed at low levels, or not at all. Analysis of chicken endogenous proviruses has shown that many are defective (Hayward et al., 1980; Hughes et al., 1981). The generally low levels of expression and/or defectiveness of the endogenous proviruses may help account for their persistance in the germ line of the animal, since the selective pressure against such benign viral genes would be minimal.

The presence of endogenous proviruses in normal cells can often cause serious problems in studies of virus infection. Not only do they provide a background of viral DNA, RNA, and proteins that complicates the analysis of contributions of exogenous viruses, but they can also interact with the infecting virus by phenotypic mixing and recombination. Some endogenous proviruses can occasionally produce infectious virus particles, either spontaneously or following chemical induction (Vogt, 1967; Vogt and

Friis, 1971; Rowe et al., 1971; Lowy et al., 1971; Aaronson et al., 1971; Klement et al., 1971; Teich et al., 1973; Aaronson and Dunn, 1974; Besmer et al., 1975; Greenberger and Aaronson, 1975; Robinson et al., 1976; Crittenden et al., 1977; Rascati and Tenant, 1978; Long et al., 1978).

## B. The Molecular Biology of RNA Tumor Viruses

The genetic map. The viral genetic map was deduced first in the avian system, using a variety of biochemical and genetic techniques. By far the most fruitful method was RNase $T_1$ fingerprinting and oligonucleotide mapping (for review, see Wang, 1978; Beemon, 1978). Fig. 2 shows the maps of some representative viruses.

Slowly transforming viruses, whose prototypes are the avian leukosis and murine leukemia viruses, contain the three structural genes gag, pol, and env. An additional region, (often called c), is located near the 3' terminus. Originally, c was defined by oligonucleotide mapping as a region "common" to all exogenous avian retroviruses (Wang and Duesberg, 1974; Wang et al., 1975). Recent investigations of proviral structure indicate that what was defined as c actually consists of 3' sequences (U3) located within the long terminal repeat (LTR) of the provirus (see below) plus other, apparently non-coding sequences (Yamamoto et al., 1980a; Czernilofsky et al., 1980). Although it does not have protein-coding information, c contains important regulatory sequences, such as signals for initiation of transcription by RNA polymerase II, a signal for poly(A) addition, and the initiation

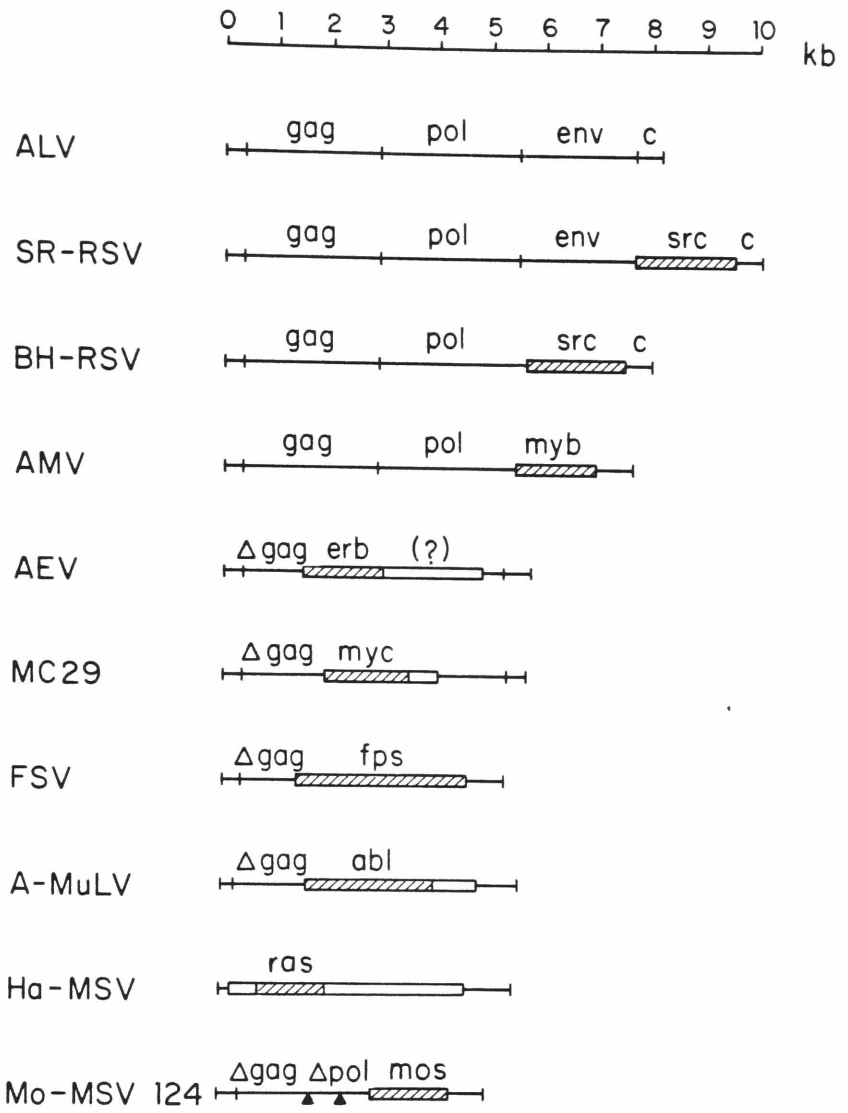



Figure 2. Genetic maps of some representative viruses. Inserted sequences, apparently of cellular origin, are shown in boxes. Sequences thought to code for transforming functions (indicated by cross-hatching) are labeled according to nomenclature proposed at the 1980 RNA tumor virus meeting (Cold Spring Harbor, New York), which is summarized in Coffin et al. (1981).

site for (+) strand DNA synthesis. Another sequence that is probably non-coding is located at the 5' terminus of the genome, and constitutes the leader sequence of viral mRNAs.

Although slowly transforming viruses from different host species have little cross homology, the gene order in all of those tested is the same: 5'-gag-pol-env-c-poly(A). Slowly transforming viruses are probably the progenitors of the rapidly transforming retroviruses, which have acquired additional genetic information from the cell.

The structure of a rapidly transforming virus is that of a slowly transforming virus except that an additional gene(s) (onc gene) is inserted. The inserted gene is derived from the normal cell genome. In most rapidly transforming viruses (e.g., Fujinami sarcoma virus, the avian acute leukemia viruses, Abelson leukemia virus, and the mammalian sarcoma viruses), this insertion replaces viral genetic information; thus most rapidly transforming viruses are replication defective. At least part of the inserted genetic information apparently codes for protein(s) responsible for cell transformation. The size and genetic content of the insert differ in different viruses, as does the site of insertion and the extent of deletion of viral sequences. All of these viruses retain viral sequences located at the extreme 5' and 3' ends of the genome, presumably because these regions contain signals vital for reverse transcription, RNA synthesis, and packaging of viral RNA. There are two isolates of nondefective rapidly transforming viruses, Rous sarcoma virus (RSV) and the closely related Bratislava virus-77 (B77). Both of these viruses contain the same additional gene, src, inserted between env and

c. Otherwise, the gene order in these viruses is the same as in the slowly transforming viruses.

The structure of the provirus. Restriction enzyme analyses have revealed several interesting structural features of the integrated viral DNA (see Figs. 3 and 4) that play important roles in viral RNA synthesis. As first demonstrated for the avian retroviruses, the viral DNA is colinear with viral genomic RNA. However, it extends beyond the limits of the RNA at both the left and right ends (Hsu et al., 1978; Shank et al., 1978b; Hughes et al., 1978; Sabran et al., 1979). RSV genomic RNA contains a short terminal repeat (R) of approximately 20 nucleotides (Haseltine et al., 1977; Shine et al., 1977; Coffin and Haseltine, 1977) (see Fig. 3). The DNA, however, contains a sequence of approximately 350 nucleotides repeated at each end. This long terminal repeat (LTR) consists of three defined segments: U3 (sequences unique to and derived from the 3' end of genomic RNA), R (the short terminal repeat of genomic RNA), and U5 (sequences unique to and derived from the 5' end of genomic RNA) (see Fig. 3). In addition to the short direct repeats within the LTRs, there are short inverted repeats of varying length (depending on the particular virus) at the left and right ends of each LTR (Yamamoto et al., 1980a; Shimotohno et al., 1980). Murine retroviruses have similar proviral structures. In MuLV and MSV, the LTRs are about 500-600 nucleotides (Vande Woude et al., 1979; Gilboa et al., 1979a,b; Hager et al., 1979; Shoemaker et al., 1980; Dhar et al., 1980; Sutcliffe et al., 1980; Van Beveren et al., 1980), whereas MMTV has much larger LTRs, about 1200 nucleo-

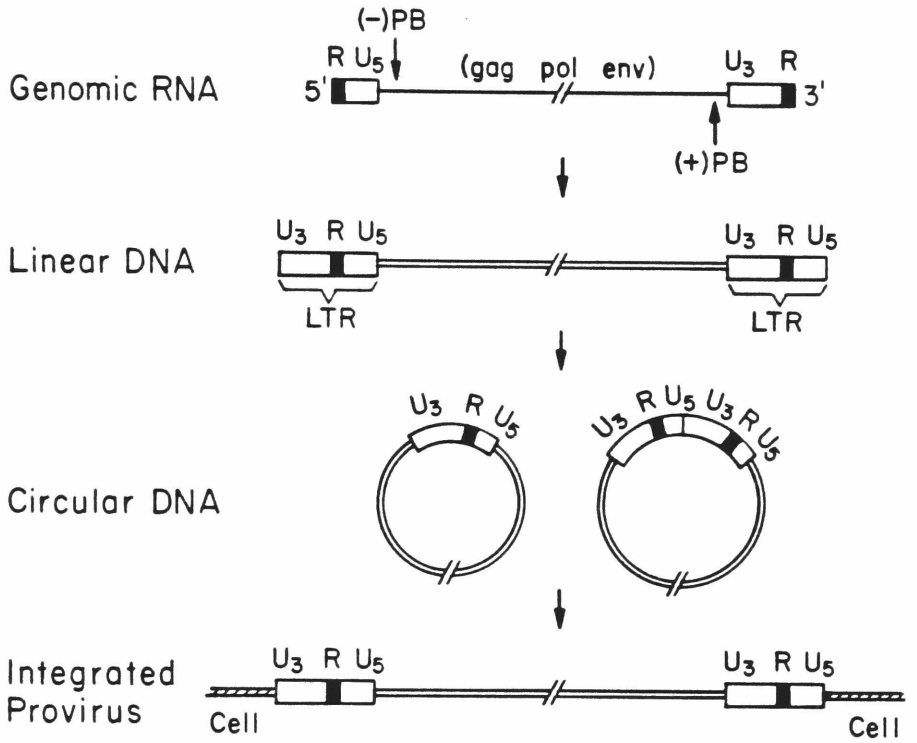



Figure 3. Structure of retroviral RNA and DNA. Termini are shown greatly enlarged to emphasize structural details: terminal redundancy on genomic RNA (R); sequences unique to the 5' (U5) and 3'(U3) ends of genomic RNA; long terminal repeat (LTR) on viral DNA. Circular DNA forms containing either one or two copies of the LTR have been identified in infected cells. The form involved in integration (linear or closed circular) has not been positively determined.

tides in size (Shank et al., 1978a; Cohen et al., 1979; Majors and Varmus, 1981).

DNA sequence analyses of integrated proviruses have revealed other interesting features. First, as mentioned earlier, integration normally involves a specific site on the virus. However, there appears to be no specificity for the cellular site of integration. The terminal two nucleotides of the LTR are deleted in integrated proviruses, whereas a short stretch of cellular sequence at sites where a provirus has integrated is duplicated (4-6 nucleotides, depending on the particular virus) (Dhar et al., 1980; Shimotohno et al., 1980; Van Beveren et al., 1980; Ju and Skalka, 1980). This duplication of host sequences results in the LTRs of integrated proviruses being flanked by short direct repeats. Thus the LTR has many structural features in common with known prokaryotic and eukaryotic transposable elements (for review, see Temin, 1980).

The precise mechanism involved in generating the LTRs is not known. It has been postulated that reverse transcription of the viral RNA into DNA involves two "jumps" from one end of the template to the other (Shank et al., 1978b; Gilboa et al., 1979b; reviewed by Coffin, 1979). The primer binding site for (-) strand synthesis [designated (-) PB; Fig. 3], is located a short distance from the 5' end of the genome. Synthesis proceeds "leftward" (to the 5' end of the RNA template). The repeated sequence (R) on the RNA provides homology for pairing between the newly synthesized (-) strand DNA and the 3' end of the RNA, thus allowing a "jump" from the 5' end to the 3' end of the RNA template. Synthesis then proceeds again towards the 5' end of the RNA.

Synthesis of (+) strand DNA probably initiates on the newly synthesized (-) strand, at a site located near the 3' end of the genome [designated (+) PB], and proceeds rightward to the 3' end. A second "jump" then occurs allowing completion of the (+) strand.

Start and stop signals for transcription of viral genes. Promoters for many prokaryotic genes have been extensively characterized. Although the sequences of these promoters are not identical, they share many features. Based on the available information from many different genes, Pribnow (1975, 1979) has defined a consensus prokaryotic promoter consisting of approximately 40 nucleotides immediately upstream from the initiation site. The sequence TATPuATPu (the "Pribnow box") is located 5-6 bases upstream from the initiation site; a second region with the idealized sequence TGTTGACAATTT is located further upstream, separated by 12-14 nucleotides from the Pribnow box. Deletion of the Pribnow box completely abolishes transcription in vivo and in vitro. At least 20 contact points are thought to be involved in the interaction between the polymerase and the DNA.

The promoter in eukaryotic systems has been less well defined. A sequence of 6-9 AT residues (the "TATA" or "Goldberg-Hogness" box) has been found 29-30 bases upstream from the cap site of many eukaryotic mRNAs (see Ziff, 1980). Originally, many investigators thought that this sequence might be analogous to the Pribnow box. This hypothesis was supported by in vitro transcription experiments, in which the presence of the TATA box was absolutely required for initiation of RNA synthesis

in various transcriptional units by RNA polymerase II (Wasylyk et al., 1980; Corden et al., 1980; Hu and Manley, 1981; Tsai et al., 1981; Wasylyk and Chambon, 1981; Grosveld et al., 1981). However, other evidence suggests that the situation is considerably more complicated. First, there are several SV40 and adenovirus transcriptional units that lack a TATA box (Fiers et al., 1978; Reddy et al., 1978; Ghosh et al., 1978; Flavell et al., 1979). These transcriptional units nevertheless are transcribed in vivo. Similarly, Grosschedl and Birnstiel (1980a) have shown that deletion of the TATA box in the sea urchin H2A histone gene does not abolish transcription in vivo. Instead, these investigators found diminished although significant transcription, but the transcripts had heterogeneous 5' termini. Heterogeneous 5' termini are also found in viral transcriptional units that lack a TATA box (Baker et al., 1979). Thus, rather than being the functional analogue of the Pribnow box, the TATA box seems to play a role in positioning the initiation site; Grosschedl and Birnstiel (1980a,b, 1982) have suggested the term "selector" to describe the function of this region. These investigators also found that deletion of a sequence much further upstream (between 110 and 140 nucleotides from the initiation site) essentially eliminated expression in vivo. They suggest the term "modulator" to describe this region (Grosschedl and Birnstiel, 1980b). A similar split organization of control regions has been found for SV40 (Benoist and Chambon, 1981; Gruss et al., 1981; Banerji et al., 1981; Moreau et al., 1981), the yeast cytochrome C gene (Guarente and Ptashne, 1981; Faye et al., 1981), and the Herpes simplex virus thymidine kinase gene (McKnight et al., 1981), and may be a

general feature of eukaryotic transcriptional units. It is interesting that in several cases these modulator regions appear to function in either orientation relative to the initiation site (Grosschedl and Birnstiel, 1980b; Benoist and Chambon, 1981; Gruss et al., 1981; Banerji et al., 1981; Moreau et al., 1981). This and other recent data (Grosschedl and Birnstiel, 1982) suggest that part of the function of the modulator sequence may be to provide an entry site for RNA polymerase II.

The location of the "promoter" for RNA tumor virus RNA was, until recently, a complete mystery. It was unclear how, if the promoter were located upstream from the initiation site, the virus could encode its own promoter. It now appears that the promoter is located within the LTR. It is encoded near the 3' end of genomic RNA (in U3), and is transferred to the left end of the provirus during reverse transcription (Fig. 3).

DNA sequencing data have demonstrated the presence of an AT-rich sequence resembling a TATA box approximately 30 nucleotides upstream from the cap site, within the LTRs of avian and murine retroviruses (see above sequencing references and Fig. 4). This sequence is located in the same relative position as the TATA box in other eukaryotic genes. In several cases, transcription has been shown to initiate in vitro in the LTR at the appropriate site relative to the TATA box (Yamamoto et al., 1980b; Fuhrman et al., 1981; Ostrowski et al., 1981; see also Results of this thesis). In addition, the LTR appears to contain sequences resembling the modulator sequences described above. The most striking example of this is a 72-base pair (bp) tandem repeat located about 180 nucleotides upstream from the initiation

site for viral RNA synthesis in the Moloney MSV LTR (Dhar et al., 1980). This sequence has no homology with the 72-bp repeat of SV40, which has been shown to positively modulate SV40 transcription. Nevertheless, the murine LTR 72-bp repeat can substitute functionally for the SV40 repeat in SV40/MSV LTR hybrid molecular clones (Levinson et al., 1982). No large tandem repeat sequence has been found within avian LTRs. However, there are several smaller repeats upstream of the TATA box in avian LTRs which might serve the same function as the murine 72-bp repeats (see sequencing references for avian LTRs).

Another important recognition signal, the AATAAA sequence, thought to code for cleavage and poly(A) addition [AAUAAA on the viral RNA] is also located within the long terminal repeat, and is encoded near the 3' end of the RNA (Fig. 4). The AAUAAA signal is located approximately 20 nucleotides upstream from the poly(A) addition site on eukaryotic mRNAs (Proudfoot and Brownlee, 1976), and is found in the same relative position on viral genomic RNA. In avian viruses, this sequence is located just to the left of the 20-nucleotide repeat (R) on the viral RNA. Thus it is transcribed into RNA at the 3' end but not at the 5' end.

In MuLV and MSV, "R" is approximately 60 nucleotides long. The AAUAAA is located within R and is thus present at both ends of the RNA. This appears to pose a logistic problem for the virus, since polyadenylation at the 5' site would presumably result in inefficient production of full-length RNA. Benz et al. (1980) have proposed a model that may explain the ability of the adenylating system to discriminate between the left and the right end AAUAAA sequences of MuLV and MSV RNAs. At the 3' end of the

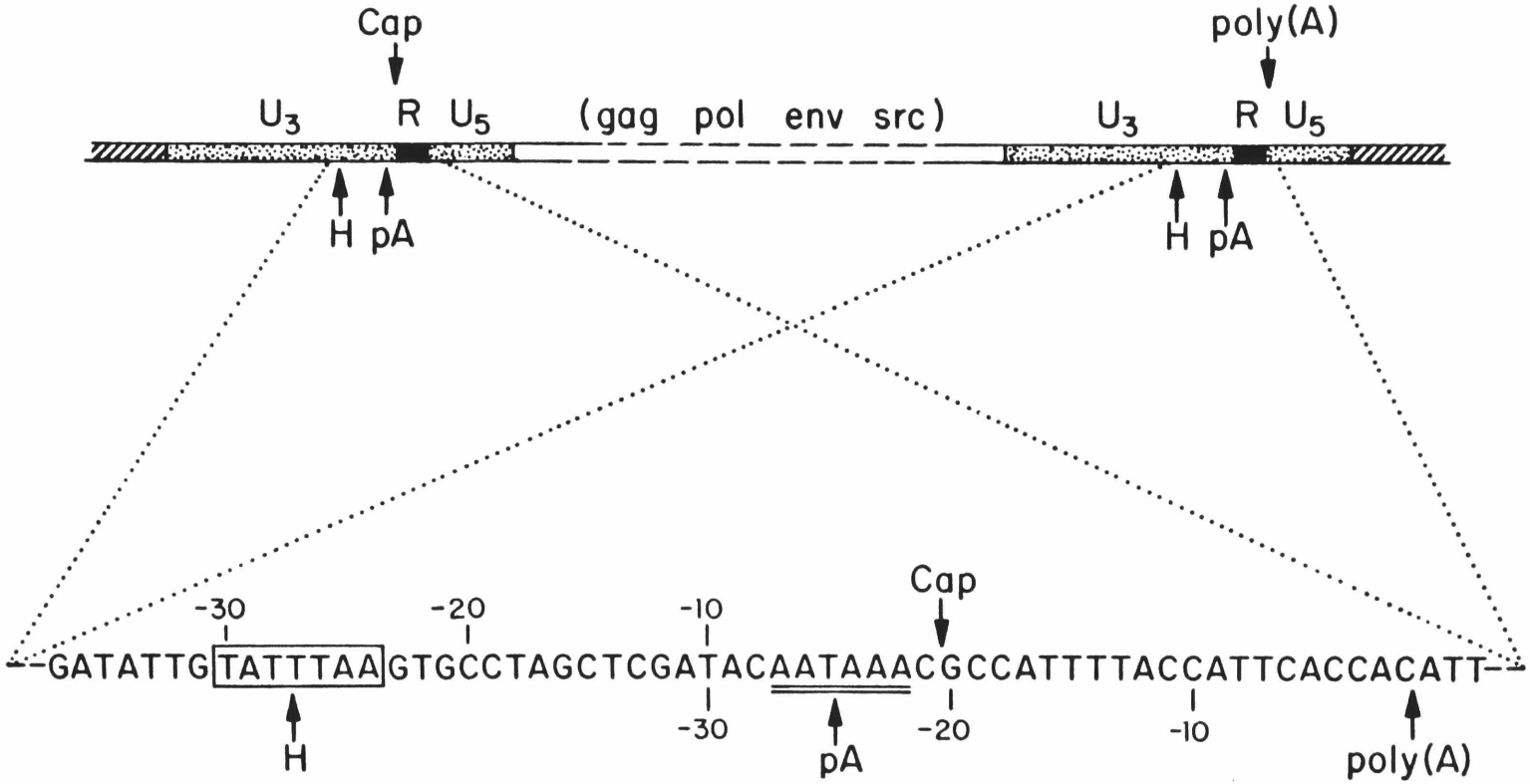



Figure 4. Nucleotide sequence of portions of RSV DNA involved in initiation of transcription and poly(A) addition. Positions corresponding to the capped and polyadenylated sequences of genomic RNA are indicated. Putative signals for capping and poly(A) addition include the Goldberg-Hogness (TATA) box (H) and the AATAAA signal (pA). Sequence shown here is that of D. Schwartz and W. Gilbert (personal communication). Similar sequences have reported by other groups and for other viruses (see text).

genome, a stable hairpin structure can be formed, ending just prior to the AAUAAA, and extending leftward some 60 nucleotides. At the 5' end, however, the last 15 nucleotides of this proposed hairpin structure are located to the left of the cap site, and are not transcribed into RNA. An alternative, less stable structure could be formed at the 5' end. The stable hairpin at the 3' end might be a necessary recognition signal for the polyadenylating enzyme(s). It is also possible, however, that adenylation does occur, in some cases, at the 5' site. This would generate a small poly(A)-containing RNA; however, such an RNA has not been reported. If such premature adenylation did occur, it might provide another type of control of the level of transcription from the LTR, similar to the attenuator mechanism in bacteria (for review, see Yanofsky, 1981).

Studies in the adenovirus (Nevins and Darnell, 1978) and SV40 (Ford and Hsu, 1978) systems indicate that AAUAAA is not a termination signal. Transcription apparently continues beyond this site, the RNA is then cleaved, and poly(A) is added. The nature of the termination signal in eukaryotes is unknown. Nevertheless, it is possible that the virus encodes a termination signal downstream from the poly(A) site, within the LTR. In order to serve as a termination signal at the right end, and not at the left end, one would have to envision some specialized mechanism, possibly involving structural features of the RNA. Alternatively, transcription may continue until it reaches a termination signal in the adjacent cellular sequences; Yamamoto et al. (1980a) have shown that this happens at least some of the time.

Synthesis of viral RNA. Although a small amount of transcription utilizing unintegrated viral DNA as the template cannot be excluded, a wide variety of experiments indicate that integrated viral DNA is the template for viral RNA synthesis (Varmus et al., 1973, 1975, 1976, 1978; Schincariol and Joklik, 1973; Guntaka et al., 1975; Jolicoeur and Baltimore 1976a,b; Sveda and Soeiro, 1976; Bishop et al., 1976; Jolicoeur and Rassart, 1980; Quintrell et al., 1980). Experiments with the specific inhibitor αamanitin indicate that viral RNA synthesis is catalyzed by RNA polymerase II (Jacquet et al., 1974; Rymo et al., 1974; Dinowitz, 1975), which also catalyzes the synthesis of cellular mRNA (Lindell et al, 1970). This is consistent with the existence of putative control sequences for RNA polymerase II within the LTR, as discussed above.

Initiation of normal viral mRNA synthesis occurs within the left LTR at the Cap site (Fig. 4) and proceeds downstream into the viral structural and replicative genes. As mentioned above, it is not clear where termination of viral RNA synthesis occurs. The rate of synthesis of viral RNA has not been measured directly, but estimates can be made from the steady-state concentration of viral RNA and the rate of RNA breakdown. Cells infected with ALV or RSV contain 5,000-20,000 copies of virus-specific RNA (Hayward, 1977). Similar levels have been reported in several other systems such as MuLV (Fan and Baltimore, 1973). Thus viral RNA falls into the "high abundance" class of eukaryotic mRNAs (Hastie and Bishop, 1976). The stability of viral mRNA has been measured in several different retroviral systems, with half-life values ranging from 6-10 hours (Levin and

Rosenak, 1976; Scolnick et al., 1976; Cabradilla et al., 1976; Stacey et al., 1977). Based on an estimated half-life for viral RNA of 8 hr, it can be calculated that viral RNA is synthesized at a rate of approximately 0.3 copies per cell per second. Assuming an average of 2-4 copies of exogenous proviral DNA per cell, this would correspond to about 0.1 copies per provirus per second. Messenger RNA chain elongation in eukaryotic cells proceeds at a rate of approximately 50-100 nucleotides per second (Sehgal et al., 1976). Thus RNA polymerase molecules on the provirus would be located at intervals of 500-1000 nucleotides, and each provirus would contain 10-20 polymerase molecules at any one time. The above calculation is subject to error because of the uncertainties in the various measurements used, but the overall error is probably no greater than 2-4 fold. Furthermore, it is likely that this value underestimates the maximum rate of synthesis, since it represents an average for all integrated proviruses. Analyses in several systems indicate that not all of the integrated proviruses are transcribed at the same rate. Even based upon the lowest value for transcriptional activity within this range, however, the provirus would have to be considered among the most active cellular transcriptional units (Hastie and Bishop, 1976).

Posttranscriptional modifications of viral RNA. Like many other eukaryotic RNAs (for review, see Shatkin, 1976) most retroviral virion RNAs studied, including RSV and ALVs (Keith and Frankel-Conrat, 1975; Furuichi et al., 1975) and MSVs and MuLVs (Bondurant et al., 1976; Rose et al., 1976) contain a cap struc-

ture at their 5' end. Both avian and murine viruses have, in addition to a terminal 7-methyl G residue, a penultimate 2-O-methyl G and a non-methylated third residue, i.e., a Cap I type structure (Shatkin, 1976). Cellular retroviral RNAs contain the same cap structure at their 5' ends (Mellon and Duesberg, 1977; Cordell et al., 1978). Evidence from the adenovirus system suggest that capping in eukaryotes is the earliest posttranscriptional modification, occurring almost simultaneously with initiation in vivo (Weber et al., 1977; Fraser et al., 1978, 1979; Evans et al., 1979; Salditt-Georgieff et al., 1980) and in vitro (Weil et al., 1979; Manley et al., 1979, 1980).

Retroviral genomic (Lai and Duesberg, 1972; Rho and Green, 1974; Quade et al., 1974; Wang and Duesberg, 1974) and messenger (Hayward, 1977; Weiss et al., 1977) RNAs contain poly(A) at their 3' ends. Kinetic studies of the adenovirus late transcriptional unit indicate that poly(A) addition occurs within 30-90 seconds of RNA synthesis, before completion of polymerase II transit (Nevins and Darnell, 1978) and before internal adenosine methylation (Chen-Kiang et al., 1979). Whether these findings will hold true in retrovirus-infected cells is as yet unclear, but it seems likely that at least the order of posttranscriptional events is the same for all eukaryotes.

Retroviral genomic RNA contains 10-12 residues of internal 6-methyladenosine internally (Furuichi et al., 1975; Stoltzfus and Dimock, 1976; Beemon and Keith, 1977). Distribution of these residues is not random (Beemon and Keith, 1977; Dimock and Stoltzfus, 1978). Instead, most of the $m^6A$ is found between 500 and 4000 nucleotides from the 3' terminus of the genome; i.e.,

the regions of the src and env genes (Beemon and Keith, 1977). Very few, if any, residues map further upstream. This observation prompted Beemon and Keith (1977) to suggest that methylated regions are conserved in further RNA processing. Although this is consistent with recent results in the adenovirus system (Chen-Kiang et al., 1979), it should be noted that the above retroviral methylation studies were done only with genomic RNA. Recently, Stoltzfus (personal communication) has shown that $m^6A$ is largely conserved in subgenomic mRNA, but precise quantitation and mapping have not yet been performed. Perhaps the most surprising discovery in eukaryotic molecular biology in recent years was the report that adenovirus-2 late mRNAs contained sequences at their 5' ends specified by non-contiguous DNA (Gelinas and Roberts, 1977; Klessig, 1977; Chow et al., 1977; Berget et al., 1977), so called "RNA splicing". Since that time, splicing has been shown to be involved in the generation of virtually all eukaryotic mRNAs.

The genetic content of retroviral mRNAs was established first for the avian viruses; similar strategies of viral gene expression are followed by the mammalian viruses. Using cDNA probes specific for different portions of the viral genome (corresponding roughly to the gag, pol, env, and src genes and the c region of RSV), Hayward (1977) and Weiss et al. (1977) performed nucleic acid hybridization experiments on size-fractionated poly(A) RNA from virus-infected cells. Three major RNA species, with sedimentation values of approximately 39S, 28S and 21S, were detected in RSV-infected cells. The 39S RNA had a genetic content essentially identical to genomic RNA. The

subgenomic 28S RNA contained env, src, and c information, whereas the 21S RNA had only src and c information. Based on this data, these workers suggested that the structures of the subgenomic RNAs were: (28S) 5'-env-src-c-poly(A), and (21S) 5'-src-c-poly(A). Similar studies for ALV-infected cells revealed only two major species: a larger, genome-length mRNA, and a subgenomic (21S) RNA of structure 5'-env-c-poly(A) (see Fig. 5).

All nondefective retroviruses have at least two mRNAs that conform to the general scheme described above. Several defective retroviruses also have subgenomic mRNAs (Anderson et al., 1980; Sheiness et al., 1981; Gonda et al., 1981; Chen et al., 1981; Chiswell et al., 1981). However, others express only a single genome-length RNA.

The finding of splicing in the adenovirus system, coupled with the description of subgenomic mRNAs in retrovirus-infected cells prompted several investigators to examine whether a splicing mechanism was involved in the genesis of these subgenomic mRNAs. Splicing has been demonstrated in several murine and avian retrovirus systems by a variety of liquid hybridization, oligonucleotide fingerprinting, and heteroduplex techniques (Weiss et al., 1977; Mellon and Duesberg, 1977; Cordell et al., 1978; Krzyzek et al., 1978; Rothenberg et al., 1978; Fan and Verma, 1978; Donoghue et al., 1978, 1979; Faller et al., 1978; Robertson and Varmus, 1979; Stoltzfus and Kuhnert, 1979). The size of the leader sequence in the avian system is approximately 390 nucleotides (Hackett et al., 1982; G. Gasic and W.S. Hayward, personal communication). Apparently the same leader is spliced to both subgenomic mRNAs in RSV-infected cells (Hackett et al.,

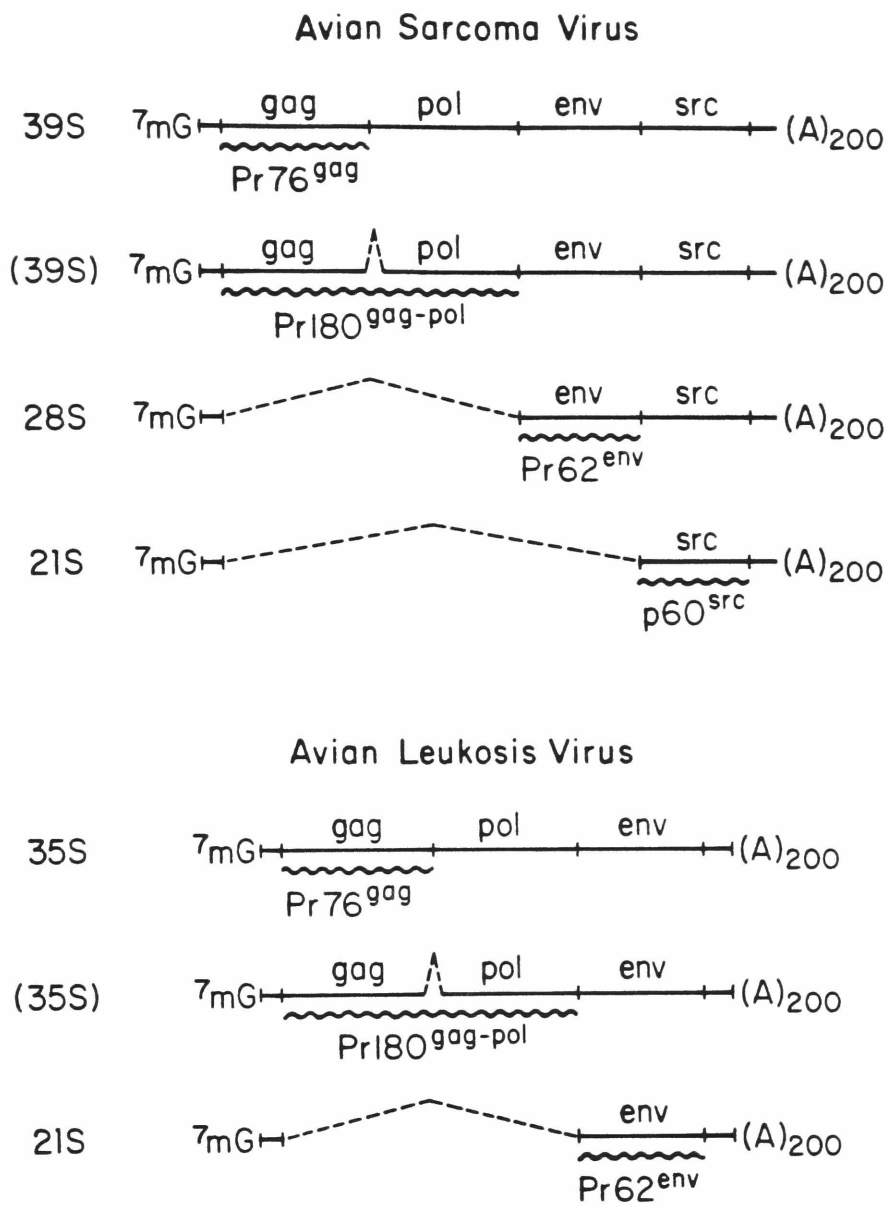



Figure 5. Avian sarcoma virus (ASV) and avian leukosis virus (ALV) mRNAs and their primary translation products. RNA sequences removed by splicing are indicated by broken lines. The putative mRNAs for $Pr180^{gag-pol}$ have not been directly identified. The indicated splice between gag and pol (to remove terminating codons at the end of the gag gene and shift the reading frame) is suggested by sequencing data of D. Schwartz and W. Gilbert (personal communication).

1982).

Translation of viral mRNA. A variety of cell-free translation and microinjection experiments have demonstrated that genome-length mRNA directs the synthesis of the gag gene product (Von der Helm and Duesberg, 1975; Naso et al, 1975a,b; Gielkins et al., 1976; Pawson et al., 1976; Kerr et al., 1976), whereas subgenomic mRNAs direct the synthesis of internal viral genes (Gielkins et al., 1976; van Zaane et al., 1977; Stacey et al., 1977; Pawson et al., 1977; Purchio et al., 1977; Pawson et al., 1980a) (see Fig. 5). For those defective transforming viruses that express only one RNA species, cell-free translation experiments result in the synthesis of a single protein, usually a fusion polyprotein between the gag gene and the onc gene of the virus (Hayman, 1981, see Fig. 5).

## C. Mechanisms of Oncogenesis by RNA Tumor Viruses

Rapidly transforming viruses. Rapidly transforming viruses contain a gene(s) that encodes a transforming protein(s). At least 12 discrete transforming genes have been identified among the various avian and mammalian rapidly transforming viruses (for list, see Coffin et al., 1981). The prototypic transforming protein is $p60^{src}$ of RSV, first identified by Erikson and his coworkers (Purchio et al., 1977; Brugge and Erikson, 1977), and extensively characterized by a number of groups (Brugge and Erikson, 1977; Purchio et al., 1977; 1978; Brugge et al., 1978; Beemon and Hunter, 1978; Levinson et al., 1978; Sefton et al., 1979; Hunter and Sefton, 1980; Collett et al., 1980). $p60^{src}$ has

an associated protein kinase activity (Collett and Erikson, 1978; Levinson et al., 1978) with specificity for tyrosine residues (Hunter and Sefton, 1980; Collett et al., 1980; Sefton et al., 1980). Recently, $p60^{src}$ synthesized in E. coli has been shown to possess tyrosine protein kinase activity (Gilmer and Erikson, 1981; McGrath and Levinson, 1982); thus the kinase activity seems to be an intrinsic property of $p60^{src}$, rather than a contaminating cellular kinase. $p60^{src}$ protein is synthesized in the cytoplasm (Brugge et al., 1977a; Lee et al., 1979; Purchio et al., 1980) but the mature protein is found mainly associated with the plasma membrane (Willingham et al., 1979; Krueger et al., 1980; Courtneidge et al., 1980; Rohrschneider, 1980). Recent evidence shows that in the plasma membrane, $p60^{src}$ is located in adhesion plaques (Rohrschneider, 1980). Several putative cellular substrates for this protein have also been identified, including vinculin (also associated with adhesion plaques) (Sefton et al., 1981) and a 34,000-36,000 major cellular protein of as yet unknown identity (Radke et al., 1980; Erikson and Erikson, 1980). Little is known, however, about the mode of action of the src protein.

Several other (but not all) rapidly transforming viruses encode tyrosine protein kinases [e.g. FSV (Hanafusa et al, 1980; Lee et al., 1980), FeSV (Barbacid et al., 1980b; Van de Ven et al., 1980; Abelson MuLV (Witte et al., 1980), Y73 (Kawai et al., 1980) and UR2 (Feldman et al., 1982). In many cases, the kinase activity resides in a gag-onc fusion polyprotein (Witte et al., 1978; Hanafusa et al., 1980; Lee et al., 1980; Barbacid et al., 1980a; Van de Ven et al., 1980; Kawai et al., 1980; Feldman et

al., 1980, 1982). However since, in many cases, different viruses encode the same transforming gene but have gag-onc fusion polyproteins containing varying amounts of gag, it seems unlikely that the gag information is involved in oncogenesis. The finding that a number of transforming proteins have protein kinase activity specific for tyrosine is interesting since the majority of cellular protein kinases have specificity for serine or threonine. Indeed, transformation is associated with a 7-10 fold increase in the level of phosphotyrosine (Hunter and Sefton, 1980). It is possible that some of the onc proteins may have related functions in the cell. This hypothesis is supported by the finding that the transforming proteins of Rous sarcoma virus($p60^{src}$) and FSV ($P140^{fps}$) phosphorylate a specific cellular protein of molecular weight 34,000-36,000 at the same site (Erikson et al., 1981). Much work remains to be done to determine whether there is a final common pathway for the action of the transforming proteins.

Some transforming proteins do not appear to be tyrosine protein kinases. For example, the avian acute leukemia virus MC29 encodes a fusion protein of molecular weight 110,000 that does not appear to have kinase activity in vitro or in vivo (Bister et al., 1977, 1980; Kitchener and Hayman, 1980; Sefton et al., 1980). Recent evidence suggests that the myc protein is located in the cell nucleus (S. Eisenman, personal communication), rather than on the cell membrane (as is the src protein). This suggests that the myc protein may exert its action directly on cellular DNA, perhaps serving as a generalized transcriptional activator or as a transposase. It seems likely that cell-membrane-

associated transforming proteins play a role in communication between the cell surface and the nucleus.

