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C-ERB B ACTIVATION AND
AVIAN LEUKOSIS VIRUS INDUCED ERYTHROBLASTOSIS

by

Maribeth Anne Raines

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ABSTRACT

C-ERB B ACTIVATION AND AVIAN LEUKOSIS VIRUS INDUCED ERYTHROBLASTOSIS

by

Maribeth Anne Raines

Both qualitative and quantitative alterations in cellular gene expression have been associated with oncogenesis. Molecular characterization of avian leukosis virus (ALV) induced erythroblastosis indicates that there is an absolute correlation between alteration of the proto-oncogene c-erb B and erythroblastosis induction. The c-erb B gene is closely related to the human epidermal growth factor receptor (hEGF-R). ALV can activate the oncogenic potential of c-erb B by two distinct mechanisms: insertional activation (IA c-erb B) and transduction of c-erb B. In the former, the majority of integrated proviruses are intact and are situated in the same transcriptional direction. C-erb B transcription initiates in the 5' LTR of the provirus and continues into erb B to generate a truncated c-erb B RNA. Alternatively, a portion of the c-erb B gene can be transduced into ALV to become new viruses capable of inducing rapid erythroblastosis. These viruses encode c-erb B products identical to the IA c-erb B products; both products lack sequences corresponding to the extracellular ligand-binding domain of hEGF-R. It is the elevated and perhaps inappropriate expression of a truncated growth factor receptor which causes the uncontrolled proliferation and differentiation of erythroblasts. The identification of two transduced c-erb B virus mutants suggest that the c-terminal portion of the c-erb B protein is also important in determining the oncogenic potential of c-erb B.

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INTRODUCTION AND LITERATURE REVIEW

A. Hematopoiesis

Leukemia is a disruption of hematopoiesis. In order to understand the role of oncogenes and retroviruses in leukemogenesis, it seems essential that the basic principles of hematopoiesis be discussed. The main focus of this discussion is concerned with avian hematopoiesis with special emphasis on erythropoiesis. The murine system is also described since most of the pioneering work has been done in this system.

Hematopoiesis involves the production and maintenance of blood cells. The site of blood formation in the chicken, like other animals, is developmentally regulated. Hematopoiesis begins in the chick embryo in the blood islands of the yolk sac (Romannoff, 1960). This remains the major site of hematopoiesis during embryogenesis. Limited hematopoietic activity has also been detected in the liver and spleen between days 7 and 9 of development. Bone marrow hematopoiesis begins as early as days 8 and 9 and becomes the major source for blood cell formation after hatching. Hematopoiesis outside of the bone marrow, known as extramedullary hematopoiesis, rarely if ever occurs in the hatched chicken. This is in contrast to mammals where the liver and spleen play an important role in embryonic hematopoieisis and during periods of hematopoietic stress in the adult animal (Rifkind et al., 1980).

The hematopoietic system is composed of blood cells which are capable of proliferating, differentiating, and maturing into several cell types. The mature cells include erythrocytes, granulocytes, macrophages, eosinophils, megakaryocytes, and B- or T-lymphocytes. These cells are morphologically distinct and each performs a different function necessary for survival. Their lifespans are relatively short and therefore, they must be continuously replaced. The mature cells, although routinely observed in the bloodstream, are thought to originate in the bone marrow from a common pluripotent stem cell. This stem cell has the ability to proliferate (or self-renew) and differentiate into the various cell types. Under normal physiologic conditions, the stem cell is quiescent, but is capable of rapid proliferation if necessary.

The most convincing evidence in support of a pluripotent stem cell was first provided by reconstitution experiments (Till et al., 1961; McCulloch et al., 1965). Recipient mice were made hematopoiesis deficient by irradiation and were injected with bone marrow cells from a donor containing a chromosome marker. Distinct hematopoietic colonies of donor origin appeared in the spleens of the recipients. Examination of individual colonies revealed a heterogeneous population of hematopoietic cells containing either erythroid or myeloid cells, or both. Each colony was shown to arise from a single cell and maintained the ability to reconstitute other irradiated mice. Based on these observations a pluripotent stem cell, sometimes referred to as CFU-S or colony forming unit-spleen, was proposed. Later experiments extended the pluripotency of the stem cell to lymphoid cells (Abramson et al., 1977). Similar types of reconstitution experiments have been performed in the chicken. In this case, colonies appear in the bone marrow rather than the spleen and are called CFU-M for colony forming unit-marrow. This observation is consistent with the idea that the liver and spleen of the chicken do not provide a suitable environment for extramedullary hematopoiesis.

As the stem cell differentiates a hierarchy of cell types and cell lineages appear. This heirarchy has been defined based on functional as well as morphological properties. Progenitor cells, the immediate progeny of stem cells, were first identified using in vitro colony assays. Colonies containing distinct progenitor forms were observed after seeding bone marrow cells in semisolid media. The in vitro colony assay is now routinely used to identify distinct progenitor cells (Pluznik et al., 1964; Bradley et al., 1966; Metcalf et al., 1979). Unlike stem cells, the progenitor cells are "committed" to differentiate along a particular cell lineage and display only limited proliferative capacity. Two of the best characterized progenitor cells are those of the erythroid and granulomyelocytic lineages. The granulomyelocytic progenitors represent a distinct subset of precursor cells which differentiate into granulocytes or macrophages in vitro and in vivo. Colonies containing both granulocytes and macrophages originate from these progenitors and are called CFU-GM for colony forming unit-granulocyte/macrophage.

When specific in vitro culture conditions were used, two types of erythroid colonies were identified (Stephenson et al., 1971; McCleod et al., 1974; Samarut et al., 1980). One is a more mature erythroid colony, termed the colony forming unit-erythroid (CFU-E). It is characterized by small diffuse colonies of 20 to 60 cells in methylcellulose or plasma clot cultures. These cells, when removed from semisolid media and suspended in culture medium, differentiate into hemaglobinized cells (mature erythrocytes) within 3 days. Another colony resembling a more primitive erythroid precursor is also observed. These colonies are called the burst forming unit-erythroid (BFU-E) since they are composed of tightly packed cells and contain as many as a thousand cells per colony. The BFU-E derived cells are also capable of differentiating into hemoglobinized cells in liquid culture, but require a longer incubation period, usually between 7 and 10 days. Thus the BFU-E and CFU-E are functionally distinct. In the chicken, the CFU-E and BFU-E can be characterized further based on the presence of specific antigenic markers (Samarut et al., 1979). BFU-E and CFU-M preferentially express a brain related antigen (Br-antigen) whereas CFU-E displays an antigen characteristic of immature red cells (Im-antigen). Antibody mediated cytolysis of cells containing the Im-antigen does not inhibit BFU-E formation (Samarut et al., 1980). Thus the BFU-E is considered an intermediate cell type between the pluripotent CFU-M (CFU-S in the murine system) and CFU-E and has the potential for limited self-renewal.

Several other erythroid cell types have been distinguished based on cell morphology and histochemical staining. These include the erythroblast, polychromatic erythrocyte, and the mature erythrocyte. This is the terminology used by avian hematologist and is somewhat different than the conventional terminology which uses normoblast as the form for most erythroid intermediates (Lucas et al., 1961; Beck, 1977). Where these cell types fit into the cell lineages defined in tissue culture is unclear. The most immature erythroid cell that can be identified morphologically is the erythroblast (or pronormoblast). Unlike the other erythroid intermediates the erythroblast expresses little if any hemoglobin, stains benzidine negative, and reacts with anti-Im. This erythroid cell type is rarely seen in the bloodstream and is observed at very low numbers in the bone marrow of the chicken (less than 0.1%) (Nelson et al., 1980). An increased number of erythroblasts has been observed in anemic birds. The erythroblast does appear to be capable of cell division since mitotic figures have been observed. These properties taken collectively suggest that the erythroblast is probably identical to the CFU-E. Thus erythropoiesis traverses the following pathway. Stem cell > BFU-E > CFU-E/erythroblast > polychromatic erythrocytes > mature erythrocytes.

The identification of several intermediates in the erythroid lineage illustrates the prevailing features of hematopoietic differentiation. That is, once a stem cell "commits" itself to a particular cell lineage, further proliferation and differentiation occur almost simultaneously. A more mature phenotype is accompanied by a progressive loss of proliferative potential. The end result is a cell with no proliferative ability but serving a very specific function to the organism. In the case of erythrocytes, it is to maintain tissue oxygen levels by transporting oxygen. The presence of committed, but intermediate cell types which can readily differentiate provides a means for rapid response to hematopoietic stress such as bleeding or infection.

As expected for any developmental system, hematopoiesis is very tightly regulated and its regulation appears to be influenced by its microenvironment. The bone marrow is composed of a complex stromal cell network consisting of several cell types (Dexter 1982; Sorrell et al., 1980). The sites of erythropoiesis and granulopoiesis are localized to two distinct compartments in the chick bone marrrow. Erythropoiesis occurs intravascularly with the more immature erythroid cells being associated with endothelial cells which line the vascular region of the marrow. Granulopoiesis, on the otherhand, occurs extravascularly, and is often associated with reticulum cells. The reticular cells are fibroblastic cells which form a loose cellular network in the marrow. Adipocytes are also found associated with macrophages and together with the reticular cells, they fill the perisinusoids of the marrow.

The role of the bone marrow microenvironment in hematopoiesis is still unclear. This microenvironment can be reconstituted _in vitro_ by establishing long term bone marrow cultures consisting of an adherent stromal cell layer (Dexter et al., 1974; Dexter et al., 1976). These cultures, known as Dexter cultures, support the proliferation and differentiation of both stem cells and their maturing progeny. Although the original Dexter cultures selectively supported only myeloid and erythroid cultures, modifications of this culture system have been used to support lymphoid cells (Whitlock et al., 1982). The adherent multilayer of stromal cells is a key element in these cultures. It has been speculated that these cells function as a matrix for stem cell attachment and that specific cell-cell interactions affect stem cell proliferation. The particular localization in the bone marrow may determine which lineage will ultimately develop, while the stromal cells serve to maintain the stem cell population in general.

It is apparent from the <u>in vitro</u> colony assays that the proliferation and development of the committed progenitor cells do not require bone marrow derived cultures. A feeder cell layer or conditioned media, however, is usually necessary for successful colony formation (Dexter 1984). The importance of conditioned media in colony formation supported the idea that humoral factors were important in regulating hematopoiesis and has become an area of intensive research in the last decade. Several hematopoietic growth factors have been purified to homogeneity and molecularly cloned (Gough et al., 1984; Fung et al., 1984 ; Kawaski et al., 1985; Wong et al., 1985; Lin et al., 1986; McDonald et al., 1986; Shoemaker et al., 1986). These growth factors can be divided into two types based on their colony stimulating activities in vitro. Some are multipotent, like interleukin-3 (IL-3) and granulocyte/macrophage-colony stimulating factor (GM-CSF). IL-3 stimulates the proliferation and development of all myeloid and erythroid precursor cells and can facilitate the self-renewal of CFU-S (Ihle et al., 1982). GM-CSF appears to be more restricted than IL-3 in its activity and promotes growth of progenitor cells of the granulocyte and macrophage lineages (Burgess, et al., 1980). The formation of erythroid bursts can be stimulated by GM-CSF but only in the presence of another growth factor, erythropoietin (Sieff et al., 1985). Erythropoietin is one of many hematopoietic growth factors whose activity is confined to a specific cell lineage. Erythropoietin acts exclusively in the development of erythroid progenitor cells (Marks et al., 1978). Other unipotent growth factors include G-CSF (granulocyte-colony stimulating factor) and CSF-1 (colony stimulating factor-1) which affects growth of granulocytes and macrophages, respectively.

All of the above mentioned growth factors are low molecular weight glycoproteins. Most of them have been purified from the media of cultured cells with the exception of erythropoietin. Erythropoietin is synthesized exclusively in the liver and kidney and has been purified from urine and serum (Miyake et al., 1977). The other CSFs are synthesized by multiple cell types (Metcalf 1985). These cells are distributed throughout most tissues and include fibroblasts, activated T-lymphocytes, and endothelial cells (Cline et al., 1979; Whetton et al., 1986). CSF-1 and GM-CSF have also been detected in the urine and serum of animals but at lower levels (Burgess et al., 1977; Stanley 1985). IL-3 was purified from a leukemic cell line and has yet to be detected _in vivo_. Continuous exposure to the growth factor appears to be required for proliferation and survival of the appropriate target cells, since without them the cells deteriorate. This poses somewhat of a problem since the bone marrow cells themselves do not secrete growth factors. G-CSF is one of the few growth factors known to be synthesized by bone marrow cells. The function of the ultrastructure of the bone marrow may be to act as a surface on which hematopoietic growth factors can be concentrated. In this way growth factors synthesized in other tissues can reach the appropriate hematopoietic cell. This interaction could be achieved via the extracellular matrix and membrane associated glycoconjugates characteristic of stromal cells. Recent evidence supports this view (Hunt et al., 1987).

Although the hematopoietic growth factors are widely distributed, they act on very specific cell types. Their action has been shown to be mediated by receptor molecules present on the cell surface. The characterization of specific hematopoietic growth factor receptors has been limited by the low number of progenitor cells and the heterogeneous nature of the bone marrow. Radio-iodonated growth factors have been used to identify specific growth factor receptors on the surface of bone marrow cells. Each growth factor binds to a receptor of different apparent molecular weight suggesting that each growth factor has its own receptor (Whetton et al., 1986). Growth factor receptors are coexpressed on cells at different stages of hematopoietic development (Metcalf, 1985). There appears to be no direct competition among growth factors. That is, different growth factors do not bind to a common receptor molecule. Regulation of growth factor receptors does, however, appear to be cooordinately regulated (Walker et al., 1985; Lotem et al., 1986). This regulation, termed transmodulation, involves the down regulation or removal of receptor molecules from the cell surface. The particular growth factors which are down-regulated coincide with the hierarchy of hematopoietic cell lineages and the multipotency of the growth factors. For example, binding of bone marrow cells with IL-3 down regulates not only its own receptor but also receptors for GM-CSF, CSF-1, and G-CSF. Similarly, GM-CSF down modulates GM-CSF, CSF-1, and G-CSF receptors. CSF-1 and G-CSF only down regulate their own receptors. Down regulation of these receptors appears to reduce the number of avalaible receptors and not the affinity of the receptor for its ligand. It is not clear how a down-regulated receptor leads to an active state of differentiation and proliferation, especially since very low levels of receptor occupancy (less than 10%) are necessary to achieve the biological effects associated with erythropoietin, GM-CSF, and G-CSF (Metcalf, 1985). Down-modulation may serve a more instructive role; where a cell once down-regulated by a particular growth factor is destined to proceed down a particular differentiation pathway.

The presence of specific cell surface receptors on hematopoietic cells raises the question of what is the signal that is transduced and what are the immediate effects on the cell? Does ligand binding signal proliferation, then differentiation or both? Studies with erythropoietin (epo) suggest that the principal biological effect of epo is not differentiation but proliferation of the target precursor cells (Marks et al., 1978). Differentiation to the mature erythrocyte appears to be a property inherent in an epo-responsive cell. If differentiation is the only consequence of epo action, one would expect to see large numbers of erythroid precursors in the bone marrow. The use of a mitogenic signal as an initial response provides an efficient way of rapidly recovering from hematopoietic stress. How a growth factor receptor signals mitogenesis and how this signal is coupled to a specific differentiation program awaits further elucidation of the specific growth factor receptors and their signal transduction pathways.

The majority of work done on hematopoietic growth factors and their receptors has been done in the human and murine systems. Similar activities have been identified in the avian system, but none have been purified to homogeneity (Samarut et al., 1976,1978). Some hematopoietic growth factors do cross react between species, albeit with diminished binding and biological effects in the heterologous system. Erythropoietin appears to be especially species specific, since murine erythropoietin is not active on rat erythroid cells and displays only limited sequence homology (McDonald et al., 1986). The establishment of long-term bone marrow cultures and the crude purification of hematopoietic growth factor-like activity from the avian system suggests that hematopoiesis in the chicken is similar to that of humans and mice. That is, there is a hierarchy of hematopoietic cells which are regulated by humoral factors. The cells and molecules which mediate these responses may prove to be physically distinct, but the general principles of hematopoietic development appear to be similar.

The above discussion illustrates the complex nature of blood cell development. It involves the multiplication of stem cells and their progressive differentiation into mature cells of various types, each having an important function. This program of events is exquisitely regulated by a variety of growth factors which can act alone or in synergy to produce a specific cell type. Leukemia results from an imbalance in the normal hematopoietic program. It is typically manifested as the accumulation of blast-like cells in the bone marrow and bloodstream of the affected animal. The leukemic blast cells are usually of a specific hematopoietic cell lineage and are analagous to the committed progenitor cells. Unlike other progenitor cells, leukemic cells have increased proliferative ability and in many cases become "immortalized" so that they can be continuously passaged in tissue cell culture. Some leukemic cells can be induced to differentiate after treatment with agents like phorbol esters and dimethylsulfoxide, but are resistant to the effects of the appropriate hematopoietic growth factors. Leukemic cells display features suggesting that the proliferative and differentiation signals have been uncoupled or perhaps one signal, i.e., the proliferative signal, is potentiated. Characterization of leukemias and the oncogenes involved should provide useful information regarding what the proliferation and differentiation signals are, and also how that signal may be transduced to complete the differentiation program.

B. Retroviruses and Cancer

Unlike the study of avian hematopoiesis, the avian system has been at the forefront of retrovirus research. Indeed it has only been in the last decade that a human retrovirus has been found associated with leukemia. It seems ironic that in 1987 one of the major threats to the human population is a retrovirus when just 10 to 20 years ago retroviruses were thought to be obscure agents restricted to the avian system and not mainstream to cancer biology. The following discussion focuses primarily on avian retroviruses, their oncogenes, and how they interact within the cell.

Retroviruses were first identified as the causative agents of avian leukemias and tumors and are commonly referred to as RNA tumor viruses. In 1911, Peyton Rous demonstrated that chicken sarcomas could be transplanted using a cell free filtrate. It is the virus from this sarcoma now known as the Rous Sarcoma Virus (RSV), which has been instrumental in establishing a large number of concepts central to retrovirology as well as cancer biology. Direct inoculation of RSV onto chorioallantoic membranes resulted in small tumors and supported the idea that a retrovirus could "transform" a normal cell into a tumor cell (Keogh, 1939). In 1958, Temin and Rubin further described a similar "transformation" in vitro using monolayers of chicken embryo fibroblasts. RSV transformed cells stood out as distinct "foci" on the cell monolayer and the number of foci correlated with the amount of virus applied to the cells. The development of conditional and transformation defective mutants of RSV suggested that RSV contained a specific gene responsible for the tumorous phenotype (Martin et al., 1972). This transforming gene, now known as v-src, was shown to originate from cellular sequences and was highly conserved among metazoans (Stehelin et al., 1976; Spector et al, 1978).

RSV is just one of many avian retroviruses. The avian retroviruses are divided into two classes based on their pathogenic properties and genetic content (Teich, 1982). The slow transforming viruses or non-acute retroviruses, as the name implies, induce neoplastic disease after a long latency period of several months. They are capable of inducing a wide spectrum of diseases and are often found associated with naturally occurring tumors. The non-acute class of retroviruses is replication competent and can be routinely found in infectious stocks of defective viruses. For this reason, they are also known as "associated" or "helper" viruses. These viruses do not transform cells <u>in vitro</u> and do not contain oncogenes. Avian leukosis virus (ALV) and reticulo-endotheliosis virus (REV) are two distinct subclasses of non-acute retroviruses. The avian leukosis virus group is further subdivided based on the expression of specific envelope coat proteins. The molecular basis of oncogenesis by ALV and REV will be discussed in detail later.

The other class of retroviruses is the acute transforming viruses. These viruses induce acute and fatal neoplastic disease within a few weeks or even within days. They usually induce one predominant neoplasm. Based on the predominant neoplasm which they induce, the acute transforming viruses are subdivided into either the sarcoma viruses or the leukemia viruses (Teich, 1984b). RSV is an example of the former subclass. Most of the acute transforming viruses are defective for replication. That is, they lack one or more of the three essential genes for virus replication (see next section). Virus production can occur if the defective genes are supplied <u>in trans</u> by a replication competent virus, i.e., the non-acute retrovirus. Unlike the non-acute viruses, the acute transforming viruses transform cells <u>in vitro</u> and contain additional oncogenic sequences. At least 12 different types of oncogenic sequences, referred to as viral oncogenes, have been found associated with avian retroviruses (Bishop et al., 1985). Like the v-src gene of RSV, normal chicken cells contain genes which are homologous with each of these viral oncogenes. These cellular sequences are called proto-oncogenes or cellular oncogenes. Acute transforming viruses presumably arose by recombination between proto-oncogenes and a replication competent, non-acute virus. The process by which these cellular oncogenes are incorporated into a retrovirus is known as transduction and will be discussed in detail later. Although cellular oncogenes were originally defined as a result of retroviral transduction, chromosomal translocation, gene amplification, and other relevant mutations of these same proto-oncogenes have been associated with cancers in other species including humans (Weinberg, 1982, Varmus, 1984, Alitalo, 1985). It is in this respect that avian retrovirology has had its most profound effect on cancer biology today.

C. Retrovirus Life Cycle

The unique way in which retroviruses interact with their hosts make them potent cancer causing agents. The following discussion focuses on aspects of the retrovirus life cycle which are relevant to oncogenesis (for a more complete review see Varmus, 1983, 1984).

Retroviruses are single-stranded RNA viruses. Their genomes are diploid, consisting of two identical single- stranded RNA molecules. All the genetic information necessary for virus replication is contained within a single RNA molecule. The retrovirus genome consists of three structural genes; *gag* encodes the group associated antigens which comprise the core structure of the virus; *pol* encodes reverse transcriptase, an RNA directed DNA polymerase; and *env* is responsible for the synthesis of the envelope glycoproteins. These three genes are arranged 5'*gag*-*pol*-*env*3' in the genome. In general viruses which do not contain any one of these three genes are defective for replication. Successful infection and replication of defective viruses can occur if complemented with a replication competent, or so-called helper virus.

The retroviral genome closely resembles a eukaryotic mRNA. Not only is it a plus strand virus, but it also contains a 5' cap site, internally methylated adenosines, and a poly (A) tract. In addition, the genome contains several noncoding regions most of which are situated at the termini of the virus. These noncoding sequences possess a variety of functions most of which are essential for a complete virus life cycle. Important noncoding sequences include: 1) the R region, repeated sequences present at both ends of the viral RNA, 2) U5 sequences, sequences unique to the 5′ end of the viral genome and represented twice in the integrated viral DNA, 3) the primer binding site (PBS), the site where the tRNA primer binds to initiate synthesis of the first DNA strand, 4) the leader sequence, sequences preceding the gag gene important in packaging of viral RNA and in some cases containing the splice donor site required for generation of subgenomic RNAs, 5) 3′ noncoding region (NT), sequences between env and the beginning of U3 which contain a purine-rich tract capable of priming plus strand DNA synthesis, and 6) U3 sequences, sequences unique to the 3′ end of the viral genome and present twice in proviral DNA as a component of the LTR. In addition U3 contains a consensus polyadenylation signal, and promoter and enhancer sequences important in the regulation of viral RNA synthesis.

The virus life cycle begins with entry into the cell. Although retroviruses adsorb rather nonspecifically to the cell, internalization is mediated through an interaction between the envelope glycoproteins and specific cell surface receptors. In the case of Avian Leukosis Viruses (ALVs), there are five envelope subgroups, A through E, which have been designated based on their host range, cross interference of receptors, and neutralization of antibodies (Payne, 1985). Four autosomal loci govern susceptibility to the viral subgroups. They are called tv- a, tv-b, tv-c, and tv-e, tv standing for tumor virus. No locus corresponding to subgroup D has been identified. Subgroup B and subgroup D viruses, however, appear to use the same set of receptors. In each case the susceptibility allele is dominant. Once the virus has bound to its receptor, it is not clear how the virion is internalized. Internalization could occur through fusion with the plasma membrane or by receptor-mediated endocytosis. It should be noted that mammalian cells do not express any of the tv loci, yet can be infected by ALVs. This process occurs with very low efficiency, and entry presumably occurs through nonspecific adsorption to the plasma membrane.

Once the cell has been infected, viral DNA is synthesized and integrated into the host genome. Successful DNA synthesis requires not only the appropriate subtrates, primers, and enzymes, but also an active cellular environment. The final product of DNA synthesis is a linear duplex DNA which contains duplicated copies of U3 and U5 at its termini. The duplicated sequence is referred to as the long terminal repeat (LTR). These sequences are necessary for integration into the host genome and subsequent expression of viral gene products.

Reverse transcriptase is responsible for synthesis of both the plus and minus strands of DNA. The minus strand is synthesized first. It is initiated by binding of a host tRNA to the primer binding site situated at the 5′ end of the viral RNA. The polymerase synthesizes only 100 to 180 nucleotides before reaching the end of the template. This short minus strand DNA, called strong stop DNA, contains R sequences complementary to the sequences present at the 3′ end of the viral genome. Hybridization to complementary DNA sequences situated at the 3′ end of the viral genome serves as primer for the synthesis of the entire minus strand. Plus strand synthesis procedes similar to minus strand synthesis. It begins in the 3′ noncoding region of the virus and is quickly halted due to termination of the template. Synthesis terminates at sequences complementary to the primer binding site of the strong stop DNA. This duplication enables the second 'jump' of the polymerase and completes plus strand synthesis. The final product is a linear duplex DNA with two copies of U3-R-U5 at each end.

Errors in reverse transcription may account for the relatively high frequency (10^{-3} to 10^{-4}) at which viral mutants are generated during viral passage. Deletion mutants may be generated by inappropriate transfer of nascent DNA strands from one template to the other (Coffin, 1979). Reverse transcriptase also shows a high rate of misincorporation in vitro (Gopinathan et al., 1979) and may account for viral mutants containing point mutations. The infidelity of reverse transcriptase may in part be due to the lack of an associated editing function. Errors introduced into the viral genome during reverse transcription are essential to the transduction of host sequences since most viral oncogenes contain multiple mutations relative to their cellular counterparts.

After the linear duplex DNA is synthesized it is transported to the nucleus where a portion of it becomes circularized. The circular

DNA is specifically cleaved by a virus associated endonuclease. As a result it is always inserted into the host chromosome colinear with the viral genome. Insertion appears to be random and there is no obvious sequence specificity at the integration sites.

The completion of the virus life cycle depends on the transcription of the provirus into progeny genomes and mRNAs. The structure of the integrated provirus is similar to that of the virus except for the presence of the LTRs at each end. The LTR provides most of the regulatory sequences necessary for transcription. The cap site is located at the 5′ boundary of R. 25-30 nucleotides upstream of the initiation site are sequences resembling the so-called "TATAA box". Also present are CCAAAT sequences 70-85 nucleotides upstream of the cap site. Both these sequences have been implicated in determining the transcriptional start site of most eukaryotic genes. A polyadenylation signal, AATAAA, is located 20 nucleotides from the U3-R boundary. In addition to these sequences, the LTR also contains sequences, called enhancer sequences, which can increase the activity of a nearby heterologous promoter (Banerji et al., 1981; Khoury et al., 1983). These sequences are situated in the 5′ two-thirds of U3, and appear to operate in a position and orientation independent manner. It is not clear whether these enhancer sequences in any way influence the promoter activity of the LTR.

Transcription is catalyzed by RNA polymerase II. Capping and other processing events are presumably performed by additional host enzymes. Transcription is initiated at the U3/R boundary of the 5′ LTR and terminates at the R/U5 boundary of the 3' LTR. Two polyadenylated RNAs are generated. One encodes a full-length genomic mRNA which can be packaged into virions or serve as mRNA for the gag and pol genes. The other RNA is generated by splicing from the leader sequence of the virus into env. This subgenomic RNA serves as template for the env polyprotein. ALV is unique in that its splice donor dite is located within the gag coding sequence. As a result, six amino acids of the gag gene are present at the amino-terminus of env. The presence of a translational start site before the splice donor site is of special significance in activating the proto-oncogene c-erb B.

The primary translation products of gag, pol, and env are precursor polyproteins which are post-translationally modified to generate mature virion proteins. Expression of the gag, pol, and env genes is required for productive infection. The majority of RNA packaged into virions is the genomic viral RNA, although some nonspecific packaging has also been reported (Boccara et al., 1982; Linial, 1987). This selection is presumbably due to the recognition of specific viral sequences. Sequences present in the leader region of the virus have been shown to contain sequences important in packaging (Shank et al., 1980; Koyama, 1984). These sequences, however, are present in both the subgenomic and genomic RNAs. Sequences present in the gag gene, or the "intron" region of ALV, have been implicated in viral packaging (Pugatsch et al., 1984). The latter case offers an explanation for preferential packaging of the genomic RNA since these sequences are spliced out in the subgenomic RNA. Copackaging of genomic RNA and cellular RNA can facilitate viral recombination and the generation of defective viruses which contain oncogenes.

D. Oncogenesis by Nonacute Retroviruses

The complex nature of retrovirus replication and its intimate interaction with the host cell suggests several ways in which non-acute retroviruses can mediate pathogenesis. Expression of the viral structural genes themselves may directly or indirectly affect the growth of the host cell. For example, the env gene product is expressed on the cell surface and can serve as antigen to activate the immune system (Teich, 1982). Continuous expression could lead to immunosuppression thereby allowing tumor cells to escape immune surveillance. The env gene product itself has been implicated in murine leukemogenesis. In this case the normal env gene is altered as a result of recombination between the infecting virus and endogeneous viral sequences. The recombinant virus, called the spleen focus forming virus (SFFV), induces rapid tumor formation (Teich, 1985). It is as yet unclear how the structurally altered env gene mediates leukemogenesis in the murine system. It seems most likely that expression of the recombinant env gene product mimics some other molecule (perhaps a growth factor receptor) which is instrumental in regulating hematopoiesis. More recently it has been demonstrated that certain viral genes of the human T-cell leukemia viruses (HTLV-1 and HTLV-2) can act in trans thereby affecting transcription and translation of other viral gene products. The tat-1 and tat-2 genes are known to affect expression of cellular genes, namely interleukin-2 and the interleukin-2 receptor (Greene et al., 1986). This illustrates yet another way in which virus infection can disrupt normal cell function and perhaps lead to oncogenesis.

Retroviruses can also act as mutagens since their DNA form, the provirus, integrates randomly into cellular DNA. Insertion of a provirus into a cellular gene can disrupt its normal expression. Inactivation of the gene by proviral insertion may not be readily detected since the host genome is diploid and only one allele would be disrupted. A number of genetic markers such as coat color and developmental disorders have been identified which are associated with retroviruses inserted within a few specific loci (Kozak, 1985; King et al., 1985). These are usually examples of recessive mutations and have only been identified through classical genetics. Tumor formation may result from a dominant mutation and would therefore select for a different type of mutation rather than gene inactivation.

