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# The Mechanism of Leukemogenesis by Avian Leukosis Virus

by

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DISSERTATION

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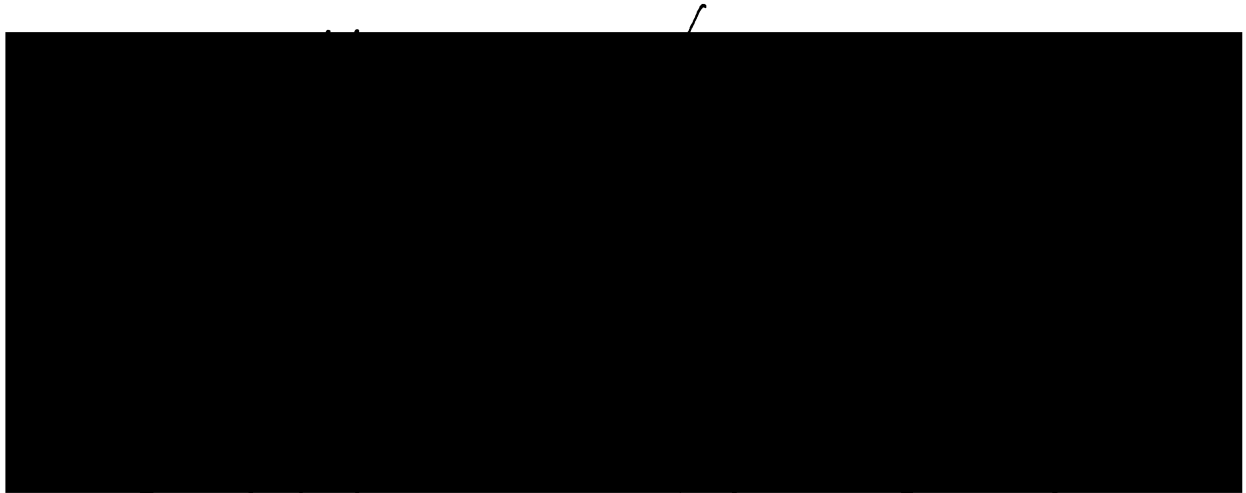

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This is dedicated to

my father

and

mother

# ACKNOWLEDGMENTS

My graduate carrer has been exciting and, in retrospect, enjoyable. I cannot possibly acknowledge all the individuals who have contributed to my education and experiments and I apologize to those who I fail to mention.

Propitious circumstances led me to Harold Varmus' lab. My decision to remain in the lab was inspired by his scientific perspicacity and intelligence, but I remember most vividly his understanding, patience and support during the difficult times.

Mike Bishop deserves special thanks for providing critical guidance and support while Harold was on sabbatical and continuing to contribute to my project throughout my time in the lab.

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Suzanne, the ultimate organizer, was also the primary initiator and companion in our escapades in the arts and humanities.

Richard, more than anyone, has instilled and shared the joy of

scientific ruminations. Our friendship has been cemented during the vicissitudes of our softball careers.

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Lyman Crittenden was a gracious collaborator. Without his knowledge of the biology of chickens and ALV and his provision of tumors, the project never would have progressed.

Steve Hughes substantially contributed to many of the ideas and experiments in this thesis.

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Finally, Enid withstood the worst and remained supportive and understanding.

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# ABSTRACT

Avian leukosis virus (ALV) is an RNA tumor virus which primarily induces B-lymphocyte neoplasms arising in the bursa of Fabricius in chickens. We have been investigating the mechanism of ALV-induced tumorigenesis by analyzing the ALV-specific DNA and RNA found in bursal lymphomas caused by viral infection. Our observations indicate that tumor induction by ALV may depend upon activation of a cellular gene by ALV proviral DNA rather than upon expression of viral genes. First, all bursal lymphomas are clonal populations of tumor cells containing at least one ALV provirus, but solitary proviruses are often defective and many tumors are devoid of virus-specific mRNA's. Second, in most ALV-induced tumors, proviral DNA is found in the same region of the host genome; Hayward et al. have identified this locus as c-myc, the cellular homologue of the putative transforming gene (v-myc) of myelocytomatosis virus-29. We have found that enhanced expression of c-myc occurs in association with proviruses positioned in any of three configurations with respect to the cellular gene: i) upstream in a transcriptional sense from c-myc, in the same transcriptional orientation, ii) upstream, in the opposite transcriptional orientation and, iii) 3' to the gene in the same transcriptional orientation. Analyses of molecularly cloned examples of ALV proviruses and c-myc from tumors displaying configurations ii) and iii) have confirmed these arrangements. These findings have suggested a novel ability of the ALV provirus to enhance the transcription of adjacent cellular DNA and thereby exert its oncogenic effects. We have attempted to recapitulate

enhanced transcription of c-myc by introducing the molecular clones into mouse L fibroblasts. Preliminary experiments revealed a modest augmentation of c-myc transcription from molecules carrying a provirus in configuration iii) when compared to transcription from an unaltered c-myc molecular clone.

Ancillary investigations confirmed the expectation that Rous sarcoma virus, which carries an oncogene v-src, produces non-clonal sarcomas.

I have also prepared molecular clones of two strains of ALV, RAV-1 and RAV-2, which yield biologically active virus after incorporation into chick embryo fibroblasts.

Hayward, W.S., Neel, B.G. and Astrin, S.M. 1981. Nature 290, 475.

# CHAPTER 1

## Introduction

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"Lymphoid leukosis" (LL) describes a disease of fowl in which neoplastic proliferation of lymphoid cells leads to solid tumors most often involving the bursa of Fabricius, spleen and liver. A group of retroviruses aptly labelled avian leukosis viruses (ALV) are the etiologic agents of lymphoid leukosis (Purchase and Burmester,1978). Although LL was described essentially concurrently with other retrovirus-induced tumors in fowl—including erythroblastosis, fibrosarcomas and myeloid leukemia—research into the biology of ALV has lagged far behind investigations of the viruses affiliated with other tumors (Burmester and Purchase,1978). Undoubtedly this situation arose because ALV-induced lymphomas require four to six months to manifest themselves and ALV does not morphologically transform cells in culture. On the other hand, these same characteristics have recently provoked interest in the possibility that ALV might employ a novel mechanism to induce lymphomas. Consequently we began a series of studies designed to address the mechanism of ALV-induced tumor formation. A histopathological description of lymphomagenesis caused by ALV and descriptions of the molecular structure of the ALV genome formed the basis for our studies.

## Pathology

A variety of different tumors can result from infection by ALV including lymphomas, erythroblastoses and nephroblastomas, and at least one hyperplasia, osteopetrosis (Burmester and Purchase,1978). The relative proportion of each tumor type in an infected chicken population is influenced by the route of infection, the dose of infecting virus, the particular virus strain, the age of the chicken at infection

and the genetic background of the chicken (Burmester and Purchase,1978). However, in general, the most prevalent disease is lymphoid leukosis originating in the bursa of Fabricius (Burmester and Purchase,1978; Crittenden,1980).

The bursa of Fabricius

The bursa of Fabricius is a gut-associated lymphoid organ which apparently serves as a primary site for B-cell development in fowl (Chang et al.,1955). The discovery of this primary lymphoid organ allowed for a relatively precise in vivo definition of B-lymphocyte development in the chicken (Glick,1977). These experiments, however, often relied on chemical or surgical bursectomy and suffer from uncontrollable variables inherent to an in vivo system traumatized by surgical or chemical manipulation. This has resulted in equivocal and often contradictory reports. I will summarize the two major views in some detail, both for reference purposes and also because it will serve as a backdrop for the description of tumor pathology which follows.

The bursal anlage begins to form at day five of embryonic development. Experiments using interspecific chimaeras (Le Douarin and Jotereau,1980) and intravenous injection of sex chromosome-marked cells (Moore and Owen,1965; Weber and Mausner, 1977) suggest that migration of a putative lymphoid stem or progenitor cell from the yolk sac and/or bone marrow to the bursa is restricted to days 8-14. These cells presumably proliferate and differentiate to form the lymphoid follicles of stem and/or progenitor cells and cells at various points along the B-lymphocyte lineage. The first cells expressing immunoglobulin appear in the bursal follicles at day 12 of development (Grossi et al.,1977). These cells exhibit both cytoplasmic and surface IgM. (This property

is distinct from mouse pre-B cell development where cytoplasmic IgM preceeds surface IgM expression (Levitt and Cooper,1980).) A number of experiments carried out by Cooper and his colleagues (Kincade and Cooper,1971,1973; Kincade et al.,1970,1973) suggest that cells expressing IgM further differentiate to cells expressing IgG then IgA perhaps in an antigen-independent manner. Bursectomy experiments indicated that this progression requires the bursal environment and that appearance of immunoglobulin expressing cells in secondary lymphoid organs such as the spleen follows the same order observed in the bursa, that is IgM to IgG to IgA; and this seeding of secondary organs was prevented by removing the bursa by day 17 of development.

The proliferative rate of lymphoid cells in the bursa is greatest during the first month after hatching. After this point organ growth slows until regression and involution commences between 3 and 4 months post-hatch (Glick,1977). A different stem or progenitor cell capable of restoring humoral responses makes its appearance in the bursa at approximately four weeks post-hatch and soon after can be found in the spleen and bone marrow (Toivanen and Toivanen,1973; Eskola and Toivanen,1977). In contrast to the bursa-dependent cell described above, this cell can differentiate in bursectomized chickens and has been designated a post-bursal stem cell (Toivanen and Toivanen,1973). However, since birds bursectomized early in life are incapable of developing a humoral immune system (but see below) the post-bursal stem cell presumably requires the bursa for some aspect of its development. Two sets of experiments provide evidence that extra-bursal sites exist for B-lymphocyte development into IgM producing cells. First, surgical and chemical bursectomy performed on embryos failed to abrogate a

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humoral response expressed as IgM (Glick,1977; Fitzsimmons et al.,1973; Jancovic et al. ,1975). Secondly, Lewis et al. (1981) have shown that reticuloendotheliosis virus (REV-T) transforms cells with pre-B characteristics and this cell is not found in the bursa

In view of the discrepancies described above it seems safest (but by no means certain) to conclude that committment of stem cells to the B-lymphocyte lineage and differentiation to IgM producing cells can occur outside the bursa but in the normal bird the bursa accepts cells which differentiate into IgM expressing cells and acts as the primary site for progression to IgG and IgA producing cells, subsequently supplying secondary lymphoid organs with these immunocompetent cells.

Tumor Pathology

The histopathological consequences of ALV infection are superimposed on the normal development of the bursa. Histological analyses of bursae from infected birds has revealed that the earliest manifestation of disease occurs in individual bursal follicles which become occupied by large lymphoblastoid cells (Peterson et al.,1964; Cooper et al.,1968). These cells appear morphologically transformed and are evident in 50-100 follicles per bursa (there are approximately 10,000 follicles per bursa) (Neiman et al.,1980). This data, in combination with the observation of apparently polyclonal antibodies in the sera from tumor-bearing birds, prompted M.D. Cooper et al. to propose that bursal lymphomas are polyclonal in origin (Cooper et al.,1968). Further histology described by Neiman et al. (1980) revealed that bursae sectioned between 6-7 weeks post infection displayed slightly reduced numbers of the abnormal follicles plus one or two macroscopic nodules of lymphoblasts. Finally, the tumors removed from 4-6 month old birds occupy

50-90% of the bursa (which normally would have regressed by this time) and metastatic lesions are sometimes apparent (Purchase and Burmester,1978; Cooper et al.,1968). This set of static observations has led to the theory (Neiman et al.,1980) that a relatively large number (50-100) of lymphocytes become transformed at early times following infection and proliferation of these cells leads to transformed follicles. The cells in only one or a few of these follicles acquire the proliferative capability necessary to form a tumor nodule and progress into a clonal lymphoma and associated metastases.

A number of investigators have addressed the nature of the target cell for ALV transformation. Since the tumors first appear in the bursa these studies have focused on the role of this organ in lymphomagenesis. Surgical bursectomy up to three months following infection at day one post-hatch by ALV eliminates the appearance of tumors comprised of cells in the B-lymphocyte lineage (Peterson et al.,1966). Purchase and Gilmour (1975) have shown that treatment of chicks with cyclophosphamide(Cy), which leaves the bursal epithelium intact but destroys the bursal lymphocyte population, eliminates subsequent lymphomagenesis. Injection of suspensions of bursa cells into Cy treated chicks can reconstitute both humoral immunity and susceptibility to ALV lymphomas. These experiments provide evidence that the target cell for ALV transformation resides in the bursa. These same authors reported further support for this localization in experiments involving bursal cells from line $6_3$ chickens which exhibit natural immunity to ALV tumorigenesis (Purchase et al.). Reciprocal reconstitution experiments revealed that bursal cells from ALV resistant chickens conferred resistance to Cy treated sensitive birds and vice versa. Humoral immunity

was successfully reconstituted in all birds. Although unlikely, these experiments cannot rule out the possibility that target cells infected outside the bursa require an interaction with bursa cells for progression to a transformed state, and line $6_3$ chicks cannot provide this interaction.

In agreement with the bursal location of ALV target cells, immunoflourescent staining of tumor cells has shown that they stain with anti-mu antisera and anti-light chain antisera (Cooper et al.,1974). No staining was observed with anti-gamma or anti-alpha antisera. The tumor cells were expressing surface IgM and variable amounts of cytoplasmic IgM. In general the tumor cells stained less intensely than mature plasma cells suggesting, in conjunction with their size, that they were arrested as immature lymphocytes. Cooper et al. (1974) cited this apparent differentiation arrest as evidence that ALV might exert its oncogenic effects on a cell undergoing the DNA rearrangements necessary (at least in mammals) for IgM expression. Given the present information describing the involvement of DNA deletion during mammalian B-lymphocyte differentiation (Leder et al.,1980), it seems improbable that a mature B-lymphocyte has been transformed by ALV and induced to dedifferentiate. Differentiation of a cell to produce IgM subsequent to transformation by ALV has not been excluded. The nature of the cell targeted for transformation remains a major question in leukosis research because of the implications its identification might have on models describing mechanisms of transformation.

## Structure and characteristics of ALV

### Replication

The avian leukosis viruses responsible for LL are replication competent retroviruses. They share common genome organizations and employ identical strategies for replication and expression. The virus particle packages two copies of the RNA genome in a protein capsid or core surrounded by a lipid envelope derived from the host cell's plasma membrane. The ALV RNA genome codes for three genes required for viral replication (Figure 1A): gag encodes a 76kd polyprotein which is cleaved during virus maturation to generate the core proteins; pol gives rise to the RNA-dependent DNA polymerase (reverse transcriptase); and env codes for the viral glycoproteins associated with the viral envelope (Vogt,1977).

Three other regions of the genome play pivotal roles during the RNA-templated DNA synthesis. The 5' and 3' terminal 16 or 21 bases (depending on the strain of avian virus) form a direct repeat designated R (Shwartz et al.,1977). The host cell tRNA used as a primer for minus strand DNA synthesis is hydrogen- bonded to 18 bases in the viral genome which lie approximately 80 bases from the 5' copy of R (Taylor,1977). The bases between R and the primer binding site are termed U5. A site at the 3' end of the viral genome defines a major initiation point for synthesis of plus strand DNA (Varmus et al.,1978; Swanstrom et al.,1981; Hishinuma et al.,1981). The sequence between this initiation site and the 3' end of the genome, excepting R, constitutes the region called U3. Thus the structure of the viral genome (outlined in Fig. 1A) is R-U5 gag pol env U3-R.

Infection commences when the virus enters the cell employing a specific interaction between the viral envelope glycoprotein and a cell surface receptor (Weiss,1982). The genome is uncoated by an as yet

undefined mechanism and DNA synthesis begins. Unique acrobatics enacted during transcription of the genome into DNA (details of which are unnecessary for this introduction) generate a linear DNA molecule with long terminal repeats (LTRs) whose structures can be represented as 5-U3 R U5-3 (Fig. 1B) (Shank et al.,1978). The linear molecules migrate to the nucleus where they apparently serve as precursors for the formation of at least two forms of circular DNA. These forms contain either one or two LTRs (Shank and Varmus,1978) (Fig. 1C). Viral DNA then becomes covalently joined to host chromosomal DNA to generate a provirus. It is presently unclear which DNA species acts as the immediate precursor to the provirus. The provirus is colinear with the linear DNA, hence it is flanked by LTRs (Fig. 1D) (Shank et al.,1978; Hsu et al.,1978). Viral DNA can integrate into a large number of locations in the host genome (For references see Varmus,1982b). Restriction endonuclease analyses and nucleotide sequencing of host-proviral junctions has failed to reveal common features at the host integration sites.

Genome and messenger RNA are transcribed from the provirus by host RNA polymerase II (Jaquet et al.,1974; Rymo et al.,1974). Structural studies of viral genome and mRNA (Weiss et al.,1977; Cordell et al.,1978; Stacey and Hanafusa,1978), sequence analyses of viral DNA (Swanstrom et al.,1981; Hishinuma et al.,1981; D. Shwartz personal communication) and in vitro transcription studies (Yamamoto et al.,1980) have elucidated regions of the provirus which are crucial for the genesis of RNA. The LTR carries sequences apparently necessary for the initiation of RNA synthesis and provides polyadenylation and transcription termination signals. The structural identity of LTR's places

these transcription initiation and termination functions at both proviral termini. Thus, the 5' LTR could act to terminate transcripts originating in cellular sequences and the 3' LTR could promote transcription of adjacent cellular DNA. Transcription of host sequences originating from a proviral LTR has been observed (chapter 2,3; Quintrell et al., 1980; Neel et al.,1981) but termination of a cellular transcript within the 5' LTR has not been reported. However, although both LTR's can function as promoters, examples of transcription from the 3' LTR are infrequent. It is unclear why identical LTR's preferentially display either initiation or termination functions depending on their position at the 5' or 3' end of a provirus. Efficient transcription promoted by the 3' LTR may require abolition of transcription from the 5' LTR (see Chapter 2, Discussion and Neel et al.,1981). The unique proviral sequences immediately flanking the LTR's could also influence their respective activities.

A site located 390 base-pairs (bp) from the initiation site for RNA synthesis acts as a splice donor site to generate the subgenomic mRNA which is translated to yield the envelope glycoprotein (Hackett et al.,1982; Swanstrom et al.,1982) (Fig. 1F). The splice acceptor site for this mRNA is as yet undefined. DNA sequence analysis has revealed that a spliced mRNA must be created to allow translation of the polyprotein precursor of reverse transcriptase (D. Shwartz, pers. comm.). The splice presumably occurs in a small region at the junction of gag and pol but the actual pol mRNA has not been detected. Once the mRNAs are formed, the viral proteins are translated (Fig. 1G,H), the genomic RNA is packaged and the cycle repeats.

Viral Oncogenesis

Although all avian retroviruses employ a similar replication cycle, they can be classified into four groups based on their genome structures and oncogenic potentials. This classification scheme illustrates the major issues which confronted us as we began our investigations into ALV lymphomagenesis.

i) the replication competent sarcoma viruses

Rous sarcoma virus (RSV) is the sole representative of a replication competent virus which produces neoplasia rapidly (sarcomas arise within two weeks following infection) and induces fibroblast transformation in culture. This virus has given tumor virologists their most detailed glimpse into a mechanism of viral oncogenesis. RSV carries a bonified oncogene (v-onc), v-src (Martin,1970), which encodes a 60kd phosphoprotein (Brugge et al.,1978; Collett and Erickson,1978; Levinson et al.,1978) exhibiting tyrosine kinase activity (Hunter and Sefton,1980). The uninfected chicken contains a homologue (c-src ) of the viral oncogene (Stehelin et al.,1976). c-src is transcribed (Spector et al.,1978b) and translated into a 60k tyrosine kinase which is similar but not identical to $pp60^{v\text{-}src}$ (Opperman et al.,1979; Collett et al.,1978). The c-src gene is conserved throughout vertebrate evolution (Spector et al.,1978a) and has even been found in Drosophila (Shilo and Weinberg,1981). This conservation is presumably indicative of the importance of $pp60^{c\text{-}src}$ in the ontogeny of (at least) vertebrate organisms. However the function of c-src remains obscure. The origin of RSV has been postulated to involve the transduction of c-src from the host genome by a replication competent retrovirus (probably ALV) (Bishop,1981; Varmus, 1982a). The transduced viral oncogene may effect transformation by either: coding for a protein with activity identical

to its cellular counterpart which is over-expressed under the influence of the strong proviral promoter or encoding a protein whose activity has been altered by mutation of the transduced gene.

ii) The defective sarcoma and leukemia viruses

Replication-defective sarcoma and leukemia viruses have transduced cellular genes distinct from c-src (Sheiness and Bishop,1979; Roussel et al.,1979) (excepting the Bryan strain of RSV) which have been implicated in oncogenesis by their respective viruses (less persuasively than c-src in most cases). The leukemia virus members of this category produce a variety of hematopoietic tumors (usually arising within one month following infection) and transform the analogous target cells in vitro. Several members also induce sarcomas and carcinomas (Graf and Beug,1978). The defective sarcoma viruses display associated tyrosine kinase activity (Bishop and Varmus,1982). On the other hand there is evidence that the v-oncs of the leukemia viruses do not encode proteins with tyrosine kinase activity (Sefton et al.,1980).

iii) The replication competent leukosis viruses.

The characteristics of these viruses have already been described above. It is clear that ALV can be distinguished from members of the first two groups on the basis of four characteristics: 1) ALV does not contain a transduced cellular oncogene; 2) it induces neoplasms which become apparent only after a latent period of several months following infection; 3) it fails to transform cells in culture; 4) the spectrum of neoplasms produced by ALV differs from the oncogenic spectra of other avian retroviruses.

In addition to ALV at least two other distinct types of replication- competent viruses are capable of inducing LL in chickens

and exhibit the four characteristics listed above (Teich et al.,1982). Myeloblastosis-associated viruses (MAV), isolated from the BAI-A complex of avian myeloblastosis viruses, have a similar genome to ALV but carry an unrelated U3 sequence (Gonda et al.,1981). Chicken syncytial virus (CSV) acts as a helper virus for the defective reticuloendotheliosis virus (REV-T). CSV is unrelated to either ALV or MAV and may have originated from mammalian retroviruses (Simek and Rice,1980). iv) The replication competent non-oncogenic viruses.

Non-oncogenic viruses originate spontaneously from certain proviruses which can be found as genetic elements in some chickens. The most well characterized virus of this category, RAV-0, is infrequently produced by cells carrying the endogenous provirus ev-2 (Vogt and Friis,1971; Astrin et al.,1980). Virus interference assays showed that the envelope glycoprotein of RAV-0 is uniquely found in endogenous viruses and on this basis these viruses are classified as subgroup E (Vogt and Friis,1971; Weiss,1969). RNase $T_1$ oligonucleotide fingerprinting and nucleotide sequencing studies indicate that differences between ALV and RAV-0 env genes consist of single base changes and small insertions and deletions (Coffin et al.,1978). The sequence divergence between the respective U3 regions is much more pronounced (Neiman et al.,1977; Hishinuma et al., 1981). Recombination experiments have shown that the non-oncogenic phenotype of RAV-0 segregates from the subgroup E envelope protein (Crittenden et al.,1980; Robinson et al.,1980). By elimination, this result implicated the U3 region as a determinant of oncogenic potential. Since U3 intimately participates in viral RNA biogenesis, these experiments provided the first clue in the quest to discover the oncogenic mechanism.

As we commenced our study three approaches were considered. 1) An investigation into the nature of the virus released from tumor cells might elucidate alterations in the genome reflecting the process of oncogenesis. This approach, applied to a murine virus analogue of ALV which induces thymomas with long latencies (murine leukemia virus or MuLV), had yielded the surprising result that viruses released from tumor cells carried a recombinant env gene (Hartley et al.,1977; Elder et al.,1977). By virtue of this recombination these viruses had acquired the ability to grow to high titres in the thymus of infected mice. Some of these viruses also accelerted tumor formation in mice already destined to suffer thymomas late in life (Cloyd et al.,1980). Since the third approach outlined below could also address these issues, we did not analyze virus released from tumors. 2) Constructing recombinant viruses using ALV and RAV-0 would further define the region necessary for tumorigenesis. This approach called for molecularly cloning DNA molecules representing ALV and RAV-0 and recombining these molecules using recombinant DNA technology. We initiated these experiments but did not progress to the point of making recombinants. The initial phases of this work yielded results which address the inherent oncogenic potential of ALV and are described in appendix 2. 3) The approach which yielded the greatest rewards consisted of an investigation into the structure of ALV proviruses present in bursal lymphomas. Several questions seemed accessible using this strategy. Were the tumors clonal? While histology and characterization of the serum immunoglobulins had failed to provide an answer, an analysis of the junctions between ALV proviruses and host cell DNA could resolve this issue in a manner demonstrated for mouse mammary tumor virus by Cohen et al.

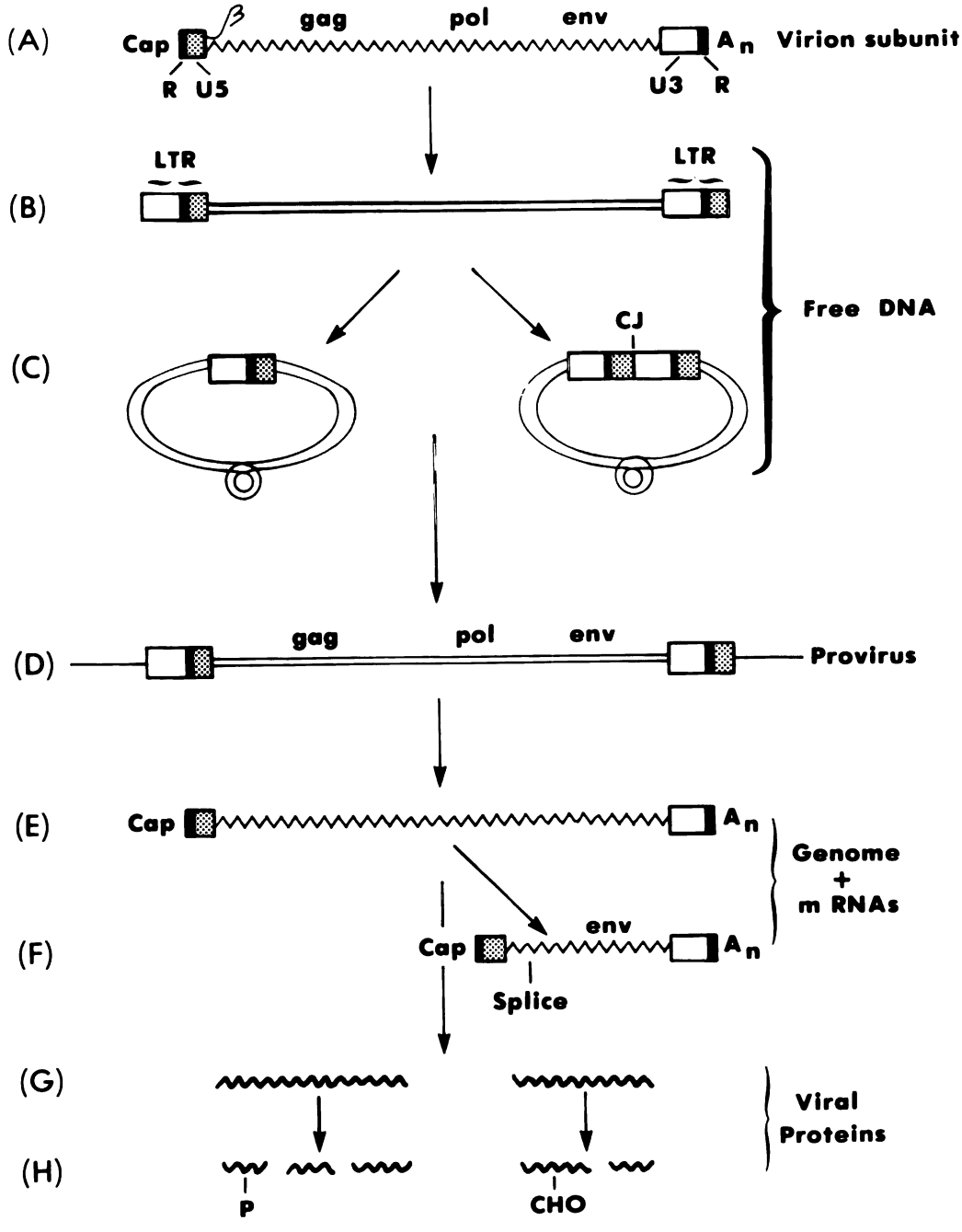
(1979). Furthermore, this type of analysis could also define the regions of the host genome occupied by ALV proviruses in the tumors, an approach pioneered by Ketner and Kelly (1976) and Botchan et al. (1976). Insertional mutagenesis is a potential corollary of viral DNA integration and the mechanism of oncogenesis could rely on an interaction of the provirus and specific flanking cellular sequences. The internal structures of proviruses resident in tumor cells could also be analyzed. Since replicating virus obtained from tumors did not display enhanced or altered tumorigenic potential the possibility existed that the integrated DNA had undergone a rearrangement necessary for oncogenesis yet destroying the ability of transcribed RNA to be packaged or replicated. Potential recombination events described in the first approach would also be present in proviruses found in the tumors.

The following two chapters describe the results of investigations into the nature of ALV proviruses present in bursal lymphomas.

Figure 1.

Replication and expression of retroviral genomes. (A) One of the two identical subunits of a viral RNA genome with its major structural and genetic features: the short sequence repeated at both termini (R, filled boxes); the unique sequence at the 5' end of the RNA which is repeated in viral DNA (U5, shaded box); host tRNA hydrogen-bonded to the genome at the boundary of U5; the coding domains for the viral structural proteins (gag, pol, env); the unique sequence found at the 3' end of the genome and repeated in viral DNA (U3, open box), and the polyadenylic acid tract (poly (A)). (B) The major product of reverse transcription, linear duplex DNA terminated by long terminal repeats (LTR's) composed of U3, R, U5. (C) Closed circular DNA, with one or two copies of the LTR. (D) Proviral DNA. (E and F) Genomic and messenger RNA's, derived from the primary transcript by capping, poly (A) addition and splicing; the splice donor and acceptor sites used to generate env mRNA are indicated in (F). (G and H) The polyproteins translated from viral mRNA's and their mature products after cleavage and, in some cases, glycosylation (CHO) or phosphorylation ($PO_4$).

# REPLICATION OF RETROVIRUSES



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# CHAPTER 2

## Analysis of Avian Leukosis Virus DNA and RNA in Bursal Tumors: Viral Gene Expression is not Required for Maintenance of the Tumor State

ABSTRACT

To investigate the mechanism of oncogenesis by avian leukosis viruses (ALVs), we have characterized the viral nucleic acids present in virus-induced bursal lymphomas using restriction endonucleases and molecular hybridization techniques.

Each of twelve tumors induced by either Rous associated virus-1 or -2 (RAV-1 or RAV-2) contained a predominant population of cells with ALV proviruses integrated at common sites. This is consistent with a clonal origin of these tumors. Seven of nine RAV-2-induced bursal tumors contained single proviruses, and all seven solitary proviruses were found to have suffered deletions. The detailed structures of four of these proviruses were investigated by comparing maps of restriction enzyme recognition sites in the proviruses to a map of restriction sites in unintegrated RAV-2 DNA. Major deletions had occurred near or at the 5' ends of these proviruses, spanning sequences potentially important in the production of viral RNA. One provirus also lacked most of the information coding for the replicative functions of the virus. Restriction maps of flanking cellular DNA suggest that these four proviruses were inserted in similar regions of the host genome.

We have studied virus-specific RNA in four bursal tumors and four cell lines derived from bursal tumors. No normal viral RNA species were detectable in three tumors containing single aberrant proviruses. However, transcripts of 2.2 kilobases which reacted only with a hybridization probe specific for the 5' end of viral RNA were observed in one of these three tumors. Analogous species, varying in length from 1.5 to 6.0 kb, were observed in a fourth bursal tumor with multiple proviruses and in all four cell lines. (This tumor and the cell lines

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The structures of the aberrant proviruses and the absence of normal viral RNA in some tumors indicate that expression of viral genes is not required for maintenance of the tumor phenotype. Furthermore, in at least some cases, the mechanism of oncogenesis may involve stimulation of transcription of flanking cellular sequences by a viral promoter.

INTRODUCTION

Avian leukosis viruses (ALVs) most frequently induce lymphatic neoplasms of the B-cell lineage arising in the bursa of Fabricius of chickens (for review, see Purchase and Burmester, 1978). The characteristics of oncogenesis by ALVs place these viruses in a unique category of avian retroviruses. ALV-induced bursal lymphomas become microscopically evident only 4-6 weeks after infection and require 4-6 months to reach macroscopic size. ALVs do not transform cells in culture, and no gene responsible for their oncogenic effects has been identified. In contrast, the other major classes of avian retroviruses, the sarcoma and the defective leukemia viruses, induce neoplasms which become grossly apparent within a few weeks post-infection and kill the chicken within 1-2 months; in addition, these viruses transform their respective target cells in culture. Rous sarcoma virus has been shown to exert its oncogenic effecs via a protein, $pp60^{src}$ (Brugge and Erikson, 1977; Purchio et al., 1978), which is not required for virus replication and is encoded in a gene (src) which has been transduced from the cellular genome (Stehelin et al., 1976; Spector et al., 1978a). Transduced host cell sequences also appear to be respon-

sible for the oncogenic effects of the defective leukemia viruses and other sarcoma viruses (Sheiness and Bishop, 1979; Rousssel et al., 1979).

The mechanism by which ALVs might exert their oncogenic effects has remained obscure. ALVs do not appear to contain transduced cellular sequences or to encode proteins other than those required for replication (Vogt, 1977). Sequences located near the 3' end of viral RNA have been implicated in ALV oncogenesis (Tsichlis and Coffin, 1980) because this region exhibits the only major divergence (Neiman et al., 1977; Coffin et al., 1978; Shank et al., 1980) from the sequences of a non-oncogenic avian retrovirus endogenous to some normal chickens, RAV-0 (Motta et al., 1975; Purchase et al., 1977).