As mentioned earlier, the viral onc genes are derived from cellular genetic information. This was first suggested by Stehelin et al. (1976), who demonstrated that the src gene of RSV has a homologous counterpart in normal, uninfected cells. Cellular homologues of the transforming genes (or putative transforming genes) of several other rapidly transforming viruses have also been identified (Frankel and Fischinger, 1976; Roussel et al., 1979; Sheiness and Bishop, 1979; Sherr et al., 1979; Chien et al., 1979; Baltimore et al., 1980; Oskarsson et al., 1980; Simek and Rice, 1980; Chen, 1980; Shibuya et al., 1982). Although it has not been rigorously shown for all rapidly transforming viruses, it is thought that all viral transforming genes have cellular counterparts (c-onc genes), which are the precursors to the viral transforming genes (v-onc genes.) The c-onc genes are expressed at low levels in most cells (Frankel and Fischinger, 1976; Wang et al., 1977; Spector et al., 1978a,b; Frankel et al., 1979; Roussel et al., 1979; Sheiness and Bishop, 1979; Chen, 1980; Shibuya et al., 1982). However, the levels of expression of a given c-onc gene may vary in different tissues at different times in the lifetime of an animal (Chen, 1980; Shibuya et al., 1982; C.-K. Shih and W.S. Hayward, personal communication; T. Gonda, personal communication; S. Saule and D. Stehelin, personal communication). Also, the possibility that there is cell-cycle dependent variation in the level of expression of c-onc genes has not been investigated. In no case has the function of a c-onc gene been determined. However, in those cases analyzed, c-onc

genes have been found to be highly conserved in all vertebrates (Spector et al., 1978c; Sheiness and Bishop, 1979; Stehelin et al., 1980; Baltimore et al., 1980; Shibuya et al., 1982). Thus it seems likely that these genes play some essential role in control of cell growth, differentiation, or development.

The simplest model for rapidly transforming virus-induced neoplastic transformation, and quite likely the correct one, is that abnormal cell growth results from the constitutive expression of a c-onc gene that is normally expressed at low (or regulated) levels. These cellular genes have become linked to the highly active viral promoter by recombination at some point in the evolution of the viruses. Hanafusa and his coworkers (Hanafusa et al., 1977; Halpern et al., 1979) have demonstrated the generation of sarcoma viruses after infection of chickens with certain transformation defective (td) mutants of RSV. Similar viruses were later obtained by Vigne et al. (1979). The so-called "recovered avian sarcoma viruses" (rASVs) contain src-related information derived from c-src (Wang et al., 1978; Karess et al., 1979; Karess and Hanafusa, 1981; T. Takeya and H. Hanafusa, personal communication). Incorporation of these sequences into rASV results in a 100- to 1000-fold increase in the level of expression of src-related mRNA and protein compared with normal fibroblasts (Karess et al., 1979). These experiments suggested that c-src is potentially oncogenic if expressed at high levels (or expressed in the wrong cell at the wrong time). It was not absolutely clear from these experiments, however, that c-src was functionally identical to v-src because generation of rASVs was only possible when the parental td mutant retained part

of the v-src gene; all of the rASVs retained part of the v-src gene of the parental td virus. Thus it was not certain that increased expression of a normal cellular gene alone was sufficient for oncogenesis.

Recently, there have been direct demonstrations that increased expression of an otherwise normal cellular gene causes neoplastic transformation. Oskarsson et al. (1980) cloned the cellular sequences (c-mos) corresponding to the transforming gene (v-mos) of Moloney MSV. If the cloned DNA is linked to the MSV LTR, neoplastic transformation of NIH/3T3 cells occurs at high efficiency in a transfection assay (Oskarsson et al., 1980; Blair et al., 1981). Thus the endogenous gene is capable of transforming the cell under appropriate conditions without any apparent alteration in the coding region of the gene and without the participation of any viral coding sequences. Since the sequence of the acquired c-mos gene in the transformants was not determined, it is not possible to rigorously exclude mutation of c-mos in addition to increased expression. However, the observed transformation efficiency is quite high, so this seems unlikely. Similar levels of transformation of NIH/3T3 cells were obtained with molecularly cloned v-mos linked to the LTR (Blair et al., 1980). DeFeo et al. (1981) have recently reported analogous results for v-ras (the transforming gene of Harvey sarcoma virus) and c-ras. The role of the LTR in these experiments is not completely clear. It seems likely that part of its function is to provide an active promoter and control signals, which induce high levels of expression of the onc gene. Blair et al. (1981) found transcripts in their c-mos transformants that were consistent

with initiation from the LTR. However, the possibility that the linked LTR plays an important role in integration, perhaps in directing integration into transcriptionally active regions of the genome, has not been excluded.

Slowly transforming viruses. Thus far, no viral protein has been shown to be directly responsible for neoplasia induced by this class of viruses. Studies of murine viruses have indicated that alterations in the envelope glycoprotein can influence oncogenesis (Elder et al., 1977; Rommeleare et al., 1978). However, if a viral protein is involved, its effect must be indirect, since neoplastic transformation is induced in MuLV-infected animals only after a latent period of several months. In many cases, neoplasia induced by ecotropic MuLV is preceded by the appearance of more highly oncogenic recombinant viruses that have dual-tropic host range. These viruses have been termed MCF viruses (so named for the ability of these viruses to cause foci on mink cells). These viruses still induce neoplasia only after a significant latent period (at least one month). Although it is likely that MCF viruses are the proximate carcinogens in MuLV-induced neoplasia, it also seems likely that the basis for their enhanced oncogenicity involves increased ability to infect certain target cells rather than the function of a specific viral protein. For the avian slowly transforming viruses , specifically the avian leukosis viruses, there is no evidence for the generation of an intermediate, more highly oncogenic recombinant virus. The mechanism of oncogenesis by slowly transforming viruses was, until recently, obscure. The basic question

remained: how could a virus lacking a transforming gene induce neoplasia?

## D. Etiology and Pathogenesis of ALV-induced Diseases

Etiologic agents. The prototypes of the slowly transforming viruses are the avian leukosis viruses (ALVs), first described by Ellerman and Bang (1909). The inability of these viruses to cause transformation in tissue culture made study of ALVs more difficult than study of avian rapidly transforming viruses, so ALVs were relegated to a secondary role in early tumor virology. Since many ALV strains were originally isolated as helper viruses associated with the env-defective Bryan strain of RSV, ALV isolates were often designated Rous-associated viruses (RAV). Similar helper viruses were found associated with the avian acute leukemia viruses, for example: MCAV (myelocytomatosis virus-associated virus), EAV (erythroblastosis virus-associated virus), and MAV (myeloblastosis virus-associated virus). More obscure nomenclature has also been used, including RPL (for regional poultry laboratory, the site of isolation of many of these viruses), and YAV ("yet another virus"). Deletion mutants of RSV that have lost the src gene (td mutants), and thus the ability to induce rapidly developing neoplasia, are still able to induce the same types of slowly developing neoplasia as other ALV (Biggs et al., 1973; Purchase et al., 1977). These deletion mutants will be classified as ALV in this thesis.

Differences in pathogenicity between exogenous and endogenous viruses: the role of env and c in ALV oncogenesis.

Although most exogenous ALVs induce neoplastic disease, the endogenous ALV, Rous-associated virus-0 (RAV-0), appears to be essentially non-oncogenic in chickens (Motta et al., 1975; Crittenden et al., 1979). Comparison of the genomes of exogenous ALVs and RAV-0 by hybridization (Neiman et al., 1977; Hayward, 1977) and oligonucleotide fingerprinting (Coffin et al., 1978) revealed differences primarily in the 3' portion of the genome, within *env* and *c*. Since endogenous viruses have envelope glycoproteins of subgroup E specificity, it seemed possible that *env* might be involved in oncogenesis.

To test this possibility, the oncogenicity of recombinants (termed RAV-60s) between RAV-0 and exogenous viruses (Hanafusa et al., 1970) was tested (Crittenden et al., 1980; Robinson et al., 1980; Weiss and Frisby, 1981). RAV-60s have acquired most of their genetic information from the exogenous virus, but have acquired the *env* gene, with subgroup E host range, from RAV-0 (Hayward and Hanafusa, 1975; Shoyab and Baluda, 1976). All RAV-60 isolates tested, unlike RAV-0, caused lymphomas in susceptible chickens. Thus, oncogenicity did not seem to be related to the *env* gene. RAV-60s have *c* information derived from the exogenous parent (Crittenden et al., 1980; Robinson et al., 1980; Tsichlis and Coffin, 1980). The only genetic region that was consistently associated with oncogenicity was *c*. Thus, attention focused on the role of the *c* region in ALV oncogenesis. Since this region does not appear to encode a protein, but contains U3, as well as additional information, the working hypothesis of many investigators (and, indeed, part of the initial reasoning behind the model for ALV oncogenesis presented below) was that the low

oncogenicity of RAV-0 reflected the relative "weakness" of the endogenous viral signals for transcription by RNA polymerase II ("weak promoter"). In support of this hypothesis was the observation that RAV-0 replicates poorly in most avian cells compared with exogenous viruses (Hanafusa et al., 1975). It is ironic, therefore, that two recent findings indicate that the situation is much more complicated. First, Tsichlis and Coffin (1980) isolated a recombinant (NTRE-7) that has c region sequences similar to RAV-60 (exogenous type), and replicates actively, but which induces neoplasia with decreased efficiency, increased latency, and of altered spectrum (see below) than exogenous viruses. Perhaps even more surprising, Weiss and Frisby (1981) have found that RAV-0 induces lymphomas with high frequency (about 50%) in Sonnerat Junglefowl. In this particular host, RAV-0 replicates to high titers. Clearly, other viral and host-related factors in addition to c are important in oncogenesis.

Spectrum of neoplastic disease induced by ALV. ALVs induce a wide variety of neoplastic and non-neoplastic diseases in chickens. The neoplasm most frequently observed in most studies is B-cell lymphoma, arising in the bursa of Fabricius, but other neoplasms, such as fibrosarcomas, nephroblastomas and erythroblastosis are also observed (Purchase et al., 1977; Purchase and Burmester, 1978).

The incidence and spectrum of diseases can vary widely, depending on such factors as virus titer, site of injection, age of the bird at the time of infection, and genetic composition of the virus and the host. In general, the incidence of neoplasia

is highest (often as high as 80-90%) in birds that are infected either in the embryo, or during the first few weeks after hatching. Burmester et al. (1959) reported both age-dependent and virus titer-dependent differences in the incidence of specific neoplasms. Using RPL-12 virus (a field isolate, containing a mixture of ALVs), they observed a high incidence of erythroleukemia in birds infected with large amounts of virus, or at early times (within one week of hatching). Lower amounts of virus, or later times of infection, favored development of lymphomas. Bacon et al. (1981) observed a high incidence (up to 25%) of erythroleukemia in line 15I chickens infected with RAV-1, a virus that induces primarily B-cell lymphomas in most other birds. These studies indicated that the incidence of erythroleukemia was strongly influenced by the B-haplotype of the chicken. The observed differences in incidence and spectrum of neoplasia disease undoubtably reflect, at least in part, a greater susceptibility to virus infection at early stages of development, when the immune system is not yet fully functional. However, other development-related phenomena, for example, possible differences in chromatin configuration of certain regions of the genome, or possible differences in permitted sites for viral integration, may play a role in differences in the pathologies observed (see Discussion).

Pathogenesis of B-cell lymphomas induced by ALV. Development of B-cell lymphomas in ALV-infected birds appears to be a multi-stage process (Cooper et al., 1968; Neiman et al., 1980a). The first morphologic change observed is the appearance of

"transformed follicles" in the bursa at about two months of age in birds infected shortly after hatching. As many as 10-100 follicles may appear in a single bursa (Neiman et al., 1980). Later, most of these follicles regress, possibly in response to normal physiologic signals that cause regression of the bursa at 4-6 months of age. One, or at most a few, of the transformed follicles, however, continue growing and give rise to transformed nodules, which are observed at about 3 months. Transformed nodules may become clearly defined bursal lymphomas, and give rise to widely disseminated metastatic disease. Metastases typically involve the liver, kidneys and spleen, but may involve other organs including the heart, lungs, pleura and peritoneum. Tumor cells contain surface IgM (Cooper et al., 1974), suggesting that the target cell for transformation is an immature B-cell. Transformation apparently results in a block of differentiation prior to heavy chain switching.

The bursa is clearly the source of target cells for B-cell lymphomagenesis; B-cell lymphomas fail to arise in bursectomized animals, although other neoplasms occur (Peterson et al., 1964, 1966; Cooper et al., 1968; Burmester, 1969; Purchase and Gilmore, 1975). Bursectomy as late as 12 weeks after infection prevents the development of metastatic disease (Cooper et al., 1968), and bursectomy as late as 5 months substantially reduces the incidence of metastases (Peterson et al., 1966). Thymectomy has no apparent influence on lymphoma development, suggesting that the thymus is not the source of either target cells for transformation, or cells vital for the host anti-tumor response.

Little in known about the early stages in development of

other ALV-induced neoplasms, such as nephroblastomas, fibrosarcomas, and erythroleukemias.

Other diseases induced by ALV. Osteopetrosis, a proliferative disorder of bone, is often found at later times in ALV-infected birds. It is unclear whether this hyper-proliferative state is non-neoplastic or pre-neoplastic at present. However, there is some evidence that birds with osteopetrosis may have a higher incidence of osteosarcoma (R. Smith, personal communication). Smith and Ivanyi (1980) have isolated a "helper" virus, MAV-2(0), from avian myeloblastosis virus stocks, which induces a high incidence of osteopetrosis, with very short latency (2-6 weeks). Recent studies (R.E. Smith, personal communication) indicate that the agent responsible for this disease is a minor component of the MAV-2(0) population. Preliminary oligonucleotide fingerprinting studies failed to discern any difference between high- and low-incidence osteopetrosis strains of MAV-2. Interestingly, the high incidence and rapid appearance of osteopetrosis induced by the MAV-2(0) agent only occurs when the virus is injected into early embryos.

The recombinant virus NTRE-7 (Tsichlis and Coffin, 1980), induces very few lymphomas, but causes osteopetrosis at late times (J. Coffin and H. Robinson, personal communication). Neither of the parental viruses of this recombinant (RAV-0 and a tdPr-B RSV isolate) are strongly oncogenic, and neither induces osteopetrosis at detectable levels.

Other non-neoplastic diseases in addition to osteopetrosis have been reported. These include anemia, cachexia, and a "non-

neoplastic syndrome" (NNS) that appears early (6-12 weeks) after infection (Crittenden et al., 1981). NNS has some characteristics of an autoimmune disease. It is characterized by a variety of lesions, including ascites, cardiomyopathy and decreased liver size, premature bursal atrophy, myositis and death. This disease is found only in birds that are not expressing endogenous proviral information. One hypothesis is that the disease is caused by a hyperimmune response directed against retroviral antigens. Birds expressing endogenous viral genes might display tolerance to exogenous retroviral antigens. Chickens that lack any endogenous proviral information (Astrin et al., 1979) are particularly susceptible to NNS. Earlier breeding studies (Astrin et al., 1979) established that endogenous proviral information was not essential for normal avian development. If the above hypothesis is correct, then endogenous viruses might have been conserved in evolution to help protect against NNS.

## E. A Model for Oncogenesis by ALV

Based on structural characteristics of the LTR, characteristics of the retrovirus life cycle, oncogenicity experiments that suggested that oncogenic potential resided primarily in c, Hayward (Hayward et al., 1981a) suggested a model for oncogenesis by ALV, and, by analogy, other slowly transforming viruses (Fig. 6). In the integrated ALV provirus, the viral structural and replicative genes are flanked by LTRs. All studies conducted thus far indicate that proviral integration is essentially random (Hughes et al., 1978; Steffen and Weinberg, 1978; Cohen et al.,

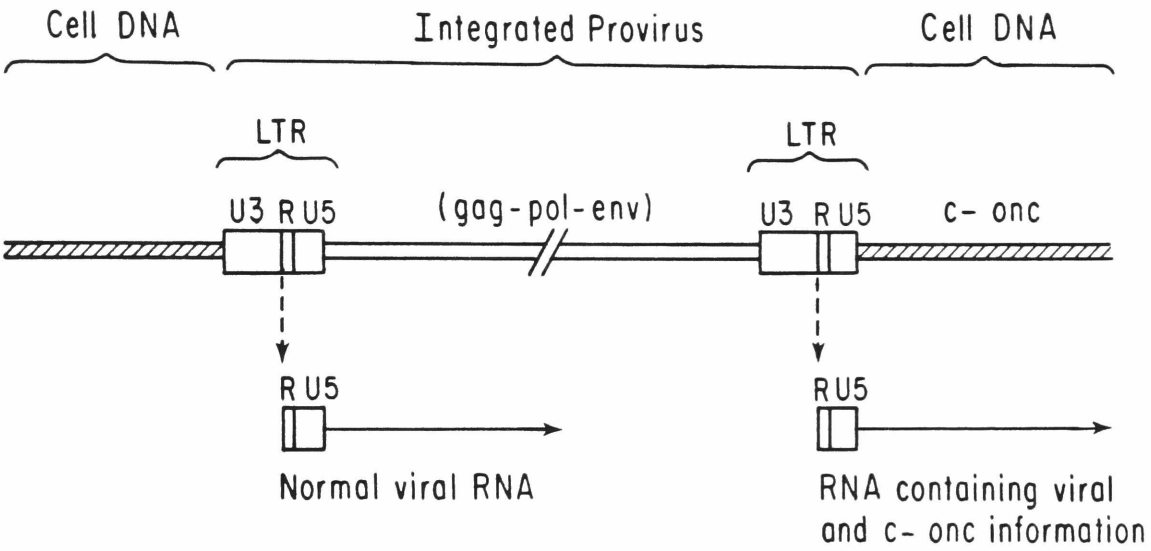



Figure 6. The promoter insertion model of oncogenesis. The integrated provirus is shown schematically, with LTRs enlarged (see also Figs. 3 and 4). $cDNA_5$, (5' probe) is complementary to the first 101 nucleotides of ALV RNA (Haseltine et al., 1977; Shine et al., 1977), and thus corresponds to R + U5 in the diagram. Synthesis of normal viral RNA (genomic and mRNA) initiates within the left LTR. Initiation within the right LTR could result in elevated transcription of adjacent cellular sequences. If, as a rare event, the provirus integrated adjacent to a potentially oncogenic cellular gene (c-onc), then transcriptional activation of this gene might lead to neoplastic transformation.

1979; Bacheler and Fan, 1979; Ringold et al., 1979; Quintrell et al., 1980; Shimotohno et al., 1980; Majors and Varmus, 1981). Normal viral genomic and mRNA synthesis utilizes the left LTR. But since the left and right LTRs are identical, it might be expected that initiation could also occur within the right LTR. Transcription initiating in the right LTR, and reading into adjacent cellular sequences, could result in enhanced expression of these cellular sequences. Transcripts apparently initiated in the right LTR had been found earlier in some RSV-transformed mammalian cell clones (Quintrell et al., 1980), and a heterogeneous population of such transcripts was found in randomly infected cell populations (W.S. Hayward, personal communication). If an ALV provirus is capable of activating an adjacent cellular gene, then integration adjacent to a cellular gene with oncogenic potential (i.e., a c-onc gene) might induce elevated expression of this gene, and result in neoplasia. Such a mechanism would be analogous in some ways to that involved in oncogenesis by rapidly transforming viruses. The essential feature in both cases is that a normal cellular gene is placed under viral transcriptional control. With the slowly transforming viruses, however, linkage involves insertion of the viral promoter (LTR) into cellular DNA; hence this model is called the "promoter insertion" model of oncogenesis (Hayward et al., 1981a,b; Neel et al., 1981)

## F. The Scope of This Thesis

The studies presented in this thesis fall into four major areas:

(i) The promoter insertion model of oncogenesis--essential elements:

I analyzed the virus-related poly(A) RNA from a large number of ALV-induced tumors. Together with analysis of the virus-related DNA (performed by Susan Astrin, The Institute for Cancer Research, Fox Chase Cancer Center) and hybridization kinetic studies of the genetic content of virus-related RNAs in these tumors (performed by William S. Hayward, Rockefeller University), these results show that in about 85% of lymphomas induced by ALV, an ALV provirus is integrated adjacent to c-myc, the cellular counterpart of the transforming gene (v-myc) of MC29 virus. Integration results in new transcripts initiating from the ALV LTR, containing viral 5' and c-myc information. These RNAs are expressed at high levels in tumors. These studies verified the essential aspects of the promoter insertion model.

(ii) Oncogene activation--molecular details:

I molecularly cloned DNA representing the provirus-host cell junction in an ALV-induced lymphoma. Charles Rogler (Fox Chase) isolated a similar clone from another, independent ALV-induced lymphoma. I compared the structures of these two clones to each other as well as to a clone of the normal c-myc gene, which I isolated from a chicken genomic library, and a clone containing the v-myc gene, which I obtained from Takis Papas (National Institutes of Health), by restriction mapping. This comparison, together with S-1 deletion mapping analysis, demonstrated that in both tumors, normal integration events had occurred adjacent to c-myc, without structural alteration of the bulk of c-myc coding

sequences. The LTR in both tumors was oriented such that transcription could initiate within the LTR and proceed downstream into c-myc. c-myc contains at least two exons and one intron, and probably at least four exons and three introns. In vitro transcription studies show that the major initiation site for RNA polymerase II in vitro in the tumor-derived clones was the viral initiation site in the LTR. In c-myc, however, there are at least two putative initiation sites in vitro.

(iii) Isolation of a new recombinant sarcoma virus from an ALV-induced lymphoma:

One tumor induced by the slowly transforming virus td107A was a fibrosarcoma. A replication-defective rapidly transforming virus was isolated from this tumor and characterized. Hybridization analysis indicated that the genome of this virus contained the fps gene. Oligonucleotide fingerprinting experiments, conducted with Lu-Hai Wang (Rockefeller University), indicate that this new virus is a recombinant between the parental td107A virus and the cellular fps gene.

(iv) Isolation of molecular clones of human c-onc genes:

Based on the implications of the promoter insertion model for ALV oncogenesis, namely that activation of a normal cellular gene can result in neoplasia, it seemed reasonable to see if activation of c-onc genes played a role in human cancers. As a first step, I have isolated parts of human myc-related and src-related DNAs and characterized them by restriction mapping. Several types of experiments with these clones are now possible and are in progress.

## Materials and Methods

Animal cells and viruses. Chicken embryo fibroblasts (CEFs) were obtained from group-specific antigen negative ($gs^-$) eggs supplied by SPAFAS (Norwich, Conn.). Primary and secondary cultures were prepared and maintained as described (Hanafusa, 1969). Cell culture conditions and virus titration methods were those of Hanafusa (1969). All fibroblast cultures used were negative for both chicken helper factor (chf) and gs antigen as determined by complement fixation (Hanafusa et al., 1972). RP-9, a continuous lymphoid cell line derived from a RAV-2-induced bursal lymphoma, was supplied by W. Okazaki (Regional Poultry Laboratory, East Lansing, Michigan). It was maintained in suspension culture in tissue culture flasks (Falcon) or spinner bottles. The media was RPMI 1640 (GIBCO) supplemented with 5% fetal bovine serum, 5% chicken serum, 10% tryptose phosphate broth, and 10 ug/ml Fungizone. Cells were passaged every 2 days, when cell densities had reached 1-3 million cells per milliliter.

The viruses used in this study included Rous-associated viruses-1 and 2 (RAV-1 and RAV-2), transformation-defective mutants of the Schmidt-Ruppin strain of Rous sarcoma virus (td107A, td103, and td109) (Kawai et al., 1977), Fujinami sarcoma virus (FSV), both the wild type strain and NY225, a temperature-sensitive mutant (Hanafusa et al., 1981), UR1, and 16L virus, which was obtained in the course of these studies. All of these viruses except 16L were obtained from the laboratory stocks of either H. Hanafusa or W.S. Hayward. Virus infection of fibroblast cultures was usually at multiplicities of one to five.

16L virus was isolated from liver tumors present in chicken 16, which was infected in embryo with td107A. The tumor, which had been frozen, was homogenized by grinding in a sterile mortar containing extraction solution (Tris-saline containing 5% fetal calf serum) to give 20% (w/v) homogenates. The resulting suspension was centrifuged, and the supernatant was used to infect secondary CEFs treated with DEAE-dextran. These cultures were overlaid with agar and examined at one week for evidence of transformation. Initial virus titers were $10^3$ to $10^4$ focus-forming units (FFU)/ml. Confluently transformed cultures were obtained following several cycles of transfer and agar overlay. Virus produced from such cultures had a titer of about $10^7$ FFU/ml. The same procedure was used to recover virus from several ALV-induced lymphomas. The viruses recovered from lymphomas in this fashion were not capable of producing foci on CEF (see Results).

Virus for RNA extraction or complementary DNA preparation was harvested and purified as described previously (Hayward and Hanafusa, 1973), except that pronase and hyaluronidase treatment were omitted.

Production of Lymphoid Leukosis. Hatched chickens were from $gs^-$ $chf^-$ eggs supplied by SPAFAS or Life Sciences (St. Petersberg, Fla.). The latter are provided through the Resource Program, National Cancer Institute. ALV ($10^6$-$10^7$ infectious units) was injected intravenously or intramuscularly between 2-7 days after hatching. Some birds were infected with ALV in embryo and were generously supplied by Teruko Hanafusa and Lu-Hai Wang

(Rockefeller University). Tumors first appeared at approximately 16 weeks post-infection. Control birds received no injections, and were maintained in isolation from infected birds. Birds were sacrificed using ether, and tissues were excised for nucleic acid extraction and histopathology. Paraffin-embedded sections were kindly performed and evaluated by James Walberg, Rockefeller University.

RNA extraction. Extraction of total nucleic acid from fibroblasts was performed as described previously (Hayward, 1977), except that incubation with proteinase K was performed for 1 hour at 37°C. $^{32}$P-labeled chicken 27S and 18S rRNAs used as size markers in gel analyses were prepared from confluent fibroblast monolayers grown in tissue culture flasks (Corning). The cells were exposed to low phosphate media for 6 hr and then 2 mCi of carrier- free $^{32}$P-orthophosphate (New England Nuclear) were added in 15 ml low phosphate media. Labeling was for 12 hr, followed by 3 changes of media at 3 hour intervals (Wang et al., 1975). RNA extraction was as above. Sucrose density gradient (15-30%) fractionation was then performed as described previously (Hayward, 1977). Ribosomal RNA peaks were located by Cerenkov counting, and the RNA was precipitated with ethanol.

Total nucleic acid was extracted from homogenized tumors essentially as described for fibroblasts.

$^{32}$P-labeled 35S RAV-2 RNA was extracted from virions purified from fibroblast cultures labeled as described above by the method of Hayward (1977). $^{32}$P-labeled 16L viral RNA was isolated by a variation of this procedure designed to minimize RNA degra-

dation. CEF cultures confluently transformed with 16L virus were labeled with 1 mCi/ml of carrier-free $^{32}$P-orthophosphate for 12 hr as described previously (Wang et al., 1975). The 12 hour supernatant was discarded and culture fluids were then collected at two hour intervals for 10 hr. Cellular debris were removed immediately as described previously (Hayward, 1977). This RNA was immediately heat-denatured and poly(A)-selected by oligo(dT)-cellulose chromatography. Poly(A) RNA was size-fractionated by sucrose density gradient centrifugation. Fractions from the 35S ("helper") and 30S ("16L") regions of the gradient were pooled separately and precipitated with ethanol.

Poly(A) selection of cellular RNA. Poly(A)-containing RNA was isolated from total cellular nucleic acid as described previously by one cycle of chromatography on poly(U)-Sepharose (Pharmacia) (Hayward, 1977), or two cycles of chromatography on oligo(dT)-cellulose T-2 (Collaborative Research) (Wang et al., 1975). In the course of these experiments, we found that the latter technique resulted in lower amounts of contamination of poly(A) RNA with ribosomal RNA, and thus better transfer in the 27S and 18S regions in subsequent filter blotting.

Glyoxal gel electrophoresis and blot hybridizations. Poly(A)-containing RNA (5-15 ug) was precipitated with ethanol in siliconized microfuge tubes (Eppendorf). The precipitate was washed with 0.5 ml of absolute ethanol and dried briefly in a lyophilizer. The pellet was resuspended in 5-8 ul of glyoxal buffer and denatured at 50$^{o}$ as described (McMaster and Carmichael, 1977). After addition of 5 X marker dyes (1 X dyes =

0.05% bromophenol blue, 0.05% xylene cyanol, 20% glycerol), the samples were separated by electrophoresis in a 1% agarose (Seakem, LE grade) gel. The running buffer was 10 mM sodium phosphate, pH 6.8. $^{32}$P-labeled 35S RAV-2 viral RNA and rRNA markers were included in each gel.

Following electrophoresis, RNA was transferred to aminophenylthioether (APT) paper (Brian Seed, personal communication) by a modification of the procedure of Alwine et al. (1977) or to nitrocellulose paper by the technique of Thomas (1980). For transfer to APT paper, base treatment of the gel was as described, but neutralization was carried out in 200 mM sodium acetate, pH 4, instead of phosphate buffer. APT paper was activated like ABM paper with $NaNO_2$ and HCl, but the final buffer washes were also with sodium acetate instead of phosphate buffer. Blotting was with 800-1000 ml sodium acetate buffer, pH 4. Completeness of transfer was monitored by Geiger counting of the $^{32}$P-marker RNA lane.

After transfer, the filters were washed with prehybrid mix (50% formamide, 5 X SSC, 5 X Denhardts, 1% glycine, 250 ug/ml carrier yeast RNA or denatured salmon sperm DNA, 50 mM sodium phosphate, pH 6.5, 0.1% SDS) for 4-8 hr at 37°. Hybridizations were carried out in 50% formamide, 5 X SSC, 1 X Denhardts, 100 ug/ml carrier nucleic acid, 20 mM sodium phosphate, pH 6.5, 0.1% SDS. [Denhardt's solution (1X) is 0.02% bovine serum albumin, 0.02% polyvinylpyrolidone, 0.02% Ficoll; 5 X SSC is 0.75M NaCl, 0.075M sodium citrate (Denhardt, 1966).] 25-50 ul of hybrid mix were used per cm$^2$ of filter; 1-4 x10$^4$ cpm of $^{32}$P-labeled probe were used per cm$^2$. Hybridizations were for 3 days at 37° in

sealed plastic bags.

Following hybridization, filters were washed three times at room temperature with 250 ml 2 X SSC/0.1% SDS for five minutes each, followed by two 15 minute washes at 37° with 250 ml prewarmed 0.1 X SSC/0.1% SDS, and finally two washes at 37° with 100 ml prewarmed 50% formamide/5 X SSC, for 30 minutes each. Filters were blotted dry with Whatman 3MM paper and exposed to Kodak XR-5 or XAR-5 film under a Dupont Lightning Plus intensifying screen at -70°.

Preparation of complementary DNA (cDNA) probes. $cDNA_5$, was synthesized from purified RAV-2 virions by a modification of the procedure of Haseltine et al. (1977), as described previously (Hayward et al., 1980). The reaction product was hydrolyzed for 8-12 hr at 37° in 0.2 N NaOH to remove the RNA primer, and then purified by electrophoresis for 7 hr at 200V in a 7.5% acrylamide/urea gel as described (Hayward et al., 1980). The 101 nucleotide "strong stop" DNA band ($cDNA_{5'}$) was identified by brief autoradiography, excised, and electroeluted for 3-4 hr at 100 mA in a dialysis bag. The eluate was extracted once with phenol/chloroform/isoamyl alcohol (1:1:0.01) and once with chloroform/isoamyl alcohol (1:0.01), and concentrated by ethanol precipitation. The resultant probe is homologous to the 5'-terminal 101 nucleotides of ALV RNA.

A probe representative of the total ALV genome ($cDNA_{rep}$) was prepared by W.S. Hayward using poly(A)-containing RNA purified from RAV-2 virions as the template in an exogenous reverse transcriptase reaction. Random DNA oligonucleotides generated by

digestion with DNase I served as primers. The conditions for cDNA synthesis and primer preparation were those of Taylor et al. (1976) except that the calf thymus DNA was treated with 0.2 N NaOH for 30 minutes at 100° before use. This eliminates contaminating cellular RNA present in commercially obtained calf thymus DNA. The product of the polymerase reaction was precipitated once with ethanol. The precipitate was resuspended in 1 ml of 0.2 N NaOH and the RNA template hydrolyzed as described above. Unpolymerized nucleotides were removed by passage over a Sephadex G-75 column equilibrated in 0.2 NETS (0.2 M NaCl, 10 mM Tris, pH 7.4, 10 mM EDTA, 0.2% SDS). Following ethanol precipitation, the cDNA was hybridized to a vast excess of non-poly(A)-containing RNA from $gs^{-}chf^{-}$ cells, and contaminating homologous sequences removed by chromatography on hydroxylapatite, as described previously (Hayward, 1977). Following removal of phosphate by chromatography over G-75 Sephadex, the cDNA product was concentrated by ethanol precipitation.

cDNA probes specific for the unique (and putative transformation-specific) regions of RSV (src), FSV (fps), avian erythroblastosis virus (erb), avian myeloblastosis virus (myb), and avian myelocytomatosis virus (myc) were prepared by W.S. Hayward. The protocol for their preparation consisted of preparing cDNA for each of these viruses using random DNA oligonucleotides as primers as above, followed by removal of helper virus-specific sequences by hybridization and selection on hydroxylapatite (Hayward et al., 1981b).

Analysis of tumor DNA. Isolation of cellular DNA from

tumors was performed by S.M. Astrin (Fox Chase). Conditions for restriction endonuclease digestion of genomic DNA, gel electrophoresis, and blot hybridization (Southern, 1975) as modified by Ketner and Kelly (1976) have been described (Neel et al., 1981; Hayward et al., 1981a,b).

Bacterial cells and viruses. Escherichia coli strain ED 8654 (Murray et al., 1977) was used for isolation and propagation of recombinant bacteriophage. A lambda library of Rhode Island Red chicken genomic DNA (Dodgson et al., 1979) was obtained from Richard Axel, Columbia University. A lambda library of human DNA from a patient with thalessemia (Ramirez et al., 1979) was obtained from Arthur Bank, Columbia University. The bacteriophage vector λgtWES-λB (Leder et al., 1977) was used to construct recombinant bacteriophage. This vector was digested with Eco R1 and Sac I, and the Eco R1 arms purified from the dispensable λB fragments by sucrose density gradient centrifugation.

Restriction endonuclease digestions. Restriction enzymes were obtained from New England Biolabs or Bethesda Research Labs, except Cla I, which was obtained from Boehringer-Manheim. Conditions for digestion were as described by the manufacturers except that excess enzyme (2-5 U/ug) was usually used.

Molecular cloning of tumor junction fragments. Tumor DNA was digested to completion with Eco R1. Approximately 600 ug of digested DNA from a splenic nodule in chicken 7 was fractionated by discontinuous electrophoresis in a "gene machine" (Edgell and Polsky, 1980). The gel was 0.5% agarose (Seakem LE, Marine Col-

loids) in E buffer (40 mM Tris, 5 mM Na acetate, 1 mM EDTA, pH 7.8). Electrophoresis was at 40V for 15 min., followed by a collection and filling cycle of 5 minutes. One fraction was collected every 20 min. and the procedure was carried out for 40 hours. Three fractions in the expected molecular weight range ( 3 kb) were precipitated and used separately in ligation reactions. C. Rogler (Fox Chase) used electrophoresis in low-melting agarose (Sea Plaque, Marine Colloids) to size-fractionate tumor DNA from chicken 10. Techniques for isolating DNA from low melting agarose have been described (Takeya et al., 1981). DNA from the appropriate size range was pooled and used for ligation. The tumor 7 insert DNA was ligated to the Eco R1 arms of λgtWES-λB (1 ug). The molar ratio of arms to insert was 1:0.2. Details of procedures for in vitro packaging (Becker and Gold, 1975; Hohn and Murray, 1977), screening (Benton and Davis, 1977), phage purification, amplification and preparation of phage DNA have been previously described (Ju et al., 1980). Similar molecular cloning procedures, including ligation ratios, were used by Rogler to molecularly clone the tumor 10 junction fragment. The efficiencies of packaging of recombinant bacteriophage ranged from $10^5$ to $10^6$ infectious particles/ug phage DNA. Recombinant bacteriophage (200,000-400,000) were plated on 150 mm Petri dishes (20,000/plate) and screened. Before hybridization, filters containing phage DNA imprints were prewashed at $37^o$ in 5 X SSC (1 X SSC is 0.15 M NaCl, 0.015 M Na citrate), 0.02 M Tris-HCl (pH 7.4), and 0.5% SDS for at least 45 min. Hybridization was to $cDNA_{5'}$, and was carried out at $37^o$ in 50% formamide, 5 X SSC, 20 mM Tris-HCl (pH 7.4), 0.5% SDS, and at least 200 ug/ml yeast RNA

for 18-24 hr. Ten ml of hybrid mix containing 2 x $10^6$ Cerenkov cpm of $cDNA_5$, probe were used for each filter. Following hybridization, filters were washed 3 times briefly in 0.2 X SSC/0.2% SDS and then incubated once or twice for 45 min each at 37° in the same solution. Washed filters were dried and exposed to Kodak XR-5 or XAR-5 film for 4-24 hr with an intensifying screen. Positive plaques were purified three times before amplification.

Subcloning of the Sal I fragments of the λtjf-7 3.1 kb Eco Rl insert into the plasmid pBR322 involved standard techniques. Subcloning of the 3.6 kb Eco Rl insert of λtjf-10 was performed by C. Rogler.

Isolation of c-myc clones. Approximately 4 x $10^5$ recombinant bacteriophage from the chicken genomic library (Dodgson et al., 1979) were plated on 150 mm Petri dishes. Screening with the appropriate probe (see Results), and phage isolation and purification were performed as described above. Subcloning of the Bam H-1 fragments of λc-myc 2 into pBR322 was performed by Gregory Gasic, Rockefeller University.

Isolation of molecular clones of human DNA homologous to onc genes. A human genomic library from a β-thalessemic patient (Ramirez et al., 1979) was generously provided by A. Bank, Columbia University. Approximately 1-2 X $10^6$ recombinant bacteriophage were plated on NUNC Bioassay plates (Vanguard) (200,000/plate). Following Benton-Davis blotting as described above, filters carrying phage DNA imprints were hybridized to probes containing either the entire v-myc or v-src gene. Isolation and purification of phage homologous to these probes was

performed as described above. The myc-related human DNA sequences were subcloned into pBR322 by digestion of the plasmid and the bacteriophage insert containing myc sequences with Eco R1 and Pst I, followed by ligation and selection by standard techniques.

For detection of human repeat DNA sequences, an Eco R1 digest of normal human genomic DNA was nick translated to high specific activity and used as a probe. Warren Jelinek (Rockefeller University) supplied a probe for the human Alu repeat family.

Preparation of $^{32}$P-labeled nick-translated probes. High specific activity nick-translated probes were prepared by the method of Maniatis et al. (1975) using aqueous $\alpha^{32}$P-dCTP (>2000 Ci/mmol., Amersham). Specific activities of 1-2 x $10^8$ Cerenkov cpm/ug were routinely obtained. Low specific activity radiolabeled restriction fragments were prepared by nick translation for use in restriction mapping. The procedure was the same as that for preparing high specific activity probes except that the reaction buffer contained all four dNTPs at 20 uM, and one-half the amount of $\alpha^{32}$P-dCTP was used per reaction. Specific activities obtained ranged from 5 x $10^5$ to 5 x$10^6$ Cerenkov cpm/ug.

Analysis of cloned DNAs by electrophoresis and blot hybridization. DNA samples were electrophoresed in submerged horizontal agarose (Seakem) gels (0.8%-2%, depending on the size range of fragments to be analyzed) in E buffer. $^{32}$P-labeled λ-Hind III fragments or ØX 174-Hae III fragments were used as molecular weight markers. DNA was blotted onto nitrocellulose filters by

the technique of Southern (1975). Prewashing and hybridization conditions were as described above for Benton-Davis filters.

Restriction mapping. Appropriate inserts from molecular clones were purified by preparative electrophoresis in low melting agarose. These fragments were nick-translated to low specific activity . Mapping was performed by standard procedures involving digestions with one or a combination of restriction enzymes, followed by electrophoresis on agarose gels. The gels were dried onto DEAE paper and autoradiographed. Size values reported in the text are accurate to within 50-100 nucleotides.