The provirus carries with it strong transcriptional regulatory elements at its termini. The introduction of these regulatory elements into the host genome by retroviral insertion has the potential to produce a dominant phenotype. A retrovirus in this context could induce tumor formation or leukemia production by placing the cellular gene under the regulation of viral sequences. This results in increased and perhaps inappropriate expression of the cellular gene. This type of alteration is often referred to as insertional activation because of the associated increase in transcription. Insertional activation of certain cellular genes, namely proto-oncogenes, appears to be the predominant mechanism by which most non-acute retroviruses induce neoplasia.

A mechanism for pathogenesis by non-acute retroviruses was first provided by studies B-cell lymphomas. The majority of lymphomas analyzed contain a provirus inserted in the c-myc locus. C-myc is the cellular counterpart of the viral oncogene, v-myc, the transforming gene found in the avian retrovirus, MC-29. All tumors showed marked increases in the relative levels of c-myc mRNA (30-100 fold; Hayward et al., 1981). Molecular analysis of B-cell lymphomas induced by avian leukosis virus (ALV) established that there are at least two distinct mechanisms by which non-acute retroviruses can activate transcription of adjacent cellular sequences.

The chicken c-myc gene contains three exons, the first of which is noncoding (Watson et al., 1983; Shih et al., 1984). Integrated proviral sequences were detected upstream of the first coding exon (exon 2) in over 80% of the B-cell lymphomas analyzed (Hayward et al, 1981; Shih et al., 1984; Fung et al., 1982b; Payne et al., 1982). Most of the insertion sites were located within the first intron, but others were observed as far as 5 kb upstream. Proviruses were usually situated in the same transcriptional orientation. Elevated levels of c-myc were detected in tumor samples containing this type of viral-c-myc arrangement. The elevated c-myc transcripts comigrated with novel viral related RNAs. These virally linked transcripts exclusively hybridized to U5 sequences of the LTR. Based on these observations, it was proposed that c-myc transcription is initiated in the 3' LTR and that transcription reads through into the c-myc gene (Hayward et al., 1981). In this situation, the 3′ LTR of the provirus acts as a promoter for the adjacent c-myc gene. This was somewhat unusual since viral transcription normally utilizes the promoter within the 5′ LTR. The deletion of additional ALV sequences within or near the 5′ LTR in most, if not all, of the tumors analyzed suggested that their removal may potentiate the use of the 3′ LTR as a promoter (Neel et al, 1981; Payne et al, 1981; Fung et al., 1981). This particular mechanism of activation is known as promoter insertion. C-myc expression is presumably deregulated by putting it under the activity of the viral promoter.

In a few other cases the proviral DNA was found either a) inserted downstream of c-myc and in the same transcriptional orientation, or b) upstream of c-myc but in the opposite orientation (Payne et al., 1982). The former case may be an exceptional one since it was observed in only one tumor sample. This particular sample contained elevated c-myc RNA levels and the c-myc related transcripts were found to terminate prematurely due to the insertion of viral sequences at the 3′ end of the gene. In the latter case, elevated levels of c-myc expression were also observed but no ALV sequences were found associated with the c-myc RNAs. The positioning of an ALV provirus in the opposite transcriptional orientation would not allow the viral promoter to be used for initiating c-myc transcription. In these cases other viral sequences are thought to be responsible for elevated c-myc expression. These sequences, now known as enhancer elements, reside in the U3 region of the viral LTR, and act independent of position and orientation to enhance the transcriptional efficiency of the normal cellular promoter. This mechanism of c-myc activation, referred to as enhancer insertion, is different from promoter insertion in that transcription is not initiated from viral sequences but uses cellular promoters. The cellular promoters utilized may not be the normal cellular promoters since several tumor samples appear to utilize cryptic promoters. Both mechanisms affect the level of c-myc transcription. The highest levels are usually associated with tumors containing promoter insertion arrangements. Thus promoter insertion may prove to be a more efficient mechanism for activating the c-myc gene in B-cell lymphomas since most of the tumors analyzed display this type of activation mechanism.

In both promoter insertion and enhancer insertion, the 5' untranslated region of the normal c-myc mRNA is removed due to integration within the first intron. This loss of untranslated sequence may further affect c-myc expression. It has been proposed that the noncoding sequences of c-myc RNA may form a potential hairpin or rho-like structure which would subsequently inhibit efficient translation of c-myc RNA (Saito et al., 1983, and Nottenburg et al., 1986). Removal of the untranslated region as a result of proviral insertion may enhance translation as well as transcription. The relative level of the c-myc protein produced may actually exceed the 30 to 100 fold estimates made based on transcription.

The truncated c-myc RNA should encode a protein virtually identical to that of the normal c-myc protein. In only one case, however, has the structure of the c-myc protein from a tumor sample been determined. When the nucleotide sequence of the activated c-myc gene was compared to that of normal c-myc, several point mutations were found (Westaway et al., 1984). Some of the differences did result in amino acid changes. It is not clear whether these structural changes in the activated c-myc gene product contribute in any way to the development of B-cell lymphomas. The prevailing theme of oncogenesis by nonacute retroviruses is one that implies an alteration in gene regulation. This observation raises the question of whether qualitative changes as well as quantitative changes in gene expression may be necessary for tumor formation.

The B-cell lymphoma studies described above are correlative and do not demonstrate that c-myc is responsible for lymphomagenesis. Experiments to directly address the disease potential of c-myc have been limited to studies using acute leukemia viruses which carry v-myc sequences. There are several strains of v-myc containing viruses. These include MC-29, MH-2, and OK-II. Like the activated c-myc gene, the v-myc genes of these viruses contain several nonconservative point mutations (Bister et al., 1986). These point mutations do not, however, affect common amino acids. Most of the v-myc proteins are synthesized as viral fusion proteins and therefore are not identical to the activated c-myc protein. Interestingly, these viruses have a different oncogenic spectrum and do not induce B-cell lymphoma.

Myelocytomatosis, the predominant disease associated with v-myc containing viruses, is quite distinct from B-cell lymphomas and affects cells of the early myeloid lineage (Graf et al., 1978). Closer examination of MC-29 infected birds has revealed small B-cell lymphomas. These are not the predominant lesion and therefore may have been missed in earlier studies (Hayward et al., 1983). A newly isolated variant of MC-29, HB-1, induces short latency lymphomas of B- and/or T-cell origin (Enrietto et al., 1983). The difference in the pathogenicity of the HB-1 virus is thought to be due to recombination between v-myc and c-myc sequences. This hypothesis needs to be tested more rigorously since the recombinant virus has also undergone additional recombination events in the helper virus sequences. Inconsistencies in other experimental parameters such as the age and strain of the chickens used, as well as the route of injection, make the results of these experiments, and experiments with other myc containing viruses difficult to compare and interpret.

C-myc activation does not appear to be limited to avian B-cell lymphomas since activated c-myc has been observed in other diseases induced by nonacute retroviruses. In one case, an adenocarcinoma induced by the ring-neck pheasant virus (RPV) was found to contain an activated c-myc gene (Simon et al., 1984). RPV is classified as an avian leukosis virus (Fujita et al., 1974; Temin et al., 1976). This virus is unusual in that it is appears to be a recombinant virus between endogenous <u>env</u> sequences of the pheasant, and ALV. Its disease potential is also unique in that it can induce a variety of short and long latency neoplasms (Carter et al., 1983). The molecular basis of oncogenesis by this virus is poorly understood; in some ways it appears to be analogous to the murine SFFV virus discussed earlier. The presence of an activated c-myc gene in this adenocarcinoma may be secondary to the initial transformation event, since c-myc was found activated in only one tumor (Simon et al., 1984). In addition, several other lesions, including fibrosarcomas were observed in this one diseased chicken. An activated c-myc gene was not detected in any of the other lesions further suggesting that c-myc activation in the isolated tumor sample may not be significant.

C-myc activation has also been implicated in T-cell lymphomas induced by REV. Over 90% of the REV induced B-cell lymphoma samples contained activated c-myc alleles (Noori-Daloii et al., 1981; Swift et al., 1985). The structural arrangement of the proviruses was consistent with a promoter insertion mechanism of activation (Swift et al., 1987). It is interesting that the activation mechanism in T-cell lymphomas was slightly different from that observed in the B-cell lymphomas induced by both ALV and REV (Isfort et al., 1987). As expected the level of c-myc expression was elevated in the T-cell lymphomas, but only 6-18 fold over normal c-myc as opposed to the 30-100 fold increase observed in B-cell lymphomas. The difference in the relative levels of c-myc expression in the two tumor types may be due to the predominance of enhancer insertion as the mechanism of c-myc activation in the T-cell lymphomas. All the REV proviruses were found to be inserted 5′ of the second exon of c-myc, and half of the proviruses were situated in the opposite transcriptional orientation with respect to c-myc. Only a subset of the proviruses inserted in the same transcriptional orientation were found to actually utilize the 3′ LTR to promote downstream transcription. In the other cases, transcription initiation mapped to a common cryptic promoter site present in the intron of c-myc. These observations indicate that activation of c-myc can occur via enhancer insertion irrespective of the transcriptional orientation. Enhancer insertion rather than promoter insertion appears to be the predominant mechanism by which c-myc is activated in REV-induced T-cell lymphomas.

Enhancer insertion of c-myc is also the predominant mode of activation observed in lymphomas induced by murine retroviruses (Steffen, 1984; Corcoran et al., 1984). Thus the enhancer element is not limited to avian retroviruses and appears to play an important role in myc activation. The fact that the same virus (REV) can alter the same gene (c-myc) in two different cell types (B-cells versus T-cells) by somewhat different mechanisms suggests that there may be other factors which dictate the precise mechanism of c-myc activation.

Activation of c-myc in ALV induced lymphomas provided the first indication that abnormal expression of proto-oncogenes could be responsible for tumorigenesis. Since that time a variety of other neoplasms induced by non-acute retroviruses have been analyzed for the activation of cellular oncogenes. In addition to the c-myc gene, the c-erb B gene has been identified as an activated proto-oncogene in ALV induced erythroblastosis (Fung et al., 1983). This leukemia and the precise mechanism of c-erb B activation will be discussed in detail later. The mechanism of c-erb B activation is similar to the promoter insertion mechanism identified in B-cell lymphomas except that c-erb B transcription initiates from the 5′ LTR of the integrated provirus rather than from the 3′ LTR. Again, these subtle variations in activation mechanisms in different cell types suggests that there may be other factors which determine the precise mechanism by which a proto-oncogene can be activated.

The c-Ha-ras gene is the only other avian proto-oncogene that has been found to be activated in an ALV induced neoplasm. The activated c-Ha-ras gene was observed in a nephroblastoma induced by MAV-1 (myeloblastosis associated virus type -1; Westaway, 1986). MAV-1 is similar to the ALVs used to induce B-cell lymphomas and erythroblastosis (Moscovici et al., 1968). A promoter insertion mechanism appears to mediate c-Ha-ras activation by MAV-1, since LTR sequences are linked to the c-Ha-ras RNA. The proviral insertion, however, appears to be distal to the c-Ha-ras gene and is not detectable as a rearranged restriction enzyme fragment by Southern analysis. This tumor represents a unique class since an activated c-Ha-ras mRNA could only be identified in one of several nephroblastomas analyzed. Thus, c-ras activation may not be crucial to the formation of kidney tumors in chickens.

A variety of tumors induced by nonacute murine retroviruses have been analyzed for activated oncogenes. Both c-myc and c-myb activation have been observed in lymphomas (Steffen, 1984; Corcoran et al., 1985;

Shen-Ong et al., 1984). The frequency of association of insertional activation is better in these cases than the correlation of c-Ha-ras and c-myc activation in avian nephroblastomas and adenocarcinomas, but is still poor. In general, an activated oncogene could be detected in less than 30% of the retrovirus induced tumors. This is in stark contrast to the lymphomas and leukemias induced in the chicken where there is a 80-100% correlation between tumorigenesis and activation of c-myc or c-erb B. While c-myc and c-erb B activation appear to play an important role in avian leukemogenesis, the weaker correlation in other systems suggests that other events may be necessary for the development of a full fledged tumor.

Finally, cellular sequences with no homology to viral oncogenes have also been implicated in oncogenesis. These potential proto-oncogenes have been identified by virtue of their linkage to proviral DNA in a number of tumors. This approach, known as transposon tagging, takes advantage of the fact that tumors induced by non-acute retroviruses are clonal with respect to the integrated proviruses. Provirus integration sites which map to a common region in several independent tumor samples suggest that the adjacent or activated gene may be critical to tumor formation. In the avian system common integration sites have been found associated with RPV proviruses in nephroblastomas and fibrosarcomas (Simon et al., 1984). The cellular sequences adjacent to the RPV proviruses, however, have not been characterized, nor has the precise mechanism of activation been determined. A similar strategy has been used to analyze a variety of tumors induced by mouse retroviruses and several common loci have been identified. These include int-1, int-2, pim-1 and mlvi-1 (Nusse et al., 1982; Peters et al, 1983; Tsichlis et al., 1983; Cuypers et al., 1984). Perhaps the most notable is the int-1 locus which has been shown to possess at least partial transforming ability *in vitro* (Brown et al., 1986). Like the c-myc and c-myb genes in murine lymphomas, the correlation between these new proto-oncogenes and the particular tumor induced is not absolute. More recent evidence suggests that some of these proto-oncogenes may work in concert with each other during tumor progression. Thus activation of more than one proto-oncogene may be necessary for the fully malignant phenotype to be manifested. The detection of activated proto-oncogenes may not be straightforward, and may be influenced by the stage of tumor development. This could explain, in part, the poor statistics observed between proto-oncogene activation and murine tumor formation. The idea that tumorigenesis is a multistep process is not a new one and is consistent with the current theories of cancer biology.

The molecular characterization of neoplasms induced by non-acute retroviruses has extended our understanding of the role of cellular genes in oncogenesis, how they can be mutated, and how retroviruses can mediate this process in a very specific manner. In summary, retroviruses insert into the host genome as an intermediate step in the virus life cycle. In addition to acting as an insertional mutagen, they introduce strong transcriptional regulatory elements into the genome. Two types of viral elements, promoters or enhancers, can be used to increase the transcription of a cellular gene. What determines the usage of one element over the other appears to be dictated by the cell type affected as well as the gene in which the crucial insertional lesion occurs. The net result is an overall increase in transcription and deregulation of the cellular gene. Although abnormal expression may be sufficient for development of the transformed phenotype, other structural changes either in the activated proto-oncogene itself or in other genes may contribute further to formation of the malignant tumor. We have seen different retroviruses induce the same neoplasm by activating the same genes. A single retrovirus can also be multipotent and induce different neoplasms by interacting with different cellular genes. It is these basic observations that have provided the foundation for our current understanding of oncogenesis. The molecular characterization of other tumors has extended these initial findings and led to the identification of yet another set of cellular genes important in retrovirus induced neoplasms. Analysis of naturally occurring tumors suggests that the same genes may be involved in nonvirally induced cancers. The question, however, remains as to what the normal functions of these genes are, and how deregulation contributes to oncogenic transformation.

E. Oncogenesis by Acute Transforming Viruses

Oncogenesis by acute transforming viruses does not require the complex virus-cell interactions described for the non-acute retroviruses. Successful infection by the acute virus into the appropriate target cell is sufficient for tumorigenesis since activated oncogenic sequences are intrinsic to their genomes. Continuous recruitment of target cells explains the rapidity and frequency of tumor formation characteristic of the acute transforming viruses. The highly tumorigenic nature of these viruses make them easy agents to isolate and monitor. It is not surprising that these retroviruses were some of the first to be characterized. How most of these viruses originated remains a mystery. Presumably they arose by recombination between a non-acute transforming virus and a cellular oncogene. This process is known as transduction. The limited number of independent acute transforming viruses isolated over the last 80 years suggest that transduction is a rare event. Analysis of the sequences and structure of known acute transforming viruses, suggest some of the events which may be involved (cf. Bishop, 1983; Varmus, 1984 for reviews of the transduction process).

Several features of retroviruses make them amenable to the transduction of cellular sequences. First, retroviruses frequently undergo recombination. Viral env sequences have been shown to be exchanged at an unusually high frequency during mixed infection (Vogt, 1971; Coffin, 1979). Second, the retrovirus genome is diploid. Heterozygote virions can occur which would allow two different genomes to be physically linked. Linkage between two different viral genomes should facilitate recombination. And finally, the DNA form of the virus integrates into the host genome. This is itself a recombination event and provides another way in which cellular and viral sequences can be physically joined.

In considering a mechanism of transduction, there are several features characteristic of viral oncogenes which should be accounted for. 1) Viral oncogenes are intrinsic to the viral genome and are therefore are always flanked by viral sequences. The positioning within the virus varies and may disrupt any of the structural genes. 2) Intron sequences that lie between the transduced exons of oncogenes are removed. This does not include sequences 5' of the first transduced exon since intron sequences presumably derived from cellular oncogenes are frequently observed at this point. 3) Viral oncogenes are not an exact copy of their cellular counterparts. In many cases only a portion of the cellular oncogene is transduced. The sequences not transduced may be untranslated sequences, like those described for c-myc, or coding sequences. The removal of coding sequences at either the amino- or carboxy-termini poses an additional problem since the initiation and/or termination sites are absent. In these cases, the oncogenic protein is expressed as a fusion protein with viral sequences at its amino- and/or carboxy-terminus. The correct reading frame of the oncogenic protein must be maintained in all cases, even when it is expressed as a hybrid protein. In addition to truncations, viral oncogenes contain other mutations internal to the coding sequences. These include non-conservative point mutations and internal in-frame deletions. 4) Examination of the recombination points between cellular sequences and the viral genome do not reveal any unique features suggestive of a recombination mechanism. In only a few isolated cases have short stretches of sequence homology been observed (Besmer, et al., 1986).

A model for transduction was proposed by Swannstrom and coworkers (1983) based on the features of RSV. It is consistent with the structure of most acute transforming viruses and is described below. First, a non-acute retrovirus inserts itself adjacent to a cellular oncogene such that it is oriented in the same transcriptional direction. This step is similar to the insertional activation mechanism described for c-myc and c-erb B. The next step requires deletion of the 3'LTR. Adjacent cellular sequences of intron or exon origin may also be deleted. Removal of the 3' LTR allows for efficient transcription of a hybrid RNA molecule. The hybrid RNA is initiated in the 5' LTR, contains both viral and cellular sequences, and extends to the 3' end of the cellular oncogene. Introns are subsequently removed, and the chimeric RNA is packaged into virions. A second recombination is required to provide 3' viral sequences and complete the transduction process. The 3' viral sequences are supplied by the parental, non-acute retrovirus and require the formation of a heterodimer. Recombination is thought to be due to template switching of the DNA polymerase during reverse transcription, a process known as copy-choice (Coffin, 1979). In transduction of cellular oncogenes, the copy-choice mechanism does not appear to be facilitated by homologous sequences. The complexity of the transduction process coupled with the probability of each step occurring correctly may explain its rarity in nature.

There is one variation on this proposed transduction mechanism which has been implied by some recent studies. It is that deletion of the 3' LTR may not be necessary to generate a functional transducing virus. Initially, a deletion in the 3' LTR was invoked to generate a fusion transcript that is packageable. It was previously thought that the subgenomic env RNAs could not be packaged because they lacked the appropriate packaging sequences (Pugatasch et al., 1983). By analogy, any viral-fusion transcript generated by a subgenomic splice would also not be suitable for virus assembly. There are now at least two cases where subgenomic, spliced mRNA molecules are not only packaged but replicate as viruses (Ikawa et al., 1986; Martin et al., 1986). Thus deletion of the 3' LTR nay not be necessary for transduction if readthrough transcription and splicing can occur such that the subgenomic splice donor and a splice acceptor in the adjacent c-onc are used. This alternative method of transduction would not be very efficient. Transcriptional readthrough of the 3' LTR of integrated proviruses accounts for less than 10% of all viral transcription (Herman et al., 1986). In addition, the spliced subgenomic viruses described above grow to low titers suggesting that the packaging efficiency is impaired. The low frequency at which readthrough transcripts are synthesized and their impaired packageability make them plausible, but unlikely candidates for intermediates in the transduction process.

It has been difficult to rigorously test aspects of this model since there are few, if any, experimental systems available. Reconstitution experiments done in tissue culture have usually been facilitated by homologous sequences and are not directly comparable to the above mechanism. There are at present few animal host systems which have been shown to transduce cellular oncogenes. The first is the ALV induced erythroblastosis system which transduces the oncogene c-erb B in 25 to 50% of the leukemia samples analyzed (Chapter 4 and Miles et al., 1985). This system has been exploited for the purpose of understanding the mechanism of transduction, and the results are discussed elsewhere. Feline leukemia virus (FeLV) associated lymphomas also frequently contain transduced c-myc genes (Levy et al., 1984; Mullins et al., 1984, Neil et al., 1984). These transductions are only observed in naturally occurring lymphomas and not in lymphomas resulting from injection of FeLV. Their analysis is consistent with the transduction scheme outlined above. In terms of c-myc activation, only very short truncated regions of exon 1 of c-myc is retained in the transduced viruses, supporting the idea that exon 1 plays a regulatory role in c-myc expression (Stewart et al., 1986).

The observation that the same cellular oncogene is transduced in several independently isolated acute transforming viruses suggests that these genes are indeed important in oncogenesis (cf. Bishop, 1983, for complete list). It is unclear how these overexpressed and potentially mutated cellular genes, be they insertionally activated or virally transduced, transform cells. Several things should be kept in mind when considering this question. First, the type of tumor produced and the specific cell types which are affected by expression of the oncogenic protein. Second, the actual oncogenic protein itself should be identified and its biochemical properties determined. Third, mutants altered in either their transforming ability or biochemical properties should be analyzed since they may provide further insight into the function of the oncogenic protein. Finally, comparison of the mutated oncogene to its normal cellular counterpart should reveal features important in determining its oncogenic potential.

The remainder of this discussion focuses on the erb B oncogene and its transforming ability. Although at least 20 other oncogenes have been identified, studies on erb B have greatly influenced our understanding of how normal cells become transformed. Erb B illustrates one of many ways in which this process may occur.

F. Structure and Function of Viral erb B

v-erb B was first identified as the transforming component of the avian erythroblastosis virus, AEV (Bister and Duesberg, 1979; Lai et. al., 1979; Roussell et. al., 1979). At this time two strains of AEV had been described, strain R (AEV-R) and strain ES4 (AEV-ES4). Both strains were shown to induce a high incidence of erythroblastosis within a short period of time. Each virus transformed fibroblasts *in vitro* and could induce fibrosarcomas if injected intramuscularly (Graf et al., 1976, 1977; Rothe-Meyer et al., 1933). Biochemical comparison of the viral proteins expressed in AEV-R and AEV-ES4 suggested that these two strains were in fact identical (Hayman et al., 1979). The remainder of this discussion will refer to these two virus strains as AEV-R. More recently, another strain of AEV, designated AEV-H, has been isolated (Hihara, et. al., 1983). Like the other strains, AEV-H is capable of inducing erythroblastosis and causing fibrosarcomas

(Hihara et al., 1983). AEV-H has also transduced c-erb B sequences. Its sequence content and biological properties, however, are distinct from AEV-R and AEV-ES4.

The AEV-R genome is 5.3 kb in length. Most of the pol gene and a portion of the gag and env genes of the helper virus have been deleted and substituted by 3 kb of AEV-specific sequence (Bister et al., 1979; Roussel et. al., 1979; Lai et. al., 1979). Two mRNAs are transcribed from the AEV-R genome, a 5.3 kb genomic RNA and a 3.5 kb subgenomic RNA (Anderson et al., 1980). These RNAs code for two distinct proteins (Hayman et al., 1979; Lai et al., 1980; Pawson et al., 1980; Privalsky et al., 1982). $P75^{erbA}$ is synthesized from the genomic RNA and is a gag-related protein of 75 kD. It contains the 5' half of the AEV-R specific sequences, designated as v-erb A. The subgenomic RNA contains the remaining 3' half of AEV-R specific sequences, designated as v-erb B, and codes for a glycoprotein of 68 kD. Molecular cloning of AEV-R proviral DNA verified the nature of the two AEV-specific genes (Vennstrom et al., 1980). V-erb A and v-erb B appear to be distinct since they are derived from cellular sequences (c-erb A and c-erb B) located on different chromosomes and coding for different mRNAs (Roussel et al., 1979; Saule et al., 1981; Symonds et al., 1984).

The AEV-H genome contains only v-erb B sequences (Yamamoto et al., 1984a). Its genome is 7.8 kb in length and contains intact gag and pol genes. A portion of the env gene has been replaced by approximately 2 kb of v-erb B specific sequences. Like the AEV-R genome, two mRNAs are synthesized, a full length genomic RNA and a 3.0 kb subgenomic RNA containing the erb B specific sequences. This subgenomic RNA synthesizes an erb B protein of ca. 78kD.

Both AEV-R and AEV-H have been molecularly cloned and their nucleotide sequence determined (Yamamoto et al., 1984b; Privalsky et al., 1984). Although only a portion of the entire v-erb B sequence from AEV-R has been published (Henry et al., 1985; Sealy et al., 1983), the entire sequence of an AEV-R ts-mutant, ts-134, allows the erb B sequences in AEV-H and AEV-R to be compared (Choi et al., 1986). Both v-erb B alleles contain common intron sequences at their 5' junctions. AEV-H, however, has transduced approximately 50 nucleotides less than AEV-R. Directly adjacent to the c-erb B intron sequence is the 5' terminus of a c-erb B exon. A consensus splice acceptor marks the intron-exon boundary (Henry et al., 1985). This splice acceptor is presumably the one utilized in synthesizing the subgenomic mRNAs in both AEV-H and AEV-R, and this same splice acceptor site is used to generate the insertionally activated c-erb B RNAs (described in Nilsen et al., 1985). Comparison of the remainder of the v-erb B alleles indicate that they are closely related. They differ at 21 nucleotides, 11 of which are conservative point mutations. AEV-R has also suffered an internal in-frame deletion of 21 amino acids near its carboxyl termini. Both AEV-R and AEV-H v-erb B genes appear to be truncated at their carboxyl terminus since their coding region is fused directly to sequences derived from the <u>env</u> gene. These features indicate that AEV-H and AEV-R resemble typical acute transforming viruses. That is, 1) they have transduced only a portion the c-erb B gene, 2) the transduced c-erb B sequences are situated internal to viral sequences, 3) recombination has occurred within intron and exon sequences, and 4) a number of point mutations have accumulated and may contribute to the acute transforming ability.

The v-erb B protein is expressed as a fusion protein which contains 6 amino-terminal amino acids of the gag gene. These sequences are fused directly to v-erb B as a result of subgenomic splicing and maintain the correct translational reading frame. In AEV-H, the subgenomic RNAs can be alternatively spliced in a manner similar to that described for the insertional activation of c-erb B (see Nilsen et al., 1986), and therefore can produce two different proteins. Use of a viral splice acceptor and a cryptic splice donor 159 nt away results in the addition of 53 amino acids of env. AEV-H and AEV-R contain 546 and 567 v-erb B codons, respectively. Both terminate in stop codons immediately 3' to the erb B-viral junction. These codons are out of frame with respect to the env coding sequence and therefore are not true fusion proteins. Comparison with the insertionally activated c-erb B coding sequence indicates that AEV-H and AEV-R lack the last 34 and 74 amino acids of erb B, respectively. Primary translation products of ca. 61,700 MW and 69,000 MW are predicted for AEV-R and AEV-H, consistent with the 62 kD and 67 kD erb B related proteins detected in immmunoprecipitates of AEV-R and AEV-H infected cell extracts (Privalsky and Bishop, 1982; Hayman et. al., 1983, Privalsky et. al., 1983).

The deduced amino acid sequence of the v-erb B gene predicts a transmembrane glycoprotein (Privalsky et al., 1983, 1984). A 23 amino acid transmembrane domain separates the amino-teminal extracellular domain (77 amino acids) from the cytoplasmic domain (430 or 473 amino acids). The orientation in the plasma membrane has been verified using antibodies specifically directed against the amino-terminus (Schatzman et al., 1986). Synthesis of the erb B protein occurs on membrane bound polysomes and is transported from the rough endoplasmic reticulum to the cell surface (Hayman et al., 1984; Beug et al., 1984). The extracellular domain contains several sites for N-linked glycosylation. Experiments using glycosylation inhibitors indicate that the v-erb B polypeptide from AEV-R is synthesized as a 62.6 kD precursor and is modified to a 66 kD and 68 kD intermediate form before becoming fully glycosylated to the 74 kD erb B protein. Correct glycosylation of v-erb B does not appear to be important in either subcellular routing or transformation since selective expression of the glycosylation intermediates results in transformation (Schmidt et al., 1985). Cell surface expression of v-erb B does appear to be important in v-erb B function since temperature sensitive (ts) mutants of AEV-R which are unable to incorporate erb B into the plasma membrane at the nonpermissive temperature also are not transforming in fibroblasts (Beug et al., 1984). In general, only a small portion of the v-erb B protein actually reaches the cell surface. This is presumably due to the absence of a "consensus" signal sequence in v-erb B as a result of amino-terminal truncation (see below).

The first clue to the function of v-erb B was provided by demonstration of homology between v-erb B and the src gene family of tyrosine specific kinases (Privalsky et al., 1983). Similar enzymatic activities among different oncogenes suggested that they may act by a common mechanism. No v-erb B associated kinase activity was initially detected in AEV-R transformed cells (Hayman et al., 1983; Privalsky et al., 1983; Hayman et al., 1984). Development of more sensitive kinase assays and the use of enriched membrane fractions verified that v-erb B did possess an intrinsic kinase activity both <u>in vivo</u> and <u>in vitro</u> (Gilmore et al., 1985; Decker, 1985; Hayman et al., 1986). In frame mutagenesis which disrupts the structural integrity of the protein kinase domain suggests that kinase activity is essential for v-erb B transforming ability.

v-erb B supplied the link between tyrosine kinases and cell proliferation when it was found to share homology with the human epidermal growth factor receptor (hEGF-R; Downward, et al., 1984; and Ullrich, et al., 1985). The human EGF-R is also a transmembrane protein possessing intrinsic tyrosine kinase activity (cf Carpenter, 1987; Schlessinger, 1986 for reviews). The tyrosine kinase activity in EGF-R however, is regulated by epidermal growth factor (EGF) and results in cell proliferation. Both v-erb B and hEGF-R contain related transmembrane and cytoplasmic sequences. V-erb B lacks the major portion of the extracellular domain responsible for binding EGF. The cytoplasmic protein kinase domain is the most highly conserved region between the two molecules displaying 95% amino acid homology. This striking homology between v-erb B and the hEGF-R suggests that the c-erb B gene is equivalent to the avian EGF-R. This is a widely accepted view, despite the fact that very little is known about the c-erb B gene product or the avian EGF-R. Ligand-stimulated tyrosine kinase activity of hEGF-R is most likely an important event in signalling mitogenesis. Similarly, the transforming ability of v-erb B appears to be related to its tyrosine kinase activity. Therefore, the removal of the amino-terminus from c-erb B is thought to be a necessary step in making it oncogenic. Such an alteration would make the kinase ligand-independent and constitutively active.