We have begun to investigate the mechanism of ALV-induced tumorigenesis by analyzing the ALV-specific DNA and RNA found in bursal lymphomas and tumor cell lines derived from ALV-induced bursal lymphomas. We have found that the tumors appear to be clonal and that several contained single proviruses, allowing us to determine the structure of the provirus presumably responsible for oncogenesis in each case. Most of these proviruses exhibited deletions which spanned regions potentially important in viral RNA biogenesis. At least one deletion also removed most of the genetic information present in the provirus.

Our analysis of virus-specific RNA in tumors and tumor cell lines has revealed RNA species which may result from transcription of host cell sequences initiated at viral promoters. In addition, in three tumors with solitary, defective proviruses, we were unable to detect normal viral mRNAs. Similar results have been obtained by Neel et al. (1981) and are presented in the accompanying paper.

RESULTS

Restriction Map of RAV-2 DNA.

In order to analyze RAV-2 proviruses present in bursal tumors, it was necessary to construct a map of restriction endonuclease recognition sites present in RAV-2 DNA. Using techniques similar to those described by Shank et al. (1978), we derived a physical map of restriction sites in RAV-2 linear DNA. The approximate relationships between restriction sites, viral RNA, viral genes, and the long (330 bp) terminal redundancy (LTR) are shown in Figure 2. Kpn I, Sac I and Hind III cleave the viral DNA once. Eco RI and Bam HI each produces three internal fragments from RAV-2 DNA, some of which comigrate with internal fragments from proviruses endogenous to chickens used in our study. However, the 2.3 kbp and 1.1 kbp Eco RI fragments and the 1.8 kbp Bam HI

fragments are derived only from exogenous RAV-2 proviruses and have been used as signature fragments diagnostic of specific regions of the RAV-2 provirus (see Figure 2).

ALV-induced tumors are clonal and contain few ALV proviruses.

In this report we present results obtained with tumors from eight SPAFAS animals and one $15I_5 \times 7_2$ animal inoculated with RAV-2. In addition, we have analyzed tumors from three $15I_5 \times 7_2$ birds infected with RAV-1 [see Experimental Procedures]. Table 1 summarizes information concerning each of the tumors used in the experiments described in subsequent sections.

Digestion of proviral DNA with restriction endonucleases produces two types of virus-specific fragments: (i) fragments containing viral sequences linked to host sequences (junction fragments) and (ii) inter-

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nal fragments common to all normal proviruses, regardless of the integration site in host cell DNA. Because retroviral DNA can enter many sites in host DNA (Hughes et al., 1978; Steffen and Weinberg, 1978), analysis of junction fragments requires a clonal population of cells, homogenous with respect to sites occupied by proviral DNA. Studies of proviruses in murine tumors induced after long latency by leukemia and mammary tumor viruses have indicated that such tumors are clonal or semi-clonal (Steffen and Weinberg, 1978; Cohen et al., 1979; Cohen and Varmus, 1980; Jahner et al., 1980) and hence amenable to full analysis of proviruses with restriction enzymes. We (see below) and others (Neiman et al., 1980; Neel et al., 1981; Y.K. Fung and H.-J. Kung, personal communication) have confirmed this observation using tumors induced by the ALVs.

The presence of ALV-related endogenous proviruses in most chickens complicates the analysis of restriction fragments of ALV DNA in tumors. Hughes et al. (1980b) and Hayward et al. (1980) have constructed physical maps of most of the endogenous proviruses identified by Astrin et al. (1980). We have thus been able to identify the endogenous proviruses present in several of the tumors analyzed (see Table 1 and Appendix 1).

To address the issue of clonality and to estimate the number of copies of ALV DNA in each tumor, tumor DNAs were initially tested with enzymes which cleave once in RAV-2 proviral DNA. Such enzymes produce two fragments from each normal RAV-2 provirus present in the tumor cells; these fragments are absent from parallel digests of DNA from uninvolved tissue from the same bird.

Analyses of DNA from two tumors are illustrated in Figure 3. Hybridi-

zation of a probe representing the entire viral genome ($cDNA_{rep}$) to a Kpn I digest of DNA from tumor LL 5 revealed two fragments (Fig. 3, lane 1) not found in a digest of DNA fom uninfected circulating red blood cells (Fig. 3, lane 3). This tumor was thus clonal or semi-clonal and probably contained only one new exogenous provirus. Annealing of $cDNA_{rep}$ to a Hind III digest of DNA from bursal tumor LL 6 produced two bands (lane 4) which were absent in a digest of DNA from uninfected red blood cells (lane 6). This tumor was clonal and also apparently contained a single RAV-2 provirus.

Proviruses in metastatic growths are identical to those in the primary tumors.

Some birds with bursal tumors were found to contain metastases in the spleen or liver.

We were thus able to ask whether the metastatic lesions were clonal and whether they contained the same RAV-2 proviruses as the primary tumors.

A Kpn I digest of DNA from a focus of tumor cells present in the liver of chicken 5 (Fig. 3, lane 2) was indistinguishable from the digest of bursal tumor DNA (lane 1)), suggesting that the metastatic cells also contained a single RAV-2 provirus integrated at the same location as the provirus in the bursal tumor cells. The liver tumor apparently resulted from proliferation of bursal tumor cells without amplification or extensive alteration of proviral DNA. These conclusions were supported by further mapping experiments with material from chicken 5, using additional enzymes and hybridization probes (data not shown), and by analysis of DNA from the bursal tumor, a splenic metastasis, and uninfected circulating red blood cells from bird 6. As revealed by

digestion with Hind III (lanes 4-6), the primary tumor and the metastatic growth appeared to harbor the same, single RAV-2 provirus.

The single RAV-2 provirus present in bursal tumor LL1 is defective

The existence of clonal tumors containing single exogeneous proviruses allowed us to construct physical maps of the proviruses presumably responsible for tumorigenesis. In Figure 4, we present a partial analysis of the single provirus in tumor LL 1. Kpn I produced two tumor-specific fragments of 11.8 and 8.2 kbp from LL 1 DNA (Fig. 4A, lanes 1 and 2), as expected for a clonal growth bearing a single new provirus. (The 11.8 fragment was clearly distinguishable from a similarly-sized fragment containing endogenous proviral DNA in an autoradiogram obtained after a shorter exposure. This result was confirmed in tests with Hind III and Sac I , both of which cleave RAV-2 DNA once (data not shown). To investigate the genetic composition of this provirus, we annealed RAV-2 $cDNA_3$, to the Kpn I digest of LL 1 DNA (Figures 4A, lanes 3 & 4). $cDNA_3$, is complementary to unique sequences located at the 3' end of the viral RNA ($U_3$; see Figure 2 and Experimental Procedures), and it should anneal to both of the tumor specific Kpn I fragments by virtue of the $U_3$ sequences located in the proviral LTRs. However, only the 8.2 kbp Kpn I fragment reacted with RAV-2 $cDNA_3$, ; the 11.8 kbp fragment did not react (Figure 4A, lanes 3 and 4). The Bam C probe, specific for sequences located in the gag gene of both exogenous and endogenous proviruses (see Figure 2), annealed to the 11.8 kbp Kpn I fragment, but not to the 8.2 kbp fragment, identifying the larger fragment as the left junction fragment (Fig. 4, lanes 5 and 6). The simplest interpretation of these data is that the RAV-2 provirus present in this tumor lacked $U_3$ sequences at the left cell-provirus

border.

Digestion of DNA from LL 1 with an enzyme, Eco RI , that cleaves RAV-2 proviral DNA at multiple sites, supported this observation. Eco RI digestion of a normal RAV-2 provirus, coextensive with linear viral DNA, should produce three internal fragments (see Figure 2) which can be detected with $cDNA_{rep}$. Only about 150 base pairs at each end of proviral DNA remain joined to cell sequences, and the Eco RI junction fragments cannot be detected with $cDNA_{rep}$. The 3.8 kbp internal fragment bearing sequences from the center of the RAV-2 provirus comigrates with a similar fragment from the endogenous proviruses present in these chickens. However, the internal restriction fragments of 2.3 and 1.1 kbp are unique to the RAV-2 provirus because the Eco RI recognition sites located in the RAV-2 LTR's do not occur in the LTR's of the endogenous proviruses (Hughes et al., 1980b).

Eco RI digestion of the RAV-2 provirus in LL 1 generated the expected 1.1 kbp Eco RI fragment, but the 2.3 kbp fragment was absent (Figure 4B, lanes 7, 8). (The 1.1 kbp fragment is difficult to see in lane 7, but it was clearly evident in the autoradiogram and readily visualized by annealing with $cDNA_3$, (lane 11).) An additional unexpected Eco RI fragment of 13 kbp was detected in the digest of the tumor DNA. The Bam C probe, composed of sequences contained entirely within the normal 2.3 kbp Eco RI fragment, hybridized to the 13 kbp fragment (lanes 9, 10). These findings suggested that a deletion which removed the Eco RI site in the $U_3$ region of the left LTR linked sequences normally found within the 2.3 kbp internal fragment to host sequences (cf. Figure 5).

This interpretation was supported by annealing with $cDNA_3$, which

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Many tumors contain abnormal proviruses

The results of the previous section suggested that one of the bursal tumors contained an abnormal provirus and demonstrated how Eco RI can be used to screen tumors for proviruses which have suffered major alterations.

We have analyzed DNA from 12 tumors with Eco RI and with at least one enzyme which cleaves once in RAV-2 DNA (data not shown). Seven of these tumors appeared to contain single RAV-2 proviruses (Table 1). All seven solitary proviruses have sustained alterations which affect

at least the region defined by the Eco RI 2.3 kbp fragment. The tumors containing multiple proviruses were more complicated. One tumor probably contained two defective exogenous proviruses; another probably contained both defective and non-defective exogenous proviruses. Finally, DNA from three other tumors yielded all of the expected Eco RI fragments but no aberrant fragments. However, this analysis was insufficient to exclude the presence of one or more abnormal proviruses in these tumors.

Physical maps of single aberrant RAV-2 proviruses in tumors LL 1-LL 4..

We have used the strategies illustrated with LL 1 to construct detailed physical maps of single aberrant RAV-2 proviruses present in four tumors (LL 1-4) (see Appendix 1). In each case, single deletions appeared to account for the mapping data; the extent of each lesion is diagrammed in Figure 5 and discussed more fully below.

Physical maps of DNA flanking proviruses in LL1, LL2, and LL 3 reveal similar integration sites.

Using the mapping data from which the proviral deletions were deduced, it was possible to construct physical maps of the regions of chicken DNA which had acquired RAV-2 proviruses in LL 1, LL 2, and LL 3; these maps are depicted in Figure 6.

The restriction enzyme recognition sites in the DNA flanking the proviruses in tumors LL 1 and LL 3 could be unambiguously mapped, based on the sizes of the host-provirus junction fragments. Since the exact size of the deletion in the provirus in tumor LL 2 was not determined, we could not be certain of the absolute distances between the recognition sites encompassing the deletion, although we were able to estimate the distances to within 1 kbp (Figure 5). Moreover, the deletion must

affect the size of each host-provirus junction fragment similarly; therefore the order and position of the sites could be determined relative to each other on each side of the provirus.

The positions of recognition sites for Bam HI , Kpn I , Eco RI and Hind III to the right of the proviruses in tumors LL 2 and LL 3 were identical. The recognition sites of these enzymes to the left of the proviruses had the same order and same position relative to each other. If we assume that the deletion in the provirus of tumor LL 2 spanned 1.2 kbp (a reasonable estimate, based upon the data), then the recognition sites were identical on both sides of the integration sites occupied in LL 2 and LL 3. (We have made this assumption in Figure 6.)

Sac I did not cleave either of these proviruses. The Sac I fragment containing the provirus in tumor LL 2 was 20 kbp, and the provirus-containing Sac I fragment from the DNA of tumor LL 3 was 24 kbp. The extent of the deletions in the two proviruses could not differ sufficiently to account for this 4 kbp difference. Thus there must have been a difference in the position of the Sac I recognition sites in the host sequences flanking the proviruses. However, the cell sequences flanking the proviruses in tumors LL 2 and LL 3 seem to be very similar and might exhibit only minor differences, including one within the six bases comprising a Sac I recognition site. Many examples of genetic polymorphism recognized in this fashion have been reported (Mandel et al., 1978; Weinstock et al., 1978; Lai et al., 1979; Hughes et al., 1979).

Comparison of the maps depicted in Figure 6 reveals that the positions of restriction sites in DNA flanking the LL1 provirus are very similar to the sites in DNA flanking the LL 2 and LL 3 proviruses, but

inverted in orientation. (Again Sac I appeared to differentiate the integration sites, but the differences may be due to sequence polymorphisms as described above.) We suggest that similar regions of the host genome have been used as integration sites in LL 1-LL 3, but that the provirus in LL 1 was inserted in an orientation opposite to that of the proviruses in LL 2 and LL 3 (see Discussion).

The limited number of useful restriction sites in the truncated provirus in LL 4 stymied efforts to generate a detailed map of the integration site. However, single and double digestions with Eco RI and Sac I have shown that sites for these enzymes were arranged on both sides of the LL 4 provirus in the same pattern as found for the LL 1 provirus (data not shown). It is thus possible that the integration site in LL 4 is similar or identical to that used in the other three tumors.

Bursal tumors may lack normal RAV-2 mRNAs and exhibit provirus-promoted transcription of flanking cellular DNA.

Proviruses contain regions which may supply sequences important in initiation, polyadenylation, and splicing of viral RNA. The deletions in the solitary proviruses LL 1-LL 4 spanned either the postulated promoter region, the env mRNA donor splice site, or both (see Fig. 5). The deletion in the LL 4 provirus also removed most of the coding nformation present in the RAV-2 provirus (Fig. 5). These results suggested that normal expression of viral genes could not occur in these tumors and that viral gene products may not be necessary for maintenance of the tumor state.

To examine this possibility, we have attempted to analyze viral RNA in bursal tumors, using gel electrophoresis to determine the size

and hybridization kinetics to measure the concentration of ALV-related RNA. In Figure 7 we present autoradiograms displaying viral RNA species, detectable with various hybridization reagents, from a tumor bearing multiple RAV-2 proviruses (LL 21) and a tumor bearing a single defective RAV-2 provirus (LL 1). Analysis of RNA from tumor LL 21 showed that all three hybridization probes, $cDNA_{rep}$, $cDNA_3$, and $cDNA_5$, detected the normal RAV-2 mRNAs of 8.4 kb ($mRNA^{fl\,gag}$ and $mRNA^{gag\text{-}pol}$) and 3.2 kb ($mRNA^{env}$) (Figure 7, lanes 1-3) expected in RAV-2 infected cells (Hayward et al., 1977; Weiss et al., 1977; Lee et al., 1979; Quintrell et al., 1980); these species were also observed in parallel analyses of RAV-2 infected fibroblasts (Figure 8, panels A-C, lane 1). However, $cDNA_5$, also detected an RNA species of about 2.4 kb which failed to anneal with the other cDNAs (lane 3).

RNA from LL 1 did not appear to contain the normal RAV-2 RNAs of 8.4 and 3.2 kb (lanes 4-6), as predicted from the structure of the provirus in this tumor (see Figure 5). Again, $cDNA_5$, recorded an RNA species (2.2kb) (lane 6) which did not anneal to $cDNA_{rep}$ or $cDNA_3$, (lanes 4 and 5). We presume that such transcripts join sequences copied from the $U_5$ region of RAV-2 DNA to sequences copied from flanking cellular DNA; mechanisms by which such transcripts might be generated are considered in the Discussion. We failed to detect any virus-specific RNA in samples of RNA from tumors LL 2 and 3 analyzed in parallel with RNA from tumor LL 1 using $cDNA_{rep}$, $cDNA_3$, and $cDNA_5$, (data not shown).

The analysis of gel-fractionated RNA from tumor LL 1 was corroborated by determining the kinetics of hybridization of $cDNA_{rep}$ and $cDNA_5$, to LL 1 RNA in solution (data not shown). Seventy percent of

$cDNA_5$, annealed to RNA from tumor LL 1 with a Crt 1/2 of $9 \times 10^{-3}$ mole-sec/l. This corresponds to approximately 10 copies of th 2.2 kb RNA per cell. Only 25% of $cDNA_{rep}$ hybridized at a Crt 1/2 value of $2 \times 10^{-4}$ mole-sec/l. This low level probably represented annealing of $cDNA_{rep}$ to transcripts from endogenous proviruses and cellular src (Wang et al., 1977; Spector et al., 1978b). Parallel tests of RNA from ALV producing cultured cells (BK 4484A) indicated that the $cDNA_5$, and $cDNA_{rep}$ used in these experiments hybridized at similar rates and to similar extents with normal viral RNA (data not shown). Again, parallel solution hybridization of RNA from tumors LL 2 and LL 3 to $cDNA_5$, and $cDNA_{rep}$ failed to reveal virus-specific RNA present in concentrations higher than that expected from transcripts of endogenous proviruses (data not shown).

Cultured bursal tumor cells also contain RNA species detected only with $cDNA_5$,.

Three tissue culture lines established by Hihara et al. (1974, 1977) (BK4484A; 1104B-1; 1104X-5) and a fourth (R2B) established by one of us (S.A.C.) from a tumor passaged in vivo by Okazaki et al. (1980) all contain multiple acquired ALV proviruses (data not shown). Viral RNA species were detected using probes specific for various regions of the viral genome (Figure 8, panels A-C, lanes 2-5). Each line contained the normal two species of viral RNAs (8.4 and 3.2 kb) which reacted with $cDNA_{rep}$, $cDNA_3$, , and $cDNA_5$, (Figure 8, panels A-C) and were indistinguishable from species in RAV-2 infected fibroblasts (Figure 8, panels A-C, lane 1). However, each tumor line also contained RNA species detected only with $cDNA_5$, ; such species were not observed in RAV-2 infected fibroblasts (lane 1 in each panel) or in uninfected tis-

sues from tumor-bearing or normal chickens (data not shown). The BK4484A cells exhibited two RNA species (2.6 and 1.5 kb) which reacted only with $cDNA_5$, (Figure 8C, lane 2). The cell lines 1104B-1 and 1104X-5, which were derived from the same tumor (Hihara et al., 1974) but had slightly different patterns of proviral restriction fragments, contained similar RNA species of 66 kb and 1.6 kb detected only with $cDNA_5$, (Figure 8C, lanes 3 and 4). Three size species of RNA (6 kb, 2.6 kb, and 1.7 kb) were identified only with $cDNA_5$, in R2B cells (Figure 8C, lane 5).

The most abundant RNA species detected only with $cDNA_5$, in each line was present at approximately 50 to 100 copies per cell, as estimated from the intensity of bands in Figure 7C and from kinetic measurements of total viral RNA in line BK4484A.

## DISCUSSION

In this report we have described the physical structure and genetic composition of proviruses and virus-specific RNAs present in bursal lymphomas from chickens with leukosis caused by avian leukosis virus and in tissue culture lines derived from ALV-induced bursal tumors. We have found that (1) all tumors appear to be clonal (i.e., each tumor consisted of a predominant population of cells containing at least one provirus integrated at a common site); (2) most (9 of 12) tumors contained proviruses which incurred major deletions detected by restriction enzyme analyses (in 7 of 12 tumors the altered provirus is the only exogenous provirus present in the tumor cells); (3) in at least three tumors no normal species of viral RNA were detectable, suggesting, in conjunction with the aberrant structure of several solitary proviruses, that expression of viral genes is not required for

maintenance of the tumor state; (4) some bursal lymphomas and cell lines derived from bursal lymphomas exhibited RNA species which probably consist of sequences from the 5' end of viral RNA joined to cell sequences; and (5) in at least three tumors, single RAV-2 proviruses were located at very similar, if not identical, sites in the host genome. This constellation of findings is consistent with the proposal that ALVs may exert their oncogenic effects by altering expression of a subset of host genes, rather than by elaboration of a viral gene product. Similar data have been set forth by Neel et al. (1981) in the accompanying paper.

Expression of replication functions is unnecessary for maintenance of the tumor state.

The detailed physical maps of four solitary proviruses in bursal tumors suggests that normal expression of replication genes could not occur and thus is not necessary for maintenance of the oncogenic state (Figure 5). Three of the proviruses sustained deletions which affected either the presumed viral promoter or the donor splice site for env mRNA. The fourth provirus incurred a deletion which removed most of the genetic information of the provirus. The apparent absence of normal species of viral RNA in the tumors LL 1-LL3 further supports the idea that expression of replication functions is unnecessary for maintenance of the tumor state. However, we cannot exclude the possibility that viral genes are instrumental in the initiation of tumor growth, since all of our experiments were performed with materials from advanced neoplasms. As expected from the structures of the proviruses in each tumor, we were unable to detect subgroup B virus in the bursal tumors LL 1, LL 3 and LL 4 (see Experimental Procedures). Subgroup B

virus was detected in tumor LL 2. We believe this virus was produced by non-tumor cells containing a complete RAV-2 provirus but present in the bursal tumor at a level undetectable by our hybridization analyses.

Novel species of RNA are present in some bursal tumors and tumor cell lines.

Although normal viral RNAs may be absent from tumor cells, we have observed RNA species which anneal only to $cDNA_5$, in tumor cell lines and in some tumors. These findings support the hypothesis that ALV induces tumors by promoting transcription of flanking cellular genes (see below), but we have also encountered two tumors (LL 2 and LL 3) in which no virus-related RNA could be detected. The findings with LL 2 and LL 3 could mean that the hypothesis is incorrect, that a second mechanism is also operative, or that late changes in provirus structure or transcriptional activity have obscured the initiating events.

Virus specific RNAs which react only with $cDNA_5$, were first observed by Quintrell et al. (1980) in lines of ASV-transformed mammalian cells. These transcripts were postulated to consist of viral $U_5$ sequences joined to host cell sequences. Synthesis of normal species of viral RNA is thought to be initiated at the site within the left LTR corresponding to the capped nucleotide at the 5' end of the viral genome (Hughes et al., 1978; Sabran et al., 1979; Tsichlis and Coffin, 1980). Putative promoter sequences have been identified in the $U_3$ region and are thus found at both ends of each normal provirus (Shimotohno et al., 1980; Dhar et al., 1980; Czernilofsky et al., 1980; Swanstrom et al., 1980; Sutcliffe et al., 1980; Van Beveren et al., 1980; Majors and Varmus, 1980a; Hager and Donehower, 1980). The RNAs which only anneal to $cDNA_{5'}$ are probably generated by transcription of host

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cell sequences using a viral promoter supplied by an LTR positioned at the right or left ends of normal or abnormal proviruses. Two models for the origin of these RNAs are diagrammed in Figure 9A. Transcription could originate within the right LTR and directly proceed into host cell sequences or could originate within the left LTR and proceed through the entire provirus into cell sequences. In the latter case, the RNA must then be processed (spliced) to remove most of the viral sequences. In the case of tumor LL 1, which lacked the left LTR, the RNA probably originated within the right LTR (Fig. 8B). In other instances in which such RNA species have been observed, we have yet to determine which LTR initiated the transcripts.

Are ALV proviruses integrated into similar regions of the genome in different bursal lymphomas?

Provirus-promoted transcription of host sequences represents a mechanism by which ALVs could exert a tumorigenic effect. Recent experiments of Cooper and his colleagues (1980) suggest that normal cell sequences taken out of the context of their normal flanking regions can exert transforming (oncogenic) effects. ALVs may subvert transcriptional control of certain cellular genes by inserting an upstream viral promoter; heightened or aberrant expression of such genes could conceivably extinguish growth control of B lymphocytes and produce leukosis. A similar mechanism might be employed by mammalian retroviruses such as MuLV, MMTV and bovine leukemia virus, which also induce clonal tumors after a long latent period (Steffen and Weinberg, 1978; Jahner et al., 1980; Cohen et al., 1979; Cohen and Varmus, 1980; Kettman et al., 1980).

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One prediction of this model is that the proviruses present in different tumors will be integrated near a specific subset of cell genes capable of exerting oncogenic effects on the B-lymphocyte. This does not imply that RAV-2 integrates into a small number of sites in the chicken genome. On the contrary, ample data suggest that retroviruses integrate into many sites in the host genome (Hughes et al., 1978; Steffen and Weinberg, 1978; Cohen et al., 1979; Bachelor and Fan, 1979; Ringold et al., 1979; Gilmer and Parsons, 1979; Canaani and Aaronson, 1979; van der Putten et al., 1979; Keshet et al., 1979; Jahner et al., 1980; Collins et al., 1980; Hughes et al., 1980; Quintrell et al., 1980; Cohen and Varmus, 19800; Groner and Hynes, 1980; Majors and Varmus, 1980b; Kettman et al., 1980). We propose that RAV-2 infects many cells in the bursa, integrating at different sites in most cells. However, only a limited number of integration events may place the provirus in a position to initiate tumorous growth. This hypothesis could account for the clonal nature and lengthy latency of ALV-induced tumors.

After completing the experiments described here, we learned from Hayward and his colleagues that the ALV proviruses in the tumors described by Neel et al. (1981) were inserted near the cellular sequences related to the putative transforming gene of myelocytomatosis virus-29 (MC-29); moreover, some of the RNA species detected with $cDNA_5$, but not with $cDNA_{rep}$ (Neel et al., 1981) appeared to anneal with cDNA specific for the transforming region of MC-29 (Hayward et al., 1981). We have therefore reexamined materials from our tumors with a probe derived from the same region of cloned MC-29 circular DNA (B. Vennstrom and J.M. Bishop, manuscript in preparation). All of our

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tumors, including LL 1-LL 4, contained proviruses closely linked to the cellular homologue of the MC-29 onc gene, confirming the results of Hayward et al. (1981). In some cases, the MC-29-specific region was positioned on the 5' side of the proviruses. The latter results are consistent with the restriction maps of the integration sites for proviruses in LL 1-LL 3 (Fig. 6) and may be related to the absence of detectable viral RNA in LL 2 and LL 3.

Cooper and Neiman (1980) have recently found that NIH-3T3 fibroblasts can be transformed at high frequencies wth DNA from ALV-induced bursal lymphomas. ALV proviral sequences (including sequences from the LTR) did not appear to be required for transformation of the NIH-3T3 cells. Further work is required to understand the relationship of these findings to those reported to us and by Neel et al. (1981).

Proviruses in metastases

Two of the tumors described in our report had metastasized either to the liver or spleen. The single abnormal provirus present in each metastatic growth was identical by restriction mapping to the provirus present in the primary bursal lymphoma. Thus provirus amplification, virus spread, or further major alterations in the sructure of the provirus are not required to confer metastatic potential on the tumor cell population residing in the bursa. Our findings are clearly inconsistent with the proposal by Neiman et al. (1980) that amplification of viral DNA is associated with metastatic potential.

Deletions may reflect selection against viral expression.

The abnormal structure of the exogenous proviruses present in the tumors was a striking feature of our results. Eight out of 12 tumors contained only proviruses with significantly altered conformation. A

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ninth tumor contained both nondefective and defective proviruses. However, it seems improbable that the provirus must undergo a structural change to exert its oncogenic capacity. We and others (Neiman et al., 1980; Neel et al., 1981; Y.K. Fung and H.-J. Kung, personal communication) have observed tumors containing only apparently normal exogenous proviruses. A more likely possibility is that cells containing abnormal proviruses defective in expression of viral antigens are selected during the process of tumor progression by the host immune response to viral proteins, especially the env glycoprotein.

We have encountered other examples of proviral deletions similar to those described here, involving sequences near or at the left LTR, under conditions which may select against the expression of viral genes. By selecting for phenotypic revertants of an ASV-transformed rat cell (Varmus et al., 1980), mutants bearing deletions affecting the left end of an ASV provirus and eliminating expression of the viral src gene have been obtained (Majors et al., 1981). Hughes et al. (1980b) and Hayward et al. (1980) have described several deletions affecting the left ends of endogenous chicken proviruses; Hughes et al. (1980b) have proposed that these mutated proviruses may pose less of an evolutionary disadvantage to their host than intact proviruses, thus accounting for their prevalence.

Detailed mapping o

# APPENDIX 1

Detailed mapping of single RAV-2 proviruses in four bursal tumors.

LL 1

A portion of the analysis of the RAV-2 provirus in LL 1 is illustrated in Figure 4 and described in the Results section of the main text. The location of the left host-provirus junction was further defined using Sac I and Bam Hl. Sac I digestion of tumor DNA produced two fragments of 8.1 kbp and 6.7 kpb from the exogenous provirus (Figure 10A, lane set 1), only one of which (8.1 kbp) hybridized to $cDNA_3$, (lane set 2) as predicted from the experiments described earlier. The presence of two virus-specific fragments indicated that the provirus had retained the Sac I site. Digestion of tumor DNA with Bam HI revealed the 1.8 kbp signature fragment from the envelope region of the provirus after annealing to $cDNA_{rep}$ (lane set 3). $cDNA_3$, detected an 8.5 kbp fragment (lane set 4) which represented the right host-provirus junction fragment. We were unable to detect the left Bam HI junction fragment with the available probes. This may have been due to the comigration of this fragment with a fragment from an endogenous provirus or to the paucity of viral DNA in the left junction fragment ($cDNA_3$, would have failed to detect a comigrating fragment because the fragment lacked the LTR). Further experiments using Bam HI (not shown) suggest that the structure of the exogenous provirus is intact to the right of the Sac I site.

The analyses of LL 1 (Figure 4, Figure 10A) indicated that the left host-provirus junction lacked $U_3$ and $U_5$ sequences (Figure 5). This structure was probably due to a deletion of the left LTR but the possibility remains that it resulted from the integration of a circular molecule which joined proviral sequences to host sequences between the LTR and the Bam HI site closest to the LTR in the gag region (see Fig-

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Figure 10B, lane sets 1-3, show Kpn I digests of DNA from LL 2. Only one new fragment (10.9 kbp) was clearly present in digests of tumor DNA as judged by hybridization to $cDNA_{rep}$ (Figure 10B, lane set 1). $cDNA_{rep}$ annealed more strongly to DNA migrating at 8.8 kbp in digests of tumor DNA than to DNA of this size in digests of uninfected spleen DNA. This suggested that a second fragment containing RAV-2 proviral sequences may have been comigrating at 8.8 kbp with the fragment from an endogenous provirus. This interpretation was supported by hybridization of tumor DNA with the Bam C probe; the result also assigned this fragment to the left end of the RAV-2 provirus (Figure 10B, lane set 2). Lane set 3 shows that the 10.9 kbp Kpn I fragment reacted with RAV-2 $cDNA_3$,. Again, the 8.5 kbp fragment in digests of tumor DNA annealed more strongly to RAV-2 $cDNA_3$, than to 8.5 kbp fragments in digests of spleen DNA (Figure 10, lane set 3). In this case, since both Kpn I fragments containing RAV-2 DNA annealed to RAV-2 $cDNA_3$, , $U_3$ sequences were at least partially retained in both LTRs. The reaction of this preparation of RAV-2 $cDNA_3$, with endogenous proviral DNA is probably due to a portion of the probe complementary to the 3' end of the env gene (Hughes et al., 1980b).

Lane set 4 shows that $cDNA_{rep}$ failed to detect the 2.3 kbp fragment in an Eco RI digest of tumor DNA but a new tumor-specific fragment appeared at 4.2 kbp. Hybridization with the Bam C probe (lane set 5) showed that sequences normally in the 2.3 kbp fragment were present in the 4.2 kbp fragment. This result suggested that the 4.2 kbp fragment was the left host-provirus junction fragment which lacked the Eco RI

site in the LTR. The 4.2 kbp fragment annealed to $cDNA_3$, (lane set 6), indicating that it still retained some $U_3$ sences. In addition, $cDNA_3$, revealed another new exogenous fragment, presumably containing the right end of the RAV-2 provirus, migrating at 12.7 kb. $cDNA_5$, (lane set 7) only reacted with the 12.7 kbp fragment. This result indicated that the deletion included the Eco RI site and the $U_5$ sequences in the left LTR, but spared some of the $U_3$ sequences.

Sac I and Bam HI digests placed the rightward end point of the deletion in the gag gene to the right of the Bam HI site closest to the left LTR. Sac I digests of DNA hybridized to either $cDNA_{rep}$ (lane set 8) or $cDNA_3$, (lane set 9) showed only one tumor-specific fragment, suggesting that the Sac I site had been deleted. A Bam HI digest of tumor DNA hybridized to $cDNA_{rep}$ (lane set 10) revealed a faint band at 6.0 kbp on the autoradiogram. This fragment reacted with $cDNA_3$, (lane set 11), identifying it as one of the junction fragments. The intensity of $cDNA_{rep}$ annealing to tumor DNA at 6.6 kbp relative to DNA migrating at 6.6 kbp in a digest of spleen DNA was consistent with the comigration of the second junction fragment with a fragment from an endogenous provirus. Hybridization with the Bam C probe showed that this was the case (lane set 12). Deletion of the Bam HI site nearest the left LTR created a host-proviral junction fragment containing the sequences represented by the Bam C probe. This explains why the Bam C probe annealed to the 6.6 kbp fragment in addition to the 1.6 kbp fragment from the endogenous proviruses. Since the Bam C probe hybridized well to the 6.6 kbp fragment, a substantial amount of these sequences must have been present in the RAV-2 provirus.

These experiments mapped a deletion which begins in $U_3$ to the 5'

side of the Eco RI site in the left LTR, spans $U_5$ sequences and the Sac I and Bam HI sites, and ends in the N-terminal half of gag.