In vitro transcription. In vitro transcription reactions were carried out by the procedure of Manley et al. (1980) as modified by Handa et al. (1981). HeLa cell extracts were generously provided by Susan Haynes, Rockefeller University. DNA samples were digested to completion with the appropriate restriction enzyme(s), extracted twice with Tris-saturated phenol, three times with water-saturated ether and precipitated twice with ethanol. Each reaction contained 10 ul HeLa cell extract, 20-50 uCi of $\alpha^{32}$P-UTP (>2,000 Ci/mmol, New England Nuclear), and 15 ul of a solution of 15 mM Tris-HCl (pH 7.9), 7 mM $MgCl_2$, 25 mM ammonium sulfate, 0.5 mM EDTA, 1.3 mM dithiothreitol, 20 mM creatine phosphate, 50 uM each of ATP, GTP and CTP, 5 uM UTP, and DNA. The amount of DNA was optimized for each DNA tested (range of concentrations for optimization: 0.3 ug-1.5 ug). α-amanitin was added to some reactions to a final concentration of 1 ug/ml. Reactions were incubated at 30° for 1 hr. Following incubation, the reaction mixes were extracted twice with

phenol:chloroform:isoamyl alcohol and once with chloroform:isoamyl alcohol. Unpolymerized nucleotides were removed by three cycles of ethanol precipitation. The final ethanol precipitates were glyoxalated as described above, and electrophoresed on either 1.2% or 1.5% agarose gels. Markers were a mixture of glyoxalated λHind III fragments and ØX174-Hae III fragments that had been 5'-end-labeled by kination.

5'-end selection of 16L viral RNA. $cDNA_5$, was prepared in large amounts as described by Wang et al. (1982). Deoxycytidylic acid (dC) residues were added to the 3' end of this cDNA with terminal transferase by L.-H. Wang, Rockefeller University. Following hybridization of $^{32}P$-labeled viral RNA to the dC-tailed cDNA, RNA molecules containing viral 5' information were selected by chromatography over oligo(dG) cellulose as described (G. Gasic and W. S. Hayward, personal communication; Wang et al., 1982).

RNase $T_1$ fingerprinting. $^{32}P$-labeled and selected "35S" and "30S" RNAs were analyzed by RNase $T_1$ fingerprinting and oligonucleotide mapping as described previously (Wang et al., 1975). The nucleotide composition of selected oligonucleotides was determined by published methods (Beemon et al., 1974; Wang et al., 1975).

Analysis of virus-related proteins in 16L virus-infected cells. Cell cultures confluently transformed by 16L virus were labeled with $^{35}S$-methionine and cell lysates prepared by Bernard Mathey-Prevot, Rockefeller University, using techniques described previously (Hanafusa et al., 1981). Virus-related proteins were

analyzed by immunoprecipitation of the cell extract with anti-gag antiserum, followed by electrophoresis on SDS-polyacrylamide gels (Laemmli, 1970), as modified by Hanafusa et al. (1981). Preparation and properties of the antisera have been described by Feldman et al. (1980). Antiserum specific for the fps gene product was prepared as described (Mathey-Prevot et al., 1982). In vitro protein kinase assay was performed on immunoprecipitates as previously described (Feldman et al., 1980).

## Results

### A. Oncogenesis by ALV; The Essentials of the Promoter Insertion Model

Tumor incidence and characteristics. Tumors were produced following injection of chicken embryos or 2-7 day-old chickens with ALV. Four different strains of virus (RAV-1, RAV-2, td103, and td107A) and two different lines of chickens (SPAFAS and Life Sciences) were used. There were no discernible strain- or line-specific differences in pathogenesis.

Tumors were first detected at four months. By six months, when all remaining birds were sacrificed, approximately 40% had developed tumors. This frequency is consistent with previously published results (Purchase et al., 1977; Purchase and Burmester, 1978). All tumors were lymphoblastic lymphomas except for one fibrosarcoma in bird 16 (see section C of Results). There were no cases of osteopetrosis or nephroblastoma. The most commonly involved lymphomatous tissues were bursa, liver, kidney, and spleen; occasional lesions were found in the gonads or the heart. There was no evidence of neural involvement, nor was there any histological evidence of Marek's disease. Several birds (10-15%) died from undetermined but nonneoplastic causes. I also analyzed a continuous lymphoid cell line, RP-9, derived from a RAV-2 induced bursal lymphoma (W. Okazaki, personal communication).

Analysis of viral DNA in lymphoid tumors. Integration in mass-infected cell populations is essentially random. However, if the promoter insertion model is correct, then integration in

tumors should be clonal and specific, because oncogenesis would only be the consequence of a rare event--proviral integration adjacent to a specific cellular gene or set of genes (Fig. 6). To examine the integration sites in ALV-induced lymphomas, Susan Astrin (Fox Chase) digested tumor DNA with the restriction endonuclease Eco R1. Virus-specific restriction fragments were detected by blotting (Southern, 1975) and hybridization either with a probe complementary to the first 101 nucleotides of ALV RNA ($cDNA_5$, or 5' probe), or with a probe representative of the total ALV genome ($cDNA_{rep}$ or rep probe). Cleavage of an integrated, nondefective ALV genome with Eco R1 should yield five fragments containing viral information (Hsu et al., 1978; Shank et al., 1978; Boone and Skalka, 1981). Two of these, one near the left end of the provirus, and one at the right end, contain 5' information. The 5'-containing fragment from the left end is an internal fragment of the provirus, and thus would be detected in an analysis of a mass-infected population of cells as well as in a clonal population. This 2.4 kb fragment hybridizes to both 5' and rep probes, and should appear in any tissue containing a nondefective, integrated exogenous provirus. Conversely, the absence of this band indicates that a tissue does not contain a complete exogenous provirus. The 5'-containing fragment from the right end contains only about 160 nucleotides of viral information (R+U5+60 nucleotides of U3) and extends into adjacent cellular sequences. This fragment thus contains the junction between proviral and host sequences and is termed a "junction fragment". A junction fragment can only be detected if a provirus is integrated at the same site in the majority of cells in a

population. Since this fragment contains only about 2% of the total viral information, it is not detected by rep probe. Thus, junction fragments can be distinguished from other proviral restriction fragments by comparing the hybridization pattern with 5' and rep probes. The size of the junction fragment serves as a basis for comparing the integration sites in different tumors. Tumors with the same proviral integration site(s) will yield identical junction fragments.

In her initial studies, Astrin found that all ALV-induced lymphomas examined (24 birds) had discrete junction fragments. Since junction fragments were detected, all or most of the cells in each tumor must be derived from a single infected cell. All tumors from a single bird contained the same tumor-specific junction fragments; thus metastatic tumors are clonally derived. This confirmed the work of Neiman et al. (1980b) who had reported that ALV-induced lymphomas were clonal.

However, Astrin also made two additional observations. First, the DNA of many of the tumors did not contain the 2.4 kb internal fragment of the integrated ALV provirus. Thus, many tumors did not contain complete proviruses. More importantly, she found that the tumor junction fragments from different tumors could be grouped into a limited number of size classes. Further restriction analyses of tumors from one of these classes (six independent tumors) using Eco R1 in combination with other restriction enzymes showed that the adjacent cellular sequences from each of these tumors possessed identical restriction maps. Since there is no known specificity in the integration process, these results suggested that oncogenesis results only after integration

at one of a limited number of sites in the cell genome.

Analysis of viral RNA in lymphoid tumors. The promoter insertion model predicts that tumors should contain new RNAs initiated from the viral promoter (located within the LTR) and proceeding downstream into cellular sequences. The first 101 nucleotides of an RNA initiated from either the left or the right LTR are encoded within the LTR (R+U5; see Fig. 6). By using 5' probe, I could identify any RNA initiated within an LTR. Transcription of normal viral mRNA initiates within the left LTR and proceeds into the viral structural genes. Because these transcripts contain this additional viral information, they can be detected with rep probe as well as 5' probe. Transcripts initiated from the right LTR would continue into cellular sequences; the only viral information present on these RNAs would be complementary to 5' probe. Since this viral information represents a small fraction of the viral genome, these new RNAs would not be detected with rep probe. Thus I could identify RNAs that contained both viral and cellular information as those that were detected by hybridization to 5' probe but not to rep probe.

I analyzed the poly(A) containing RNA from many tumors by glyoxal gel electrophoresis, blotting (Alwine et al., 1977) and hybridization to 5' probe. Nonneoplastic cells infected with ALV produce two poly(A)-containing RNAs, a genome length 35S (8.1 kb) species and a subgenomic 21S (3.2 kb) species (Hayward, 1977; Weiss et al., 1977). Some of the tumors (Fig. 7) have these normal viral mRNAs (for example, 11, 19, 33, 34, RP-9). However, many of the tumors lack any detectable 35S or 21S RNA (for exam-

ple, 7, 9, 10, 35, 36). These results are consistent with the tumor DNA analysis in which many tumors were found to lack a complete provirus. Together, the DNA and RNA data show that expression of viral structural and replicative genes is not required for maintenance of neoplastic transformation.

In addition to the normal viral mRNAs found in some tumors, nearly all (19/21) of the lymphomas I examined, and the lymphoid cell line RP-9, had a discrete new RNA species homologous to 5' probe (examples shown in Fig. 7A). The failure to detect a new RNA species in the other two lymphomas may have been due to technical problems such as RNA degradation, or due to oncogenesis by a variation of the promoter insertion mechanism (see Discussion). Such new RNAs were not found in uninfected tissue or in infected but nonneoplastic tissue (Fig. 7B).

Most of the tumors contained a new 5'-containing 2.5 kb RNA (Fig. 7A, arrow). A 2.9 kb species was found in two of the tumors (17B, 23L) as well as in RP-9. There may be slight size variations within each RNA size class. The 2.5 kb or 2.9 kb RNA species were not detected with rep probe (Fig. 8), and therefore must contain viral 5' information covalently linked to cellular information. This conclusion was confirmed by hybridizations with retroviral gene-specific probes (data not shown).

In addition, several tumors contained additional 5'-containing RNAs. A 5.5 kb RNA appeared to be present in several tumors. This RNA species hybridized to rep probe as well as to 5' probe; thus this species contains viral coding sequences. It is not clear whether the 5.5 kb RNAs in different tumors have similar structure and genetic content, or whether these RNAs play

Figure 7. New virus-related RNAs in lymphomas. Poly(A) RNA (5-15 ug) from normal and tumor tissues was glyoxalated, (McMaster and Carmichael, 1977), size-fractionated on 1% agarose gels, and transferred to APT paper (Brian Seed, personal communication). Hybridizations were performed with $^{32}$P-labeled, 5' probe (see text) (1-4 x $10^4$ Cerenkov cpm/$cm^2$). The positions of normal viral 35S and 21S mRNAs are indicated by open arrows. New tumor-specific RNAs (2.5 kb, 2.9 kb, 5.5 kb) that are common to more than one tumor are indicated by solid arrows. (A) Lymphoma samples from 17 different birds (B=bursa, L=liver, K=kidney). (B) Control tissues from uninfected birds (12B, 12L, 13B), and from non-neoplastic muscle of infected birds (10M, 11M). Note that tissue 11M is infected (contains 35S and and 21S mRNA), but nonneoplastic. In all cases, $^{32}$P-labeled viral 35S RNA and 27S and 18S ribosomal RNAs were electrophoresed in parallel lanes as size markers (not shown).

A
7L
7K
10B
10L
11B
11L
19B
33L
34L
35L
36B
36L
17B
23L
9B
9K
RP9
5.5kb
2.9kb
2.5kb
B
12B
12L
13B
10M
11M

a role in oncogenesis. One possibility is that they merely represent defective viral genomes.

In nearly all cases where more than one tumor from the same animal was analyzed (birds 7, 10, 11, 36), the same tumor-specific RNA was found in each. Together with the DNA data, this is consistent with a clonal origin for metastases. In one bird (#9), the DNA and RNA patterns were different in bursal tumors and kidney metastases. Whether this is the result of two independent primary tumors is unclear.

William Hayward (Rockefeller University) examined the RNA from several tumors by RNA-excess liquid hybridization ($C_rt$ analysis) to determine the relative abundance of virus-related RNA. Four of the tumors selected contained low or undetectable amounts of viral mRNAs in the blot hybridization analysis. For these tumors, determining the amount of 5'-containing RNA is essentially equivalent to determining the amount of the new, tumor-specific RNA. In each of these tumors, the 5'-containing RNA was present at levels of at least 100 copies per cell, and there was at least a 50-fold higher level of RNA containing 5' information than RNAs hybridizing to rep probe.

Recovery of virus from ALV-induced tumors. Consistent with the experiences of previous workers (T. Hanafusa and H. Hanafusa, personal communication), I have been unable to recover a virus from ALV-induced lymphomas that transforms fibroblasts. I did recover helper virus (presumably the original infecting strain) from two tumors that express 35S and 21S RNAs (19B and 34L), as well as RP-9 (data not shown). These viruses do not, however,

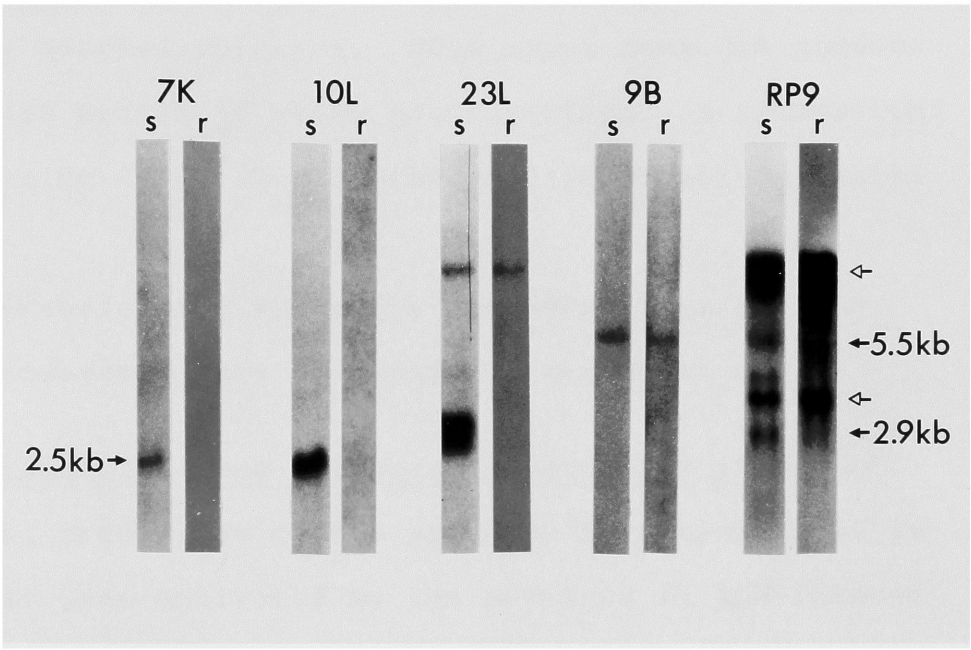



Figure 8. New virus-related lymphoma RNAs contain non-viral information. Poly(A)-containing RNAs from lymphomas were analyzed as in Fig. 7. Hybridizations were performed on the same blots with either 5' probe (labeled "s" for strong stop DNA, see text) or cDNA$_{rep}$ (r), a probe representative of the total RAV-2 genome. The positions of 35S and 21S viral mRNAs (open arrows) and the 2.5 kb, 2.9 kb and 5.5 kb 5'-containing RNAs (solid arrows) are as indicated. The presence of non-viral information in the new RNAs was inferred by comparing the pattern of hybridization with the two probes (see text).

transform fibroblasts in tissue culture (see Fig. 18, and Sect. C). It is possible that if a rapidly transforming recombinant virus did exist, it might only transform lymphoid cells. However, I injected virus isolated from the supernatants of RP-9 cells into newly hatched chickens. This virus does not produce neoplastic disease before 16 weeks postinjection. A recombinant rapidly transforming virus should produce disease with a faster course.

A rapidly transforming virus was recovered from the one ALV-induced fibrosarcoma that we obtained (see Sect. C).

Identification of a gene activated by promoter insertion. The most critical prediction of the promoter insertion model is that the cellular gene activated by the provirus in ALV-induced lymphomas is capable of transforming cells when fixed into a state of elevated expression. It seemed possible that the normal cellular gene(s) activated might be one of the cellular counterparts of a v-onc gene. William Hayward prepared cDNA probes for five v-onc genes of avian viruses, and used these probes to determine the level of expression of their cellular counterparts in ALV-induced lymphomas by $C_rt$ analysis. With one exception, the expression of four of the five c-onc genes tested (c-src, c-fps, c-myb, and c-erb) was the same in normal and tumor tissues. In contrast, levels of myc-specific RNA were 30-100 higher in about 85% of ALV-induced lymphomas (90-330 copies per cell). An even higher level of c-myc expression was found in RP-9 (1,000 copies/cell). Infection alone was not sufficient to induce myc expression, since infected but nonneoplastic tissues had low lev-

els of myc RNA in the presence of high levels of viral mRNAs. These data suggested that c-myc is the cellular gene responsible for ALV-induced neoplastic transformation in these tumors (Hayward et al., 1981b). In RP-9 cells, myb-specific RNA was also present at elevated levels, but to a much lesser extent (40 copies/cell); the significance of this finding remains unclear.

In each of the tumor samples containing low amounts of RNA homologous to rep probe (i.e., those tumors containing low amounts of viral mRNAs), viral 5' sequences were expressed at levels similar to that of myc-specific RNA. This is consistent with the interpretation that 5' and c-myc sequences are present on the same RNA molecules in ALV-induced lymphomas. To test directly whether the myc-specific sequences and viral 5' sequences are encoded on the same RNA transcript, the myc probe was hybridized to the blots of tumor poly(A) RNA (Fig. 9). Six of the seven samples shown in Fig. 9 contained a single major myc-specific RNA; in each case, this RNA corresponded to a species detectable with 5' probe. This strongly suggests that 5' and myc sequences are present on the same RNA molecule. These RNAs did not hybridize to rep probe. Both 2.5 kb and 2.9 kb 5'-specific RNAs have myc information. RP-9 contains multiple myc-specific RNA species, including one at 2.9 kb. This 2.9 kb RNA, as well as the four additional myc-specific RNAs each corresponded to a 5'-containing RNA. The high degree of specificity of the $cDNA_{myc}$ probe is indicated by the failure of this probe to hybridize to 35S viral RNA in tumor 35L, even though this species is much more abundant than the 2.5 kb RNA detected with this probe. RP-9 and tumor 34L also contain several smaller

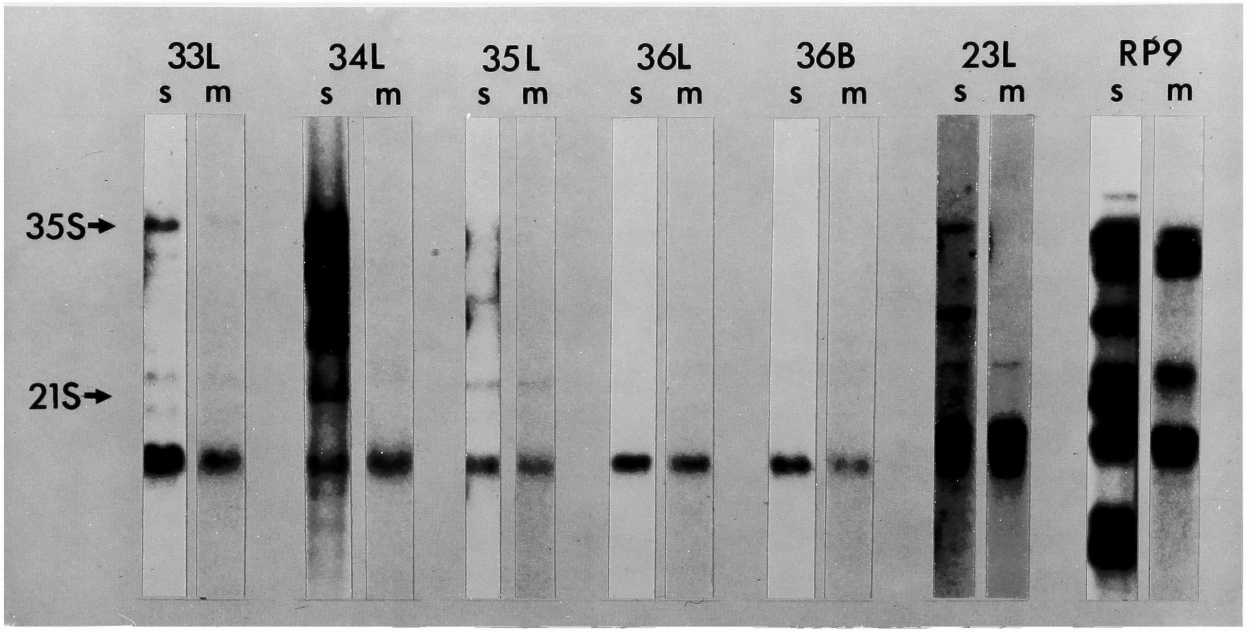



Figure 9. myc-specific and viral 5'-specific RNAs in ALV-induced lymphomas. Poly(A)-containing RNAs were analyzed as in Figs. 7 and 8. RNAs containing viral 5'-specific and myc-specific sequences were identified by hybridization to 5' probe (s) or $cDNA_{myc}$ (m). The blot was first hybridized to 5' probe and exposed to X-ray film; it was then washed under denaturing conditions to remove this probe and rehybridized with $cDNA_{myc}$. Positions of viral 35S and 21S RNAs are indicated by open arrows; 2.5 kb and 2.9 kb myc-specific RNAs are indicated by closed arrows. Tissues are designated as in Figs. 7 and 8.

RNAs that hybridize with 5' and rep probes, but not with $cDNA_{myc}$; these RNAs are presumably aberrant viral transcripts.

Susan Astrin used the $cDNA_{myc}$ probe to see whether ALV proviruses were integrated adjacent to c-myc in ALV-induced lymphomas. In normal cells, c-myc sequences are contained entirely within a 14 kb Eco R1 fragment (Sheiness et al., 1980; Hayward et al., 1981b). Lymphoma DNAs contained a new Eco R1 fragment detected with $cDNA_{myc}$ that is not present in normal cells (Hayward et al., 1981b). In each case this fragment corresponded to a tumor junction fragment detected with 5' probe (but not with rep probe). This suggests that integration in these tumors has occurred adjacent to the c-myc gene. Furthermore, tumor junction fragments of different sizes contained myc information. This suggests that integration at several sites upstream of the same cellular gene (c-myc) can result in activation of that gene and oncogenesis. In a larger sampling of lymphomas (S.M. Astrin, personal communication), utilizing a molecular clone of the cell-derived part of a tumor junction fragment (see Sect. B), it was found that 30 out of 36 ALV-induced lymphomas contained proviral information integrated adjacent to c-myc. The 14 kb normal cell myc-specific Eco R1 fragment was also found in the lymphomas. This was expected because integration probably occurs adjacent to only one of the two copies of c-myc present in the diploid chicken genome. Those samples that did not contain elevated levels of myc-specific RNA also did not contain proviruses integrated adjacent to c-myc or new, tumor-specific myc RNA species.

Taken together, this DNA and RNA data confirms the

essentials of the promoter insertion mechanism. In the majority of tumors studied, an ALV provirus was located adjacent to the c-myc gene. The tumors synthesized new, tumor-specific mRNAs that initiate within the viral LTR and continue downstream into the adjacent c-myc locus. This leads to enhanced c-myc expression and neoplasia.

B. The Promoter Insertion Mechanism--Molecular Details

Molecular cloning of tumor junction fragments. Tumor DNA from birds 7 and 10 was digested with the restriction endonuclease Eco R1, blotted onto nitrocellulose filters (Southern, 1975), and hybridized to 5' probe. As described above, Eco R1 fragments that represent ALV provirus-host cell junctions ("junction fragments") can be identified by virtue of their hybridization to 5' probe, but not to rep probe. Tumor 7 had a 3.1 kb junction fragment and tumor 10 had a 3.6 kb junction fragment. Both tumors contained 2.5 kb tumor-specific poly(A) RNAs that contained ALV 5' and myc information (Fig. 9).

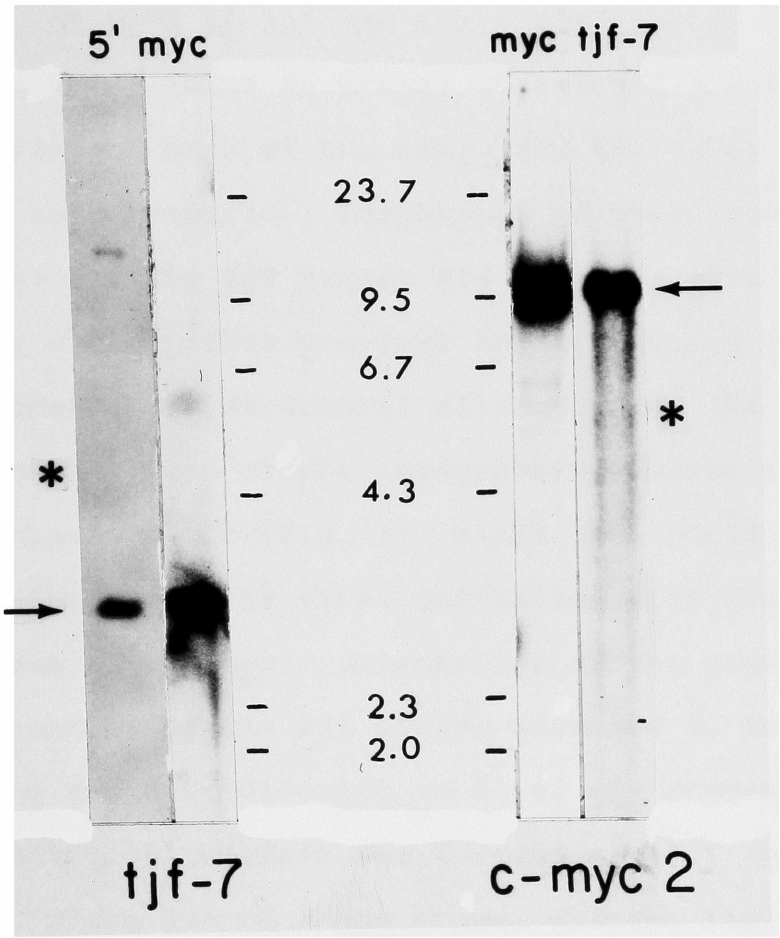
Based on this size analysis, Eco R1 digested tumor DNA was size-fractionated, ligated to Eco R1-digested λgtWES-λB (Leder et al., 1977), packaged in vitro (Becker and Gold, 1975; Hohn and Murray, 1977), and screened by the procedure of Benton and Davis (1977), using 5' probe. Two of the clones obtained, λtjf-7 (tumor junction fragment from bird 7) and λtjf-10 (tumor junction fragment from bird 10), were chosen for further analysis. λtjf-7 contains a 3.1 kb Eco R1 fragment that hybridizes to 5' probe (Fig. 1, left panel, arrow); this fragment is the tumor

junction fragment. In addition, this clone contains a 4.8 kb Eco Rl fragment that does not hybridize to 5' probe (Fig. 10, left panel, asterisk); this fragment has not been analyzed further. λtjf-10, which was constructed by Charles Rogler (Fox Chase), contains the expected 3.6 kb tumor junction fragment, which hybridizes to 5' probe (data not shown).

The 3.1 kb Eco Rl fragment of λtjf-7 hybridizes to myc probe, as well as to 5' probe (Fig. 10). A similar result was obtained for the 3.6 kb λtjf-10 insert (not shown). This confirms unambiguously that viral 5' and c-myc sequences are covalently linked in the DNA of these tumors.

Isolation of a clone of the normal c-myc gene. A chicken genomic library (Dodgson et al., 1979) was kindly provided by Richard Axel, Columbia University. This library consists of 15-20 kb fragments of a partial Hae III/Alu-I digestion of chicken DNA that have been "tailed" with Eco Rl oligonucleotide linkers and inserted into the lambda bacteriophage vector Ch 4A (Blattner et al., 1977). Recombinants were screened by the Benton-Davis method. The probe was the 2.4 kb fragment of a Sac I digest of the λtjf-7 insert. This fragment contains c-myc sequences but not ALV sequences (see Figs. 11 and 12). Several clones that hybridized to this probe were obtained, and one of these, λc-myc 2, was selected for further analysis because it contained the largest myc-specific fragment. An Eco Rl digest of λc-myc 2 produces two restriction fragments in addition to the Ch 4A arms: a 9.6 kb fragment that contains all the c-myc sequences homologous to v-myc (Fig. 10, right panel, arrow), and a 5.5 kb fragment

Figure 10. Molecular cloning of a tumor junction fragment and the normal c-myc gene. Left panel: The Eco R1-generated tumor junction fragment from a lymphoma in chicken 7 was molecularly cloned into λgtWES (Leder et al., 1977) to give clone λtjf-7. Bacteriophage DNA was isolated, digested with Eco R1, transferred to a nitrocellulose filter (Southern, 1975), and hybridized to either 5' probe or v-myc probe (see text). The arrow indicates the 3.1 kb tumor junction fragment. The asterisk (*) indicates the position of a 4.8 kb band identified by ethidium bromide staining, which is present in the tjf-7 clone but does not hybridize to 5' or myc probes. The Hind III fragments of lambda DNA were used as size markers.
Right panel: A molecular clone containing the c-myc gene from normal chickens was selected from a chicken genomic library (see text). DNA from this clone (λc-myc 2) was digested with Eco R1, transferred to nitrocellulose and hybridized to either v-myc or to the 2.4 kb Sac I fragment of the tjf-7 clone, which lacks ALV sequences (see text and Fig. 2). The arrow indicates the myc-containing Eco R1 fragment. The position of the fragment of λc-myc 2 that does not contain myc sequences is indicated by the asterisk (*).


5' myc
myc tjf-7
23.7
9.5
6.7
4.3
2.3
2.0
*
*
tjf-7
c-myc 2

that does not contain myc-related sequences (Fig. 10, right panel, asterisk). The restriction map of the 9.6 kb insert (see below) corresponds with earlier restriction data on the c-myc locus (Sheiness et al., 1980; S.M. Astrin, personal communication).

Restriction mapping of tjf and c-myc clones. To compare the structure of the c-myc locus in normal tissue and ALV-induced lymphomas, restriction maps of the c-myc and two tumor junction fragment clones were prepared. Comparison of these maps (Fig. 11) reveals that: (1) The tjf clones are colinear with the right end of the c-myc clone. Thus proviral integration in birds 7 and 10 occurred without gross structural alteration of the c-myc locus. (2) The orientation of the integrated provirus in the tjf clones is such that the LTR (Fig. 11, black box) could be used to initiate transcription at the viral initiation site that would proceed downstream into c-myc. Orientation of the provirus was confirmed by digestion of the tjf clones with Sac I, followed by Southern blotting and hybridization to 5' or myc probes. As expected (see Fig. 11), the left Sac I fragment (0.7 kb) hybridized both to 5' probe and to v-myc probe, whereas the right Sac I fragment (2.4 kb) hybridized only to myc probe (data not shown).

Localization of putative coding domains in c-myc. The c-myc and v-myc genes share extensive nucleotide homology (Roussel et al., 1979; Sheiness and Bishop, 1979). It is generally thought that MC29, which contains v-myc, arose as a result of recombination between a slowly transforming virus and c-myc. The v-myc

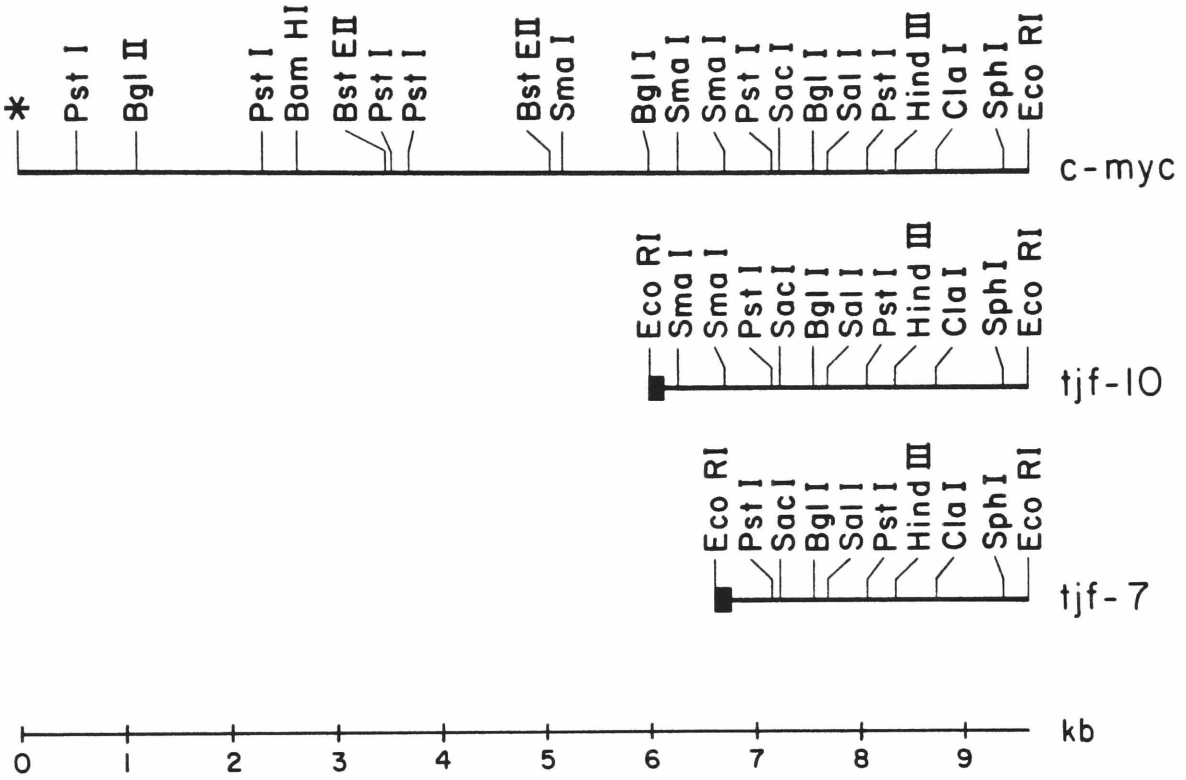



Figure 11. Restriction maps of tumor junction fragments: comparison with the c-myc gene. The 3.1 kb Eco Rl insert of tjf-7 and the 3.6 kb insert of tjf-10 were isolated by preparative electrophoresis, as was the 9.6 kb myc-specific fragment from c-myc 2. Following low specific activity labeling of the fragments with $^{32}$p-dCTP, the restriction endonuclease cleavage maps of these inserts were determined and compared. LTR sequences in the tumor junction fragments are represented by solid boxes. The left end of the c-myc clone (*) is defined by either a Hae III or an Alu I site. The cleavage positions are accurate to within 50 nucleotides. There are no Kpn I, Pvu I, Xba I or Xho I sites in any of the clones.

gene is translated as part of a gag-myc fusion protein in MC29-infected cells; thus the entire v-myc gene may represent protein coding sequences (Bister et al., 1977; Kitchener and Hayman, 1980). I reasoned that sequences in common between v-myc and c-myc might represent coding sequences in the c-myc locus. Therefore, I prepared a detailed restriction map of a clone containing the v-myc gene and a more detailed restriction map of tjf-10 than is shown in Fig. 11.

The clone containing the v-myc gene has a 2.9 kb Bam H-1 fragment of an integrated MC29 provirus (Lautenberger et al., 1981) inserted into pBR322. The map of this insert is shown in the upper part of Fig. 12. It contains some viral gag sequences at the left end, followed by the v-myc gene, and a small amount of sequence to the right of v-myc. Comparison of this insert with the tjf-10 insert shows that: (1) There is a region on the left ($\sim$0.7 kb) and a region on the right ($\sim$0.85 kb) of tjf-10 in which its restriction map corresponds exactly to the left and right of v-myc. (2) These two regions in tjf-10 are separated by a region of about 1 kb that appears to be absent from the v-myc clone. The simplest interpretation of these results is that there is a 1 kb non-coding domain separating two coding domains. S-1 deletion mapping experiments using the technique of Shenk et al. (1975) with the v-myc and tjf-10 clones indicate that the sizes of the putative coding domains are 0.72 kb and 0.85 kb, respectively, whereas the non-coding domain is 0.96 kb (data not shown). (3) The v-myc and tjf-10 clones also diverge at their extreme left ends. This suggests that proviral integration in tjf-10 occurs upstream from the bulk of c-myc coding sequences.

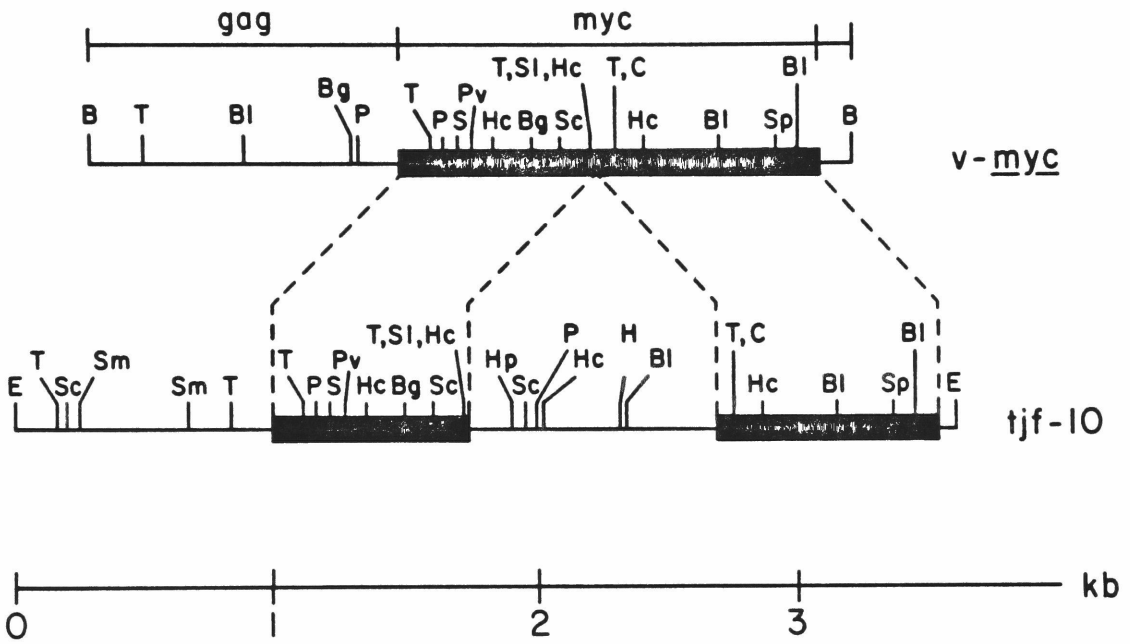



Figure 12. Identification of putative coding and non-coding domains in c-myc. A more detailed restriction map of the 3.6 kb λtjf-10 insert was determined. It was compared to a map of part of an integrated MC29 provirus containing the entire v-myc gene (Lautenberger et al., 1981). The positions of the MC29 gag and myc genes are indicated above the clone. Regions in the two clones having identical restriction maps are shown as thick bars. Restriction sites were determined using low specific activity-labeled insert fragments as in Fig. 2. [ B=Bam Hl, Bg=Bgl I, Bl=Bal I, C=Cla I, E=Eco Rl, H=Hind III, Hc=Hinc II, Hp=Hpa I, P=Pst I, Pv=Pvu II, S=Sac I, Sc=Sac II, Sl=Sal I, Sm=Sma I, Sp=Sph I, T=Taq I ]

There may, however, be coding and/or leader sequences in c-myc that are not represented in v-myc.

Gregory Gasic (Rockefeller University) obtained the DNA sequence of the left ends of the tjf 7 and tjf 10 clones. These sequencing studies indicated that in both tumors a normal proviral integration event had occurred, placing a right LTR upstream of the bulk of the c-myc coding sequences. In addition, in both tumors, integration had occurred slightly upstream from cellular sequences bearing strong resemblance to the "consensus" splice donor sequence of eukaryotes (Lerner et al., 1980; Rogers and Wall, 1980) and the adenovirus-2 major late leader splice donor sequence (Akusjarvi and Pettersson, 1979; Zain et al., 1979). If these are functional splice donor sites in vivo, then they may serve as splice sites for processing the primary transcript of the c-myc gene of normal cells. In this case, integration in both tumors 7 and 10 has occurred in exons. In tjf-7, there is a pyrimidine-rich stretch beyond the putative splice donor site consisting of TTA and ATT repeats, which continues for about 100 nucleotides. This region is probably not translated, lending further credence to the identification of the immediately preceding sequence as a splice donor site.

This finding suggests a possible explanation for why tumors with proviruses integrated at different sites upstream from c-myc produce new, tumor-specific RNAs of approximately the same size, 2.5 to 2.9 kb . It may be that c-myc can only be activated when the provirus integrates into a c-myc exon. The exon could then provide a splice donor site to enable transcripts initiated in the LTR (which lacks a splice donor site) to be spliced to the

bulk of c-myc coding information located further downstream. If splicing eliminated the majority of the sequence between the integration site and the 0.72 kb coding domain, the mRNAs from tumors with proviruses integrated at different sites could be about the same size.