Other aspects of the v-erb B protein with regard to its regulation and function have been extrapolated from studies with hEGF-R. The hEGF-R contains three tyrosine residues which are major sites for autophosphorylation. These sites have been mapped to tyrosine residues 1173 (P1), 1148 (P2) and 1068 (P3), all within the carboxy-terminal region of hEGF-R (Downward et al., 1984b). Autophosphorylation at these sites has been shown to enhance the kinase activity of EGF-R (Weber et al., 1984, and Bertics et al., 1985). Tyrosine 1173 is the major site of phosphorylation <u>in vivo</u> and is thought to play an important role in regulation. Analogous tyrosine residues have been located in v-erb B, although phosphorylation at these residues has not been documented. The v-erb B protein does, however, appear to be autophosphorylated (Kris et al., 1985; Gilmore et al., 1985; Hayman et al., 1986; Decker, 1985). The major tyrosine residue (P1) is missing from both viral erb B products and is thought to be relevant in determining oncogenicity (Downward et al., 1984b). An analogous P1 site was identified in the insertionally activated c-erb B gene (Nilsen et al., 1985). Therefore removal of P1 does not appear to be necessary for erythroblast transformation (this topic is discussed in detail in the accompanying chapters).

hEGF-R is also known to be phosphorylated at serine and threonine residues (Carlin et al., 1982; Hunter et al., 1981). Threonine phosphorylation is thought to regulate tyrosine kinase activity. Treatment with 12-0-tetradecanoylphobol-13-acetate (TPA) stimulates protein kinase C resulting in an overall increase in serine and threonine phosphorylation. EGF-R kinase activity is also reduced by TPA treatment, and DNA synthesis is inhibited (Decker, 1984; McCaffrey et al., 1984). Threonine residue 654 is a predominant site of phosphorylation associated with TPA treatment and may negatively regulate tyrosine kinase activity (Hunter et al., 1984; Lin et al., 1986). An analagous threonine residue is present in v-erb B at the same position -- near the cytoplasmic side of the transmembrane domain. The proximity of this residue to the plasma membrane makes it a likely target for regulating receptor activity and mitogenesis. Threonine phosphorylation provides a mechanism of down regulating EGF-R activity and may be important in regulating other cell surface receptors (i.e., the platelet derived growth factor receptor, PDGF-R). The importance of serine phosphorylation in receptor regulation has not been addressed but may be important since it has been shown to play an important role in transmodulation of the adrenergic receptors (cf. Sibley et al., 1985, for review).

Although the biochemistry of the v-erb B gene product has not been extensively characterized, a great deal is known about the properties of AEV-R transformed cells. The primary target cell for erb B activity appears to be the erythroblast, although given the appropriate modifications other cell types can also be transformed (see Chapter 5). The majority of data regarding erythroblast transformation has been obtained from studies using AEV-R, which has transduced v-erb A as well as v-erb B. Although v-erb A is not required for erythroblast or fibroblast transformation, it has been shown to potentiate the transforming ability of v-erb B as well as other oncogenes in the tyrosine kinase family (Frykberg et al., 1983; Sealy et al., 1983b; Damm et al., 1987). This difference is manifested as an inhibition in differentiation and/or increased proliferation between AEV-R and AEV-H (or AEV-R mutants defective in v-erb A) transformed cells (Gandrillon, et al., 1987). The nucleotide sequence of v-erb A shows striking homology to various steroid receptors including the human glucocorticoid receptor (Weinberger et al., 1985), chicken estrogen (Krust et al., 1986), and chicken progesterone receptors Conneely et al., 1986). Recent cloning of the c-erb A proto-oncogene indicates additional homology with the tri-iodothyronine receptor (Sap et al., 1986; Weinberger et al, 1986). The precise role of v-erb A in transformation, however, remains unclear. The transduction of c-erb A sequences in addition to c-erb B sequences into the AEV-R genome further illustrates the cooperation between potentially oncogenic sequences and their importance in selecting for a more malignant phenotype.

The target cell for AEV transformation has been characterized using selective immunolysis of bone marrow cells. Antisera specific for various stages of erythroid cell differentiation reveals that AEV-R exclusively transforms cells at the BFU-E stage (Samarut, 1982). Further characterization of the transformed cells indicate that they display features similar to normal CFU-E and therefore must differentiate before becoming transformed. Transformed erythroblasts synthesize small amounts of hemoglobin, express erythroid specific H5, and contain erythroblast specific cell surface antigens (Beug et al., 1979). Erythroblasts from leukemic animals form CFU-E colonies in semisolid media. Normal bone marrow cells can also induce CFU-E colonies if infected with AEV-R _in vitro_ (Graf, 1973). Colony formation does not require the addition of erythropoietin or other erythroid specific growth factors which may be present in anemic serum (Beug et al., 1982a). This is in contrast to normal CFU-E cells which absolutely require erythropoietin (epo) for colony formation. AEV-R infected erythroblasts are unique from erythroblasts transformed by other viruses in that they can be maintained in culture for prolonged periods. A number of continuous chicken erythroblast cell lines have been isolated from AEV-R transformed cells (Beug et al., 1982b). Expression of v-erb A is presumably responsible for the increased proliferative capacity of these cells. Erythroblasts transformed by v-erb B alone (i.e., AEV-H) require special culturing conditions and a portion of these cells spontaneously differentiate (Beug et al., 1985; Frykberg et al., 1983). Interestingly, embryonic erythroid cells transformed by AEV-R also spontaneously differentiate in culture indicating that v-erb A expression may not be effective during early embryogenesis (Moscovici et al., 1983).

A number of AEV mutants have been isolated and characterized (Graf et al., 1978; Beug et al., 1982b). Most notable is the temperature sensitive mutant, ts-34, which contains a point mutaion in the protein kinase domain (Choi et al., 1986). Erythroblasts transformed by this virus behave similar to normal CFU-E when shifted to the non-permissive temperature (Beug et al., 1980). That is they require erythropoietin to differentiate and display antigenic markers characteristic of terminally differentiated erythrocytes. These cells have been especially useful in studying avian erythropoiesis. The fact that the ts mutation maps to the protein kinase domain further suggests the importance of tyrosine kinase activity in transformation.

Several AEV mutants defective in erythroblast transformation have also been isolated (Royer-Pokora et al., 1979; Beug et al., 1980; Yamamoto et al., 1984a). These viruses retain their capacity to transform fibroblasts. The mutations of two host range mutants, td-359 and td-130, have been mapped to the carboxy-terminal portion of v-erb B. The v-erb B proteins synthesized by these two viruses are severely truncated at their c-terminus. The truncations, howvever, do not extend into the protein kinase domain and therefore should not directly affect kinase activity. The ability of the v-erb B protein to transform one cell type and not another suggests that different configurations and/or interactions at the c-terminus may determine functionally significant differences in kinase activity in specific cell types. Recent identification of other host range mutants further support this notion (see Chapter 5).

AEV-R produces all the phenotypic alterations associated with oncogenic transformation of fibroblasts. These traits include focus forming ability of cell monolayers, elevated levels of hexose transport, increased production of plasminogen activator proteases, and the formation of colonies in soft agar. Similar to the search for kinase activity, special culturing conditions appear to be required to detect these properties. This is in contrast to some of the other tyrosine kinase-related oncogenes which readily transform fibroblasts (Ng et al., 1986; Tracey et al., 1985; Graf, 1973). Although this point is not dealt with in the literature it should be kept in mind when considering the function of erb B in the cell. Although the difference in fibroblast transforming ability may be a subtle one, it is in agreement with the weak kinase activity associated with it. This kinase activity may be less than normal due to inefficient transport of the protein to the cell surface. In either case, the kinase activity in erythroid cells must be sufficient for transformation. It is interesting that other src-related oncogenes can also transform erythroblasts but not any more efficiently than v-erb B (Kahn et al., 1984; Palmieri et al., 1984). Thus the v-erb B molecule may transduce the most efficient proliferation signal in erythroid cells but not in others. This is further supported by the observation that AEV-R can infect and replicate in other hematopoietic cells, but only transforms erythroblasts. The mitogenic signal transduced by erb B, therefore may be specific for certain cell types and is not functional in other cell types. Different cell types may use different signalling pathways since subtle changes in the erb B protein alter its specificity. In view of its homology to a growth factor receptor, namely hEGF-R, there remain only a few possiblilities for what occurs in c-erb B mediated transformation. 1) c-erb B is not the EGF-R, but some other erythroid specific growth factor receptor; perhaps the erythropoietin receptor since AEV-transformed cells are epo-independent; 2) Erythroblastosis results from the inappropriate expression of a truncated EGF-R molecule, such that erb B mimics an erythroid (or cell) specific growth factor receptor; 3) Cell specific factors may regulate the kinase activity of v-erb B and therefore its transforming potential. The oncogenic potential of erb B in this case, would be defined by these factors. The latter two possibilities are not mutually exclusive and appear to be the most likely since hEGF-R is not expressed in murine or human hematopoietic cells.

CHAPTER 1: NONACUTE RETROVIRUS INDUCTION OF AVIAN ERYTHROBLASTOSIS IN DIFFERENT STRAINS OF CHICKENS

Results:

A. Incidence of erythroblastosis in different chicken lines

Susceptibility of erythroblastosis was tested by injecting RAV-1 or RAV-2 (Rous associated virus type 1 and 2), prototype ALVs, into different chicken lines. We routinely injected 100 to 1000 infectious units of virus into the peritoneum of one-day old chicks. Our initial studies focused on line 151 since it had been shown to be highly susceptible to RAV-1 induced erythroblastosis (Bacon et al., 1981; Fung, et al., 1983). Hatchability and egg production of this line, however, is consistently low and has limited the number of eggs available for inoculation at one time. Therefore, several hatchings were required to obtain sufficient numbers for sample collection. Data from similar hatches (hatches occuring within a two to three week time period) are grouped together. In addition to the low number of hatched 151 chicks available, a large number of chicks died of nonspecific lesions within two weeks of hatching. Death due to nonspecific lesions did not appear to be due to virus injection since both uninfected and infected 151 birds died of nonspecific lesions, but not other birds derived from other chicken lines (Table 1) and may be a feature characteristic of this particular inbred chicken line. Eighty to one-

Table 1. Incidence of Erythroblastosis in Different Inbred Chicken Lines

	Chicks Inoculated		Lesions (%)			Average
Inoculum[1]	Strain	Number	Non-Specific	Ery[2]	LL[2]	Latency Period[3] in days
None	151	13	54	0	0	N/A
	151 X 15I4	0	0	0	0	N/A
AEV	151	7	26	100	0	19 (13-24)
	15I5 X 72	10	0	67	0	22 (25-41)
RAV-1	151	49	51	87	0	60 (34-100)
	151	45	46	96	0	65 (53-100)
	151	22	77	100	0	64 (49-85)
	15I5	22	0	23	18	72 (52-90)
	151 X 15I4	21	1	100	0	61 (37-90)
	151 X 15I4	20	0	90	0	66 (55-77)
RAV-2	15I4	35	9	28	0	80 (41-110)
REV	151 X 15I4	25	0	0	0	N/A

[1] Chicks were injected intraperitoneally with approximately 10^3 infectious units of virus at one-day of age.

[2] Ery - erythroblastosis; LL - lymphoid leukosis. Incidence is neglecting death due to non-specific lesions. Experiments were terminated at 15 weeks post inoculation.

[3] Average latent period is expressed as geometric mean. Numbers in parentheses are shortest and longest latency period observed. N/A - not applicable.

hundred percent of the 151 birds which survived nonspecific diseases and were injected with RAV-1, developed erythroblastosis. This level of susceptibility was maintained over a three year period of sample collection.

The low viability of line 151 chicks prompted us to survey other chicken lines. We chose two lines, line 15I5 and 15I4, since they were healthier and maintained a genetic background similar to line 151. These lines are sublines of 15I (this line was identical to 151 until 1941) and differ in their subgroup specificity. Line 15I4 is resistant to subgroup A virus (ie., RAV-1) infection, while line 15I5 chicks are resistant to infection by subgroup B viruses (ie., RAV-2). As expected both lines showed little if any death due to nonspecific lesions, but were only moderately susceptible to erythroblastosis induction (Table 1). Twenty-six percent of 15I4 and twenty-three percent of 15I5 chicks developed erythroblastosis. Line 15I5 was the only line tested in which lymphomas developed.

In an effort to maintain the healthy characteristics of the 15I sublines but select for increased susceptibility to erythroblastosis, we tested the progeny of 151 X 15I4. These chicks are healthy and maintain the high susceptibilty of RAV-1 induced erythroblastosis characteristic of the parental 151 line. Ninety to one-hundred percent of RAV-1 infected 151 X 15I4 chicks developed erythroblastosis when tested over a two year period (Table 1).

The latency of erythroblastosis induction is also shown in Table 1. Latency was determined as the number of days post inoculation required for severe leukemia to develop. In many cases this was identical to the death date. The latencies for all the erythroblastosis varied between 34 and 100 days. The latencies in the more susceptible chicken lines, 151, and 151 X 15I4, averaged approximately 65 days or 8 weeks. Lines 15I5 and 15I4 had slightly longer latencies, 72 and 80 days, respectively. Experiments were terminated at 15 weeks since most of the birds had died of erythroblastosis by this time.

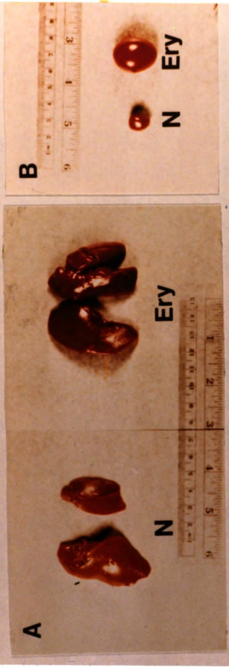
The above description focuses on erythroblastosis induced by ALV. In order to compare this leukemia to that induced by AEV-R, we have injected AEV-R into two different chicken lines. Two week old chicks from either line 151 or a resistant line, line 15I5 X 71, were inoculated with AEV-R. The erythroblastosis induced by AEV-R was pathologically indistinguishable from that of ALV. The kinetics of erythroblast accumulation and anemia induction were also similar. The latency of erythroblastosis development, however, was shorter than that observed for ALV. On average, erythroblastosis developed 19 or 22 days after AEV-R injection. Both lines were susceptible to erythroblastosis induction by AEV-R since all of the 151 and 67% of the 15I5 X 71 birds developed erythroleukemia.

We have used the highly susceptible 151 X 15I4 chicken line to determine whether another class of non-acute retroviruses is capable of inducing erythroblastosis. We chose chicken synctial virus (CSV), a member of the reticuloendotheliosis virus family, since it induces B-cell lymphomas indistinguishable from those induced by ALV (Witter et al., 1979). Of the 25 birds inoculated, none developed erythroblastosis or any other neoplasm within the 15 week test period (Table 1). Thus avian erythroblastosis induction by non-acute retroviruses may be limited to the avian leukosis viruses.

B. Gross lesions

Erythroblastosis is characterized by the uncontrolled proliferation of erythroblasts. The disease originates in the bone marrow, the only site of hematopoiesis in the chicken (Romanoff, 1960), but metastasizes to other tissues at the terminal phase of the disease (see below). As a result, gross morphological changes are observed in the bone marrow, liver, spleen, and occasionally in the kidney. The bone marrow turns red and loses its fat cells. Breakdown of the stromal network is apparent and the consistency of the marrow is increased. The liver becomes enlarged and reddened (Figure 1A Ery, compared with control, N). Enlargement of the spleen (Figure 1B) and kidney are also usually apparent. These organs can be enlarged two to three times that of their normal size. This produces the greatest burden to the animal and is presumably responsible for death. The alterations in gross tissue morphology are characteristic of erythroblastosis and are routinely used as criteria for diagnosis.

Figure 1. Gross lesions of ALV induced erythroblastosis. Liver (A) and spleen (B) from a typical erythroleukemic (Ery) and a normal (N) uninfected control were excised and photographed. Both samples were taken from line 151 chickens at 69 days post-hatching.


A
N
Ery
B
N
Ery

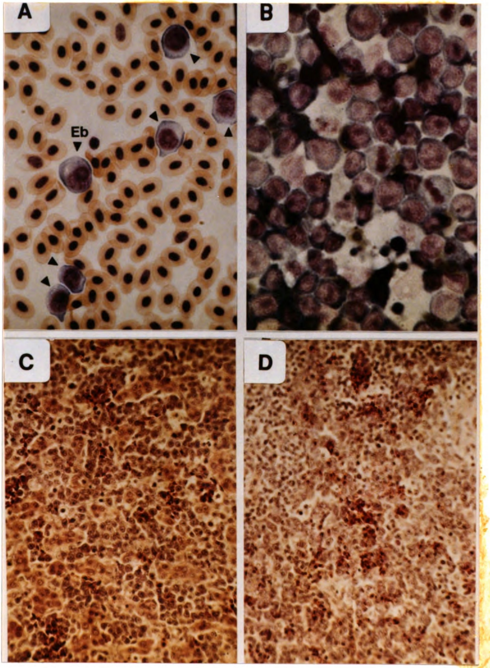
C. Histology and Hematology

Microscopic examination of leukemic tissues indicates that the changes in gross morphology are due to an infiltration of erythroblasts. Erythroblasts are immature erythroid cells and can be distinguished from other blast cells by their basophilic cytoplasm, large round nucleus, prominent nucleoli, and characteristic perinuclear halo. Typical examples are shown in Figure 2A, Eb. Mature erythrocytes, in contrast, are smaller, ovoid in shape, contain a tightly packed nucleus, and show no cytoplasmic basophilia. Histological examination of the liver and spleen from leukemic chickens indicate that the intravascular spaces of the liver and spleen, namely the hepatic sinusoids and splenic red pulp region, are heavily infiltrated with basophilic erythroblasts (Figure 2C and 2D). Similar infiltrations are observed in the bone marrow and bloodstream (Figure 2A, 2B). Erythroblasts comprise only a minor population of cells in normal bone marrow (<1%) and are rarely observed in the bloodstream of uninfected animals (<0.01%; Nelson et al., 1980). However, they are the predominant cell type observed in leukemic bone marrow (Figure 2B) and constitute as much as 90% of all bone marrow cells. The number of erythroblasts circulating in the bloodstram can increase as much as 10,000 fold in severely leukemic chickens.

D. Development of Erythroblastosis

Erythroblastosis development can be monitored by analyzing the blood of infected animals. Differential cell counts of blood smears

Figure 2. Histological examination of ALV induced erythroblastosis. Peripheral blood (A) and bone marrow (B) smears showing erythroblasts (Eb). Infiltration of liver sinuses (C) and the red pulp region of the spleen (D) with basophilic erythroblasts. Panels A and B, 100X; panels C and D, 40X.

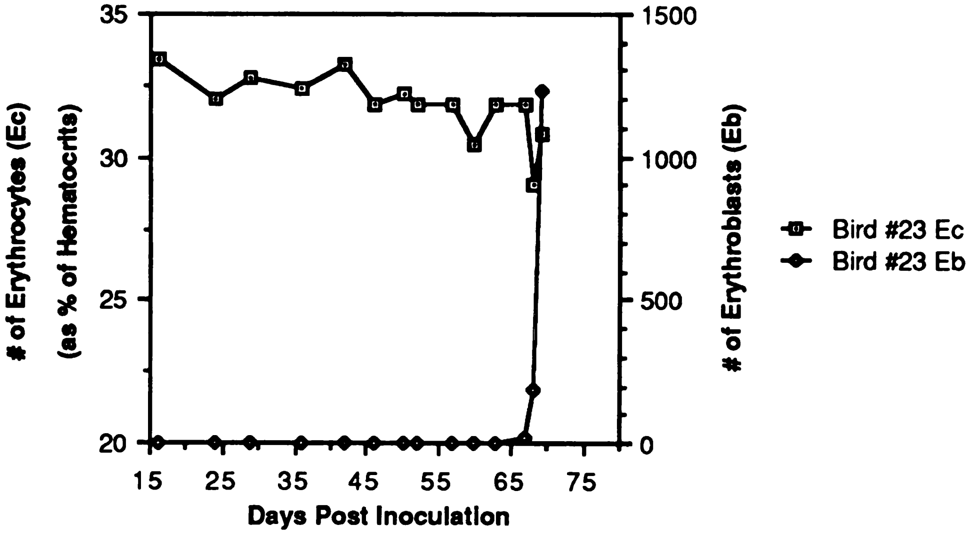

A
B
C
D
Eb

were used to follow the development of the leukemia. Blood samples were taken at regular time intervals after virus inoculation. The number of erythroblasts were scored per 100 white blood cells (WBC). Little if any changes in the white blood cell population could be detected throughout leukemia development. Any gross changes in the percent of packed white blood cells (buffy coat) correlated with an increase in the number of erythroblasts. Examples of typical erythroblastosis development induced by ALV are shown for birds 23 and 63 (circles; Figure 3A, 3B). Erythroblasts are not seen in the circulating bloodstream for the first 67 or 70 days (Figure 3A, 3B, respectively). These numbers are similar to erythroblast counts from uninfected animals. Erythroblastosis develops rapidly such that the number of erythroblasts increase dramatically over a two to five day period. During this period, referred to as the leukemic phase, as many as 1000 erythroblasts per 100 WBC can be observed in the bloodstream. Leukemic animals with erythroblast counts this high usually die within 24 hours. In only one case was there evidence of leukemia regression, and this particular animal relapsed into a fatal leukemic phase within three weeks of the initial appearance of erythroblasts in the bloodstream.

Anemia development was also followed by measuring hematocrits or the percent of packed red blood cells (RBC) per unit volume of blood. Uninfected chickens have a hematocrit of 32% which decreases with age to approximately 30%. The development of anemia closely parallels erythroblastosis development (boxes, Figure 3A). No change in

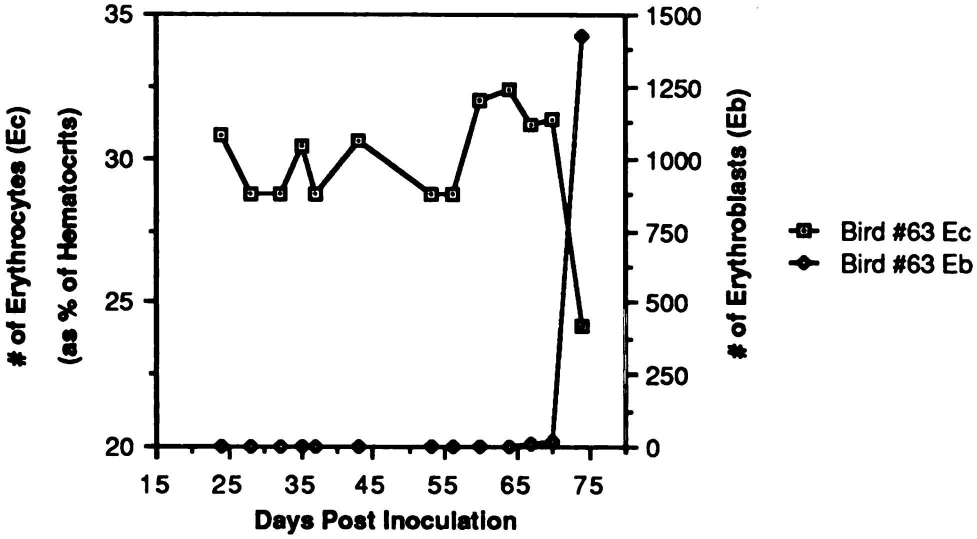
Figure 3. Development of erythroblastosis. The number of erythrocytes (boxes) and erythroblasts (circles) in the circulating blood were measured at regular time intervals. The erythrocyte count is expressed as the percentage of packed red blood cells per unit volume (or hematocrit). A hematocrit of 32% is taken as a standard value for uninfected chickens. Erythroblast counts were determined from peripheral blood smears and scored as the number of erythroblasts per 100 white blood cells. Erythroblasts appeared in the blood of bird #23 (A) with no change in the number of erythrocytes. Bird #63 (B) also developed erythroblastosis with similar kinetics and was accompanied by severe anemia (decrease in hematocrit).

A.



35
30
25
20
1500
1000
500
0
15
25
35
45
55
65
75
Days Post Inoculation
# of Erythrocytes (Ec)
(as % of Hematocrits)
# of Erythroblasts (Eb)
Bird #23 Ec
Bird #23 Eb

B.



35
30
25
20
1500
1250
1000
750
500
250
0
15
25
35
45
55
65
75
Days Post Inoculation
# of Erythrocytes (Ec)
(as % of Hematocrits)
# of Erythroblasts (Eb)
Bird #63 Ec
Bird #63 Eb

hematocrit is observed until erythroblasts appear in the bloodstream. At this time, the percent of packed RBCs rapidly decreases and can reach levels as low as 10%. Anemia is routinely observed in erythroblastosis and accompanies approximately 80% of the leukemias induced by ALV. No significant change in the hematocrit level is observed in the remaining 20% of the ALV induced erythroleukemias (for example see boxes, Figure 3B).

Discussion:

In an effort to characterize the molecular basis of oncogenesis by non-acute retroviruses, we have identified two inbred chicken lines, line 151 and line 151 X 15I4, that are highly susceptible to erythroblastosis induction after injection of RAV-1. The incidences described here are higher than those previously reported and may be due to inoculation at an earlier time. Erythroblastosis samples from line 151 chickens have been extensively characterized (Fung et al., 1983; Raines et al., 1985; Beug et al., 1986; Miles et al., 1985). Indeed AEV-H, was originally isolated from an erythroblastosis sample induced in line 15I (Hihara et al., 1983). We report here the identification of another line, line 151 X 15I4, which is healthier and maintains the high susceptibility phenotype. We have used this line for all of our later studies and have found no variations at either the biological or molecular level.

The high susceptibility to erythroblastosis in line 151 chickens appears to be dominant since only one of the parental strains carries this trait. This observation is supported by backcrosses done in other laboratories (Robinson et al., 1985b). It is somewhat puzzling that two sublines of 15I, namely 15I4 and 15I5 display only marginal susceptibility to erythroblastosis. Progeny of 15I5 X 71 crosses are entirely resistant to erythroblastosis induction by ALV and induce predominantly lymphomas (Bacon et al., 1981). These observations suggest that susceptibility to erythroblastosis is most likely determined by more than one gene.

The only genes which have been implicated in erythroblastosis susceptibility are the B5 and the B15 haplotype of the chicken major histocompatability complex (MHC; Bacon et al., 1981, 1983). B5 and B15 are the two most prevalant haplotypes observed in line 151 and 15I4. Again their influence appears to be indirect since erythroblastosis correlates with recessive B5 alleles or heterozygote B5/B15 alleles. They do not appear to be essential for erythroblastosis induction since two instances have been identified where the animals were homozygous for B13 (Robinson, et al., 1985b). The presence of the B5 or B15 alleles, however, may contribute indirectly to erythroblastosis induction. Other host genes besides the B-haplotype of the MHC must be involved in erythroblastosis susceptibility, although the nature of these genes has yet to be determined.

Lymphoid leukosis (LL) is the predominant neoplasm associated with ALV infection, yet LL was detected in only 4 birds. The latency of erythroblastosis (5 to 14 weeks) appears to be much shorter than that of lymphoid leukosis (4 to 10 months). In most cases, the high incidence of erythroblastosis prevented detection of lymphomas. Assessment of the incidence of lymphomas in the surviving birds is also difficult since they were terminated prior to the reported latency of LL. Indeed, the few cases that were detected had latencies near the termination date (13 weeks). Therefore, the susceptibility of these chicken lines to ALV induced lymphomas remains undetermined.

We have previously demonstrated a strong correlation between ALV induced erythroblastosis and activation of the c-erb B gene (Fung et al., 1983, and Raines et al., 1985). The acute transforming virus AEV-R also induces erythroblastosis and has transduced a portion of the c-erb B gene. The histological and hematological characterization of erythroblastosis induced by ALV and AEV-R suggests that they are similar. That both the cellular and viral erb B genes can induce the same neoplasm suggests they may play similar roles in oncogenesis. The erb B protein has been shown to represent a truncated epidermal growth factor receptor (EGF-R) molecule which retains the intracellular domain (Downward et al., 1984). All activated c-erb B genes insert near a common c-erb B exon. This is also the first exon transduced by AEV-R. This exon marks the beginning of homology between EGF-R and erb B (Raines et al., 1985: Nilsen et al., 1985). Based on these observations we have suggested that interruption of the c-erb B locus at this particular exon is critical to activation of its oncogenic potential. This interruption removes the EGF-binding portion of the molecule and enables the transformed erythroblast to bypass its normal regulatory signals. The result is uncontrolled proliferation of erythroblasts and ultimately the development of erythroblastosis.

In addition to v-erb B, AEV-R contains another oncogene, v-erb A. Mutants of AEV-R suggest that functional v-erb B, but not v-erb A, are required for erythroblastosis induction (Frykberg, et al., 1983 and Sealy et al., 1983). No evidence for the involvement of c-erb A, the cellular homologue of v-erb A, has been detected in ALV induced erythroblastosis (unpublished result). AEV-H, another retrovirus which induces acute erythroblastosis, contains only a v-erb B gene. V-erb A, therefore, does not appear to be required for erythroblastosis induction. Subtle differences in the phenotypes of erythroblasts transformed in vitro by AEV-R and AEV-H, however, have been identified. Both AEV-H and ALV transformed erythroblasts spontaneously differentiate when cultured in vitro. AEV-R transformed erythroblasts, in contrast, maintain their proliferative ability (Beug et al., 1985, Beug et al., 1986). This data suggests that although erb A is not required for erythroblast transformation, it may play a role in arresting erythroid differentiation. These affects are not apparent in the leukemic animal and therefore may not be important _in vivo_. Anemia does not appear to be directly involved in erythroblastosis since it does not always accompany its development. A similar phenomenon has been described for AEV-R (Graf et al., 1976). Therefore, anemia may represent a non-specific response to either ALV infection or erythroblastosis induction. Indeed some ALVs are known to induce anemia with no other apparent lesions (Smith et al., 1982).

Although there are many similarities between ALV and AEV induced erythroblastosis, two major differences remain - latency and lack of strain specificity. The latency for AEV induced erythroblastosis is short and ranges from 13 to 41 days. Erythroblastosis induced by ALV develops between 34 and 100 days, but on the average requires 8 weeks. In addition to its short latency, AEV-R induces a high frequency of erythroblastosis irrespective of chicken strain. ALV induces high incidence erythroblastosis in only two known strains (151, and 151 X 15I4). The difference in strain specificity between AEV-R and ALV is best illustrated by the comparison of erythroblastosis induction in line 15I5 X 71. ALV induces predominantly lympoid leukosis and virtually no erythroblastosis in this chicken line (Bacon et al., 1981, 1983), while AEV-R is 67% susceptible to erythroblastosis induction. This may be a minimal estimate since older, more immunocompetent chicks were injected with AEV-R. The difference in latency and incidence of leukemia induction are what distinguish acute transforming viruses from the non-acute viruses and reflect the different mechanisms by which these two viruses transform cells. AEV-R carries its own oncogene and can readily transform target cells upon infection. Leukemia development results from continuous recruitment of newly transformed cells. Transformation by ALV, on the other hand, is a statistical event, requiring successful infection of the target cell as well as correct proviral insertion within the c-erb B locus. The increased latency and restrictive susceptibility associated with ALV induced erythroblastosis can be explained by the greatly reduced probability of these two events occurring.