LL 3

The third panel displays an analysis of Kpn I digests of DNA from chicken 3. The patterns of restriction fragments detected with different probes were remarkably similar to those of bird 2 (compare Figure 10C, lane sets 1-3 to Figure 10B, lane sets 1-3), and a similar argument applies. The similarity of the restriction fragment patterns of proviruses in these two tumors using enzymes which cleave once within the provirus suggested that these proviruses may be located at similar sequences in the host genome. $cDNA_{rep}$ revealed no new Eco RI fragments other than the 1.1 kbp fragment in tumor DNA (Figure 10C, lane set 4). Hybridization with the Bam C probe (lane set 5) indicated that the sequences normally in the 2.3 kbp fragment migrated at 1.1 kbp. A probe made from the 3.0 kbp Eco RI fragment of SR-A, which contains the sequences in the RAV-2 Eco RI 1.1 kbp fragment plus src, also reacted with the 1.1 kbp fragment (data not shown). Since internal regions of the provirus are intact (e.g., Kpn I sites are retained), two fragments of 1.1 kbp must have comigrated, a fragment containing sequences normally in the 2.3 kbp Eco RI fragment and the wild-type 1.1 kbp fragment. Assuming that a deletion of contiguous sequences had occurred, one of three explanations could account for these results. Since the 2.3 kbp Eco RI fragment was absent, either one or both of the Eco RI sites defining this fragment could have been deleted, or the deletion might have occurred within the two sites. If both Eco RI sites were deleted by a single lesion, we would not expect to see hybridization of the Bam C probe to fragments from the exogenous pro-

virus (Figure 2). Therefore, at least one of the Eco RI sites and some of the sequences normally in the 2.3 kbp fragment were probably present. Hind III cleaves a normal RAV-2 provirus about 800 base pairs to the right of the Eco RI site at the gag/pol junction. Lane set 8 shows that Hind III produced 2 fragments, both of which reacted with $cDNA_{3'}$. Thus, the Hind III site was present and at least some 3' sequences were retained at both ends of the provirus. A deletion which lay to the left of the Hind III site and to the right of the host/provirus junction could only produce a 1.1 kbp fragment if it were contained entirely within the 2 Eco RI sites normally defining the 2.3 kbp Eco RI fragment. The following results were consistent with this interpretation. As described in the analysis of tumor LL 1, Eco RI produces four fragments (from a wild-type RAV-2 provirus) which react with $cDNA_{3'}$. Two of these fragments should react with $cDNA_{5'}$: the internal fragment from the left end of the provirus and the right host/provirus junction fragment. Lane sets 6 and 7 show that the 12.7 kbp Eco RI fragments reacted with both $cDNA_{3'}$ and $cDNA_{5'}$. The 2.7 kbp Eco RI fragment reacts only with $cDNA_{3'}$. Hybridization of $cDNA_{5'}$ to a 1.1 kbp fragment is consistent with the suggestion that one of the two 1.1 kbp fragments contained sequences to the right of the Eco RI site in the left LTR. Since this band contained both internal Eco RI fragments, the 12.7 kbp fragment represented the right host cell/provirus junction and the 2.7 kbp fragment represented the left junction.

The deletion spanned the Sac I and Bam HI sites near the left LTR. Sac I digests revealed only one fragment after hybridization to $cDNA_{rep}$ and $cDNA_{3'}$ (lane sets 9 and 10). Using an argument analogous to that described in the analysis of the previous provirus, the Bam HI site was

also missing (data not shown). Thus the deletion began to the right of the left LTR (or within the $U_5$ sequence) and proceeded for 1.2 kbp.

LL 4

Panel D, lane set 1 shows a Hind III digest of DNA from bird 4. Only one tumor specific fragment annealed weakly to $cDNA_{rep}$. $cDNA_3$, detected only this 10 kbp fragment (lane set 2). Pvu II-B probe, containing sequences from the 3' end of the env gene and the 5' end of the src gene in SR-A (see Experimental Procedures), failed to detect any tumor specific fragments (Lane 3). These observations suggested that this provirus incurred a large deletion spanning the Hind III recognition sequence and the env gene.

Bam HI digestion of LL 4 DNA and hybridization to $cDNA_{rep}$ failed to reveal the 1.8 kbp signature fragment from the env gene (lane set 4). $cDNA_3$, detected only one tumor-specific Bam HI fragment (10 kbp) in lane set 5 which hybridized weakly to $cDNA_{rep}$ in lane set 5. Neither Pvu II-B or Bam C probe (lane sets 6 and 7) revealed tumor specific fragments.

Both Eco RI signature fragments (2.3 and 1.1 kbp) were missing when digests of tumor DNA were annealed to either $cDNA_{rep}$ (lane set 8) or $cDNA_3$, (lane set 9). $cDNA_3$, detected only two fragments with sizes of 9 kbp and 3.1 kbp (lane set 8) which were specific for the tumor. Digestions of tumor DNA with Sac I and Kpn I produced only one tumor specific fragment detectable with $cDNA_{rep}$ or $cDNA_3$, (data not shown). The provirus thus lacked these two recognition sequences.

This provirus had most likely suffered a deletion which removed sequences extending from the Sac I recognition site approximately 400 bp from the left host cell-provirus junction to the 3' end of the env

gene. The presence of $U_3$ sequences and only one Eco RI recognition site suggested that the RAV-2 provirus in this tumor consisted primarily of only one LTR. However, we are not certain whether this LTR originated at the left or right end of the RAV-2 provirus.

Legend to Table 1.

[1] See Experimental Procedures.

[2] B, Bursa; L, Liver; S, Spleen.

[3] ev-1 and ev-4 are endogenous proviruses associated with the $gs^-$ and $chf^-$ phenotype of certain chicken lines and have been defined by restriction endonuclease analyses and breeding experiments (Astrin et al., 1980).

[4] Not determined.

[5] Eco Rl digestion of DNA from this tumor produced the expected 2.3 kbp and 1.1 kbp fragments in addition to other Eco Rl fragments containing sequences normally found in the 2.3 kbp Eco Rl fragment (see text).

[6] Eco Rl digestion of DNA from these tumors did not reveal aberrant fragments. This does not preclude the presence of abnormal RAV-2 proviruses (see text).

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|---|---|---|---|---|---|---|---|
| RPRL | $15I_5 \times 7_2$ | 21 | RAV-2 | ND | | | Non-defective[6] |
| RPRL | $15I_5 \times 7_2$ | 31 | RAV-1 | ND | B,S | 4 | Non-defective[6] ND |
| RPRL | $15I_5 \times 7_2$ | 32 | RAV-1 | ND | B,S | 3 | Non-defective[6] ND |
| RPRL | $15I_5 \times 7_2$ | 33 | RAV-1 | ND | B | 3 | Non-defective[6] ND |

TABLE 1. Number and structure of Proviruses in ALV-Induced Tumors.

| Source | Parental Lines | Chicken Number | Virus Inoculated | Virus Isolated from Bursal Tumor[1] | Tissues Containing Transformed Lymphocytes[2] | Number of Exogenous Proviruses | Structure of Exogenous Proviruses | Endogenous Proviral Loci[3] |
|---|---|---|---|---|---|---|---|---|
| SPAFAS | 11 | 1 | RAV-2 | - | B | 1 | Defective | ev-1, ev-4 |
| SPAFAS | 11 | 2 | RAV-2 | + | B | 1 | Defective | ev-1, ev-4 |
| SPAFAS | 11 | 3 | RAV-2 | - | B | 1 | Defective | ev-1, ev-4 |
| SPAFAS | 11 | 4 | RAV-2 | - | B | 1 | Defective | ev-1 |
| SPAFAS | 11 | 5 | RAV-2 | - | B,L | 1 | Defective | ND[4] |
| SPAFAS | 11 | 6 | RAV-2 | + | B,L,S | 1 | Defective | ND |
| SPAFAS | 11 | 7 | RAV-2 | - | B,L,S | 1 | Defective | ND |
| SPAFAS | 11 | 11 | RAV-2 | - | B | 2(?) | Defective | ev-1 |
| RPRL | $15I_5x7_2$ | 21 | RAV-2 | ND | B,L,S | 3 | Defective and Non-defective[5] | ND |
| RPRL | $15I_5x7_2$ | 31 | RAV-1 | ND | B,S | 4 | Non-defective[6] | ND |
| RPRL | $15I_5x7_2$ | 32 | RAV-1 | ND | B,S | 3 | Non-defective[6] | ND |
| RPRL | $15I_5x7_2$ | 33 | RAV-1 | ND | B | 3 | Non-defective[6] | ND |

Legend to Figure 2.

Diagramatic representation of restriction enzyme recognition sites in RAV-2 DNA.

The positions of the restriction sites were determined as described by Shank et al., 1978. Unintegrated RAV-2 DNA was prepared from the Hirt supernatant fraction of chicken cells acutely infected by the virus stock also used for induction of tumors in chickens. Restriction fragments were ordered using probes specific for various regions of the viral RNA, sequential digestions with two restriction enzymes, and comparison of the restriction fragment patterns of form I and form III viral DNA. These data were supplemented with information obtained from restriction enzyme digests of RAV-2 DNA cloned in bacteriophage λ gtWES·λB (see Experimental Procedures). R = Eco RI; B = Bam HI; S = Sac I; K = Kpn I; H = Hind III. The terminal repeats (LTR), approximately 300 bp, are drawn as boxes at the ends of the DNA. The open box represents sequences specific to the 3' end of the viral RNA ($U_3$), the shaded boxes represent sequences specific to the 5' end of viral RNA ($U_5$). The approximate locations of viral genes are indicated on the diagram of viral RNA. "Signature fragments" which distinguish RAV-2 DNA from proviruses endogenous to the chickens used in this study are marked by lines connecting the two restriction sites and contain the sizes of these fragments in kilobase pairs (kbp). Some of the probes used in our studies represent the regions delineated by the labelled lines between the diagrams of viral RNA and DNA. Descriptions of the content of each probe are included in the Results section.


gag
pol
env

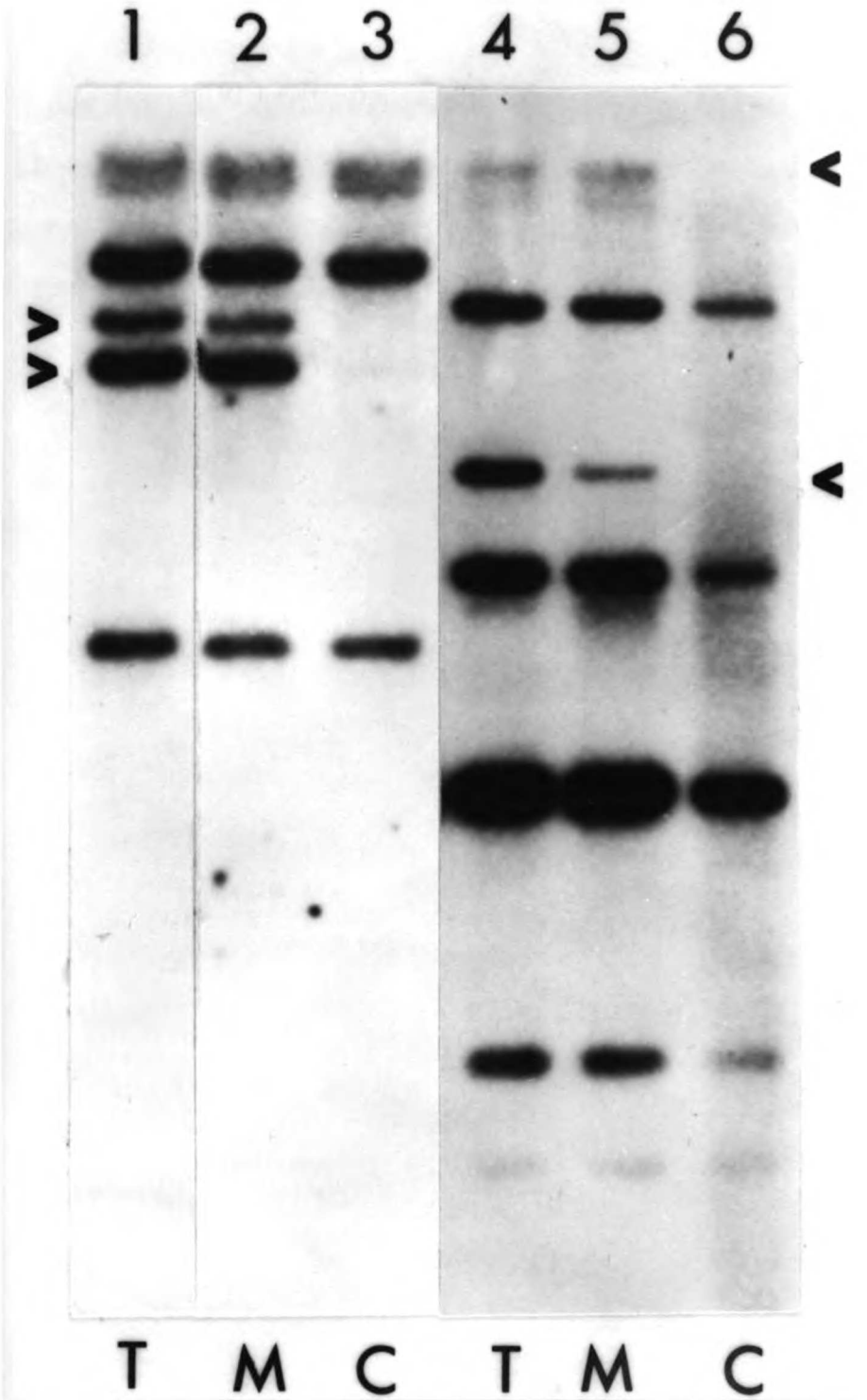
R
S
B
2.3kbp
B
R
H
B
1.8kbp
K
B
R
1.1kbp
R
DNA
cDNA 5′
BAM C
cDNA 3′
PROBES
gag
pol
env
RNA

Legend to Figure 3.

Demonstration of clonality of bursal tumors and metastases by digestion of tumor DNA with restriction enzymes which cleave once within the RAV-2 provirus.

Five micrograms of DNA from primary tumors (T), metastatic tumors (M), and control tissues (C) were digested to completion with Kpn I or Hind III, electrophoresed through 0.8% agarose, transferred to nitrocellulose and annealed to $cDNA_{rep}$ as described in Experimental Procedures.

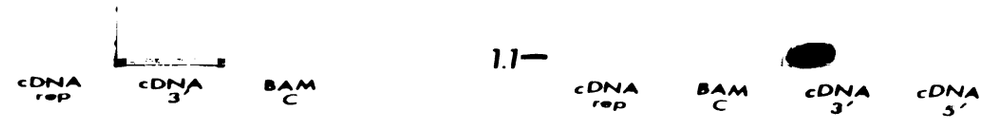
Lanes 1-3: Kpn I digests of DNA from tumor, hepatic metastasis, and normal liver from bird 5. Lanes 4-6: Hind III digests of DNA from tumor, splenic metastases and circulating red blood cells from bird 6. The arrows denote the positions of fragments specific to tumor tissue.

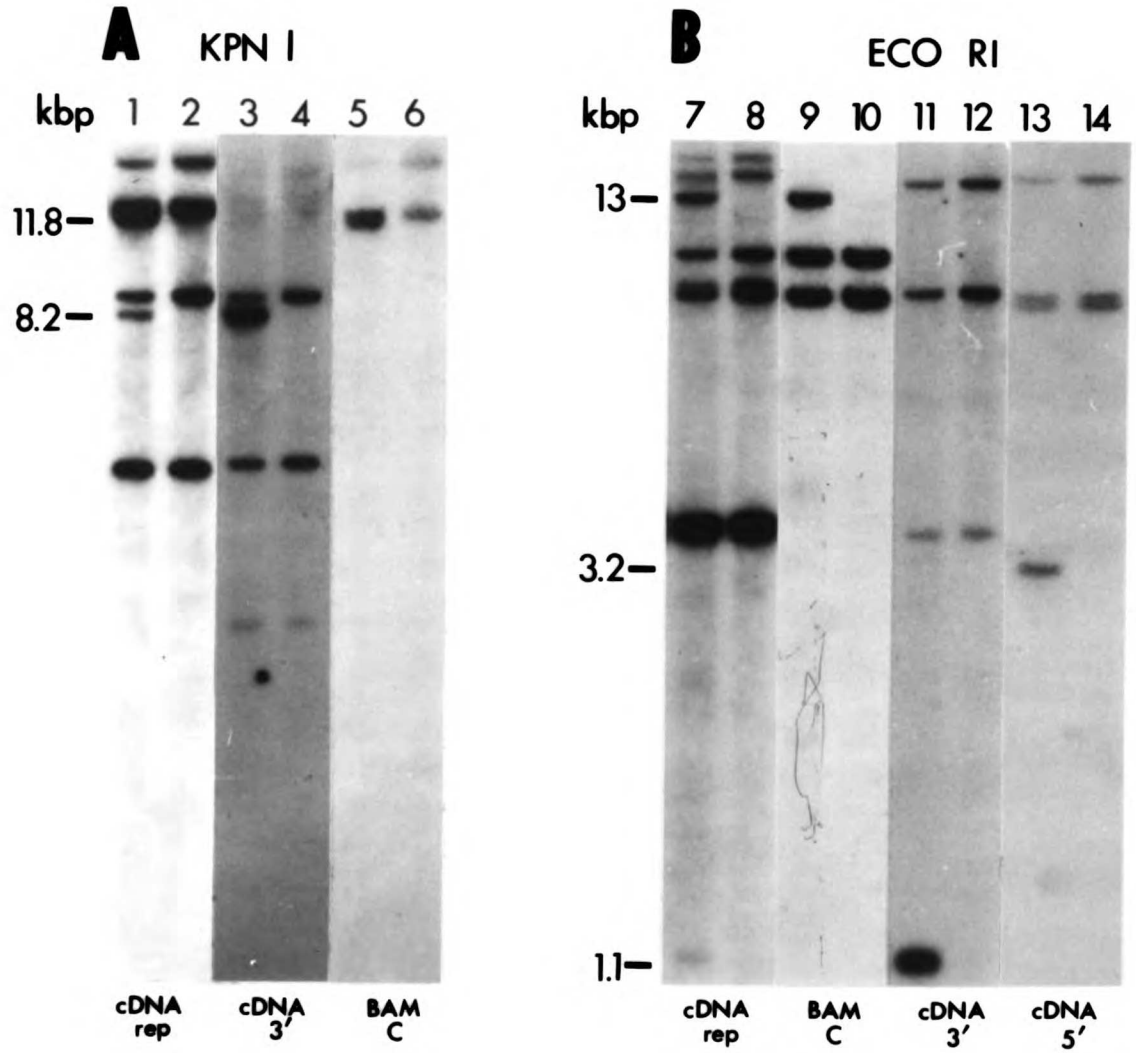

1
2
3
4
5
6
T
M
C
T
M
C

Legend to Figure 4.

Definition of a deletion in the single RAV-2 provirus in tumor LL 1.

After digestion with Kpn I (Panel A) or Eco RI (Panel B), DNA from bird 1 was analyzed as described in the legend to Figure 3. Each set of 2 lanes shows the results with bursal tumor DNA (even numbered lanes) and uninvolved spleen DNA (odd numbered lanes) hybridized to the virus-specific probes indicated below the lanes. The composition of each probe is described in the text and illustrated in Figure 3. Tumor-specific bands are marked with their lengths in kbp.

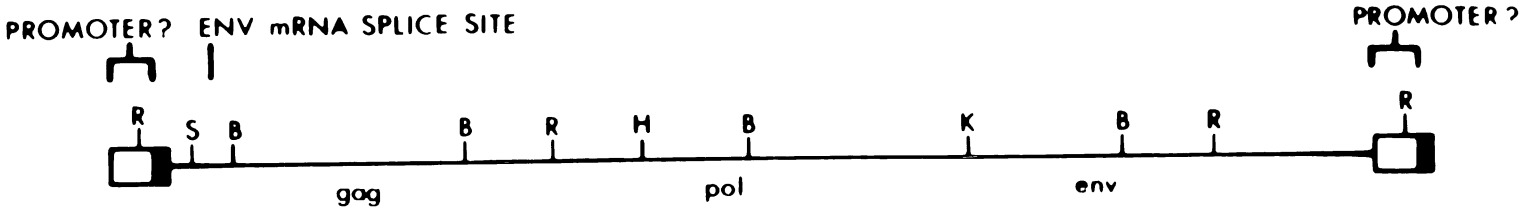

cDNA rep
cDNA 3'
BAM C
1.1—
cDNA rep
BAM C
cDNA 3'
cDNA 5'


A
KPN I
kbp
1
2
3
4
5
6
11.8
8.2
cDNA rep
cDNA 3′
BAM C
B
ECO RI
kbp
7
8
9
10
11
12
13
14
13
3.2
1.1
cDNA rep
BAM C
cDNA 3′
cDNA 5′

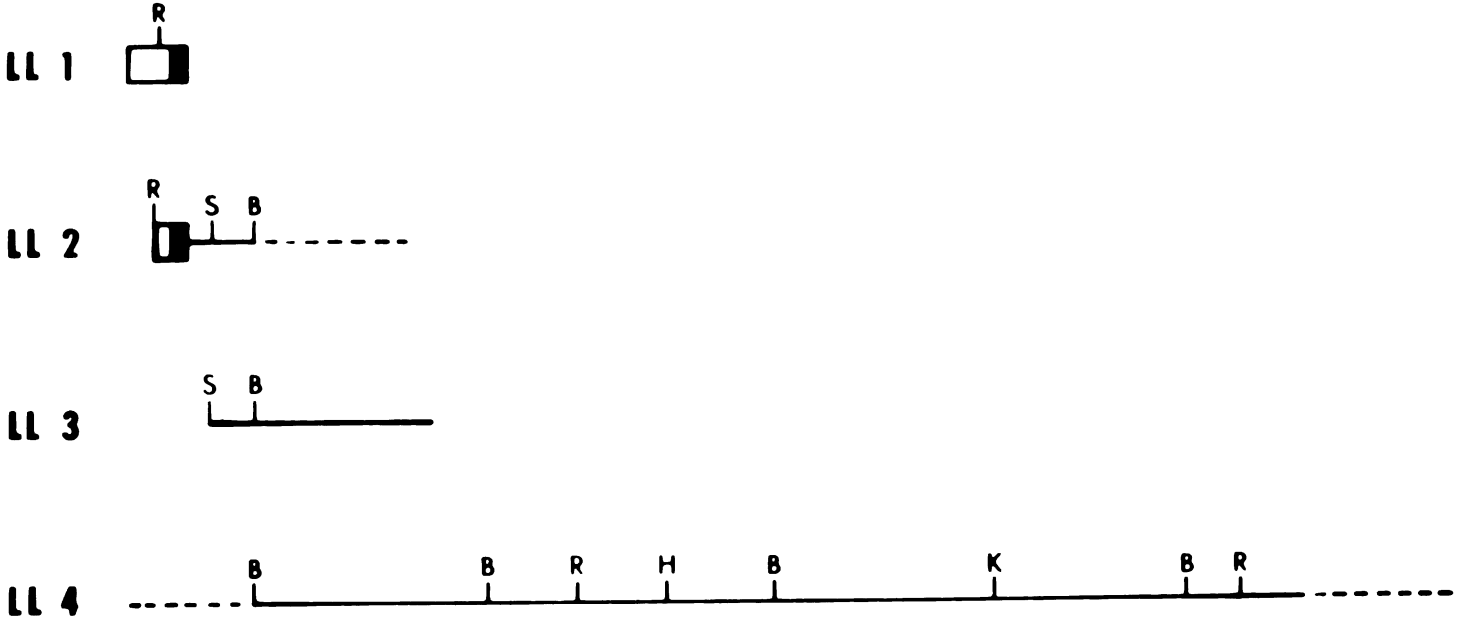
Legend to Figure 5.

Physical maps of regions deleted from single proviruses present in four bursal tumors.

The diagram of RAV-2 DNA at the top of the figure illustrates the LTRs, relevant restriction sites, and approximate gene locations. The region which may contain the proviral promoter and polyadenylation signals (Shank et al., 1978; Hughes et al., 1978; Sabran et al., 1979; Tsichlis and Coffin, 1980; Swanstrom et al., 1980; Czernilofsky et al., 1980), and the sequences containing the env mRNA splice donor site (P. Hackett, G. Gasic, personal communications) are indicated. The region deleted from the provirus in each tumor (LL 1-LL 4) is drawn below the RAV-2 DNA. These diagrams represent minimal estimates of the extent of each deletion. The deletions could extend into regions which are dotted in the diagrams of the proviruses from LL 2 and LL 4. The RAV-2 DNA in LL 4 retained one LTR, but we are uncertain whether it originated at the left or right end of the provirus.




## REGIONS DELETED FROM RAV-2 PROVIRUSES



Legend to Figure 6.

Maps of restriction sites in host cell DNA flanking the RAV-2 provirus in three tumors.

The sites of integration are marked by diamonds. The restriction enzyme symbols are the same as in Figure 2. The maps are oriented so that transcription of the sense strand of proviral DNA would proceed from left to right (cf. Fig. 2).

LL 1
R
H
K
S
S
H
R
K
B
LL 2
B
K
R
H
B
K
H
R
LL 3
B
K
R
H
B
K
H
R
Sac I 20 kbp
Sac I 24 kbp
2 kbp

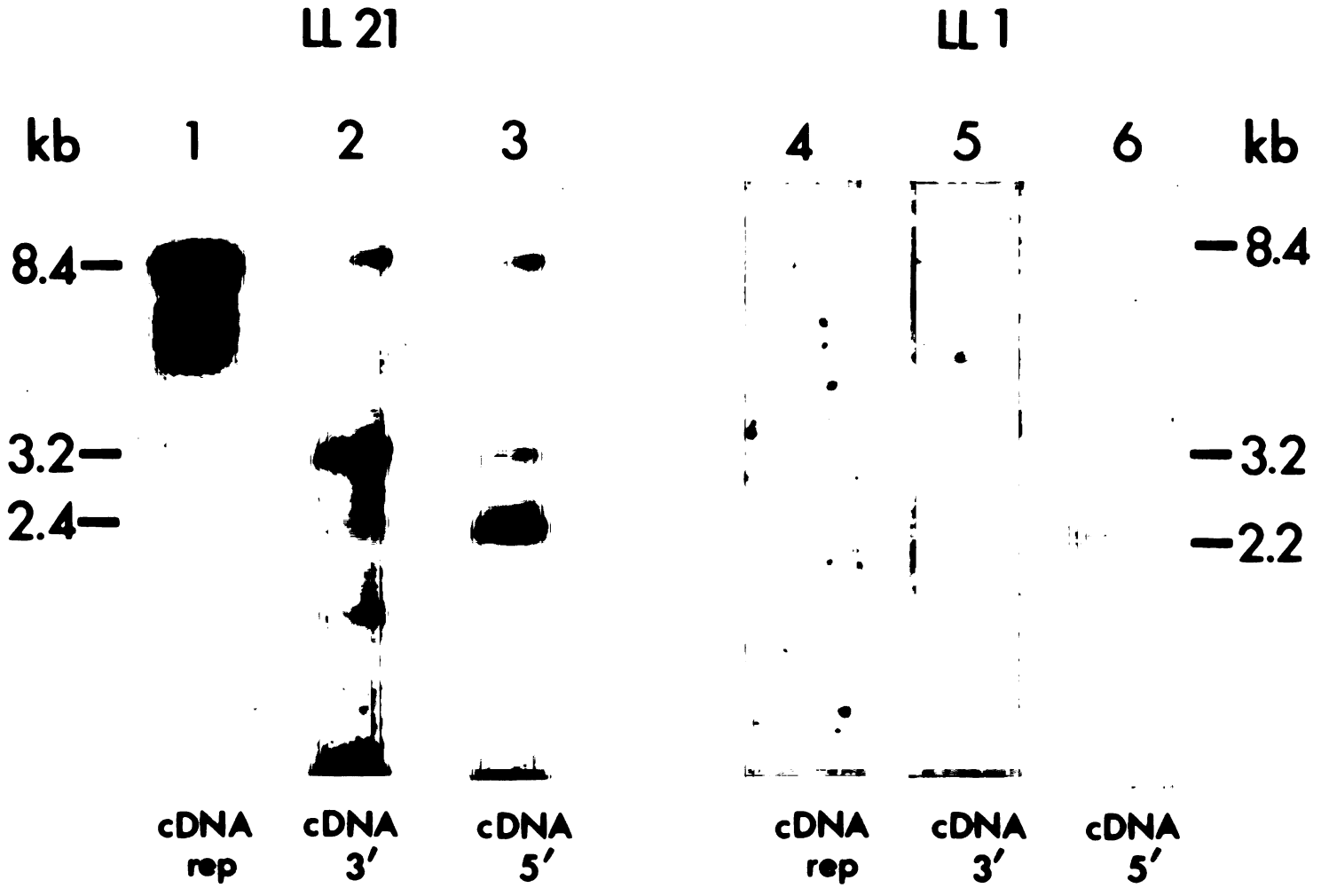
Legend to Figure 7.

Atypical species of viral RNA from a tumor harboring multiple proviruses and a tumor harboring a single provirus.

Total RNA (80 ug) from tumor LL 21 and tumor LL 1 was electrophoresed through 1.2% agarose containing methyl mercury hydroxide, transferred to activated DBM-cellulose paper, and annealed to virus-specific probes as described in Experimental Procedures. Results shown in lanes 2 and 3 and in lanes 4-6 were obtained by annealing different probes sequentially to RNA on the same filter.

The left hand panel shows total RNA from tumor LL 21; the right hand panel shows RNA from tumor LL 1. The probes employed for annealing in each panel are indicated underneath the panel. The RNAs are marked by arrows and their lengths are indicated in kb.

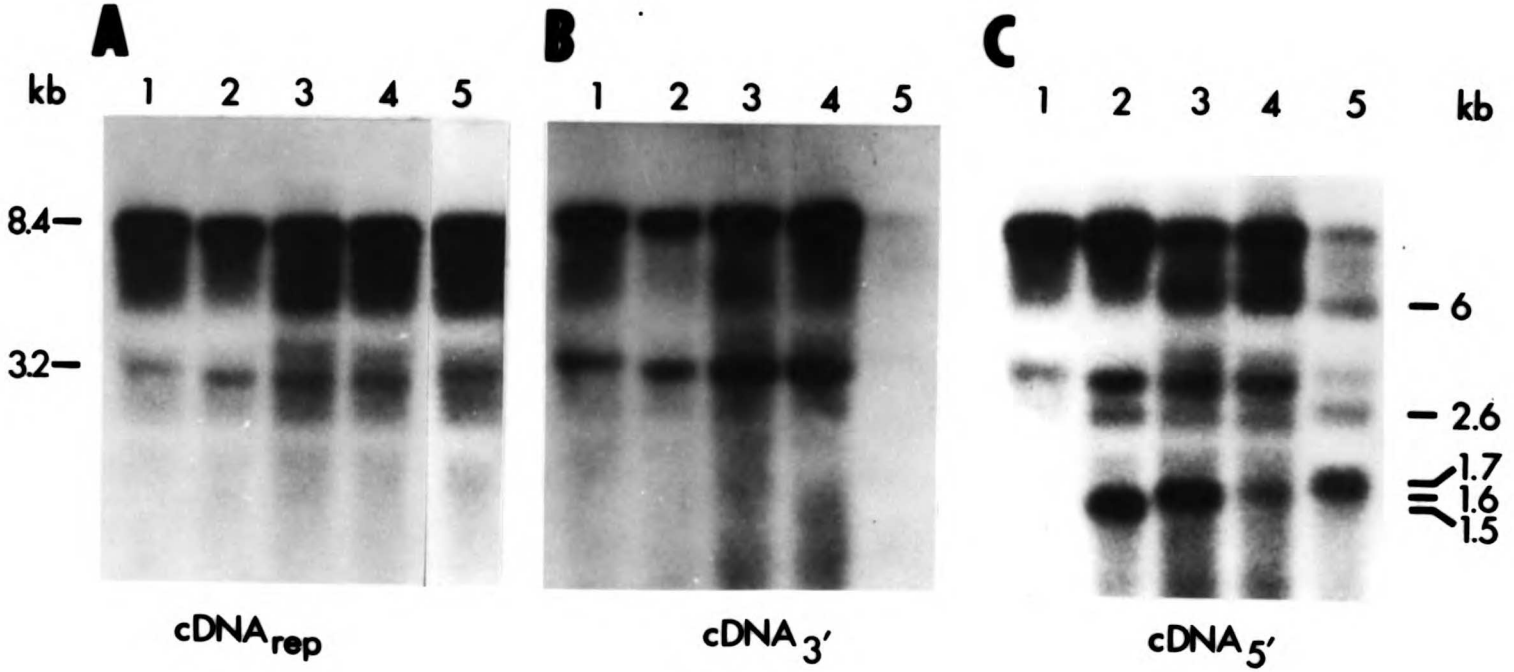

cDNA rep
cDNA 3′
cDNA 5′
cDNA rep
cDNA 3′
cDNA 5′


LL 21
LL 1
kb
1
2
3
4
5
6
kb
8.4
3.2
2.4
8.4
3.2
2.2
cDNA rep
cDNA 3′
cDNA 5′
cDNA rep
cDNA 3′
cDNA 5′

Legend to Figure 8.

Detection of atypical viral RNA species in cell lines derived from ALV-induced bursal lymphomas.