Identification of possible precursors to tumor-specific RNAs. As a first step towards exploring this hypothesis, we overexposed the blots of tumor poly(A)-containing RNAs that had been hybridized to myc probe. In these long exposures (Fig. 13), the tumor-specific 2.5 kb and 2.9 kb myc-containing transcripts are now seen as heavy bands. However, there are also several larger myc-containing RNAs in each tumor that are present at 10-20 fold lower levels. In those cases where there was enough of these larger tumor-specific RNAs to be detected with 5' probe, most of the larger RNAs (Fig. 13, asterisks) had 5' information as well. These RNAs presumably represent precursors of the smaller RNAs. The size range of these larger RNAs (3.2 to 4.0 kb) is consistent with the size expected for the primary transcript and processing intermediates of the tumor junction fragments in these tumors (see Discussion). Fig. 13 is organized such that RNAs from tumors with ALV proviruses integrated further upstream from the Eco R1 site (i.e, those with larger tumor junction fragments) are shown towards the right of the figure. Tumors 34L, 36B, and 36L, which have proviruses integrated at approximately the same site (Hayward et al., 1981b), have many similarly-sized putative precursor RNAs. Tumors with proviruses integrated further downstream (35L and 33L) have smaller myc-

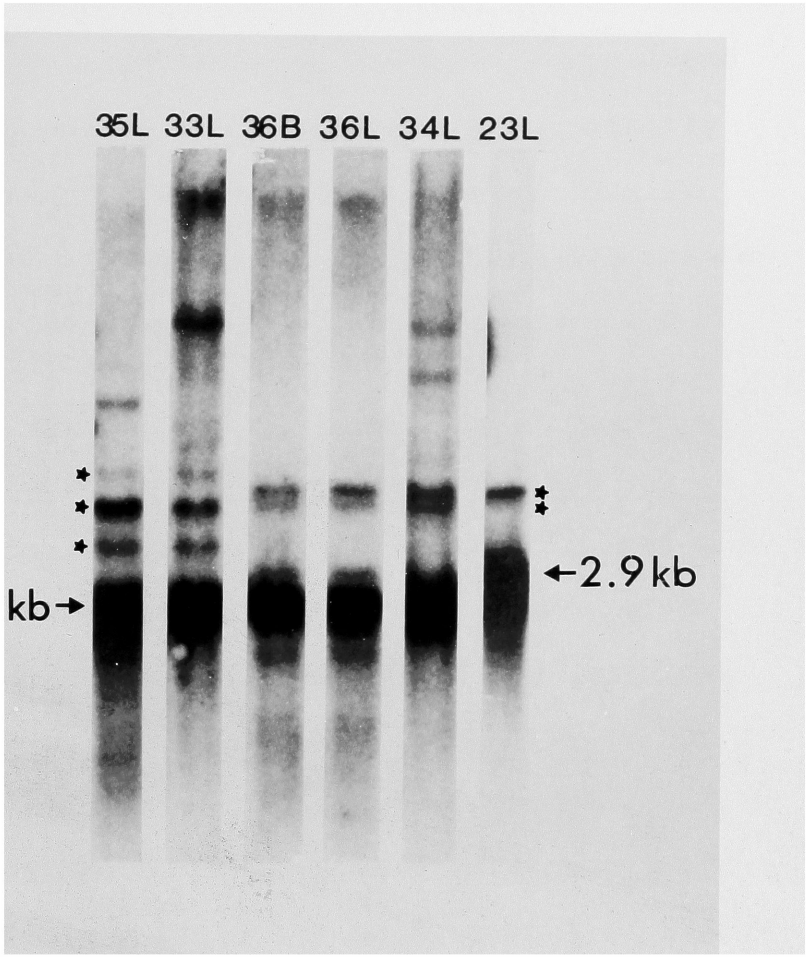



Figure 13. Identification of putative tumor-specific precursor RNAs in ALV-induced lymphomas. Poly(A)-containing RNAs from tumor tissues were analyzed as in Fig. 9. The blots were initially exposed for 1-2 days to identify the tumor-specific 2.5 or 2.9 kb myc-myc-specific RNAs (Fig. 9), and then exposed for 1-2 weeks. Tumors with ALV proviruses integrated closer to the right Eco R1 site of c-myc (those containing smaller tumor junction fragments) are at the left of the figure. Tumor junction fragment sizes were determined previously by S. Astrin (Neel et al., 1981; Hayward et al., 1981b). The positions of the 2.5 and 2.9 kb RNAs are indicated by arrows. Putative tumor-specific transcripts or processing intermediates are indicated by asterisks (*).

specific RNAs, even though all of these tumors (33L, 34L, 35L, 36B and 36L) produce 2.5 kb mRNAs. These results are consistent with the hypothesis that integration must occur in c-myc exons for a tumor to result. However, there are some substantially larger myc-specific transcripts whose origins are not easily accounted for by this interpretation. A more conclusive resolution of this issue awaits S-1 mapping of the tumor-specific mRNAs.

In vitro transcription of tumor junction fragments and c-myc. To examine transcription of the c-myc locus in normal and ALV-transformed cells in more detail, in vitro transcription experiments were performed. The templates for these reactions were a subclone of λtjf-7 in pBR322 containing the 1.1 kb Sal I fragment (the left Sal I fragment) of the λtjf-7 3.1 kb Eco Rl insert, and pc-myc, a subclone of λcmyc-2 in pBR322 containing the 6.7 kb Bam Hl fragment (the right Bam Hl fragment) of the c-myc 9.5 kb Eco Rl insert. These subclones were digested with various restriction enzymes and incubated with HeLa cell extracts (Manley et al., 1980). The "runoff" transcription products were denatured with glyoxal (McMaster and Carmichael, 1977) and displayed on agarose gels.

Under the conditions used, the production of runoff transcripts was extremely sensitive to the amount of input template DNA. Changing the DNA concentration by as little as two-fold (Fig. 14) had dramatic effects on the efficiency of the reaction. Thus, the optimal concentration of DNA for transcription of each subclone was determined (Fig. 14) and used for subsequent experi-

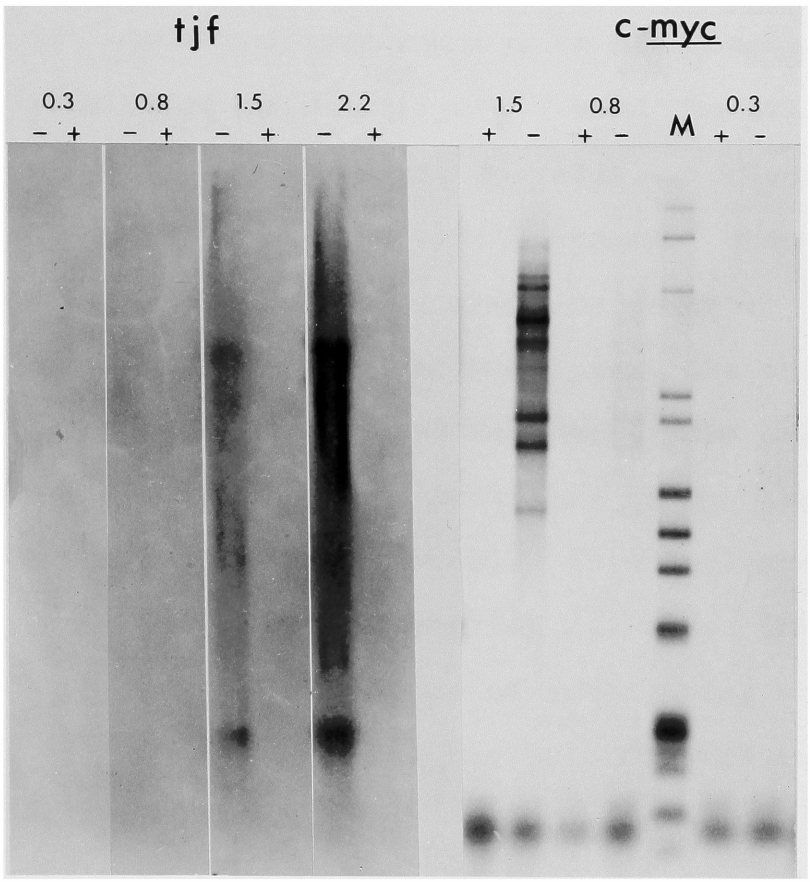



Figure 14. Dependence of in vitro transcription reactions on DNA concentration. Varying amounts of either the tjf-7 (left) or c-myc (right) subclones (see text) were incubated in a 25 ul in vitro transcription reaction (Manley et al., 1980), as modified by Handa et al. (1981). The numbers above the lanes indicate the amount of template DNA (in ug) added to each reaction. Reactions were carried out with (+) or without (-) the addition of α-amanitin to a final concentration of 1 ug/ml. M=glyoxalated mixture of λ-Hind III and ϕX174-Hae III size markers.

ments.

The results of in vitro transcription of the tjf-7 subclone are shown in Fig. 15. As a positive control to demonstrate the fidelity of initiation under my conditions, I used the plasmid pSmaF (generously supplied by J. Nevins, Rockefeller University), which contains the adenovirus-2 major late promoter. When cut with Sma I, transcription initiating at the late promoter in pSmaF should result in 0.53 kb runoff transcripts. The observed transcript (Fig. 15) is 0.52 kb, in good agreement with the expected value. This transcription is sensitive to the addition of α-amanitin at a concentration of 1 ug/ml. This indicates that transcription was catalyzed by RNA polymerase II, since this enzyme is sensitive to low concentrations of α-amanitin, whereas RNA polymerase III is only inhibited at higher concentrations of the inhibitor (Lindell et al., 1970).

The normal initiation site in the LTR is about 60 nucleotides to the right of the Eco Rl site in the tjf-7 insert. There is a Sac I site at 0.65 kb, a Bgl I site at 0.98 kb, and a Sal I site at 1.18 kb to the right of the Eco Rl site. If the tjf-7 subclone is digested with Sac I and used as the template in an in vitro transcription reaction, transcripts initiating at the normal viral initiation site should be 0.65 minus 0.06 kb or 0.59 kb in size. The observed transcript is 0.56 kb, in good agreement with the value expected for initiation within the LTR. Addition of α-amanitin to the transcription reaction completely eliminates the 0.56 band (Fig. 15). When Bgl I-digested tjf-7 subclone is used as the template, an α-amanitin-sensitive 0.89 kb band is observed, compared with the expected 0.92 kb, and a Sal I-

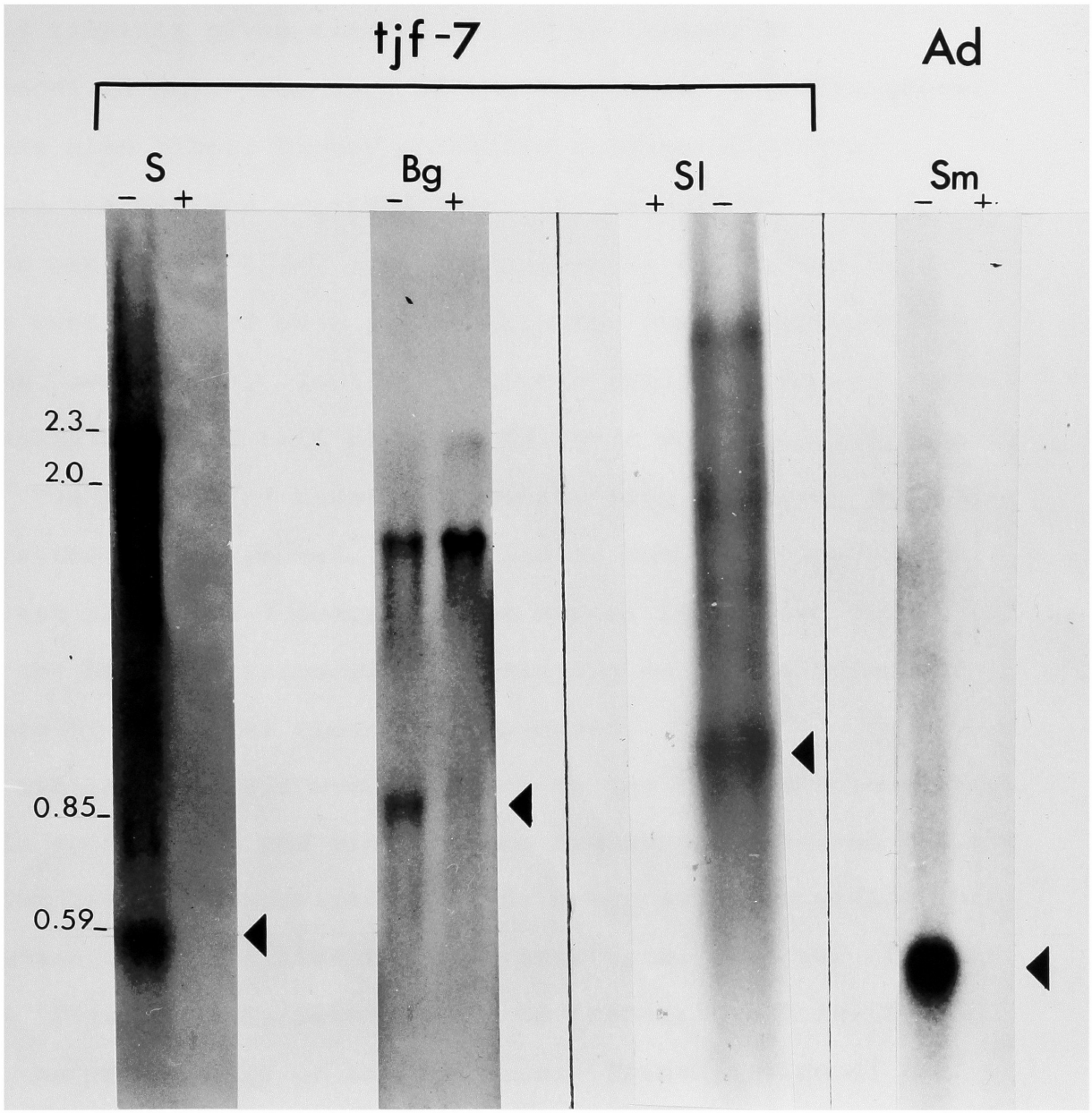



Figure 15. In vitro transcription of a tumor junction fragment. 1.5 ug of the tjf-7 subclone, digested with the indicated restriction enzyme, were added to a 25 ul in vitro transcription reaction in the presence (+) or absence (-) of α-amanitin at 1 ug/ml. On the right (Ad), 0.3 ug of pSmaF, a subclone containing the major late promoter of Ad-2 was digested with Sma I and tested in a 25 ul in vitro transcription reaction as a control for fidelity of initiation (see text). Arrowheads indicate the positions of α-amanitin-sensitive runoff transcripts. Numbers indicate the position of migration and sizes of glyoxalated DNA fragments. [S=Sac I, Bg=Bgl I, Sl=Sal I, Sm=Sma I]

digested template gives rise to a 1.05 kb transcript (expected=1.12 kb). For each of the templates described above, there are also other, higher molecular weight α-amanitin-sensitive transcripts observed (Fig. 15, asterisks). The restriction map of the tjf-7 subclone indicates that these transcripts must initiate at a site within the pBR322 part of the subclone (see below). Initiation within pBR322 in Manley extract experiments has also been reported by other workers (Fire et al., 1981). The results of these experiments with different templates indicate that, as expected, initiation of RNA synthesis by RNA polymerase II in tjf-7 occurs at the normal initiation site within the LTR. No transcripts initiating in the cell-derived sequences of the tjf-7 clone were observed.

A similar analysis was performed on the c-myc subclone (Fig. 16). In contrast to the single major transcript observed for the tjf-7 templates, transcription of the c-myc subclone yields multiple α-amanitin-sensitive runoff transcripts. One set of transcripts (Fig. 16, asterisks) is due to transcription initiating within the pBR322 part of the subclone. Transcription of Eco R1-digested pBR322 DNA under the conditions used here gives rise to a major 1.7 kb band as well as a few minor bands (Fig. 16). Knowledge of the restriction sites in pBR322 (Sutcliffe, 1978) as well as in c-myc allows localization of the major pBR322 initiation site to a map position of about 2.6 kb in pBR322. In addition to the pBR322-derived transcripts, though, there are two distinct initiation sites reading rightward in the c-myc insert. These initiation sites are separated by about 1 kb, and give rise to the two sets of runoff transcripts indicated by the arrows in

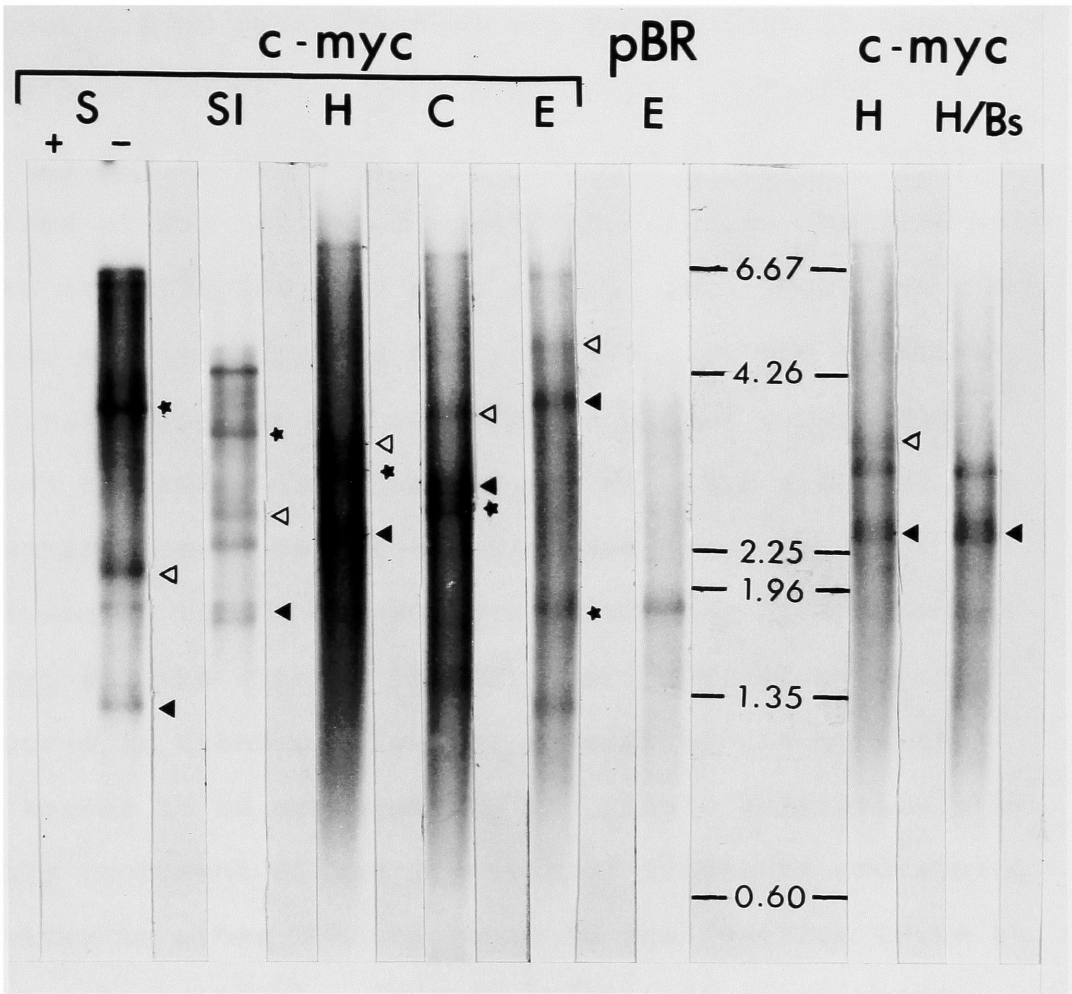



Figure 16. In vitro transcription of c-myc. 1.5 ug of pc-myc digested with the indicated restriction enzyme were added to a 25 ul in vitro transcription reaction. The presence (+) or absence (-) of 1 ug/ml α-amanitin is indicated. Transcripts initiating from $I_1$ (solid arrows) or $I_2$ (open arrows) within the insert of pc-myc are indicated, as are transcripts initiating at a specific site within the pBR322 part of the subclone (*). The lane labeled pBR is a reaction primed with 1.5 ug of Eco R1-digested pBR322. The migration positions of a few glyoxalated λ-Hind III or ϕX174-Hae III DNA fragments are indicated. [S=Sac I, Sl=Sal I, H=Hind III, C=Cla I, E=Eco R1, H/Bs=Hind III + Bst E II].

Fig. 16. The solid arrows indicate transcripts initiated at a site ($I_1$) about 3.6 kb upstream from the Eco Rl site at the right of c-myc; the open arrows indicate transcripts initiated at a site ($I_2$) about 4.6 kb upstream from this Eco Rl site. Table 1 shows the sizes of the indicated runoff transcripts observed with each template shown in the left part of Fig. 16. These are compared with the results expected for initiation at the putative initiation sites, based on the restriction map of c-myc (Fig. 11). For each template, the observed and expected sizes of the runoff transcript are in very good agreement.

In addition to runoff transcripts initiating at the two sites in c-myc and the site in pBR322, there are larger transcripts observed in transcriptions of several of the templates that do not appear to be explained by any single initiation site. These probably represent either ligation of fragments containing initiation sites to other DNA fragments in the reaction (Fire et al., 1981) or, alternatively, cryptic initiation sites whose existence might be uncovered (for unclear reasons) by digestion of the pc-myc plasmid with a particular enzyme. There is also an apparent initiation site about 600 nucleotides upstream of $I_2$ that reads leftward. A transcript initiating from this initiation site ($I_L$) can be seen using Bam Hl-digested pc-myc as the template (data not shown). Transcripts from $I_L$ cannot be seen with any of the templates in Fig. 16 because there are no restriction sites for these enzymes upstream of $I_L$.

The restriction enzyme Bst EII digests pc-myc very close to the putative initiation site $I_2$. Thus, digestion with Bst EII might be expected to eliminate transcription initiating at $I_2$.

Table 1. In vitro transcription of the c-myc locus.

| Template[1] | Transcripts(kb)[2] | Expected Transcripts(kb)[3] |
|---|---|---|
| Sac I | 2.1 | --- |
| | 1.2 | --- |
| Sal I | 2.6 | 2.6 |
| | 1.7 | 1.7 |
| Hind III | 3.2 | 3.2 |
| | 2.35 | 2.3 |
| Cla I | 3.5 | 3.65 |
| | 2.7 | 2.75 |
| Eco Rl | 4.35 | 4.5 |
| | 3.6 | 3.6 |

[1] DNA templates were pc-myc (see text) digested with the indicated restriction endonucleases.

[2] Observed size values refer to the transcripts indicated by the solid and open arrows in Fig. 16.

[3] Expected size values for a given template were calculated based on the observed transcripts for the Sac I template and known restriction sites in c-myc (Fig. 11). Therefore, no expected values are listed for the Sac I template.

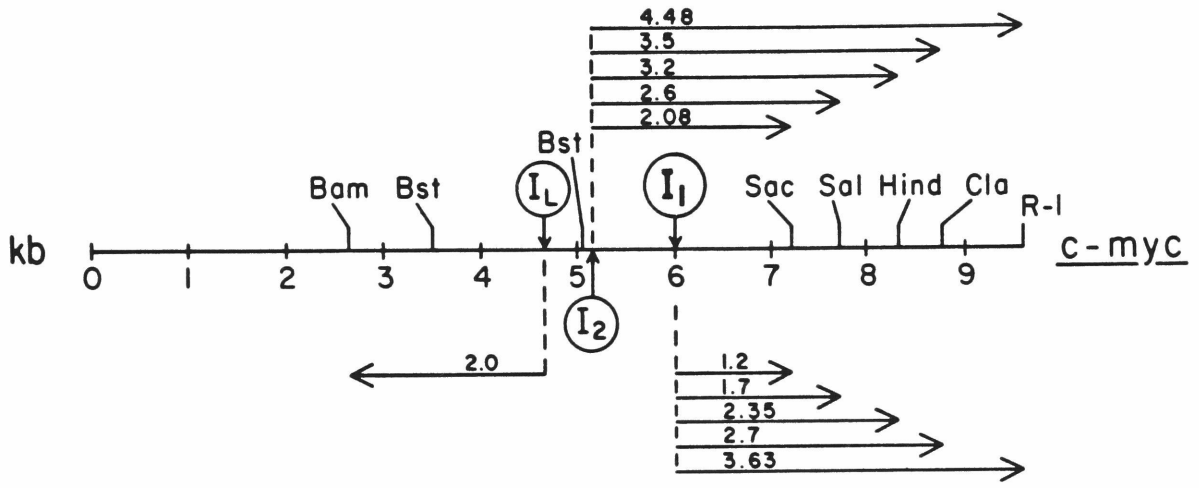



Figure 17. Map of transcripts initiated in vitro from c-myc. A map of c-myc showing the sites of cleavage of restriction enzymes used in the in vitro transcription experiments is shown. The sizes of runoff transcripts initiating at the two sites within c-myc ($I_1$ and $I_2$) utilizing various truncated templates are indicated (see Fig. 16). $I_L$ indicates the position of an initiation site that is oriented upstream (data not shown in Fig. 16). [Bam=Bam H1, Bst=Bst EII, Cla=Cla I, Hind=Hind III, R-1=Eco R1, Sac=Sac I, Sal=Sal I]

Fig. 16 (right) shows that pc-myc digested with Hind III gives rise to two transcripts (corresponding to initiation at $I_1$ and $I_2$), whereas pc-myc digested with Bst EII and Hind III yields only a single transcript in vitro (corresponding to initiation at $I_1$). Similarly, Sma I digestion of pc-myc eliminates transcription initiating at $I_2$ and results in the expected truncated transcript initiating at $I_1$ (data not shown). The results of the pc-myc in vitro transcription experiments are summarized in Fig. 17, which shows the position of the putative initiation sites relative to the c-myc restriction map.

C. Isolation of a Recombinant Rapidly Transforming Virus from an ALV-induced Fibrosarcoma

In the course of my studies of ALV lymphomagenesis, there was one chicken (#16) that developed a fibrosarcoma instead of a lymphoma. Tumor supernatants from this bird contained a new rapidly transforming virus (16L virus). This section presents data on the biological and biochemical characterization of this virus.

Biological properties of 16L Virus. Several chickens were infected in embryo with td107A. td107A is a deletion mutant of subgroup A Schmidt-Ruppin Rous sarcoma virus (Kawai et al., 1977) that lacks most, if not all, of the src gene, and does not give rise to recovered avian sarcoma virus (rASV) (Hanafusa et al., 1977). Instead, chickens infected with td107A usually develop B-cell lymphomas after a long latent period. However, upon

sacrifice at 19 weeks, one chicken (#16) had multiple large nodules in the liver, kidneys, heart, and peritoneal wall, with no bursal involvement. Histopathologic analysis revealed that the tumors were fibrosarcomas.

When a liver nodule was homogenized and an aliquot of the supernatant fluid was plated on CEF, discrete foci of transformed cells were observed (see Fig. 18). Interference assay (data not shown) revealed the presence of a subgroup A retrovirus. Tumor supernatants from two ALV-induced lymphomas (19B and 34L) and the lymphoma cell line RP-9 did not cause morphologic transformation of fibroblasts (Fig. 18) even though infectious slowly transforming virus (presumably the initial strain used for infection of these birds) was recovered (data not shown). Injection of the supernatants into the wing webs of 1-2 day old chickens led to palpable tumors in 5-7 days and death in 10-14 days (10/10 birds). No FSV strains or other fps-containing retroviruses were being injected into laboratory flocks at this time, nor were any other unexplained sarcomas noted in other birds.

Size and genetic content of 16L RNA. William Hayward performed hybridization kinetic analysis on tumor 16L RNA as well as on a few other representative tumors induced by td107A and other leukosis viruses. Whereas the vast majority of ALV-induced tumors are lymphomas, which show elevated myc expression, tumor 16L expressed fps-related information at about 300 times normal level. A kidney nodule taken from the same bird (16K) also exhibited increased expression of fps. The level of expression of fps in normal muscle tissue from bird 16 was not elevated. In

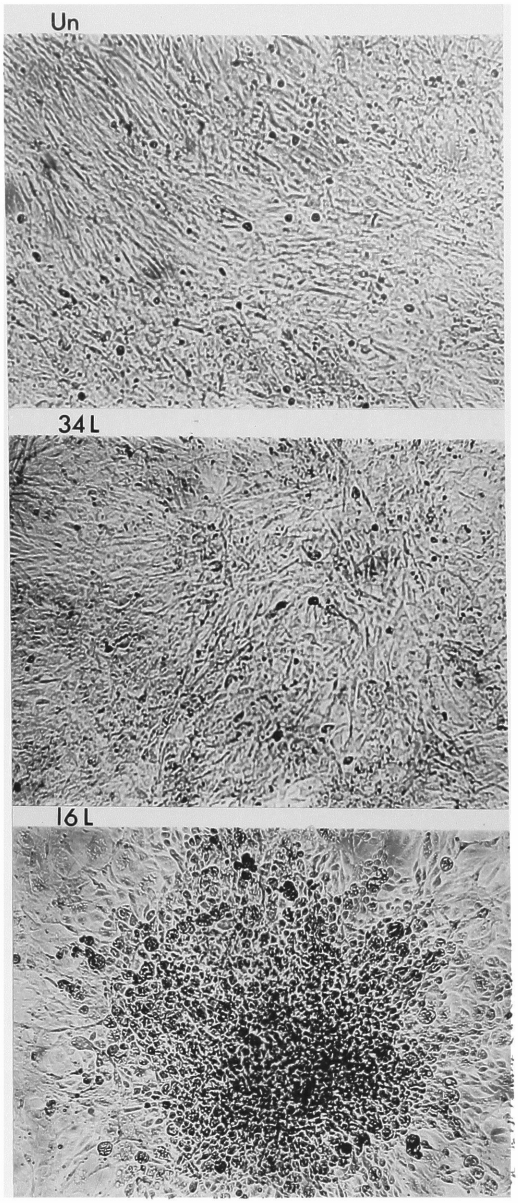



Figure 18. Morphologies of cells infected with tumor supernatants. Secondary cultures of chicken embryo fibroblasts were treated with DEAE-dextran and infected with dilutions of extracts of tumor 34L or 16L. Uninfected cells (Un) are shown at the top of the figure.

addition, several other onc genes were expressed at normal levels in bird 16.

Poly(A) RNA from tumor 16L was analyzed by blot hybridization (Alwine et al., 1977). A new 6.0 kb RNA was found in addition to the expected 8.1 and 3.2 kb td107A mRNAs (Hayward, 1977; Weiss et al., 1977). However, unlike the new RNAs found in ALV-induced lymphomas, which have only viral 5'-specific information, the 6.0 kb RNA hybridized to a probe ($cDNA_{rep}$) representative of the total ALV genome. In addition, this RNA hybridized to a molecularly cloned probe for the v-fps gene of FSV (data not shown).

Oligonucleotide fingerprinting of 16L RNA. Together with Lu-Hai Wang (Rockefeller University), I further characterized the genetic content of 16L viral RNA by oligonucleotide fingerprinting. Following poly(A) selection and sucrose density gradient centrifugation, 35S (helper viral RNA) and 30S (16L transforming component) peaks were observed. Preliminary fingerprint experiments demonstrated that 30S RNA isolated in this fashion was significantly contaminated with fragments of the 35S species (data not shown). To reduce this contamination, cDNA homologous to the 5' end of ALV RNA was prepared, extended with poly(dC), and hybridized to 30S RNA. The resulting hybrids were selected by oligo(dG) cellulose chromatography. Approximately 60% of the poly(A)-containing 30S RNA was recovered following this procedure.

Fig. 19 shows the RNase $T_1$ oligonucleotide fingerprints of 16L 35S and 30S RNAs; the oligonucleotide compositions of spots

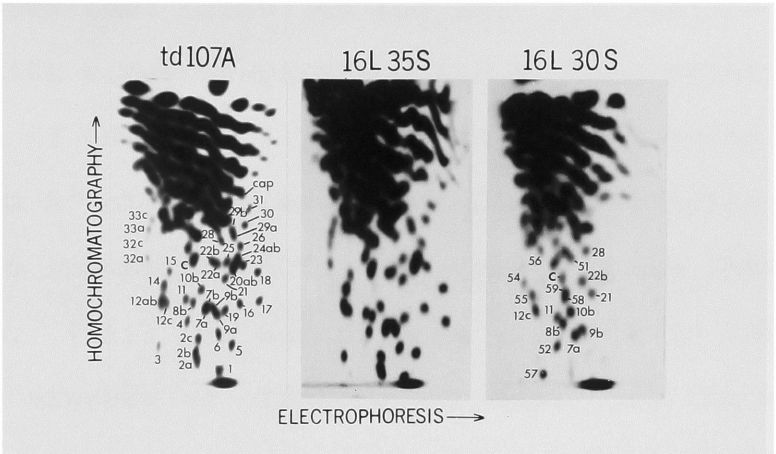



Figure 19. Fingerprint patterns of RNase $T_1$-resistant oligonucleotides of td107A and 16L viral RNAs. $^{32}P$-labeled viral RNA was purified by sucrose density gradient centrifugation and oligo(dT)-cellulose column chromatography. The 30S component of 16L viral RNA was further purified by hybridization to poly(dC)-tailed $cDNA_5$. Numbering of td107A oligonucleotides followed that of Wang et al. (1978).

found in 16L but not in td107A are shown in Table 2. Comparing these fingerprints with fingerprints of td107A, FSV, and UR1 RNAs [UR1 is a recently characterized independent avian sarcoma virus isolate that contains the fps gene. (Balduzzi et al., 1981; Wang et al., 1981)] shows that (Figs. 19 and 20): (i) The 35S (helper) component of the 16L virus preparation has a fingerprint pattern identical to that of td107A; (ii) The 30S (transforming component) has 5' and 3' oligonucleotides indistinguishable from td107A but a large substitution for sequences in the interior of the td107A genome. (iii) The substituted sequence in 16L viral RNA contains four classes of oligonucleotides: (a) the majority of the oligonucleotides are present in the fps-specific sequences of both FSV and UR1; (b) one spot, no. 58, is present in FSV, but not in UR1; (c) another spot, no. 55, is present in UR1 but not in FSV, and (d) one spot, no. 59, is not present in td107A, FSV, or UR1 RNAs. (iv) The oligonucleotide differences found among 16L, FSV, and UR1 cannot be explained by single base changes (Hanafusa et al., 1980; Wang et al.,1981 and Table 2). The simplest interpretation of these data is that the substituted sequence in 16L virus is derived from cellular fps (c-fps) information. FSV and UR1 presumably also acquired c-fps information and subsequently diverged slightly.

Analysis of virus-specific proteins in 16L-infected cells. FSV (Hanafusa et al., 1980) and UR1 (Wang et al., 1981) encode gag-fps fusion polyproteins that have associated protein kinase activities specific for tyrosine residues, and which are the putative transforming proteins of these viruses (Pawson et al.,

Figure 20. Oligonucleotide maps of 16L virus and related viruses. The orders of RNase $T_1$-resistant oligonucleotides of td107A, UR1 and FSV have been published previously (Wang et al., 1978, 1981; Hanafusa et al., 1980). The oligonucleotide maps of 16L "35S" and "30S" RNAs were deduced from the fingerprints shown in Figure 19. The scales at the left and right represent the genomes of td107A and the three sarcoma viruses. The size of each genetic region is indicated. 16L RNA contains oligonucleotides 9b and 11, which are derived from the gp37 coding region of td107A (T.Takeya and H. Hanafusa, personal communication). However, it is not clear whether any of the 3' oligonucleotides of UR1 or FSV are derived from the *env* gene. 16L virus oligonucleotides are shown in bold-face, as are identical oligonucleotides present in td107A, FSV or UR1. Oligonucleotides within the brackets of each sarcoma viral RNA map are derived from *fps*-related sequences of the corresponding sarcoma virus. In addition to being designated by bold-face numbers, shared *fps*-specific oligonucleotides are connected by dashed lines. The base compositions of *fps*-specific oligonucleotides in 16L are shown in Table 2. (-) indicates the absence of a particular oligonucleotide from the corresponding position of the map. The actual positions of *fps*-related oligonucleotides in 16L viral RNA were not determined in this study. The indicated order of oligonucleotides common to FSV and UR1 is based on the previously determined oligonucleotide maps of these viruses (Hanafusa et al., 1980; Wang et al., 1981). Oligonucleotide 46 has a base composition similar to that of oligonucleotide 56 (Table 2 and Hanafusa et al., 1980) except that 46 is two nucleotides longer. It is not certain that 46 and 56 represent homologous sequences, so 46 is shown in parentheses. Cap I and Cap IV are cap-containing oligonucleotides of different length and composition.

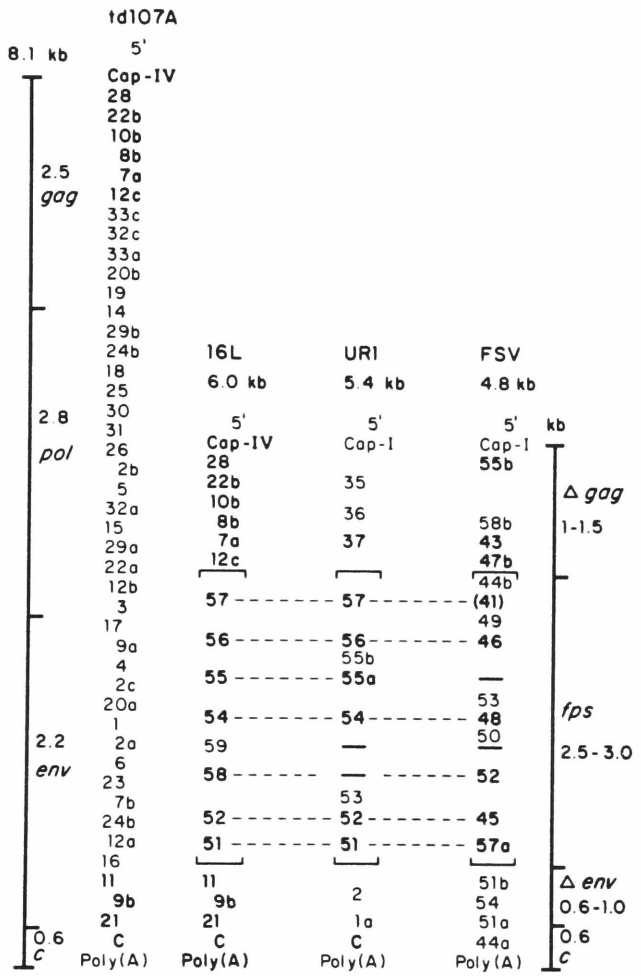

td107A
8.1 kb
5'
Cap-IV
28
22b
10b
8b
2.5
7a
gag
12c
33c
32c
33a
20b
19
14
29b
24b
18
25
2.8
30
31
pol
26
2b
5
32a
15
29a
22a
12b
3
17
9a
4
2c
20a
1
2.2
2a
env
6
23
7b
24b
12a
16
11
9b
21
0.6
C
Poly(A)
16L
6.0 kb
5'
Cap-IV
28
22b
10b
8b
7a
12c
57
56
55
54
59
58
52
51
11
9b
21
C
Poly(A)
URI
5.4 kb
5'
Cap-I
35
36
37
57
56
55b
55a
54
—
—
53
52
51
2
1a
C
Poly(A)
FSV
4.8 kb
5'
Cap-I
55b
58b
43
47b
44b
(41)
49
46
—
53
48
50
52
45
57a
51b
54
51a
44a
Poly(A)
kb
Δ gag
1-1.5
fps
2.5-3.0
Δ env
0.6-1.0
0.6
C

Table 2. fps-related large RNase $T_1$-resistant oligonucleotides present in 16L viral RNA.

| Spot No. | RNase-resistant fragments |
|---|---|
| 51 | 1-2U, 4C, G, AC, 2AU |
| 52 | U, 4C, G, AC, 2AU, 1.5AAC |
| 54 | 4C, G, 2AC, AAC |
| 55 | 6C, 2AC, AU, AG |
| 56 | U, 7C, G, 0.4AC, AU |
| 57 | 2U, 8C, 4AC, AU, AG |
| 58 & 59 | 4U, 10C, G, 2AC, 3AU, AG |

The composition analysis of oligonucleotides was done with spots recovered from the fingerprint of purified 31S 16L viral RNA shown in Fig.19.

1980b; Wang et al., 1981). In order to analyze 16L virus-specific proteins, Bernard Mathey-Prevot (Rockefeller University) labeled 16L-infected CEFs with $^{35}$S-methionine, and virus-specific proteins in the cell lysate were immunoprecipitated and electrophoresed on SDS-polyacrylamide gels. 16L-infected cells synthesized a 142K protein (P142) that was precipitated by anti-gag antiserum. Electrophoresis of P142, FSV P140, and UR1 P150 in adjacent lanes indicates that the 16L protein is larger than the FSV protein and smaller than the UR1 protein. P142 was also specifically immunoprecipitated by antibody specific for the fps component of P140 (Mathey-Prevot et al., 1982). To see whether P142 has protein kinase activity, cell extracts were immunoprecipitated with anti-gag anti-serum in the presence of $\alpha^{32}$P-ATP. In such an experiment, P142 was phosphorylated (data not shown). A similar result was previously obtained with the FSV and UR1 polyproteins (Feldman et al., 1980; Wang et al., 1981).