The inability of REV to induce erythroblastosis in line 151 X 15I4 chickens is surprising. Although REV resembles a mammalian retroviruses and is unrelated to ALV, it does induce B-cell lymphomas indistinguishable from those induced by ALV (Witter et al., 1979). Therefore ALV and REV should infect common target cells. It is not clear whether REV can infect erythroid progenitor cells, the presumed target cell of this leukemia, since no virus of REV origin has been identified which is capable of transforming erythroblasts. B-cell lymphomas induced by REV and ALV contain activated c-myc genes. Both proviruses use similar mechanisms to augment c-myc transcription (Swift et al., 1985). The structure of the REV genome and the mechanism of c-erb B activation suggest that REV may be unable to direct the synthesis of a functional erb B protein and therefore would not induce erythroblastosis. In erythroblastosis samples, ALV inserts itself into the middle of the c-erb B coding sequence thereby removing the c-erb B initiation codon. A translational start site present in the ALV provirus replaces the normal one. REV does not contain an analogous initiation codon. The activated c-erb B mRNAs resulting from REV insertion would lack a suitable initiation codon and therefore may not be translated. An alternate, more inefficient initiation codon within c-erb B itself may also be used to initiate translation, but in this case, the levels of erb B protein expressed may not be sufficient to transform erythroblasts. The inability of REV to infect the appropriate target cell and correctly activate c-erb B expression are two possible explanations for why injection of REV does not induce erythroblastosis in chickens of known susceptibility. REV based retroviral vectors containing activated c-erb B genes should be useful in testing these two possibilities.

ALV and AEV induced erythroblastosis represents the first example where the same identical neoplasm is induced by an acute and non-acute retrovirus through the action of the same oncogene. Although the modes of activation may differ, the end result is the same - elevated expression of an altered oncogenic gene product and development of the same neoplasm. Although retroviruses carrying other oncogenes can induce erythroblastosis, erb B is only associated with leukemias of erythroid origin (Palmieri et al., 1985 ; Kahn et al., 1984). The specificity of erb B for this cell type suggests that it may act to disrupt an important component of the erythropoietic differentiation machinery.

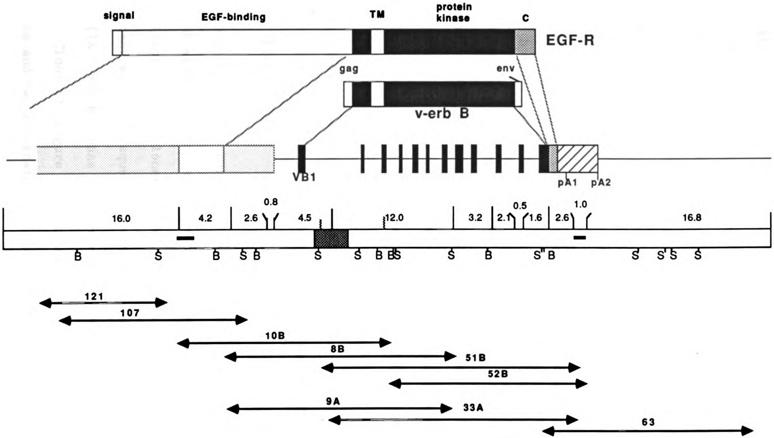
CHAPTER 2: STRUCTURE AND EXPRESSION OF THE CHICKEN C-ERB B GENE.

Results and Discussion:

A. <u>Structure of the chicken c-erb B gene</u>

The chicken c-erb B gene was initially identified by its homology to v-erb B, the transforming gene of the avian erythroblastosis virus (AEV). Isolation and characterization of genomic clones from chicken DNA located this homology to 12 exons contained within a 24 kb region (Vennstrom et al., 1982, and Sergeant et al., 1982). A survey of inbred chicken DNAs, however, suggested heterogeneity in the c-erb B gene (Raines and Lewis, unpublished, and Fung et al., 1983). The most obvious difference was characterized by the presence of three new Eco RI fragments (2.3, 5.1, and 6.2 kb in size) in some birds and not in others. In order to define this heterogeneity more precisely we constructed genomic libraries and isolated a variety of c-erb B containing clones. A summary of the structure of the c-erb B locus deduced from mapping genomic clones is shown in Figure 4. Analysis of these clones indicated that there were two alleles of c-erb B in the chicken, designated the alpha-and beta-alleles. The alpha-allele is identical to that previously described (Vennstrom et al., 1982). The beta-allele, however, contains a 2.5 kb deletion in the intron region downstream of VB1, the first exon homologous to v-erb B. Additional Eco RI restriction enzyme site polymorphisms occur near the deletion

Figure 4. Molecular cloning of the alpha and beta-alleles of c-erb B. Molecular clones of the alpha and beta alleles of c-erb B were isolated from several different lambda phage libraries of line 151 and line 6_3 DNA. The insert regions of the clones are shown by arrows. The Eco Rl restriction map of the alpha allele is shown (vertical lines above open box). Additional Eco Rl sites present in the beta-allele clones (B clones) are marked by dotted lines (above open box). Other restriction enzyme sites (below box) are similar for both the alpha and beta-alleles and define the region of deleted sequences in the beta-allele (heavily dotted box). Coding sequence of v-erb B and the human EGF-R, and various domains are shown for comparison. Regions related to v-erb B (filled boxes) are based on heteroduplex analysis of the alpha-allele (Vennstrom et al., 1982, and Sergeant et al., 1982). Location of VB1, the first exon homologous to v-erb B, was defined by fine restriction mapping of the 4.5 and 2.3 kb Eco Rl fragments of the alpha and beta-alleles, respectively. Regions displaying homology to human EGF-R sequences are indicated: EGF-binding domain, open box; carboxy-terminal region of hEGF-R (C), moderately stippled box; 3′ untranslated region of the insertionally activated c-erb B cDNAs, hatched box; erb B exons upstream of VB1 as defined by clone 167 (see text), lightly stippled box; approximate location of polyadenylation sites, pA1 and pA2. Horizontal bars denote probes used in Figure 6. Restriction enzyme sites shown are Bam H1 (B), Sac I (S), and Eco Rl (vertical lines above open box).



signal
EGF-binding
TM
protein kinase
C
EGF-R
gag
env
v-erb B
VB1
pA1
pA2
16.0
4.2
2.6
0.8
4.5
12.0
3.2
2.1
0.5
1.6
2.6
1.0
16.8
B
S
B
S
B
S
S
B
B
S
S
B
S
B
S
S
S
S
121
107
10B
8B
51B
52B
9A
33A
63

and further downstream. The deletion and restriction enzyme site polymorphisms account for the additional Eco RI fragments observed in certain chickens and not in others. The beta-allele appears to be functional since chickens homozygous for this allele have been identified (Raines and Lewis, unpublished, and Miles et al., 1985). Insertional activation of the beta-allele of cerb B has also been observed in ALV induced erythroblastosis (Raines et al., 1985).

The c-erb B locus is now known to extend beyond the homology defined by v-erb B. The first indication of this was provided by the demonstration of homology between the verb B gene and the human epidermal growth factor-receptor (EGF-R). The region of v-erb B homology spans a small portion of the extracellular domain of the EGF-R and extends through the transmembrane domain into the cytoplasmic domain. V-erb B lacks the last 74 amino acids of the EGF-R (Privalsky et al., 1984). The striking homology between the protein kinase domain of EGF-R and v-erb B (98%) strongly suggests that c-erb B is identical to the chicken EGF-R.

In an attempt to establish this identity we have characterized both the structure and expression of the normal c-erb B locus. Analysis of genomic clones extending beyond the 3′ end of c-erb B is consistent with the sequence analysis of the insertional activated c-erb B (IA c-erb B) cDNAs (Nilsen et al., 1985; and Goodwin et al., 1986). Clone 63 (Figure 4) contains a large portion of untranslated sequence and two alternate polyadenylation sites, pA1 and pA2. The use of both of these polyadenylation signals has been verified using S1 analysis of IA c-erb B containing and normal RNAs (data not shown). As predicted by the IA c-erb B sequences, the genomic locus also contains sequences encoding the additional 74 amino acids not present in the c-terminus of v-erb B. These carboxyl terminal sequences share 60% homology to the human EGF-R. Most notable is the the conservation of three tyrosine residues in c-erb B corresponding to the major autophosphorylation sites of the EGF-R (Downward et al., 1984b; Ullrich et al., 1984, Nilsen et al., 1985). The conservation of c-terminal regulatory sequences in the c-erb B gene provides further evidence in support of c-erb B being the chicken EGF-R.

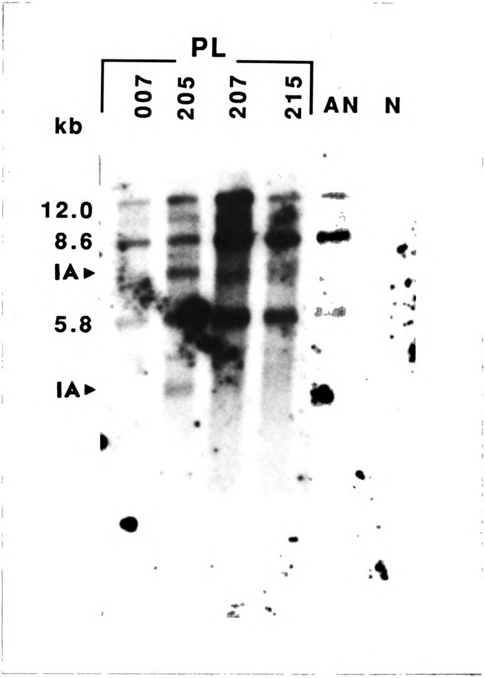
The primary difference between EGF-R and c-erb B is the presence of a large extracellular EGF-binding domain at the amino-terminus of EGF-R (Downward et al., 1984b; Ullrich et al., 1984). It has been proposed that the normal c-erb B gene also encodes a similar amino-terminus, but that removal of the ligand binding domain is necessary for oncogenesis. If this hypothesis were true, sequences 5' of c-erb B should be homologous to EGF-R. Since VB1 defines the point of divergence between c-erb B and EGF-R, we have focused our studies on sequences 5' of this exon. As an initial attempt to identify homology, we probed genomic clones spanning the region 5' of VB1 (clones 10B and 107) with an EGF-R cDNA probe specific for the ligand-binding domain (LBD probe). Only a 4.2 kb Eco RI fragment hybridized at low stringency. Subsequent experiments, however, revealed additional hybridization 5' of this Eco RI fragment but not in the 3' 2.6 and 0.8 kb Eco RI fragments (Raines and Callaghan, unpublished) suggesting a very large intron region between VB1 and the next 5′ exon (EB-1). The homology in this region was not comparable to that in the v-erb B region suggesting that perhaps this region is more divergent. This is somewhat surprising in view of the fact that chicken cells contain a receptor which binds murine EGF and therefore should contain a similar ligand binding domain (Kris et al., 1985). Alternatively, this result could indicate that c-erb B is not the EGF receptor but some other related growth factor receptor. The weak and spurious homology between the two probes could be explained by the presence of a common cysteine-rich region in hEGF-R. This clustering of cysteine-residues is a motif common to other receptor molecules (Carpenter, 1987).

The Drosophila EGF-R (DER) has recently been cloned and sequenced (Schejter et al., 1986; Livneh et al., 1985;). Although the kinase domain displays 55% homology, the extracellular domain is more divergent (33%). The similarity in the extracellular domain was comparable to that observed between DER and the human NEU gene, another human receptor molecule distinct from hEGF-R (Coussens et al., 1985; Yamamoto et al., 1986). Interestingly DER does not bind murine EGF and was cloned using v-erb B as a molecular probe. The physiological function of this Drosophila receptor molecule has yet to be identified as well as its ligand. If one assumes that a receptor is named based on the ligand it binds, then DER has been inappropriately assigned a name based on sequence homology. The same remains to be seen for the avian c-erb B gene which appears to be the most homologous to hEGF-R.

B. Expression of the chicken c-erb B locus

In order to define the exons and extracellular coding region of c-erb B more precisely, we have analyzed several different tissue samples for elevated c-erb B expression. Of the limited number of normal tissues screened, low levels of c-erb B expression were consistently observed (data not shown). These results were consistent with levels of c-erb B expression reported by others (Gonda et al., 1982). Liver RNA from leukemic birds that were prematurely sacrificed exhibited the highest levels of normal c-erb B expression (Figure 5, PL lanes). A ten to twenty-fold increase in expression is observed when compared to normal uninfected liver samples (Figure 5, lane N liv). Transformed erythroblasts at this stage of leukemia development are confined almost exclusively to the bone marrow and have not yet metastasized to the liver (see Chapter 6 for a detailed description of these samples). This is verified by detection of very low levels of the 3.6 and 7.0 kb IA c-erb B RNAs in these samples (Figure 5, lane 205; cf. Nilsen et al., 1985 and Goodwin et al., 1986, for a detailed description of these RNAs). The nature of the apparent elevation in c-erb B expression is unclear. Analysis of anemic liver RNA (Figure 5, lane AN) also reveals a moderate elevation in c-erb B expression suggesting that perhaps induction of c-erb B is related to hematopoietic stress induced by either successive bleeding or erythroblast transformation. In this case c-erb B expression may be elevated by production of its ligand and subsequent down regulation of the erb B receptor. An analagous down modulation of EGF-R by EGF

Figure 5. *Northern blot analysis of c-erb B related RNAs in preleukemic and uninfected liver tissue.* Poly (A)+ RNA was isolated from liver tissues of either ALV infected chickens prior to the development of erythroleukemia (PL samples, see Chapter 6 for description), normal uninfected chicken (N), or anemic chickens (AN). 5 ug of poly (A)+ RNA was fractionated on 1% formaldehyde agarose gels and electrotransferred to GeneScreen (New England Nuclear Corp., Boston, Mass.). A 1.7 kb Apa I-Sac I fragment from the insertional activated c-erb B cDNA was nick-translated and used as an erb B specific hybridization probe (for description see Figure 11, probe T). Hybridization and washing were as previously described (Raines et al., 1985). The size of the transcripts were determined relative to the mobility of ^{32}P-labelled, Hind III digested lambda DNA markers. The location of the 7.0 and 3.6 kb erb B related transcripts typical of insertionally activated c-erb B leukemia samples are indicated by arrows.

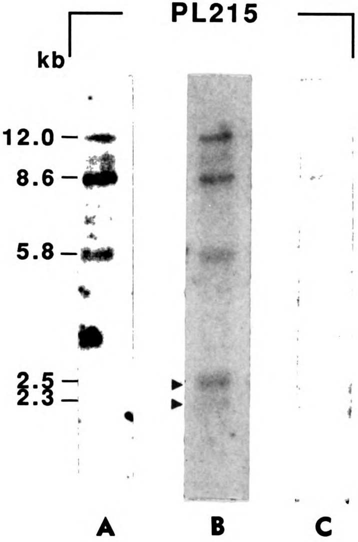

PL
007
205
207
215
AN
N
kb
12.0
8.6
IA ▸
5.8
IA ▸

treatment has been shown to increase the level of EGF-R mRNA (Clark et al., 1985).

Three c-erb B related RNAs, 5.8, 8.6, and 12.0 kb in size, were detected in all of the samples analyzed. This is in contrast to the c-erb B RNAs reported by others (Vennstrom et al., 1982; Gonda et al., 1984; Nilsen et al., 1986). In order to identify potential differences in coding sequences we hybridized normal RNA to a probe specific for erb B RNAs terminating at the second polyadenylation site (probe C). This probe hybridized to only the 8.6 and 12.0 kb c-erb B RNAs (Figure 6C) and not to the 5.8 kb RNAs (Figure 6A) suggesting that the 5.8 kb RNA terminates at the first polyadenylation signal and the 8.6 and 12.0 kb RNAs terminate at pA2. The difference in size between the 5.8 and 8.2 kb RNAs is consistent with the use of similar promoters and different polyadenylation signals. We assume the difference between the 8.6 and 12.0 kb is due to a difference in 5′ c-erb B sequences since they both contain similar 3′ untranslated sequences.

In order to document transcription of sequences 5′ to VB1, we hybridized Northerns to a 0.6 kb Eco RI-Pst I genomic fragment (RP probe). This was the smallest genomic fragment identified which hybridized to the hEGF-R derived LBD probe, and therefore was expected to produce the best hybridization signals. All three c-erb B related transcripts hybridized to the RP probe suggesting that this fragment did indeed contain exon sequences (Figure 6B). Two new size classes of RNAs, 2.3 and 2.5 kb, were detected with the RP probe. These transcripts appear to hybridize exclusively to sequences upstream of

Figure 6. Northern blot analysis of normal c-erb B RNAs. Northern analysis of poly (A)+ RNA (5ug) from preleukemic sample 215 was performed using three different hybridization probes: a) a 1.7 kb Apa I-Sac I fragment identical to the erb B specific probe in Figure 5; b) RP probe, a 0.6 kb Eco R1-Pst I fragment derived from a portion of a 4.2 kb Eco R1 fragment of genomic DNA (see Figure 4, vertical bar, and text); and c) a 1.0 kb Eco R1 fragment of genomic DNA situated 3' of the first polyadenylation signal of c-erb B (Figure 5, vertical bar).


PL215
kb
12.0
8.6
5.8
2.5
2.3
A
B
C

VB1 since they were not detected with erb B specific probes. The levels of expression of these novel transcripts is similar to the other erb B related RNAs in both preleukemic and normal tissues suggesting that they may be coordinately regulated.

The presence of multiple c-erb B related transcripts suggests that the c-erb B transcription unit is a complex one. The differential hybridization described above suggests that more than one promoter is utilized and alternate polyadenylation is involved, as well as possible alternate splicing. This is slightly different from the hEGF-R which produces only two transcripts in normal cells, 5.6 and 10.5 kb (Ullrich, et al., 1984; Ishii, et al., 1984; Merlino, et al., 1985). These RNAs have been shown to differ in their 3' untranslated sequence. No transcripts analagous to the 12.0 kb c-erb B RNA have been detected in human tissues. Most of the hEGF-R studies have been done in A431 cells, an epidermoid carcinoma cell line, which contains an amplified EGF-R gene. An abherrant 2.9 kb EGF-R RNA is observed in A431 cells. This RNA hybridizes exclusively to the ligand-binding domain of EGF-R and encodes a secreted protein (Lin et al., 1984; Merlino et al., 1985). The detection of an analagous transcript in normal as well as leukemic chicken tissues suggests that c-erb B may be alternately processed such that the smaller 2.3 and 2.5 kb transcripts encode a secreted ligand-binding domain. Further hybridization experiments, cDNA cloning and sequence analysis should determine whether this gene is related to c-erb B, or encodes another closely related gene.

C. cDNA cloning of the extracellular domain of the chicken c-erb B related RNAs

RNA from one of the preleukemic liver samples (sample 215) was used to synthesize a cDNA library in lambda gt-10. We selectively enriched for erb B sequences upstream of VB1 by using a synthetic primer complementary to sequences within the transmembrane domain of IA-cerb B (Figure 7A). Approximately 100,000 clones were screened with the RP probe and yielded three positive clones. Restriction mapping and Southern hybridization indicated that one clone (clone 166) contained an 80 bp insert, while the other two clones (clones 165 and 167) contained 1.6 and 2.0 kb Eco RI inserts, respectively. The restriction enzyme maps of these two clones are shown in Figure 7B. Based on the restriction enzyme map and hybridization data, clone 167 was the only clone which contained erb B sequences. The remainder of our studies have focused on the characterization of this clone since it overlapped with erb B sequences and was the largest clone identified.

The insert DNA was used to probe southern blots of both genomic DNA as well as DNA from the genomic lambda clones. The pattern of hybridization on Eco RI restricted DNA from clones 10B, 107, and 121 is shown in Figure 8A. This pattern is consistent with the presence of erb B sequences since both the 2.3 and 5.1 kb Eco RI fragments of the beta-allele of c-erb B are detected. In addition all Eco RI fragments situated 5' of VB1 also hybridize. These include the 0.8, 2.6, and 4.2 kb fragments as well as other 5' sequences. This is in contrast to our initial analysis using the hEGF-R LBD probe which only detected the

Figure 7. Selective cDNA cloning of c-erb B sequences located 5' of VB1. A). Nucleotide sequence of a 25 residue synthetic oligonucleotide primer used to prepare a lambda gt-10 cDNA library from poly (A)+ RNA (2ug) from PL215 liver tissue. The complementary c-erb B sequence was taken from Nilsen et al., (1985), and situated 236 nt downstream from the beginning of erb B coding sequences. B). Restriction enzyme map of two c-erb B cDNA clones (167 and 165) obtained from the PL 215 cDNA lambda library. Clones are aligned based on common restriction enzyme sites. The erb B related region is represented by a solid box. The restriction enzymes shown include: A, ApaI; B, Bam H1; Sp, Sph I; and St, Stu I.

A.

```
      237                       261
      |                         |
5'    CCTGTGCCTGGTTGTGGTTGGTCTA     3'   cDNA sequence
3'    GGACACGGACCAACACCAACCAGAT     5'   synthetic primer
```

B.

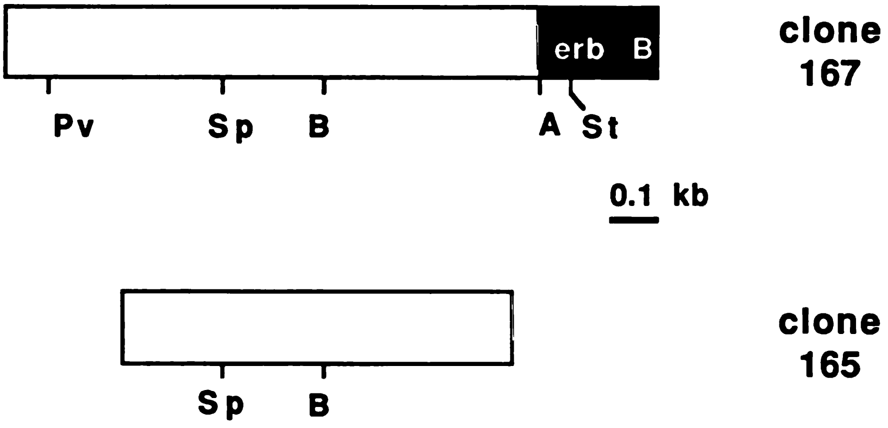
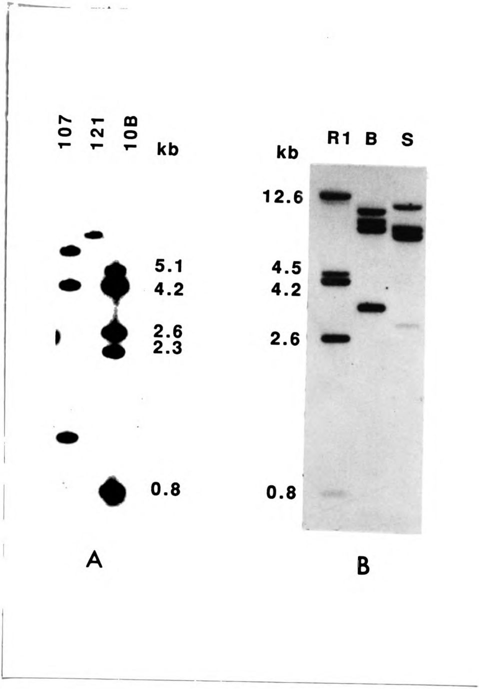


Figure 8. Southern blot analysis using cDNA clone 167. The 2.0 kb insert from clone 167 was gel purified, nick-translated, and used to probe Southern blots containing genomic lambda DNA clones (A), or genomic chicken DNA (B). Lambda phage DNA was prepared from clones 10B, 107, and 121 (see Figure 4). Cloned inserts were liberated by digestion with Eco R1 (10B) or Eco R1 and Sal I. Genomic DNA was extracted from an uninfected chicken tissue and was digested with either Eco R1 (R1), Bam H1 (B), or Sac I (S). The digested DNAs were electrophoresed, transferred to nitrocellulose and probed with the insert from clone 167. Molecular weights were based on relative mobility compared to Hind III/Eco R1 digested lambda DNA markers. The 2.3 kb and 5.1 kb bands hybridizing to clone 10B correspond to the beta-allele of c-erb B (see Figure 4, and text). The genomic chicken DNA is homozygous for the alpha-allele, and therefore recognizes only the 4.5 kb and 12.0 kb bands of c-erb B.


107
121
10B
kb
5.1
4.2
2.6
2.3
0.8
A
kb
R1
B
S
12.6
4.5
4.2
2.6
0.8
B

4.2 kb Eco RI fragment in these clones. A similar hybridization pattern was obtained when the 167 insert of clone 167 was hybridized to Eco RI digested genomic DNA (Figure 8B). Therefore the sequences present in clone 167 correspond to c-erb B sequences and span the region 5′ to VB1. These sequences appear to be derived from a single locus since no additional hybridization was detected in genomic DNA aside from that which can be accounted for by c-erb B (figure 8B).

We have recently subcloned the insert from this clone into the Bluescript phagemid vector for subsequent sequence analysis. Preliminary sequencing data indicate that clone 167 begins precisely at the site of the synthetic oligonucleotide primer (and therefore, contains 260 nt of erb B) and extends 5′ into c-erb B and the extracellular sequences. Further sequence analysis should indicate whether the c-erb B gene is homologous with the ligand-binding domain of the hEGF-R.

Based on the size of the 167 clone, we estimate that approximately 1.0 kb of 5′ c-erb B sequence is missing, a portion of which is probably coding. Recent immunoprecipitation data suggests that the primary translation product of c-erb B is 150 kD, 30 kD larger than the hEGF-R (N. Maihle, unpublished). If this estimate is correct, approximately 800 bp of the c-erb B coding region is missing in clone 167. C-erb B coding sequence does appear to be present since a portion of this clone can be expressed as a fusion protein in bacteria. We are currently making antisera in order to further characterize the nature of the extracellular domain of the c-erb B gene product.

The identification of c-erb B sequences 5′ to VB1 support the idea that amino-terminal truncation is necessary for activation of the oncogenic potential of c-erb B. Activation of c-erb B, then, results in both qualitative and quantitative changes in its expression. The fact that it is a membrane associated tyrosine-specific kinase makes it a likely candidate for signalling proliferative responses in normal and transformed cells. The remaining chapters deal primarily with the mechanism of c-erb B activation and variations on that theme. In most cases the c-erb B gene is assumed to be the chicken analogue of the hEGF-R. Although this assumption may not be correct, the concept remains the same; truncation and ligand-independence of the normal receptor can lead to constitutive enzymatic activity and oncogenesis.

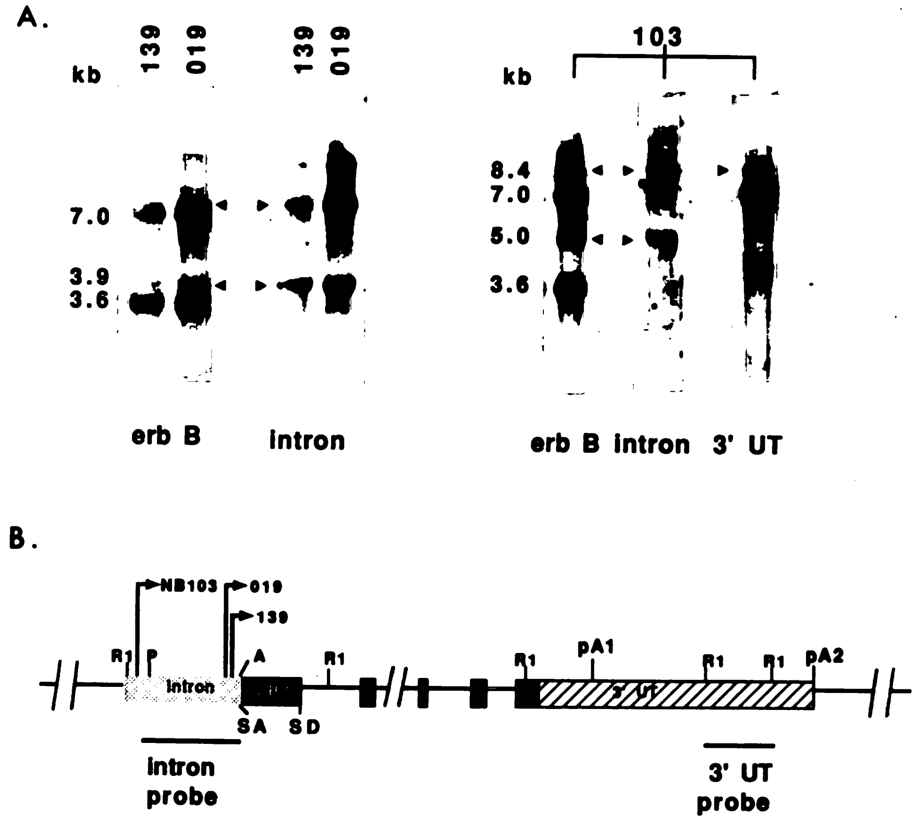
CHAPTER 3: TRANSCRIPTION FROM THE 3′ LONG TERMINAL REPEAT OF INTEGRATED ALV PROVIRUSES

Results:

A. Identification of two novel erb B related RNAs in ALV induced erythroblastosis

We have previously analyzed the RNA from two ALV induced erythroblastosis samples containing insertionally activated c-erb B genes. These samples contain two size classes of c-erb B mRNA, a 3.6 and 7.0 kb, which differ primarily in their 3′ untranslated regions (Nilsen et al., 1985; Goodwin, et al., 1986). In an effort to extend these initial findings we have analyzed c-erb B expression in six other ALV induced erythroblastosis samples known to contain insertionally activated c-erb B (IA c-erb B) alleles. Hybridization of Northern blots with an erb B specific probe was consistent with our initial findings. A 3.6 and 7.0 kb erb B mRNA was detected in all of the leukemic RNA samples analyzed (Figure 9A). Upon closer examination, other minor erb B related transcripts could be seen (Figure 9A, arrow). This RNA is a minor erb B species in samples 139 and 019 and comprises approximately 2 to 5% of the total erb B related RNAs. Sample 103 is unique in that two novel erb B species are present which constitute as much as 25% of all of the erb B mRNAs.

Figure 9. Detection of novel c-erb B related RNAs in leukemic samples containing insertionally activated c-erb B genes. Poly (A)+ RNA was isolated from three ALV induced erythroblastosis samples (019,139,103) known to contain insertionally activated c-erb B alleles (see Raines et al., 1985). Poly (A)+ RNA (5ug) was fractionated on 1% formaldehyde agarose gels and electrophoretically transferred to GeneScreen (New England Nuclear, Inc., Boston, Mass.). The blots were probed with sequences specific for erb B, 5' intron sequences, or 3' untranslated sequences (panel A). The erb B specific probe has been previously described (Figure 5), and contains 1.7 kb of erb B coding sequences. The intron specific probe and 3' untranslated probes (3'UT) are diagrammed in panel B. The intron probe is a 1.4 kb Pst I-Apa I fragment located upstream of the first exon homologous to v-erb B (designated VB1). It spans the region where the ALV provirus is known to insert (dotted boxes). The approximate site of integration for ALV proviruses in leukemic samples 019, 139, and 103 are indicated by arrows, and are taken from Raines et al. (1985). The 3' UT probe is a 1.0 kb Eco R1 fragment containing untranslated sequences (hatched box) downstream of the first polyadenylation site of the c-erb B gene (pA1). pA1 and pA2 are identical to the sites defined by Goodwin et al., (1986). Restriction enzyme sites shown include: A, Apa I; P, Pst I; RI, Eco RI. Solid boxes represent coding exons.