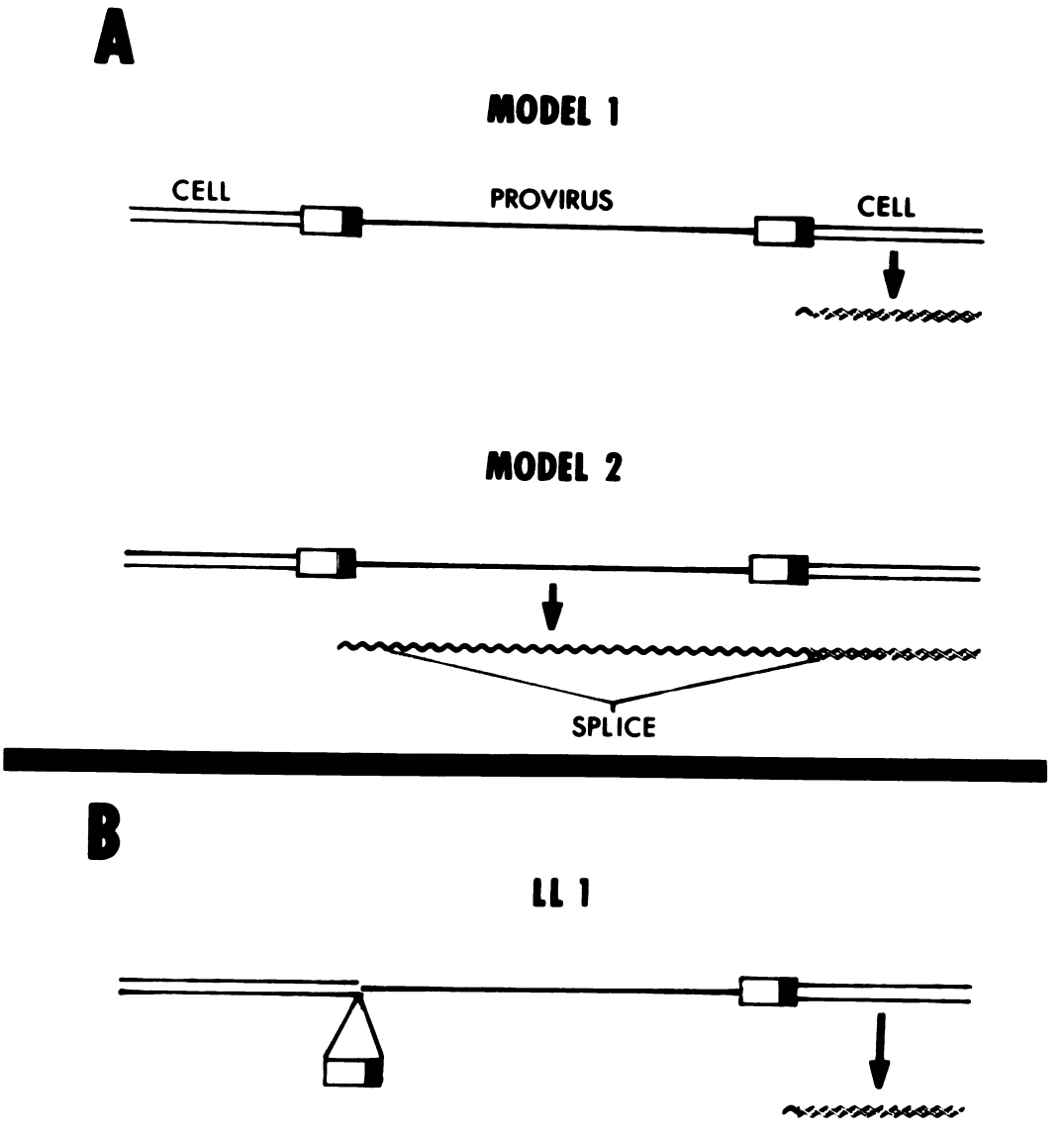
Total RNA (50 ug) from RAV-2 infected fibroblasts and from tumor lines described in the text was electrophoresed through 1.2% agarose containing methyl mercury hydroxide, transferred to activated DBM- cellulose paper and annealed to virus-specific probes as described in Experimental Procedures. The probes are indicated beneath each panel. Lane 1, RAV-2 infected fibroblast RNA; lane 2, line BK 4484A RNA; lane 3, line 1104B-1 RNA; lane 4, line 1104X-5 RNA; lane 5, line R2B RNA. The sizes of various RNA species (in kb) detected only with $cDNA_5$, are marked at the right; the sizes of the normal species of RAV-2 mRNA are marked at the left. The RNA on the DBM-cellulose filter was sequentially annealed to each probe as described in Experimental Procedures.


A
kb
1
2
3
4
5
8.4
3.2
cDNA rep
B
1
2
3
4
5
cDNA 3'
C
1
2
3
4
5
kb
6
2.6
1.7
1.6
1.5
cDNA 5'

Legend to Figure 9.

Possible mechanisms for generating transcripts of host cell DNA from a viral promoter.

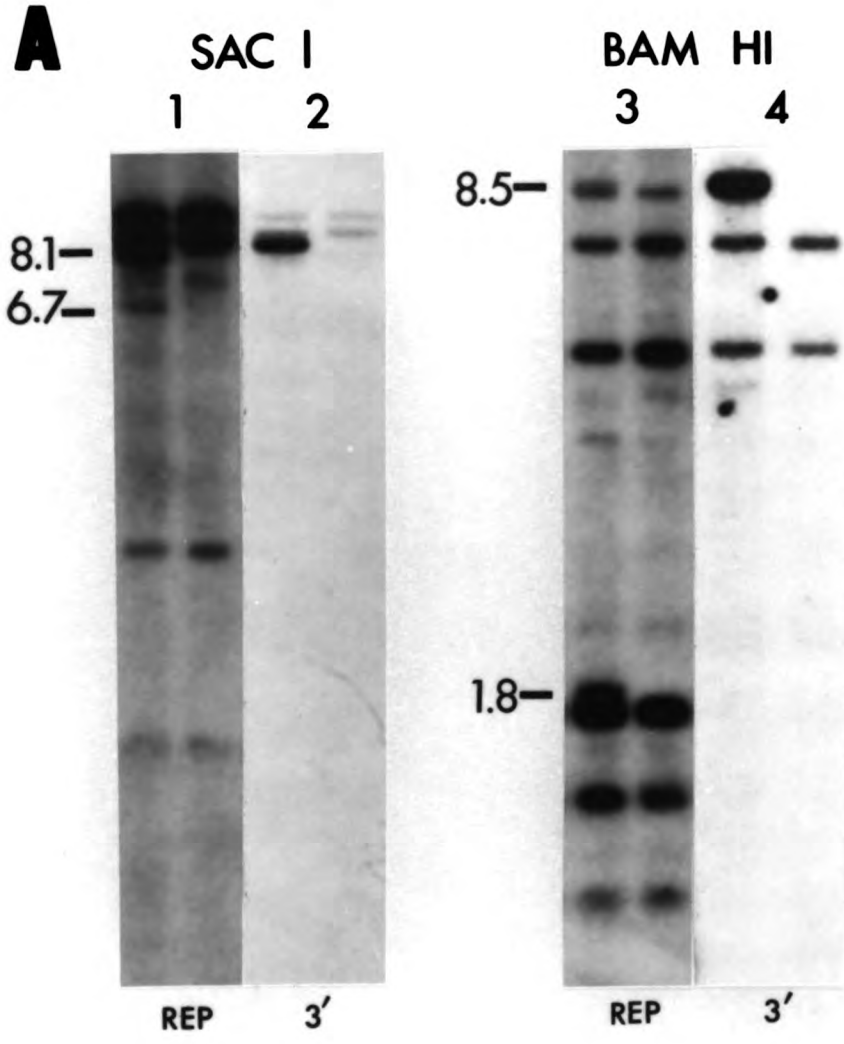
In the first model, transcription initiates in the right LTR and proceeds directly into flanking cell DNA. The second model postulates that transcription originates in the left LTR and reads through possible termination signals in the right LTR. The final transcript is formed by a processing event which removes most of the viral sequences. The lower diagram shows the structure of the provirus in tumor LL 1 from which the left LTR has been deleted. The probable origin of the RNA which annealed only to $cDNA_5$, is drawn below the provirus.

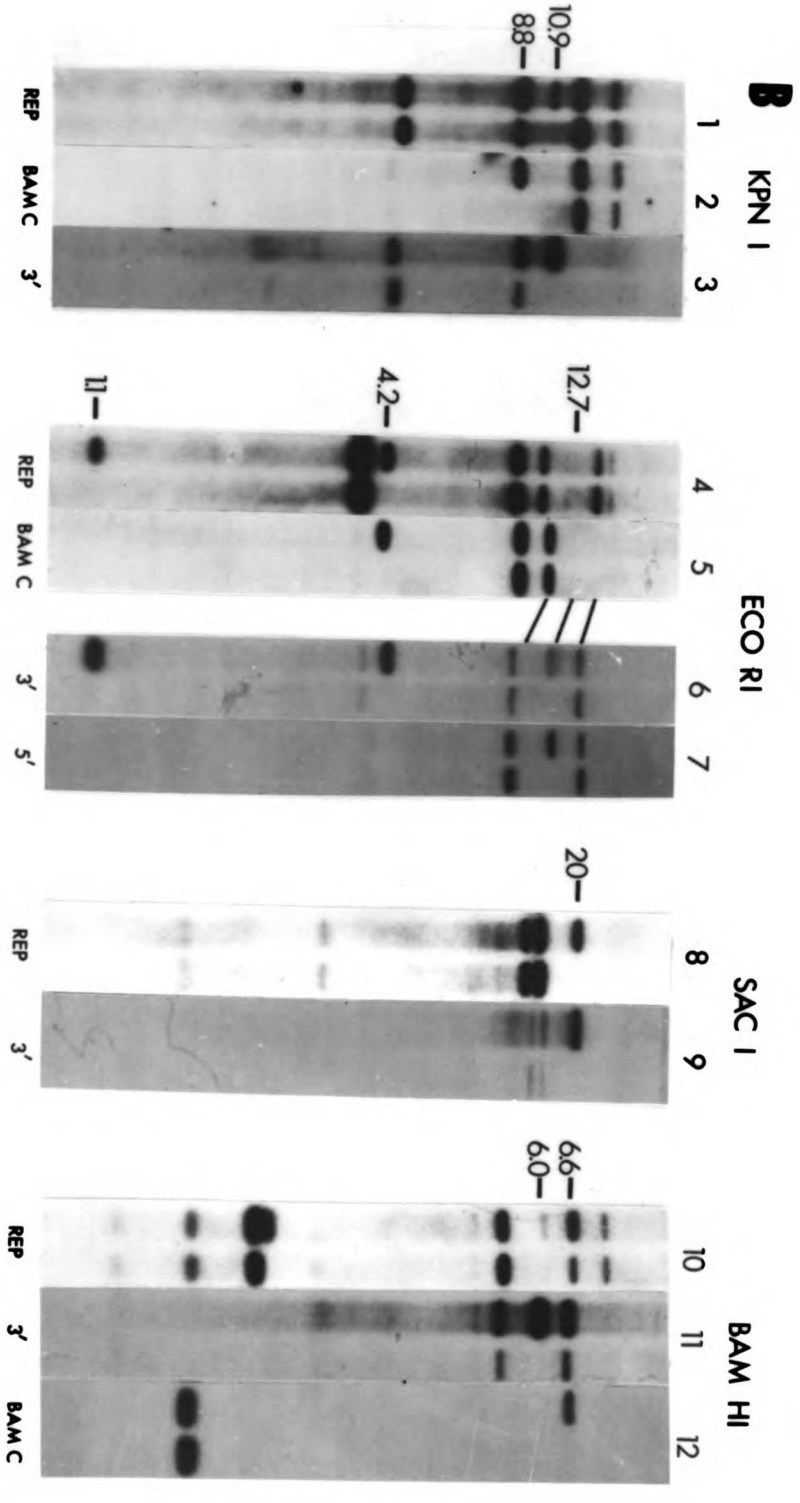

A
MODEL 1
CELL
PROVIRUS
CELL
MODEL 2
SPLICE
B
LL 1

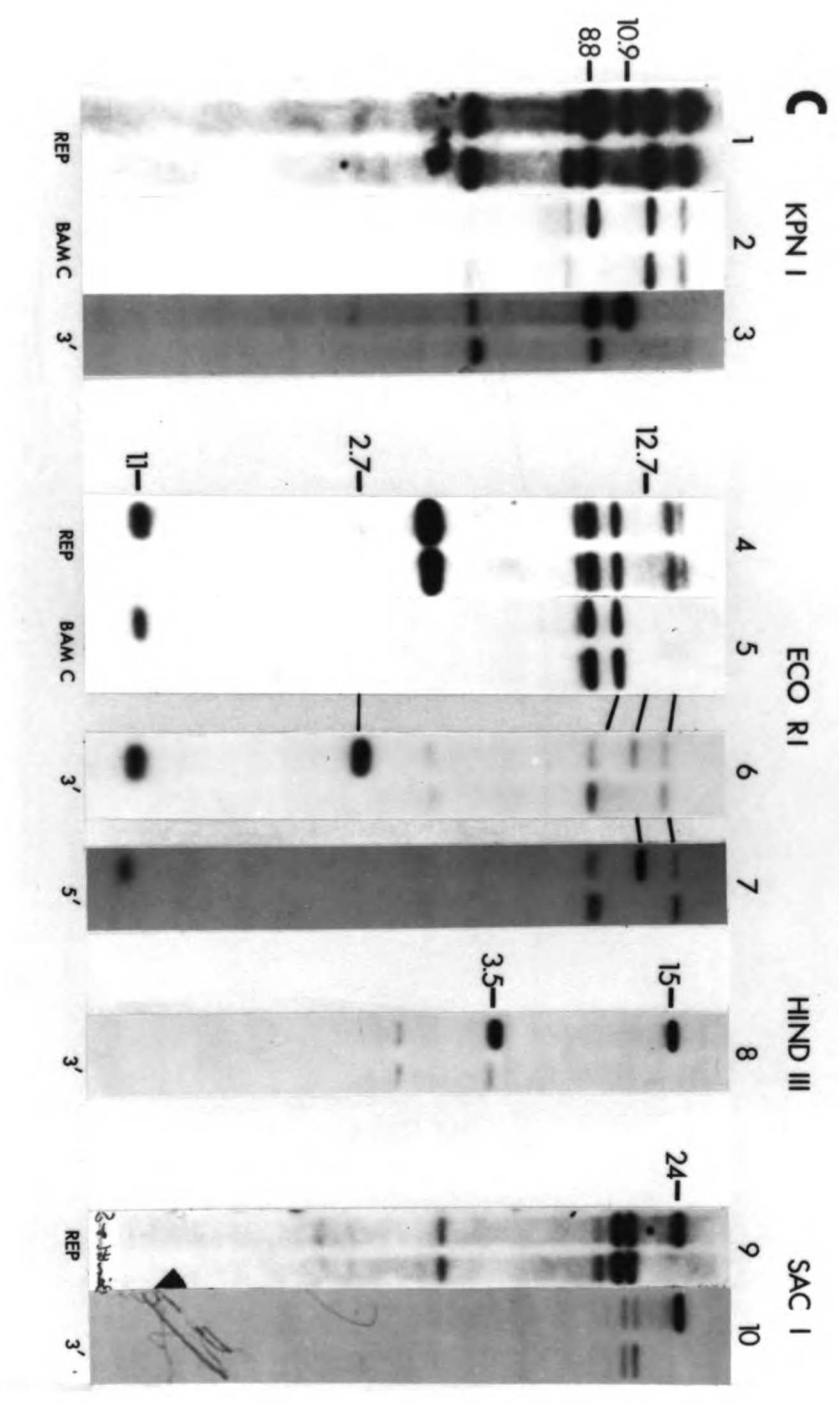
Legend to Figure 10.

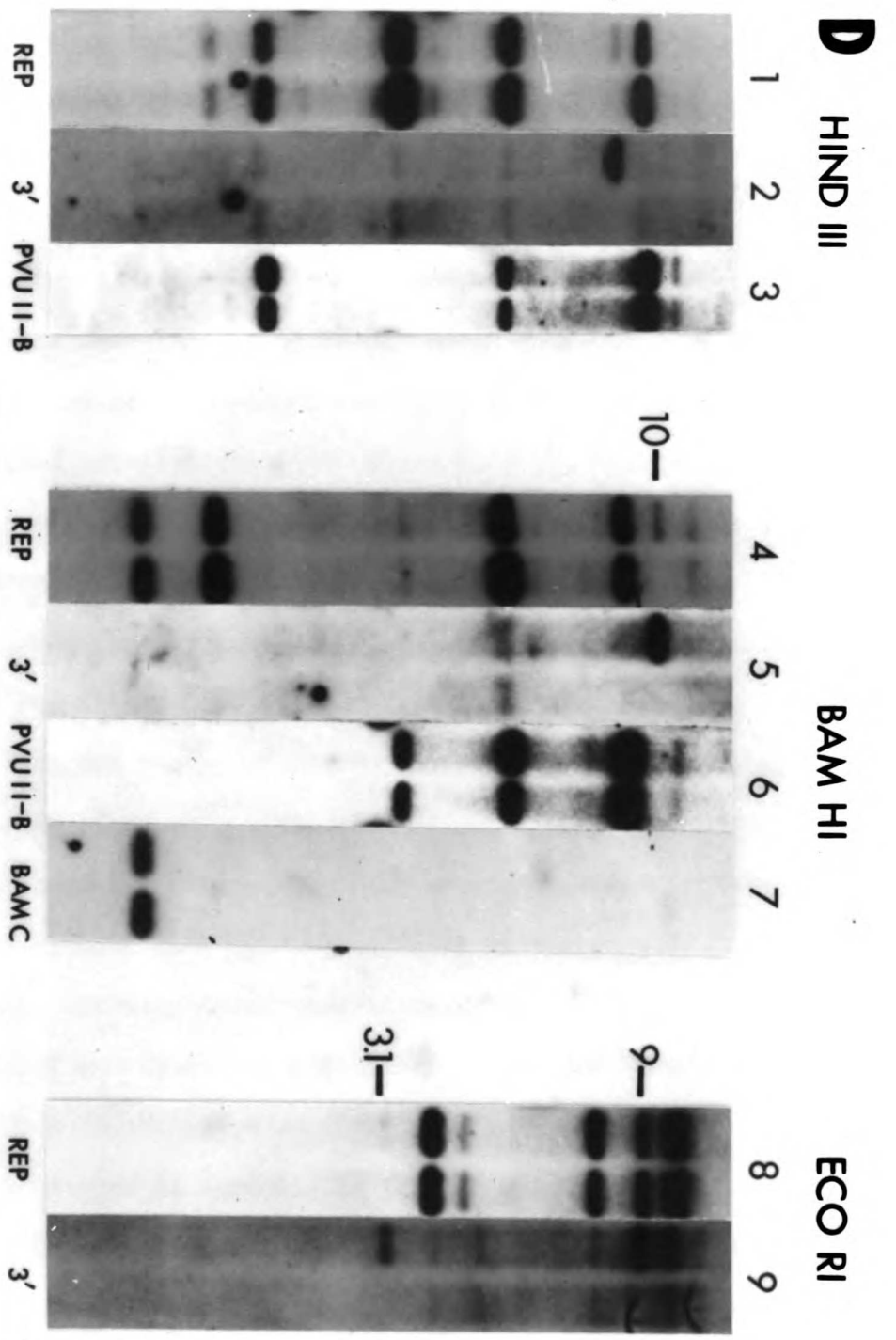
Detailed restriction enzyme analyses of altered proviruses in four tumors.

Each panel represents analyses of DNA from one tumor (A = LL 1; B = LL 2; C = LL 3; D = LL 4). The restriction enzymes and probes used in each experiment are indicated. Each number refers to a pair of lanes; the left hand lanes contain a digest of tumor DNA, while the right hand lanes contain a digest of DNA from uninfected tissue. The sizes (in kbp) of tumor-specific fragments are shown.


A
SAC I
1
2
8.1
6.7
REP
3′
BAM HI
3
4
8.5
1.8
REP
3′


B
KPN I
ECO RI
SAC I
BAM HI
1
2
3
4
5
6
7
8
9
10
11
12
10.9
8.8
12.7
4.2
1.1
20
6.6
6.0
REP
BAM C
3'
5'

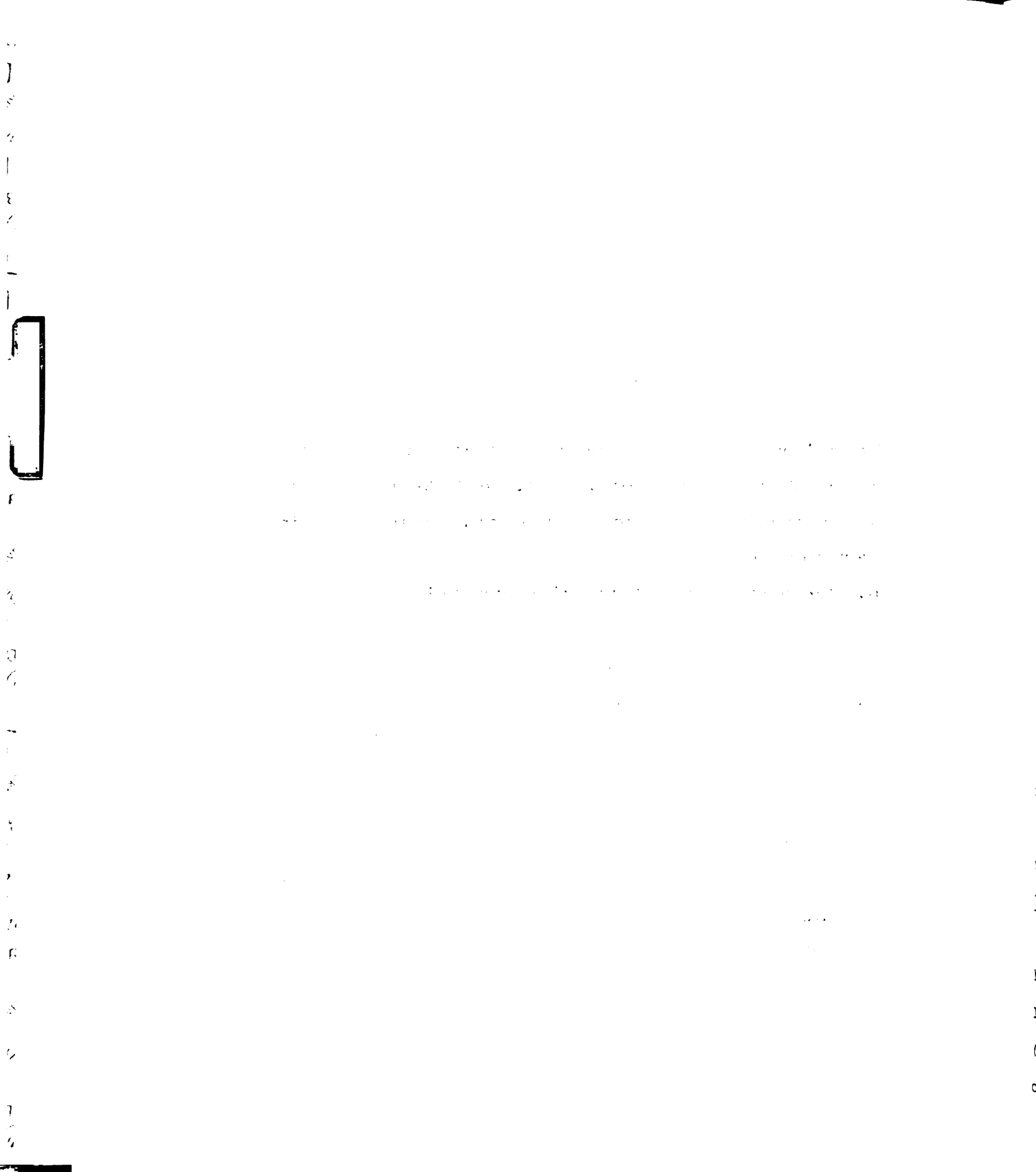

C
KPN I
ECO RI
HIND III
SAC I
10.9
8.8
12.7
2.7
1.1
3.5
15
24
REP
BAM C
3'
5'
1
2
3
4
5
6
7
8
9
10


D
HIND III
BAM HI
ECO RI
1
2
3
4
5
6
7
8
9
10—
10—
9—
3.1—
REP
3'
PVU II-B
REP
3'
PVU II-B
BAM C
REP
3'

EXPERIMENTAL PROCEDURES

Source of tumors.

Eighty SPAFAS line 11 and thirty-five RPRL $15I_5x7_2$ day-old chicks were hatched and placed in plastic canopy isolators. Both lines of chickens are maintained under specific pathogen-free conditins and are free of common avian pathogens, particularly lymphoid leukosis viruses. At one week of age, heparinized blood was collected and packed cells were frozen for DNA analysis. Forty SPAFAS and thirty-five $15I_5x7_2$ chickens were inoculated at one week of age with $10^5$ infectious units of a stock of Rous associated virus-2 (RAV-2) propagated from plaque purified material obtained from P.K. Vogt. The SPAFAS line 11 birds were maintained in isolators to 281 days of age. The inoculated birds were palpated for bursal tumors at least weekly from 140 to 240 days of age. When bursal enlargement was noticed by palpation, the birds were bled and killed. Portions of the bursa and any gross tumors were quick-frozen in liquid nitrogen. Portions of the brain, pancreas, muscle, and blood were also saved. Portions of each tissue, except blood, were saved for DNA analysis and were also fixed in formalin and examined for histopathological evidence of tumor cells. Sex and age matched non-inoculated control birds were killed and corresponding material was taken for extraction of DNA and RNA. The incidence of leukosis in the infected SPAFAS chickens was 34% (11 of 34 survivors). The incidence of leukosis in the $15I_5x7_2$ chickens was 48.5% (16 of 34 survivors). Three $15I_5x7_2$ tumor bearing birds were also killed for analysis. The presence of subgroup B virus in the bursa was determined as in Crittenden et al. (1979). Bursal tumors, spleen, and muscle were also prepared from an additional group of $15I_5x7_2$ chickens inoculated



with RAV-1 (obtained from P.K. Vogt) one day after hatching.

Tumor cell lines.

Three tumor cell lines derived from lymphomas induced by subgroup A ALV were kindly provided by Dr. H. Hihara. The isolation and maintenance of these lines has been described (Hihara et al., 1977). Briefly, 1104B-1 and BK 4484A were passaged by 1:10 dilution every 2-3 days into RPMI 1640 containing 10% fetal calf serum (FCS) (Gibco), 10% tryptose-phosphate broth (TPB), and 5 µg/ml gentamycin. 1104X-5 was passaged in the same manner except that trypsin was used to harvest the cells.

The RAV-2 infected transplantable tumor line (LSCT-RP6) was generously provided by Dr. W. Okazaki (Okazaki et al., 1980). The tumor line was maintained by inoculation of $10^6$-$10^7$ cells into the pectoral muscle of one day old $15I_5x7_1$ chicks. A single cell suspension was made from one such tumor by teasing it apart with needles; the cells were grown in RPMI 1640 supplemented with 10% FCS, 10% TPB and 5 µg/ml gentamycin. This cell line was designated R2B. The 10th in vitro passage of these cells was tumorigenic when inoculated onto the pectoral muscle of one day old chicks (unpublished results of S.A.C.). All of the cell lines were grown in an atmosphere of 10% $CO_2$ and 7% $O_2$ in nitrogen.

Preparation and cloning of viral DNA.

RAV-2 viral DNA was obtained from chick embryo fibroblasts 48 hours after infection with RAV-2 at a high multiplicity (moi = 1 IU/cell). Unintegrated viral DNA was prepared by Hirt fractionation (Hirt, 1967) as described previously (DeLorbe et al., 1980). Supercoiled viral DNA was isolated from Hirt supernatant fractions by acid

phenol exraction (Zasloff et al., 1978). Supercoiled RAV-2 DNA was digested with Sac I and cloned into bacteriophage λgtWES·λB as described by DeLorbe et al. (1980).

Preparation of high molecular weight DNA from chicken tissue

Frozen tissue (approx. 1 gm.) was minced with a razor blade and added to 10 mls of buffer containing 10 mM Tris-HCl pH 8.1, 250 mM $Na_3EDTA$ and 25% v/v glycerol. The tissue was dispersed by homogenization (one stroke) using a motor-driven (Talboys Eng., Inc.) Dounce homogenizer. Protease K (Merck) (200 µg/ml) and SDS (1%) were added, and the solution was incubated at 50°C for 3 hrs then extracted with phenol"chloroform (1:1) until the aqueous phase cleared. The solution was then extracted one time with chloroform and the nucleic acid precipitated with two volumes of ethanol. Precipitated DNA was removed by spooling the fibers around a glass rod, drained, and resuspended in TE (10 mM Tris-HCl pH 8.1, 1mM $Na_3$ EDTA).

Preparation of high molecular weight DNA from cell lines.

Cells were pelleted and washed twice with Tris-glucose (0.14 M NaCl, 5 mM KCl, 5 mM glucose, 25 mM Tris-HCl pH 7.4). The cells were resuspended in TE buffer at a final concentration of approximately $10^7$/ml. The cells were lysed by adding Protease K (200 ug/ml) and 1% SDS and incubating as described above. The solution was extracted with phenol:chloroform, precipitated, and resuspended as described above.

Preparation of RNA from chicken tissue.

Whole cell RNA was prepared using a modification of the guanidinium thiocyanate procedure developed by Chirgwin (Ullrich et al., 1977) and described by Robertson and Varmus, 1979.

Preparation of RNA from tissue culture cells.

We extracted RNA from whole cells as described previously (Weiss et al., 1977).

Analysis of cellular DNA and viral DNA with restriction endonucleases.

DNA prepared as described above was cleaved with restriction endonucleases and fractionated by electrophoresis through agarose gels. The fractionated DNA was transferred to nitrocellulose membranes for subsequent analysis with radioactive cDNAs (Southern, 1975). We have described the details of these procedures elsewhere (Shank et al., 1978).

Analysis of viral RNAs

RNAs were fractionated through 1.2 % agarose gels containing methyl mercury hydroxide, transferred to diazobenzyloxymethyl cellulose paper (Alwine et al., 1979), and hybridized sequentially to multiple radioactive cDNAs as described by Quintrell et al. (1980).

Molecular hybridization in solution

RNA was hybridized with radioactive cDNAs in solution as described previously (Leong et al., 1972). The percent hybridization was normalized to the maximum hybridization achieved using RNA from ALV-infected bursal lymphocytes derived from a bursal lymphoma (line BK 4484A, see above). The data was expressed as a function of Crt (mole/sec/l) corrected to standard conditions. The number of copies of RNA per cell was calculated as described in Spector et al. (1978b).

Preparation of molecular hybridization probes.

We have described previously the preparation and characterization of $cDNA_{rep}$ (Shank et al., 1978) and $cDNA_3$, (Tal et al., 1977). The Bam C probe and, in some cases, $cDNA_{rep}$ were prepared using restriction fragments derived from SR-A RSV cloned in bacteriophage and subcloned

into pBR 322 (DeLorbe et al., 1980). The appropriate restriction fragments were purified from pBR 322 either by centrifugation through a sucrose gradient (DeLorbe et al., 1980) or by electrophoresis through low melting (Sea-plaque) agarose gels. The restriction fragments were localized in the gel by transillumination with ultraviolet light and the appropriate region of the gel removed and resuspended in a large volume, usually 20 mls, of STE (0.3M NaCl, TE) plus 0.5% SDS and incubated at 68°C for 30 minutes. The solution was extracted with one volume of phenol, and the phenol phase was re-extracted with STE. This solution was extracted three times with butanol:isopropyl alcohol (7:3) and the DNA was precipitated with two volumes of ethanol.

Probes were synthesized using these restriction fragments as templates as described by Shank et al. (1978), with the following changes: a denatured DNA restriction fragment was substituted for the RNA template, and a ratio of 50 ug of calf thymus primers to 1 ug of restriction fragment was employed.

$cDNA_5$, was prepared by incubating 9 ug of 70S viral RNA in a 300 ul reaction mix, similar to that used in the preparation of $cDNA_3$,, except that oligo dT was omitted, the concentration of the unlabelled nucleotides was 25 uM, and 300 uCi of a $^{32}$P-dCTP (2000-3000 Ci/mmole, Amersham or NEN) was used. The reaction product was separated from unincorporated nucleotides by gel filtration with Sepharose G-50 and loaded on an 8% sequencing gel (Maxam and Gilbert, 1977). Bands representing $cDNA_{5',(100)}$ and $cDNA_{5',(70)}$ (Friedrich et al., 1977) were located by autoradiography and eluted from gel slices according to the procedure of Maxam and Gilbert (1977). $cDNA_{5',(100)}$ was used for analysis of cellular DNA and $cDNA_{5',(70)}$ for analysis of RNA.

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# CHAPTER 3

## Multiple Arrangements of Viral DNA and an Activated Host Oncogene in Bursal Lymphomas

ABSTRACT

Proviruses of avian leukosis virus (ALV) are located in the vicinity of a putative cellular oncogene (c-*myc*) in ALV-induced bursal lymphomas. Enhanced expression of c-*myc* occurs in association with proviruses found in any of three configurations: (i) on the 5' side ("upstream") of c-*myc* in the same transcriptional orientation; (ii) on the 3' side ("downstream") of c-*myc* in the same orientation; and (iii) upstream, in the transcriptional orientation opposite to that of c-*myc*. Thus activation of adjacent cellular genes by retroviral DNA can involve mechanisms other than provision of a transcriptional promoter.

Avian leukosis viruses (ALVs) are replication-competent retroviruses which lack transforming genes but cause tumors, most commonly bursal (B-lymphocyte) lymphomas, after a lengthy latent period[1,2]. Three recently reported observations indicate that tumor induction by ALVs may depend upon activation of cellular genes by proviral DNA, rather than upon expression of viral genes. (i) All bursal lymphomas are clonal populations of tumor cells containing at least one ALV provirus[3-6], but solitary proviruses are often defective and many tumors are devoid of normal virus-specific mRNAs[4,5]. (ii) In most ALV-induced bursal lymphomas, proviral DNA is found in the same region of the host genome[4,5]; Hayward et al have identified this locus as c-myc [7], the cellular homologue of the putative transforming gene (v-myc) of myelocytomatosis virus-29 (MC-29)[8,9]. (iii) Many tumors contain unusual species of polyadenylated RNA which anneal with cDNA specific for the U5 domain of the long terminal repeat (LTR) in proviral DNA ($cDNA_{5'}$, see Fig.11C), but not with other viral probes[4,5]. These novel RNA's, presumed to be initiated in proviral LTRs and extended into flanking cellular DNA, appear to contain c-myc sequences and are many-fold more abundant than the usual transcriptional product of the c-myc locus[7].

Hayward et al[7]. reported that 31 of 37 lymphomas contained proviral DNA linked to c-myc, and the structures of proviral DNA and RNA in many of these tumors were consistent with a model in which the abundant c-myc transcripts were initiated within a proviral LTR positioned upstream from c-myc in the same transcriptional orientation. Six tumors did not show enhanced expression of c-myc. On the basis of these findings, it was proposed that a "promoter insertion" mechanism operates to

enhance expression of cellular oncogenes such as c-myc during ALV tumorigenesis.

In our previous study of four defective ALV proviruses in bursal lymphomas[4], all four appeared to be located in the same region of the host genome, but two (from tumors LL1 and LL4) were in one orientation with respect to flanking DNA and two (from tumors LL2 and LL3) were in the opposite orientation. Further analysis of the tumor DNA's with probes for c-myc has shown that all four proviruses are located in or near the c-myc locus, upstream from sequences homologous to v-myc. The two proviruses (in LL1 and LL4) which conform to the "promoter insertion" model were associated, as predicted, with RNA species detectable with $cDNA_{5'}$, but similar species were not observed in LL2 or LL3 in which the proviruses and c-myc had opposite transcription orientations.