## D. Isolation of Molecular Clones of Human DNA Related to v-onc Genes

The promoter insertion model implies that activation of one of a set of normal cellular genes (c-onc genes) can result in neoplasia. This suggests that activation of c-onc genes by agents other than viruses, such as chemical carcinogens and radiation, might also result in neoplasia. Since c-onc genes are present in all vertebrates, including man, it seemed reasonable that activation of c-onc genes might be involved in human cancer. As a first step towards investigating this question, I have

selected molecular clones of human DNA related to two c-onc genes, myc and src.

Molecular cloning of human myc-related DNA. A human genomic library (Ramirez et al., 1979) was kindly provided by Arthur Bank, Columbia University. This library consists of 15-20 kb fragments of a partial Eco R1 digestion of human DNA from a patient with β-thalessemia that have been ligated to the bacteriophage Ch 4A (Blattner et al., 1977). Recombinants were screened by the procedure of Benton and Davis (1977). Initially, the probe was the 1.9 kb fragment (right fragment) of a Sal I digest of the λtjf-7 3.1 kb Eco R1 insert (see Fig. 11). Several clones showed homology to this probe. Restriction analysis indicated that none of the clones analyzed were overlapping (data not shown). When these clones were tested for their ability to hybridize to v-myc probe, surprisingly, only one clone, λHm-10, showed detectable homology. It is not clear why so many other clones were detected with the tjf-7 Sal I fragment. Apparently there is some sequence within the putative intron of tjf-7 that is homologous to a sequence present in multiple copies in the human genome. This sequence is not present in multiple copies in chicken DNA, however, since genomic blots of chicken DNA hybridized with this probe show only a single band of hybridization (S. M. Astrin, personal communication).

Digestion of λHm-10 with Eco R1 gives three fragments in addition to the lambda arms: a 5.4 kb fragment, which comigrates with a 5.1 kb fragment, and a 3.2 kb fragment. Blotting and hybridization to v-myc probe (Fig. 21) show that only the comi-

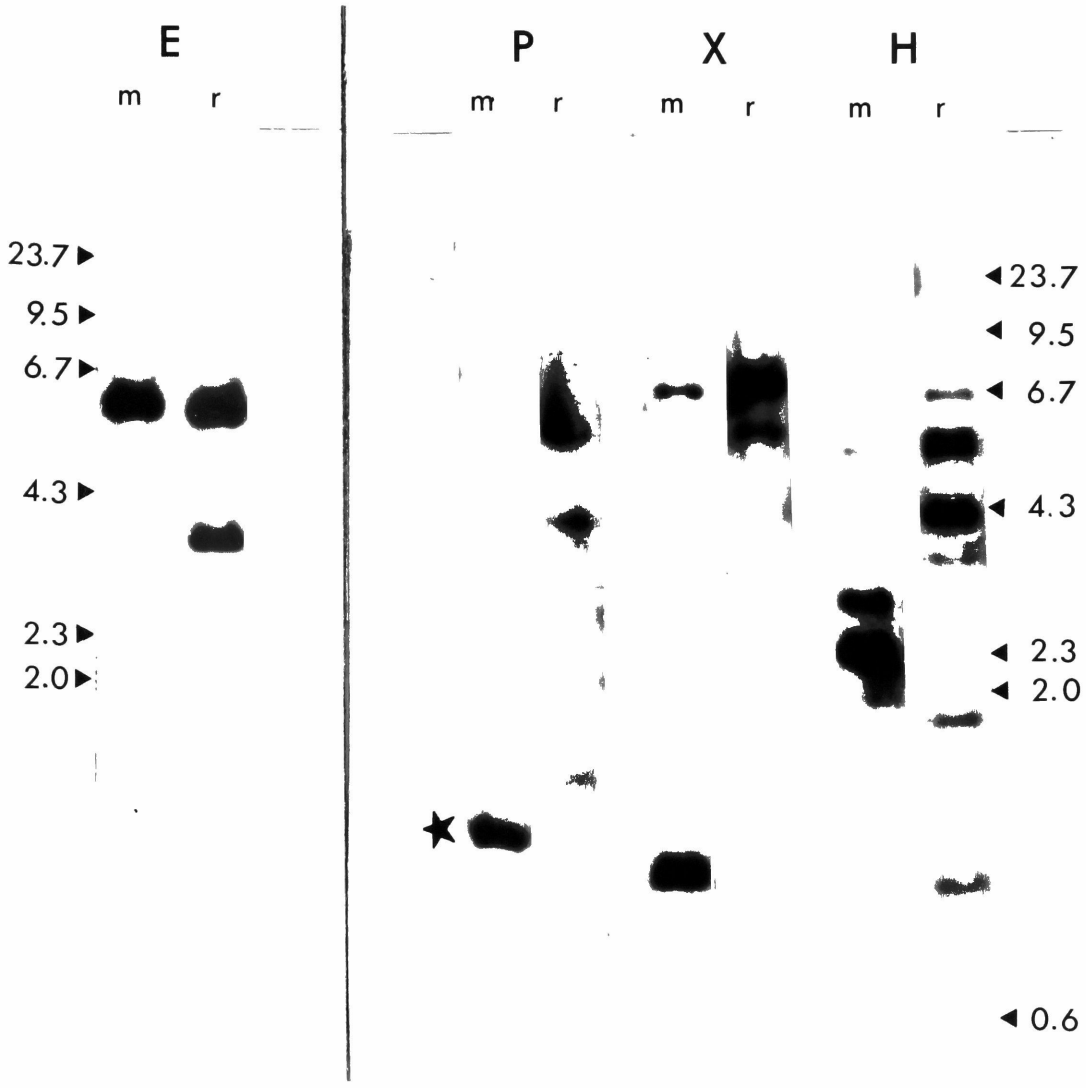
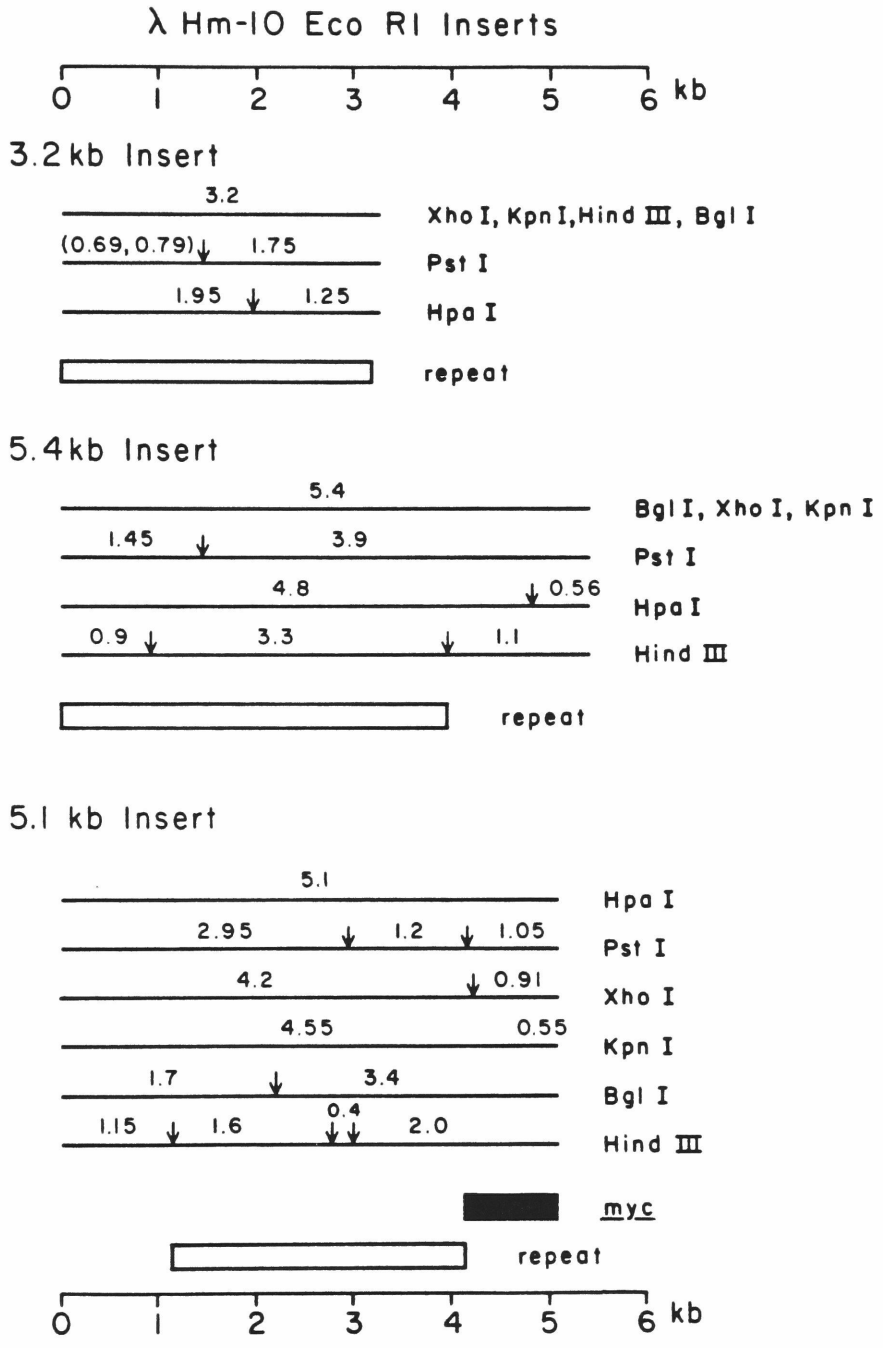



**Figure 21. Localization of myc-specific and human repeat** sequences in λHm-10. Left panel: λHm-10 DNA was digested with Eco R1 (E), electrophoresed in an agarose gel, and transferred to a nitrocellulose filter. The blot was hybridized successively to $^{32}$P-labeled v-myc probe (m) and $^{32}$P-labeled total human DNA, which is effectively a probe for repeated sequences (r).
Right panel: The 5.0-5.5 kb doublet generated by Eco R1 digestion of λ Hm-10 DNA was eluted from a preparative gel, digested with various restriction enzymes, electrophoresed, and transferred to nitrocellulose. The blot was hybridized successively to v-myc (m) and repeat (r) probe. The asterisk indicates the 1 kb myc-specific, repeat-free Eco R1/Pst I fragment that has been subcloned into pBR322 (see text). [P=Pst I, X=Xho I, H=Hind III] For each panel, the numbers indicate the position of migration of $^{32}$P-labeled λ-Hind III markers.

grating bands at 5.0-5.5 kb contain v-myc-related sequences; there is no hybridization to the 3.2 kb fragment. By isolating the 5.0-5.5 kb doublet and digesting with a number of different restriction enzymes, it was possible to derive limited restriction maps for the 5.1 kb and 5.4 kb fragments. For example, digestion of the mixture of fragments with Kpn I gave two new bands, of size 4.55 and 0.55 kb, but left a 5.4 kb band as well. Digestion with Pst I gave 5 bands, of sizes 3.9 kb, 2.95 kb, 1.45 kb, 1.2 kb and 1.05 kb. Digestion with both Kpn I and Pst I resulted in the 1.05 kb Pst I fragment being digested with Kpn I, with no other changes in the restriction pattern observed in digestions with Pst I alone (data not shown). This indicated that the Pst I 1.05 kb fragment and the Kpn I 4.55 kb and 0.55 kb fragments were derived from the same (5.1 kb) Eco Rl fragment in the original λHm-10 clone. Similar analyses with other restriction enzymes allowed the construction of the maps shown in Fig. 22. A few restriction sites were also determined for the 3.2 kb Eco Rl fragment (Fig. 22, top), which was first purified by gel electrophoresis. In λHm-10, the 5.1 kb Eco Rl fragment is linked to the left arm of the Ch 4A vector (data not shown), but the relative orientation of the other two Eco Rl fragments is not clear. However, preliminary experiments with partial digests of λHm-10 indicate that the order is 5.1-5.4-3.2 (data not shown).

Human DNA contains several types of repeated sequences dispersed throughout the genome (Houck et al., 1979; Jelinek et al., 1980). The presence of repeated sequences in Hm-10 would prevent its use as a probe for levels of myc-related RNA in normal and tumor tissues. Hybridization of radiolabeled total human

Figure 22. Restriction maps of λHm-10 Eco R1 inserts. Maps of the three Eco R1 inserts of λHm-10 were determined as described in the text. The order of fragments listed within parentheses is uncertain. v-*myc*-related information is located within the indicated region (solid bars), whereas human repeat-related information is located within the regions indicated by open bars. The precise location of *myc*- or repeat-related information within these regions is not yet known.


λ Hm-10 Eco RI Inserts
0 1 2 3 4 5 6 kb
3.2 kb Insert
3.2
Xho I, Kpn I, Hind III, Bgl I
(0.69, 0.79) 1.75
Pst I
1.95 1.25
Hpa I
repeat
5.4 kb Insert
5.4
Bgl I, Xho I, Kpn I
1.45 3.9
Pst I
4.8 0.56
Hpa I
0.9 3.3 1.1
Hind III
repeat
5.1 kb Insert
5.1
Hpa I
2.95 1.2 1.05
Pst I
4.2 0.91
Xho I
4.55 0.55
Kpn I
1.7 3.4
Bgl I
1.15 1.6 0.4 2.0
Hind III
myc
repeat
0 1 2 3 4 5 6 kb

DNA to a blot of Eco R1-digested λHm-10 indicated that both the 5.0-5.5 kb doublet and the 3.2 kb fragment had homology to human repeat DNA. Since single copy DNA represents a very small fraction of the total human genome, a radiolabeled total human DNA probe would only detect repeated sequences.

In an effort to localize myc-related and repeat-related information within Hm-10, λHm-10 DNA was digested with a variety of restriction enzymes and blotted. The blots were then hybridized consecutively to v-myc and total human DNA probe (Fig. 21, right). From this analysis, it is clear that all of the myc-related sequences in λHm-10 reside in the 5.1 kb insert within the 1.05 kb Pst I fragment. This fragment (Fig. 21, asterisk) is free of repeat information. Sequences in Hm-10 related to repeat DNA have been localized to the regions indicated in Fig. 22 (open bars). The precise position(s) of the repeat sequences within the indicated regions in Fig. 22 has not yet been determined.

The 1.05 kb Pst I fragment, which contains all of the myc-related information in λHm-10, is too small to encode all of the information present in the chicken c-myc gene (at least 1.6 kb, see Fig. 12). To determine what part of chicken c-myc is present in Hm-10, the 1.05 kb Pst I fragment was subcloned into pBR322 to give clone pHm-10, radiolabeled, and hybridized to a blot of λc-myc 2 DNA digested with Eco R1 in combination with other restriction enzymes. The results are shown in Fig. 23. The pHm-10 probe detects only the 1.9 kb Sal I fragment, the 0.74 kb Bal I fragment, the 0.83 kb Hinc II fragment, and the 0.85 kb Cla I fragment of c-myc. The other, faint bands in Fig. 23 do not correspond to c-myc restriction fragments and probably represent

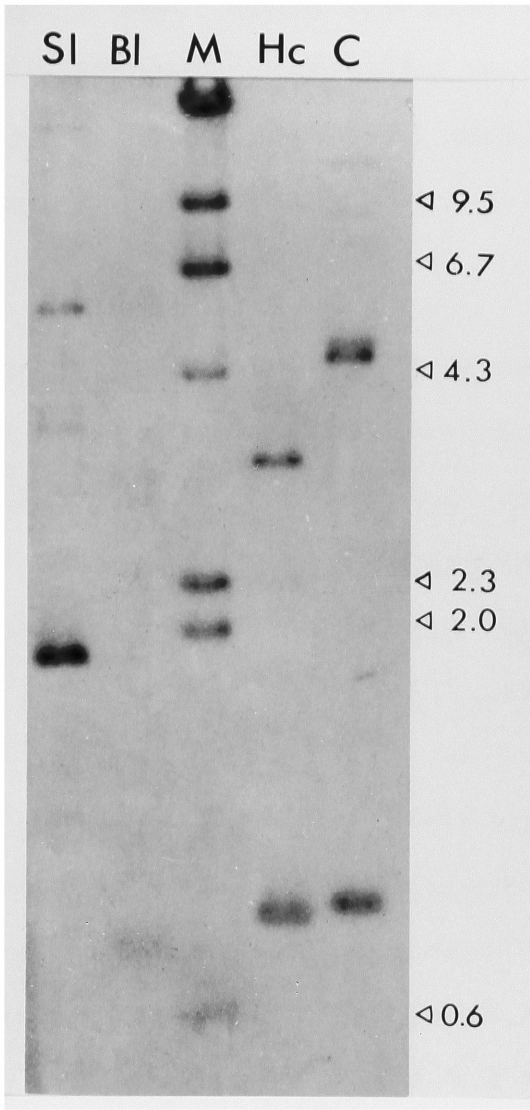



Figure 23. The extent of myc-related information present in λHm-10. λc-myc 2 was digested with Eco Rl in combination with the indicated restriction enzyme, electrophoresed and transferred to nitrocellulose. The blot was hybridized to $^{32}$P-labeled pHm-10, a subclone of λHm-10 that contains all of the v-myc-related information present in λHm-10 (see text and Figs. 21, 22). The positions of migration of $^{32}$P-labeled λ-Hind III fragments and their sizes (in kb) are indicated by open arrows. [Sl=Sal I, Bl=Bal I, M=marker fragments, Hc=Hinc II, C=Cla I]

partial digestion of the λcmyc 2 DNA. Since the 0.83 kb Hinc II fragment and the 0.85 kb Cla I fragment overlap by only 100-150 nucleotides (see Fig. 11), there are only 100-150 nucleotides of sequence within Hm-10 that are strongly homologous to chicken c-myc.

Molecular cloning of human src-related DNA. Recombinant bacteriophage from the human library were also screened with a probe for the v-src gene (that also included a small amount of env information), generously provided by Tatsuo Takeya, Rockefeller University. From 800,000 bacteriophage screened, two identical clones, λHs-9 and λHs-14, were obtained. Only λHs-9 was further characterized.

Digestion of λHs-9 with Eco Rl yields two restriction fragments in addition to the vector arms: a 9.4 kb fragment and a 5.3 kb fragment. Both of these fragments hybridize to the v-src probe in a Southern (1975) analysis (Fig. 24). The 9.4 kb fragment apparently has more src information than the 5.3 kb fragment, judging by the relative intensities of the bands in Fig. 24. Both fragments also contain human repeat sequences, as seen by their hybridization to total human DNA probe. The relative intensities of the 9.4 kb and 5.3 kb bands with this probe are approximately the same.

To begin to localize src- and repeat-related sequences within Hs-9, the 9.4 kb and 5.3 kb fragments were purified by gel electrophoresis and their restriction maps determined (Fig. 25). As can be seen by the relative intensities of hybridization using src and repeat probes on the same blot of λHs-9 digested

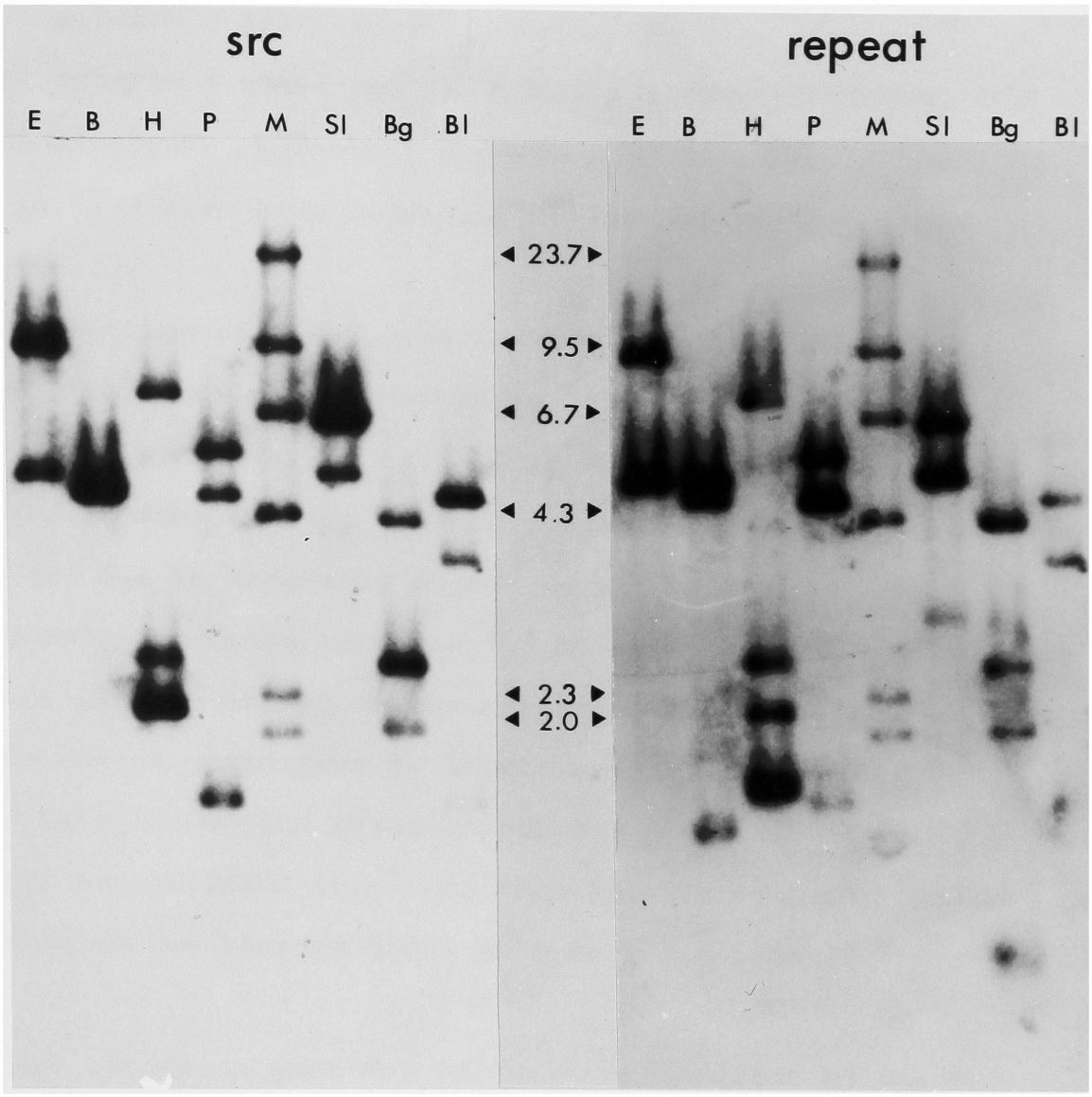


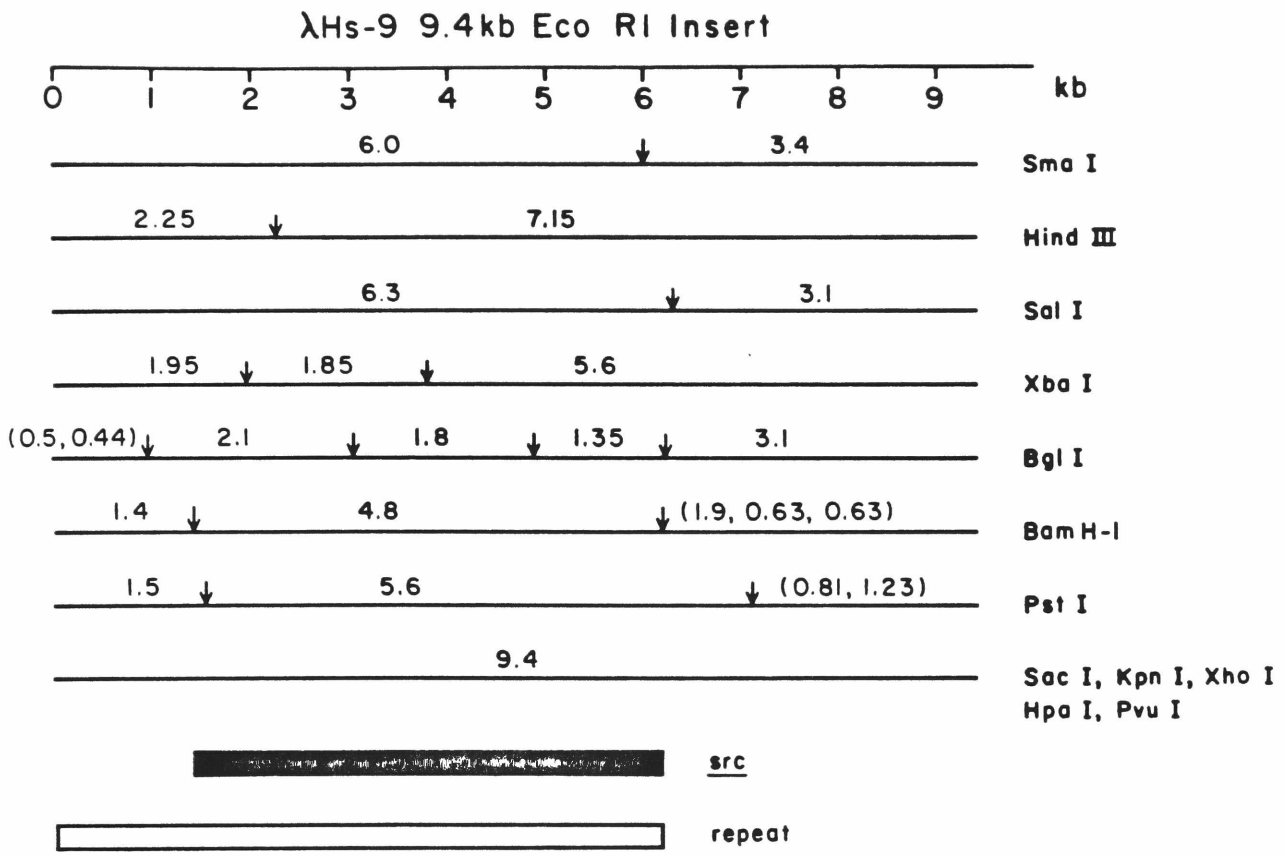

Figure 24. Localization of src- and repeat-related sequences in λHs-9. λHs-9 was digested with Eco Rl alone (E) or in combination with various other restriction enzymes electrophoresed, and transferred to nitrocellulose. Blots were hybridized sequentially to v-src (src) and total human DNA (repeat) probes (see text). The positions of migration of λ-Hind III size markers are shown. [B=Bam Hl, H=Hind III, P=Pst I, M=marker fragments, Sl=Sal I, Bg=Bgl I, Bl=Bal I]

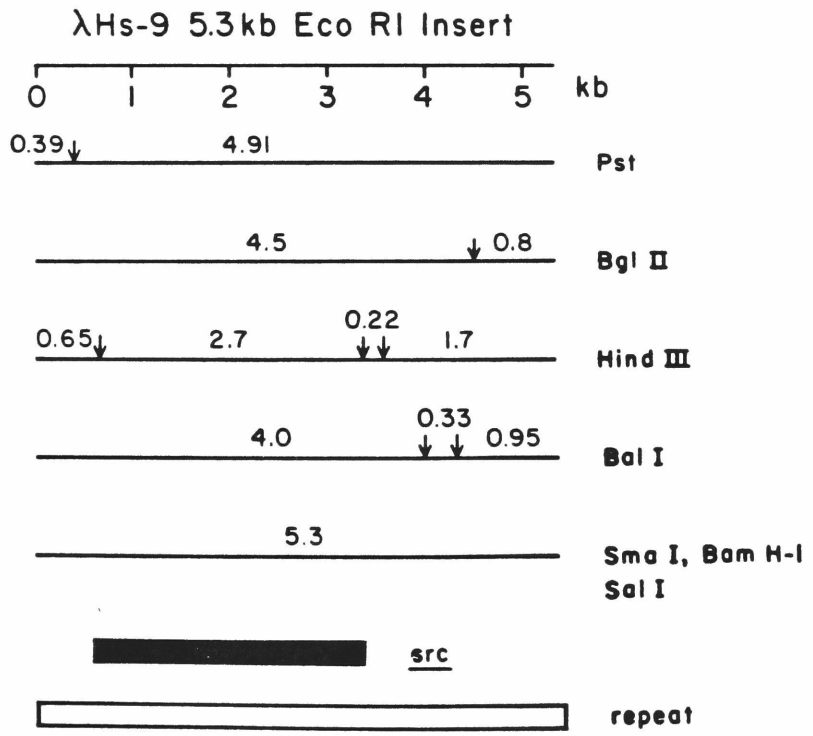
with Eco R1 alone, or in combination with other restriction enzymes (Fig. 24), src-related and repeat-related sequences in Hs-9 have a different distribution. Using the same type of analysis as described above for the human myc-related clones, the src-related (cross-hatched bars) and repeat-related (open bars) sequences in Hs-9 have been localized within the regions indicated in Fig. 25.

One implication of these localization experiments is that the human src-related locus probably has introns, as the chicken c-src gene does (Shalloway et al., 1981; Takeya et al., 1981). This follows from the finding that the src-related regions in the 9.4 kb and the 5.3 kb fragments do not extend to the ends of either fragment. Assuming that the 5.3 kb and the 9.4 kb fragments were originally linked in the chicken genome, the src-related information would thus be interrupted by non-src sequences. This assumption seems reasonable since it is highly unlikely that two unlinked sequences that ligated randomly during the generation of the library would both have src-related sequences.

It is not yet clear what the relative orientation of the 9.4 kb and the 5.3 kb fragments is in λHs-9. Preliminary experiments suggest that the 5.3 kb fragment contains sequences homologous to the extreme left end of chicken c-src, but further analysis is necessary to determine the precise representation and localization of chicken c-src-related sequences in the λHs-9 clone.

Figure 25. Restriction maps of λHs-9 Eco R1 inserts. Restriction maps were determined for the 9.4 kb and 5.3 kb Eco R1 inserts of λHs-9. The order of fragments shown in in parentheses is uncertain. Regions containing v-src-related (solid bars) or repeat-related (open bars) information are indicated. The precise location of src- and repeat-related sequences within these regions is not known.


λHs-9 9.4kb Eco RI Insert
0 1 2 3 4 5 6 7 8 9 kb
6.0 3.4 Sma I
2.25 7.15 Hind III
6.3 3.1 Sal I
1.95 1.85 5.6 Xba I
(0.5, 0.44) 2.1 1.8 1.35 3.1 Bgl I
1.4 4.8 (1.9, 0.63, 0.63) Bam H-I
1.5 5.6 (0.81, 1.23) Pst I
9.4 Sac I, Kpn I, Xho I Hpa I, Pvu I
src
repeat


λHs-9 5.3kb Eco RI Insert
0 1 2 3 4 5 kb
0.39 4.91 Pst
4.5 0.8 Bgl II
0.65 2.7 0.22 1.7 Hind III
4.0 0.33 0.95 Bal I
5.3 Sma I, Bam H-I Sal I
src
repeat

## Discussion

### A. Lymphomagenesis by ALV

Virus-related DNA and RNA in ALV-induced lymphomas. Analysis of the virus-related DNA and RNA leads to the following general conclusions: (1) Viral gene products are not required for maintenance of neoplastic transformation. Many of the proviruses studied by S. Astrin were found to be extensively defective, as shown by the absence of the 2.4 kb internal 5'-containing Eco R1 fragment in these tumors. ALV proviruses in lymphomas examined by other investigators were also defective (Payne et al., 1981, 1982; Fung et al., 1981). Similarly, I have found that over 50% of ALV-induced lymphomas fail to express 35S and/or 21S viral mRNAs (Fig. 7). (2) At least one copy of the LTR is present in all tumors. Astrin has identified 5'-containing tumor junction fragments in all tumors. Similarly, all ALV-induced tumors examined by Payne et al. (1981, 1982), Fung et al. (1981) and Cooper and Neiman (1981) had at least one copy of the LTR. (3) ALV-induced lymphomas are clonal. Unique proviral integration sites were detected by restriction analysis in the DNA from all primary bursal tumors. Metastases had the same restriction pattern as bursal tumors in the same animal, suggesting that all of the tumor cells are descendants of a single, infected cell. This result has also been obtained by others (Neiman et al., 1980b; Payne et al., 1981; Fung et al., 1981). Thus, the initiating event in ALV lymphomagenesis is probably a rare event. (4) Tumors from different birds have integration

sites in common. Restriction analysis revealed that the same tumor junction fragments were present in tumors from different birds. This suggested that proviruses in tumors were integrated adjacent to a limited number of cellular genes or, alternatively, at a limited number of sites adjacent to the same cellular gene. (5) ALV-induced lymphomas contain discrete new poly(A) RNAs consisting of viral sequences (R+U5) covalently linked to cellular sequences. Most tumors contained either a 2.5 or 2.9 kb new RNA that had no viral information other than sequences homologous to 5' probe (Figs. 7 and 8). Since these new RNAs fall into a limited number of size classes, they most likely represent transcription of the same or a limited number of cellular genes. (6) These new RNAs are expressed at high levels in lymphomas. Hybridization kinetic analysis by W.S. Hayward indicated that the levels were 30-100 times higher than the levels of expression of c-onc genes in uninfected cells (Hayward, 1977; Wang et al., 1977; Spector et al., 1978a,b; Sheiness and Bishop, 1979; Chen, 1980; Shibuya et al., 1982). (7) There is no evidence for the participation or generation of of a rapidly transforming virus in ALV-induced lymphomas. No virus capable of transforming fibroblasts was recovered from any of the lymphomas tested (e.g. 34L, Fig. 18). Viruses containing the v-myc gene transform fibroblasts with high efficiency (Langlois et al., 1970; Graf, 1973). Virus from one sample, RP9, was further tested by injection into 1-7 day old chickens. Lymphomas appeared after a long latent period (>4 months), but no rapidly developing neoplasia was observed. In addition, the structure of the myc-specific RNAs in lymphomas is not consistent with that of a viral genome. All

known defective transforming viruses contain viral sequences at the 5' and 3' ends of their genome (see Fig. 2). Sequences at the 3' end encode recognition signals for reverse transcription of viral RNA, integration of proviral DNA and synthesis of viral mRNA. Analysis of RNA from ALV-induced lymphomas revealed with rep probe (Fig. 8) and with a probe specific for the c region (data not shown) revealed no viral 3' information. Furthermore, sequences thought to be involved in RNA packaging (Linial et al., 1978), located in the region from about 100 to 350 nucleotides from the 5' end (Shank and Linial, 1980), are not present in the tumor-specific RNAs (Fig. 8 and G. Gasic and W.S. Hayward, personal communication).

Activation of c-myc by promoter insertion. These data suggest that lymphomagenesis by ALV requires proviral integration adjacent to a specific cellular gene and "read-off" from the viral promoter in the LTR, resulting in enhanced expression of the cellular gene. This can account, at least in part, for the observed latency in ALV-induced oncogenesis. In the vast majority of ALV-induced lymphomas (>80%; Hayward et al., 1981b; S.M. Astrin, unpublished), this cellular gene is c-myc, the cellular counterpart of v-myc, the transforming gene of MC29 virus (Fig. 26). This follows from three observations: (1) W.S. Hayward's finding that c-myc expression is 30-100 times higher in ALV-induced lymphomas than in normal tissue; (2) that the new RNAs containing viral 5' information also contain c-myc information (Fig. 9); and (3) that tumor junction fragments contain viral 5' information as well as c-myc information (S.M. Astrin's results

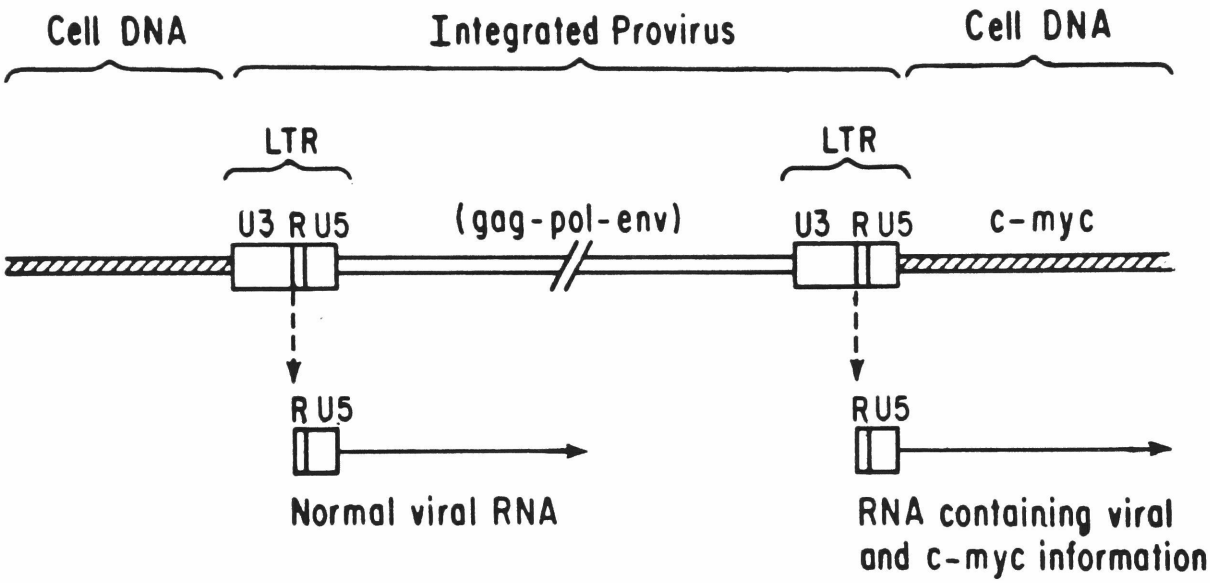



Figure 26. Activation of c-myc by promoter insertion in ALV-induced lymphomagenesis. The ALV provirus is shown integrated upstream of the bulk of c-myc coding information in an orientation such that new RNAs could initiate within the LTR and continue downstream into c-myc. This orientation is observed in the vast majority of ALV-induced lymphomas (see text).

and the results of molecular cloning experiments; see Fig. 10). Since tumor junction fragments of different sizes have myc information, integration at several sites upstream of c-myc can apparently lead to activation of this gene.

It is unclear why c-myc should be the gene activated in such a high percentage of ALV-induced lymphomas. Many other c-onc genes have been identified (see Introduction), and others undoubtably remain to be discovered by either tumor virologists or tumor viruses. One possible reason for preferential involvement of c-myc is tissue specificity for neoplastic transformation by a given onc gene. In tissue culture, MC29 transforms both hematopoietic cells (Graf, 1973) and fibroblasts (Langlois et al., 1970; Graf, 1973). Until recently, it was thought that MC29 and other v-myc-containing viruses did not cause lymphomas. Earlier workers reported that MC29 caused myelocytomatosis, fibrosarcomas, and carcinomas in infected birds (Langlois et al., 1970; Graf and Beug, 1978), with the relative incidence of these neoplasms depending mainly on the route of injection of the virus. Recent experiments, however, have demonstrated a fairly high incidence (~20%) of lymphomas in MC29-infected chickens (C. Moscovici and W.S. Hayward, personal communication). These lymphomas developed more slowly (1-3 months) than other MC29-induced neoplasms; presumably earlier workers failed to report lymphomas induced by ALV because many of the birds died first from other neoplastic diseases. The lymphomas observed were clearly not due to the helper virus MCAV (MC29 virus-associated virus), because (1) no lymphomas were observed during the first four months in birds infected only with MCAV, and (2) the MC29-induced lymphomas

expressed high levels of 5.5 kb MC29 RNA, which is easily distinguished from the 2.5-2.9 kb myc-specific RNA in ALV-induced tumors (C.-K. Shih and W. S. Hayward, personal communication).

Tissue specificity alone, however, may not be adequate to explain the preferential involvement of c-myc in B-cell lymphomas induced by ALV. Abelson murine leukemia virus, also induces B-cell lymphomas and transforms lymphocytes in vitro (Rosenberg and Baltimore, 1980), yet there is no evidence that c-abl is activated in ALV-induced lymphomas. It is possible that other c-onc genes are activated by ALV in the ALV-induced lymphomas that do not show enhanced myc expression. However, these account for a small percentage of the lymphomas. Similarly, since MCAV induces primarily lymphomas (after the appropriate latent period), and since the infectivity of MC29(MCAV) is determined by MCAV, it is unclear why the spectrum of neoplastic disease induced by MCAV and MC29/MCAV would not be the same, were all c-onc genes equally susceptible to activation by ALV. But lymphomas account for a relatively small fraction of the neoplasms induced by MC29(MCAV) (C. Moscovici and W.S. Hayward, personal communication).