A.
kb
139
019
139
019
7.0
3.9
3.6
erb B
intron
103
kb
8.4
7.0
5.0
3.6
erb B
intron
3' UT
B.
NB103
019
139
R1
P
intron
A
R1
SA
SD
R1
pA1
3' UT
R1
R1
pA2
intron
probe
3' UT
probe

Initially we thought that these minor erb B related RNAs represented expression of the normal uninterrupted cerb B gene. This, however, did not appear to be the case since the sizes of the minor erb B RNAs differed from the sizes of the normal c-erb B transcripts identified in uninfected tissues (Vennstrom et al., 1982; Gonda et al., 1982; Goodwin et al., 1986). The sizes of the additional erb B transcripts were different in various leukemic samples and ranged from 3.8 kb to 5.0 kb. Our previous DNA analysis revealed that the approximate size of the novel transcripts correlated with its proviral integration site within the c-erb B gene. Both samples 139 and 019 contain ALV proviruses inserted within 400 bp of the first exon of c-erb B (designated VB1 since it is the first exon showing homology to the v-erb B oncogene). These samples contain a similar 3.9 kb novel erb B RNA. Sample 103, on the otherhand, contains an ALV provirus inserted approximately 1.3 kb 5′ of VB1 and synthesizes a 5.0 kb novel RNA species. Thus samples containing proviruses inserted close to VB1 consistently contained smaller erb B transcripts than those known to contain proviruses inserted further upstream of VB1.

The above observations suggested that the difference in size between these novel erb B transcripts and the 3.6 kb erb B RNA could be due to the presence of intron sequences. To test this possibility, we hybridized parallel northern blots with a probe specific for the intron region 5′ to VB1 (Figure 9A and 9B). Indeed the novel 3.9 kb erb B RNA of samples 139 and 019 comigrate with an RNA containing c-erb B intron sequences. Similarly, the 5.0 and 8.4 kb erb B RNAs unique to sample

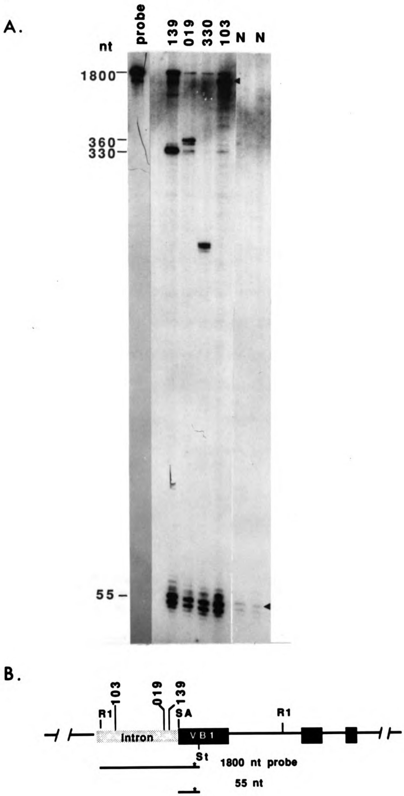
103 also hybridized with the intron specific probe. The intron specific probe detected an additional 7.2 kb RNA species in samples 139 and 019. Detection of this species with an erb B probe may have been obscured by the predominance of the 7.0 kb erb B related mRNA.

The size difference between the two intron containing RNAs of all three samples was similar to that seen between the 7.0 and 3.6 kb RNAs. Since the latter two RNAs were known to differ in their 3′ untranslated regions, we hybridized a blot containing sample 103 to a probe which would distinguish between the untranslated region present in the two erb B transcripts (Figure 9B). Only the novel 8.4 kb and 7.0 kb erb B transcripts were detected suggesting that the smaller 3.6 kb and 5.0 kb erb transcripts utilize the first polyadenylation signal of c-erb B (pA1) while the other larger transcripts terminate at the second polyadenylation site (pA2; Figure 9A).

B. Transcription of intron sequences in insertionally activated c-erb B samples

S1 analysis was used to determine the precise amount of intron sequence present in the novel erb B transcripts. A 1.9 kb Eco R1-Stu I fragment spanning the intron region was labelled at the 5′ Stu I site and used to probe leukemic RNA samples. The Stu I site is located 55 nt 3′ to the splice acceptor site of VB1 (Figure 10B), therefore the labelled probe should hybridize to all erb B related mRNAs. A 55 nt S1 resistant fragment is detected in all leukemic samples as well as in uninfected tissues (Figure 10A), and indicates removal of intron

Figure 10. S1 nuclease analysis of erb B related RNAs in erythroleukemic and normal tissues. (A) A 5' end-labelled Eco R1-Stu I fragment was hybridized to 1 ug or 5 ug poly (A)+ RNA from several erythroleukemic samples (139 to 103), or normal uninfected (N) tissues, respectively, and digested with S1 nuclease. S1 nuclease resistant fragments were separated on a 5% denaturing polyacrylamide gel, and detected by autoradiography. Undigested probe is also shown (probe). (B) Diagram of the 1.8 kb Eco R1-Stu I fragment used as probe in (A). This fragment contains 55 nt of VB1, including the splice acceptor (SA), and 1.7 kb of upstream intron sequences (dotted box). The star denotes location of radioactive label. The location of inserted ALV proviruses in erythroleukemic samples 103, 019, and 139 are designated designated as arrows and are taken from Raines et al., (1985). Restriction enzyme sites are RI, Eco RI; St, Stu I.


A.
nt
probe
139
019
330
103
N
N
1800
360
330
55
B.
R1
103
019
139
SA
Intron
VB1
R1
St
1800 nt probe
55 nt

sequences due to splicing. Additional nuclease resistant fragments are present in each leukemic sample and are larger than the 55 nt fragment. This observation verified our initial results suggesting that the novel erb B related RNAs contain intron sequences.

The size of the nuclease resistant fragment should provide an estimate of the amount of intron sequence transcribed. Sample 139 and 019 protect a 330 and 360 bp fragment while sample 103 protects a 1.3 kb fragment. Interestingly, this amount of intron sequence corresponds to the approximate site of integration relative to VB1 and can account for the size differences between the 3.6 and 7.0 kb transcripts. The relative intensities of the intron containing fragment and the 55 nt exon fragment, however, do not correlate with the ratios observed in the Northern analysis. We suspect this difference is due to inefficient formation of the 55 nt hybrid relative to the longer hybrids and is not representative of the true abundance of the corresponding RNAs.

Discussion:

Insertional activation of c-erb B results in the expression of two size classes of c-erb B RNAs, 3.6 and 7.0 kb. Synthesis of these RNAs is initiated in the 5′ LTR, proceeds through the entire viral sequence and into the cerb B gene, and terminates at one of two possible polyadenylation sites. Splicing removes all of the erb B introns and most of the viral sequences. The resulting 3.6 and 7.0 kb erb B mRNAs contain 5′ viral sequences linked directly to erb B coding sequences.

The transcripts differ only in the amount of 3′ untranslated sequence present. We report here the detection of novel erb B related transcripts in several other ALV induced erythroblastosis samples which contain insertionally activated c-erb B alleles. These samples contain the previously characterized 3.6 and 7.0 kb mRNAs as well as other novel transcripts. These transcripts are not the predominant erb B mRNAs and are distinct from the previously described transcripts in that they contain intron sequences. The amount of intron transcribed directly correlates with distance between the inserted ALV provirus and the first exon of cerb B (VB1). Two novel transcripts are consistently observed which, like the other erb B mRNAs, differ in their 3′ untranslated regions. Based on these observations we speculate that these novel erb B RNAs are the result of transcription initiated in the 3′ LTR of the inserted provirus. This is in contrast to the 3.6 and 7.0 kb erb B transcripts which are transcribed from the 5′ LTR. 3′ LTR promoted transcripts would readthrough the downstream intron sequence, into the cerb B gene, and terminate at one of the two c-erb B polyadenylation sites (pA1 or pA2). Splicing of these transcripts would remove all the internal erb B intron sequences but not those situated between the inserted provirus and the first exon (VB1). As a result the 3′ LTR promoted transcripts would contain intron sequences and although two size classes of RNAs would be synthesized in a single leukemic sample, the amount of intron sequence present would determine the relative size variability among different leukemic samples.

The level of expression of the novel erb B transcripts in samples 019 and 139 appears to be approximately 2 to 5% of the total erb B mRNAs. The predominant erb B mRNAs present in these samples result from transcriptional readthrough of the 3′ LTR. Other studies indicate that these transcripts account for 5 to 20% of all virus related transcription (see chapter 4). Extrapolation from these estimates indicate that the transcriptional activity of the 3′ LTR is less than 1% of the total viral transcription. This estimate is in agreement with the level of 3′ LTR promoted transcripts from randomly integrated ALV proviruses (Herman et al., 1986). We cannot, however, rule out the possibility that the 3′ LTR promoted erb B RNAs are less stable than those initiated in the 5′ LTR. The inclusion of intron sequences or the absence of additional viral sequences could contribute to RNA instability. This does not seem likely since other 3′ LTR promoted transcripts, namely those of activated c-myc, lack additional viral sequences and are known to be stable. In addition, c-erb B intron sequences are included in all of the erb B containing retroviruses with no apparent affect on their levels of expression (Yamamoto et al. 1984b; Henry et al., 1985; and unpublished).

In contrast to the 019 and 139 samples, the novel transcripts of sample 103 were expressed at relatively high levels compared to the 3.6 and 7.0 kb transcripts. This sample is atypical and differs from 019 and 139 in that the ALV provirus associated with the c-erb B locus is deleted (Raines et al., 1985). Southern analysis indicates that the 103 provirus is missing approximately 1.2 kb of viral sequences, but both LTRs appear to be intact. Northern analysis is consistent with this observation and reveals an aberrant genomic viral RNA of 6.5 kb (unpublished). Although the precise location of this deletion has not been mapped, it does not appear to encompass sequences 5′ of the viral splice donor site (up to nucleotide 326) since the 3.6 and 7.0 kb erb B mRNAs indicate that the transcripts are properly spliced. This internal deletion in the provirus may be responsible for the relative increase in the 3′ LTR promoter activity.

Internal proviral deletions and a subsequent increase in 3′ LTR promotion are not unique to this erythroleukemia sample. Both ALV and REV proviruses inserted in the c-myc locus of B-cell lymphomas are deleted. In most cases the deletions do not affect the LTRs and the 3′ LTR is used to initiate c-myc transcription. Unlike sample 103, no viral transcripts initiated from the 5′ LTR have been detected in B-cell lymphomas (Hayward, et al., 1981; Robinson, et al., 1985; Swift et al., 1985; Ridgeway et al., 1985). These studies suggested that internal deletions within the provirus could suppress transcription from the 5′ LTR.

In vitro studies indicate that retroviruses may contain a positive regulatory element which affects the promoter activity of the 5′ LTR. Transfection experiments using plasmid which contain variable amounts of 5′ viral sequences linked to an indicator gene suggest that the 5′ end of the gag gene (up to nucleotide 532) is required for efficient viral transcription (Norton et al., 1985). The gag gene has also been shown to contain an enhancer sequence 900 nt downstream from the promoter site (Arrigo et al., 1987). Interestingly, the deletion of this sequence inhibits transcription of the 5' LTR but does not totally suppress it. Deletion of these sequences in sample 109 could account for the relative levels of expression of the erb B related transcripts. The apparent enhancement of the novel erb B mRNAs would not be due to increased transcription from the 3' LTR, but rather from the reduction of transcription from the 5' LTR.

The exclusion of 5' LTR promoted transcripts in proviruses associated with activated c-myc genes suggest that other factors must also be important in determining which LTR is predominantly active. Other studies also indicate that certain promoters arranged in tandem are preferentially used. They suggest that epigenetic factors actually select for transcription from one promoter and in doing so supress the activity of the other (Emerman et al., 1984). Changes in chromatin structure may mediate this epigenetic suppression by exposing the preferred promoter and closing the other. In the case of c-myc activation, extremely high levels of c-myc expression may be necessary for oncogenesis. The inefficiency of transcriptional readthrough from viral RNAs initiated in the 5' LTR may not produce sufficient amounts of c-myc RNA to transform B-cells. C-erb B activation, on the otherhand, may not require high levels of expression. Instead the 5' viral sequences may be necessary for expression of a functional protein since these sequences contain the presumed translational start site for erb B (Nilsen et al., 1985). Therefore the use of one LTR over the other may be dictated by the particular oncogene that is activated and the tumor samples which result select for these epigenetic factors.

Insertion of the ALV provirus into the c-erb B locus disrupts synthesis of the normal c-erb B gene product, i.e., the chicken epidermal growth factor receptor (EGF-R) such that its amino terminal sequences are removed. The normal translational start site is presumably replaced by one found in the gag gene. Only truncated c-erb B mRNAs initiating from the 5′ LTR would contain the gag start codon. Another potential initiation codon is located 6 amino acids from the beginning of the erb B coding sequences. In vitro transcription-translation analysis of the insertionally activated c-erb B cDNAs indicate that this AUG can be utilized to initiate translation however, it does not appear to be the preferred start site for translation (N. Maihle and H. J.Kung, unpublished results). This observation raises the question of whether the 3′ LTR promoted c-erb B RNAs are important in oncogenesis. We believe that they are not important based on the following data. REV, a virus whose splice donor site is situated 5′ of the gag translational start site, is unable to induce erythroblastosis in our highly susceptible 151 X 15I4 chicken line (see chapter 1). REV efficiently infects and activates the c-myc locus resulting in B- or T-cell lymphomas (Swift et al., 1985; Isfort et al., 1987). By analogy, REV may insert into the c-erb B locus and activate c-erb B transcription. Translation of the activated c-erb B mRNAs, however, may be inefficient due to the absence of an appropriate initiation codon. A sufficient amount of erb B protein is not synthesized and therefore erythroblast transformation does not occur. Although we have not tested this hypothesis directly, in vitro construction of c-erb B containing REVs may be useful in determining whether infectivity or translational efficiency is responsible for the inability of REV to induce erythroblastosis.

ALV proviruses inserted into the c-erb B locus resemble other randomly integrated proviruses. Approximately 15% of all viral transcripts readthrough into the downstream sequences and less than 2% are initiated from the 3' LTR. This study represents the first example where the transcriptional activity of an intact ALV provirus can be be determined for a specific integration site, namely the c-erb B locus. We have obtained a molecular clone of one such provirus and are in the process of determining its transcriptional activity in vitro. Mutational analysis of this and other ALV proviruses such as those in sample 103 should provide further insight into the differential transcriptional activity of the ALV LTRs.

CHAPTER 4: ON THE MECHANISM OF C-ERB B TRANSDUCTION: NEWLY RELEASED TRANSDUCING VIRUSES RETAIN POLY A TRACTS OF ERB B TRANSCRIPTS AND ENCODE C-TERMINALLY INTACT ERB B PROTEINS

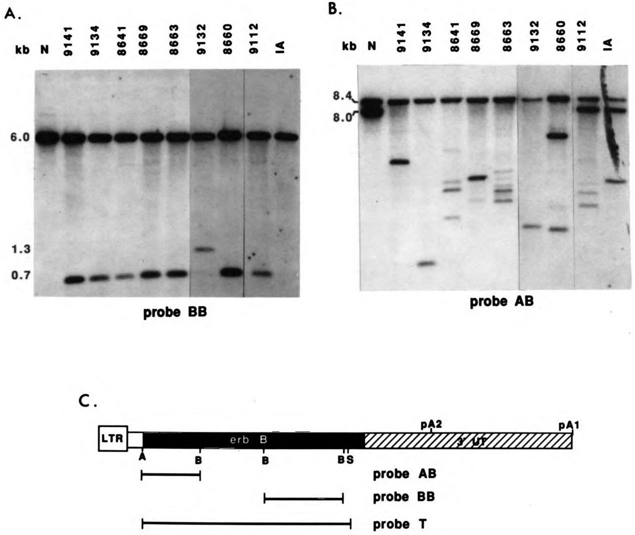
Results:

A. Structure of the Transduced Proviruses

We have previously shown that the majority of erythroblastosis samples induced by ALV contain proviral insertions within the c-erb B locus (Raines et al., 1985). The proviral insertion sites all map at or near the 5' end of the VB1 exon, an exon where homology with v-erb B begins. About 25% of the erythroblastosis samples, however, revealed the presence of transduced erb B viruses. The present report is concerned specifically with the structural characterization of these transduced proviruses.

Transduced erb B proviruses can be readily identified by their lack of introns and have a restriction map more compatible with c-erb B cDNA or v-erb B than with the c-erb B genomic locus. Here, we present the Bam HI digestion data to illustrate the approaches used to screen tumor samples for the presence of transduced proviruses. Bam HI cleaves c-erb B cDNA three times (Figure 11C); an internal 0.72 kb Bam H1 fragment (probe BB) was used as a probe. As shown in figure 1A, samples carrying the transduced erb B gene (lanes with tumor numbers) reveal an additional 0.72 kbp fragment, whereas in an uninfected (lane

Figure 11. The presence of transduced erb B proviruses in ALV induced erythroblastosis. A and B). DNA isolated from ALV induced erythroblastosis samples were digested with Bam HI and analyzed by Southern blotting (Raines et al., 1985) with probe BB (A) and probe BB (B). N: normal uninfected line 151 chicken DNA; IA: representative leukemic samples which carry a c-erb B allele which is insertionally activated by an ALV provirus. Numericals indicate erythroblastosis samples. C). The structure of the 7.0 kb insertional activated c-erb B cDNA (Goodwin et al., 1986). Probe AB and probe BB correspond tho the Apa I-Bam HI and Bam HI-Bam HI fragments of the cDNA. Probe T is derived from a 1.7 kb Apa I-Sac I fragment and contains the majority of the erb B coding sequence (black box). Regions and sites diagrammed include: A, Apa I; B, Bam HI; S, Sac I; 3' UT, 3' untranslated region (hatched box); pA1, first polyadenylation signal in c-erb B; pA2, last polyadenylation site in c-erb B; LTR-gag, ALV-derived sequences (open box).


A.
kb
N
9141
9134
8641
8669
8663
9132
8660
9112
IA
6.0
1.3
0.7
probe BB
B.
kb
N
9141
9134
8641
8669
8663
9132
8660
9112
IA
8.4
8.0
probe AB
C.
LTR
erb B
3' UT
pA2
pA1
A
B
B
BS
probe AB
probe BB
probe T

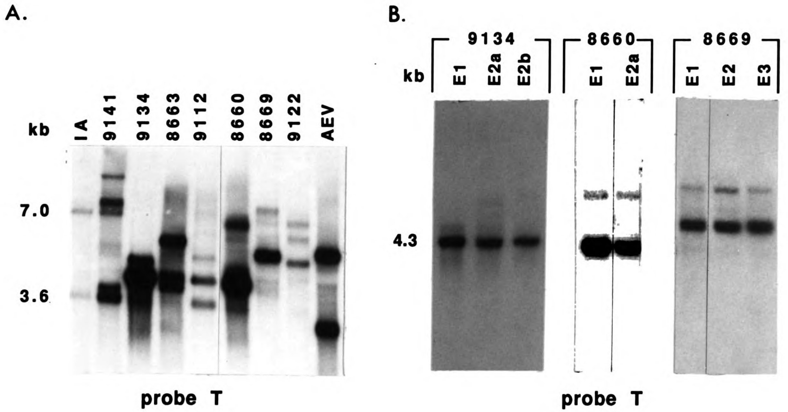
N) sample or a sample containing an insertional activated c-erb B gene (lane IA) the exons covered by BB probe remain in an unspliced form and are distributed over a 6.0 kbp Bam HI fragment. Sample 9132 has a Bam HI fragment slightly larger than 0.72 kb. More detailed analysis revealed structural rearrangement within the transduced erb B sequences (data not shown).

The 0.72 kb Bam H1 fragment represents a common diagnostic fragment for all the transduced erb B proviruses. To differentiate between individual transduced proviruses, we used probe AB, which was derived from the very 5′ end (a 0.35 kb Apa I-Bam H1 fragment) of c-erb B (Figure 11C) and should detect the variable 5′ viral-erb B junctions in different transducing viruses. Indeed, junction fragments of different sizes (0.8 to 4.5 kb) are detected in transduced samples and in most of them, multiple bands are seen, indicating the presence of more than one transduced erb B provirus.

B. Expression of Transduced erb B Proviruses

We then analyzed the expression of these transduced proviruses. A complex pattern of erb B related transcripts is detected in many of the samples (Figure 12A). This is in stark contrast to the simple pattern observed in leukemic samples resulting from insertional activation of c-erb B locus (cf. lane IA), where, as previously shown (Nilsen et al., 1985), only two transcripts, 7.0 and 3.6 kb in size, are seen. The complex expression pattern of transduced proviruses, however, is not unexpected, since for a given provirus, at least two transcripts (a

Figure 12. Expression of erb B transducing viral RNA in ALV induced erythroblastosis samples and secondary leukemias. RNA samples isolated from the original leukemia (panel A and E1 samples in panel B) or secondary leukemias (E2 and E3 samples in panel B), were subjected to Northern analysis (Radinsky et al., 1987) using probe T (Figure 11C). E2 samples are acute erythroblastosis samples derived from injection of E1 leukemic extracts. E3 samples are acute erythroblastosis samples derived from the E2 leukemic extracts. AEV, leukemic liver sample from a bird infected with AEV-R. Other notatations are similar to those described in Figure 11.


A.
kb
7.0
3.6
IA
9141
9134
8663
9112
8660
8669
9122
AEV
probe T
B.
kb
4.3
9134
E1
E2a
E2b
8660
E1
E2a
8669
E1
E2
E3
probe T

genomic and a spliced RNA) can be generated (see below) and since in many samples, multiple transduced proviruses are present. Another striking difference between the transduced and insertional activated samples is the level of expression. In general, the former is about 10-20 fold higher than the latter and reaches a level comparable to AEV infected samples (lane AEV). This is most likely due to the fact that transduced erb B transcripts result from direct transcription of the 5' LTR, whereas additional readthrough of the 3' LTR is required to generate the IA c-erb B transcripts. The latter process is estimated to occur in only 15% of the viral transcripts (Herman et al., 1986).

To verify that the observed novel transcripts did originate from the transduced proviruses, viral extracts from some of these samples were made and inoculated into embryos. Of the four samples tested, all but one (9141) induced erythroblastosis. RNA from the resulting erythroblastosis was compared to that of the original inoculum. As shown in Figure 12B, erb B related transcripts of similar size are observed in the original samples (9134E1, 8660E1, 8669E1) as well as the newly generated leukemic samples (E2 and E3). This conclusively documents the viral nature of these transcripts.

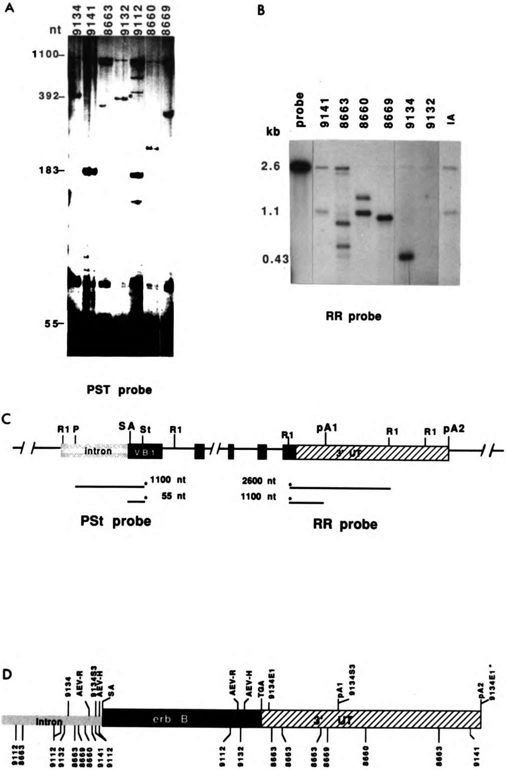
B. <u>The 5' and 3' junctions of the transduced c-erb B sequences</u>

To probe the possible mechanism underlying the frequent transduction of the c-erb B gene, we sought first to identify the 5' and 3' boundaries of the erb B genes in these viruses. S1 protection analysis of the leukemic RNAs was performed using genomic probes; probe

PSt for the 5′ boundary and probe RR for the 3′ boundary. Probe PSt is a 1.1 kb Pst I-Stu I fragment which contains 55 nucleotides of the VB1 exon including the splice acceptor (SA) and approximately 1.1 kb of the intron sequences 5′ of VB1 exon. RNA from all the transduced leukemic samples protected a 55 nt fragment, which represents a spliced erb B RNA (Figure 13A). Importantly, all the transduced samples also contain protected fragments larger than 55 nt. These fragments are likely to be derived from the transduced viral genomic RNA and provide a measurement of the extent of intron sequences incorporated into the individual viral genomes. These data are diagrammatically summarized in Figure 13D. The 9134E1 provirus was subsequently sequenced (see below) and shown to contain 326 nt of intron sequence, in complete agreement with the S1 analysis data presented here.

A similar strategy was taken to define the amount of 3′ sequences of the c-erb B locus incorporated into the transduced viral genomes. A 2.6 kb Eco R1 fragment (probe RR) which encompasses the last 260 nt of the erb B coding sequences and 2.3 kb of the 3′ untranslated region was used to porbe leukemic RNA. A summary of the protected fragments and where they map relative to the erb B coding sequence is shown in Figure 13D. All except 9112 and 9132 protected fragments larger than 260 nt indicating that the carboxy-terminus of the erb B protein expressed by these viruses is intact, a feature different from the existing AEV-H and AEV-R isolates. The 9132 sample did not protect any fragments; further mapping analysis located the breakpoint 5′ of the Eco R1 site and the junction point of AEV-R and AEV-H (data not shown).

Figure 13. The 5′ and 3′ junctions of erb B transducing viruses. A and B). S1 nuclease protection analysis of RNA samples isolated from ALV erythroleukemias. Sample notations are the same as those described in Figure 11; probe, is undigested probe. C). Diagram of the 5′ and 3′ exon-intron map of the chicken c-erb B locus (not drawn to scale) showing probes used to for S1 analysis. PSt probe and RR probe are derived from a 1.1 kb Pst I-Stu I fragment and a 2.6 kb Eco RI-Eco RI fragment, respectively, and are situated in the region indicated. Regions and sites depicted include: exon sequences, solid boxes; VB1, the first exon with homology to v-erb B; 3′UT, 3′ untranslated region (hatched box); pA1 and pA2, major polyadenylation sites; *, location of radioactive label; R, Eco RI; P, Pst I; St, Stu I. D). A summary of the extent of 5′ and 3′ erb B sequences incorporated into the transducing viral genomes. This is a composite summary based on S1 protection analysis (A and B), restriction enzyme mapping, cloning, and sequencing. Numericals below the diagram represent data derived solely from S1 analysis. The numericals above the diagram represent data either deduced or confirmed by cloning and sequencing of the proviruses. Only the most intense bands which were reproduced in several independent experiments are depicted in panel D. The 2.6 and 1.1 kb bands present in panel B could result from polyadenylation and therefore may not represent 3′ junction points; only those verified by sequencing are included in D. 9134E1* denotes a breakpoint due to deletion rather than recombination. The dotted line adjacent to 9141 indicates the the 3′ boundary of 9141 proviral derived DNA clones. The AEV-R and AEV-H boundaries are based on published sequence (Henry et al., 1985; Yamamoto et al., 1984b).


A
nt
9134
9141
8663
9132
9112
8660
8669
1100
392
183
55
PST probe
B
probe
9141
8663
8660
8669
9134
9132
IA
kb
2.6
1.1
0.43
RR probe
C
R1
P
SA
St
R1
R1
pA1
R1
R1
pA2
Intron
VB1
3' UT
1100 nt
55 nt
2600 nt
1100 nt
PSt probe
RR probe
D
9134
AEV-R
9134S3
AEV-H
SA
AEV-R
AEV-H
TGA
9134E1
PA1
9134S3
PA2
9134E1
Intron
erb B
3' UT
9112
8663
9112
9132
8663
8669
8660
9141
9112
9112
9132
8663
8663
8663
8669
8660
8663
9141

One limitation of the S1 analysis described above is that it measures contiguous sequences present at the 5′ and 3′ ends of erb B and does not distinguish whether the breakpoint is due to recombination with viral sequences, deletion (see below) or polyadenylation. Thus, they represent the minimal amount of erb B flanking sequences incorporated into the viral genone. Also, due to the presence of multiple proviruses in the same leukemic samples, we cannot draw individual maps of the erb B containing viruses.