The findings with LL2 and LL3 were thus inconsistent with a "promoter insertion" mechanism and suggested that other kinds of regulatory events might occur in the apparent oncogenic collaboration between proviral DNA and c-myc. We have therefore surveyed additional ALV-induced lymphomas for the disposition of proviral DNA with respect to c-myc and for the composition of ALV and c-myc-related RNA. Our results demonstrate that at least three arrangements of proviral DNA in the c-myc locus are associated with enhanced levels of c-myc RNA: one of these arrangements is consonant with a "promoter insertion" mechanism, but the other two---viral DNA downstream from c-myc in the same orientation and viral DNA upstream from c-myc in the opposite orientation---imply that hypotheses other than "promoter insertion" are required to account for the enhancement phenomena.

Strategies for studying c-myc activation.

We have used the restriction mapping procedures described in our previous report[4] (cf. Fig.11C) to characterize the structure of ALV proviruses and their integration sites in a total of 12 chicken bursal lymphomas and 4 lymphoid cell lines. In the present studies, cloned restriction fragments from the v-myc region of MC-29 viral DNA (see Fig.11B) served as additional hybridization reagents to determine the location and transcriptional orientation of ALV proviruses with respect to at least part of the coding domain of c-myc. In every case examined, an ALV provirus was situated at the c-myc locus. Physical maps of the interrupted c-myc loci from lymphoma DNA were compared to a map of the normal c-myc locus generated from restriction endonuclease digests of chicken DNA and from digests of a cloned fragment of chicken DNA containing most or all of the c-myc locus (Fig.11A). With these methods, we have identified 7 tumors and 1 cell line with proviruses positioned on the 5' side of c-myc in the same transcriptional orientation (configuration I); one tumor with a provirus on the 3' side of c-myc in the same orientation (configuration II); and 4 tumors with proviruses on the 5' side of c-myc but in the opposite transcriptional orientation (configuration III). We are unsure of the proviral orientation in the remaining three cell lines, each with multiple proviruses, although there is a provirus upstream from c-myc in very case.

We have explored the functional consequences of these arrangements of proviral and c-myc DNA by identifying species of polyadenylated RNA from representative tumors with hybridization probes for myc and for ALV sequences (particularly U3 and U5, see Fig.11C). In some cases we also determined whether multiple hybridization reagents were annealing

with separate comigrating RNA species or with a single species. In the ensuing sections, we present analyses of tumor DNA and RNA from samples illustrating the three arrangements of viral and c-myc sequences.

Configuration I: "Promoter Insertion".

Eco RI digestion of DNA from uninfected chicken cells produces a 14 kbp fragment detected by the Pst I fragment of cloned MC-29 DNA which served as our v-myc probe (referred to hereafter as myc probe, cf. Fig.11B; Fig.12B, lane 2). myc probe anneals with two fragments from Eco RI digests of DNA from tumor LL4 - a new fragment of 3.1 kbp, as well as the normal 14 kbp fragment (Fig.12B, lane 1). The 3.1 kbp fragment was apparently generated by an Eco RI site in an ALV LTR adjacent to c-myc, since coincident bands were produced by hybridization to either $cDNA_3$, or $cDNA_5$, (Fig.12B, lanes 3 and 5). (Fragments of other sizes observed after hybridization of $cDNA_5$, and $cDNA_3$, to parallel digests of control DNA [lanes 4 and 6] contain sequences from ALV-related endogenous provirus[10,11].) The Eco RI site in the ALV LTR is known to be in the U3 domain[4,12]; if the same Eco RI restriction fragment anneals to probes for myc, U3, and U5, the provirus must be oriented so that it is transcribed in the same direction as c-myc. We have previously shown that the single ALV provirus in LL4 is highly defective, composed mostly, if not entirely, of a single LTR; hence only two new ALV-related Eco RI fragments were detected with $cDNA_3$, and only one with $cDNA_5$, (Fig.12B lanes 3-6). Tests with several additional endonucleases have confirmed the conclusions drawn from the Eco RI data and have located the integration site of the defective provirus in LL4 approximately 0.5 kbp to the left of a Sac I site near the 5' end of the c-myc sequences detectable with our myc probe (data not

shown). A diagram illustrating the arrangement of this provirus with respect to c-myc is provided in Figure 12A.

The configuration of the ALV provirus in LL4 suggests that transcription originating in the proviral LTR could proceed into c-myc, in the manner proposed by the "promoter insertion" model [4,5,7]. The data presented in Fig. 12C substantiate this prediction. A single polyA$^+$ RNA species of
2.5 kb was detected with $cDNA_5$, (Fig.12C, lane 7). No other species of RNA were observed with probes for other regions of the viral genome, including U3 (Fig.12C lane 8), a result compatible with the truncated structure of the LL4 provirus. The myc probe also anneals to a 2.5 kb species of RNA from LL4 (Fig.12C lane 9), suggesting that the transcripts observed with $cDNA_5$, also carry myc sequences. Uninfected chick embryo fibroblasts also contain 2.5 kb transcripts which anneal with myc probe and presumably represent the normal transcription products of c-myc [13] (Fig.12C lane 10);
c-myc RNA of the same size and abundance is present in normal bursal tissue (D. Sheiness and T. Gonda, personal communication). The concentration of
c-myc RNA in LL4 was approximately 70-fold greater than in normal tissues as determined by densitometry, with adjustments for the amounts of RNA and the autoradiographic exposure times for lanes 9 and 10 (see legend to Fig.12). Although the transcripts revealed in lanes 9 and 10 are similar in size, we do not know the precise composition of the stable species. It is likely that the transcripts in tumor LL4 have been initiated at a novel promoter provided by the ALV LTR, elongated through the c-myc locus, and processed to remove at least some inter-

vening sequences.

Configuration 2: Proviral insertion downstream from c-myc.

Eco RI digests of DNA from tumor LL6 do not contain a novel c-myc fragment (Fig.13B lanes 1, 2). Hybridization with $cDNA_3$, (Fig.13B lanes 3, 4) confirmed that c-myc and ALV proviral sequences are not present in the same Eco RI restriction fragment. However, Kpn I digestion of tumor DNA produced a new myc-specific fragment of approximately 19 kbp, in addition to the normal c-myc fragment of 13 kbp (Fig.13B, lanes 5, 6). The relative intensity of the bands representing the two Kpn I fragments implies that the abundance of the uninterrupted c-myc allele is reduced compared to that of the normal locus. This finding has been confirmed with other enzymes (cf. the analysis with Hind III in lane 11) and observed with several other tumors in our collection; we presume that the tumor cells in these cases are aneuploid for the chromosome bearing c-myc. The annealing of $cDNA_3$, to the Kpn I fragment of 19 kbp (Fig.13B lane 7) provides evidence for an ALV provirus between the Eco RI site at the 3' end of c-myc and a Kpn I site positioned further to the right. Confirmatory data for an insertion on the 3' side of c-myc were derived from Bam HI, Bgl I, and Hind III digests (not shown). Further support was obtained using Sal I-Pst I fragments of v-myc as probes to distinguish between the 5' and 3' exons of c-myc. When uninfected chicken DNA is digested with an enzyme (e.g., Hind III) which cleaves within the only identified c-myc intron (B. Vennstrom et al. manuscript in preparation), the Sal A probe (from the 3' end of fIyc) detects a 1.8 kbp Hind III fragment in digests of normal chicken DNA, whereas the Sal B probe (from the 5' end of v-myc) detects a 10 kbp Hind III fragment (Fig.13B lanes 9, 10). Using the Sal A probe to

analyze digests of LL6 DNA, we identified a new Hind III fragment (6.6 kb) that also contains ALV sequences (Fig.13B lanes 11-14), confirming the position of the ALV provirus near the 3' end of c-myc. Data not presented here show that the ALV provirus in LL6 has incurred a large deletion, leaving only the 3' LTR and ca. 0.7 kb of adjacent viral DNA. Furthermore, a large deletion spanning at least 15 kb of cellular DNA on the 3' side of c-myc has occurred in this tumor, removing the Hind III, Kpn I, and Sac I sites normally proximal to the 3' exon of c-myc (cf. Fig.11A). (This deletion explains why the apparent increments in size of Kpn I and Hind III fragments in LL6 greatly exceed the size of the proviral insert.) Additional mapping results, also not shown, place the defective provirus within 500 bp of the Eco RI site on the 3' side of the v-myc-related sequences in the same transcriptional orientation, as diagrammed in Fig.13A.

LL6 contains polyadenylated RNA of 3.7 kb which anneals to myc probe and $cDNA_{3'}$, but not to $cDNA_{5'}$ (Fig.13B lanes 15-17). The increase in transcript size over the size of normal c-myc transcripts may be attributed in part to ALV sequences; a probe homologous to the 3' end of env also hybridizes to the 3.7 kb transcripts (not shown). The concentration of this RNA was estimated to be 20 times greater than the concentration of c-myc RNA in normal bursa or fibroblasts. The arrangement of the template and the size and content of the RNA suggest that transcription of c-myc in LL6 may originate upstream from c-myc, perhaps at the normal initiation site, and proceed into the ALV provirus, with termination or polyadenylation occuring in or near the end of U3 (see diagram, Fig.13A). The ALV provirus has therefore altered the normal biogenesis of c-myc RNA, most likely by replacing the c-myc

sequences normally recognized as signals for transcript termination or polyadenylation.

Configuration 3: Proviral insertion upstream from c-myc in the "opposite" orientation.

Hybridization of Eco RI digested LL3 DNA with myc probe reveals a 2.9 kb fragment in addition to the normal 14 kbp fragment (Fig.14, lanes 1 & 2). Unlike the new Eco RI fragment seen in digests of LL4 DNA (see Fig.12B), the 2.9 kbp fragment from LL3 DNA anneals with $cDNA_3$, but not $cDNA_5$, (Fig.14B lanes 3-6). The most likely interpretation of this data (Fig.14A) is that the LTR adjacent to c-myc has assumed a transcriptional orientation opposite that of c-myc. Digestion of LL3 tumor DNA with Kpn I and Hind III followed by sequential annealings with Bam C probe and myc probe confirmed the proposed orientation (data not shown). In addition, our conclusion is supported by previously reported evidence that the ALV proviruses in LL3 and LL4 are in opposite orientations within the same chromosomal context[4].

We have constructed a recombinant DNA library of LL3 DNA by inserting tumor DNA partially digested with Sau 3A into the bacteriophage $\lambda$ vector 1059 (ref. 14). Three recombinant phage containing the ALV provirus and flanking cell sequences were isolated from the library. Fig. 14B shows a photograph of ethidium bromide-stained, Eco RI-digested DNA from one of our isolates (lane 7) and the results after Southern transfer and hybridization with myc probe, $cDNA_3$, and $cDNA_5$, (lanes 8-10). The 2.9 kbp fragment derived from the cloned DNA anneals to $cDNA_3$, and myc probe but not $cDNA_5$,. These data and a more detailed analyses of our three isolates (not shown) are in complete agreement with the results obtained using tumor DNA.

Three other bursal tumors (LL2 [ref. 4], LL5, and LL7) have an arrangement of ALV DNA and c-myc similar to that described for LL3. We previously reported that LL2 and LL3 lack polyadenylated RNA detectable with $cDNA_{rep}$ or $cDNA_{5'}$. These results, in concert with the conclusions from restriction mapping, imply that if the c-myc locus is activated by the adjacent ALV DNA in these tumors, a viral promoter is not directly responsible. To address this issue with reagents specific for c-myc as well as ALV sequences, we examined RNA from primary tumor and a large hepatic metastasis of LL7. myc probe reveals a species of RNA in the LL7 bursal tumor migrating at 2.5 kb (Fig.15 lane 1). Surprisingly, this RNA also appeared to react with $cDNA_{5'}$ (Fig.15 lane 2) but not with probes for other regions of the genome, including $cDNA_{3'}$ (Fig.15 lane 3). Since our $cDNA_{5'}$ probe is strand specific (it only detects RNA with the same polarity as the viral RNA genome), the orientation of the ALV provirus and c-myc in tumor LL7 precludes the possibility that ALV U5 sequences from this provirus are physically joined to a transcript of the c-myc coding strand (see figure 14A). This type of transcript might be produced by a subset of cells in LL7 containing a provirus upstream from c-myc in the same orientation. These cells could produce high levels of transcripts with U5 sequences joined to c-myc sequences and remain undetected in our DNA analyses if they represented less than 10% of the total tumor cell population.

In an attempt to isolate a homogeneous population of tumor cells we examined a liver metastasis from the LL7 tumor. Metastases are often clonal expansions of single tumor cells[15,4]; restriction enzyme analyses of the metastasis DNA (not shown) indicated that the arrangement of ALV and

c-myc DNA in the LL7 metastasis was identical to that in the predominant cell population in the primary tumor. However, the major 2.5 kb species of RNA from the metastasis which annealed to the myc probe did not react with $cDNA_5$, (Fig.15 lane 5) or to probes for other regions of the ALV genome (data not shown). Thus the metastatic process appeared to isolate a population of tumor cells with an ALV provirus upstream from c-myc in the opposite orientation and enhanced expression of c-myc in the absence of a fused transcript. The analyses of metastasis RNA in Figure 15 revealed minor transcripts (3.7 and 3.2 kb in lane 4 and 8.4 and 3.2 kb in lane 5) which were apparently absent from the primary tumor. However, long autoradiographic exposures of the filters carrying primary tumor RNA revealed faint bands corresponding to these species (not shown). The 8.4 and 3.2 kb transcripts revealed by $cDNA_5$, represent normal ALV mRNAs and imply the presence of a few cells which harbor non-defective ALV proviruses, either superinfected metastatic lymphocytes or infected normal liver cells. The coincidence of the 3.2 kb transcript detected by the probes in Figure 15 lanes 4 and 5 can be attributed to comigrating transcripts (see below).

myc and ALV sequences are covalently joined in RNA from tumors with configurations I and II but not III.

To assess whether ALV U5 sequences were physically linked to myc sequences in tumor transcripts, we employed the "sandwich blot" procedure of Dunn and Hassell[16] (Fig.16). Bam HI-Bgl I double digests of cloned chicken c-myc DNA were electrophoresed (Fig.16A lane 1) and transferred to nitrocellulose. Unlabelled tumor RNA was annealed to the filter-bound DNA, the filters were washed, and RNA containing ALV U5 or U3 sequences was detected by hybridization to labelled $cDNA_5$, or

$cDNA_3$,. An autoradiographic signal in a position corresponding to one of the Bam HI-Bgl I restriction fragments indicates physical linkage in RNA of sequences homologous to both the restriction fragment and the ALV LTR. When we used RNA from a tumor cell line (BK 4484A) containing an ALV provirus upstream from c-myc in a parallel orientation (Configuration I), we observed bands corresponding to fragments A and C but not B when $cDNA_5$, was used as a radioactive probe (Fig.16A lane 2). This result proves that these cells contain transcripts consisting of ALV U5 sequences physically linked to the c-myc sequences in fragments C and A. This conclusion is diagrammed in configuration I in Figure 16B and conforms to the "promoter insertion" model.

Figure 16A, lane 3 demonstrates that ALV U3 sequences and myc sequences are physically linked in RNA from tumor LL6. The signal observed with $cDNA_3$, at the position of fragment B locates the 5' end of these transcripts at least 1 kb upstream from the Sac I site that marks the 5' end of the
c-myc sequences detected by our probe (see Figure 11A and 16B). If these transcripts originate at the normal c-myc promoter, then these data place that promoter to the left of the Bgl I site which defines the right end of fragment B (see configuration II in Figure 16B). We have determined the position of proviruses relative to this Bgl I site in three tumors (LL4, LL5, and LL7) and one cell line (BK 4484A). Each provirus is integrated to the right of this site. Thus proviruses integrated 5' to c-myc in configurations I and III (see below) would be positioned between the normal promoter and the myc sequences that we have detected at increased concentrations in tumor RNA; this could mean that a secondary initiation site is used for myc RNA in these cases.

We cannot, however, exclude other possibilities; for example, the transcripts in tumor LL6 may be initiated aberrantly, thus obscuring the location of the true c-myc promoter, or viral sequences could be transcribed in Configuration III and removed from RNA by splicing.

RNA from the LL7 metastasis contains transcripts with U5 joined to sequences in fragments C and B but not A (Fig. 16A lane 4). Since restriction mapping data (not shown) place the ALV provirus in LL7 5' to the c-myc sequences detected by our probe, the "sandwich blot" indicates that transcription probably originates in the proviral LTR and proceeds through the region represented in fragment C and into that contained in fragment B. It also confirms that ALV U5 sequences are not joined to the identified c-myc coding sequences in transcripts (Figure 15). Thus the 3.2 kb RNA species revealed in Figure 15 lanes 4 and 5 most likely represents comigration of env mRNA and a minor c-myc transcript containing no ALV sequences.

The ALV provirus can act as an insertional mutagen.

We have assessed the relative configurations of ALV proviruses and c-myc in a total of 12 ALV-induced bursal lymphomas and 4 cell lines derived from tumors caused by ALV. Each tumor or cell line harbored an ALV provirus situated in the region of c-myc, and, in every case examined (five tumors and four cell lines), this insertion was associated with levels of myc-containing transcripts elevated 20-100 fold over levels of c-myc transcripts in uninfected bursa. These data further support the proposal[7] that ALV exerts its oncogenic effects by increasing the expression of c-myc.

Based upon the structures of c-myc DNA and RNA in their tumors, Hayward et al.[7] have argued that the increased level of c-myc tran-

scription is uniformly due to the insertion of an ALV promoter upstream from the gene, thus subverting the expression of c-myc by supplying a promoter more efficient than its own. In contrast, we have shown that ALV proviruses can assume three different configurations with respect to c-myc, and each configuration is associated with increased transcription of c-myc. We have been unable to identify a significant divergence in experimental protocol that might explain the differences between our results and those of Hayward et al. In view of our results, we propose that the ALV provirus (specifically the LTR) can act as an insertional mutagen capable of enhancing the expression of a cellular gene, c-myc, by presently unknown mechanisms, one aspect of which may be reflected in "promoter insertion".

The belief that these phenomena are instrumental in lymphomagenesis is founded primarily upon the extraordinary frequency with which proviral insertions in the c-myc locus and high levels of c-myc RNA are encountered in ALV-induced bursal tumors. However, the techniques employed thus far have not identified the stage of tumor induction at which c-myc is affected, nor have they fully assessed the possibility that sequential rearrangements have occurred at the proviral integration sites within the c-myc locus. (In fact, deletions affecting proviral or flanking cellular sequences have been encountered at a much greater frequency in this experimental context[4-6] than in others [cf. ref. 19].) In addition, transformation experiments with DNA from bursal lymphomas indicate that oncogenic loci devoid of viral or c-myc sequences may also be activated during the neoplastic process[17,18].

The transcriptional enhancement exerted by the ALV provirus during leukemogenesis constitutes one of two possible mutagenic effects on the host genome consequent to retroviral DNA insertion: proviruses can also inactivate host genes. The abolition of v-src expression due to the insertion of Moloney murine leukemia virus (Mo-MLV) DNA into a Rous sarcoma virus provirus has recently been observed in culture[19]. Also, the mouse genotype dilute may be due to an endogenous MLV provirus inserted at a coat color-determining locus.[20] The mutagenic properties of retroviral proviruses, as well as structural features[21], are shared with transposable elements found in bacteria and yeast. Although transposable elements have usually been reported to inactivate genes when they induce mutations[22], this most likely reflects the genetic selection employed; IS2 in bacteria[23] and Ty 1 in yeast[24,25] have also been shown to alter the expression of adjacent genes in a positive manner by mechanisms still incompletely defined.

The mechanism of transcriptional enhancement.

The mechanism of ALV-induced enhancement of c-myc expression also remains obscure. However, two general, non-exclusive proposals can be considered. First, the increase of c-myc expression in each of the three observed configurations may reflect properties of c-myc. c-myc could be under control of cis-acting regulatory elements either 5' or 3' to the gene. Integration of ALV DNA into these sequences could then release c-myc from their influence. Experiments of Cooper et al.[26] suggest that cis-acting sequences may regulate expression of endogenous chicken proviruses, and cis-acting regulators on the 3' side of a gene have been reported for the mating type locus in yeast[27] and the fetal globin genes in humans[28]. A second, more likely proposal postulates

that enhancement may reflect a distinctive property of the provirus rather than a property of c-myc. For example, the provirus could increase the accessibility of c-myc to the host cell's transcriptional machinery by altering the nucleosome positions in the region of c-myc. This could account for increased c-myc expression in each configuration. Alternatively, the mechanism of enhancement could be unique for each configuration. The proviral LTR could act as a c-myc promoter if inserted in the correct orientation upstream from c-myc. If inserted in the proper orientation downstream from c-myc, the provirus could act to stabilize the c-myc transcripts. If inserted upstream from c-myc but in an opposite orientation, it could affect chromatin structure or bind accessory transcriptional factors in ways which might increase transcription in both directions.

The hypothesis that a retroviral LTR can enhance the expression of a linked gene may find support in experiments which introduce selectable genes into cultured cells either as a calcium phosphate precipitate or by microinjection. The Moloney murine sarcoma virus LTR markedly augments the frequency of morphological transformation of NIH-3T3 cells by a restriction fragment containing the putative transforming gene of murine sarcoma virus (v-mos). This augmentation occurs when the LTR is positioned either on the 5' or 3' side of the viral oncogene[29]. When the LTR has been positioned downstream from v-mos with the same transcriptional polarity, transcripts which proceed through v-mos and terminate in the U3 region are observed in the transformed cells (T. Wood and G. Vande Woude, personal communication). This situation is analogous to the transcriptional pattern found in tumor LL6. Placement of the Rous sarcoma virus

LTR at either the 5' or 3' end of a complete Herpes simplex virus thymidine kinase (HSV-*tk*) gene including the true HSV-*tk* promoter enhances the number of HSV-*tk* positive colonies after microinjection into *tk*$^-$ mouse L cells (P. Luciw & M. Capecchi, personal communication). It is not yet clear whether the effect of the retroviral LTR in these cell culture experiments is to increase transcription of the adjacent genes.

In conclusion, we have proposed that the ALV provirus contains a previously unknown ability, independent of its configuration, to affect the transcriptional activity of adjacent cellular DNA. The transcriptional enhancement provided by the ALV provirus suggests that novel mechanisms may control the expression of eukaryotic genes.

Legend to Figure 11.

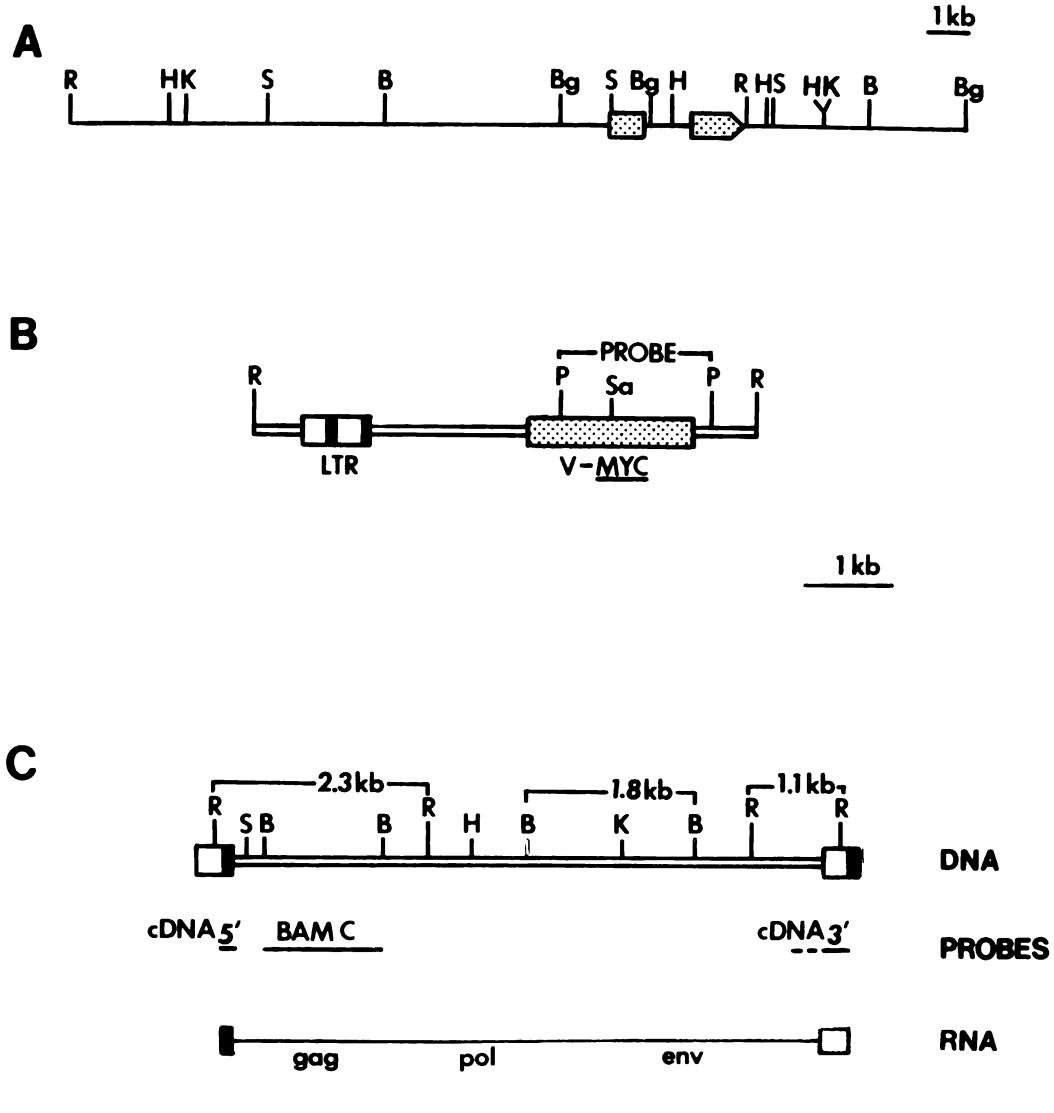
A. Map of restriction endonuclease sites in the region of the chicken genome containing c-myc. The physical map of c-myc was generated using restriction endonuclease digests of chicken DNA. An identical map has been obtained from a cloned DNA fragment isolated from a recombinant phage library of chicken DNA (B. Vennstrom, manuscript in preparation). The domains homologous to v-myc are shown as shaded boxes and were determined using as probe a Pst I fragment of cloned MC-29 viral DNA[30], which was subcloned in pBR322. This Pst I fragment, as diagrammed in panel B, is 1.5 kilobase pairs (kbp) in length and contains, in addition to v-myc sequences, a short (<0.3 kbp) sequence from the env regions of MC-29. As a result, the v-myc probe anneals weakly to restriction fragments of ALV-related proviral DNA containing env sequences, but the signal is too weak to interfere with our analyses (see Figs.12B,13B,14B). The Pst I site which marks the 5' end of the flyc fragment cleaves within the v-myc sequences (cf. Fig.11B); thus a small segment (probably less than 200 bp) of v-myc specific nucleotides are not represented in the probe, and the shaded boxes do not represent the entirety of c-myc coding sequences. The v-myc sequences in MC-29 are joined to gag sequences encoding viral structure protein[31] generating, upon translation, a fusion protein, $p110^{gag-myc}$ (ref. 32). Thus the structure of the c-myc locus is still incompletely defined, and the map does not indicate transcriptional or translational boundaries for the cellular gene. The direction of transcription of c-myc is indicated by the arrow-like shape of the righthand exon and was determined by preparing restriction fragments from a Sal I digest of the Pst I v-myc fragment as probes specific for the 5' and 3' domains of v-myc.

The length of the intervening sequence was determined by heteroduplex analysis of the cloned c-myc DNA fragment and MC-29 viral DNA (B. Vennstrom et al. in preparation). (This analysis also confirmed the transcriptional polarity of c-myc.)

R: Eco RI; B: Bam HI; S: Sac I; K: Kpn I; H: Hind III; Bg: Bgl I

B. A diagram of cloned MC-29 viral DNA. The MC-29 DNA was cloned by linearizing circular MC-29 viral DNA with Eco RI and inserting the DNA into λgtWES·λB (ref. 35). The Pst I sites (P) and Sal site (Sa) used to generate v-myc specific probes are shown. The v-myc region is shown as a shaded box.

C. Maps of restriction endonuclease sites in RAV-2 DNA. This map was generated as described in Payne et al.[4]. The long terminal repeats (LTR), approximately 330 bp, are drawn as boxes at the ends of the DNA. The open box represents sequences specific to the 3' end of the viral RNA (U3); the shaded boxes represent sequences specific to the 5' end of viral RNA (U5). The approximate location of viral genes are indicated on the diagram of viral RNA. "Signature fragments", which distinguish RAV-2 DNA from proviruses endogenous to the chickens used in this study, are marked by lines connecting the two restriction sites. Some of the probes used in our studies represent regions delineated by the labeled lines between the diagrams of viral RNA and DNA. Descriptions of each probe are included in the text and in Payne et al.[4].

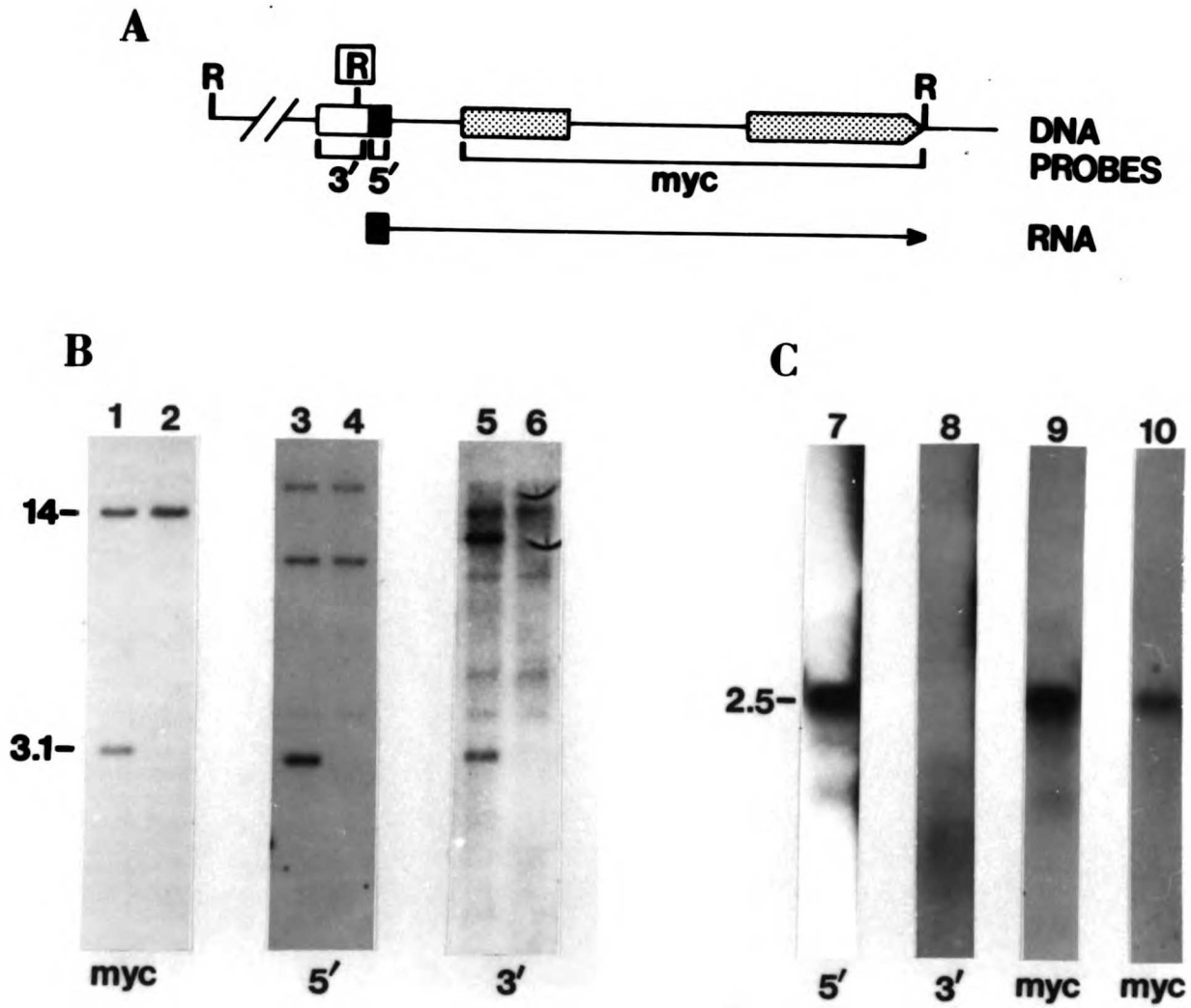

A
1kb
R
HK
S
B
Bg
S
Bg
H
R
HS
HK
B
Bg
B
PROBE
R
P
Sa
P
R
LTR
V-MYC
1kb
C
2.3kb
1.8kb
1.1kb
R
S
B
B
R
H
B
K
B
R
R
DNA
cDNA 5'
BAM C
cDNA 3'
PROBES
gag
pol
env
RNA

Legend to Figure 12. Configuration I: ALV and c-myc DNA and RNA in tumor LL4.

A. Diagram of the structure of the single ALV provirus in tumor LL4[4] and its position and transcriptional orientation relative to c-myc. The symbols for the proviral LTR and c-myc DNA are as described in the legend to Fig.11. Cell DNA is represented as a single line. The proviral Eco RI site responsible for the 3.1 kbp fragment detected in Fig.12B is enclosed in a box. The sequences detected by the probes used in Fig.12B and Fig.12C are delineated under the diagram of the DNA. The virus-specific RNA in this tumor is diagrammed below the probes. (Since these transcripts are equivalent in size to normal c-myc ___ transcripts, we presume the hybrid RNA is processed but we are unsure of its precise composition.)