It seems likely, then, that other factors favor activation of c-myc by ALV. Breindl et al. (1980) have suggested that proviruses integrate preferentially into transcriptionally active regions of chromosomes. If c-myc is in a transcriptionally active conformation at some stage of B-cell differentiation, integration within this region might occur with higher than random frequency. It is interesting that c-myc appears to be expressed at higher levels in the bursae of 20-30 day-old

chickens than in either older or younger chickens (C.-K. Shih and W.S. Hayward, personal communication). However, there is as yet no evidence that this increased expression correlates with an altered chromosomal conformation of c-myc. Alternatively, c-myc may be more readily activated by the promoter insertion mechanism due to some structural feature(s) of this gene. Integration at several sites upstream from c-myc can result in its activation. This multiplicity of potentially "productive" sites might increase the probability of activating this gene over other c-onc genes, which might only be activated by integration at a more limited number of sites. Another possibility is that proviral integration might occur with higher than random frequency in the c-myc locus because of some as yet undetermined sequence presence in c-myc. However, it should be emphasized that no specificity for cellular acceptor sites has been demonstrated for any retrovirus (see Introduction).

Defectiveness of the integrated provirus. As mentioned above, most of the proviruses integrated in the vicinity of c-myc in ALV-induced lymphomas have been found to be defective. In every case where only a single integrated provirus was detected, that provirus was found to be defective based on restriction analysis (results of S.M. Astrin; see also Payne et al., 1981, 1982; Fung et al., 1981) and/or the absence of 35S and/or 21S viral mRNAs. Many tumors with multiple integrated proviruses also lacked the internal fragment of the provirus, and, in a large proportion of those that contained this fragment, it was present at substantially less than one copy per cell (as

estimated by band intensity). The internal fragments in these tumors probably represented proviruses introduced by secondary infections of a minor portion of the cells at some later stage in tumor development. In many tumors, only one of the two proviral LTRs was detected. Noori-Daloii et al. (1981) reported that one lymphoma induced by reticuloendotheliosis virus (REV) contained an essentially intact provirus integrated adjacent to the c-myc gene (see below). However, these investigators did not determine whether a small part of the REV provirus (for example, the left LTR) was absent in this tumor. Payne et al. (1981) have reported an ALV-induced lymphoma in which only the left LTR may be missing from the provirus integrated adjacent to c-myc.

Several tumors contained proviruses integrated at multiple sites, and in roughly equimolar amounts. Since none of the integration sites, other than those adjacent to c-myc appeared to be common to more than one tumor, it is likely that only proviruses adjacent to c-myc are at all relevant to neoplastic transformation. The determination of the restriction map of c-myc (Fig. 10) along with knowledge of the restriction map of several ALV strains has permitted the analysis of proviral sequences covalently linked to the c-myc gene. This was done by identifying restriction fragments that contained both myc-specific and viral sequences (S.M. Astrin, personal communication). All of the proviruses analyzed contained substantial deletions, which ranged in length from 0.5 kb to more than 7 kb. The minimum amount of viral information found in any of the lymphomas was about 0.4 kb--approximately the size of the LTR-- suggesting that the LTR alone is sufficient for activation of c-myc.

The high frequency of deletions within these proviruses suggests that defectiveness may play an essential role in ALV-induced lymphomagenesis. One possible explanation is that cells lacking any complete proviruses (and thus not expressing viral antigens) might be more likely to escape detection by the host immune system. These cells might then enjoy a selective advantage and could thus proliferate more rapidly and develop into tumors. This possible advantage might not apply to those tumors that contain complete proviruses integrated at other sites and synthesize apparently normal viral mRNA. It is possible, though, that these viral mRNAs are synthesized by proviruses that were introduced into the cells at more advanced stages of tumor development, or that the mRNAs are defective in ways not discernible by the hybridization techniques used. It is interesting that several tumors apparently expressed normal amounts of 35S mRNA but no detectable 21S mRNA. These tumors could contain almost intact proviruses and still fail to express the major viral antigenic determinant, the env gene product, since env is translated from 21S mRNA (see Introduction). There may be more subtle defects in 21S mRNA, rendering it untranslatable, in those tumors where it is expressed. A second possible explanation for a requirement for proviral defectiveness is that the viral promoter within the right LTR cannot be used efficiently if normal transcription (from the left LTR) is taking place. During normal viral RNA synthesis, chain elongation proceeds into the right LTR, beyond the site where RNA polymerase would bind in initiation of downstream transcription. Thus the binding of polymerase or other proteins involved in transcription from the right LTR

might be disrupted. It should be possible to test this idea by examining the relative efficiency in vivo or in vitro of a template containing tandem copies of the LTR. Finally, the observed defectiveness may reflect some property of the c-myc locus at a particular stage of development (perhaps the time when integration occurs). It may thus be related to the high frequency of integration adjacent to c-myc in ALV-induced lymphomas. Many of the proviruses integrated at sites other than adjacent to c-myc were also defective. Perhaps this high frequency of deletions reflects the functioning of some recombination/transposition activity in bursal cells.

It is not known whether the observed proviral defectiveness is generated before or after integration. Thus far no common features have been found among the many different defective proviruses that might suggest a specific mechanism for generating these deletions. The cases where a single LTR remains integrated adjacent to c-myc could be generated by homologous recombination between the LTRs of a complete provirus, but other deletions would presumably involve non-homologous events. It is clear, however, that if defectiveness is an essential step in ALV-induced oncogenesis, the probability that infection would lead to tumor formation would be substantially reduced. This would be another contributing factor to the latency of the ALV-induced neoplasia. If the deletion event occurs after integration, it might help explain the multi-stage kinetics of lymphoma induction (see Introduction).

The two-gene model of ALV lymphomagenesis. Cooper and Nei-

man (1980, 1981) used a different approach aimed at identifying genes responsible for ALV-induced lymphomas. High molecular weight tumor DNA was tested in transfection experiments for its ability to induce morphologic transformation of NIH/3T3 cells. They found that DNA from ALV-induced tumors caused morphologic transformation of recipient cells with a frequency substantially higher than that found with normal cell DNA. However, no viral LTR or coding sequences were detected in the DNA of the recipient transformed cells. This led them to conclude that proviral DNA sequences were not directly involved in oncogenesis (Cooper and Neiman, 1980).

Following the observation that c-myc is activated in most ALV-induced lymphomas, Cooper and Neiman (1981) analyzed their transformants to determine if c-myc had been transferred in their experiments. Tests of the DNA and RNA of donor lymphoma cells confirmed the existence of proviral integration adjacent to c-myc as well as the presence of RNA transcripts containing viral 5' and c-myc information. However, no new c-myc sequences were detected in the DNA of the transformed recipient cells transfected with the lymphoma DNA.

To reconcile these apparently conflicting observations, the authors proposed that two cellular genes are involved in lymphomagenesis: the c-myc gene, activated by proviral integration, and a second, unlinked gene, activated by an unknown mechanism that does not involve direct proviral integration. One possibility is that the activated c-myc gene causes, either directly or indirectly, further rearrangements of cellular genes, and that one of these rearrangements results in activation of the cellular

gene that is detected in the transformation assay. This is consistent with the multi-stage nature of lymphoma development. However, there are other possible explanations that account for the observed kinetics without invoking the involvement of another onc gene (see below). There are estimates that as many as 1,000 genes may be activated in transformation by retroviruses (Groudine and Weintraub, 1980) and papovaviruses (A. Levine, personal communication); one or more of these genes may be capable of causing transformation of NIH/3T3 cells. Cooper and Neiman's results do imply that the activated gene is structurally altered in cis. Nevertheless, the activity detected in the transfection assay may reflect a secondary consequence of c-myc expression that is not directly involved in lymphomagenesis.

The latency of lymphoma induction by ALV. The long latency of tumor induction by ALV and other slowly transforming viruses is only partly explained by the low probability of integration events that could lead to the activation of a potentially oncogenic cellular gene. As mentioned above, either a requirement for proviral defectiveness and/or the two gene model of Cooper and Neiman could contribute to the long latent period observed. It is also possible that the host immune response plays some important role; there may be a dynamic process between transformation and immune surveillance. Alternatively, it is possible that the target cell for transformation is only present at specific times in development. For example, if integration occurs preferentially within transcriptionally active regions as suggested by Breindl et al. (1980), integration near a specific

c-onc gene would occur only when this gene is normally expressed.

Structure of the c-myc gene in normal cells and in ALV-induced lymphomas. The structures of molecular clones containing provirus-host cell junctions from the DNA of two independent ALV-induced lymphomas were compared to the structure of a clone of the normal c-myc gene. The results of restriction mapping (Fig. 11), Southern blot-hybridizations (not shown) and DNA sequencing (G. Gasic, Rockefeller University) show that, in these two tumors, the right LTR of an ALV provirus has integrated adjacent to and upstream from the bulk of c-myc coding sequences. Integration has occurred without gross structural rearrangement of the c-myc locus. The provirus is oriented such that the LTR could activate downstream transcription of c-myc.

Because the clones analyzed contained proviral information at their right ends, it was not possible to analyze the effect of proviral integration on the integrity of upstream sequences (which probably include the normal c-myc RNA polymerase II initiation site). Noori-Daloii et al. (1981) have reported that integration of REV proviruses adjacent to c-myc is accompanied by deletion of c-myc sequences. However, it was not possible to determine from their data whether the deletions occurred upstream or downstream from the integrated provirus. This point is of interest because the region upstream from the right LTR may be a region of frequent deletion in ALV-induced lymphomas since proviral defectiveness occurs nearly all of the time as well. Alternatively, if the deletions in the REV-induced lymphomas involved sequences downstream from the integrated provirus, it

would be interesting to know if the bulk of c-myc coding sequences were perturbed.

In order to help localize potential coding sequences in the c-myc locus, the tjf-10 clone was compared to a clone containing the v-myc gene by restriction mapping (Fig. 12). There are two regions of v-myc-related sequences, which are probably exons, separated by about 1 kb of sequences unrelated to v-myc, which is probably an intron. The exon sizes are 0.72 kb (left) and 0.85 kb (right). The total amount of coding information ( 1.6 kb) shared between v-myc and c-myc is essentially identical to previously reported estimates of the size of the v-myc gene (Mellon et al., 1978; Sheiness et al., 1978). Also, Robins et al. (1982) have recently reported similar values for these putative c-myc exons based upon heteroduplex mapping of c-myc/v-myc hybrids. The restriction maps in the v-myc-related regions of the c-myc clone (derived from a Rhode Island Red chicken) and the tjf clones (derived from White Leghorn chickens) are identical to each other and to the v-myc clone. This suggests that c-myc information is highly conserved in chickens, in agreement with previous data (Sheiness and Bishop, 1979).

It is formally possible that the 1 kb of sequence between the two coding regions in c-myc that is not present in v-myc, which I have suggested is an intron, is actually present in the tumor-specific mRNAs. However, this is highly unlikely. The size of the tumor-specific mRNA produced in tumors 7 and 10 is 2.5 kb (Figs. 7 and 9). This RNA is not big enough to encode all of the information present in the 0.72 kb, 0.85 kb, and 0.96 kb regions in addition to 100-200 nucleotides of poly(A) and at

least 99 nucleotides (R+U5) from the ALV LTR. It seems highly unlikely that sequences in c-myc that are also present in v-myc would be absent from c-myc mRNA. Current evidence (S. Braverman and W.S. Hayward, personal communication) suggests that recombination between RNA tumor viruses occurs after packaging of the heterologous RNAs into virus particles. It is likely that recombination between slowly transforming viruses and c-onc genes involves packaging of the c-onc mRNA. If recombination involves a processed RNA intermediate, introns could not be regenerated, and v-myc should only contain sequences present in c-myc mRNA.

Based on these studies, I have constructed a model of what is now known about the c-myc locus and its putative transcript following integration of an ALV provirus (Fig. 27). The 0.85 kb and 0.72 kb "exons" are positioned relative to a few restriction sites. Together, they account for about 1.6 kb of information in the 2.5 kb mRNA produced in tumors 7 and 10. Whether or not the mRNA proceeds past the right end of the 0.85 kb "exon" is not as yet established. Payne et al. (1982) have presented some evidence that tumor-specific mRNAs may extend beyond the Eco R1 site in c-myc. Also, the tumor-specific RNA contains at least 99 nucleotides from the ALV LTR and 100-200 nucleotides of poly(A). Therefore, the entire region from the LTR to the 0.72 kb "exon" probably is not present in the 2.5 kb RNA. Instead, there is probably at least one more exon and one more intron, both of unknown length, within this region of c-myc. The finding of putative splice donor sites in both tjf-7 and tjf-10, in which integration has occurred 500 nucleotides upstream from tjf-7 (data of G. Gasic), suggests that there may be at least four

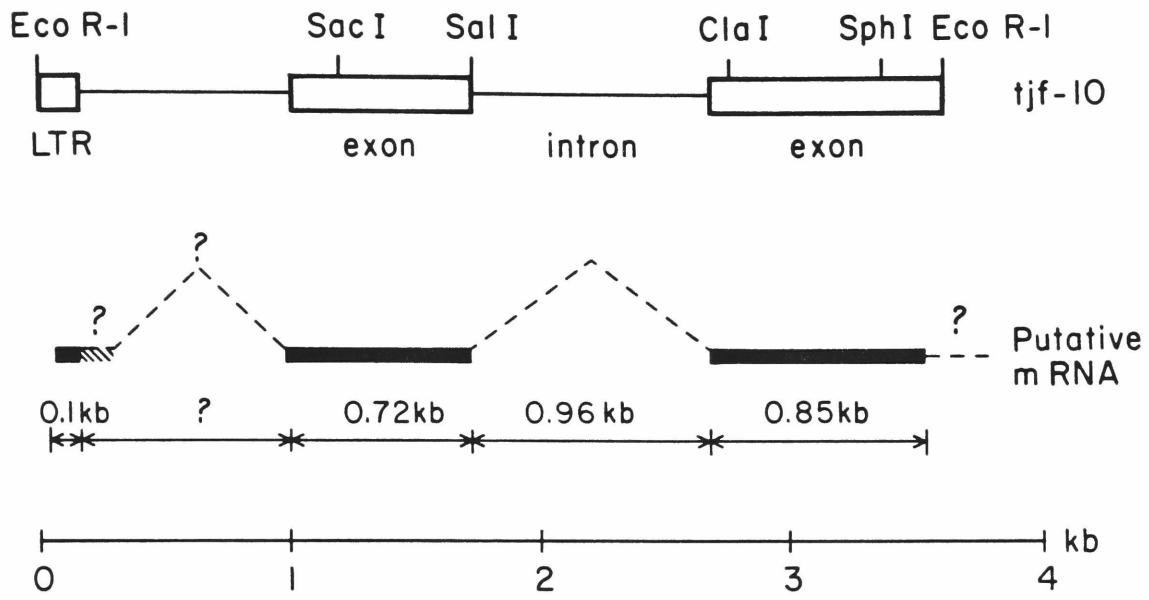



Figure 27. Model of the c-myc locus and its putative transcript following integration of an ALV provirus upstream from c-myc. The top of the figure shows a schematic view of the structure of the c-myc locus in tumor 10. Putative coding (exon) and non-coding (intron) domains are indicated, as are the ALV LTR and a few restriction sites. The bottom of the figure shows a model for the genesis of the tumor-specific 2.5 kb mRNA (see text). Transcription initiates at the Cap site in the viral LTR and proceeds downstream. Regions indicated by dashed lines are presumably removed by splicing. Uncertainties regarding the existence and sizes of additional exons and introns, as well as the precise 3' end of the mRNA are indicated by question marks (?).

exons and three introns in c-myc. The additional c-myc exons are probably small (not more than a few hundred nucleotides) and may form part of a leader sequence in c-myc mRNA. This suggestion is consistent with the multiple putative precursor RNAs observed in long exposures of Northern blots of tumor RNAs (Fig. 13). Since the exact number and sizes of additional exons and introns are as yet unclear, there are question marks in the appropriate regions of Fig. 27. Conclusive resolution of this issue awaits S-1 mapping of the tumor-specific mRNAs.

The data suggest that proviral integration in tumors 7 and 10 occurs upstream of all of the c-myc information that is found in v-myc, which probably represents the bulk of c-myc coding sequences as well. All other tumors studied by Astrin had Eco R1 tumor junction fragments of at least 2.7 kb in length. Assuming that integration in these tumors also occurred without gross alteration of c-myc, then integration in these tumors also occurred upstream of these putative coding sequences. However, the data also suggest that there are additional exons in c-myc. It is unclear whether these exons contain coding or leader sequences. This issue cannot be resolved conclusively at present because the protein encoded by the c-myc gene has not yet been identified and the DNA sequence of the entire c-myc locus (and thus the start codon for c-myc protein) has not been determined. I cannot unambiguously state that there is no alteration in c-myc coding information in tumors 7 and 10. However, there are a few tumors that had considerably larger tumor junction fragments--on the order of 5 kb (S.M. Astrin, personal communication). In vitro transcription experiments using the p-cmyc subclone as the

template (Fig. 16) indicate that the furthest putative initiation site for RNA polymerase II is located 4.4 kb upstream from the Eco Rl site at the right of c-myc (Fig. 17). Although it has not yet been demonstrated that either of the two putative initiation sites functions in vivo, comparisons of initiation sites determined by in vitro and in vivo experiments in other systems (Cepko et al., 1981; Fire et al., 1981) have, in general, been in good agreement. In particular, I am aware of no example in which specific initiation is detected in vitro and yet none of the initiation sites defined in this manner are utilized in vivo. In addition, Payne et al. (1982) have provided suggestive evidence that a potential initiation site in vivo is located about 3.5 kb upstream from the right Eco Rl site in c-myc. This corresponds with the site $I_1$ as defined in my in vitro transcription experiments. Preliminary studies aimed at examining precursor RNAs to the normal c-myc mRNA suggest that a c-myc promoter is located no more than 4 kb from the Eco Rl site. Thus it is likely that, at least in these few tumors with larger junction fragments, proviral integration has occurred without any alteration of c-myc.

The finding that there are at least two potential initiation sites within the c-myc locus is interesting. If both sites are used in vivo, they may be important in regulation of transcription from the normal c-myc locus. Multiple initiation sites that are differentially regulated have been identified in other eukaryotic systems such as the yeast invertase gene (Carlson and Botstein, 1982) and the mouse α-amylase gene (Young et al., 1981), as well as in prokaryotes. It will be particularly important to define the 5' ends of c-myc mRNA when c-myc expression is

slightly higher than normal (i.e., in the bursae of normal 20-30 day old chickens), compared with the 5' end of c-myc mRNA at other times.

Although my in vitro transcription results using the tjf-7 subclone are consistent with initiation occurring at the appropriate site (the normal viral Cap site) in the LTR, there does not seem to be any striking quantitative difference in the efficiency of transcription of the tjf-7 and c-myc templates. This could be because the in vitro transcription system lacks certain factors necessary for proper quantitative regulation of transcription. Alternatively, it may be that the tjf-7 clone, which begins at the Eco Rl site located about 60 nucleotides upstream of R, lacks LTR sequences upstream of this Eco Rl site that are positive modulators of transcription. This latter possibility is particularly interesting in light of the recent results of Grosschedl and Birnstiel (1982) in the sea urchin histone gene system. Previous workers had been unable to demonstrate any effect of sequences upstream from the TATA box on transcription in vitro. However, as mentioned previously (see Introduction), the major modulating sequence for transcription efficiency in vivo appears to be more than 60 nucleotides upstream from the normal initiation site. Grosschedl and Birnstiel, however, demonstrated that a sequence located 110-140 nucleotides upstream of the initiation site for the H2A mRNA was a positive modulator of transcription in vitro when circular DNA was used as the template and start sites were determined by the S1 mapping procedure of Berk and Sharp (1977). Using the truncated template assay for initiation sites, this upstream sequence

had no effect on the efficiency of in vitro transcription. They suggest that this is because free ends can mimic the function of the upstream modulator sequence, which they suggest is to provide an entry site for RNA polymerase II. It will be important to test the relative efficiency of in vitro transcription of the tjf-7 clone following ligation to the rest of the U3 sequences using an S1 assay.

The gene product of c-myc. The protein encoded by the c-myc gene has not yet been identified in either ALV-induced lymphomas or normal cells. This presumably is due to a technical difficulty, the inability to prepare antisera specific for the myc part of the gag-myc fusion protein of MC29 and related viruses. The myc fusion protein does not appear to have protein kinase activity (Bister et al., 1980; Sefton et al., 1980), unlike the many other v-onc proteins that have kinase activity. Recent evidence suggests that the myc fusion protein is located in the cell nucleus (R. Eisenman, personal communication), instead of on the cell membrane (as are several other onc proteins). This suggests the possibility that the c-myc protein may function by interacting directly with DNA. Perhaps it has transposase or "recombinase" activities, in light of the Cooper-Neiman "two gene" model. It is interesting in this regard that expression of c-myc may be somewhat higher in cells and tissues undergoing genetic rearrangements involving the immunoglobulin genes (T and B cells: T. Gonda, J.M. Bishop and H. Varmus, personal communication; early bursal tissue: C.-K. Shih and W.S. Hayward, personal communication).

Orientation of the provirus: the role of the LTR. In the majority of ALV-induced lymphomas analyzed thus far by our group, as well as other groups (Payne et al., 1981, 1982; Fung et al., 1981), the provirus is integrated in an orientation that would permit transcription to initiate at the viral promoter and continue into c-myc. Payne et al. (1982), however, have identified several tumors in which the provirus is integrated in other orientations. Of twelve tumors analyzed, four contained proviruses integrated upstream of c-myc but in the opposite transcriptional orientation. Another tumor contained a provirus integrated in the same transcriptional orientation, as c-myc, but downstream from the bulk of c-myc coding sequences (as defined by v-myc probe, see Fig. 12). Several of these tumors contained elevated levels of myc-specific RNA that was not linked to viral 5' sequences, suggesting that transcription of c-myc in this tumor did not initiate at the viral promoter.

Thus it appears that sequences within the provirus can exert a regulatory influence over c-myc without providing an initiation site. In the tumor in which integration had occurred downstream from c-myc, this regulatory influence acts at a distance several kilobases from the initiation site (presumably the c-myc promoter). In this tumor the myc-specific transcript appears to terminate in the viral LTR. It is possible that c-myc activation in this tumor results from a slightly different mechanism, related to poly(A) addition or termination signals in the LTR. Control of gene expression at the level of termination has been documented in several prokaryotic systems. Perhaps the most relevant system to downstream LTR activation of c-myc is the

phenomenom of retroregulation in bacteriophage lambda (Schindler and Echols, 1981). In this system, termination at alternative sites results in the presence or absence of a processing signal for RNase III, with resultant effect on mRNA stability. By providing alternative termination [or poly(A) addition] sites, the LTR could be functioning in a similar fashion. In the case of the upstream LTRs, the LTR could be functioning as an antiterminator, analogous to the regulatory elements described for several prokaryotic amino acid biosynthesis operons (see Yanofsky, 1981). This would require proviral integration downstream from the normal c-myc initiation site for RNA synthesis. It is not clear from the published data of Payne et al. (1982) exactly where the ALV provirus is integrated relative to the putative c-myc initiation sites in those cases where the provirus is upstream of c-myc but inverted.

The finding that alternative orientations of the LTR can lead to activation of c-myc is reminiscent of several studies in which putative regulatory elements have been linked in various orientations to cellular genes and their effects assayed in vivo. Vande Woude and his collaborators (Blair et al., 1980, 1981; Oskarsson et al., 1980) have cloned the transforming gene (v-mos) of Moloney murine sarcoma virus (MoMSV) and its cellular counterpart (c-mos). These cloned genes do not induce transformation at significant frequency in an NIH/3T3 cell transfection assay. However, when either c-mos or v-mos DNAs are linked to the MoMSV LTR, transformation occurs at high frequency. With v-mos, enhancement of transforming activity was observed when the LTR was linked either upstream or downstream from the coding

sequences (Blair et al., 1980). With c-mos, however, high frequency transformation was observed only when the LTR was located upstream from the coding sequences (Oskarsson et al., 1980; Blair et al., 1981). Recently DeFeo et al. (1981) have shown that ligation of an LTR upstream from the c-$ras_H$ gene (cellular counterpart of the transforming gene of Harvey murine sarcoma virus) can also cause transformation of NIH/3T3 cells. Ligation of LTRs at several sites downstream from this gene did not cause activation (DeFeo et al., 1981 and D. Lowy, personal communication). The reason for the different behavior of the v-onc and c-onc genes in the transfection assay is unclear at present. The LTR appears to be functioning, both in these transfection experiments and in ALV-induced lymphomas, in a manner similar to that of the SV40 72-bp repeat (Benoist and Chambon, 1981; Gruss et al., 1981; Banerji et al., 1981; Moreau et al., 1981), which appears to activate transcription in a bi-directional manner. The recent observation that a 72-bp repeat in the MoMSV LTR can replace the SV40 repeats functionally (Levinson et al., 1982) strengthens this comparison. With the report of Payne et al. (1982) that alternative orientations of the LTR can activate c-myc, S. Astrin and I rechecked all of our tumors for which sample was still available. In about 85% of those tumors where the provirus was integrated adjacent to c-myc, proviral orientation was such that the LTR could initiate downstream transcription. In about 15% of the tumors, it is still possible that alternative orientations exist. In one tumor, 9K, there are new myc-specific RNAs that do not correspond to bands detected by 5' probe. These RNAs are also not detected with a U3 probe (data not shown). Although the

DNA data for this tumor is confusing, the RNA data suggest that 9K may have an ALV provirus inserted upstream of c-myc but in opposite transcriptional orientation. An LTR located downstream of c-myc might be expected to give rise to myc-specific RNAs containing U3 information, as reported by Payne et al. (1982). In any case, it is clear that the majority of our ALV-induced tumors contain proviruses integrated upstream from c-myc and in the same transcriptional orientation. Kung and his collaborators (personal communication) also find that about 85% of ALV-induced lymphomas have the LTR upstream of and in the same transcriptional orientation as c-myc. If one accepts the notion that the LTR encodes both modulator (which can function in either transcriptional orientation) and selector (functional only in one transcriptional orientation) elements, then the observed orientation frequencies could be explained. An LTR integrating upstream of c-myc and in the same transcriptional orientation would already have a functional modulator/selector apparatus assembled. If integration occurred upstream but in inverted orientation, activation might require the recruitment of a cellular sequence that could replace the selector function. The number of potential sites at which the proper modulator/selector apparatus could be assembled might be limited and thus activation by this mechanism would be a less frequent event. Why Payne et al. (1982) have found such a high percentage (~40%) of alternative orientations compared to our group and Kung's group is unclear at present. The only differences between their protocols and ours were in time of injection and particular virus strains used.

Comparison of oncogenesis by ALV and MC29. Figure 28

compares the mechanism of oncogenesis by ALV with that of the rapidly transforming virus MC29, which contains the v-myc gene. The ALV provirus is shown integrated upstream of c-myc and in the same transcriptional orientation, as it is in the majority of ALV-induced lymphomas. Several important differences exist between these two viral/cell systems:

(1) MC29 virus and other rapidly transforming viruses contain a strong viral "promoter" already covalently linked to a c-onc gene. Thus, the site of integration is not important in transformation by these viruses. For the same reason, neoplasia occurs fairly rapidly. In ALV-induced lymphomas, however, transformation is only a rare consequence of integration occurring adjacent to a c-onc gene, which is usually c-myc. Thus ALVs are slowly transforming viruses.

(2) MC29 mRNA is apparently unspliced (Sheiness et al., 1981). The mRNA for the ALV-activated c-myc gene is processed to remove at least one, and probably more intervening sequences.

(3) MC29 RNA can serve as a viral genome that is packaged in virus particles and reverse-transcribed in the presence of a helper virus. The myc-specific RNA in ALV-induced lymphomas lack essential viral sequences at its 3' end (U3+R) required for reverse transcription, for transcription, and presumably for integration; it also lacks the (-) strand primer binding site (Taylor and Illmensee, 1975; Taylor, 1979) and sequences essential for packaging (Linial et al., 1978; Shank and Linial, 1980) located near the 5' end of the viral genome.

(4) The v-myc gene product of MC29 virus is a fusion protein containing both viral gag and myc sequences. Although the myc

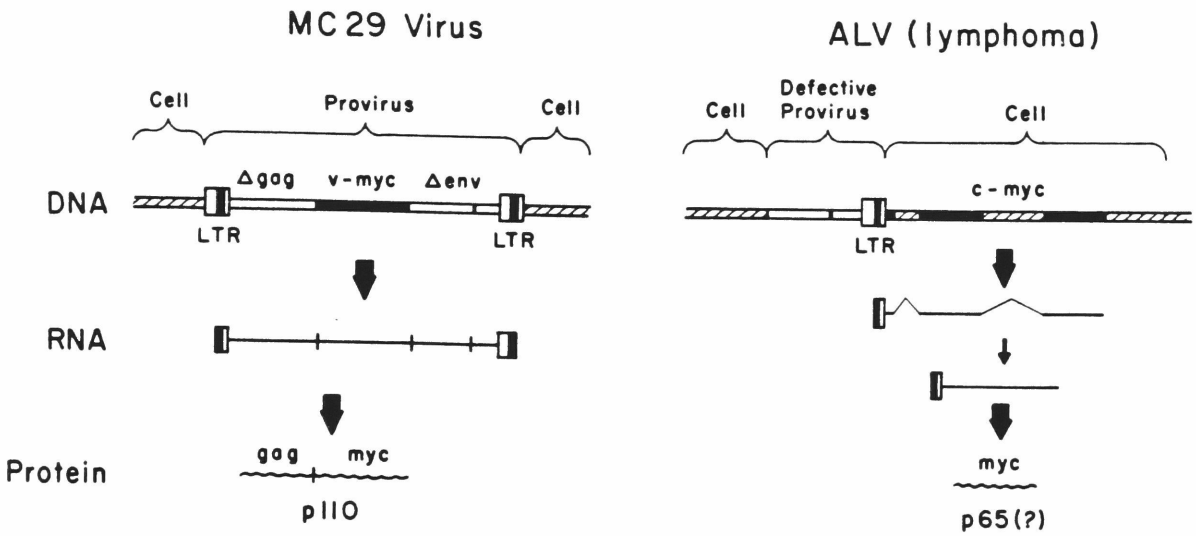



Figure 28. Comparison of oncogenesis by ALV and by the rapidly transforming virus MC29. The site of integration does not play a major role in transformation by MC29 since the onc gene (v-myc) is incorporated into the viral genome. Transformation by ALV occurs only when the provirus is inserted adjacent to a c-onc gene. A defective ALV is shown adjacent to c-myc, as found in most, if not all ALV-induced lymphomas (see text). The putative product ("p65") of the c-myc gene has not been isolated. Its size is an estimate based on the apparent coding capacity of the c-myc gene (see text).

protein in ALV-induced tumors has not been isolated, DNA and RNA analyses presented here indicate that it must lack all viral structural gene sequences. Thus the viral part of the MC29 fusion protein is probably not required for transformation by this virus, at least in B-cells. In all likelihood, the myc protein in ALV-induced lymphomas strongly resembles the normal c-myc gene product.

## B. The Generation of 16L Virus

This thesis also reports the generation of an acute transforming retrovirus containing sequences homologous to the fps gene following infection of a chicken with an avian leukosis virus. This virus presumably arose via recombination between the infecting leukosis virus and a source of fps information.

Several lines of evidence suggest that the fps information is of cellular origin:

(1) The tumor from which the virus was isolated arose at a site distal from virus injection, and only after a long latent period. If there was a contaminating acute virus such as FSV or URl in the initial virus stock, neoplasia would be expected to develop much more rapidly (2-4 weeks), and at the site of injection.

(2) No similar neoplasms were noted in other birds infected at the same time with the same virus stock. The virus stock used to infect these birds was cloned several times, and was not capable of producing foci on CEFs (Kawai et al., 1977).

(3) No fps-containing virus-infected birds were present in

this laboratory at the time of these experiments.

(4) 16L virus contains some RNase $T_1$ oligonucleotides present in the fps sequences of FSV but not UR1, and some present in UR1 but not FSV, as well as sequences not present in either of these viruses. Thus, if 16L virus were derived from a viral contaminant, there would have to have been either three contaminating viruses or a new, as yet undescribed contaminating virus. UR1 was not even available in this laboratory when tumor 16L was obtained. There are some oligonucleotides present in FSV or UR1 or both that are not present in 16L virus (Figs. 19 & 20). This could represent divergence from the c-fps sequence. Alternatively, only part of the c-fps gene (or its flanking sequences) may have been transduced in 16L virus. 16L virus appears to be more closely related to UR1 than to FSV. This may reflect the more recent isolation and less extensive passage history of UR1 (Balduzzi et al., 1981) compared with FSV (Fujinami and Inamoto, 1914).

(5) All of the non-fps oligonucleotides in 16L that correspond to retroviral structural and replicative genes are found in td107A. These oligonucleotides are distinct from those of the non-fps sequences present in either FSV or UR1. If recombination with a viral contaminant had occurred, one might expect 16L virus to contain non-fps information from the contaminant as well.

(6) No evidence was obtained for the presence in chicken 16 of another fps-containing virus, which should have its original 5' and 3' terminal sequences.

16L virus is a direct example of an avian rapidly

transforming virus arising from recombination in vivo between a slowly transforming virus and a cellular gene. Earlier, it was shown that viruses [termed recovered avian sarcoma viruses (rASVs)] that are recombinants between certain transformation-defective deletion mutants of Rous sarcoma virus and the cellular src gene could be isolated following injection of td mutants of Rous sarcoma virus into birds (Hanafusa et al., 1977; Wang et al., 1978; Halpern et al., 1979; Karess et al., 1979). However, rASVs were isolated only from chickens infected with mutants that retained a significant part (20-30%) of the viral src gene. The genetic structure of the other known avian rapidly transforming viruses (cell-related information inserted between viral 5' and 3' sequences) suggests that these viruses also arose via recombination between a slowly transforming virus and a cellular gene. However, in many cases, extensive passage of virus strains in vivo and in vitro occurred before their biochemical analysis was possible. While this work was in progress, Stavnezer et al. (1981) reported the isolation of defective rapidly transforming viruses following passage of ALV in CEFs derived from a certain line (SPAFAS) of chickens. These viruses presumably arose via a mechanism similar to that involved in the generation of 16L. The reason for the recovery of these viruses at such high efficiency in this particular tissue culture system is unclear.

In the murine system, the Harvey (Harvey, 1964) and Kirsten (Kirsten and Mayer, 1967) strains of murine sarcoma virus were isolated following serial passage of murine leukemia viruses in rats; Moloney murine sarcoma virus was isolated following leukemia virus passage in mice (Moloney, 1966). Similarly, the

feline sarcoma viruses (Snyder and Theilen, 1969; Gardner et al., 1970) were isolated from tumors induced in cats infected with feline leukemia viruses. As with the avian rapidly transforming viruses, however, biochemical analysis of these viruses was not performed at early passage, so no direct comparison could be made with the parent viruses. Recently, recombinant mammalian rapidly transforming viruses have also been obtained in tissue culture (Rasheed et al., 1978; Rapp and Todaro, 1980; Goldfarb and Weinberg, 1981a,b).

The promoter insertion model of ALV lymphomagenesis suggests a mechanism for the generation of rapidly transforming viruses that is consistent with the structure of 16L virus and other defective rapidly transforming viruses. Similar models have been suggested by others (Goldfarb and Weinberg, 1981b; Wei et al., 1981). If an abnormal integration event left a defective or permuted td107A provirus adjacent to c-fps, then transcripts containing td107A information at their 5' end and c-fps information at their 3' end would be generated. Large amounts of such transcripts would be produced since the viral long terminal repeat contains sequences implicated in efficient transcription by RNA polymerase II. Also, these transcripts should be efficiently packaged into virus particles since they would contain sequences known to be required for viral RNA packaging. These RNA molecules themselves could not serve as genomic RNA because they lack viral 3' sequences (U3+R) necessary for efficient replication. Non-homologous recombination between td107A RNA and these transcripts could generate a virus like 16L. Strong evidence in support of such proposed recombination events is found in the

work of Goldfarb and Weinberg (1981a,b). They demonstrated that MuLV superinfection of cells transfected with DNA of Harvey sarcoma virus mutants carrying deletions of varying length at their 3' ends resulted in the generation of recombinant sarcoma viruses of similar structure to 16L virus.

It is interesting that the cellular gene incorporated into 16L virus is c-fps. fps-related information is found in a number of independently derived rapidly transforming viruses of both avian (FSV, UR1, PRCII, 16L) and mammalian (Snyder-Theilen strain of feline sarcoma virus) origin (Hanafusa et al., 1980; Lee et al., 1980; Neil et al., 1980; Shibuya et al., 1980; Wang et al., 1981, and this report). In contrast, most other c-onc genes have been identified in relatively few independently isolated viruses. Whether this is merely due to the chance isolation of an abnormally large number of fps-containing viruses or, instead, reflects some specific property of the c-fps gene or its flanking sequences awaits further analysis of the c-fps locus.

Although several transforming viruses contain fps, the extent and the precise organization of fps information differs in different viruses. FSV, UR1, and 16L virus all appear to have approximately the same amount of fps, but PRCII has only about 60% of this (Shibuya et al., 1980 and this report). Comparison of the sizes of the fps-related inserts in feline sarcoma virus (Sherr et al., 1980) and FSV (Shibuya et al., 1982) suggests that feline sarcoma virus encodes only part of fps as well. Apparently only part of fps is necessary to cause cell transformation. The amount of gag-related information in the gag-fps fusion proteins differs as well (Ghysdael et al., 1981; B.

Mathey-Prevot and H. Hanafusa, personal communication). Since different viruses have different gag-fps junctions, it is unlikely that gag has specific regions necessary for recombination with cellular sequences.

Avian leukosis viruses, and presumably other slowly transforming viruses, thus appear to be able to induce oncogenesis via two routes. In either case, the fundamental oncogenic mechanism appears to be placing a cellular gene under viral transcriptional control. More commonly, random proviral integration places an ALV long terminal repeat adjacent to the c-myc gene of a lymphoid cell. The resultant activation of c-myc leads to lymphomagenesis. Much more rarely, the infecting ALV can recombine with a c-onc gene, for example c-fps, and generate a new rapidly transforming virus such as 16L virus. The failure of earlier workers to isolate such recombinants from chickens experimentally infected with ALV is probably because the combination of events necessary to generate such a virus occurs only rarely. One major unanswered question is why recombinant myc-containing rapidly transforming viruses are not recovered from ALV-induced lymphomas. At the present time, we are left with the unsatisfying explanation that the isolation of 16L was a chance occurrence of a rare event, and that not enough ALV-induced lymphomas were examined to come across a recombinant myc-containing virus.

C. The Relevance of ALV-induced Lymphomagenesis

Oncogenesis by slowly transforming viruses. Two ALV-induced

nephroblastomas have been analyzed (Cooper and Neiman, 1981; H.-J. Kung, personal communication); both contained proviruses integrated adjacent to the c-myc gene. Thus, elevated expression of the same c-onc gene appears to be able to induce neoplasia in two different tissue types. It should be noted, however, that Varmus (personal communication) has analyzed ALV-induced nephroblastomas and does not find proviral integration adjacent to c-myc.

Fung, Crittenden, and Kung (personal communication) have analyzed erythroblasts from leukemic line 15I chickens infected with ALV. ALV induces a high incidence of erythroblastosis in this chicken line (Bacon et al., 1981). Proviruses were found integrated in the vicinity of c-erb in erythroblasts from approximately 50% of the leukemic chickens. Levels of erb-specific RNA were elevated in these cells, although a complete analysis of the transcripts has not yet been performed. Erythroleukemia is the major neoplasm induced by viruses carrying v-erb. The observation that c-erb rather than c-myc is activated in ALV-induced erythroleukemias is important because it shows that c-onc gene activation by ALV is not restricted to the c-myc gene. Instead, the particular gene involved may be more related to the target tissue.

REV (helper virus) induces a long latency B-cell lymphoma in birds, similar to that induced by ALV. As mentioned above, Noori-Daloii et al. (1981) have recently found that REV-induced lymphomas, like ALV-induced lymphomas, contain proviruses integrated adjacent to c-myc. Although the expression of c-myc was not analyzed by these authors, it seems likely that c-myc is

activated by REV in the same way as by ALV. Although REV has a structure similar to that of ALV (i.e., the structure of a slowly transforming retrovirus), it is unrelated by nucleic acid homology. REV seems to be more closely related to mammalian viruses than to the ALV group (Hunter et al., 1978; Cohen et al., 1981; Rice et al., 1981).

In both ALV-induced erythroblastosis and REV-induced lymphomas, there is some evidence that the provirus and adjacent c-onc sequences have undergone selective gene amplification (Noori-Daloii et al., 1981; H.-J. Kung , personal communication). Whether this reflects aneuploidy of the c-myc containing chromosome or amplification of only a segment of this chromosome is unclear at present. It is interesting, though, that lymphomagenesis by MuLV is accompanied by a specific trisomy (see Klein, 1981).