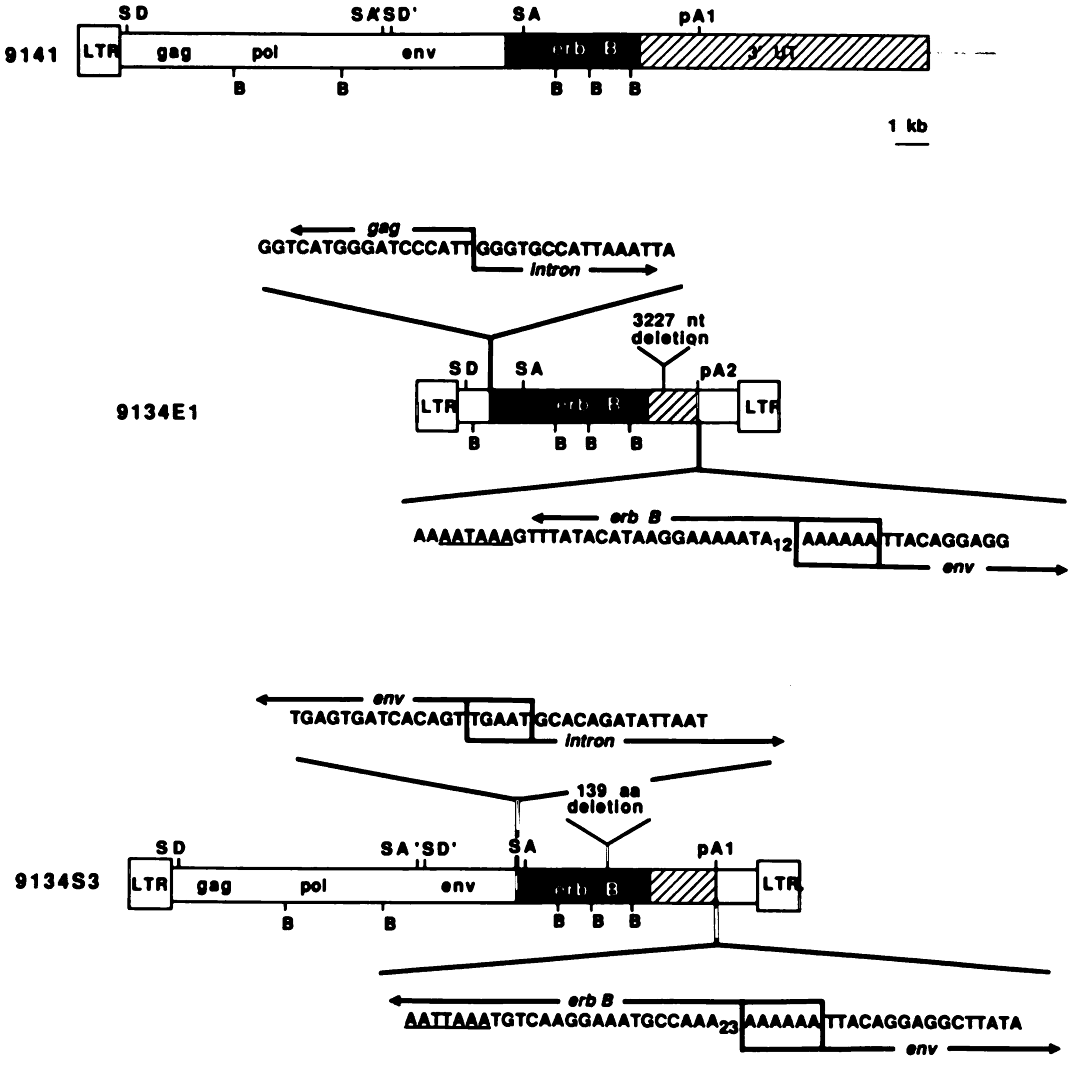
C. Molecular cloning and sequence analysis of the transduced proviruses

The limitation of the analysis described above prompted us to isolate individual proviral clones for detailed characterizations. From genomic libraries of the leukemic samples, we have isolated clones representing three transduced proviruses, 9141, 9134E1, 9134S3. Detailed restriction mapping and sequencing were performed for these proviruses and in two cases (9134E1 and 9134S3), all the erb B derived sequences including the sites of recombination have been determined. In this paper, we will focus on the recombination sites of these proviruses, in the hope to better understand the transduction process. In chapter 5 the transforming potentials of 9134E1 and 9134S3 will be described.

1. Structure of the 9141 Provirus

The 9141 provirus possibly represents the largest transducing genome thus far identified (Figure 14). It is at least 12 kb long and contains 6.0 kb of ALV sequence at its 5′ end, interrupted by a small

Figure 14. Structure of c-erb B transduced proviral clones 9141, 9134E1, and 9134S3. Molecular clones carrying transduced c-erb B proviral sequences were isolated from genomic DNA of erythroleukemic samples (9134E1 and 9141) or transformed fibroblast DNA (9134S3). The 9134S3 transformed fibroblast cell line is described in detail in Chapter 5. Viral and erb B regions were located by restriction enzyme digestion and Southern blot analysis with ^{32}P-labelled probes corresponding to either viral sequences (open box), erb B containing sequences (solid box), or 3' untranslated sequences of c-erb B (hatched box). Only the Bam H1 restriction map is shown here. The 9134E1 and 9134S3 intron sequences (dotted box) were determined by DNA sequence analysis, and the precise junction between viral sequences and erb B sequences are shown (arrow, above provirus). The amount of c-erb B untranslated sequence (hatched box) and its 3' recombination point (arrow, below provirus) was similarly determined. The intron region of 9141 was estimated based on S1 analysis (Figure 12A and 12D). The 9141 provirus clones did not extend beyond the 3' untranslated sequences. The approximate point of divergence was estimated based on Southern analysis of 9141 DNA and Northern and S1 analysis of 9141 RNA. The sizes of erb B related transcripts in Figure 11 are similar to the insertionally activated cDNAs suggesting that the viral transcripts terminate in cellular sequences near the estimated point of divergence. Larger transcripts may result from alternate splicing or polyadenylation at yet another site downstream from the point of divergence. The viral and erb B splice donor (SD), and splice acceptor (SA) are shown as well as the cryptic splice donor ('SD', see Nilsen et al., 1985), and the two c-erb B polyadenylation signals (pA1 and pA2, Goodwin et al., 1986) are also shown.


9141
LTR
SD
gag
pol
SA'SD'
env
SA
erb B
pA1
3' UT
B
1 kb
gag
GGTCATGGGATCCCATTGGGTGCCATTAAATTA
intron
3227 nt deletion
9134E1
LTR
SD
SA
erb B
pA2
LTR
erb B
AAAATAAAGTTTATACATAAGGAAAAATA12AAAAAATTACAGGAGG
env
env
TGAGTGATCACAGTTGAATGCACAGATATTAAT
intron
139 aa deletion
9134S3
LTR
SD
gag
pol
SA'SD'
env
SA
erb B
pA1
LTR
B
erb B
AATTAAATGTCAAGGAAATGCCAAA23AAAAAATTACAGGAGGCTTATA
env

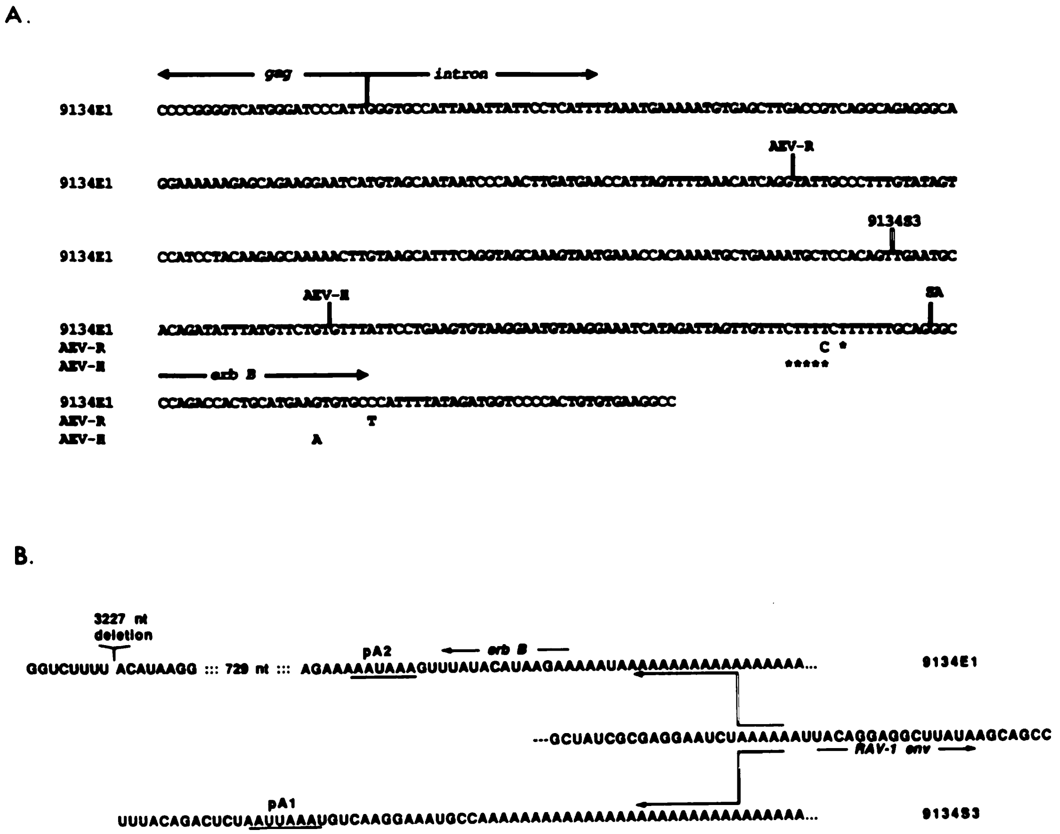
deletion of about 1.0 kb in the env region. The 5' junction point between ALV and erb B lies in the intron region about 150 bp 5' of the VB1 exon. The entire erb B coding sequences downstream from VB1 are retained in a completely spliced form. Interestingly, virtually the entire 3' untranslated region of the 7.0 kb IA cerb B transcript (Figure 11C) is transduced, suggesting that the transcript co-terminal with the 7.0 kb transcript is the precursor engaged in recombination. Our clone terminates at a site 0.3 kb 5' from the downstream poly A (pA2) signal. The upstream poly A signal, pA1, is retained. Since both LTRs are required for integration, we presumed that there should be a 3' LTR at the 3' end of this provirus. Exhaustive screening as well as Southern analysis of the 9141 leukemic DNA failed to provide such an evidence. It is thus likely, that 9141 provirus represents a vestige of a transduced virus which has lost the 3' LTR following integration. The defective nature of the 9141 provirus together with the unusually large size explain why extracts of 9141 failed to induce acute erythroleukemia.

The defective provirus, however, is responsible for the development of the original 9141 leukemia. Southern analysis reveals that the 9141 provirus is the only transduced genome in the 9141 leukemic sample. This provirus contains multiple splice sites; at least eight transcripts can be generated via differential processing. The predicted sizes match closely with those identified in Northern analysis. The two major transcripts, the 7.0 and 3.6 kb message, are virtuallly identical in size and content to the oncogenic transcripts found associated with IA c-erb B. It is, therefore, not surprising that 9141 provirus is leukemogenic, despite, the fact that no infectious virus can be produced. The monoclonal nature of 9141 provirus (unpublished data) further supports this notion.

2. Structure of the 9143E1 provirus

The 9134E1 provirus (Figure 14) is the predominant component of the original 9134 erythroblastosis and of leukemia samples resulting from injection of 9134 extracts. It has one of the smallest leukemic viral genomes thus far identified with a total size of 4.5 kb. This size is consistent with the RNA analysis (Figure 12A). The 5′ ALV sequence constitutes only 541 bp; the breakpoint being situated 161 bp downstream of the gag AUG, a region that has previously implicated in encapsidation of ALV RNA (Pugatesh et al., 1983). The ALV sequence is juxtaposed with 326 bp of erb B intron with no significant homology at the junction. The erb B intron sequences retained in 9134 (Figure 14 and 15A) is larger than those of AEV-R and AEV-H, and also differs at several nucleotide positons. We have also sequenced the relevant portion of intron from a c-erb B genomic clone derived from line 151 chicken DNA and found that they are identical to those associated with 9134E1. Thus, the intron sequence of 9134E1 is a faithful copy of the c-erb B locus from line 151 chickens. The erb B coding portion is identical to that of the IA c-erb B cDNA indicating that no point mutations have occurred. The most intriguing part of this provirus perhaps lies at its 3′ end, where 929 bp of the 3′ UT (untranslated) region of the c-erb B locus adjoins the last 44 bp of the env coding

Figure 15. Nucleotide Sequence of the 5' and 3' viral-erb B junctions in 9134E1 and 9134S3. A). Sequences surrounding the 5' recombination point of the 9134E1 provirus including viral sequences and all of the erb B intron sequences. Vertical lines indicate the junction between viral and intron sequences in AEV-R, AEV-H, 9134S3 and 9134E1. Only divergent sequences are indicated; * denotes deletion; SA, splice acceptor site. B). The 3' recombination points of 9134E1 and 9134S3 viruses. The boundary of the 3227 nt deletion present in the 9134E1 provirus and the two erb B polyadenylation signals pA1 and pA2 (underlined) are indicated. Arrows illustrate the possible scheme whereby the DNA synthesized by reverse transcriptase of wild type RAV-1 RNA switches template to the polyadenylated erb B RNA via oligo(A) homology.


A.
gag
intron
9134E1 CCCCGGGGTCATGGGATCCCATTGGGTGCCATTAAATTATTCCTCATTTTAAATGAAAAATGTGAGCTTGACCGTCAGGCAGAGGGCA
AEV-R
9134E1 GGAAAAAAGAGCAGAAGGAATCATGTAGCAATAATCCCAACTTGATGAACCATTAGTTTTAAACATCAGGTATTGCCCTTTGTATAGT
9134S3
9134E1 CCATCCTACAAGAGCAAAAACTTGTAAGCATTTCAGGTAGCAAAGTAATGAAACCACAAAATGCTGAAAATGCTCCACAGTTGAATGC
AEV-H
SA
9134E1 ACAGATATTTATGTTCTGTGTTTATTCCTGAAGTGTAAGGAATGTAAGGAAATCATAGATTAGTTGTTTCTTTTCTTTTTTGCAGGGC
AEV-R C *
AEV-H *****
erb B
9134E1 CCAGACCACTGCATGAAGTGTGCCCATTTTATAGATGGTCCCCACTGTGTGAAGGCC
AEV-R T
AEV-H A
B.
3227 nt
deletion
GGUCUUUU ACAUAAGG ::: 729 nt ::: AGAAAAAUAAAGUUUAUACAUAAGAAAAAUAAAAAAAAAAAAAAAAAA...
pA2
erb B
9134E1
---GCUAUCGCGAGGAAUCUAAAAAAUUACAGGAGGCUUAUAAGCAGCC
RAV-1 env
pA1
UUUACAGACUCUAAUUAAAUGUCAAGGAAAUGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA...
9134S3

region of ALV (Figure 15B). The recombination point is within a string of 23 A's (interrupted by one T). This oligo (A) is apparently derived from the poly A sequence of an erb B transcript utilizing the pA2 signal. We have also sequenced the env gene of the RAV-1 provirus that was originally used to generated the erythroblastosis. Most interestingly, the point of recombination lies within a stretch of 6 A's which are found in the RAV-1 genome. These 6 A's can thus potentially serve as a homologous recombination site during reverse transcription. The 9134E1 provirus has suffered a large deletion of 3227 bp including the pA1 signal. As a consequence, the virus is more compact and contains one internal polyadenylation signal (pA2). The 9134E1 provirus is fully infectious and leukemogenic (figure 12B and chapter 5), despite the presence of pA2 internal to the virus.

3. Structure of the 9134S3 provirus

9134S3 probably represents an independent virus isolate present in the 9134 leukemia extracts. It was undetectable in the original leukemia sample by DNA or RNA analysis and became apparent only upon selection for fibroblast-transformation or sarcomagenesis. The structure and the transforming properties of the 9134S3 and 9134E1 provirus are distinct. 9134S3 has much larger 5' ALV-related sequences; it includes intact gag and pol genes, and a segment of env, which is joined to 98 bp of erb B intron sequence. The 5' junction point contains an overlapping pentanucleotide common to both env sequences of ALV and erb B intron sequences. These pentanucleotides are likely to be involved in homologous recombination. The erb B coding portion contains an in-frame deletion of 417 bp, which renders the virus sarcomagenic, but not leukemogenic (see chapter 5). Most strikingly, the erb B sequence also terminates within a stretch of poly A residues and the 3' recombination point occurs in ALV env sequences identical to that shown for 9134E1 (Figure 14 and 15B). One important difference, however, is that the poly A tracts in this case is longer (31 residues) and is derived from an erb B transcript utilizing pA1 rather than pA2 signal. This suggests that 9134E1 and 9134S3 evolved through independent recombination events and that the oligo A homology represent a site of frequent recombination.

Discussion:

A. The Proposed Mechanisms for Oncogene Transduction

Viral oncogene transduction is a well-documented phenomenon but the mechanism is still not fully understood. Several models have been proposed (Goldfarb et al., 1981; Swannstrom et al., 1983; Herman and Coffin, 1987; and Roebroek et al., 1987). A multi-step model that has received general acceptance (Swannstrom et al., 1983) contains the following features: 1) Proviral integration within or upstream of a protooncogene in the same transcriptional orientation; 2) deletion of the 3' proviral DNA sequences including the 3' LTR thereby fusing the proto-oncogene to the viral transcriptional unit; 3) transcription from the 5' LTR to generate a fusion transcript encompassing both proviral and proto-oncogene sequences; 4) packaging of the chimeric transcripts and wild-type viral genomes into heterodimeric virus particles; and 5) template switching between the heterodimer by reverse transcriptase during viral DNA synthesis, such that the 3′ viral sequence is restored to the chimeric molecule. The overall consequence of this process is the capturing of the proto-oncogene into the central portion of the viral genome, and retention of the terminal cis-acting viral elements, essential for replication. This model predicts that the 5′ joining of viral and cellular sequences occurs at the DNA level (Step 2) and the 3′ joining at the RNA level (Step 5). The majority of transduced viral genomes analyzed contain 5′ recombination points which are situated in the intron region of a proto-oncogene (thus retaining a splice acceptor site) and a 3′ recombination point lying within an exon region (for review see Besmer, 1983) or, in one case, a poly A tract (Huang et al., 1986), supporting this model. There are, however, several interesting exceptions. For instance, in some recently identified myc and src variants (Ikawa et al., 1986; Martin et al., 1986), the 5′ recombination point coincides with sequences generated by splicing. This raises the possibility that RNA splicing across the viral and cellular sequences may serve as an alternative pathway for gene fusion (i.e., Step 2). Herman and Coffin (1987) recently demonstrated that readthrough transcripts carrying the entire ALV provirus and the adjacent downstream cellular sequences can be synthesized and packaged efficiently. This raises another interesting possibility since the readthrough transcript can serve as a precursor for recombination at both the 5′ and 3′ end, presumably during reverse transcription. This model is attractive in its simplicity since no proviral deletion step needs to be invoked. It also predicts a higher frequency of oncogene transduction in cases where readthrough transcription into the proto-oncogene is a necessary step, such as in c-erb B activation. In the following paragraphs, we shall discuss the structural data of erb B transducing viruses obtained in this report in the context of these models.

B. The Mechanisms of c-erb B Transduction

1. Insertional Activation as the First Step in c-erb B Transduction

All the transduction models described above postulate that proviral insertion near a cellular oncogene is the first step involved in oncogene transduction. Yet for most of the transduced viral oncogenes, it is difficult to experimentally recreate this rare evolutionary step. Thus, ALV induced erythroblastosis, where both insertional activation and transduction are frequently observed, presents a unique opportunity to examine such an issue. Our findings that all the proviruses involved in the insertional activation of erb B are in the same orientation as the erb B gene, and cluster in the intron region which coincides with the 5′ junctions of all the transduced erb B viruses analyzed in this report lends strong support to this thesis.

2. Formation of the 5′ Junction: Deletion of Proviral and erb B Sequences at the DNA Level

As discussed above, the second step of oncogene transduction involves the fusion of the viral and erb B sequence into a single transcriptional unit. This is where the proposed models differ most significantly. The model of Herman and Coffin (1987) which suggests that the readthrough viral-erb B transcript is a transduction precursor is appealing and may account for the unusually high frequency of erb B transducrion. The large size of the primary viral-erb B readthrough transcript (35 kb), however, makes it unlikely that this hybrid RNA transcript can be efficiently packaged. The second model which suggests 5' fusion via RNA splicing is also attractive for erb B transduction, since c-erb B is activated through splicing and the viral-erb B fusion RNAs are present in all leukemic samplews. We, however, have no evidence that this is the case. Our data, especially based on direct cloning and sequencing analysis, clearly show that most, if not all, transducing erb B viruses retain intron sequences and intact 5' splice acceptor sites. We interpret this to mean that efficient packaging of the ALV-erb B transcript requires a viral "encapsidation signal", which at least in part resides in a region between the splice donor (SD) site and the 5' junction of 9134E1. Our results are most consistent with the generation of a fusion transcript by deletion of proviral and erb B intron sequences at the DNA level. Examination of the 5' junction structures, however, does not indicate a unified mechanism for such a process. In the case of 9134S3, homologous recombination involving a pentanucleotides, has occurred. By contrast, no obvious homology is detected at the junction of 9134E1.

3. Formation of the 3' Junction: Template Switching Within Poly A Tracts by Reverse Transcriptase

Once deletion of the 3' proviral sequence has occurred, the ALV-erb B fusion transcript can be generated by initiation at the 5' viral LTR promoter and termination at one of the erb B polyadenylation sites, either pA1 or pA2. By virtue of maintaining the encapsidation sequence, this fusion message should be effectively packaged into virions together with the wild-type ALV RNA, which are amply abundant in the leukemic cells since most of them harbor more than one provirus. Upon subsequent infection, template switching by reverse transcriptase permits the incorporation of 3' viral sequence into the fusion transcript. It is most interesting that in two different isolates, 9134E1 and 9134S3, the template switch occurs within the poly A tract of erb B RNA and at a common site within the ALV genome. The six A residues present in the ALV env gene apparently facilitate the switching process. While we cannot completely rule out the possibility that 9134E1 and 9134S3 arose through a common ancestral virus, the distinct 5' junctions and the different poly A tracts used in 3' recombination strongly suggest that they involved independent transduction processes. The presence of a poly A tract within a retroviral genome as demonstrated here, together with a similar finding in the Fujinami virus (Huang et al., 1986), provide strong evidence for a 3' recombination that takes place at the RNA level. Both 9134E1 and 9134S3 contain internal polyadenylation signals, a feature that in theory should impede the transcription of viral genomic RNA.

Literature reports on this point, however, are not converging. Some internal poly A signals appear to be detrimental to virus production (Shimotohno et al., 1981; Sylla et al., 1986; Joyner et al., 1983; Bandyopadhyay et al., 1984), whereas others have no effect. We found that at least in the 9134E1 case, RNA transcripts terminating prematurely at the internal poly A site could be identified in infected cells yet, the virus still was released at moderately high levels (unpublished data).

It is also significant that, unlike AEV-H and AEV-R, all but two of these newly generated transduced erb B proviruses retain the c-terminal coding sequences including the putative major tyrosine autophosphorylation site. This suggests that the loss of the c-terminus of c-erb B is not a necessary step involved in transduction and more likely is a consequence imposed by selection for fibroblast-transformation properties. This subject is addressed more fully in the next chapter.

In summary, our analysis of ten newly generated erb B transducing viruses reveal that the 5′ junction site occurs in the intron region of erb B, thereby retaining a functional splice acceptor site. This suggests that the insertional activated erb B transcripts, despite their abundance in leukemic cells, are probably not the immediate precursors for transduction. The 3′ junction site resides predominantly in the 3′ untranslated region of c-erb B and, in two cases, within the poly A tracts of the c-erb B RNA molecules. This reinforces the view that the second recombination occurs at the RNA level and suggests that it may be facilitated by homologous sequences.

CHAPTER 5: IDENTIFICATION OF NEW C-ERB TRANSDUCING VIRUSES: DELETIONS IN THE C-TERMINAL DOMAIN ACTIVATE SARCOMAGENIC POTENTIAL

Results:

A. Disease potential of leukemia samples containing new c-erb B transducing viruses

We have identified several new c-erb B transducing viruses from different ALV induced erythroblastosis samples. We have shown that most of these retroviruses carry the entire c-erb B coding region including an intact carboxy-terminal domain similar to the insertional activated c-erb B gene product (IA c-erb B). This is in contrast to the v-erb B containing retroviruses, AEV-R and AEV-H, which lack c-terminal erb B sequences and terminate in the *env* gene of the ALV genome. We have used the leukemia samples carrying the new c-erb B transducing viruses to test the oncogenic potential of c-terminally intact c-erb B. Plasma and liver homogenates from three different leukemia samples were injected into chick embryos. All induced short latency erythroblastosis but at low incidences (Table 2). Because of the apparent loss of virus titer from the original tumor extracts, we used homogenates from the secondary leukemias (E2) for all subsequent experiments. One E2 extract, 9134E2a, contained an unusually high titer of focus forming units, when assayed on chick embryo fibroblasts (CEF). This was surprising since most of the newly released erb B transducing viruses were unable to transform fibroblasts (Tracy et al.,

Table 2. Induction of Erythroblastosis From Leukemia Samples Containing Transduced c-erb B Viruses

Originating Chicken	Source	Number With Erythroblastosis/ Number Inoculated	Latency Period⁺
8669	Plasma	4/9	14-19
8660	Plasma	1/10	6
9134	Liver	5/8	7-15

0.1 ml of liver homogenates or plasma was injected intravenously into 16-day embryos.

⁺Latent period is days required for erythroblastosis to develop.

1985 and Beug et. al., 1986). In order to characterize the fibroblast transforming component of 9134E2a more closely, we attempted to amplify and purify this component through further propagation and selection by transformation as illustrated in Figure 16. Transformation was selected for in vitro by colony formation in soft agar, and in vivo by the ability to induce sarcomagenesis.

Northern analysis was used to monitor the c-erb B transducing viruses during purification. As previously described (see chapter 4), the original 9134E1 sample contains two erb B-related transcripts (4.3 kb and 4.5 kb) which correspond to the genomic and subgenomic viral transcripts (Figure 17). Similarly sized erb B transcripts are only observed in the secondary erythroblastosis (E2a and E2b) and not in transformed fibroblasts or sarcomas (S3a, S3b, S4a, S4b, or F3). Instead smaller erb B RNAs are present suggesting that a new smaller erb B virus is present. The erb B transcripts in the fibroblast selected samples (F3, S4a, and S4b) differ from those directly selected in vivo (S3a and S3b) suggesting that two distinct viruses are responsible for the high focus forming ability of the inoculum.

B. Detection of deletions in the erb B sequences of the transduced viruses

The smaller erb B transcripts in the sarcoma and fibroblast samples suggested that they may have undergone structural rearrangements. In order to address this possibility we determined the integrity of the erb B sequences using S1 analysis. We focused

Figure 16. Purification scheme for three different c-erb B transducing viruses in erythroleukemic chicken 9134. Erythroblastosis (E) samples, fibrosarcomas (S), and infected chicken embryo fibroblasts (F) were used to purify three different c-erb B transducing viruses. The route of injection or infection is indicated, and the number of tumor bearing animals generated/number injected is noted in brackets. Only samples which were used for passage were characterized further and are as illustrated.

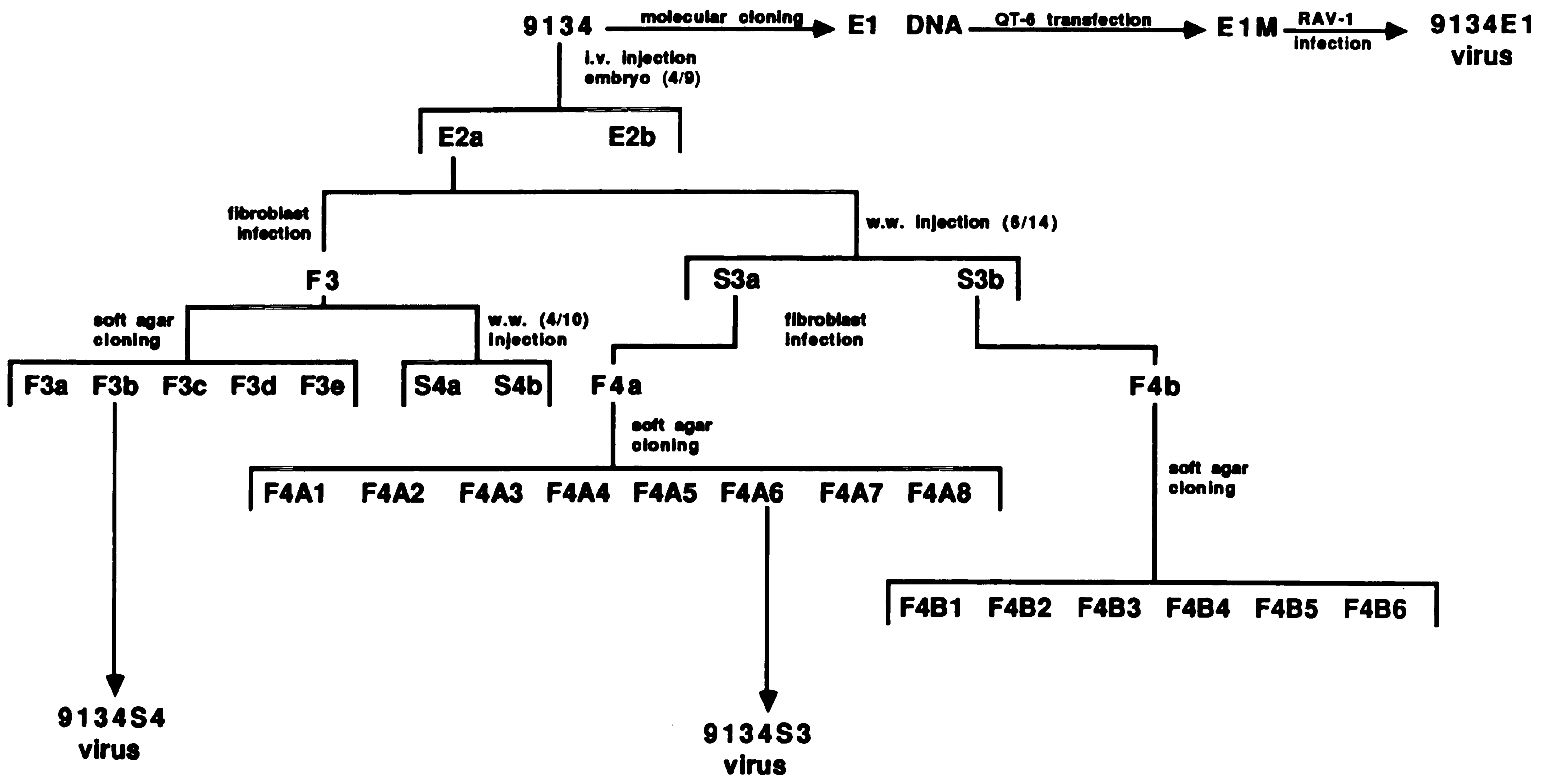
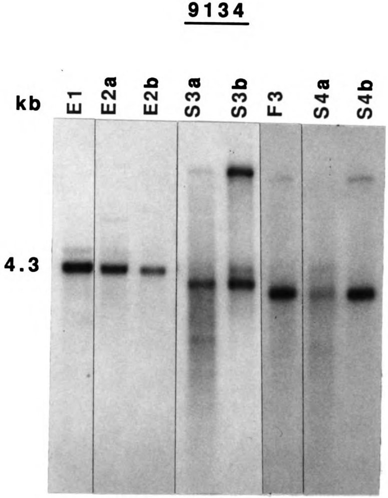

9134
molecular cloning
E1 DNA
QT-6 transfection
E1M
RAV-1
infection
9134E1
virus
i.v. injection
embryo (4/9)
E2a
E2b
fibroblast
infection
w.w. injection (6/14)
F3
S3a
S3b
soft agar
cloning
w.w. (4/10)
injection
fibroblast
infection
F3a
F3b
F3c
F3d
F3e
S4a
S4b
F4a
F4b
soft agar
cloning
F4A1
F4A2
F4A3
F4A4
F4A5
F4A6
F4A7
F4A8
soft agar
cloning
F4B1
F4B2
F4B3
F4B4
F4B5
F4B6
9134S4
virus
9134S3
virus

Figure 17. Northern analysis of erb B related RNAs in sarcomas induced by 9134 viral extracts. Poly (A)+ RNA was isolated from either the original 9134 erythroleukemic sample (E1), or secondary erythroleukemias (E2a and E2b), sarcomas (S3a, S3b, S4a, S4b) or transformed fibroblasts (F3). Poly (A)+ RNA (2 ug) was fractionated on 1% formaldehyde agarose gels and electrotransferred to GeneScreen (New England Nuclear, Boston, Mass.). Hybridization was done using an erb B specific probe (a 1.7 kb Apa I-Sac I fragment derived from c-erb B coding sequences). The position of the 4.3 kb subgenomic 9134E1 virus is indicated.