B. 5 micrograms (μg) each of LL4 tumor DNA (odd numbered lanes) and DNA from uninfected tissue (even numbered lanes) from the same bird were digested with Eco RI, electrophoresed through 0.8% agarose, transfered to nitrocellulose and annealed sequentially to the probe indicated beneath the lanes[4]. Sizes in kilobases are shown beside the lanes.

C. Lanes 7-9: Poly-$A^+$ RNA was prepared from the tumor as described by Varmus et al.[19] except that frozen tissue was homogenized using a tissumizer (Tekmar Industries, Cincinnati, Ohio), and 20 μg samples were electrophoresed through 1.2% methyl-mercury agarose, transfered to diazobenzlomethoxy-cellulose paper[33] and annealed sequentially to the designated probes[34]. Lane 10: 50 μg poly $A^+$ RNA from uninfected chick embryo fibroblasts was prepared and analyzed as described above. The autoradiogram of lane 10 was exposed 8.7 times as long as the autora-

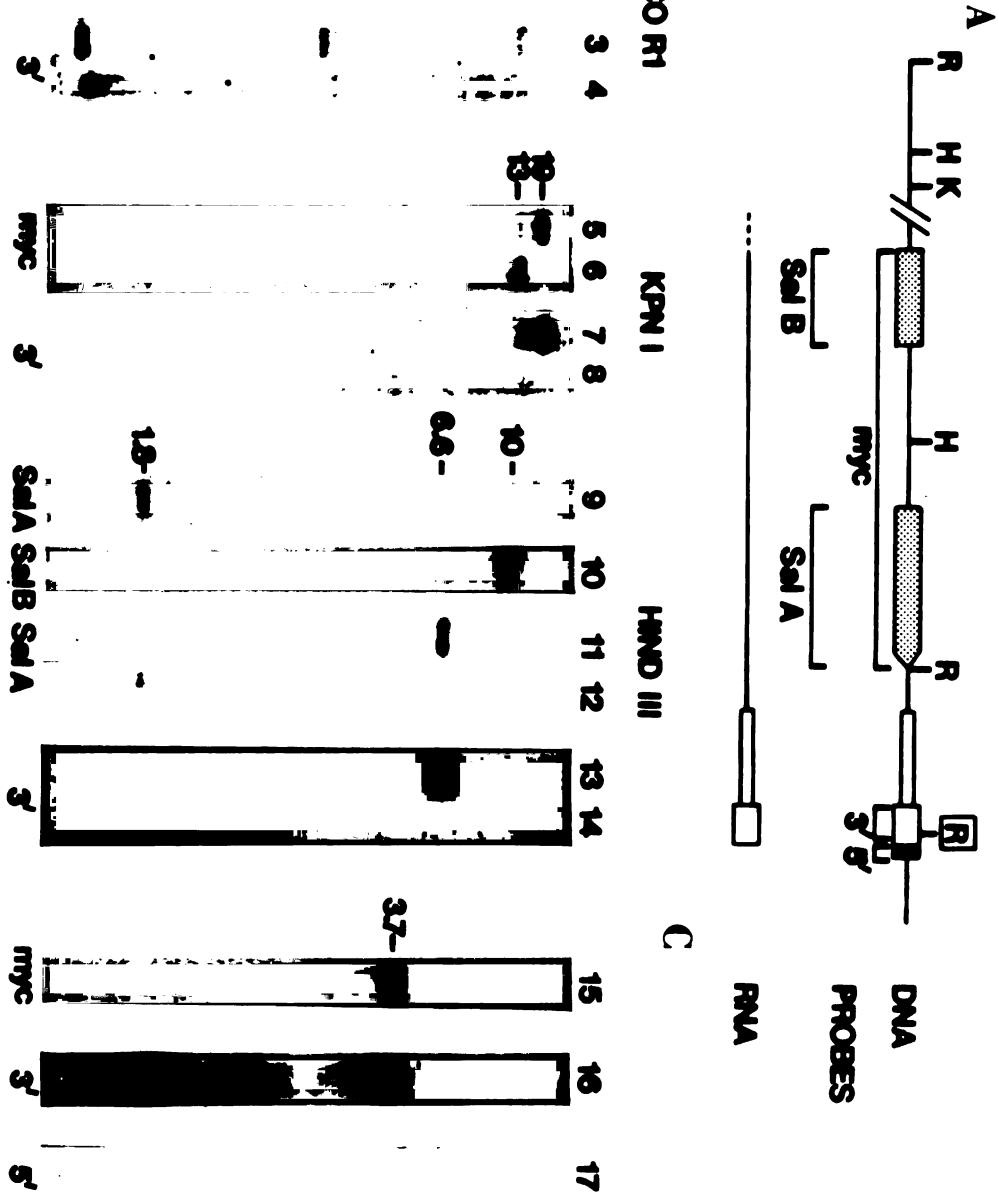

A
R
R
R
3′ 5′
myc
DNA PROBES
RNA
B
1 2 3 4 5 6
14–
3.1–
myc
5′
3′
C
7 8 9 10
2.5–
5′
3′
myc
myc

Legend for Figure 13. Configuration II: ALV and c-myc DNA and RNA in tumor LL6.

A. Diagram of the provirus and c-myc and the resulting transcripts in LL6 as revealed using the indicated probes in analyses shown in panels B & C. The symbols and the preparation of the Sal A and Sal B probes are described in the legend to Fig.11. Proviral DNA is represented as 2 parallel lines, cell DNA as 1 line. The Eco RI site introduced by the provirus is boxed. As explained in the text, a large deletion of cellular DNA has occurred on the 3' side of c-myc but the precise position and size of the deleted DNA is not certain. The Hind III, Kpn I, and Eco RI sites on the right hand side of the provirus lie beyond the region depicted and have been omitted here.

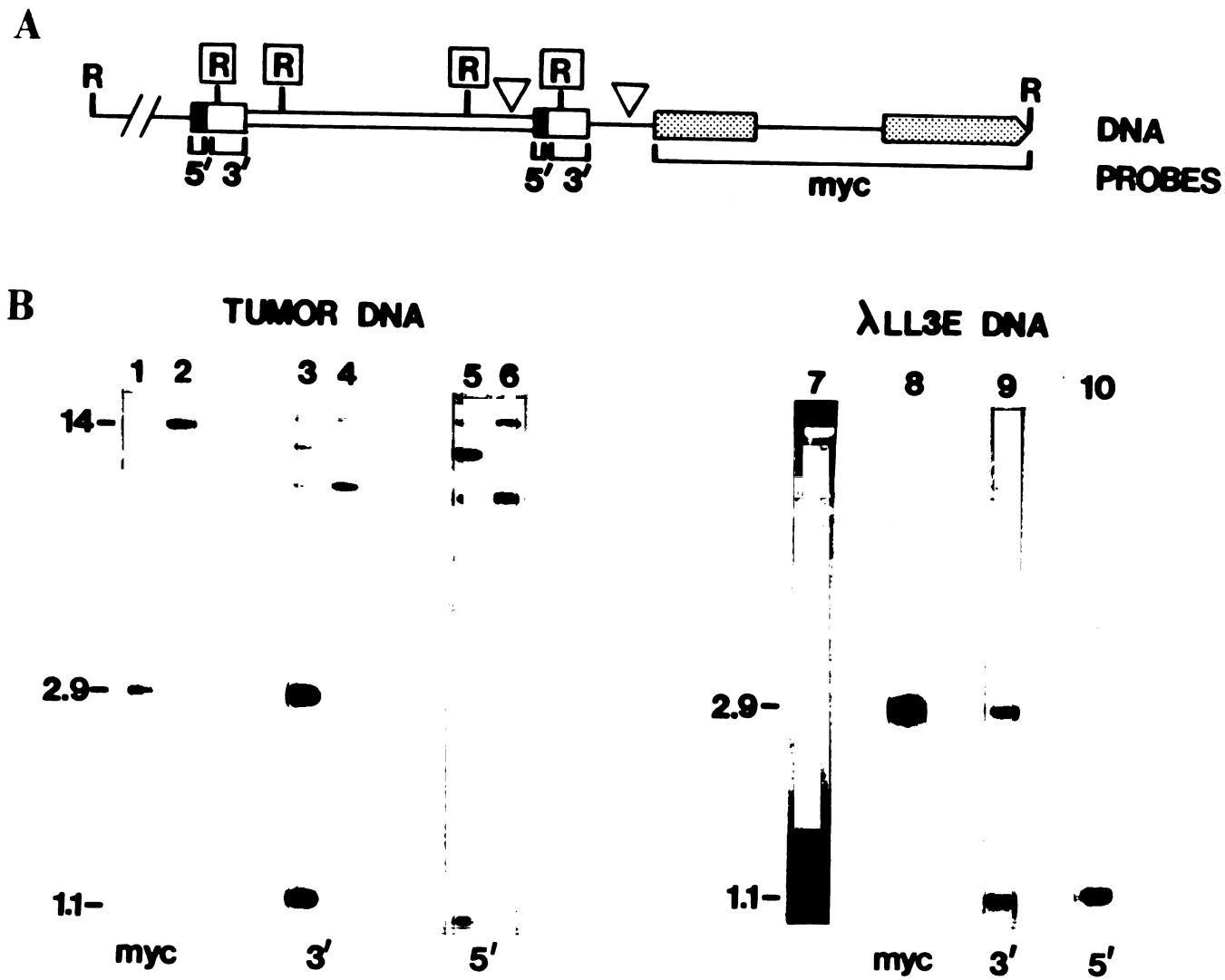
B. Lanes 1-8, 11-14: Tumor DNA (odd numbered lanes) and uninfected DNA (even numbered lanes) were analyzed with Eco RI, Kpn I and Hind III using methods described in the legend to Fig.12B. Lanes 9, 10: uninfected chicken embryo DNA analyzed as in Fig.12B.

C. Tumor RNA was analyzed as in Fig.12C.


A
R
H K
Sal B
myc
H
Sal A
R
R
3'
DNA PROBES
RNA
B
ECO R1
KPN I
HIND III
1 2 3 4
5 6 7 8 9 10 11 12 13 14
15 16 17
14
3.6
1.4
1.9
0.6
10
3.7
myc
3'
Sal A
Sal B
Sal A
C

Legend to Figure 14. Configuration III: ALV and c-myc DNA in tumor LL3. A. Diagram of the ALV provirus and c-myc in tumor LL3; the triangles represent deletions. The provirus in the primary tumor LL7 and its liver metastasis has a similar configuration to that in LL3 except there is no deletion in the cell DNA and the deletion within the ALV provirus is smaller.

B. Lanes 1-6 represent analysis of Eco RI digests of tumor DNA (odd numbered lanes) and uninfected tissue DNA (even numbered lanes) using probes for myc, U3, and U5 as described in the legends to previous figures. Lanes 7-10 contain DNA from LL3E, a recombinant bacteriophage carrying c-myc and part of the ALV provirus from LL3 tumor DNA. Lane 7, a photograph of the ethidium bromide-stained Eco RI digest of λLL3E DNA visualized by transillumination with ultraviolet light; lanes 8-10, autoradiograms of the results of sequential hybridizations with myc probe, $cDNA_3$,, and $cDNA_5$, to cloned DNA transferred to nitrocellulose. LL3E and two others containing different DNA fragments from the c-myc region of LL3 tumor DNA were isolated from a recombinant DNA library of partial products of Sau 3A digestion of tumor DNA cloned in the bacteriophage λ vector 1059[14]. Size-fractionated DNA, approximately 20 kb long, was selected by preparative electrophoresis of 200 µg of digested DNA through low-melting agarose (Seaplaque)[4]. After recombinant molecules were packaged in vitro[35] and used to infect E. coli Q359[14], recombinant phage carrying the ALV provirus were detected using $cDNA_3$,[36].


A
R
R
R
R
R
R
5′ 3′
5′ 3′
myc
DNA
PROBES
B
TUMOR DNA
λLL3E DNA
1 2 3 4 5 6
7 8 9 10
14–
2.9–
1.1–
2.9–
1.1–
myc 3′ 5′
myc 3′ 5′

Legend to Figure 15. Configuration III; ALV and c-myc RNA in tumor LL7 and its metastasis.

Lanes 1-3; analysis of virus-specific RNA from bursal tumor LL7 was performed as in Fig.12C. Lanes 4-5 represent an analysis of RNA from a liver metastasis from tumor LL7. 5 µg of poly-$A^+$ RNA was electrophoresed through 1.2% formaldehyde agarose and transfered to nitrocellulose[37]. The sequential hybridizations using the indicated probes were performed as with the DBM-filter bound RNA.

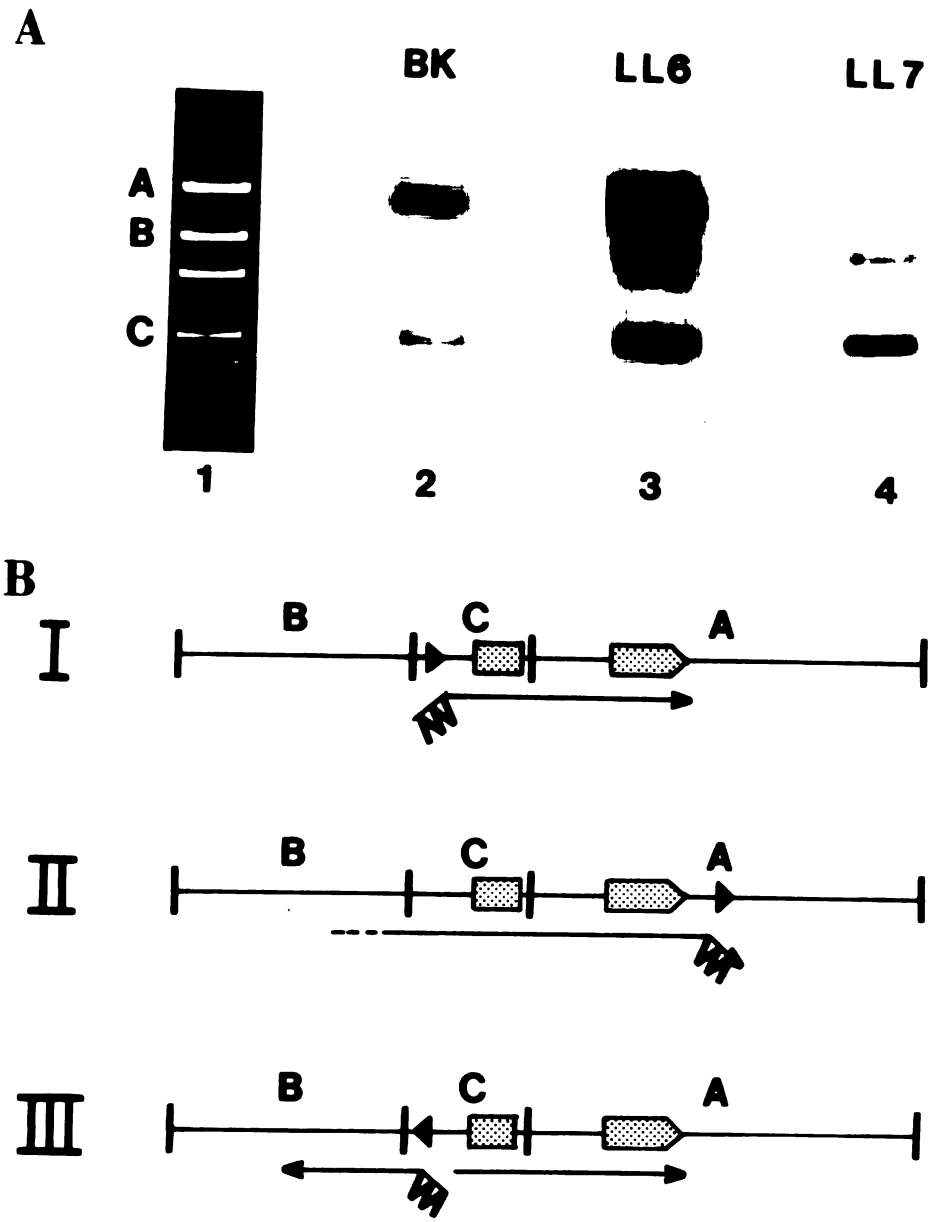
TUMOR
METASTASIS
1
2
3
4
5
2.5–
8.4–
3.7–
3.2–
2.5–
myc
5′
3′
myc
5′

Legend to Figure 16. Tests for linkage of ALV and myc sequences on tumor RNAs.

"Sandwich blot" procedure of Dunn and Hassell[16], modified as described below, was used to assess physical linkage of ALV sequences and myc sequences in RNA prepared from BK 4484A cell line RNA (lane 3), LL3 RNA (lane 6) and LL7 liver metastasis RNA (lane 4). The 10 kbp Bam HI fragment containing c-myc (see Fig.11A), obtained from a digest of the recombinant DNA described in the legend to Fig.11A, was subcloned into pBR322 (D. Sheiness, unpublished). 1 µg of this plasmid, pCMC-2, was digested with Bam HI and Bgl I and electrophoresed through 1.2% agarose. The DNA was detected as in the legend to Fig.14, then transfered to nitrocellulose. 5 µg of poly-$A^+$ RNA was resuspended in 200 microliters of an annealing mix consisting of 50% formamide, 0.5 M NaCl, 20 mM Pipes pH6.8, 5 mM $Na_2$ EDTA, 0.4%SDS, 250 µg/ml poly A, 2 x Denhardt's solution[38], 200 µg/ml carrier yeast RNA. This solution was applied to the filter-bound DNA and incubated for 24 hrs at 41°C. The filter was washed twice at 53°C for 15 minutes each with 0.1 X SSC. 0.1% SDS. The filter was blotted dry and annealed to $cDNA_5$, or $cDNA_3$, as described by Quintrell et al.[34]. This filter was washed twice for 1 hr each time at 37°C with 0.1 X SSC, 0.1% SDS, dried and autoradioraphed. Lane 1. Photograph of ethidium bromide stained Bgl I, Bam HI digest of pCMC-2 DNA. The bands labeled A, B, and C result from the c-myc insert and are diagrammed in Fig.16B. The other bands consist of pBR322 DNA sequences. Lane 2 and 4: Filters annealed to $cDNA_5$,. Lane 3: Filter annealed to $cDNA_3$,.

B. Diagrams of transcriptional patterns in configurations I, II, and III based on "Sandwich blots" shown in Fig.16A and analyses of tumor

RNA from BK cell line (not shown), LL6 (Fig.13C) and LL7 (Fig.15, lanes 4 and 5). The restriction sites are labelled as in Fig.11A and the fragments designated A, B and C in Fig.16A are shown. The arrowhead marks the position of the ALV provirus and indicates its transcriptional orientation. The transcriptional pattern of each configuration is shown as an arrow below the diagram. The inclined tail of an arrow marked with a serrated line indicates ALV U5 sequences detected with $cDNA_5$, in configurations I and III, and ALV U3 sequences detected with $cDNA_3$, in configuration II.


A
BK
LL6
LL7
A
B
C
1
2
3
4
B
I
B
C
A
II
B
C
A
III
B
C
A

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# APPENDIX 2

## The Oncogenic Spectra of Molecularly Cloned Avian Leukosis Viruses

## Introduction

ALV infection of chickens results predominantly in lymphoid leukosis arising in the bursa of Fabricius. However, ALV can induce a variety of other neoplasms including erythroblastosis, nephroblastomas and endotheliomas (hemangiomas) (Purchase et al.,1977; Crittenden,1980). In general the proportion of tumors which are not LL resulting from ALV infection can be influenced by the age, physiological status, and genetic background of the chicken, the route of virus infection and the dose of virus (Crittenden,1980). Two opposing hypotheses have been considered to account for the multi-oncogenic potential of ALV: either the infecting virus stock contains a mixture of genetically different viruses, each of which produces one type of neoplasm, or one genetically homogeneous virus stock can induce the entire spectrum of neoplasms.

Many early reports of multiple tumor types associated with virus stocks were due to copropagation of non-defective and defective leukemia viruses. For instance, RPL-12, an ALV which caused both a long latency leukosis and an early onset erythroblastosis, was apparently a mixture of ALV and the defective avian erythroblastosis virus (AEV) (Crittenden,1980). Also, non-defective leukosis viruses, myeloblastosis- virus associated viruses (MAVs), isolated from the BAI-A complex of avian myeloblastosis viruses consistently induce primarily either LL (MAV-1), nephroblastomas (MAV-2(N)) or osteopetrosis (MAV-2(O)) (Smith and Moscovici,1969; Watts and Smith,1980). On the other hand, in spite of the different stable oncogenic propensities of these isolates, a low level of other tumor types arise after infection. Similarly, Rous-associated virus-1 (RAV-1) cloned by endpoint dilution

and RAV-2 cloned by plaque- purification caused a variety of tumors in addition to LL (see table 2).

We have molecularly cloned RAV-1 and RAV-2 DNA and harvested virus produced upon introduction of the cloned DNA into chick embryo fibroblasts. Each stock thus results from transcription, packaging and propagation of RNA templated from a homogeneous population of cloned DNA molecules. This procedure should generate pure virus stocks (refered to hereafter as "cloned" virus) free from any associated viruses present in the parental virus stocks. The "cloned" RAV-1 and RAV-2 stocks were injected into chicks and the pathogenic consequences were compared to those observed in a cohort of chicks infected with the parental stocks.

Results

Eight independent plaques of bacteriophage λgtWES.λB carrying inserts of either RAV-1 or RAV-2 DNA were isolated and amplified as described in chapter 2. The inserted ALV DNA was purified by sucrose gradient sedimentation following Sac I digestion. Purified DNA, permuted with respect to the normal viral linear DNA, was ligated in order to reconstitute the 5' end of the genome and the multimeric DNA was introduced into chick embryo fibroblasts by calcium phosphate coprecipitation (Graham and Van der Eb,1973; Stowe and Wilkie,1976). The media was then assayed for viral reverse transcriptase activity at various cell passages (Tereba and Murti,1977). Each isolate proved infectious by this assay (data not shown). Both "cloned" and parental stocks of RAV-2 were cytopathic to chick embryo fibroblasts in tissue culture while both RAV-1 stocks were innocuous (data not shown). Media was harvested from one RAV-1 and one RAV-2 producing culture. These

harvests were used for oncogenicity tests. The yolk sacs of six day old 15Exev-0 embryos were injected with $10^4$ infectious units of virus from each of the "cloned" and parental stocks. As can be seen in table 2 the "cloned" virus generated neoplasms other than LL at frequencies identical to the parental stocks. There also appear to be strain related differences between the parental stocks of RAV-1 and RAV-2 which are reproduced in the "cloned" stocks. A large percentage of non-LL tumors induced by both stocks of RAV-1 is erythroblastosis whereas the RAV-2 stocks caused hemangiomas and no erythroblastosis.

## Discussion

We have thus shown that isolation of a single DNA copy of viral RNA by molecular cloning and subsequent propagation of virus using this DNA as template generates a virus stock with oncogenic properties identical to the parental virus stock.

Our results argue strongly that the array of neoplasms induced by a non-defective ALV is an inherent property of the virus and not due to heterogeneity in the virus stock. We can exclude as extremely unlikely the possibility that rescue of a cellular oncogene by ALV generates a virus responsible for the non-LL tumors since these transduction events are probably very rare and the "cloned" virus was harvested after only 5-7 cell passages. It also seems unlikely that such a recombinant virus would reach a titre in the "cloned" stock which would result in non-LL neoplasms at frequencies identical to those observed using the parental stocks. In contrast to the frequency of oncogene transduction, retrovirus genomes display rapid genetic variation during propagation (Coffin et al.,1980). The virus propagated from cells harboring cloned DNA undoubtedly experienced this sort of variation possibly

accounting for viruses capable of inducing non-LL tumors. The characteristic oncogenic spectra of the two different strains would argue against this possibility since the major tumors (LL) induced by the two strains are identical.

Our data also indicates that different strains of virus stably express different oncogenic tendencies as a result of genetic variation between the strains. Presently, the viral determinant that targets specific cells (i.e., lymphocytes or erythroblasts) for transformation is obscure. Integration of RAV-1 near c-erb (the cellular homologue of the transforming gene carried by AEV) has recently been observed in RAV-1 induced erythroblastosis (T. Fung personal comm.). This result, observed in tumors from chickens with erythroblastosis induced by either "cloned" or parental virus stocks, suggests that similar insertional mechanisms may be employed by ALVs during induction of different tumors. If similar strategies are deployed by ALVs inducing nephroblastomas and hemangiomas then the genetic variations responsible for the oncogenic differences between ALV strains may provide important clues to the viral determinant of target cell specificity.

Table 2. Induction of Neoplasms after inoculation with cloned virus.

| Inoculcum | Embryos Inoculated | Viremic[1] at: 5 wks. | 10 wks. | Number of Neoplasms LL[2] | ERY[3] | ON[4] | MD[5] | Percentage Neoplasms LL | ERY and ON |
|---|---|---|---|---|---|---|---|---|---|
| RAV-1 parental | 33 | 8/10 | 7/7 | 19 | 3 | 4 | 1 | 58 | 21 |
| RAV-1 cloned | 32 | 10/10 | 10/10 | 18 | 6 | 2 | 1 | 56 | 25 |
| RAV-2 parental | 39 | 10/10 | 10/10 | 22 | 0 | 5 | 0 | 56 | 13 |
| RAV-2 cloned | 32 | 10/10 | 10/10 | 19 | 0 | 3 | 0 | 59 | 9 |
| None | 38 | 0/9[6] | 0/10 | 2 | 1 | 0 | 0 | 5 | 3 |

1 Virus assayed as described in Crittenden et al. 1977. Virology 76,90.
2 Lymphoid Leukosis
3 Erythroblastosis
4 Other Neoplasms
5 Marek's Disease
6 Exogenous virus assayed

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Stowe, N.D. and Wilkie, M.M. 1976 J. Gen. Virol. 33,447.

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# APPENDIX 3

## Sarcomas induced by Rous sarcoma virus are not clonal

## Introduction

Rous sarcoma virus has transduced a cellular gene (c-src) which, in the context of the viral genome, is necessary for the initiation and maintenance of the transformed phenotype of infected chick embryo fibroblasts (Martin,1970). The incidence of transformed cells in vivo and in vitro is linearly proportional to the dose of infectious virus suggesting that infection by one infectious virus particle is sufficient for transformation (Temin and Rubin,1958). These properties of RSV predict that sarcomas arising in infected chickens will be comprised of a large number of independently transformed fibroblasts and thus will not be clonal. We have verified this prediction by performing restriction endonuclease analyses of the RSV proviruses present in tumor cell populations.

## Results and Discussion

Twelve $15I_5x7_1$ two day old chicks were injected in the wing-web with the Schmidt-Ruppin D strain of RSV. The chicks received either 300, 30, or 3 focus-forming units of virus. Wing-web sarcomas appeared in all the birds by three weeks after infection. DNA was prepared from five tumors, one metastasis and uninfected control tissue, digested with either Eco Rl or Bam Hl, electrophoresed through agarose, transferred to nitrocellulose paper and annealled to a cDNA probe representative of the entire RSV genome (see chapter 2 for methods). Digestion of the RSV provirus with Eco Rl produces two internal fragments, 2.3kb and 3.0kb in length, which are unique to the exogenous provirus, in addition to a 4.0 kb internal fragment which comigrates with a fragment of similar size from the endogenous proviruses present in these chickens (Fig.17A, lanes9,10). The proviral sequences which

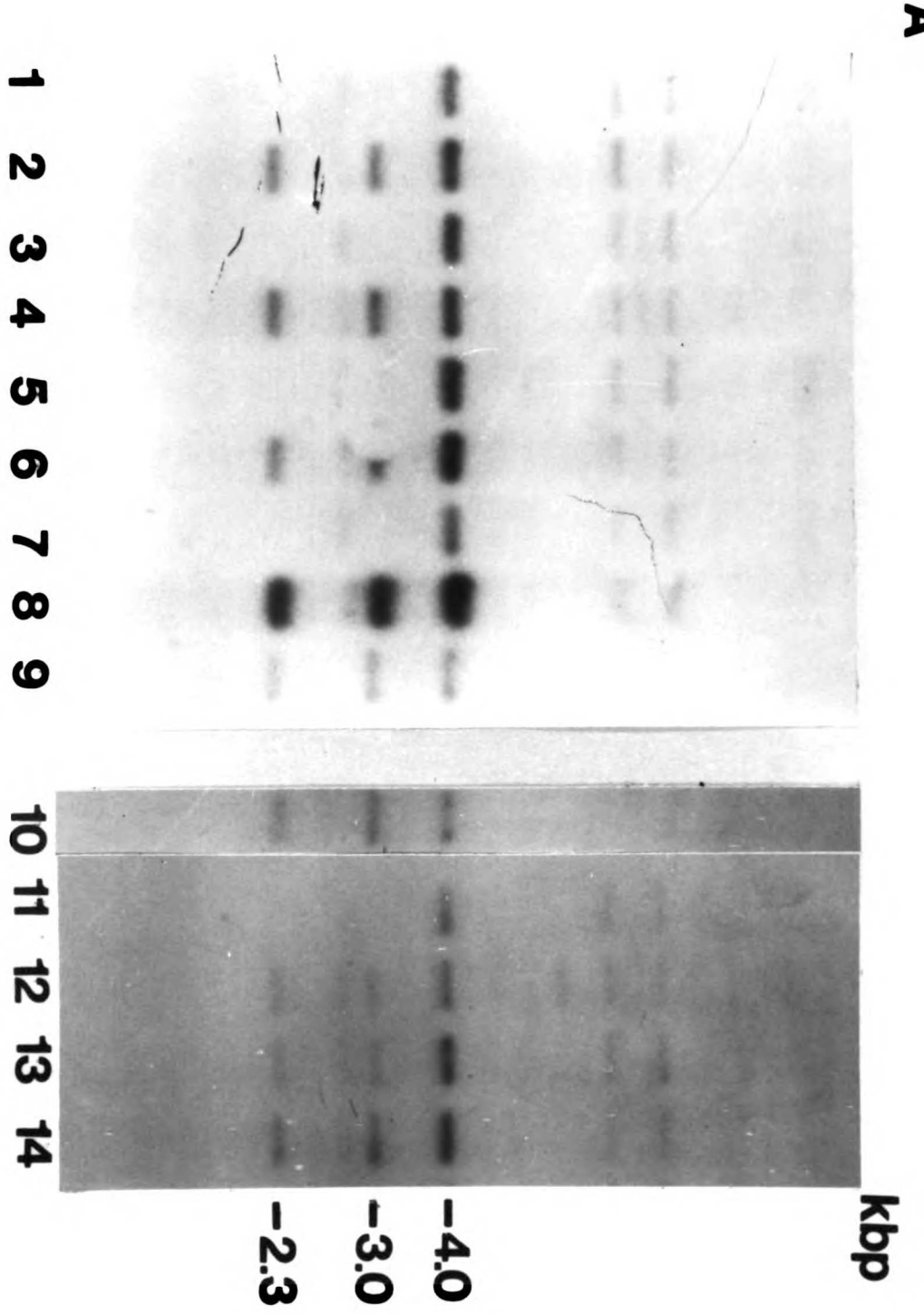
remain joined to flanking host DNA are too few to detect with the cDNA probe. The detection of the unique internal DNA fragments indicates the presence of SR-D proviruses; the intensities of the autoradiographic signals produced by the probe annealled to these fragments relative to the intensities resulting from detection of endogenous provirus fragments gives an estimate of the number of SR-D proviruses per cell. Figure 17A presents results obtained with Eco Rl digested DNA from five primary tumors, one liver metastasis and uninfected control tissue. All the tumors contain at least one SR-D provirus per cell.