As yet, no strong evidence is available demonstrating activation of c-onc genes by any of the mammalian retroviruses. It would be surprising, though, if this mechanism was not involved in oncogenesis by the slowly transforming mammalian retroviruses since they are similar to ALV in their basic structure and lack of a transforming gene. Several studies with mammalian retroviruses demonstrate striking parallels with the ALV system. Kettman et al. (1981) found that tumors induced by bovine leukemia virus contain apparently defective proviruses and do not express viral mRNA. As with ALV, the tumors are clonal with respect to integration sites, but integration at specific sites or activation of cellular genes has not been shown. Defective proviruses and absence of expression of viral gene products

have also been demonstrated for FeLV-induced neoplasia (Hardy et al., 1980). H. Varmus and his colleagues (personal communication) have found that a high percentage of MMTV-induced tumors contain proviruses integrated within a single 20 kb domain. Enhanced expression from this domain has not yet been demonstrated. It is possible, of course, that MMTV may be functioning as a negative modulator of some sequence within this domain (i.e., as an insertional mutagen) whose function is required for proper control of cell growth. The ability of retroviruses to inactivate genes has also been demonstrated (Varmus et al., 1981).

Two reports (Jahner et al., 1980; van der Puten et al., 1981) have claimed that MuLV integration in tumors is clonal, but not specific, and thus that a promoter-insertion mechanism is not operative in oncogenesis by this virus. However, these experiments had two major flaws. First, in order to eliminate the high background of endogenous viral sequences in these tumors, an "exogenous virus-specific" probe was prepared. It is not clear from their experiments to what part of the viral genome this probe corresponds. In the ALV system, it is clear that only a very small part of the viral genome (the LTR) is required to activate a c-onc gene. It is thus not clear whether the probe used by the above investigators was sensitive enough to detect such a small part of the viral genome. These investigators may therefore not have been detecting the "relevant integration". Another problem arises from their choice of restriction enzyme in their Southern blot analysis. They did not choose enzymes that cut near the end of the viral genome. Thus the different sized

virus-specific restriction fragments they observed may, in fact, have represented integration adjacent to the same or a limited set of cellular genes, but with various amounts of virus-related sequences deleted. It is clear in the ALV system that proviral deletions of various lengths occur adjacent to c-myc. It is also possible that integrations in tumors may be taking place at so many sites adjacent to the same cellular gene that discrete size classes of tumor juncton fragments would not be found in a small sample of tumors. Because the high background of endogenous viruses is difficult to overcome in the murine system, it seems that experimental approaches other than restriction analyses of tumor proviruses will prove more fruitful. These might include screening for enhanced expression of c-onc genes in MuLV-induced tumors or attempts to identify the transforming genes of these tumors by transfection experiments.

Gene activation by "promoter insertion" is not restricted to c-onc genes. Quintrell et al. (1980) found RNA transcripts containing both viral 5' and non-viral sequences in some biologically cloned mammalian cell lines transformed by RSV. A heterogeneous population of such RNAs was also found in RAV-2 mass-infected cell cultures (W.S. Hayward, personal communication). The heterogeneity of the cellular portion of these transcripts reflected the integration of RAV-2 proviruses at different cellular sites.

The molecular details of ALV-induced lymphomagenesis suggest that some previously unsuccessful attempts to rescue retroviruses associated with human cancer may have been doomed to failure. Most of these studies were aimed at recovering a transforming

activity or at detecting viral gene products. Yet it is clear that oncogenesis by slowly transforming viruses can occur with only a small part of the viral genome (the LTR) present in the resultant tumor. It will be interesting to see whether the human cutaneous T-cell lymphoma virus recently described by Gallo's group (Poiesz et al., 1981; Reitz et al., 1981) and independently in Japan (Miyoshi et al., 1981) functions via a promoter insertion mechanism. Studies already strongly suggest that this virus is a slowly transforming virus, i.e., it lacks a specific transforming gene (Reitz et al., 1981). The recent finding that hepatitis B virus has a life cycle and structure similar to retroviruses (J. Summers, personal communication), combined with the known association of this virus with hepatocellular carcinoma suggests that it too be closely examined for a promoter insertion mechanism.

Oncogenesis by non-viral agents. Most human cancer appears not to involve human tumor viruses. Nevertheless, the promoter-insertion model of oncogenesis is likely to be extremely relevant to the molecular mechanism of initiation of human cancer. At least 12 independent v-onc genes have been defined in various avian and mammalian retroviruses (see Introduction). Although it has not been rigorously demonstrated in each case, it is likely that there is a strongly conserved c-onc sequence corresponding to each v-onc. Sequences in human DNA homologous to v-onc genes have been identified and in some cases cloned (see Results, Sect. D and also Wong-Staal et al. 1981; Eva et al., 1982; D. Lowy, personal communication; G. Vande Woude, personal communication).

It is clear from the mechanism of oncogenesis of ALV as well as the mechanism of rapidly transforming virus-induced neoplasia that "inappropriate" expression of a normal cellular gene can be the critical event in oncogenesis. "Inappropriate" expression can mean either an abnormally high level of expression, or expression at a time when the gene would be normally inactive, or both. Presumably, c-onc genes are under tight regulatory control in the normal cell, and are turned on and off at specific times in the cell cycle or during stages of normal development.

Other agents, such as radiation and chemical carcinogens share the ability to induce regulatory alterations of cellular genes. It seems likely that these agents could cause alterations in c-onc gene regulatory sequences that would result in inappropriate expression of c-onc genes and neoplasia. Specific chromosomal rearrangements are correlated with many neoplastic diseases in both humans and experimental animals (for reviews, see Rowley, 1980; Cairns, 1981; Klein, 1981). Any such rearrangement that joined an active regulatory element to the coding sequences of a c-onc gene could cause activation of the gene. In this regard, it is interesting that a specific translocation involved in murine lymphoma (Weiner et al., 1978; Ohno et al., 1979) appears to involve a chromosome segment known to encode immunoglobulin genes (Meo et al., 1980). Screening for enhanced expression of c-onc genes in human tumors is in progress in many laboratories including our own. In addition, I am currently attempting to identify the chromosomal locations of several c-onc genes on human metaphase chromosomes using in situ hybridization techniques. By comparing the locations obtained with the known

neoplasia-associated chromosome abnormalities, it may be possible to gain insight into which c-onc genes (if any) are activated in a particular tumor, and by what mechanism. A complementary approach is being taken to identify potentially onc genes in human tumors using transfection techniques (Murray et al.,1981; Krontiris and Cooper, 1981; Perucho et al., 1981).

It seems likely that the common denominator in initiation of oncogenesis will prove to be the activation of one of a number of c-onc genes. The study of these viral genes, particularly factors that either alter their expression or the function of their gene products will be vital if rational approaches to the treatment of neoplasia are to be developed. It will be of particular importance to determine when and how in development and/or the cell cycle these genes function, and how disruption of their function might affect normal cell physiology. It may be quite difficult to selectively decrease the expression or function of c-onc genes in tumor cells, since the expression of these genes is generally higher in tumor cells than in normal cells. Nevertheless, with the identification of molecular targets for oncogenesis, a significant inroad will have been made to the problem of the molecular basis of cancer.

Aaronson, S., Todaro, G.J. and Scolnick, E.M. (1971). Induction of murine C-type viruses from clonal lines of virus-free BALB/3T3 cells. Science 174, 155-159.

Aaronson, S.A., Dunn, C.Y. (1974). High frequency C-type virus induction by inhibitors of protein synthesis. Science 183, 422-424.

Aaronson, S.and Stephenson, J. (1977). Endogenous Type-C RNA viruses of mammalian cells. Biochem. Biophys. Acta 458, 323-354.

Akusjarvi, G. and Pettersson, U. (1979). Sequence analysis of adenovirus DNA: complete nucleotide sequence of the 5' non-coding region of adenovirus 2 hexon messenger RNA. Cell 16, 841-850.

Alwine, J.C., Kemp, D.J., and Stark, G.R. (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.

Anderson, S.M., Hayward, W.S., Neel, B.G. and Hanafusa, H. (1980). Avian erythroblastosis virus produces two messenger RNAs. J. Virol. 36, 676-683.

Astrin, S.M. (1978). Endogenous viral genes of the White Leghorn chicken: common site of residence and sites associated with specific phenotypes of viral gene expression. Proc. Natl. Acad. Sci. U.S.A. 75, 5941-5945.

Astrin, S.M., Buss, E.G., and Hayward, W.S. (1979). Endogenous viral genes are nonessential in the chicken. Nature 82, 339-341.

Astrin, S.M., Robinson, H.L., Crittenden, L.B., Buss, E.G., Wy-

ban, J., and Hayward, W.S. (1980). Ten genetic loci in the chicken that contain structural genes for endogenous avian leukosis viruses. Cold Spring Harbor Symp. Quant. Biol. 44, 1105-1109.

Bacheler, L.T. and Fan, H. (1979). Multiple integration sites for Moloney leukemia virus in productively infected mouse fibroblasts. J. Virol. 30, 657-667.

Bacon, L.D., Witter, R.L., Crittenden, L.B., Fadly, A.M., and Motta, J. (1981). B-haplotype influence on Marek's disease, Rous sarcoma, and lymphoid virus-induced tumors in chickens. Poultry Sci. 60, 1132-1139.

Bader, J.P. (1964). The role of deoxyribonucleic acid in the synthesis of Rous sarcoma virus. Virology 22, 462-468.

Baker, C. C., Herisse, J., Courtoise, G., Galibert, F. and Ziff, E. (1979). Messenger RNA for the Ad-2 DNA Binding Protein: DNA sequences encoding the first leader and heterogenity at the mRNA 5' end. Cell 18, 569-580.

Balduzzi, P.C., Notter, M.F.D., Morgan, H. and Shibuya, M. (1981). Some biological properties of two new avian sarcoma viruses. J. Virol. 40, 268-275.

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

Baltimore, D., Shields, A., Otto, G., Goff., S., Besmer, P., Witte, O., and Rosenberg, N. (1980). Structure and expression of the Abelson murine leukemia virus genome and its relationship to a normal cell gene. Cold Spring Harbor Symp. Quant. Biol. 44, 849-854.

Banerji, J., Rusconi, S. and Schaffner, W. (1981). Expression of a β-globin gene is enhanced by remote SV40 sequences. Cell 27, 299-308.

Barbacid, M., Beemon, K. and Devare, S.G. (1980b). Origin and functional properties of the major gene product of the Snyder-Theilen strain of feline sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 77, 5158-5162.

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

Becker, A. and Gold, M. (1975). Isolation of the bacteriophage lambda A-gene protein. Proc. Natl. Acad. Sci. U.S.A. 72, 581-585.

Beemon, K. (1978). Oligonucleotide fingerprinting with RNA tumor virus RNA. Current Topics Mirobiol. Immunol. 74, 73-110.

Beemon, K. and Hunter, T. (1978). Characterization of Rous sarcoma virus src gene products synthesized in vitro. J. Virol. 28, 551-566.

Beemon, K. and Keith, J. (1977). Localization of $N^6$methyladenosine in the Rous sarcoma virus genome. J. Mol. Biol. 113, 165-179.

Benoist, C. and Chambon, P. (1981). In vivo sequence requirements of the SV40 early promoter region. Nature 290, 304-310.

Benton, W.D. and Davis, R.W. (1977). Screening λ gt recombinant clones by hybridization to single plaques in situ. Science 2196, 180-182.

Benz, E.W., Wydro, R.M., Nadal-Ginard, B., Dina, D. (1980). Moloney murine sarcoma proviral DNA is a transcriptional unit. Nature 288, 665-668.

Berget, S.M., Moore, C. and Sharp, P.A. (1977). Spliced segments at the 5' terminus of adenovirus 2 late mRNA. Proc. Natl.

Acad. Sci. U.S.A. 75, 1274-1278.

Berk, A.J. and Sharp, P.A. (1977). Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S-1 endonuclease-digested hybrids. Cell 12, 721-732.

Besmer, P., Smotkin, D., Haseltine, W., Fan, H., Wilson, A.T., Paskind, M. Weinberg, R. and Baltimore, D. (1975). Mechanism of induction of RNA tumor viruses by halogenated pyrimidines. Cold Spring Harbor Symp. Quant. Biol. 39, 1103-1107.

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

Bishop, J.M., Deng, C.T., Mahy, B.W.J., Quintrell, N., Stavnezer, E. and Varmus, H.E. (1976). Synthesis of viral RNA in cells infected by avian sarcoma virus. In Animal Virology, ICN-UCLA Symposium on Molecular and Cellular Biology, 4. Baltimore D., Huang, A. S. and Fox, C. F., eds. (New York: Academic Press) pp. 1-20.

Bishop, J.M., Levinson, W.E., Quintrell, N., Fanshier, L. and Jackson, J. (1970). The low molecular weight RNAs of Rous sarcoma virus. I. The 4S RNA. Virology 42, 182-195.

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

Bister, K., Lee, W.-H, and Duesberg, P.H. (1980). Phosphorylation of the nonstructural proteins encoded by avian acute leukemia viruses and by avian Fujinami sarcoma virus. J. Virol. 36, 617-621.

Blair, D.G., McClements, W.L., Oskarsson, M.K., Fischinger, P.J., and Vande Woude, G.F. (1980). Biological activity of cloned

Moloney sarcoma virus DNA: terminally redundant sequences may enhance transformation efficiency. Proc. Natl. Acad. Sci. U.S.A. 77, 3504-3508.

Blair, D.G., Oskarsson, M., Wood, T.G., McClements, W.L., Fischinger, P.J. and Vande Woude, G.F. (1981). Activation of the transforming potential of a normal cell sequence: a molecular model for oncogenesis. Science 212, 941-943.

Blattner, F.R., Williams, B.G., Blechl, A.E., Thompson, K.D., Faber, H.E., Furlong, L.-A., Grunwald, D.J., Kiefer, D.O., Moore, D.D., Schumm, J.W., Sheldon, E.L. and Smithies, O. (1977). Charon phages: safer derivatives of bacteriophage lambda for DNA cloning. Science 196, 161-169.

Bondurant, M., Hashimoto, S.I. and Green, M. (1976). Methylation pattern of genomic RNA from Moloney murine leukemia virus. J. Virol. 19, 998-1005.

Boone, L.R. and Skalka, A.M. (1981). Proviral DNA synthesized in vitro by avian retrovirus particles permeabilized with mellitin. II. Evidence for a strand displacement mechanism in (+) strand synthesis. J. Virol. 37, 117-126.

Breindl, M., Bacheler, L., Fan, H. and Jaenisch, R. (1980). Chromatin conformation of integrated Moloney leukemia virus DNA sequences in tissues of BALB/Mo mice and in virus-infected cell lines. J. Virol. 34, 373-382.

Brugge, J.S. and Erikson, R.L. (1977). Identification of a transformation-specific antigen induced by an avian sarcoma virus. Nature 45, 346-348.

Brugge, J.S., Purchio, A.F., and Erikson, R.L. (1977). Virus-specific RNA species present in the cytoplasm of Rous sarcoma virus-infected chicken cells. Virology 83, 16-26.

Brugge, J.S., Steinbaugh, P.J. and Erikson, R.L. (1978). Charac-

terization of the avian sarcoma virus protein $p60^{src}$. Virology 91, 130-140.

Burmester, B.R. (1969). The prevention of lymphoid leukosis with androgens. Poult. Sci. 48, 401-408.

Burmester, B.R., Gross, M.A., Walter, W.G., and Fontes, A.K. (1959). Pathogenicity of a viral strain (RPL 12) causing avian visceral lymphomatosis and related neoplasms. II. Host-virus interrelations affecting response. J. Natl. Cancer Inst. 22, 103-127.

Cabradilla, C.D., Robbins, K.C. and Aaronson, S.A. (1976). Induction of mouse type-C virus by translational inhibitors: evidence for transcriptional derepression of a specific class of endogenous virus. Proc. Natl. Acad. Sci. U.S.A. 73, 4541-4545.

Cairns, J. (1981). The origin of human cancers. Nature 289, 353-357.

Carlson, M. and Botstein, D. (1982). Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. Cell 28, 148-154.

Cepko, C.L., Hansen, U., Handa, H. and Sharp, P.A. (1981). Sequential transcription-translation of simian virus 40 by using mammalian cell extracts. Mol. Cell Biol. 1, 919-931.

Chen, J.-H. (1980). Expression of endogenous avian myeloblastosis virus information in different chicken cells. J. Virol. 36, 162-170.

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

Chen-Kiang, S., Nevins, J.R. and Darnell, J.E. (1979). N-6

methyl-adenosine in Adenovirus type 2 nuclear RNA is conserved in the formation of messenger RNA. J. Mol. Biol. 135, 733-752.

Cheung, K.-S., Smith, R.E., Stone, M.P. and Joklik, W.K. (1972). Comparison of immature (rapid harvest) and mature Rous sarcoma virus particles. Virology 50, 851-864.

Chien, Y.-H., Lai, M., Shih, T.Y., Verma, I.M., Scolnick, E.M., Roy-Berman, P., and Davidson, N. (1979). Heteroduplex analysis of the sequence relation ships between the genomes of Kirsten and Harvey sarcoma viruses, their respective parental murine leukemia viruses and the rat endogenous 30S RNA. J. Virol. 31, 752-760.

Chiswell, D. J., Ramsay, G., and Hayman, M.J. (1981). Two virus-specific RNA species are present in cells transformed by defective leukemia virus OK10 . J. Virol. 40, 301-304.

Chow, L.T., Gelinas, R.E., Broker, T.R. and Roberts, R.J. (1977). An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNA. Cell 12, 1-8.

Coffin, J.M. (1979). Structure, replication, and recombination of tumor virus genomes: some unifying hypotheses. J. Gen. Virol. 42, 1-46.

Coffin, J.M. and Haseltine, W.A. (1977). Terminal redundancy and the origin of replication of Rous sarcoma virus RNA. Proc. Natl. Acad. Sci. U.S.A. 74, 1908-1912.

Coffin, J.M., Champion, M., and Chabot, F. (1978). Nucleotide sequence relationships between the genomes of an endogenous and an exogenous avian tumor virus. J. Virol. 28, 972-991.

Coffin, J.M., Varmus, H.E., Bishop, J.M., Essex, M., Hardy, W.D., Martin, G.S., Rosenberg, N.E., Scolnick, E.M., Weinberg, R.A. and Vogt, P.K. (1981). Proposal for naming host-

derived inserts in retrovirus genomes. J. Virol. 40, 953-957.

Cohen, J.C. and Varmus, H.E. (1979). Endogenous mammary tumor virus DNA varies among wild mice and segregates during inbreeding. Nature 278, 418-423.

Cohen, J.C., Shank, P.R., Morris, W.L., Cardiff, R. and Varmus, H.E. (1979). Integration of the DNA of mouse mammary tumor virus in virus-infected normal and neoplastic tissue of the mouse. Cell 16, 333-345.

Cohen, R.S., Wong, T.C. and Lai, M.M.C. (1981). Characterization of transformation- and replication-specific sequences of reticuloendotheliosis virus. Virology 113, 672-685.

Collett, M.S. and Erikson, R.L. (1978). Protein kinase activity associated with the avian sarcoma virus src gene product. Proc. Natl. Acad. Sci. U.S.A., 75, 2021-2024.

Collett, M.S., Purchio, A.F., and Erikson, R.L. (1980). Avian sarcoma virus transforming protein pp60$^{src}$ shows protein kinase activity for tyrosine . Nature 285, 656-659.

Cooper, G.M. and Neiman, P.E. (1980). Transforming genes of neoplasms induced by avian lymphoid leukosis viruses. Nature 287, 656-659.

Cooper, G.M. and Neiman, P.E. (1981). Two distinct candidate transforming genes of lymphoid leukosis virus-induced neoplasms. Nature 292, 857-858.

Cooper, M.D., Payne, L.N., Dent, P.B., Burmester, B.R., and Good, R.A. (1968). Pathogenesis of avian lymphoid leukosis. I. Histogenesis. J. Natl. Cancer Inst. 41, 373-389.

Cooper, M.D., Purchase, H.G., Brockman, D.E., and Gathings, W.E. (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.

Cordell, B., Weiss, S.R., Varmus, H.E., and Bishop, J.M. (1978). At least 104 nucleotides are transposed from the 5' terminus of the avian sarcoma virus genome to the 5' termini of smaller viral mRNAs. Cell 15, 79-91.

Corden, J., Wasylyk, B., Buchwalder, A., Sassone-Corsi, P., Kedinger, C.and Chambon, P. (1980). Expression of cloned genes in new environment. Science 209, 1406-1414.

Courtneidge, S.A., Levinson, A.D., Bishop, J.M. (1980). The protein encoded by the transforming gene of avian sarcoma virus ($pp60^{src}$) and a homologous protein in normal cells ($pp60^{proto\text{-}src}$) are associated with the plasma membrane. Proc. Natl. Acad. Sci. U.S.A. 77, 3783-3787.

Crittenden, L. B., Motta, J. V. and Smith, E. J. (1977). Genetic control of RAV-0 production in chickens. Virology 76, 90-97.

Crittenden, L.B., Fadly, A.M., and Smith, E.J. (1981). Effect of endogeneous leukosis virus genes on response to infection with avian leukosis and reticuloendotheliosis viruses. Avian Dis., in press.

Crittenden, L.B., Hayward, W.S., Hanafusa, H. and Fadley, A.M. (1980). Induction of neoplasms by subgroup E recombinants of exogenous and endogenous avian retroviruses (Rous-associated virus type 60). J. Virol. 33, 915-919.

Crittenden, L.B., Witter, R.L. and Fadly, A.M. (1979). Low incidence of lymphoid tumors in chickens continuously producing endogenous virus. Avian Dis. 23, 646-653.

Czernilovsky, A.P., DeLorbe, W., Swanstrom, R., Varmus, H.E., Bishop, J. M., Tischer, E. and Goodman, H.M. (1980). The nucleotide sequence of an untranslated but conserved domain

at the 3' end of the avian sarcoma virus genome. Nucl. Acids Res. 13, 2967-2984.

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

DeFeo, D., Gonda, M.A., Young, H.A., Chang, E.H., Lowy, D.R., Scolnick, E.M. and Ellis, R.W. (1981). Analysis of two divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 78, 3328-3332.

Denhardt, D. (1966). A membrane filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Comm. 23, 641-646.

Dhar, R., McClements, W.L., Enquist, L.W. and Vande Woude, G.F. (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.

Dimock, K. and Stoltzfus, C.M. (1978). Sequence specificity of internal methylation in B77 avian sarcoma virus RNA subunits. Biochemistry 16, 471-478.

Dinowitz, M. (1975). Inhibition of Rous sarcoma virus by alpha-amanitin: possible role of cell DNA-dependent RNA polymerase form II. Virology 66, 1-9.

Dodgson, J.B., Strommer, J. and Engel, J.D. (1979). Isolation of the chicken β-globin gene and a linked embryonic β-like globin gene from a chicken DNA recombinant library. Cell 17, 879-887.

Donoghue, D.J., Rothenberg, E., Hopkins, N., Baltimore, D. and

Sharp, P.A. (1978). Heteroduplex analysis of the nonhomology region between Moloney MuLV and the dual host range derivative HIX virus. Cell 14, 959-970.

Donoghue, D.J., Sharp, P.A. and Weinberg, R.A. (1979). An MSV-specific subgenomic mRNA in MSV-transformed GS-124 cells. Cell 171, 53-63.

Duesberg, P.H., Canaani, E., Van der Helm, K., Lai, M.M.C. and Vogt, P.K. (1973). News and views in avian tumor virus RNA. In Possible episomes in eukaryotes. Silverstein, L.G., ed. (Amsterdam: North-Holland), pp. 142-152.

Edgell, M.H. and Polsky, F. (1980). Use of preparative gel electrophoresis for DNA fragment isolation. Methods Enzymol. 65, 319-327.

Eisenman, R.N. and Vogt, V.M. (1978). The biosynthesis of oncovirus proteins. Biochim. Biophys. Acta. 473, 187-239.

Elder, J., Gautsch, J., Jensen, F., Lerner, R., Hartley, J. and Rowe, W.P. (1977). Biochemical evidence that MCF murine leukemia viruses are envelope (env) gene recombinants. Proc. Natl. Acad. Sci. U.S.A. 74, 4676-4680.

Ellermann, V. and Bang, O. (1909). Experimentelle Leukamie bei Huhnern. Z. Bacteriol. Parisitenk Abt. I. Orig. 46, 595-609.

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

Erikson, E. and Erikson, R.L. (1976). The origin of 7S RNA in virions of avian sarcoma viruses. Virology 72, 518-522.

Erikson, E. and Erikson, R.L. (1980). Identification of a cellular protein substrate phosphorylated by the avian sarcoma virus-transforming gene product. Cell 21, 829-836.

Erikson, E., Cook, R., Miller, G.J. and Erikson, R.L. (1981).

The same normal cell protein is phosphorylated after transformation by avian sarcoma viruses with unrelated transforming genes. Mol. Cell Biol. 1, 43-50

Erikson, E., Erikson, R.L.: Association of 4S ribonucleic acid with oncornavirus ribonucleic acids. J. Virol. 8, 254-256 (1971)

Eva, A., Robbins, K.C., Andersen, P.R., Srinivasan, A., Tronick, S.R., Reddy, E.P., Ellmore, N.W., Galen, A.T., Lautenberger, J.A., Papas, T.S., Westin, E.H., Wong-Staal, F., Gallo, R.C. and Aaronson, S.A. (1982). Cellular genes analogous to retroviral onc genes are transcribed in human tumor cells. Nature 295, 116-119.

Evans, R., Weber, J., Ziff, E. and Darnell, J.E. (1979). Premature termination of adenovirus transcription. Nature 278, 367-370.

Faller, D.V., Rommeleare, J. and Hopkins, N (1978). Large Tl oligonucleotides of Moloney leukemia virus missing in an env gene recombinant HIX, are present on an intracellular 21S Moloney viral RNA species. Proc. Natl. Acad. Sci. U.S.A. 75, 2964-2968.

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

Fan, H. and Verma, I.M. (1978). Size analysis and relationship of murine leukemia virus-specific mRNAs: evidence for transposition of sequences during synthesis and processing of subgenomic mRNA. J. Virol. 26, 468-478.

Faras, A.J., Garapin, A.C., Levinson, W.E., Bishop, J.M. and Goodman, H.M. (1973). Characterization of low-molecular-

weight RNAs associated with the 70S RNA of Rous sarcoma virus. J. Virol. 12, 334-342.

Faye, G., Leung, D.W., Tatchell, K., Hall, B.D. and Smith, M. (1981). Deletion mapping of sequences essential for in vivo transcription of the iso-1 cytochrome c gene. Proc. Natl. Acad. Sci. U.S.A. 78, 2258-2262.

Feldman, R.A., Hanafusa, T. and Hanafusa, H. (1980). Characterization of protein kinase activity associated with the transforming gene of Fujinami sarcoma virus. Cell 22, 757-766.

Feldman, R.A., Wang, L.-H., Hanafusa, H. and Balduzzi, P.C. (1982). Avian sarcoma virus UR2 encodes a transforming protein which is associated with a unique protein kinase activity. J. Virol., in press

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

Fire, A., Baker, C.C., Manley, J.L., Ziff, E.B. and Sharp, P.A. (1981). In vitro transcription of adenovirus. J. Virol. 40, 703-719.

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

Ford, J.P. and Hsu, M.T. (1978). Transcription pattern of in vivo-labeled late Simian virus 40 RNA: equimolar transcription beyond the mRNA 3' terminus. J. Virol. 28, 795-801.

Frankel, A.E. and Fischinger, P.J. (1976). Nucleotide sequences in mouse DNA and RNA specific for Moloney sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 73, 3705-3709.

Frankel, A.E., Gilbert, J.H., Porzig, K.J., Scolnick, E.M. and Aaronson, S.A. (1979). Nature and distribution of feline sarcoma virus nucleotide sequences. J. Virol. 30, 821-827.

Fraser, N., Sehgal, P.B. and Darnell, J.E. (1978). DRB-induced premature termination of late adenovirus transcription. Nature 272, 590-593.

Fraser, N.W., Sehgal. P.B. and Darnell, J.E. (1979). Multiple discrete sites for premature RNA chain termination late in adenovirus-2 infection: enhancement by 5,6-dichloro-1-B-D-ribofuranosyl benzimidazole. Proc. Natl. Acad. Sci. U.S.A. 76, 2571-2575.

Fuhrman, S.A., Van Beveren, C.V. and Verma, I.M. (1981). Identification of a RNA polymerase II initiation site in the long terminal repeat of Moloney murine leukemia viral DNA. Proc. Natl. Acad. Sci. U.S.A. 78, 5411-5415.

Fujinami, A. and Inamoto, K. (1914). Uber Geschwulste bei japanischen Haushuhnern, insbesondere uber einen transplantablen Tumor. Z. Krebsforsch 14, 94-119.

Fung, Y.-K. T., Fadly, A.M., Crittenden, L.B. and Kung, H.-J. (1981). On the mechanism of retrovirus-induced avian lymphoid leukosis. deletion and integration of the proviruses. Proc. Natl. Acad. Sci. U.S.A. 78, 3418-3422.

Furuichi, Y., Shatkin, A.J., Stavnezer, E. and Bishop, J.M. (1975). Blocked methylated 5' terminal sequence in avian sarcoma virus RNA. Nature 257, 618-620.

Gardner, M.B., Rongey, R.W., Arnstein, P., Estes, J.D., Sarma, P., Huebner, R.J. and Richard, C.G. (1970). Experimental transmission of feline fibrosarcoma to cats and dogs. Nature 226, 807-809.

Gelinas, R.E. and Roberts, R.J. (1977). One predominant 5' unde-

canucleotide in adenovirus 2 late mRNAs. Cell 11, 533-544.

Ghosh, P., Reddy, U., Swinscoe, J., Lebowitz, P. and Weissmann, S. (1978). The heterogeneity and 5' terminal structure of the late RNAs of Simian virus 40. J. Mol. Biol. 126, 813-846.

Ghysdael, J., Neil, J.C. and Vogt, P.K. (1981). Cleavage of four avian sarcoma virus polyproteins with virion protease p15 removes gag sequences and yields two large fragments that function as tyrosine phosphoacceptors in vitro. Proc. Natl. Acad. Sci. U.S.A. 78, 5847-5851.

Gielkens, A.L.J., van Zaane, D., Bloemers, H.P.J. and Bloemendal, H. (1976). Synthesis of Rauscher murine leukemia virus-specific polypeptides in vitro. Proc. Natl. Acad. Sci. U.S.A. 73, 356-360.

Gilboa, E., Goff, S., Shields, A., Yoshimura, F., Mitra, S. and Baltimore, D. (1979a). In vitro synthesis of a 9 kbp terminally redundant DNA carrying the infectivity of Moloney murine leukemia virus. Cell 16, 863-874.

Gilboa, E., Mitra, S.W., Goff, S. and Baltimore, D. (1979b). A detailed model of reverse transcription and tests of crucial aspects. Cell 18, 93-100.

Gilmer, T.M. and Erikson, R.L. (1981). Rous sarcoma virus trnasforming protein, $p60^{src}$, expressed in E. coli, functions as a protein kinase. Nature 294, 771-773.

Goldfarb, M.P. and Weinberg, R.A. (1981a). Structure of the provirus within NIH 3T3 cells transfected with Harvey sarcoma virus DNA. J. Virol. 38, 125-135.

Goldfarb, M.P. and Weinberg, R.A. (1981b). Generation of novel, biologically active Harvey sarcoma viruses via apparent illegitimate recombination. J. Virol. 38, 136-150.

Gonda, T.J., Sheiness, D.K., Fanshier, L. and Bishop, J.M. (1981). The genome and intracellular RNAs of avian myeloblastosis virus. Cell 23, 279-290.

Graf, T. (1973). Two types of target cells for transformation with avian myelocytomatosis virus. Virology 54, 398-413.

Graf, T. and Beug, H. (1978). Avian leukemia viruses: interaction with their target cells in vivo and in vitro. Biochem. Biophys. Acta 516, 269-299.

Greenberger, J.S. and Aaronson, S.A. (1975). Cycloheximide induction of xenotropic Type C virus from synchronized mouse cells: metabolic requirements for virus activation. J. Virol. 15, 64-70.

Grosschedl, R. and Birnstiel, M.L. (1980a). Identification of regulatory sequences in the prelude sequences of an H2A histone gene by the study of specific deletion mutants in vivo. Proc. Natl. Acad. Sci. U.S.A. 77, 1432-1436.

Grosschedl, R. and Birnstiel, M.L. (1980b). Spacer DNA sequences upstream of the T-A-T-A-A-A-T-A sequence are essential for the promotion of H2A histone gene expression in vivo. Proc. Natl. Acad. Sci. U.S.A. 77, 7102-7106.

Grosschedl, R. and Birnstiel, M.L. (1982). Delimitation of far upstream sequences required for maximal in vitro transcription of an H2A histone gene. Proc. Natl. Acad. Sci. U.S.A. 79, 297-301.

Grosveld, G.C., Shewmaker, C.K., Jat, P. and Flavell, R.A. (1981). Localization of DNA sequences necessary for transcription of the rabbit β-globin gene in vitro. Cell 25, 215-226.

Groudine, M. and Weintraub, H. (1980). Activation of cellular genes by avian RNA tumor viruses. Proc. Natl. Acad. Sci.

U.S.A. 77, 5351-5354.

Gruss, P., Dhar, R. and Khoury, G. (1981). Simian virus 40 tandem repeated sequences as an element of the early promoter. Proc. Natl. Acad. Sci. U.S.A. 78, 943-947.

Guarente, L. and Ptashne, M. (1981). Fusion of Escherichia coli lac Z to the cytochrome C gene of Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 78, 2258-2262.

Guntaka, R.V., Mahy, B.W., Bishop, J.M. and Varmus, H.E. (1975). Ethidium bromide inhibits the appearance of closed circular viral DNA and integration of virus-specific DNA in duck cells infected by avian sarcoma virus. Nature 253, 507-511.

Hackett, P.B., Swanstrom, R., Varmus, H.E. and Bishop, J.M. (1982). The leader sequence of the subgenomic mRNAs of Rous sarcoma virus is approximately 390 nucleotides. J. Virol. 41, 522-534.

Hager, G.L., Chang, E.H., Chan, H.W., Garon, C.F., Israel, M.A., Martin,M.A., Scolnick, E.M. and Lowy, D.T. (1979). Molecular cloning of the Harvey sarcoma virus closed circular DNA intermediates: initial structural and biological characterization. J. Virol. 31, 795-809.

Halpern, C.C., Hayward, W.S. and Hanafusa, H. (1979). Characterization of some isolates of newly recovered avian sarcoma virus. J. Virol. 29, 91-101.

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

Hanafusa, H. (1975). Avian RNA tumor viruses. In Cancer, Vol 2. Becker, F.F., ed. (New York: Plenum Press), pp. 49-90.

Hanafusa, H. (1977). Cell transformation by RNA tumor viruses. In Comprehensive Virology, Vol. 10. Fraenkel-Conrat, H. and Wagner, R. R., eds. (New York: Plenum Press), pp.401-483.

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

Hanafusa, H., Hayward, W.S., Chen, J.-H. and Hanafusa, T. (1975). Control of expression of tumor virus genes in uninfected chicken cells. Cold Spring Harb. Symp. Quant. Biol. 39, 1139-1144.

Hanafusa, H., Miyamoto, T. and Hanafusa, T. (1970). A cell associated-factor essential for formation of an infectious form of Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 66, 314-321.

Hanafusa, T., Hanafusa, H., Miyamoto, T., and Fleissner, E. (1972). Existence and expression of tumor virus genes in chick embryo cells. Virology 47, 475-482.

Hanafusa, T., Mathey-Prevot, B., Feldman, R.A. and Hanafusa, H. (1981). Mutants of Fujinami sarcoma virus which are temperature sensitive for cellular transformation and protein kinase activity. J. Virol. 38, 347-355.

Hanafusa, T., Wang, L.-H., Anderson, S.M., Karess, R.E. Hayward, W.S. and Hanafusa, H. (1980). Characterization of the transforming gene of Fujinami sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 77, 3009-3013.

Handa, H., Kaufman, R., Manley, J., Gefter, M. and Sharp, P.A. (1981). Transcription of simian virus 40 DNA in a HeLa whole cell extract. J. Biol. Chem. 256,. 478-482.

Harada, F., Sawyer, R.C. and Dahlberg, J.E. (1975). A primer ribonucleic acid for initiation of in vitro Rous sarcoma virus deoxyribonucleic acid synthesis. J. Biol. Chem. 250, 3487-3497.

Hardy, W.D., McClelland, A.J., Zuckerman, E.E., Snyder, H.W., MacEwen, E.G., Francis, D. and Essex, M. (1980). Development of virus non-producer lymphosarcomas in pet cats exposed to FeLV. Nature 288, 90-92.

Harvey, T.T. (1964). An unidentified virus which causes the rapid production of tumors in mice. Nature 204, 1104-1105.

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

Hastie, N.D. and Bishop, J.O. (1976). The expression of three abundance classes of messenger RNA in mouse tissue. Cell 9, 761-774.

Hayman, M.J. (1981). Transforming proteins of avian retroviruses. J. Gen. Virol. 52, 1-14.

Hayward, W.S. (1977). Size and genetic content of viral RNAs in avian oncovirus-infected cells. J. Virol. 24, 47-63.

Hayward, W.S. Neel, B.G. and Astrin, S.M. (1981b). Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. Nature 290, 475-480.

Hayward, W.S. and Hanafusa, H. (1973). Detection of avian tumor virus RNA in uninfected chicken embryo cells. J. Virol. 11, 157-167.

Hayward, W.S. and Hanafusa, H. (1975). Recombination between endogenous and exogenous RNA tumor virus genes as analyzed by nucleic acid hybridization. J. Virol. 15, 1367-1377.

Hayward, W.S. and Neel, B.G. (1981). Retroviral gene expression. Current Top. Microbiol. Immun. 91, 217-276.

Hayward, W.S., Braverman, S.B. and Astrin, S.M. (1980). Transcriptional products and DNA structure of endogenous avian proviruses. Cold Spring Harbor Symp. Quant. Biol. 44, 1111-1121.

Hayward, W.S., Neel, B.G., Fang, J., Robinson, H.L. and Astrin, S.M. (1981a). Avian lymphoid leukosis is correlated with the appearance of discrete new RNAs containing viral and cellular genetic information. In Modern Trends in Human Leukemia, Vol 4. Neth, R. and Gallo, R., eds. (Munich: Bergmann-Verlag), pp.439-444.

Hohn, B. and Murray, K. (1977). Packaging of recombinant DNA molecules into bacteriophage particles in vitro. Proc. Natl. Acad. Sci. U.S.A. 74, 3259-3263.

Houck, C.M., Rinehart, F.P. and Schmid, C.W. (1979). A ubiquitous family of repeated DNA sequences in the human genome. J. Mol. Biol. 132, 289-306.

Hsu, T.W., Sabran, J.L., Mark, G.E., Guntaka, R.V. and Taylor, J.M. (1978). Analysis of unintegrated avian RNA tumor virus double-stranded DNA intermediates. J. Virol. 28, 810-818.

Hu, S.-L. and Manley, J. (1981). DNA sequence required for transcription in vitro from the major late promoter of adenovirus 2. Proc. Natl. Acad. Sci. U.S.A. 78, 820-824.

Hughes, S.H., Shank, P.R., Spector, D.H., Kung, H.-J., Bishop, J.M., Varmus, H.E., Vogt, P.K. and Breitman, M.L. (1978). Proviruses of avian sarcoma virus are terminally redundant, co-extensive with unintegrated linear DNA, and integrate at many sites. Cell 15, 1397-1410.

Hughes, S.H., Toyoshima, K., Bishop, J.M., Varmus, H.E.: Organization of the endogenous proviruses of chickens: implications for origin and expression . Virology, 108, 189-207 (1981)

Hunter, E., Bhown, A.S. and Bennett, J.C. (1978). Amino terminal amino acid sequence of the major structural polypeptides of avian retroviruses: sequence homology between reticuloen-

dotheliosis virus p30 and p30s of mammalian retroviruses. Proc. Natl. Acad. Sci. U.S.A. 75, 2708-2712.

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

Jacquet, M., Groner, Y., Monroy, G. and Hurwitz, J. (1974). The in vitro synthesis of avian myeloblastosis viral RNA sequences. Proc. Natl. Acad. Sci. U.S.A. 71, 3045-3049.

Jaenisch, R. (1980). Germ line integration and Mendelian transmission of exogenous Type C viruses. In Molecular Biology of RNA Tumor Viruses. Stephenson, J.R., ed. (New York: Academic Press), pp. 131-162.

Jahner, D., Stuhlmann, H. and Jaenisch, R. (1980). Conformation of free and of integrated Moloney leukemia virus proviral DNA in preleukemic and leukemic BALB/Mo mice. Virology 101, 111-123.

Jelinek, W.R., Toomey, T.P., Leinwand, L., Duncan, C.H., Biro, P.A., Choudary, P.V., Weissman, S.M., Rubin, C.M., Houck, C.M., Deininger, P.L. and Schmid, C.W. (1980). Ubiquitous, interspersed repeated sequences in mammalian genomes. Proc. Natl. Acad. Sci. U.S.A. 77, 1398-1402.