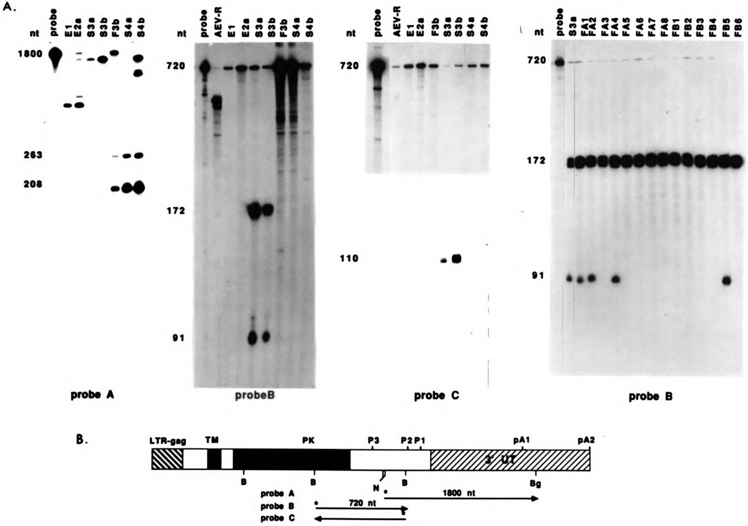

9134
kb
E1
E2a
E2b
S3a
S3b
F3
S4a
S4b
4.3

primarily on sequences outside of the protein kinase domain since mutations in this region have been shown to abolish fibroblast transformation (Ng et al., 1986). Probe A, a 1.8 kb Nco I-Bgl II fragment, was used to locate deletions within the 3' end of c-erb B (Figure 18B). Two Nco I sites are located 57 nt apart and are situated 277 and 334 nt upstream of the c-erb B termination codon. No S1 nuclease resistant fragments smaller than 277 nt were observed in E1, E2b, S3a, and S3b samples indicating that these viruses had retained their c-terminal coding sequences. The transforming viruses that were selected _in vitro_ (samples F3b, S4a and S4b) did protect smaller fragments of 208 nt and 263 nt. A deletion at this point would remove approximately the last 20 amino acids of erb B.

A 720 bp Bam HI fragment (probe B) was used to detect mutations occurring between the protein kinase domain and the c-terminal region (Figure 18B). As expected for viruses containing intact erb B sequences, only the full length probe was protected in most samples. In this analysis we have included AEV-R RNA, a virus known to be deleted in this region, as a control for hybridization due to reannealing. Two novel S1 resistant fragments are observed in the S3a and S3b samples suggesting that two deleted c-erb B transducing viruses are present. Based on their size (91 nt and 172 nt) we would predict that approximately 240 or 268 amino acids would be removed from their c-termini.

The results using probe A suggested that one of the erb B viruses present in the S3 samples contained intact c-terminal sequences;

Figure 18. S1 nuclease analysis of transduced erb B sequences in RNA from newly isolated sarcomas and transformed fibroblasts. A). RNA was isolated from erythroleukemic samples (E), sarcomas (S), or infected fibroblasts (F). RNA from AEV-R infected (leukemic) chicks was used as control. 1 ug poly (A)+ or 30 ug total RNA were hybridized to either probe A, a 3' end-labelled Nco I-Bgl II fragment, probe B, a 3' end-labelled Bam H1 fragment, or probe C, a 5' end-labelled Bam H1 fragment, treated with S1 nuclease, and separated on a 6% denaturing polyacrylamide gel. Sizes of protected fragments were determined relative to the mobility of ^{32}P-labelled Hinf I and Hae III digested PhiX174 markers. Undigested probe (probe) is also shown. B). Diagram of insertionally activated c-erb B cDNA indicating probes used to detect deletions in erb B sequences. * denotes the location of the radioactive label; dotted box, protein kinase domain; black box, transmembrane domain; hatched box 3' untranslated region; thick hatched box, 5' viral derived sequences; pA1 and pA2, first and last polyadenylation sites of c-erb B; P1, P2, P3, tyrosine residues analagous to the autophosphorylation sites present in EGF-R. Restriction enzyme sites include B, Bam HI; N, Nco I; Bg, Bgl II.


A.
nt
probe
E1
E2a
S3a
S3b
F3b
S4a
S4b
1800
263
208
probe A
nt
probe
AEV-R
E1
E2a
S3a
S3b
F3b
S4a
S4b
720
172
91
probeB
nt
probe
AEV-R
E1
E2a
F3b
S3a
S3b
S4a
S4b
720
110
probe C
nt
probe
S3a
FA1
FA2
FA3
FA4
FA5
FA6
FA7
FA8
FB1
FB2
FB3
FB4
FB5
FB6
720
172
91
probe B
B.
LTR-gag
TM
PK
P3
P2
P1
pA1
pA2
3' UT
B
B
N
B
Bg
probe A
probe B
probe C
720 nt
1800 nt

therefore it seemed likely that one of the deletions identified with probe B was internal. S1 analysis with the 720 Bam HI fragment labelled at its 5' end (Figure 18, probe C) was used to map the 3' boundary of this internal deletion. A 110 bp fragment was protected indicating that one of the two deleted c-erb B transducing viruses in S3a and S3b contained an internal deletion.

C. Establishment of cell lines expressing a single transduced erb B virus.

The detection of multiple S1 resistant fragments in the tumor RNAs indicates that they are heterogeneous with respect to the c-erb B transducing viruses they contain. It is interesting that similar viruses of erb B transducing viruses were consistently observed only after their initial selection and that viruses selected in vitro (F3 and S4) were distinct from those selected in vivo (S3). We purified the predominant transforming component from each of these two selection procedures by cloning in soft agar. F3b is one of three independently isolated colonies which were identical. A single virus, containing the 20 amino acid c-terminal truncation in c-erb B, was expressed in this cell line and was the predominant c-erb B transducing virus in both S4a and S4b samples. We have designated the c-erb B transducing virus in this cell line 9134S4. The F3b cell line which contains the S4 virus has been used for all of our subsequent studies. Two other soft agar clones were also isolated. The virus in these clones was distinct from 9134S4 but was unstable with passaging and therefore has not been characterized further.

The sarcomagenic components present in S3a and S3b were similarly isolated by first infecting fibroblasts with sarcoma homogenates and selecting for colonies in soft agar. Clones containing a single erb B virus were determined using S1 analysis with probe B (Figure 18B). This probe detected both deleted c-erb B transducing viruses in the S3a and S3b inoculum (Figure 18, probe B, lane S3a). Several clones retained both of these viruses even after soft agar selection. A common erb B virus was present in all of the clones suggesting that this virus was responsible for fibroblast transformation. We have designated this virus as 9134S3 and have used cell line F4A6, a cell line containing only this virus, as a source of the 9134S3 virus. Further S1 analysis using probe C indicated that this virus was the one which contained an internal deletion within erb B (data not shown). We estimate that this deletion encompasses approximately 438 nt.

In order to compare the mutant viruses to one that contained all of the erb B coding sequence, we established a cell line (E1M) expressing the molecularly cloned 9134E1 virus (the molecular cloning of the 9134E1 virus is described in the chapter 4). We have verified the intactness of the erb B coding region by sequencing and have found no nucleotide differences between it and the insertionally activated c-erb B (IA c-erb B) sequence. A chemically transformed cell line, Qt-6, was cotransfected with a plasmid carrying the entire 9134E1 provirus and SV2-neo. G418 resistant cells were selected, pooled, and infected with RAV-1. Immunoprecipitations of ^{35}S-labelled E1M cells with an erb B specific antisera indicates that 9134E1 synthesizes two major erb B related proteins, 82 kD and 84kD (Figure 19, lane E1M), which are indistinguishable from those expressed in cell lines containing IA c-erb B cDNA clones (Maihle, Raines, and Kung, unpublished results).

D. Immunoprecipitation of erb B related proteins

We have also immunoprecipitated the erb B related proteins from the 9134S3 and 9134S4 containing cell lines (Figure 19). All of these experiments used antisera directed against the protein kinase domain of erb B and therefore should not affect the immunoreactivity of the c-terminal mutants. The slightly smaller erb B related proteins detected in the 9134S4 cell line was in agreement with our S1 analysis; approximately 20 amino acids are missing at the c-terminus of c-erb B. We assume that the truncated protein terminates in the adjacent viral sequences, similar to the erb B products of AEV-R and AEV-H. The 62 kD and 64 kD erb B related proteins in 9134S3 suggest that the internal deletion within c-erb B is in frame, since a frameshift into the adjacent erb B sequences predict an even smaller protein product.

E. Molecular cloning and nucleotide sequence analysis of erb B transducing viruses.

In order to verify the presence of an internal in-frame deletion we molecularly cloned the 9134S3 provirus from infected CEF DNA and sequenced the entire erb B derived region. The structure of the 9134S3 provirus is shown in Figure 20 and is consistent with that predicted by Northern and S1 analysis. The virus is approximately 10.3 kb long and

Figure 19. Immunoprecipitations of erb B related proteins in cell lines infected by different c-erb B containing viruses. Uninfected fibroblasts (CEF), and fibroblasts infected with AEV-R, AEV-H, 9134S3, 9134S4, or 9134E1 were labelled with ^{35}S-methionine. Extracts were immunoprecipitated with anti-erb B serum. The positions of erb B related proteins are indicated.

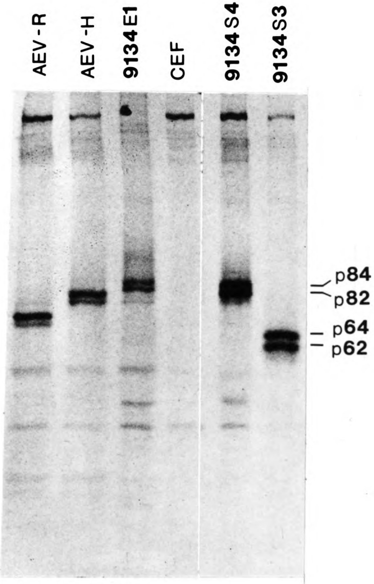
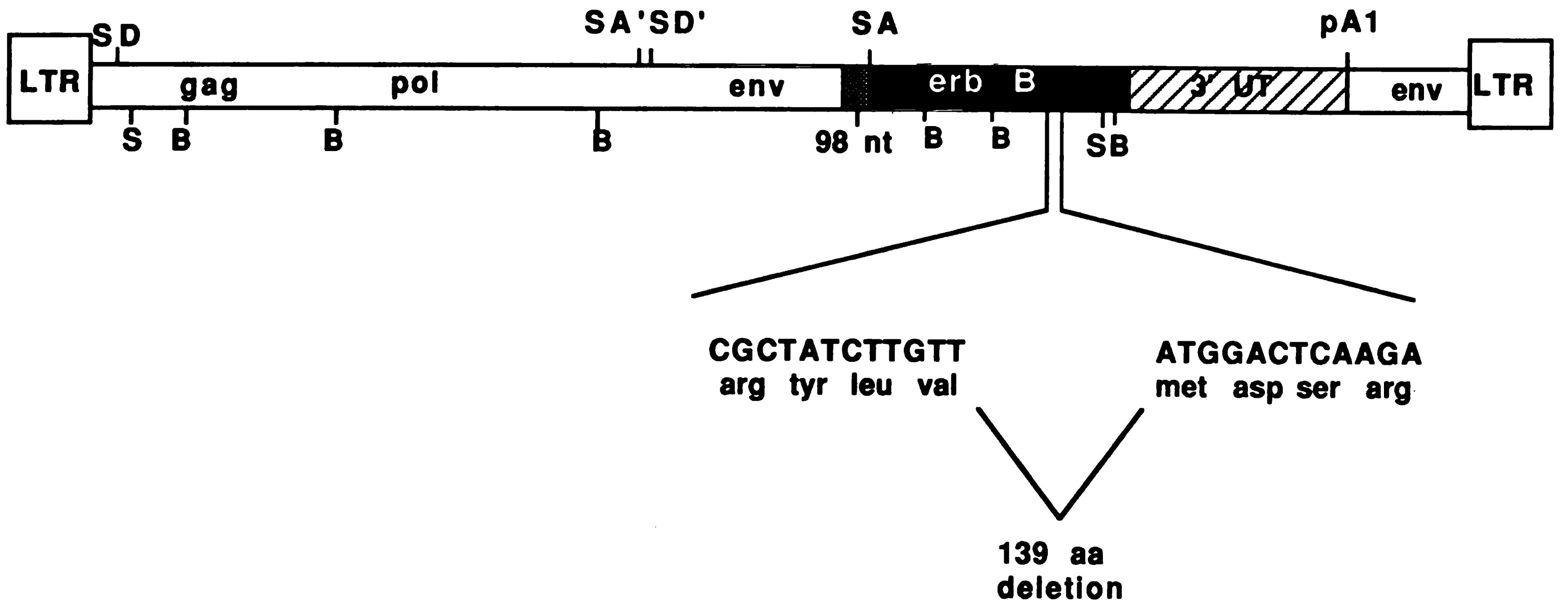

AEV-R
AEV-H
9134E1
CEF
9134S4
9134S3
p84
p82
p64
p62

Figure 20. Structure and sequence of the 9134S3 provirus. The 9134S3 provirus was molecularly cloned from infected chicken embryo fibroblast DNA. The structure of the provirus is shown. Viral and c-erb B related regions were determined by restriction enzyme mapping and Southern analysis. The precise erb B content was determined by DNA sequence analysis. The 5′ and 3′ viral-erb B junction sequences are described in Chapter 6. The sequences defining the 139 amino acid in-frame deletion within erb B are shown below the diagram. Other sequences include: viral sequences (open box), erb B intron sequences (dotted box), erb B coding sequences (solid box), 3′ untranslated region of c-erb B (hatched box). The position of potential splice acceptors (SA), splice donors (SD), and polyadenylation signals (pAl) are also shown. B, Bam HI; S, Sac I.


SD
SA'SD'
SA
pA1
LTR
gag
pol
env
erb B
3' UT
env
LTR
S
B
B
B
98 nt
B
B
SB
CGCTATCTTGTT
arg tyr leu val
ATGGACTCAAGA
met asp ser arg
139 aa
deletion

contains intact gag and pol genes. A portion of the env gene has been replaced by 2.5 kb of erb B derived sequences. The nucleotide sequence in the erb B coding region was identical to that of 9134E1 and IA c-erb B except 417 nt were missing (Figure 20). The deletion of these sequences did not affect the reading frame and fuses a valine directly to a lysine residue thereby removing 139 amino acids of erb B coding sequence. In addition to the coding sequence 98 nt of intron sequence and 1107 nt of 3' untranslated sequences including the first polyadenylation signal, pA1, of the c-erb B locus have been incorporated into the 9134S3 virus. The precise recombination points are described in detail in chapter 4.

F. Disease potential of purified c-erb B transducing viruses

Both 9134S3 and 9134S4 viruses transformed fibroblasts *in vitro*. No foci or soft agar colonies were observed in 9134E1 infected fibroblasts despite detection of erb B related viruses in our inoculum. In order to verify our *in vitro* results, we injected viral supernatants into the wing-web (w.w.) or peritoneum (i.p.) of one-day old chicks. The results are summarized in Table 3. Consistent with transformation by an acute transforming virus, apparent diseases developed within a short period of time (3 to 6 weeks). As expected, fibrosarcomas developed at the site of wing-web injection using both the 9134S3 and 9134S4 viruses. Intraperitoneal injection of 9134S3 and 9134S4 routinely also resulted in the development of disseminating firbrosarcomas of the liver and omentum adjacent to the site of injection. No evidence of fibrosarcomas, however, was detected in any

Table 3. Disease Potential of Clone Purified Virus Stocks

Inoculation		Number Diseased/Number Tested[2]		
Virus	Route of Injection[1]	Ery	Sarcoma	Hem
9134E1	i.p.	9/9	0/9	0/9
	w.w.	0/5	0/5	0/5
9134S4	i.p.	9/10	10/10	0/10
	w.w.	5/5	5/5	0/5
9134S3	i.p.	0/10	6/10	10/10
	w.w.	0/5	5/5	3/5

---

[1] i.p. - intraperitoneally; w.w. - wing web.

[2] Ery - erythroblastosis; Sarcoma - fibrosarcomas; Hem - hemangiosarcoma.

of the 9134E1 injected chicks. The presence of infectious 9134E1 virus was verified by the induction of erythroblastosis in all the i.p. injected and one of the w.w. injected chicks. Although the development of fibrosarcomas was not lethal to the 9134S3 and 9134S4 infected chickens, the spread of infectious virus to other tissues was. The 9134S4 infected birds succumbed to erythroblastosis, while 9134S3 infected birds developed hemangiosarcomas. No evidence of erythroblastosis was observed in the 9134S3 infected birds. Instead a disease characterized by the infiltration of blood cysts in the liver, spleen, and kidney was observed. Numerous blood cysts ranging in size from 1 to 20 mm in diameter were observed in these tissues. A large hemorrhagic cyst associated with the spleen was routinely observed and was the apparent cause of death. This disease appears to be distinct from the angiosarcomas induced by other c-erb B transducing viruses (Tracy et al., 1986) in that it has a shorter latency period and affects primarily the liver and spleen. Both lesions affect cells of endothelial origin.

Discussion:

A. Origin of 9134S3 and 9134S4

Twenty-five to fifty percent of ALV induced erythroblastosis samples contain c-erb B transducing viruses. We have previously shown that these leukemias typically contain multiple c-erb B viruses (Chapter 4). We report here the isolation of three c-erb B transducing viruses from a single ALV induced erythroleukemia sample 9134. Both

9134S3 and 9134S4 were isolated by virtue of their ability to transform fibroblasts or induce sarcomas. 9134E1, however, does not transform fibroblasts and was subsequently purified by molecular cloning since it was the predominant c-erb B transducing virus in the original 9134 inoculum. The 9134S3 and 9134S4 viruses must have been present at very low levels (less than 1%) in the original inoculum since they were not detected using S1 analysis. The same virus (i.e., 9134S3), however, was identified in two independent sarcomas indicating a common origin. The 9134S3 and 9134S4 viruses do not appear to be derived from the 9134E1 virus since their structures are quite different. Moreover, examination of the nucleotide sequence spanning the 5′ and 3′ viral-erb B junction regions reveal that sepearate, unrelated recombination events were involved in their generation. 9134S3 and 9134E1, therefore, are independent examples of c-erb B transducing viruses. We cannot rule out however, that these viruses shared a common ancestry prior to recombination. Both viruses may have been generated from a single ALV provirus insertion event into the c-erb B gene, which underwent different recombination events resulting in multiple c-erb B transducing viruses. Our analysis of sample 9134 verifies our initial observation indicating the heterogeneity of c-erb B transducing viruses present in ALV induced erythroblastosis.

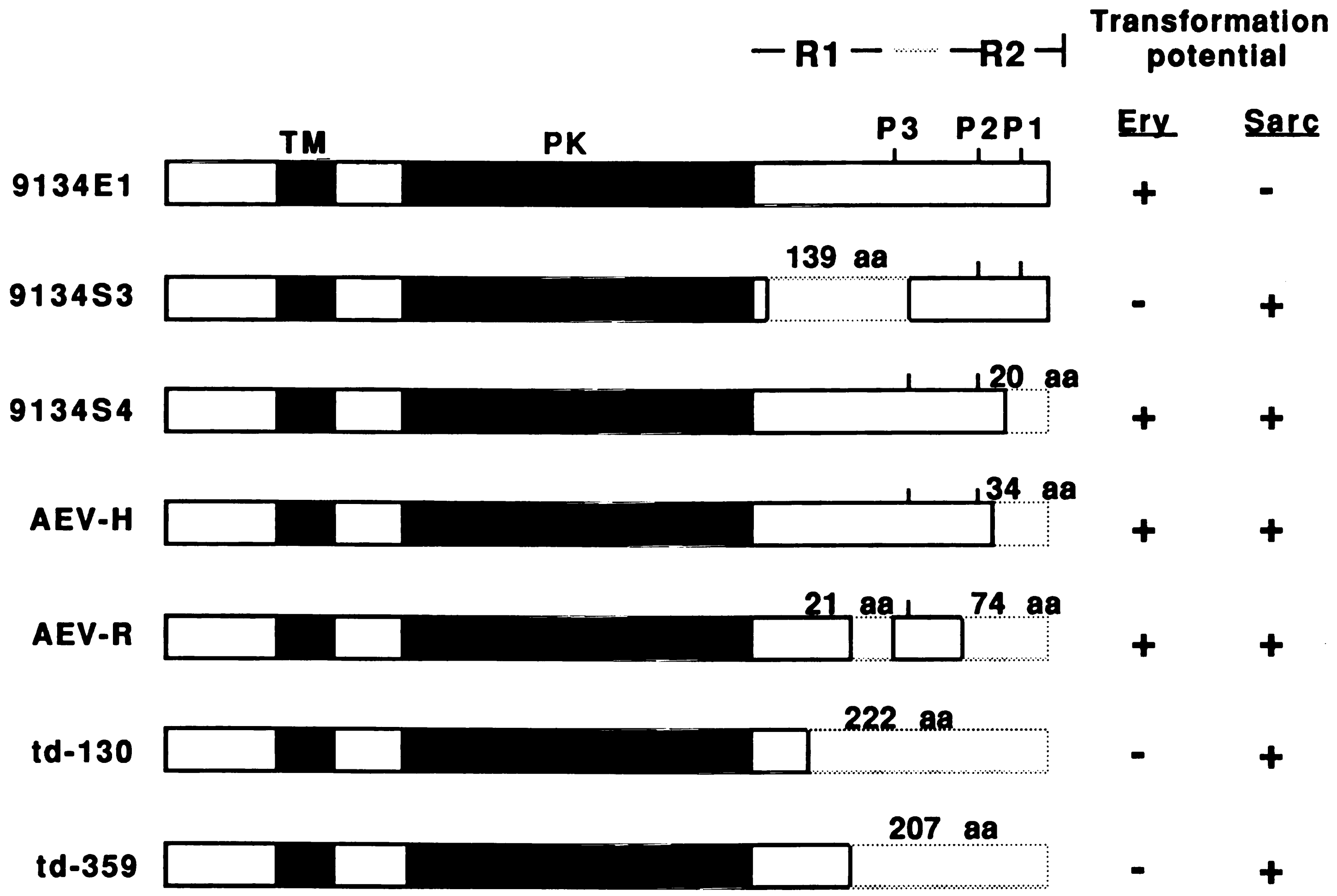
B. Expression of 9134E1 gag-erb B protein is sufficient for erythroblast transformation

Insertional activation of c-erb B results in two alternately spliced mRNAs that encode slightly different protein products (Nilsen et al., 1985; Goodwin et al., 1986). These products differ by the presence of an additional 53 amino acids of _env_ between the first 6 amino acids of _gag_ and erb B (designated as _env_+ or _env_- IA c-erb B). The nucleotide sequence of the 9134E1 virus indicates that only the _env_- IA c-erb B form can be expressed. Exclusive expression of this IA c-erb B protein is sufficient for erythroblast transformation since 9134E1 induces erythroblastosis. We are currently constructing a 9134E1-like virus expressing only the _env_+ IA c-erb B gene product to test its role in inducing erythroblastosis. This experiment is especially interesting in light of recent biochemical data which suggests that the _env_ sequence influences protein processing and therefore may affect its transforming ability.

C. Mutant c-erb B transducing viruses define two domains of erb B which influence disease potential

Each of the newly isolated c-erb B transducing viruses are distinct from previously identified viruses in that they encode different c-erb B proteins which alter their disease specificities. The structure of these viruses and the diseases they induce are shown in Figure 21, along with several other c-erb B transducing viruses. Comparison of these viruses indicates that the 264 amino acids situated between the protein kinase domain and the c-terminus are important in determining the oncogenic potential of c-erb B. This regulatory region appears to contain two subdomains, R1 and R2, based on the disease potential of each virus. Disruption of either R domain by deletion,

Figure 21. Schematic comparison of the erb B proteins displaying different oncogenic potentials. The predicted erb B protein for each virus is shown and is based on nucleotide sequence analysis (except 9134S4) of the virus. The ability to induce sarcomas (sarc) or erythroblastosis (ery) is indicated by a + or - . The transmembrane domain (solid box) and protein kinase domain (dotted box) are indicated as well as the location of tyrosine residues analagous to the major autophosphorylation sites of the human EGF-R, designated P1, P2, and P3 (Hunter, 1984). The location of deleted sequences are bounded by dashed lines. Gene structure data for AEV-R, AEV-H, td-130, and td-359 are from Choi et al., (1986), Yamamoto et al., (1984), and Damm et al., (1987).



— R1 — — R2 ⊣
Transformation potential
Ery
Sarc
TM
PK
P3
P2
P1
9134E1
+
-
9134S3
139 aa
-
+
9134S4
20 aa
+
+
AEV-H
34 aa
+
+
AEV-R
21 aa
74 aa
+
+
td-130
222 aa
-
+
td-359
207 aa
-
+

irrespective of whether it is terminal or internal, is sufficient to alter the oncogenic potential of c-erb B.

The R2 region does not appear to be required for erythroblast transformation since AEV-H and AEV-R, two extensively characterized erythroblastosis inducing viruses, lack the last 37 and 74 amino acids of erb B, respectively (Yamamoto et al., 1984b; Choi et al., 1986). Similarly the c-erb B gene product of 9134S4 lacks the ultimate 20 c-terminal amino acids. The R1 region, on the otherhand, is required for erythroblast transformation since erythroblastosis defective mutants of AEV-R and AEV-H (td359 and td130) contain deletions in this region (Damm, et al., 1987; Yamamoto et al., 1983b). Truncations do not appear to be necessary for erythroblast transformation since 9134E1 encodes a c-terminally intact protein and induces erythroblastosis. This observation has been recently verified using a replication competent retrovirus expressing IA c-erb B sequences (Pelley, Moscovici, and Kung, unpublished).

An intact R2 region has been implicated in determining host susceptibility to erythroblastosis induction (Gammett, 1986). C-terminally intact transducing viruses similar to 9134E1 were found to only induce erythroblastosis in 151 related chickens and not K28 chickens. C-erb B transducing viruses missing the R2 region, such as AEV-R, do not display this specificity. We have not observed a difference in host susceptibility in SPAFAS versus line 151 X 15I4 chickens. Erythroblastosis has been induced in both lines using 9134 homogenates as well as others which are known to contain c-terminally intact transducing viruses. Therefore host susceptibility to c-terminally intact c-erb B transducing viruses does not appear to be restricted to 151 related chickens.

The disease specificity of 9134E1 supports the idea that intact R2 sequences are inhibitory to fibroblast transformation. Indeed their removal is sufficient to activate sarcomagenesis (illustrated by 9134S4 or AEV-H). The 9134S4 virus maps the inhibitory sequence to the last 20 amino acids of erb B. This region contains a tyrosine residue analagous to the major autophophorylation site (P1) of the human epidermal growth factor receptor (hEGF-R). Phosphorylation on this tyrosine appears to be important in regulating EGF-dependent kinase activity of EGF-R (Downward et al., 1984b). By analogy, removal of this tyrosine residue from c-erb B, may regulate fibroblast transformation. The tyrosine autophosphorylation sites for IA c-erb B have not yet been determined. However, they do appear to be important, since replacement of the P1 analog with 3 other amino acid residues render it weakly sarcomagenic (Pelley and Kung, unpublished).

The P1 autophosphorylation site may not be the only tyrosine residue responsible for regulating sarcomagenesis since the P1 site is retained in 9134S3. The 9134S3 virus contains an internal deletion of 139 amino acids which encompasses an alternate tyrosine residue (P3) which is also autophosphorylated in EGF-R (Downward et al., 1984b). Therefore, removal of any one of these autophosphorylation sites may be sufficient to alter the transforming ability of c-erb B. Site-directed mutagenesis of this residue should determine its significance in sarcomagenicity. Alternatively, an internal deletion may disrupt the structure of erb B protein in such a way that the P1 site is no longer functional. The above possibilities are not mutually exclusive. The observation of other c-erb B transducing viruses which do not transform fibroblasts, yet contain internal deletions indicate that only select deletions are capable of activating the transforming potential of c-erb B. Analysis of the c-erb B kinase activity and the respective sites of autophosphorylation in the newly isolated c-erb B transducing viruses should provide insight into the potential role of P1 in sarcomagenesis.

The internal deletion in 9134S3 not only activated the sarcomagenic potential of c-erb B but also altered its disease specificity. 9134S3 infected birds succumbed to hemangiosarcomas shortly after injection. Other c-erb B transducing viruses containing similar internal deletions affecting the R1 domain induce angiosarcomas (Gammett et al., 1986), a similar disease of endothelial cell origin. Of these viruses only 9134S3 has been shown to transform fibroblasts and induce sarcomas. This variation may account for the different manifestations of endothelial cell transformation observed *in vivo*.

The erb B protein is a truncated version of the human epidermal growth factor receptor. Although it lacks the amino-terminal EGF-binding region, it retains the transmembrane domain (Downard et al., 1984; Ullrich et al., 1984). Oncogenesis is presumably due to the expression of a truncated receptor kinase molecule whose activity has become ligand-independent. Our studies suggest that the erb B molecule is active in at least three cell types - erythroblasts, fibroblasts, and endothelial cells. The ability to transform these cell types is regulated by distinct domains of the c-terminus suggesting that different signal transduction pathways may be involved. The analysis of the erb B proteins displaying altered disease specificity should aid in defining the role of c-erb B in signal transduction.

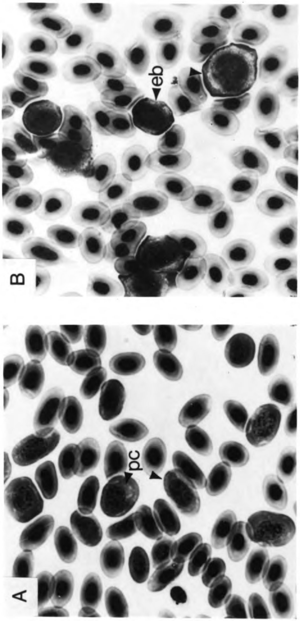
CHAPTER 6: EVIDENCE FOR DIFFERENTIATION OF ALV TRANSFORMED ERYTHROBLASTS *IN VIVO*

Results:

A. Identification of polychromatic erythrocytes in preleukemic phase of ALV induced erythroblastosis

The primary cell type observed in ALV induced erythroblastosis is the erythroblast (Eb). It is an immature erythroid cell which can easily be identified from other blast cells by its large nucleus, basophilic cytoplasm and perinuclear halo (Figure 22A). In addition to the erythroblast, other erythroid cell types can be observed in the peripheral blood of leukemic chickens. Most notable is the polychromatic erythrocyte (PC) which is characterized by its condensed (polychrome) nucleus, slightly basophilic cytoplasm, and somewhat ovoid shape (Figure 23B). It represents an intermediate in erythroid differentiation and occurs between the immature erythroblast and the terminally differentiated erythrocyte. The PC observed in erythroblastosis contains a larger cytoplasm than the polychromatic erythrocytes seen in normal uninfected peripheral blood. In monitoring for erythroblastosis development, we observed an unusually large number of these cells in the bloodstream prior to the appearance of erythroblasts. A typical example is shown in Figure 22B. Differential counts of PC and Eb erythroid cells indicate that the PCs appear two to three weeks prior to the development of leukemia

Figure 22. Blood smears from preleukemic and leukemic chickens. Blood smears of the same bird taken at 50 (A) and 68 days (B) post inoculation. Polychromatic erythrocytes (PC) and erythroblasts (Eb) are indicated, 100X.