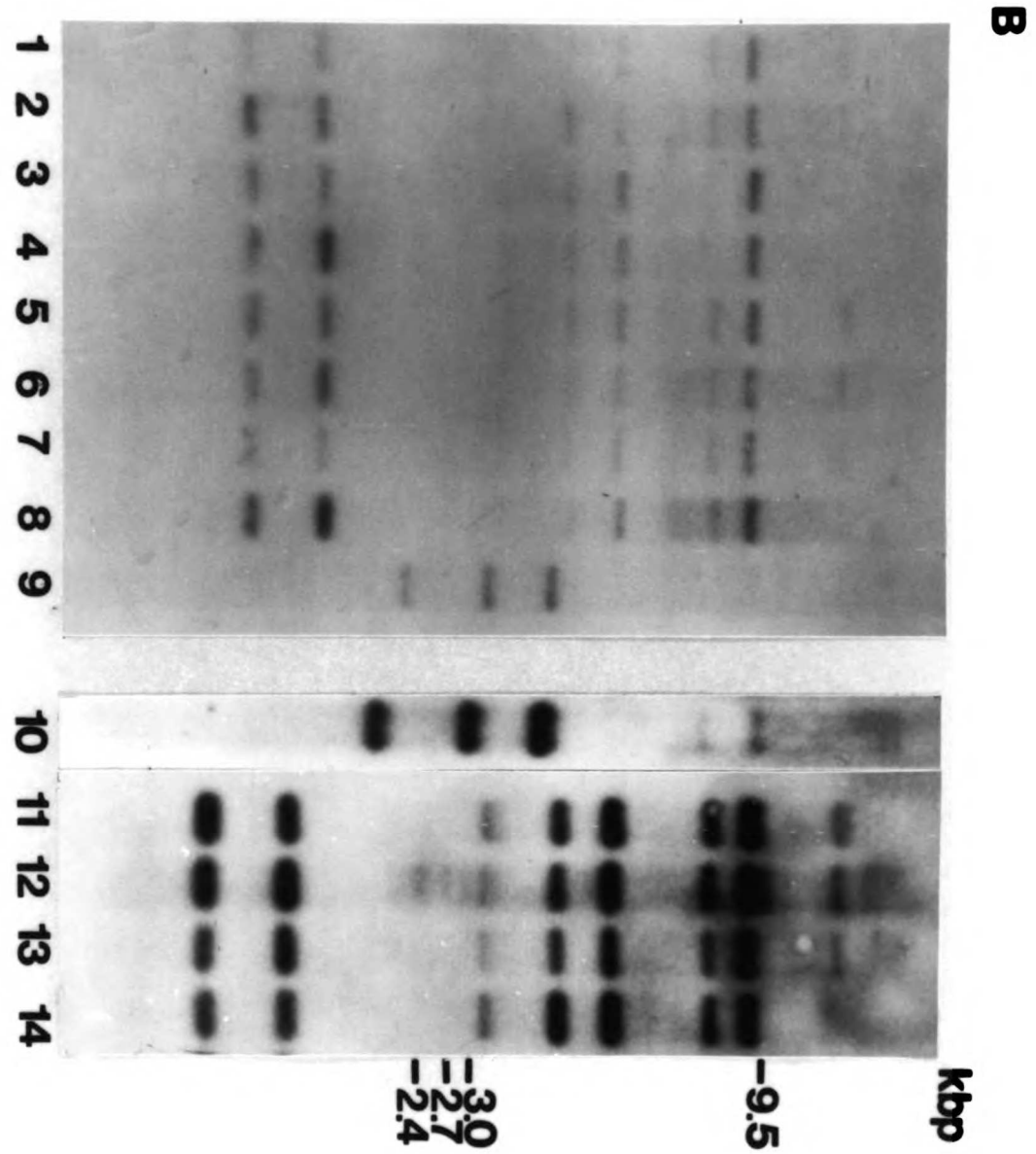
Bam Hl digestion of the SR-D provirus produces internal fragments which comigrate with similar fragments from the endogenous proviruses. However, two proviral-host junction fragments are generated, one containing approximately 6kbp from the 3' end of the provirus and one containing 600 bp from the 5' end (Hughes et al.,1978). The size of these junction fragments depends on the location of the Bam Hl sites in the flanking host DNA nearest the provirus. If the tumors are semi-clonal or clonal then tumor- specific junction fragments will be observed since most cells carry SR-D proviruses at the same location. If the tumors are not clonal then the large number of junction fragments resulting from independent integration events in each tumor cell will not resolve and no tumor-specific fragments will be observed. Figure 17B shows that in all cases excepting the liver metastasis, the tumor DNAs lack specific junction fragments and thus are not clonal. The liver metastasis does contain specific fragments (Fig.17B, lane12), although they appear to be present in lower quantities than fragments from the endogenous proviruses. Thus, the metastasis appears to be composed of cells which are the progeny of only a few parental tumor

cells. This observation is consistent with previous experiments (Poste and Fidler,1980) demonstrating that metastatic cells evolve as a sub-population from the primary tumor cells. The sub-molar ratio of tumor-specific fragments to endogenous proviral fragments indicates that more than one progenitor tumor cell participated in metastasis formation or a number of independently transformed cells have been recruited into the metastatic lesion or a number of infected, non-transformed cells were present in the tumor specimen. The analysis of DNA from the liver metastasis proves that our procedure is capable of detecting proviruses present in clonal populations and further strengthens the conclusion that the primary sarcomas are not clonal. It is interesting to note that even chicks receiving only 3 focus-forming units of virus did not exhibit semi-clonal tumors (Fig.17B,lane 4) presumably because of virus spread and efficient transformation of many cells during tumor formation. Thus, the potential growth advantage of particular tumor cells and other unknown variables in tumor progression do not distort the tumor cell population enough to allow the progeny of a few tumor cells to predominate.

Legend to Figure 17.

(A) 5 micrograms of DNA from sarcomas and uninfected control tissue was cleaved with Eco Rl, electrophoresed through 0.8% agarose, transfered to nitrocellulose and hybridized to $cDNA_{rep}$ as described in chapter 2. (lanes 1,3,5,7) uninfected control tissue DNA. (lanes 2,4,6,8) wing-web sarcoma DNA. (lanes 1,2) DNA from bird injected with 30 focus-forming units (FFU) of the Schmidt-Ruppin D strain of RSV. (lanes 3,4) bird injected with 3 FFU. (lanes 5,6) bird injected with 300 FFU. (lanes 7,8) bird injected with 300 FFU. (lanes 11-14) bird injected with 30 FFU. (lane 11) uninfected liver DNA; (lane 12) liver tumor DNA; (lane 13) wing-web sarcoma DNA; (lane 14) infected non-tumorous spleen DNA. (lanes 9,10) DNA from SR-A transformed chick embryo fibroblasts cleaved with Eco Rl. The sizes in kbp of RSV internal Eco Rl fragments are indicated. (B) Lanes as in (A) but samples digested with Bam Hl except DNA in lanes 9 and 10 were cleaved with Eco Rl. sizes in kbp of liver tumor-specific fragments in lane 12 are indicated.


A
1 2 3 4 5 6 7 8 9
10 11 12 13 14
kbp
4.0
3.0
2.3


B
1 2 3 4 5 6 7 8 9
10 11 12 13 14
3.0
2.7
2.4
9.5
kbp

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# APPENDIX 4

Molecular cloning of the ALV provirus and activated c-myc from tumor LL6

Analyses of viral-specific RNA and DNA from tumor LL6 indicated that the single ALV provirus was situated downstream from c-myc in the same transcriptional orientation (see chapter 3). In order to confirm this interpretation I have cloned a 9kbp Bam Hl restriction fragment containing the c-myc locus and nearby ALV provirus.

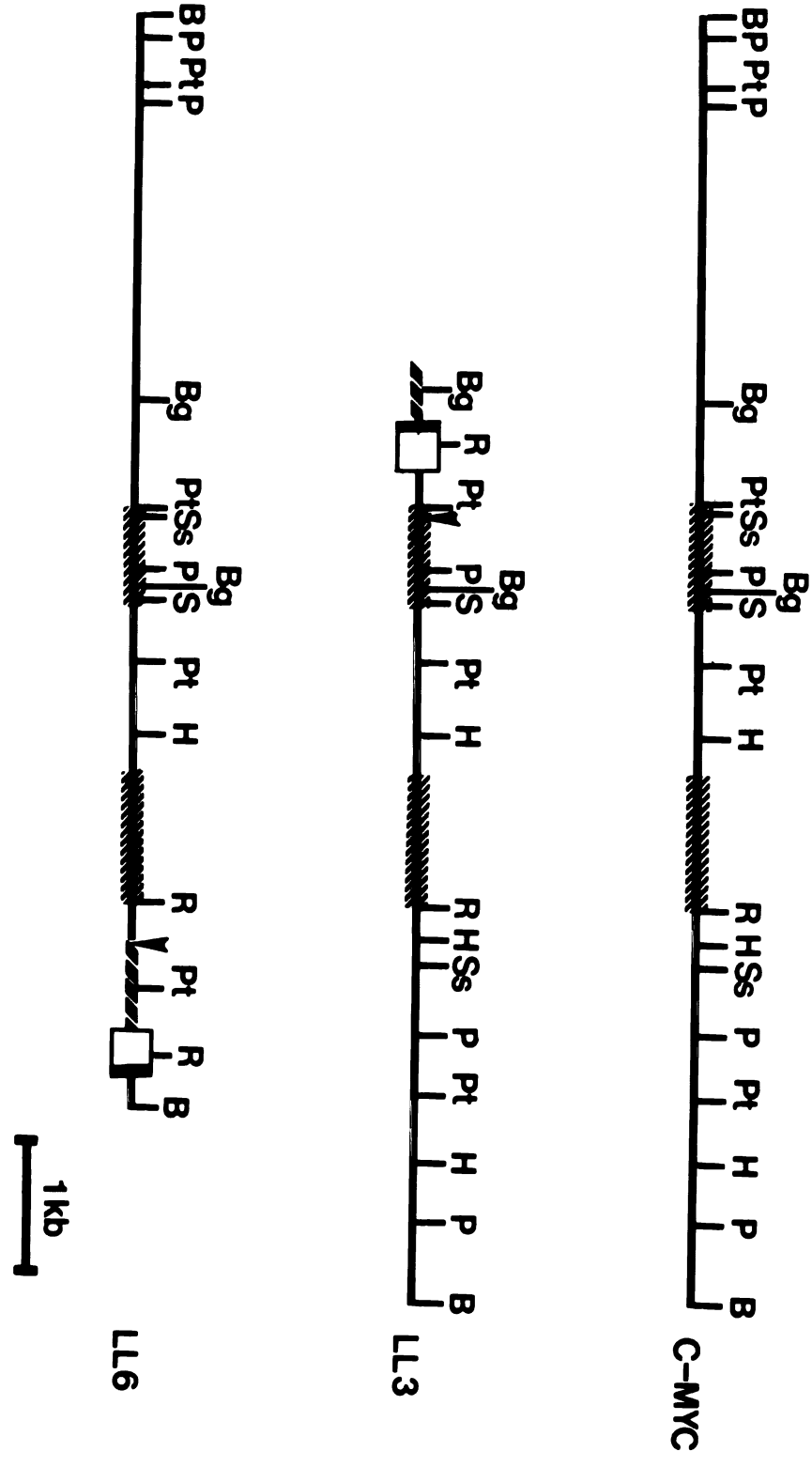
200 micrograms of LL6 DNA were digested with Bam Hl and fractionated by electrophoresis through 0.8% low-melting (Seaplaque) agarose. The gel was sliced into 4mm sections and the DNA isolated by phenol extraction as described in chapter 3. 5% of each DNA fraction was electrophoresed, transfered to nitrocellulose and annealled to myc probe (see chapter 3). 10% of the fraction containing the 9kb ALV-c-myc fragment was ligated to 2 micrograms of bacteriophage Charon 30, the recombinant molecules were then packaged in vitro (Hohn,1979) and used to infect E. coli DP 50. Recombinant phage carrying c-myc were detected (Benton and Davis,1977) using myc probe. The ALV-c-myc fragment was subcloned into pBR 322 and then subjected to digestion by a variety of restriction endonucleases and hybridized to myc probe, $cDNA_{3'}$, $cDNA_{5'}$, and $cDNA_{rep}$. This analysis was employed to generate the restriction endonuclease map presented in Fig.18. This map is compared to maps of DNA fragments subcloned into pBR322 from λLL3E (generation of λLL3E described in Chapter 3) and a recombinant bacteriophage carrying an uninterrupted c-myc locus. The plasmid containing LL3E DNA was constructed by isolating the 7.5kbp Bgl II restriction endonuclease fragment from LL3E extending from the 5' most Bgl II site in ALV to the Bgl II site 2kbp from the 3' end of the second identified c-myc exon and ligating this fragment to the Bam Hl site in pBR322. The c-myc locus from a tumor-free bird was isolated by B. Vennstom (see

chapter 3) and the 10kbp Bam Hl fragment subcloned into pBR322 was kindly provided by D. Sheiness.

The comparison of the three restriction maps presented in Fig.18 confirms the relative configurations of ALV proviruses and c-myc deduced from analyses of tumor DNA.

Legend to Figure 18.

Restriction endonuclease maps of DNA segments inserted into pBR322 to generate pCMC-B (c-myc), pLL3E (LL3) and pLL6 (LL6). B:Bam Hl; P:Pvu II; Pt:Pst I; Bg:Bgl I; Ss: Sst I (Sac I); H:Hind III; R:Eco Rl.


BPPtP
Bg
PtSs
P S
Pt
H
R
HSs
P
Pt
H
P
B
C-MYC
Bg
R
Pt
P S
Pt
H
R
HSs
P
Pt
H
P
B
LL3
BPPtP
Bg
PtSs
P S
Pt
H
R
Pt
R
B
Bg
LL6
1kb

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# APPENDIX 5

## Expression of cloned DNA fragments carrying ALV proviruses adjacent to c-myc in mouse L fibroblasts

In an attempt to reconstitute enhanced expression in tissue culture, I have introduced plasmids carrying the DNA fragments described in Appendix 4 into mouse LTK$^-$ fibroblasts by calcium phosphate coprecipitation (This technique is hereafter refered to as transfection) (Graham and Van der Eb,1973; Stowe and Wilkie,1976). Plasmids pLL6, pLL3E, pCMC-B contain the fragments shown in Fig.18 inserted into the pBR322 Bam Hl site. These plasmids and a recombinant bacteriophage containing an unaltered c-myc locus (see Chapter 3) were each mixed at a 5:1 mass ratio with 140 nanograms of a plasmid carrying the Herpes simplex virus thymidine kinase gene (HSV-TK) (kindly provided by P. Luciw). The mixtures were coprecipitated with calcium phosphate in the presence of ten micrograms of salmon sperm carrier DNA and the precipitate applied to approximately $3X10^{-6}$ LTK$^-$ fibroblasts on a 100mm dish (Wigler et al.,1979). TK$^+$ colonies were selected in HAT medium (see Wigler et al., 1979) and mass cultures obtained by propagating ten to forty TK$^+$ colonies from each experiment.

DNA and RNA were prepared from each culture as described in chapter 3. The relative amounts of incorporated chicken c-myc DNA in each culture was assessed by digesting DNA with a restriction endonuclease that cleaves the bacteriophage or plasmid DNA more than once. Eco Rl cleavage of pLL3E generates a 2.9kbp fragment encompassing the c-myc exons (see Fig.18). Sac I digestion of pCMC-B and $\lambda$CMC-2 produces in each case a 3.0kbp fragment which hybridizes to myc probe (see Fig.18). Fig.19A shows that digestion of DNA from each culture reveals the predicted fragments after annealing to myc probe. Comparison of the relative intensities of the bands suggests that cells receiving pLL3E (Fig.19A,lane 1) and pCMC-B (Fig.19A,lane 2) contain equivalent

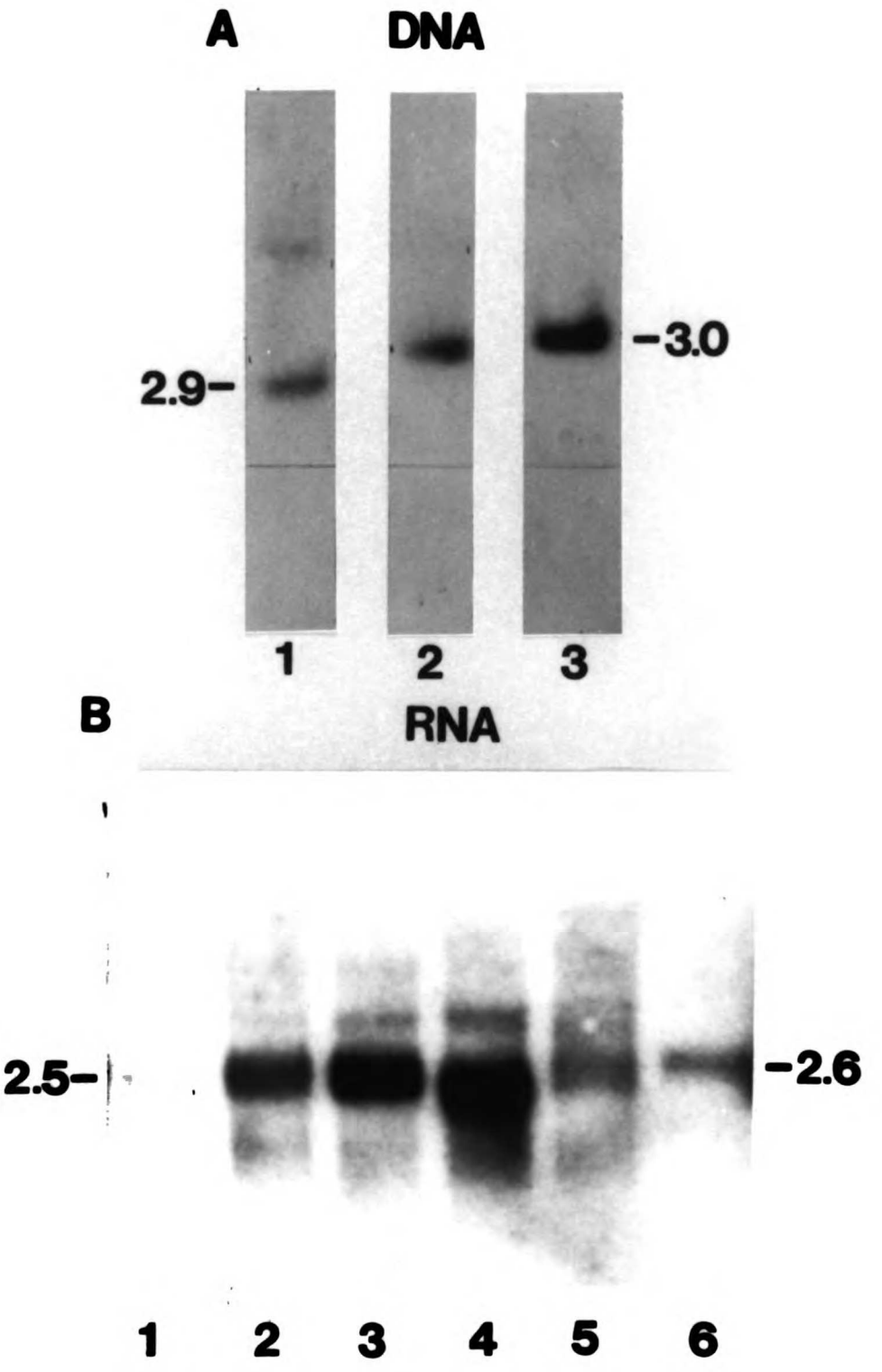
amounts of introduced DNA whereas cells receiving λCMC-2 DNA (Fig.19A,lane3) exhibit several fold more chicken c-myc DNA. We do not understand the basis for the this difference since the L cells were actually exposed to more plasmid molecules than phage molecules.

Expression of the exogenous DNA was determined by electrophoresing 5 micrograms of poly $A^+$ from each of the cultures through 1.2% formaldehyde-agarose, transfering the RNA to nitrocellulose and hybridizing the filter- bound RNA to myc probe. The results of this analysis are presented in Fig.19B. Transcripts of discreet size are apparent in cultures transfected with pLL3E, pCMC-2 and λCMC-2 (Fig.19B,lanes 2-4). Both pCMC-B and λCMC-2 produce 2.5kb transcripts which comigrate with the prominent 2.5kb c-myc RNA observed in RNA from chick embryo fibroblasts (compare Fig.19B,lanes 1-3). This result suggests that the signals required for generating the c-myc transcripts are present within the 10kbp Bam Hl fragment. Further definition of the c-myc transcriptional unit could be obtained by deleting regions of this fragment and monitoring transcription after introducing the DNA into L cells. The relative intensities of the bands representing c-myc transcripts from pCMC-B and λCMC-2 presumably reflect the relative amounts of templates in the cells. The transcript from pLL3E is slightly smaller than the authentic c-myc transcript (compare Fig.19B, lanes 1 and 4). Although we were unable to assess the transcriptional pattern in the original tumor LL3, RNA of similar size was observed in tumor LL7 which harbored an ALV provirus in a similar configuration with respect to c-myc. We presume but cannot prove that the transcript generated from pLL3E mimicks the transcriptional pattern in the original tumor. The amount of c-myc RNA produced from pLL3E is only

slightly elevated (3-5 fold) over the levels of c-myc RNA found in cells harboring pCMC-B although the number of templates in each culture is similar. Several explanations could account for this meagre enhancement: 1) the original tumor may have exhibited only a limited elevation of c-myc transcription; 2) the subcloned Bgl II fragment may lack viral and/or cellular sequences which would further augment the transcriptional activity of c-myc; 3) the enhancing ability of the ALV provirus may be diminished in the heterologous mouse fibroblasts. These possibilities may be distinguished by investigating the transcriptional activity of DNA cloned from tumors where the level and composition of c-myc transcripts is known, such as pLL6. However, analysis of RNA transcribed from pLL6 in L cells yielded equivocal results. pLL6 DNA apparently produces a population of heterogeneous transcripts concentrated around 2.5kb (Fig.19B,lane 5). In contrast, the transcript observed in the original tumor was 3.7kb (see Chapter 3). This discrepancy remains a mystery but may reflect an absence of either transcription initiation or termination signals in the cloned DNA fragment. D. Westaway (personal communication) has cloned the ALV provirus and adjacent c-myc from tumor LL4 and this DNA could prove useful in addressing the problems described above.

Legend to Figure 19.

(A) Analysis of HAT resistant mouse L fibroblasts transfected with pLL3E, PCMC-B or λCMC-2 (see text for details). (lane 1) 5 micrograms of DNA from cells transfected with pLL3E cleaved with EcoRl. (lane 2) 5 micrograms of DNA from cells receiving pCMC-B digested with Sac I. (lane 3) 5 micrograms of DNA from cells transfected with λCMC-2 digested with Sac I. The samples were run on the same gel, transfered to nitrocellulose and annealed to myc probe. The numbers represent the size in kbp of the myc-containing DNA fragments. (B) RNA from HAT resistant L cells was prepared and analyzed as described in chapter 3. Each lane contains 5 micrograms of poly $A^+$ RNA from (lane 1) uninfected chick embryo fibroblasts; or L cells transfected with (lane 2) pCMC-B; (lane 3) CMC-2; (lane 4) pLL3E; (lane 5) pLL6; (lane 6) salmon sperm carrier DNA alone.


A
DNA
2.9–
–3.0
1
2
3
B
RNA
2.5–
–2.6
1
2
3
4
5
6

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# CHAPTER 4

## Discussion

Detailed discussion of the experiments presented in chapters 2 and 3 and the appendices are included therein. This chapter will be devoted to a more general overview including: 1) a summation of the potential events occurring during lymphomagenesis and consideration of questions, dilemmas and experimental inconsistencies pervading the proposed sequence of molecular and cellular changes leading to the formation of a tumor; 2) a survey of neoplastic disease in other organisms that might involve enhanced expression of host genes; 3) a discussion of possible mechanisms by which ALV might enhance transcription of c-myc; 4) an speculation on the role of myc gene products in tumorigenesis.

I. Lymphomagenesis by ALV

A model of lymphomagenesis should consider a number of experimental observations in addition to molecular paradigms that govern our view of the retroviral life cycle.

An analysis of bursal DNA taken from birds soon after infection revealed that most cells were infected and integration occurred at many sites in the host genome (Fung et al.,1981,1982). The results of this study are in accordance with a large number of studies showing that retroviruses display little or no preference in their choice of integration sites (reviewed in Varmus,1982). It also is apparent that retroviral DNA integrates efficiently and specifically by joining sequences at the ends of LTRs to host DNA.

ALV DNA integration is followed by the appearance of clonal tumors after a lengthy latent period. The vast majority of these tumors harbor ALV proviruses adjacent to c-myc in association with levels of stable c-myc transcripts which are much higher than levels found in normal

bursa (chapter 3; Neel et al.,1981; Fung et al.,1981, Hayward et al.,1981). All of the extensively characterized proviruses in the vicinity of c-myc have incurred deletions which reduce or abolish transcription of the provirus. A large subset of the tumors which carry deleted proviruses fail to express viral RNA and consequently don't produce virus (chapters2,3; Neel et al.,1981; Hayward et al.,1981). However, there exist tumors that contain apparently normal viral transcripts and produce virus (chapter2; Neel et al.,1981). Finally, the little data which shed light on the stability of retrovirus proviruses in vertebrate cells suggests that deletions should occur at approximate frequencies of $10^{-5}$ to $10^{-6}$ per cell-generation (Varmus et al.,1981).

These observations suggest the following sequence of events during ALV-induced lymphomagenesis. ALV infects most of the bursal lymphocytes (probably $10^{6}$ to $10^{7}$ cells during the first few days after hatching). The viral DNA integrates at a large number of sites and inserts next to c-myc in only a few cells, activating c-myc and initiating transformation. These cells are now continuously dividing and at some point one or a few cells within this expanded semi-clonal or clonal population will experience deletions within the provirus. The new cell(s) acquires a growth advantage by virtue of the deletion and progresses to form the tumor. The growth stimulus provided by the initial transformation would be required to observe deletions at the expected frequencies. (If the dividing time of these cells is 24hrs then 20 days of growth will yield approximately $10^{6}$ cells.)

This working model raises a number of dilemmas and questions. The first question addresses the nature and number of cells targeted for transformation in the bursa. I have assumed that most, if not all,

bursal lymphocytes are potential targets. This arbitrary assumption (see chapter 1) applies to the following discourse, especially when a proposal requires a large number of infected cells (see the model described above). However, where proposals postulate non-random or high frequency events (i.e. site-specific integration), the lymphocytes targeted for transformation could represent a small subset of the bursal lymphocyte population.

My model proposes that proviruses with intact LTRs can activate c-myc. However, deletions encompassing LTRs are prevalent in the relevant proviruses. Under conditions selecting for loss of v-src expression Varmus et al. (1981) obtained deletions in a single RSV provirus in transformed rat fibroblasts at low frequencies (approximately $10^{-5}$ per cell-generation). Therefore, proviruses are probably not inherently unstable. This suggests that the deletions affecting the ALV proviruses confer a growth advantage on the nascent tumor cells. We have postulated (see chapter 2) that deletions which abolish viral gene expression reduce the probability that the tumor cells will be recognized and eliminated by the immune system. However, this cannot be the entire explanation since tumors expressing apparently normal viral RNA have been observed. Perhaps the use of the 3' LTR as a promoter requires the inactivity of the 5' LTR. The enhancing activity of proviruses in other configurations might also rely on the silence of 5' LTR promoter activity. This hypothesis is inconsistent with the model's suggestion that an intact provirus initiates c-myc activation and consequent transformation. The model would thus require modification to resolve this inconsistency. Perhaps integration in B-lymphocytes is imprecise with respect to viral sequences generating aberrant proviral

structures. This seems unlikely since many proviruses have properly aligned LTRs as judged by restriction endonuclease analysis. Sequence analysis of LTR-host DNA junctions will address the issue of integration precision.

Another possibility arises here: c-myc activation is not the initiating event in transformation. This concept gains support from my interpretation that the provirus in LL6 (see chapter 3) initially integrated far downstream from c-myc (at least 17kbp) and c-myc activation by the ALV provirus at such a distance seems improbable.

If transcriptional enhancement of c-myc is not an initiating event then it must be presumed that integration of ALV DNA near c-myc permits initiation by some other means and c-myc activation may participate in tumor maintenance. One approach towards determining the importance of activated c-myc in tumorigenesis consists of introducing cloned examples of each ALV-c-myc configuration into bursal cell suspensions and injection of these cells into syngeneic hosts. In addition, the level of c-myc in the 50-100 transformed follicles apparent at about four weeks post-infection could be assayed by in situ hybridization. These follicles may represent an early phase in tumorigenesis by acting as precursors to the solo tumor nodules which arise later (see chapter 1). The presence of high levels of c-myc RNA would argue for a role of activation early in the transformation process.

If c-myc activation is the initiating event then other assumptions must be challenged. First, perhaps ALV DNA does not randomly associate with host DNA but prefers the region surrounding c-myc. If integration of viral DNA occurs at c-myc in one out of twenty cells the analyses performed so far probably would not detect this degree of specificity.

Site-specific integration would provide a large population of cells in which deletion could occur to activate c-myc. Based on restriction endonuclease maps, the integration sites surrounding c-myc are not randomly placed but fall into distinct regional groups; the most common sites lie approximately 400-600 bp upstream from the 5' end of the first known exon (data not shown and S. Astrin and H. Robinson personal comm.). This phenomenon could either reflect integration specificity or a position requirement for efficient c-myc activation. Site-specific integration in bursal lymphocytes is testable by an experiment proposed by H. E. Varmus (which I disregarded). Bursal DNA prepared early after infection could be fragmentd, selected on filters containing c-myc DNA and subsequently tested for enrichment of ALV sequences.

Secondly, B-lymphocytes might display a high frequency of deletions. B-lymphocytes are known (in mammals) (reviewed in Leder et al. ) to undergo DNA rearrangements during maturation. If DNA deletion occurred at high frequencies then the probability of proviral alteration and subsequent c-myc activation might reach levels compatible with a model proposing random integration and requiring proviral deletion for c-myc activation. However, if rearrangement of the provirus next to c-myc is frequent it seems probable that this region must be normally targeted for rearrangement since high frequencies of random genome alterations would likely be deleterious to the cell. These possibilities could be approached by extending structural analyses in search of rearrangements in the region surrounding c-myc in tumor cells. It would also be relevant to assess possible linkage relationships between c-myc and the immunoglobulin loci (L. Chen in J.M. Bishop's lab is currently attempting to molecularly clone the chicken

immunoglobulin genes.). Regions flanking other known genes could also be examined for rearrangement. The activation of c-erb by ALV proviruses in erythroblastosis (see appendix 1) presents a possible dilemma here. The DNA regions in transformed erythroblasts harboring c-erb and an ALV provirus have incurred rearrangements in many instances (T. Fung personal comm.). Are erythroblasts also characterized by high frequencies of DNA rearrangements in the region of c-erb?

A third idea challenges the hypothesis that an intact provirus is incapable of c-myc activation.

Slight transcriptional augmentation of c-myc by an intact provirus could act as sufficient mitogenic stimulus to generate a cell population large enough for deletions to occur in proviruses at expected frequencies. These deletions would then produce efficient and continuous transcription of c-myc and generate the progressing tumor cell. Unfortunately, this model cannot be easily tested unless transcriptional enhancement can be recapitulated by introducing molecularly cloned substrate DNA into cultured cells (see below). For instance, the effects of various provirus structures on the transcription of an adjacent gene could be assayed using molecules constructed to contain an intact or deranged provirus adjacent to c-myc or the Herpes simplex virus thymidine kinase gene.

Taking into account the dilemmas expressed above, the activation of c-myc most likely plays a pivotal role in lymphomagenesis. (Other models are considered below). This view gained striking support when Noori-Daloii et al. (1981) discovered that bursal lymphomas induced by chicken syncytial virus (CSV), a non-defective leukosis virus unrelated to ALV, displayed enhanced transcription of c-myc in conjunction with

proviral integration adjacent to c-myc. Bursal lymphomas produced by MAV (see chapter 1) also harbor proviruses adjacent to c-myc (D. Westaway personal communication).

The participation of c-myc activation in tumorigenesis leaves several unresolved issues. The host factor(s) which determine the tropism of the virus towards cells targeted for transformation remains obscure (see appendix 2). The range of cell types infected by ALV is probably not an important determinant since ALV is capable of replicating in most tissues of the chicken (Purchase and Burmester,1978). Furthermore, there are precedents which establish that cells supporting the replication of avian retroviruses can be refractory to transformation: certain defective leukemia viruses replicate in hematopoietic cell lineages which do not appear in tumors produced by the viruses (Graf et al.,1980); RSV fails to transform macrophages although $pp60^{v-src}$ is expressed at high levels and displays apparently normal kinase activity (Durban and Boettiger,1981;A. Betkowski, pers. comm.). Also, ALV replicates in the bursa of line $6_3$ chickens but does not induce lymphomas in these birds (Fung et al.,1982). Therefore, the cellular context may be crucial in determining the efficacy of a particular onc gene product. Applying this view, ALV may integrate adjacent to a number of different c-oncs during infection but lymphomas would occur most frequently because B-lymphocytes are particularly sensitive to the oncogenic effects of c-myc. Erythroblastosis would arise less frequently because erythroblasts might be comparatively less sensitive to the effects of c-erb and impervious to the effects of c-myc. Evidence contradictory to this suggestion may be found in observations that MC-29 (which carries v-myc) can transform fibroblasts, macrophages ,

epithelial cells and perhaps lymphoid cells *in-vivo* (Graf and Beug,1978; W. Hayward, pers. comm.). However, Hayman and his colleagues (pers. comm.) have found that their strain of MC-29 does not produce lymphomas whereas an MC-29 deletion mutant which apparently recovered *c-myc* during propagation acquires a propensity for generating bursal lymphomas. If *v-myc* and *c-myc* exhibit distinguishable oncogenic properties then the contradiction evaporates.

Cell-specific properties influencing the frequency and specificity of viral DNA integration into regions surrounding *c-oncs* could determine target cell tropism. For example, the chromatin configuration of the *c-myc* locus in B-lymphocytes might be particularly condusive to viral DNA insertion or subsequent transcriptional activation. Chromatin structures in other cell types would prevent integration or transcriptional enhancement. Analysis of the chromatin conformation of *c-myc* in different cell types and at different times in development would address this possibility. In particular, determining the structure of *c-myc* in line $6_3$ lymphocytes might be informative.

An issue which confronts all models for viral oncogenesis has become important in leukosis research: does a *v-onc* (or *c-onc* in leukosis) transform cells as a result of over-expression or is oncogenic activity acquired as a result of mutation. Over-expression of *c-mos* and *c-ras* is sufficient for transformation of NIH 3T3 fibroblasts (Oskarsson *et al.*,1980; Blair *et al.*, 1981; DeFeo *et al.*,1981; Chang *et al.*,1982·). These results have not yet been extended to other *c-oncs* in spite of intensive efforts and the results of Hayman *et al.*, described above, point to oncogenic differences between *v-myc* and its cellular homologue. In the case of leukosis, 20-200 fold transcrip-

tional enhancement is found every time an ALV provirus is located in the vicintiy of c-myc but in at least some cases there are hints that the over-expressed protein may be abnormal. The first known exon of c-myc adjacent to the ALV provirus in tumor LL3 is missing a Sac I site. This Sac I site is present in both homologues in control tissue from the same bird and is absent both in situ and after molecular cloning. We do not know the extent or functional significance of this alteration (although if it is a deletion it is less than 100bp). Additionally, integration of ALV DNA may occur in c-myc coding regions. The structure of c-myc and uncertainties concerning terminal coding domains have been discussed in chapter 3. Two lines of evidence suggest the existence of further exons upstream from those already identified. Hybridization studies of cellular RNA has revealed minor c-myc species which are larger than the predominant species and are preferentially located in nuclear fractions ( D. Shieness unpublished). These nuclear molecules may represent precursors to the mature c-myc mRNA. Determination of the nucleotide sequences of proviral-host junctions in lymphoma DNA led Neel. et al. (pers. comm.) to propose that ALV DNA had inserted in c-myc exons. Taken together these results indicate that the N-terminal domain of c-myc protein may be altered in lymphomas. This possibility awaits the identification and characterization of the c-myc gene product.

The preceding discussion illuminates the hypothetical character of leukemogenic mechanisms invoking activation of adjacent host genes by proviral DNA. Two different experimental approaches have provided evidence which may link lymphomagenesis to mechanisms which are distinct from c-myc activation by proviral DNA.