Jolicoeur, P. and Baltimore, D. (1976a). Effect of Fv-1 gene product on proviral DNA formation and integration in cells infected with murine leukemia virus. Proc. Natl. Acad. Sci. U.S.A. 73, 2236-2240.

Jolicoeur, P. and Baltimore, D. (1976b). Effect of Fv-1 gene product on synthesis of N-tropic and B-tropic murine leukemia viral RNA. Cell 7, 33-39.

Jolicoeur, P. and Rassart, E. (1980). Effect of Fv-1 gene product on synthesis of linear and supercoiled viral DNA in

cells infected with murine leukemia virus. J. Virol. 33, 183-195.

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

Ju, G., Boone, L. and Skalka, A.M. (1980). Isolation and characterization of recombinant DNA clones of avian retroviruses: size heterogeneity and instability of the direct repeat. J. Virol. 33, 1026-1033.

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

Karess, R.E., Hayward, W.S. and Hanafusa, H. (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.

Kawai, S., Duesberg, P.H., and Hanafusa, H. (1977). Transformation-defective mutants of Rous sarcoma virus with src gene deletions of varying length. J. Virol. 24, 910-914.

Kawai, S., Yoshida, M., Segawa, K., Sugiyama, Ishikazi, R. and Toyoshima, K. (1980). Characteristics 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.

Keith, J. and Fraenkel-Conrat, H. (1975). Identification of the 5' end of Rous sarcoma virus RNA. Proc. Natl. Acad. Sci. U.S.A. 72, 3347-3350.

Kerr, I.M., Olshevsky, U., Lodish, H.F. and Baltimore, D. (1976).

Translation of murine leukemia virus RNA in cell-free systems from animal cells. J. Virol. 18, 627-635.

Ketner, G. and Kelley, T.J., Jr. (1976). Integrated SV40 sequences in transformed cell DNA: analysis using restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. 73, 1102-1106.

Kettmann, R., Marbaix, G., Cleuter, Y., Portetelle, D., Mammerickx, M. and Burny, A. (1981). Genomic integration of bovine leukemia provirus and lack of viral RNA expression in target cells of cattle with different responses to BLV infection. Leuk. Res., in press.

Kirsten, W.H. and Mayer, L.A. (1967). Morphologic responses to a murine erythroblastosis virus. J. Natl. Cancer Inst. 39, 311-319.

Kitchener, G. and Hayman, M.J. (1980). Comparative tryptic peptide mapping studies suggest a role in cell transformation for the gag-related protein of avian erythroblastosis virus and avian myeloblastosis virus strains CM II and MC 29. Proc. Natl. Acad. Sci. U.S.A. 77, 1637-1641.

Klein, G. (1981). The role of gene dosage and genetic transpositions in carcinogenesis. Nature 294, 313-318.

Klement, V., Nicolson, M.O. and Heubner, R.J. (1971). Rescue of the genome of focus-forming virus from rat nonproductive lines by 5'-bromodeoxyuridine. Nature 234, 12-14.

Klessig, D.F. (1977). Two adenovirus mRNAs have a common 5' terminal leader sequence encoded at least 10 kb upstream from their main coding regions. Cell 12, 2-12.

Krontiris, T.G. and Cooper, G.M. (1981). Transforming activity of human tumor DNAs. Proc. Natl. Acad. Sci. U.S.A. 78, 1181-1184.

Krueger, J.G., Wang, E. and Goldberg, A.R. (1980). Evidence that

the src gene product of Rous sarcoma virus is membrane associated. Virology 101, 25-40.

Krzyzek, R.A., Collett, M.S., Lau, A.F., Perdue, M.L., Leis, J.P. and Faras, A.J(1978). Evidence for splicing of avian sarcoma virus 5'-terminal genomic sequences onto viral-specific RNA in infected cells. Proc. Natl. Acad. Sci. U.S.A. 75, 1284-1288.

Kung, H.-J., Bailey, J.M., Davidson, N., Nicolson, M.O. and McAllister, R.M. (1975). Structure, subunit composition, and molecular weight of RD-114 RNA. J. Virol. 16, 397-419.

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

Lai, M. M. C. and Duesberg, P. H. (1972). Adenylic acid-rich sequence in RNAs of Rous sarcoma virus and Rauscher mouse leukemia virus. Nature 235, 383-386.

Langlois, A.J., Beard, D. and Beard, J.W. (1970). Strain MC29, an avian leukosis virus of unique properties. In Comparative Leukemia Research. Dutcher, R.M., ed. (Basel: Karger), pp. 96-105.

Lautenberger, J.A., Schulz, R.A., Garon, C.F., Tsichlis, P.N. and Papas, T. (1981). Molecular cloning of avian myelocytomatosis virus (MC29) transforming sequences. Proc. Natl. Acad. Sci. U.S.A. 78, 1518-1522.

Leder, P., Temier, D. and Enquist, L. (1977). EK2 derivatives of bacteriophage lambda useful in the cloning of DNA from higher organisms: the gtWES system. Science 196, 175-177.

Lee, J., Varmus, H.E. and Bishop, J.M. (1979). Virus-specific mRNAs in permissive cells infected by avian sarcoma virus. J. Biol. Chem. 254, 8015-8022.

Lee, W.-H., Bister, K., Pawson, A., Robins, T., Moscovici, C. and Duesberg, P.H. (1980). Fujinami sarcoma virus: an avian RNA tumor virus with a unique transforming gene. Proc. Natl. Acad. Sci. U.S.A. 77, 2018-2022.

Lerner, M.R., Boyle, J.A., Mount, S.M., Wolin, S.L. and Steitz, J. (1980). Are snRNPs involved in splicing? Nature 283, 220-224.

Levin, J.G. and Rosenak, M.J. (1976). Synthesis of murine leukemia virus proteins associated with virions asembled in actinomycin D-treated cells: evidence for persistence of viral messenger RNA. Proc. Natl. Acad. Sci. U.S.A. 73, 1154-1158.

Levinson, A.D., Oppermann, H., Levintow, L., Varmus, H.E. and Bishop, J.M. (1978). Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. Cell 15, 561-572.

Levinson, B., Khoury, G., Vande Woude, G. and Gruss, P. (1982). Activation of SV40 genome by 72-base pair tandem repeats of Moloney sarcoma virus. Nature 295, 568-572.

Lindell, T.J., Weinberg, F., Morris, P.W., Roeder, R.G. and Rutter, W.J. (1970). Specific inhibition of nuclear RNA polymerase II by alpha-amanitin. Science 170, 447-449.

Linial, M., Medeiros, E. and Hayward, W.S. (1978). An avian oncovirus mutant (SE21Q1b) deficient in genomic RNA: biological and biochemical characterization. Cell 15, 1371-1381.

Long, C.W., Suk, W.A., and Greenwalt, C. (1978). Activation of endogenous type C virus by amino acid alcohols. Virology 88, 194-196.

Lowy, D.R., Rowe, W.P., Teich, N. and Hartley, J.W. (1971). Murine leukemia virus: high-frequency activation in vitro by

5-iodeoxyuridine and 5-bromodeoxyridine. Science 174, 155-156.

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

Maniatis, T., Jeffrey, A. and Kleid, D.G. (1975). Nucleotide sequence of the rightward operator of phage lambda. Proc. Natl. Acad. Sci. U.S.A. 72, 1184-1188.

Manley, J.L., Fire, A., Cano, A., Sharp, P.A. and Gefter, M.L. (1980). DNA-dependent transcription of adenovirus genes in a soluble whole cell extract. Proc. Natl. Acad. Sci. U.S.A. 77, 3855-3859.

Manley, J.L., Sharp, P.A. and Gefter, M.L. (1979). RNA synthesis in isolated nuclei: in vitro initiation of adenovirus 2 major late mRNA precursor. Proc. Natl. Acad. Sci. U.S.A. 76, 160-164.

Mathews, R.E.F. (1979). Classification and nomenclature of viruses. Intervirology 12, 131-280.

Mathey-Prevot, B., Hanafusa, H. and Kawai, S. (1982). A cellular protein is immunologically crossreactive with and functionally homologous to the Fujinami sarcoma virus transforming protein. Cell 28, 897-906.

McGrath, J.P. and Levinson, A.D. (1982). Bacterial expression of an enzymatically active protein encoded by RSV src gene. Nature 2925, 423-425.

McKnight, S.L., Gavis, E.R., Kingsbury, R. and Axel, R. (1981). Analysis of transcriptional regulatory signals of the HSV thymidine kinase gene: identification of an upstream control region. Cell 25, 385-395.

McMaster, G.K., and Carmichael, G.G. (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.

Mellon, P. and Duesberg, P. (1977). Subgenomic cellular Rous sarcoma virus RNAs contain oligonucleotides from the 3' half and the 5' terminus of virion RNA. Nature 270, 631-634.

Mellon, P.A., Pawson, A., Bister, K., Martin, G.S. and Duesberg, P.H. (1978). Specific RNA sequences and gene products of MC29 avian acute leukemia virus. Proc. Natl. Acad. Sci. U.S.A. 75, 5874-5878.

Meo, T., Johnson, J., Beechey, C.V., Andrews, S.J., Peters, J. and Searle, A.G. (1980). Linkage analyses of murine immunoglobulin heavy chain and serum prealbumin genes establish their location on chromosome 12 proximal to the T (5;12) 31H breakpoint in band 12F1. Proc. Natl. Acad. Sci. U.S.A. 77, 550-553.

Miyoshi, I., Kubonishi, I., Yoshimoto, S., Akagi, T., Ohtsuki, Y., Shiraishi, Y., Nagata, K. and Hinuma, Y. (1981). Type C virus particles in cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukemic T cells. Nature 294, 770-771.

Moloney, J.B. (1966). Biological studies on a lymphoid leukemia virus extracted from sarcoma 37. I. Origin and introductory investigations. J. Natl. Cancer Inst. 24, 933-951.

Moreau, P., Hen, R., Wasylyk, B., Everett, R., Gaub, M.P. and Chambon, P. (1981). The SV40 72 base pair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants. Nucl. Acids Res. 9, 6047-6069.

Motta, J.V., Crittenden, L.B., Purchase, H.G., Stone, H.A. and Witter, P.L. (1975). Low oncogenic potential of endogenous

RNA tumor virus infection or expression. J. Natl. Cancer Inst. 55, 685-689.

Murray, M.J., Shilo, B.-Z., Shih, C., Cowing, D., Hsu, H.W. and Weinberg, R.A. (1981). Three different human tumor cell lines contain different oncogenes. Cell 25, 355-363.

Murray, N.E., Brammer, W.J. and Murray, K. (1977). Lambdoid phages that simplify the recovery of in vitro recombinants. Mol. Gen. Genet. 150, 53-61.

Naso, R.B., Arcement, L.J. and Arlinghaus, R.B. (1975b). Biosynthesis of Rauscher leukemia viral proteins. Cell 4, 31-36.

Naso, R.B., Arcement, L.J., Karshin, W.L., Jamjoom, G.A. and Arlinghaus, R.B. (1976a). The cell-free translation of Rauscher leuekemia virus RNA into high molecular weight polypeptides. Proc. Natl. Acad. Sci. U.S.A. 73, 2326-2330.

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

Neiman, P., Payne, L.N., and Weiss, R.A. (1980b). Viral DNA in bursal lymphomas induced by avian leukosis viruses. J. Virol. 34, 178-186.

Neiman, P.E., Das, S., Macdonnel, D. and McMillin-Helsel, C. (1977). Organization of shared and unshared sequences in the genomes of chicken endogenous and sarcoma viruses. Cell 11, 321-329.

Neiman, P.E., Jordan, L., Weiss, R. and Payne, L.N. (1980a). Malignant lymphoma of the bursa of Fabricius and analysis of early transformation. Cold Spring Harb. Conf. Cell Prolif. 7, 519-528.

Nermut, M.V., Frank, H. and Schafer, W. (1972). Properties of

mouse leukemia virus. III. Electron microscopic appearance as revealed after conventional preparation techniques as well as freeze-drying and freeze-etching. Virology 49, 345-358.

Nevins, J.R. and Darnell, J.E. (1978). Steps in the processing of Ad 2 mRNA: poly(A)$^+$ nucleic acid sequences are conserved and poly(A) addition preceeds splicing. Cell 15, 1477-1493.

Noori-Daloii, M.R., Swift, R.A., Kung, H.-J., Crittenden, L.B., and Witter, R.L. (1981). Specific integration of REV proviruses in bursal lymphomas. Nature 294, 574-576.

Ohno, S., Babonits, M., Weiner, F., Spira, J., Klein, G. and Potter, M. (1979). Nonrandom chromosome changes involving the Ig gene-carrying chromosomes 12 and 6 in pristane-induced mouse plasmacytomas. Cell 18, 1001-1007.

Oskarsson, M., McClements, W.L., Blair, D.G., Maizel, J.V. and Vande Woude, G. F. (1980). Properties of a normal mouse cell DNA sequence (sarc) homologous to the src sequence of Moloney sarcoma virus. Science 207, 1222-1224.

Pawson, T., Guyden, J., Kung, T.-H., Radke, K., Gilmore, T. and Martin, G.S. (1980b). A strain of Fujinami sarcoma virus which is temperature-sensitive in protein phosphorylation and cellular transformation. Cell 22, 767-776.

Pawson, T., Harvey, R. and Smith, A.E. (1977). The size of Rous sarcoma virus mRNAs active in cell-free translation. Nature 268, 416-420.

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

Pawson, T., Mellon, P., Duesberg, P.H. and Martin, G.S. (1980a).

*env* gene of Rous sarcoma virus: identification of the gene product by cell-free translation. J. Virol. 33, 993-1003.

Payne, G.S., Bishop, J.M. and Varmus, H.E. (1982). Multiple arrangements of viral DNA and an activated host oncogene in bursal lymphomas. Nature 295, 209-214.

Payne, G.S., Courtneidge, S.A., Crittenden, L.B., Fadly, A.M., Bishop, J.M. and Varmus, H.E. (1981). Analyses 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.

Perucho, M., Goldfarb, M., Shimizu, K., Lama, C., Fogh, J. and Wigler, M. (1981). Human-tumor-derived cell lines contain common and different transforming genes. Cell 27, 467-476.

Peters, G., Harada, F., Dahlberg, J.E., Panet, A., Haseltine, W.A. and Baltimore, D. (1977). Low molecular-weight RNAs of Moloney murine leukemia virus: identification of the primer for RNA-directed DNA synthesis. J. Virol. 21, 1031-1041.

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

Peterson, R.D., Purchase, H.G., Burmester, B.R., Cooper, M.D. and Good, R.A. (1966). Relationships among visceral lymphomatosis, bursa of Fabricius, and bursa-dependent lymphoid tissue of the chicken. J. Natl. Cancer Inst. 36, 585-595.

Poiesz, B.J., Ruscetti, F.W., Reitz, M.S., Kalyanaran, V.S. and Gallo, R.C. (1981). Isolation of a new type C virus (HTLV) in primary uncultured cells of a patient with Sezary T-cell leukemia. Nature 294, 268-270.

Pribnow, D. (1975). Nucleotide sequence of an RNA polymerase

binding site at an early T7 promoter. Proc. Natl. Acad. Sci. U.S.A. 72, 784-788.

Pribnow, D. (1979). Genetic control signals in DNA. In Biological regulation and development Vol. 1. Goldberger, R., ed. (New York: Plenum Press), pp. 209-277.

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

Purchase, H.G. and Gilmore, D.G. (1975). Lymphoid leukosis in chickens chemically bursectomized and subsequently innoculated with bursa cells. J. Natl. Cancer Inst. 55, 851-855.

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

Purchase, H.G., and Burmester, B.R. (1978). Neoplastic diseases: leukosis/sarcoma group. In Diseases of Poultry, 7th edition. Hofstad, M.S. and Calnek, B.W., Helmboldt, C.F., Reid, W.M., and Yoder, H.W. Jr., eds. (Ames, Iowa: Iowa State University Press), pp. 418-468.

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

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

Purchio, A.F., Jovanovich, S. and Erikson, R.L. (1980). Sites of synthesis of viral proteins in avian sarcoma virus-infected chicken cells. J. Virol. 35, 629-636.

Quade, K., Smith, R. E. and Nichols, J. L. (1974). Poly(riboadenylic acid) and adjacent nucleotides in Rous sarcoma virus RNA. Virology 62, 60-70.

Quintrell, N., Hughes, S.H., Varmus, H.E. and Bishop, J.M. (1980). The structure of viral DNA and RNA in mammalian cells infected with avian sarcoma virus. J. Mol. Biol. 143, 363-393.

Radke, K., Gilmore, T. and Martin, G.S. (1980). Transformation by Rous sarcoma virus: a cellular substrate for transformation-specific protein phosphorylation contains phosphotyrosine. Cell 21, 821-828.

Ramirez, F., Burns, A.L., Mears, J.G., Spence, S., Starkman, D. and Bank, A. (1979). Isolation and characterization of cloned human fetal globin genes. Nucl. Acids. Res. 7, 1147-1161.

Rapp, U.R. and Todaro, G.J. (1980). Generation of oncogenic mouse type C viruses: in vitro selection of carcinoma-inducing variants. Proc. Natl. Acad. Sci. U.S.A. 77, 624-628.

Rascati, R.J. and Tennant, R.W. (1978). Induction of endogenous murine retrovirus by hydroxyurea and related compounds. Virology 87, 208-211.

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

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

Reitz, M.S., Poiesz, B.J., Ruscetti, F.W. and Gallo, R.C. (1981). Characterization and distribution of nucleic acid sequences

of a novel type C retrovirus isolated from neoplastic human T lymphocytes. Proc. Natl. Acad. Sci. U.S.A. 78, 1887-1891.

Rho, H. M. and Green M. (1974). The homopolyadenylate and adjacent nucleotides at the 3'-terminus of 30-40S RNA subunits in the genome of murine sarcoma-leukemia virus. Proc. Natl. Acad. Sci. U.S.A. 71, 2386-2390.

Rice, N.R., Bonner, T.I. and Gilden, R.V. (1981). Nucleic acid homology between avian and mammalian type C viruses: relatedness of reticuloendotheliosis virus cDNA to the cloned proviral DNA of the endogenus Colobus virus CPC-1. Virology 114, 286-290.

Ringold, G.M., Shank, P.R., Varmus, H.E., Ring, J. and Yamamoto, K.R. (1979). Integration and transcription of mouse mammary tumor virus DNA in rat hepatoma cells. Proc. Natl. Acad. Sci. U.S.A. 76, 665-669.

Robertson, D.L. and Varmus, H.E. (1979). Structural analysis of the intracellular RNAs of murine mammary tumor virus. J. Virol. 30, 576-589.

Robins, T., Bister, K., Garon, C., Papas, T. and Duesberg, P. (1982). Structural relationship between a normal chicken DNA locus and the transforming gene of the avian acute leukemia virus MC29. J. Virol. 41, 635-642.

Robinson, H.L. (1978). Inheritance and expression of chicken genes that are related to avian leukosis sarcoma virus genes. Curr. Top. Microbiol. Immun. 83, 1-36.

Robinson, H.L., Pearson, M.N., DeSimone, D.W., Tsichlis, P.N. and Coffin, J.M. (1980). Subgroup-E avian-leukosis virus-associated disease in chickens. Cold Spring Harbor Symp. Quant. Biol. 44, 1133-1142.

Robinson, H.L., Swanson, C.A., Hruska, J.F. and Crittenden, L.B.

(1976). The production of unique C type viruses by chicken cells grown in bromodeoxyuridine. Virol. 69, 63-74.

Rogers, J. and Wall, R. (1980). A mechanism for RNA splicing. Proc. Natl. Acad. Sci. U.S.A. 77, 1877-1879.

Rohrschneider, L.R. (1980). Adhesion plaques of Rous sarcoma virus-transformed cells contain the src gene product. Proc. Natl. Acad. Sci. U.S.A. 77, 3514-3518.

Rommeleare, J., Faller, D.V. and Hopkins, N. (1978). Characterization and mapping of RNAse $T_1$-resistant oligonucleotides derived from the genomes of AKR and MCF murine leukemia viruses. Proc. Natl. Acad. Sci. U.S.A. 75, 495-499.

Rose, J. K., Haseltine, W. A. and Baltimore, D. (1976). 5' terminus of Moloney murine leukemia virus 35S RNA is $m^7$5'pppGmpCp. J. Virol. 20, 324-329.

Rosenberg, N. and Baltimore, D. (1980). Abelson virus. In Viral Oncology Klein, G., ed. (New York: Plenum Press), pp. 187-201.

Rothenberg, E., Donoghue, D.J. and Baltimore, D. (1978). Analysis of a 5' leader sequence on murine leukemia virus 21S RNA: heteroduplex mapping with long reverse transcriptase products. Cell 13, 435-451.

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

Rowe, W.P., Hartley, J.W., Lander, M.L., Pugh, W.E. and Teich, N. (1971). Noninfectious AKR mouse embryo cell lines in which each cell has the capacity to be activated to produce infectious murine leukemia virus. Virology 46, 866-876.

Rowley, J. (1980). Chromosome abnormalities in human leukemia.

Ann. Rev. Gen. 14, 17-39.

Rymo, L., Parsons, J.T., Coffin, J.M. and Weissman, C. (1974). In vitro synthesis of Rous sarcoma virus is catalyzed by a DNA-dependent RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. 71, 2782-2786.

Sabran, J.L., Hsu, T.W., Yeater, C., Kaji, A., Mason, W.S. and Taylor, J.M. (1979). Analysis of integrated avian RNA tumor virus DNA in transformed chick, duck, and quail fibroblasts. J. Virol. 29, 170-178.

Salditt-Georgieff, M., Harpold, M., Chen-Kiang, S. and Darnell, J.E. (1980). The addition of 5' cap structures occurs early in hnRNA synthesis and prematurely terminated molecules are capped. Cell 19, 69-78.

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

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

Schincariol, A.L. and Joklik, W.K. (1973). Early synthesis of virus-specific RNA and DNA in cells rapidly transformed with Rous sarcoma virus. Virology 56, 532-548.

Schindler, D. and Echols, H. (1981). Retroregulation of the int gene product of bacteriophage : Control of translation completion. Proc. Natl. Acad. Sci. U.S.A.. 78, 4475-4479.

Scolnick, E.M., Young, H.A. and Parks, W.P. (1976). Biochemical and physiological mechanisms in glucocorticoid hormone induction of mouse mammary tumor virus. Virology 69, 148-156.

Sefton, B.M., Hunter, T. and Beemon, K. (1979). Product of in

vitro translation of the Rous sarcoma virus src gene has protein kinase activity. J. Virol. 30, 311-318.

Sefton, B.M., Hunter, T., Ball, E.H. and Singer, S.J. (1981). Vinculin: a cytoskeletal target of the transforming protein of Rous sarcoma virus. Cell 24, 165-174.

Sefton, B.M., Hunter, T., Beemon, K. and Eckhart, W. (1980). Evidence that phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. Cell 20, 807-816.

Sehgal, P.B., Derman, E., Molloy, G.R., Tamm, I. and Darnell, J.E. (1976). 5,6-Dichloro-1-B-D-ribofuranosyl-benzimidazole inhibits initiation of nuclear heterogeneous RNA chains in HeLa cells. Science 194, 431-433.

Shalloway, Zelenetz, A.D. and Cooper, G.M. (1981). Molecular cloning and characterization of the chicken gene homologous to the transforming gene of Rous sarcoma virus. Cell 24, 531-541.

Shank, P.R. and Linial, M. (1980). An avian oncovirus mutant (SE21Q1B) deficient in genomic RNA: characterization of a deletion in the provirus. J. Virol. 36, 450-456.

Shank, P.R., Cohen, J.C., Varmus, H.E., Yamamoto, K.R. and Ringold, G.M. (1978a). Mapping of linear and circular forms of mouse mammary tumor virus DNA with restriction endonucleases: evidence for a large specific deletion occuring at high frequency during circularization. Proc. Natl. Acad. Sci. U.S.A. 75, 2112-2116.

Shank, P.R., Hughes, S.H., Kung, H.-J., Majors, J.E., Quintrell, N., Guntaka, R.V., Bishop, J.M. and Varmus, H.E. (1978b) Mapping unintegrated avian sarcoma virus DNA: termini of linear DNA bear 300 nucleotides present once or twice in two

species of circular DNA. Cell 15, 1383-1395.

Shatkin, A. (1976). Capping of Eukoryotic mRNAs. Cell 9, 645-653.

Sheiness, D. and Bishop, J.M. (1979). DNA and RNA from uninfected vertebrate cells contain nucleotide sequences related to the putative transforming gene of avian myelocytomatosis virus. J. Virol. 31, 514-521.

Sheiness, D., Fanshier, L. and Bishop, J.M. (1978). Identification of nucleotide sequences which may encode the oncogenic capacity of avian retrovirus MC29. J. Virol. 28, 600-610.

Sheiness, D., Vennstrom, B. and Bishop, J.M. (1981). Virus-specific RNAs in cells infected by avian myelocytomatosis virus and avian erythroblastosis virus. Cell 23, 291-300.

Sheiness, D.K., Hughes, S.H., Stubblefield, E. and Bishop, J.M. (1980). The vertebrate homologue of the putative transforming gene of avian myelocytomatosis virus: characteristics of the DNA locus and its RNA transcript. Virology 105, 415-424.

Shenk, T.E., Rhodes, C., Rigby, P.W.J. and Berg, P. (1975). Biochemical method for mapping mutational alterations in DNA with S1 nuclease: the location of deletions and temperature-sensitive mutations in simian virus 40. Proc. Natl. Acad. Sci. U.S.A. 72, 989-993.

Sherr, C.J., Fedele, L.A., Donner, L. and Turek, L.P. (1979). Restriction endonuclease mapping of unintegrated proviral DNA of Snyder-Theilen feline sarcoma virus: localization of sarcoma-specific sequences. J. Virol. 32, 860-875.

Sherr, C.J., Fedele, L.A., Oskarsson, M., Maizel, J. and Vande Woude, G.F. (1980). Molecular cloning of Snyder-Theilen feline leukemia and sarcoma virus: comparative studies of fe-

line sarcoma virus with its natural helper virus and with Moloney murine sarcoma virus. J. Virol. 34, 200-212.

Shibuya, M., Hanafusa, H., and Balduzzi, P.C. (1982). Cellular sequences related to three new onc genes of avian sarcoma virus (fps, yes, and ros) and their expression in normal and transformed cells. J. Virol, in press.

Shibuya, M., Hanafusa, T., Hanafusa, H. and Stephenson, J.R. (1980). Homology exists among the transforming sequences of avian and feline sarcoma viruses. Proc. Natl. Acad. Sci. U.S.A. 77, 6536-6540.

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

Shine, J., Czernilovsky, A.D., Friedrich, R., Bishop, J.M. and Goodman, H.M. (1977). Nucleotide sequence at the 5' terminus of the avian sarcoma virus genome. Proc. Natl. Acad. Sci. U.S.A. 74, 1473-1477.

Shoemaker, C., Goff, S., Gilboa, E., Paskind, M., Mitra, S.W. and Baltimore, D. (1980). Structure of a cloned circular Moloney leukemia virus DNA molecule containing an inverted segment: implications for retrovirus integration. Proc. Natl. Acad. Sci. U.S.A. 277, 3932-3936.

Shoyab, M. and Baluda, M.A. (1976). Ribonucleotide sequence homology among avian oncoviruses. J. Virol. 17, 106-113.

Simek, S. and Rice, N. (1980). Analysis of the nucleic acid components in reticuloendotheliosis virus.. J. Virol. 33, 320-329.

Smith, R.E., and Ivanyi, J. (1980). Pathogenesis of virus-induced osteopetrosis in the chicken. J. Immunol. 125, 523-530.

Snyder, S.P. and Theilen, G.H. (1969). Transmissible feline fibrosarcoma. Nature 2261, 1074-1075.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517.

Spector, D.H., Baker, B., Varmus, H.E. and Bishop, J.M. (1978b). Characteristics of cellular RNA related to the transforming gene of avian sarcoma viruses. Cell 13, 381-386.

Spector, D.H., Smith, K., Padgett, T., McCombe, P., Roulland-Dussoix, D., Moscovici, C., Varmus, H.E., and Bishop, J.M. (1978a). Uninfected avian cells contain RNA related to the transforming gene of avian sarcoma viruses. Cell 13, 371-379.

Spector, D.H., Varmus, H.E. and Bishop, J.M. (1978c). Nucleotide sequences related to the transforming gene of avian sarcoma virus are present in DNA of uninfected vertebrates. Proc. Natl. Acad. Sci. U.S.A. 75, 4102-4106.

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

Stavnezer, E., Gerhard, D.S., Binari, R. and Balazs, I. (1981). Generation of transforming viruses in cultures of chicken embryo fibroblasts infected with an avian leukosis virus. J. Virol. 39, 920-934.

Steffen, D. and Weinberg, R.A. (1978). The integrated genome of murine leukemia virus. Cell 15, 1003-1010.

Stehelin, D., Soule, S., Roussel, M., Langrou, C. and Rommens, C. (1980). Three new types of transforming genes in acute defective avian leukemia viruses. I. Specific viral nucleotide

sequences correlating with distinct phenotypes of virus-transformed cells. Cold Spring Harb. Symp. Quant. Biol. 44, 1215-1224.

Stehelin, D., Varmus, H.E., Bishop, J.M. and Vogt, P.K. (1976). DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA. Nature 260, 170-173.

Stoltzfus, C. M. and Dimock, K. (1976). Evidence for methylation of B77 avian sarcoma virus genome RNA subunits. J. Virol. 18, 586-595.

Stoltzfus, C. M. and Kuhnert, L. K. (1979). Evidence for the identity of shared 5'-terminal sequences between genome RNA and subgenomic mRNAs of B77 avian sarcoma virus. J. Virol. 32, 536-545.

Sutcliffe, J.G. (1978). pBR322 restriction map derived from the DNA sequence: accurate DNA size markers up to 4361 nucleotide pairs long. Nucl Acids Res. 5, 2721-2738.

Sutcliffe, J.G., Shinnick, T.M., Verma, I.M. and Lerner, R.A. (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.

Sveda, M.S. and Soeiro, R. (1976). Host restriction of Friend leukemia virus: synthesis and integration of the provirus. Proc. Natl. Acad. Sci. U.S.A. 73, 2356-2360.

Takeya, T., Hanafusa, H., Junghans, R.P., Ju,. G. and Skalka, A.M. (1981). Comparison between the viral transforming gene (src) of recovered avian sarcoma virus and its cellular homolog. Mol. Cell. Biol. 1, 1024-1037.

Taylor, J.M. (1979). DNA intermediates of avian RNA tumor

viruses. Curr. Top. Microbiol. Immunol. 87, 23-41.

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

Taylor, J.M., Illmensee, R. and Summers, J. (1976). Efficient transcription of RNA into DNA by avian sarcoma virus polymerase. Biochem. Biophys. Acta 442, 324-330.

Teich, N., Lowy, D.R., Hartley, J.W. and Rowe, W.P. (1973). Studies of the mechanism of induction of infectious murine leukemia virus from AKR mouse embryo cell lines by 5'-iododeoxyuridine and 5'-bromodeoxyuridine. Virology 51, 163-173.

Temin, H. M. (1964a). The nature of the provirus of Rous sarcoma. Natl. Cancer Inst. Monogr. 17, 557-570.

Temin, H. M. (1964b). The participation of DNA in Rous sarcoma virus production. Virology 23, 486-494.

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

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

Thomas, P. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. 77, 5201-5205.

Tsai, S., Tsai, M.J. and O'Malley, B. (1981). Specific 5' flanking sequences are required for faithful initiation of in vitro transcription of the ovalbumin gene. Proc. Natl. Acad. Sci. U.S.A. 78, 879-883.

Tsichlis, P.N. and Coffin, J.M. (1980). Recombinants between endogenous and exogenous avian tumor viruses: role of the c

region and other portions of the genome in the control of replication and transformation. J. Virol. 33, 238-249.

Van Beveren, C., Goddard, J.G., Berns, A. and Verma, I.M. (1980). Structure of the Moloney murine leukemia viral DNA: nucleotide sequence of the 5' long terminal repeat and adjacent cellular sequences. Proc. Natl. Acad.Sci. U.S.A. 77, 3307-3311.

van Zaane, D., Gielkens, A.L.J., Hesselink, W.G. and Bloemers, H.P.J. (1977). Identification of Rauscher murine leukemia virus-specific mRNAs for the synthesis of gag- and env- gene products. Proc. Natl. Acad. Sci. U.S.A. 74, 1855-1859.

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

van der Putten, H., Quint, W., van Raaij, J., Maandag, E.R., Verma, I.M. and Berns, A. (1981). M-MLV-induced leukemogenesis: integration and structure of recombinant proviruses in tumors. Cell 24, 729-740.

Vande Woude, G.F., Oskarsson, M., Enquist, L.W., Nomura, S., Sullivan, S. and Fischinger, P.J. (1979). Cloning of integrated Moloney sarcoma proviral DNA sequences in bacteriophage lambda. Proc. Natl. Acad. Sci. U.S.A. 76, 4464-4468.

Varmus, H. E., Guntaka, R. V., Deng, C. T. and Bishop, J. M. (1975). Synthesis, structure and function of avian sarcoma virus-specific DNA in permissive and nonpermissive cells. Cold Spring Harbor Symp. Quant. Biol 39, 987-996.

Varmus, H.E., Heasley, S., Kung, H.J., Oppermann, H., Smith, V.C., Bishop, J.M. and Shank, P.R. (1978). Kinetics of syn-

thesis, structure and purification of avian sarcoma virus-specific DNA made in the cytoplasm of acutely infected cells. J. Mol. Biol. 120, 55-82.

Varmus, H.E., Heasley, S., Linn, J. and Wheeler, K. (1976). Use of alkaline sucrose gradients in a zonal rotor to detect integrated and unintegrated avian sarcoma virus-specific DNA in cells. J. Virol. 18, 574-585.

Varmus, H.E., Quintrell, N. and Ortiz, S. (1981). Retroviruses as mutagens: insertion and excision of a nontransforming provirus alters expression of a resident transforming virus. Cell 25, 23-36.

Varmus, H.E., Vogt, P.K. and Bishop, J.M. (1973). Integration of Rous sarcoma virus-specific DNA following infection of permissive and nonpermissive hosts. Proc. Natl. Acad. Sci. U.S.A. 70, 3067-3071.

Vigne, R., Breitman, M.L., Moscovici, C. and Vogt, P.K. (1979). Restitution of fibroblast-transforming ability in src deletion mutants of avian sarcoma virus during animal passage. Virology 93, 413-426.

Vogt, P. K. and Friis, R.R. (1971). An avian leukosis virus related to RSV(0): properties and evidence for helper activity. Virology 44, 223-234.

Vogt, P.K. (1967). A virus released by "non-producing" Rous sarcoma cells. Proc. Natl. Acad. Sci. U.S.A. 58, 101-108.

Vogt, P.K. (1977). Genetics of RNA tumor viruses. In Comprehensive Virology, Vol. 9, Fraenkel-Conrat, H., and Wagner, R.R., eds. (New York: Plenum Press), pp. 341-455.

Von der Helm, K. and Duesberg, P.H. (1975). Translation of Rous sarcoma virus RNA in cell-free systems from ascites Krebs II cells. Proc. Natl. Acad. Sci. U.S.A. 72, 614-618.

Wang, L. H. and Duesberg, P. (1974). Properties and location of poly(A) in Rous sarcoma virus RNA. J. Virol. 14, 1515-1529.

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

Wang, L.-H., Duesberg, P., Beemon, K. and Vogt, P.K. (1975). Mapping of RNase $T_1$-resistant oligonucleotides of avian tumor virus RNAs: sarcoma-specific oligonucleotides are near the poly(A) end and oligonucleotides common to sarcoma and transformation-defective viruses are at the poly(A) end. J. Virol. 16, 1051-1070.

Wang, L.-H., Feldman, R., Shibuya, M., Hanafusa, H., Notter, M.F.D. and Balduzzi, P.C. (1981). Genetic structure, transforming sequence and gene product of avian sarcoma virus UR1. J. Virol. 40, 258-267.

Wang, L.-H., Halpern, C.C., Nadel, M. and Hanafusa, H. (1978). Recombination between viral and cellular sequences generates transforming sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. 75, 5812-5816.

Wang, L.-H., Hanafusa, H., Notter, M.F.D. and Balduzzi, P.C. (1982). Genetic structure and transforming sequence of avian sarcoma virus UR2. J. Virol. 41, 833-841.

Wang, S.Y., Hayward, W.S. and Hanafusa, H. (1977). Genetic variation in the RNA transcripts of endogenous virus genes in uninfected chicken cells. J. Virol. 24, 64-73.

Wasylyk, B. and Chambon, P. (1981). A T to A base substitution and small deletions in the conalbumin TATA box drastically decrease specific in vitro transcription. Nucl. Acids Res. 9, 1813-1824.

Wasylyk, B., Kedinger, C., Corden, J., Brison, O. and Chambon, P.

(1980). Specific in vitro initiation of transcription on conalbumin and ovalbumin genes and comparison with adenovirus-2 early and late genes. Nature 285, 367-373.

Waters, L.C. (1975). Transfer RNAs associated with the 70S RNA of AKR murine leukemia virus. Biochem. Biophys. Res. Commun. 65, 1130-1136.

Waters, L.C. and Mullin, B.C. (1977). Transfer RNA in RNA tumor viruses. Prog. Nucl. Acid Res. Mol. Biol. 20, 131-160.

Waters, L.C., Mullin, B.C., Ho, T. and Young, W.K. (1974). Ability of tryptophan tRNA to hybridize with 35S RNA of avian myeloblastosis virus and prime reverse transcription in vitro. Proc. Natl. Acad. Sci. U.S.A. 72, 2155-2159.

Weber, J., Jelinek, W. and Darnell, J.E. (1977). The definition of a large viral transcription unit late in Ad-2 infection of HeLa cells: mapping of nascent RNA molecules labelled in isolated nuclei. Cell 10, 611-617.

Wei, C.-M., Gibson, M., Spear, P.G. and Scolnick, E.M. (1981). Construction and isolation of a transmissible retrovirus containing the *src* gene of Harvey murine sarcoma virus and the thymidine kinase gene of *Herpes simplex* virus Type 1. J. Virol. 39, 935-944.

Weil, P. A., Luse, D. S., Segoll, J. and Roeder, R. G. (1979). Selective and accurate initiation of transcription at the Ad 2 major late promoter in a soluble system dependent on purified RNA polymerase II and DNA. Cell 18, 469-484.

Weiner, F., Ohno, S., Spira, J., Haran-Ghera.. N., and Klein, G. (1978). Chromosome changes (trisomy No. 15 and 17) associated with tumor progression in leukemia induced by radiation leukemia virus. J. Natl. Cancer Inst. 60, 227-237.

Weiss, R.A. and Frisby, D.P. (1981). Are avian endogenous

viruses pathogenic? In Xth International Symposium for Comparative Research on Leukemia and Related Disease. Yohn, D.S., ed. (New York: Elsevier-North-Holland), in press.

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

Willingham, M.C., Jay, G. and Pastan, I. (1979). Localization of the ASV src gene product to the plasma membrane of transformed cells by electron microscopic immunocytochemistry. Cell 18, 125-134.

Witte, O.N., Dasgupta, A. and Baltimore, D. (1980). Abelson murine leukemia virus protein is phosphorylated in vitro to form phosphotyrosine. Nature 283, 826-831.

Witte, O.N., Rosenberg, N., Paskind, M., Shields, A. and Baltimore, D. (1978). Identification of an Abelson murine leukemia virus-encoded protein present in transformed fibroblast and lymphoid cells. Proc. Natl. Acad. Sci. U.S.A. 75, 2488-2492.

Wong-Staal, F., Dalla-Favera, R., Franchini, G., Gelmann, E.P. and Gallo, R.C. (1981). Three distinct genes in human DNA related to the transforming genes of mammalian sarcoma retroviruses. Science 213, 226-228.

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

Yamamoto, T., deCrombrugghe, B. and Pastan, I. (1980b). Identification of a functional promoter in the long terminal repeat of Rous sarcoma virus. Cell 22, 787-798.

Yanofsky, C. (1981). Attenuation in the control of expression of bacterial operons. Nature 289, 751-758.

Young, R.A., Hagenbuchle, U. and Schibler, U. (1981). A single mouse α-amylase gene specifies two different tissue-specific mRNAs. Cell 23, 451-458.

Zain, S., Sambrook, J., Roberts, R.J., Keller, W., Fried, M. and Dunn, A.R. (1979). Nucleotide sequence analysis of leader segments in a cloned copy of adenovirus 2 fiber mRNA. Cell 16, 851-861

Ziff, E.B. (1980). Transcription and RNA processing by DNA tumor viruses. Nature 287, 481-499.

PRO·BONO·HUMANI·GENERIS
The Rockefeller University
1901

THE LIBRARY

19010000005084

End