Cooper and Neiman (1980,1981) introduced bursal lymphoma DNA into mouse NIH-3T3 fibroblasts by calcium phosphate coprecipitation and subsequently assayed for transformation by counting either foci or soft-agar colonies. Lymphoma DNA evoked the transformed phenotype in the mouse fibroblasts at frequencies far greater than normal bursa DNA. The transformed phenotype and presumably the responsible gene could be transferred by preparing DNA from the transformed mouse cells and repeating the experiment. Similar results were obtained with tumor nodule DNA. Using hybridization probes they discovered that neither ALV proviral DNA or c-myc from the chicken DNA were present in the transformed mouse cells even though an ALV provirus was situated near c-myc in the bursal tumor. A DNA segment responsible for transformation has been molecularly cloned using a library of recombinant bacteriophage carrying inserted DNA from the progeny of an NIH-3T3 cell transformed by lymphoma DNA (G. Cooper, pers. comm.). The bacteriophage capable of transformation contained a fragment of DNA with sequences repeated many times in both the chicken and mouse genomes. By paring away regions of the inserted fragment, a sequence has been identified that is found as a unique sequence in the chicken genome although present in many copies in the mouse genome. The nucleotide sequence of this region displays an open reading-frame capable of encoding a small peptide. RNA homologous to the unique copy chicken DNA is found at similar levels in normal bursa and a cell line established from an ALV-induced bursal lymphoma. This perplexing set of experiments leaves the origin of the DNA fragment and its role in lymphomagenesis unclear. Although discovered using a functional assay, DNA molecules capable of transforming mouse fibroblasts may be meaning-

less in B-lymphocyte transformation. On the other hand, the histological pathology (see chapter 1) and an experiment determining the frequency of death as a function of time after infection suggest that lymphomagenesis by ALV is a multi-step process (Neiman et al.,1980). Cooper and Neiman have postulated that their assay might detect alterations in the chicken genome which are secondary (but necessary) steps in tumor formation (Cooper and Neiman,1981).

McGrath and Weissman (1978a) have proposed a theory of murine leukemia virus oncogenesis based on their characterization of the virus-binding properties of leukemic cells. They found that leukemic cells (from thymomas) bind the particular strain of virus used to produce the tumors with higher affinity than other MLVs. Also, in a population of cells from a single thymoma, the cells which display the highest affinity for the inducing virus are the most tumorigenic when injected into syngeneic hosts. (Although the authors failed to demonstrate the donor origin of the tumors) (McGrath and Weissman,1978a,b,1979). A model was proposed stating that tumorigenesis was a consequence of continuous autologous mitogenic stimulation of T-lymphocyte clones which carried receptors specific for the inducing virus. Since the T-lymphocyte antigen receptor was unidentified they began an investigation of an in vivo passaged murine B-lymphocyte tumor line, BCL-1. A number of provacative findings emerged from their research (M. McGrath, pers. comm.). A retrovirus was produced by the tumor but splenic stromal cells, not leukemic cells were the major virus-producing cells. The BCL-1 tumor cells bound this virus more efficiently than other MuLVs, although the virus failed to induce tumors after exogenous infection of mice. Monoclonal IgM

obtained after fusing BCL-1 tumor cells with myeloma cells bound virus. The hybridoma cells, grown as ascites tumors, produced IgM which copurified with retroviral (presumably BCL-1 virus) envelope glycoprotein further suggesting a specific interaction. Anti-idiotypic antibody raised against BCL-1 IgM blocked binding of virus to BCL-1 cells. These results have been cited as further support for the model which, when extended to B-cell tumors suggests that a resting immunocompetent B-lymphocyte recognizes the infecting virus through an interaction of viral antigen and cell-surface antibody. Clonal expansion proceeds and the continuos mitogenic stimulus provided by the virus-producing stromal cells initiates transformation. McGrath (personal comm.) is presently attempting to extend these observations to ALV-induced lymphomas, although the presence of non-immunoglobulin receptors for the viral envelope glycoprotein may complicate the analyses. It is already clear, though, that some ALV-induced tumors are comprised of cells that are incapable of producing viral antigens and virus cannot be recovered from some tumor explants (most likely these explants contained antigen-presenting cells). This contradictory evidence can be rationalized by proposing that antigen stimulation is the initiating event of tumor formation but is not required (and may be detrimental to) tumor progression. The location of ALV proviruses near c-myc presents another dilemma for this theory. Very few resting B-lymphocytes specific for a given antigen are present before antigen stimulation, once again raising questions about random integration and the common presence of proviral deletions in tumors. At present, since all B-lymphocytes probably express receptors for ALV, viral mitogenesis (either general or idiotype specific) must still be considered poten-

tially important during tumor initiation.

II. Tumorigenesis in other systems

Three genetically distinct viruses-ALV, MAV and CSV- apparently employ c-myc activation to induce bursal lymphomas (see above). Additionally, ALV proviruses can be found adjacent to c-erb in some ALV-induced erythroblastosis. Thus, mounting evidence indicates that host gene activation may play a general role in avian neoplasms produced by these non-defective retroviruses.

How ubiquitous is the phenomenon of host gene activation, in particular c-oncs, in neoplasms affecting other species? Retroviruses analogous to ALV, which do not carry v-onc genes and induce tumors only after a long latent period following infection can be found in most mammalian species. Extensive molecular investigations have focused on mouse mammary tumor virus (MMTV), bovine leukemia virus (BoLV) and murine leukemia viruses (MuLV).

MMTV produces mammary carcinomas which harbor exogenous proviruses and apparently are clonal (Cohen et al.,1979). Restriction endonuclease analyses of the sort described in chapter 2 failed to reveal common proviral integration sites in different tumors (Cohen et al.,1979). The presence of many exogenous proviruses in most tumors and the possibility of structural rearrangements precluded conclusive results. Recently Roel Nusse and Harold Varmus commenced a rigorous approach to the question of common integration events by screening a large number of MMTV-induced carcinomas for a tumor harboring a single exogenous provirus. Although tedious, this strategy proved fruitful. When a single provirus-containing tumor was found, provirus-host junction fragments were molecularly cloned and the flanking host DNA used

as a hybridization reagent to test for the presence of MMTV proviruses in this region of the mouse genome in a battery of tumors. In addition, this probe was used to isolate molecular clones representing regions of the mouse genome adjacent to the initial isolate. Using these clones, Nusse screened tumors for the presence of proviruses in a 30kb region encompassing the location of the provirus in the original tumor. He discovered that 18 of 28 tumors do indeed contain MMTV proviruses within this genome segment. These insertions have occurred at numerous positions spanning at least 20kbp. This finding might reflect the ability of retrovirus proviruses to effect expression of distal flanking sequences. Although no consistent observation has been made of virus-specific transcripts other than genome-length and env mRNA, Nusse is searching for enhanced levels of transcripts lacking viral sequences. Further results reported by Lane et al. (1981) provide insight into the extent of similarity between ALV and MMTV tumorigenesis. They have found that MMTV-induced mammary carcinoma DNA transforms NIH-3T3 cells. In analogy to the ALV systemn the sequences responsible for transformation are unlinked to MMTV proviral DNA.

The B-lymphocyte tumors induced by BoLV resemble ALV produced lymphomas. BoLV causes persistent lymphocytosis which is a non-clonal proliferation of lymphocytes postulated to be a pre-neoplastic stage of leukemia (Kettmann et al.,1980). The induced lymphomas are clonal, often containing only one provirus and in approximately 25% of the cases examined the proviruses suffered deletions involving their 5' sequences (Kettmann et al.,1980,1982). Viral gene expression is not required for maintenance of the tumor state but no other unusual virus-specific transcripts are found in the tumors. Cloning of host

DNA abutting the single proviruses in two tumors and hybridization of these sequences to DNA and RNA from a number of tumors failed to reveal common integration domains or expression of 3' flanking sequences (Kettmann et al.,1982). Analyses extended to more distal flanking regions will be required to unequivically rule out adjacent gene activation as a mechanism of leukemia induction by BoLV.

In spite of intense scrutiny, thymomas produced by MuLVs do not display molecular features reminiscent of ALV-induced tumors. Restriction endonuclease experiments have not identified common integration sites (Steffen and Weinberg,1978) and unusual virus-specific or c-onc specific transcripts have not been observed. On the contrary, recombination events in the env gene precede tumor appearance and have been postulated to play a key role in tumorigenesis (Hartley et al.,1977). At present there is no convincing proof of this postulate and experiments of the type carried out by Nusse have not been reported.

Recently, a preliminary characterization of feline leukemia virus (FeLV) proviruses present in cat lymphosarcomas has been carried out by Casey et al. (1981). Each lymphosarcoma expressing FeLV antigens was comprised of cells harboring exogenous FeLV U3 sequences at common sites suggesting that the tumors had clonal origins. Rudimentary restriction endonuclease analyses did not reveal common integration sites in different tumors. Lymphosarcomas lacking viral antigens were free of exogenous FeLV U3 sequences in spite of an epidemiological association between FeLV-negative lymphosarcomas and exposure to FeLV. Data are too scant to draw further comparisons between this system and the viral- induced tumors in other species described above.

The involvement of gene activation in tumors of non-viral etiology

has been even less experimentally accessible. Aaronson, Gallo and their colleagues (Eva et al.,1982; Westin et al.,1982) have recently surveyed a large number of human solid tumors and leukemias for the expression of c-oncs. There is suggestive evidence that transcripts of c-sis (the cellular counterpart of the simian sarcoma virus putative transforming gene) are present in elevated amounts in some sarcoma and glioblastoma cell lines and elevated amounts of c-myc RNA are found in some sarcoma and carcinoma cell lines and one promyelocytic leukemia line. These results are not compelling since particular c-onc activation is not a universal feature of a given tumor type and appropriate control tissues were not always analyzed.

Weinberg, Cooper, Wigler, Barbacid and their colleagues have searched for genes involved in tumor formation by introducing DNA from tumors or cell lines into NIH-3T3 mouse fibroblasts and assaying for focus formation or soft-agar colonies (Shih et al.,1979; Cooper and Neiman,1980; Shih et al.,1981; Murray et al.,1981; Lane et al.,1981; Cooper et al.,1981; Krontiris and Cooper,1981; Perucho et al.,1981; Lane et al.,1982; Pulciani et al.,1982; Goldfarb et al.,1982; Parada et al., 1982; Der et al.,1982). DNA from some, but not all, cell lines can induce transformation in these assays. The DNA sequences responsible for this effect are different among different types of tumor cells with at least one exception. By using human repetitive DNA as a hybridization probe for human sequences , Perucho et al. (1981) have shown that the same human sequences are retained from a colon carcinoma line and a lung carcinoma line when DNA from primary foci of NIH-3T3 cells is used to produce secondary foci. The DNA from a human bladder carcinoma cell line which is responsible for transformation has been

cloned (Pulciani et al.,1982; Goldfarb et al,1982). Remarkably, this gene appears to be the human homologue (c-ras) of the transforming gene of the rat Harvey sarcoma virus (Parada et al.,1982; Der et al.,1982). The gene is not transcribed in normal NIH-3T3 cells and is transcribed in the bladder tumor cells and NIH-3T3 transformants at higher levels than found in Hela cells (Goldfarb et al.,1982). Since DNA from normal cells works inefficiently or not at all in this assay, these results suggest a stable (although unidentified) alteration of the DNA in the tumor cells confers the transforming ability.

Gene activation and its hypothetical participation in the formation of non-viral tumors could result from chromosome rearrangements regularly observed in tumor cells. Karyotype analysis and chromosome banding techniques have been used to identify two types of chromosome alterations correlated with malignancy: homogeneous staining regions (HSRs) and small apparently acentric chromosome fragments termed double minutes (DMs) (Levan et al.,1977; Barker and Hsu,1979; Kovacs, 1979; Miller et al.,1979). A number of reports suggest that these abnormalities reflect DNA amplification. The selection of methotrexate resistant cells often results in the amplification of the dihydrofolate reductase gene (dhfr) and the amplified DNA can be localized to HSRs (Nunberg et al.,1978; Dolnick et al.,1979). In addition, the appearance of DMs is also associated with dhfr gene amplification (Kaufman et al.,1979). Recently DMs found in a mouse pituitary cell line have been fractionated and DNA from this fraction molecularly cloned (George and Powers,1981). DNA represented in some of the clones is amplified in the DM containing cell line compared to a DM-free control cell line. The cloned DNA is also localized in HSR's found in a related subline of

the pituitary cell line (George and Powers,1982).

The amplification events accompanying the appearance of DMs and HSRs could enhance the expression of an oncogene producing a neoplastic phenotype in a fashion analogous to the generation of methotrexate resistant cells by dhfr gene amplification. Varshavsky has proposed a theory of tumorigenesis involving gene amplification produced by misfirings of replicons encompassing particular genes (Varshavsky,1981a). This abnormal replicon activity was postulated to be inducible by external factors, such as tumor promoters. Tumor promoters are defined by their abilities to potentiate the activity of carcinogens in an assay involving application of chemicals to mouse skin (Berenblum,1975). In support of this notion, he has recently shown that tumor promoters increase the frequency of methotrexate-resistant colonies and associated dhfr gene amplification after a single selection step (Varshavsky,1981b).

Non-random chromosome translocations are apparent in many human hematopoietic malignancies (reviewed by Rowley,1980). The cells from 85% of patients with chronic myelogenous leukemia carry a reciprocal translocation between chromosomes 9 and 22. Many other leukemias display lower incidences of different non-random translocations. Of particular interest to this discussion is the finding of specific translocations in B-lymphocytes from patients with Burkitt's lymphoma or Epstein-Barr virus negative acute lymphocytic leukemia. These translocations involve the transfer of part of chromosome 8 to chromosome 14,2 or 22 (Rowley,1980). The genes for the immunoglobulin heavy chains and kappa and lambda light chains have been previously mapped to chromosomes 14,2 and 22 respectively (Croce et al.,1979; Erickson et

al.,1981; Malcolm et al.,in press). Also, translocations of a segment of chromosome 15 to the chromosomes carrving the genes for the immunoglobulin loci have also been observed in mouse plasmacytoma cells (Klein,1981). These findings have raised the possibility that genes located on chromosome 14 in humans and 15 in mice are activated by placing them under the influence of proficient immunoglobulin gene promoters functioning in the lymphocytes. Consistent with this theory is the localization of the human heavy chain immunoglobulin genes by in situ hybridization to the chromosome 14 band defining the break point in the 8:14 translocation (Kirsch et al.,1982).

Thus there exists provacative but inconclusive data suggesting a role for gene activation in the formation of a variety of different neoplasms. Future molecular analyses, especially addressing the sequences present in DMs and at break points in translocations, should clarify this hypothesis.

III. ENHANCEMENT

Chapter 3 describes experiments delineating the relative configurations of c-myc and ALV proviruses found in association with high levels of stable c-myc transcripts. Since ALV proviruses appear to enhance transcription by inserting 5' to c-myc in either transcriptional orientation or downstream in the same transcriptional orientation as c-myc we proposed that the provirus, in particular the LTR, could act as a transcription modulator (enhancer) relatively independent of its position or orientatin with respect to the modified gene. (Other possible interpretations are outlined in chapter 3). In addition to endowing ALV with a heretofore unknown mutagenic activity, the phenomenon of enhanced transcription may reflect a functional element

common to some eukaryotic promoters.

As eukaryotic genes are cloned, sequenced and used for transcription studies, the elements of eukaryotic promoters are being elucidated. Goldberg and Hogness (Goldberg,1979) noted a conserved nucleotide sequence located approximately 30-35bp 5' to the mRNA cap site in several genes (*). This "TATA" box was postulated to function in transcription initiation by analogy to a similar sequence found by Pribnow (1975) in front of prokaryotic genes. Deletion studies have defined a qualitative role for the "TATA" box in fixing the transcriptional initiation site in vivo (reviewed in Breathnach and Chambon,1981). Another less commonly conserved sequence located approximately 70-80bp 5' to the mRNA cap site has been implicated in quantitatively regulating transcription in at least three genes (Dierks et al., 1981; McKnight et al.,1981; Mellon et al.,1981). The RSV LTR (essentially identical by hybridization analysis to the ALV LTR) contains both sets of conserved sequences but their functional significance has not been tested. Another cis-regulatory element (not expected by analogy with prokaryotic promoters) is present upstream from several eukaryotic genes. Deletion of sequences between 139 and 242bp 5' to the start site of the mRNA for iso-1-cytochrome c (CYC1) in yeast reduces the transcription of CYC1 15 fold ( Faye et al.,1981; Guarente and Ptashne,1981). Deletion of sequences located 115 to 155bp upstream from the his3 gene in yeast has a similar effect (Struhl,1981). The mouse metallotheinein gene also contains sequences several hundred base-pairs upstream from the mRNA start site which are necessary for efficient transcription after injection into mouse oocytes (Brinster et al.,1982). The region starting 184bp upstream from the sea urchin H2A

gene is required for transcription of this gene after injection into X. laevis oocytes (Grosschedl and Birnstiel,1980). A 72bp repeat found 115bp upstream from the start site of early transcription in SV-40 and an analogous sequence found approximately 300bp upstream from the early transcript start site in polyoma play similar modulating roles (Benoist and Chambon,1981; Gruss et al.,1981; Tyndall et al.,1981). Both of these viral regulatory elements also fall within regions which, when deleted, prohibit viral replication. These elements augment transcription when juxtaposed to promoters from other eukaryotic genes (Moreau et al.,1981; Banerji et al., 1981; deVilliers and Schaffner,1981). The viral and H2A modulating sequences function irrespective of their relative orientation to adjacent promoters (Grosschedl and Birnstiel,1980; Moreau et al.,1981; Banerji et al.,1981; deVilliers et al.,1981). The viral sequences will act from the 3' end of a gene and function over large distances; the SV-40 72bp repeat can enhance transcription at a distance of approximately 4kbp from the SV-40 early promoter, the chicken conalbumin gene promoter and the major late promoter of Adenovirus type 2 (Moreau et al.,1981). The functional attributes of the papova virus enhancer sequences parallel the effects of proviral insertions in LL tumors. Whereas the experiments with the papova virus sequences were all carried out by artificially adjusting the position of the enhancers in vitro and then introducing the engineered molecules into mammalian cells, the ALV proviral insertions represent the only natural in vivo example of position independent transcriptional modulation in vertebrates.

In view of the structural similarities between retroviruses and transposable elements it is germane to note that in yeast, insertions

of the transposable element Ty-1 are responsible for increased expression of iso-2-cytochrome c (CYC7) (Errede et al.,1980) and the alcohol dehydrogenase II isozyme gene (ADHII) (Williamson et al.,1981). Surprisingly, all the insertions that have been structurally characterized are upstream from the gene but in the opposite transcriptional orientation (J. Elder, pers. comm.). It is not completely clear that these mutations result from an enhancing property of Ty-1. For instance, insertions upstream from the ADHII gene produce constitutive expression of the normally glucose-repressible gene and therefore may simply destroy a repressor binding site (Williamson et al.,1981).

A number of models have been proposed to account for the activity of enhancing sequences. Moreau et al. (1981) favor the interpretation that enhancing sequences act as bidirectional chromatin entry sites for RNA polymerase II. This view is difficult to reconcile with elements functioning from the 3' end of a gene, although their experiments employed circular molecules and thus circumvented this objection. Banerji et al. (1981) list a number of possible mechanisms: 1) Enhancing sequences might favor association of adjacent regions of chromatin and the nuclear matrix. The nuclear matrix, a proteinaceous network which remains after detergent and high salt extraction of nuclei (Berezney and Coffey,1974), may act as a scaffold for transcription and DNA replication machinery (Buckler-White et al.,1980; Pardoll et al.,1980, Nelkin et al.,1980; Jackson et al.,1981; Robinson et al.,1982); 2) Transcriptional augmentation may result from a conformational change in chromatin flanking enhancer elements, perhaps by an adjustment of nucleosome positions. The 72bp repeats in SV-40 normally reside in a region of the viral genome devoid of nucleosomes (Varshavsky et

al.,1979; Saragosti et al.,1980; Jakobovits et al.,1980), however relocation of the repeat does not create new nucleosome-free areas (Moreau et al.,1981). Obviously a nucleosome positioning effect could be more subtle than the creation of nucleosome-free DNA; 3) Enzymes which alter the superhelical density of DNA have been implicated in prokaryotic gene regulation (Smith,1981). Enhancer regions may function as recognition sites for similar enzymes or other unidentified RNA polymerase II auxilliary protiens in eukaryotes. Ty-1 insertions upstream from CYC7 represent examples of this mechanism for transcriptional modulation. The Ty-1 element adjacent to CYC7 places the expression of the gene under the control of factors which respond to the mating type of the yeast strain (Errede et al.,1980). This type of mutation has been termed ROAM and the augmented transcription of the mutant gene and resident Ty-1 elements occur only in strains capable of mating.

The models presented above suffer from the lack of available knowledge describing transcription in eukaryotic cells. Further analysis of enhancement by ALV proviruses would be facilitated if the phenomenon could be recapitulated by introducing cloned molecules into tissue culture cells. We have not yet achieved more than a five-fold increase of c-myc transcription when a molecular clone of the provirus and c-myc from LL3 was compared to a clone of c-myc alone by an assay involving cotransfection with HSV-TK into mouse $LTK^-$ cells and selection for acquisition of the HSV-TK DNA (see Appendix 5). Experiments which attempt to introduce DNA into cultured cells are limited by the available cell types and their propensity to take up DNA; ideally we would like to study transcription in B-lymphocytes. Cultured lymphocytes from ALV-induced tumors are an attractive system for these exper-

iments but analyses are complicated by the pre-existing high levels of c-myc transcription. Transcription of cloned DNA in extracts from these cells has not been attempted. In light of these complications, it may be important that the ASV LTR joined to HSV-TK which retains its natural promoter has been shown to increase the frequency of stable $TK^+$ transformants compared to HSV-TK alone after micro- injection into $LTK^-$ cells (P. Luciw personal comm.). Recent experiments argue against a role for the LTR in stabilizing the HSV-TK gene in the mouse cells or augmenting the frequency that DNA is taken up. However, transient expression of HSV-TK is identical in the absence or presence of the LTR when coprecipitated with calcium phosphate onto the $LTK^-$ cells. It is thus unclear in the case of the LTR whether enhancement of stable transformation reflects enhanced transcription. If these two phenomena prove to result from the same LTR function then this system would probably be more accessible to experiments designed to address mechanisms. The papova virus enhancer elements have been shown to augment both phenotypic transformation and transient transcription (Moreau et al.,1981; Banerji et al.,1981; Capecchi,1980).

IV. EPILOGUE: a speculation

Transcriptional activation of c-myc by an adjacent ALV provirus and the transforming activity in NIH-3T3 cells of another DNA sequence from bursal lymphomas present major unreconciled observations. The following discussion will present a model which attempts to incorporate both results.

A recent report localized the v-myc fusion protein, $P110^{gag\text{-}myc}$, to the nuclei of cells transformed by MC-29 (Donner et al.,1982). These investigators also present evidence that p110 binds DNA. These

observations suggest the possibility that c-myc and its viral homologue might encode a bifunctional protein with activities similar (but not identical) to the recA protein found in bacteria. The recA gene is a component of an error-prone inducible DNA repair system in E. coli (reviewed by Radding,1981; Little and Mount,1982). Genetic analyses and biochemical studies on purified recA protein indicate that the protein has two activities: 1) it functions as a protease which cleaves the repressor molecule (lexA protein) apparently responsible for repressing recA and a number of other genes inducible by DNA damage. The activation of the protease has been shown to require products of DNA damage; 2) the recA protein also functions to promote homologous recombination. My model for tumorigenesis invoking recA-like activities for c-myc proposes that over-expression of c-myc (or expression of v-myc) leads to two events. First, over-expression of c-myc protein exhibiting anti-repressor activity would derepress a number of genes. The induced gene products could function to stimulate cell division and, in conjunction with c-myc protein, promote recombination and/or mutator activity. The DNA alterations which result from the postulated activities would genetically fix the cell into continuous division thereby producing the neoplastic phenotype. The induced incursions on the DNA structure allow for continued evolution of the tumor cells during tumor progression.

This model can explain the absence of activated c-myc transfer in the NIH-3T3 transfection assays. If an activated c-myc gene were transfered then both the induction of DNA "repair" or recombination enzymes followed by alteration of specific DNA sequences would be required for appearance of a focus. These events would generate foci

at lower frequencies than transfer of an already competent transforming sequence previously generated in the primary tumor. It might be expected from this arguement that morphological transformation of mammalian cells by v-myc DNA would be less efficient than transformation by v-src DNA since expression of pp60$^{v\text{-}src}$ is sufficient for the initiation and maintenance of the transformed phenotype. This expectation has been verified in experiments comparing the transforming potential of cloned MC-29 and RSV DNA (P. Luciw personal communication) and comparing DNA from MC-29 and RSV transformed chick embryo fibroblasts (Copeland and Cooper,1980). However one published report claims a high efficiency transformation of NIH-3T3 cells using cloned MC-29 viral DNA (Lautenberger et al.,1981). This discrepancy remains unresolved. Also, MC-29 and RSV infection of chick embryo fibroblasts produce transformation with similar frequencies, an observation at variance with mv proposal.

Complete identity with recA need not exist. In fact, induction of the wild-type c-myc locus in MC-29 transformed cells and in ALV transformed lymphocytes probably does not occur, whereas recA protein is known to induce the recA gene.

A number of further predictions can be made. The model proposes that v-myc expression is necessary for initiation but not maintenace of the transformed phenotype. This idea can be tested by isolating an MC-29 virus which is temperature-sensitive (ts) for transformation. If the prediction is valid then the mutant should be defective for transformation when infection is carried out at the non-permissive temperature but not when infection is performed at the permissive temperature and then the temperature is raised. Unfortunately, since mainte-

nance of the transformed phenotype is predicted to be independent of v-myc expression, standard procedures would not suffice to isolate a ts mutant. Isolation of a ts transformation mutant, although tedious, could be attempted by mutagenizing an MC-29 stock and adding wild-type helper (to avoid picking up replication mutants). Supernatants from isolated foci produced by infection with this stock at the permissive temperature could then be tested for ts transformation properties by infecting cells at either the permissive or non-permissive temperature. This procedure would prove unnecessary if MC-29 mutants were ts for both initiation and maintenance of transformation. Existence of such mutants would refute the model. Tumors from MC-29 infected chickens are expected to be clonal because the genetic events necessary to fix the cell into a neoplastic state would most likely occur at relatively low frequencies.

This model also introduces enzymatic activities (such as protease activity) that can be assayed once either v-myc or c-myc proteins are identified and purified. Incentives are also defined for isolating and characterizing genes induced during tumorigenesis by MC-29 and ALV. Tissue-culture cells transformed by MC-29 present an attractive system for this type of endeavor and are perhaps preferable to RSV-transformed cells since the genes induced by v-myc are potentially necessary for some step in transformation whereas $pp60^{v\text{-}src}$ is sufficient for both initiation and maintenance of transformation.

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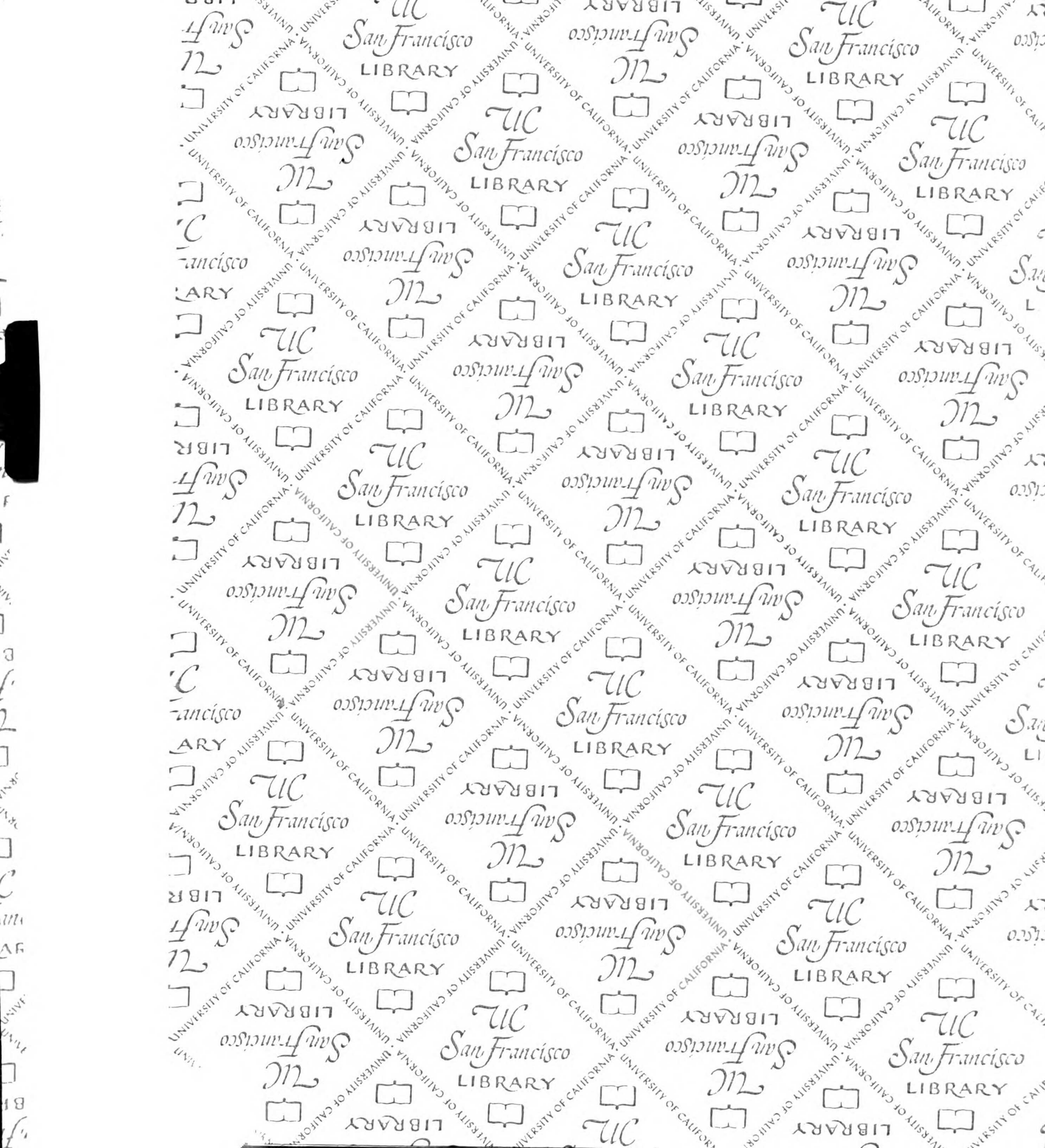
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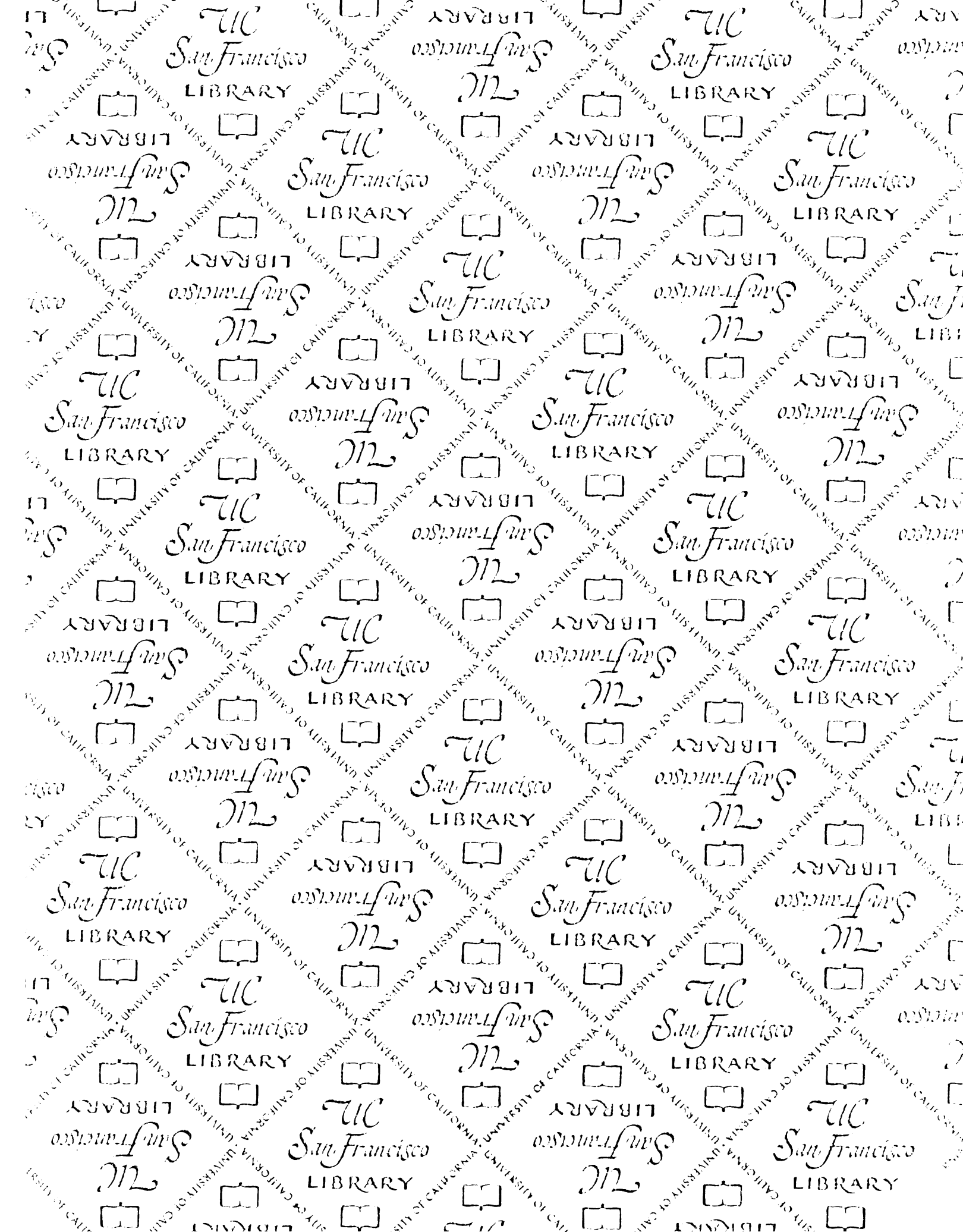
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