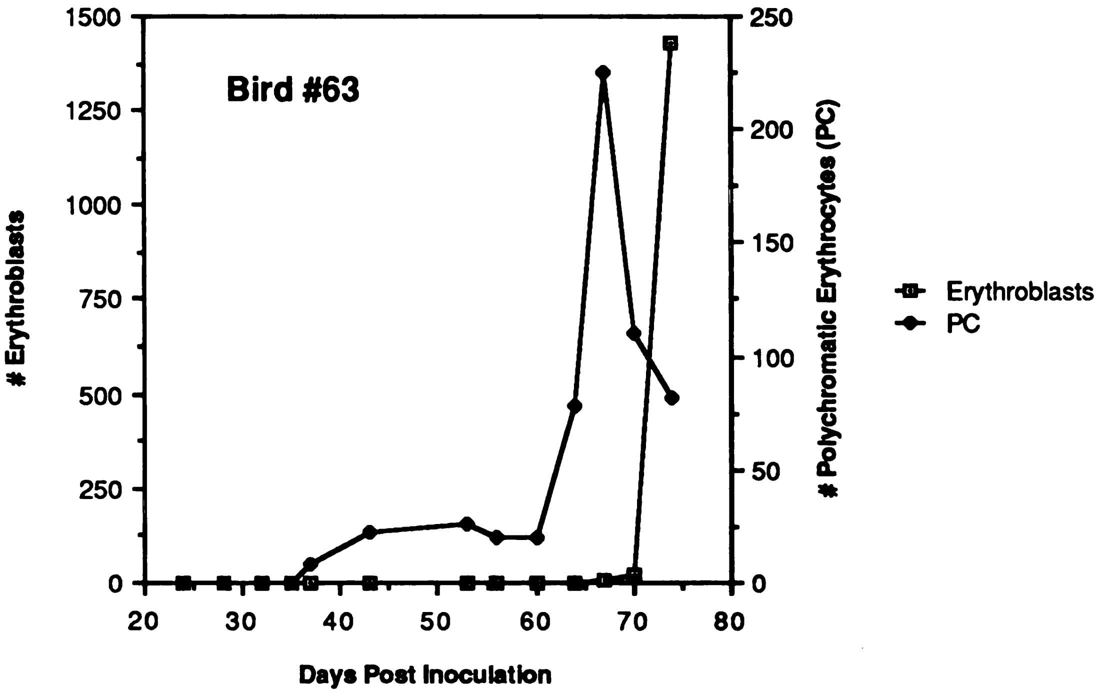

A
pc
B
eb

development of leukemia (Figure 23). The number of PCs reaches peak levels one to two weeks before the appearance of the first erythroblasts. As the erythroblasts start to appear in the bloodstream, the number of PCs diminishes becoming a minor component of the total erythroid precursors (less than 10%) at the terminal stage of leukemia. No changes are observed in the erythrocyte population during the accumulation of PCs in the bloodstream since hematocrit values remain the same during this time. This is in contrast to the appearance of erythroblasts which is usually associated with a concomitant decrease in hematocrit levels. The period over which erythroblasts infiltrate the bloodstream and metastasize to other hematopoietic organs, we define as the leukemic phase. The two to three week period where the polychromatic erythrocyte is the predominant erythroid precursor observed in the bloodstream, we refer to as the preleukemic phase. A preleukemic phase was consistently observed in all birds developing ALV induced erythroblastosis. The number of PCs and the time at which they appeared in the bloodstream varied between individual cases.

B. Gross morphology and histology of preleukemic chickens

In order to characterize this phenomena more closely, we sacrificed ALV infected birds at the preleukemic stage. Gross examination and histology indicated that the liver, spleen, and kidney were normal and showed no evidence of leukemia. This is consistent with the differential counts which detected few if any erythroblasts during this time period. The bone marrow, on the otherhand, was

Figure 23. <u>Appearance of circulating polychromatic erythrocytes prior to erythroleukemia development define a preleukemic phase.</u> The number of polychromatic erythrocytes (circles) or erythroblasts (boxes) per 100 white blood cells were determined at regular time intervals after ALV inoculation. The transient increase in polychromatic erythrocytes in the circulation of bird 63 illustrates the two phases of erythroleukemia. Day 60 tc 69, the period over which the polychromatic erythrocytes increase defines the preleukemic phase and day 70 to 74, the period in which erythroblasts accumulate, define the leukemic phase.

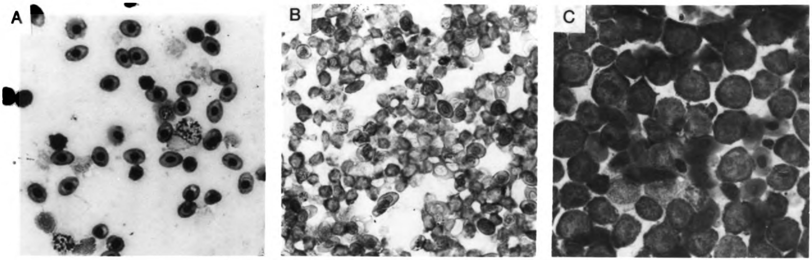

Bird #63
Erythroblasts
Polychromatic Erythrocytes (PC)
Days Post Inoculation
0
250
500
750
1000
1250
1500
0
50
100
150
200
250
20
30
40
50
60
70
80
Erythroblasts
PC

morphologically similar to that of erythroleukemic birds as evidenced by the deep red color, increased consistency, and loss of fat. Examination of bone marrow smears revealed a mixture of erythroid precursors consisting of primarily erythroblasts and polychromatic erythrocytes (Figure 24B). The number of erythroblasts in preleukemic bone marrow is much less than in the leukemic bone marrow (Figure 24C). Similar to the blood, the PCs are the predominant erythroid precursor. This is in contrast to normal bone marrow which consists primarily of more mature reticulocytes and erythrocytes (Figure 24A). The gross appearance of the bone marrow is presumably due to the destruction of the stromal network as a result of the abnormal proliferation of PCs. The sudden increase in PCs in the bloodstream may be a consequence of the deterioration of this stromal network.

C. Insertional activation of the c-erb B gene in preleukemic samples

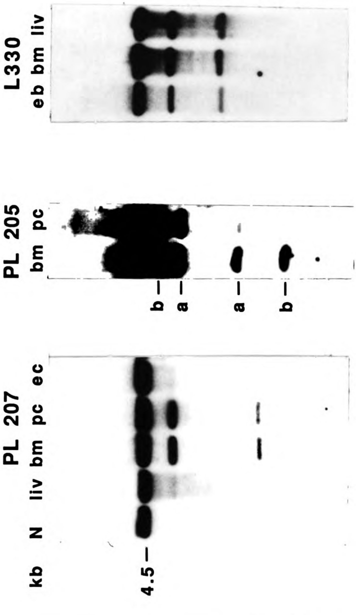
Analysis of the preleukemic birds indicated that dramatic changes were occurring in the erythroid population prior to the onset of erythroblastosis. In order to determine whether these changes were related to erythroblastosis we analyzed DNA samples from preleukemic and leukemic tissues. We have previously shown that the erythroblastosis induced by ALV is due to the activation and mutation of the host oncogene c-erb B by proviral DNA insertion into a 4.5 kb Eco RI fragement situated at the 5' end of c-erb B (Fung et al., 1983; Raines et al., 1985). As a consequence, the restriction enzyme pattern of the activated c-erb B gene in the transformed erythroblasts is different from that of the normal c-erb B gene. The presence of an

Figure 24. Comparison of bone marrow smears from birds in preleukemic and leukemic phases. Bone marrow smears of preleukemic (B), leukemic (C), and uninfected chickens (A) showing the predominance of polychrome erythrocytes, erythroblasts, or erythrocytes. A and B, 60X; C, 100X.


A
B
C

altered c-erb B fragment readily distinguishes transformed erythroblast DNA from normal DNA and thus provides a molecular marker for erythroblast transformation. Furthermore, the altered c-erb B fragments vary in size between different leukemia samples, due to the fact that the sites of proviral insertion differ. A clonal population of transformed erythroblasts originating from a single proviral integration event is identified by its characteristic altered c-erb B fragment. Thus identification of this altered c-erb B fragment should allow us to define the relationship between preleukemia and leukemia during erythroblastosis development. DNA from bone marrow, liver, and enriched PCs or Ebs or erythrocytes from the blood were digested with Eco RI and probed with the 4.5 kb Eco RI fragment (R4.5). ALV has Eco RI sites at each of its termini, therefore insertion into the 4.5 kb Eco RI fragment will generate two altered c-erb B fragments rather than one. This type of analysis has been extensively used to map proviral integrations sites and is described in detail elsewhere (Raines et al., 1985). Leukemic DNA from liver, bone marrow, and enriched erythroblasts contain the same altered c-erb B fragments (Figure 25, L330 samples). This is consistent with the gross morphology and histology of erythroblastosis (see Chapter 1) and indicates that a clonal population of erythroblasts has infiltrated these organs. Also in agreement with the histology is the absence of altered c-erb B fragments in preleukemic liver samples indicating that erythroblasts have not yet metastasized to this organ. Altered c-erb B fragments were observed in the bone marrow and enriched PCs of preleukemic birds. This suggest that the PCs present in the bone

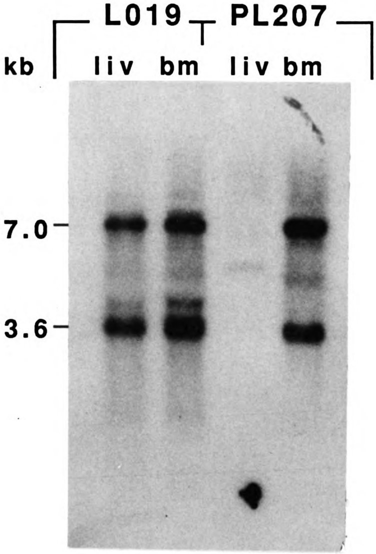
Figure 25. Structural alterations in the c-erb B locus of preleukemic and leukemic DNA samples. DNA was extracted from the bone marrow (bm), liver (liv), or enriched polychromatic erythrocytes (PC), erythroblasts (Eb) or erythrocytes fractions (ec), of peripheral blood from leukemic (L330) or preleukemic (PL207, PL205) chickens. DNAs were digested with Eco R1 and subjected to Southern blot analysis using a genomic 4.5 kb Eco R1 fragment as probe. The endogenous 4.5 kb fragment is indicated. Two novel erb B related Eco R1 fragments are indicative of an insertion by a single ALV provirus (see Raines et al., 1985, and text). Two pairs of additional fragments (a and b) are indicated in bm sample PL207.


kb
4.5
PL 207
N liv bm pc ec
b
a
a
b
PL 205
bm pc
L330
eb bm liv

marrow and bloodstream are indeed related to erythroblastosis. The PCs most likely represent a more differentiated form of the transformed erythroblast since it is a more mature erythroid cell than the erythroblast. Therefore, transformed erythroblasts may differentiate into PCs as an initial step toward erythroblastosis development. Little if any terminal differentiation appears to occur since no altered c-erb B fragments were detected in the erythrocyte population (lane ec). This, however, may be misleading since the transformed PCs and Ebs represent only a minor fraction of the total red cell population. Interestingly, sample PL207 contained two clonal populations of transformed erythroblasts as evidenced by four altered c-erb B fragments in the bone marrow (figure 25, PL207). Only one clone, however, appeared to be released into the bloodstream as PCs. This may represent a case where the structure of the bone marrow has been destroyed at one site of erythroblast transformation but not at the other leading to selective release into the bloodstream. This may explain the apparent abundance of the nonreleased clone (a) over the released clone (b) in the bone marrow.

To verify that the altered c-erb B fragments associated with the preleukemic samples resulted in c-erb B activation, we compared the erb B related RNAs from leukemic and preleukemic tissues (Figure 26B). As previously described (Nilsen et al., 1985), both the liver and bone marrow of leukemic birds expressed high levels of the 3.6 and 7.0 kb insertionally activated c-erb B RNAs (IA c-erb B). Only normal c-erb B transcripts (5.8, 9.0, and 12.0 kb) were detected in preleukemic liver

Figure 26. Northern blot analysis of leukemic and preleukemic tissues samples. Poly (A)+ RNA was extracted from liver (liv) and bone marrow (bm) tissues of preleukemic (PL 207) and leukemic (L019) chickens. 5 ug poly (A)+ RNA was subjected to Northern analysis. Hybridization was done with an erb B specific probe encompassing 1.7 kb of the erb B coding sequence (probe T, defined in Figure 11). The 7.0 and 3.6 kb erb B related RNAs typical of insertionally activated c-erb B erythroleukemia samples are indicated. Note that at this exposure little, if any, of the normal c-erb B transcripts can be detected.


L019
PL207
kb
liv
bm
liv
bm
7.0
3.6

samples after long exposures (data not shown, see Chapter 2). No IA c-erb B RNAs were observed in preleukemic liver RNA as would be expected from the absence of transformed erythroblasts in this organ. The c-erb B related RNAs in the preleukemic bone marrow were similar to those in the leukemic animal; both contain the 3.6 and 7.0 kb IA c-erb B transcripts and are expressed at comparable levels. Thus the predominant cell type in the bone marrow of preleukemic chickens, the polychromatic erythrocyte, contains an activated c-erb B gene similar to the transformed erythroblast. The timely appearance of this cell type and its obvious relation to transformed erythroblasts suggests that it is an intermediate step in the manifestation of erythroblastosis.

Discussion:

Erythropoiesis occurs almost exclusively in the bone marrow of hatched chicks (Romanoff, 1960) and it is there where the target cells for erytrhoblast transformation reside. Studies with acute transforming viruses indicate that erythroid progenitor cells at the BFU-E stage are the target cells for erythroblast transformation (Samarut, 1982). We assume the erythroblasts which accumulate in the bone marrow and bloodstream of leukemic birds, however, resemble CFU-E cells indicating that transformation of erythroid cells does not arrest erythroid differentiation. In addition to displaying differentiation markers similar to CFU-E cells (Beug et al., 1985a), ALV tranformed erythroblasts retain their capacity to differentiate *in vitro*. They differ from normal CFU-E in that they display an increased proliferative capacity and do not require erythropoietin for differentiation or self-renewal. As a result, only a portion of the cells become committed to differentiate and mature into erythrocytes. In view of these properties, it is not surprising that polychromatic erythrocytes are observed *in vivo*. The presence of the activated c-erb B gene in PC cells strongly suggests that they originated from transformed erythroblasts and are the result of partial differentiation *in vivo*. Analysis of preleukemic blood samples and leukemic blood samples from the same chicken should indicate whether the two cell types are directly related.

The large number of PCs and the destruction of the bone marrow in preleukemic chickens suggests that these cells display an increased proliferative capacity in addition to the capacity to differentiate. Like the erythroblasts, PCs from the peripheral blood of preleukemic animals differentiate in culture (Beug and Raines, unpublished). The predominance of the polychromatic erythrocytes and not erythroblasts at this stage suggest that the balance between the ability to self-renew and differentiate typical of leukemic erythroblasts is disrupted. In the preleukemic stage this balance appears to be similar to normal erythroid homeostasis in that proliferation is coupled directly to differentiation. At the leukemic stage the proliferative signal is favored over the differentiation signal, as if the two had perhaps become uncoupled. This uncoupling may be related to the erythropoietin independence characteristic of transformed erythroblasts.

The distinction between the cell types present at the leukemic and preleukemic stages suggests that some other event may be necessary for erythroblast transformation. This event may be unrelated to erythroblastosis and may be a nonspecific response due to disruption of normal hemostatic regulation. Perhaps a specific growth factor required for erythroid differentiation is depleted by the leukemic phase. Alternatively, a second event may occur in the transformed erythroblast itself, resulting in the selection of a highly proliferative subclone of erythroblasts. This latter possibility is consistent with the multistep models of tumor progression and metastasis (Buick et al., 1984). Indeed a similar selection appears to occur in ALV induced lymphoid leukosis where transformed follicles are detected as early as 4 to 8 weeks post inoculation, but only one or two develop into a lymphoma 4 to 6 weeks later (Cooper et al., 1969; Neiman et al., 1980). Selection of a subpopulation of transformed erythroblasts may also explain why preleukemia has not been reported for erythroblasosis induced by acute transforming viruses. ALV induced erythroblastosis is due to the clonal expansion of a transformed erythroblast population and requires a long latency period. Erythroblastosis induced by acute transforming viruses is polyclonal and occurs over a short latency period. The continuous recruitment and selection of highly proliferative transformed erythroblasts by acute transforming viruses may therefore, obscure the detection of a well defined preleukemia in birds infected with acute transforming viruses.

C-erb B activation appears to be a key step in erythroblast transformation since it has been associated with all ALV induced erythroblastosis samples analyzed to date (Raines et al., 1985, and Chapter 4). The presence of an insertionally activated c-erb B gene in the preleukemic polychrome erythrocytes suggests that it is one of the first steps in erythroblast transformation. Most significantly, the IA c-erb B protein bears a striking resemblance to a truncated form of the epidermal growth factor-receptor (EGF-R). The erb B protein lacks the extracellular EGF-binding domain of EGF-R, and therefore is unable to bind EGF. It retains, however, the protein kinase domain of EGF-R and a c-terminal regulatory region. As a result of amino-terminal truncation, the erb B protein is constitutively active and can therefore trigger a proliferative response in the transformed erythroid cells. The large number of PCs and erythroblasts present in leukemic and preleukemic birds is indicative of an increase in the proliferative response of erythroid cells. Interestingly, several other oncogenes of the tyrosine kinase family are also capable of inducing erythroblastosis. This suggests that an active protein kinase activity is involved in producing this proliferative signal. The constitutively active protein kinase molecules are thought to mimic the erythropoietin receptor since the transformed erythroblasts are epo-independent and a portion of the leukemic cells terminally differentiate. Further characterization of the polychromatic erythrocytes in preleukemic chickens should aid in identifying these signals and how they are coupled in normal erythropoiesis.

SUMMARY

Avian leukosis virus (ALV) is a naturally occurring virus which induces a variety of neoplasms in chickens. B-cell lymphoma is the predominant neoplasm induced, although nephroblastomas, hemangiosarcomas, and erythroblastosis have also been observed. Characterization of B-cell lymphomas suggested that proto-oncogenes, upon activation, could be oncogenic. In this case the proto-oncogene c-myc was activated by proviral insertion. In an effort to explain the multipotency of ALV we have identified two chicken strains, line 151 and 151 X 15I4, displaying an unusually high susceptibility to ALV induced erythroblastosis. Molecular characterization of these leukemic samples (over 50 total) indicate an absolute correlation between erythroblastosis induction and the activation of the proto-oncogene c-erb B. The c-erb B gene was first identified by homology to v-erb B, the transforming gene of the avian erythroblastosis virus. More recently it has been shown to be related to the gene encoding the human epidermal growth factor receptor (hEGF-R). ALV induced erythroblastosis results from two different types of c-erb B activation - insertional activation and transduction.

Insertional activation of c-erb B is the predominant mechanism of c-erb B activation and accounts for 75% of the samples analyzed. These samples contain an ALV provirus inserted within one of two c-erb B alleles, alpha and beta. The proviral integration sites are clustered

in a region very close to the first exon displaying homology to v-erb B (designated VB1). Unlike the proviruses associated with activated c-myc genes, the c-erb B associated proviruses are intact and always situated in the same transcriptional orientation as c-erb B. Transcription of the insertional activated c-erb B gene initiates in the 5' LTR of the integrated provirus, although a small amount of 3' LTR promoted erb B RNAs can also be detected (less than 1%). Transcription terminates at one of two polyadenylation sites and can utilize alternate splicing. Two different splicing reactions predict the synthesis of different insertional activated erb B proteins. Both are identical in erb B content, but contain either six amino acids of gag or six amino acids of gag plus 53 amino acids of env at their amino terminus. The erb B sequences of both proteins begin precisely at the 5' boundary of VB1 and terminate in sequences displaying homology to the carboxy-terminal portion of hEGF-R.

Transduction of c-erb B is the other mechanism of c-erb B activation and accounts for approximately 25% of the erythroblastosis samples analyzed. This is an unusually high frequency for oncogene transduction and has enabled us to study the mechanism of transduction more closely. Our results are in agreement with the model of transduction proposed by Swannstrom et al. (1983), and suggests that insertional activation of c-erb B is probably a key step in c-erb B transduction as well as deletion of the 3' LTR. The 5' recombination site consistently mapped to the intron region 5' to VB1, while the 3' recombination occurred in the last exon of c-erb B. Disruption of c-terminal sequences were observed in only two cases. Nucleotide sequence analysis of the precise junction site at the 5′ and 3′ end of two transduced retroviruses indicate that homologous sequences may be involved. Most notable is the frequent recombination between the poly (A) tract of c-erb B mRNA and a AAAAAA sequence in the <u>env</u> gene of RAV-1. This provides direct evidence for an RNA intermediate in transduction and strengthens the notion that the second recombination utilizes a "copy-choice" mechanism.

The insertional activated and transduced erb B genes encode virtually identical proteins. The c-erb B coding sequences of both begin precisely at VB1 and end at a common termination codon. This protein bears a striking homology to the hEGF-R. The hEGF-R contains three domains, an extracellular EGF-binding domain, a short transmemebrane domain, and a cytoplasmic domain. The activated c-erb B sequences are homologous to the carboxy-terminal half of hEGF-R and lack the EGF-binding domain. The cytoplasmic protein kinase domain is retained as well as the ultimate c-terminal regulatory sequences. This homology suggests that c-erb B is the avian counterpart of EGF-R. Our preliminary characterization suggests that the normal c-erb B product does contain a large extracellular domain, although it remains to be seen whether this domain is homologous to the EGF-binding domain. In any case, activation of c-erb B appears to result from a very specific amino-terminal truncation such that this extracellular (ie., ligand-binding domain) is removed. The effect of this truncation on c-erb B function is not known but is presumably due to constitutive protein kinase activity. Further biochemical analysis of the activated and normal c-erb B gene products should address this question.

The involvement of c-erb B in ALV induced erythroblastosis was correlative and did not demonstrate that activated c-erb B was responsible for erythroblastosis. We have used the transduced erb B viruses to determine more directly the oncogenic potential of c-erb B. Our results indicate that introduction of an activated c-erb B protein into the appropriate target cell is sufficient for erythroblast induction. In addition we have isolated two mutant c-erb B transducing viruses which display altered oncogenic potentials. Molecular characterization of these viruses indicate that the c-terminal sequences of c-erb B are important. These studies support the notion that c-terminal sequences of EGF-R are important in regulating receptor function. The c-erb B mutants suggest that this regulation may vary in different cell types. The differential regulation of the c-erb B protein has far reaching implications and suggests that a single growth factor receptor may use different signal transduction pathways in different cell types, or different receptors may transduce similar signals. Indeed the biology of erythroblastosis and the phenotype of transformed erythroblasts suggest that the activated c-erb B protein can mimic an erythroid specific growth factor receptor. Further characterization of both the normal and activated c-erb B products should extent our current knowledge of growth factor receptors and their role in leukemogenesis.

APPENDIX A:

C-ERB B ACTIVATION IN AVIAN LEUKOSIS VIRUS INDUCED ERYTHROBLASTOSIS: CLUSTERED INTEGRATION SITES AND THE ARRANGEMENT OF ALV PROVIRUS IN THE C-ERB B ALLELES

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c-*erbB* activation in avian leukosis virus-induced erythroblastosis: Clustered integration sites and the arrangement of provirus in the c-*erbB* alleles

(retrovirus/cellular oncogene/epidermal growth factor receptor/promoter insertion)

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ABSTRACT **There is considerable evidence that links the activation of cellular genes to oncogenesis. We previously reported that structural rearrangements in the cellular oncogene c-*erbB* correlate with the development of erythroblastosis induced by avian leukosis virus (ALV). c-*erbB* recently has been shown to be related to the gene encoding epidermal growth factor receptor. We now have characterized the detailed mechanisms of c-*erbB* activation by ALV proviruses. We report here that the ALV proviral integration sites are clustered 5′ to the region where homology to v-*erbB* starts, suggesting that interruption in this region of c-*erbB* is important for its activation. The proviruses are oriented in the same transcriptional direction as c-*erbB* and usually are full-size. The latter finding is in contrast to the frequent deletions observed within the c-*myc*-linked proviruses in B-cell lymphomas. We have also identified a second c-*erbB* allele, which differs from the previously known allele primarily by a deletion in an intron region. This allele is also oncogenic upon mutation by an ALV provirus.**

Avian leukosis virus (ALV), a naturally occurring cancer virus of chickens, can induce a variety of neoplasms, including B-cell lymphomas, erythroblastosis, nephroblastomas, fibrosarcomas, etc. (1, 2). In the past few years, the mechanisms of ALV oncogenesis have been characterized in some detail (3–8). It was shown that ALV induces B-lymphomas by activation of the host oncogene c-*myc* (3). This activation of c-*myc* is accomplished by the insertion of an ALV provirus, which carries strong promoter/enhancer sequences, near the c-*myc* gene. We recently reported evidence suggesting that ALV induces erythroblastosis by a similar mechanism, with proviruses inserted near another host oncogene, c-*erbB* (9). c-*erbB* is the cellular homolog of one of the oncogenes carried by avian erythroblastosis virus (AEV), an acute oncogenic retrovirus known to induce rapid erythroblastosis in chickens (1, 10). The data indicate that, upon activation, c-*erbB* can assume an oncogenic role similar to that of its viral counterpart. In our previous communication, we showed a strong correlation between ALV-induced structural alterations of the c-*erbB* gene and the development of erythroblastosis (9). No alteration of c-*erbA* (the cellular homolog of the other oncogene of AEV) was found in any of the samples analyzed. Furthermore, in all the leukemic samples analyzed to date, transcription of c-*erbB* but not c-*erbA* is highly elevated (unpublished data). The data suggest that activation of the c-*erbB* gene alone is sufficient to cause erythroblast transformation. Although these studies provided important insights into the involvement of the c-*erbB* locus in the development of erythroblastosis, little was known about its activation mechanism, since the position, orientation, and structure of the adjoining ALV proviruses had not been examined. In addition, the c-*erbB* gene appeared to be more complex than previously reported. Using restriction endonuclease analysis, we had detected polymorphism in the c-*erbB* locus, a feature atypical of most cellular oncogenes. At least seven different *Eco*RI-digestion patterns of c-*erbB* were identified (ref. 9 and unpublished data). Several of the *erbB*-related fragments could not be accounted for by the published map of c-*erbB* (10). The nature of these polymorphic elements—whether they represented different alleles, members of a gene family, or pseudogenes—had not been explored.

We report here our detailed characterization of an additional 37 erythroblastosis samples induced in line 15_1 chicks by ALV infection. Our data may be summarized as follows: (*i*) A 100% correlation of c-*erbB* structural alteration with the development of erythroblastosis was observed. The great majority of the proviral integration sites are clustered in a region at the 5′ end of the first exon homologous to v-*erbB*, suggesting that disruption of the c-*erbB* locus in this region is important for its activation. (*ii*) Most of the proviruses appear to be full-length and oriented in the same transcriptional direction as c-*erbB*. One such provirus was molecularly cloned and shown to be completely intact. This finding contrasts with the analogous studies with B-lymphomas, where c-*myc*-linked proviruses usually carry large deletions. (*iii*) A second c-*erbB* allele was identified. This allele is also potentially oncogenic and can be mutated by an ALV provirus to cause erythroblastosis.

MATERIALS AND METHODS

Collection and Analysis of Erythroleukemic Samples. RAV-1, a prototype ALV, was used to inoculate 1-day-old line 15_1 chicks. The development of erythroblastosis and collection of leukemic samples were similar to those described previously (9).

DNA was extracted from quick-frozen bone marrow or liver samples as described by Maniatis *et al.* (11). DNA samples (25 μg) were digested with restriction enzymes under conditions recommended by the supplier (Bethesda Research Laboratories). Digested DNAs were ethanol-precipitated, dissolved in 10 mM Tris Cl, pH 8.0/1 mM EDTA, and then electrophoresed in 0.7% agarose gel, transferred to nitrocellulose, and hybridized with the appropriate radioactive probes (9, 11).

Abbreviations: ALV, avian leukosis virus; AEV, avian erythroblastosis virus; LTR, long terminal repeat; EGF, epidermal growth factor; kb, kilobase(s).

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Radioactive Probes and Molecular Hybridization. All restriction fragments were purified by agarose gel electrophoresis and electroelution prior to radiolabeling (11). Hybridization probes were synthesized from the isolated DNA fragments by nick-translation, and hybridizations were carried out under conditions identical to those previously reported (9). Filters were washed in 30 mM NaCl/3 mM sodium citrate, pH 7/0.1% NaDodSO$_4$ at 65°C, dried, and exposed to x-ray film.

Molecular Cloning and Restriction-Enzyme Mapping. Partial *Eco*RI digests of liver DNA were size-selected on sucrose density gradients and ligated to the arms of phage vector EMBL-4 (12). The recombinant phages were packaged and screened by probes specific for v-*erbB* and the ALV long terminal repeat (LTR) as described (11, 13). Restriction-enzyme mapping of recombinants was performed by single and double digestions, followed by Southern blot (14) analysis with ALV- and v-*erbB*-specific probes.

RESULTS

α and β Alleles of c-*erbB*. Vennstrom and Bishop (10) previously have isolated and characterized c-*erbB* clones, derived from a genomic library of an outbred Leghorn chicken. The *Eco*RI and exon maps at this allele, designated the α allele of c-*erbB*, are shown in Fig. 1*A*. Our subsequent studies revealed restriction-fragment polymorphisms of c-*erbB* in different inbred lines of chickens (9). The most obvious difference is the presence of the 4.5- and 12.0-kilobase (kb) *Eco*RI fragments in some birds and the presence of the 2.3-, 5.3-, and 6.4-kb *Eco*RI fragments in others. By cloning and fine-structure mapping, we now have identified a second c-*erbB* allele, β, that can adequately account for these polymorphic variations (data to be published elsewhere). The *Eco*RI map of the β allele is summarized in Fig. 1*A*. The major difference between α and β lies in the intron region next to VB1, the first exon homologous to v-*erbB*. The β allele has a deletion of ≈2.5 kb in this region, with the appearance of a new *Eco*RI site near the boundary of this deletion. An additional *Eco*RI site specific for the β allele is located further downstream and splits the 12-kb fragment present in the α allele into 5.3- and 6.4-kb fragments. Aside from these two differences, the α and β alleles are very much alike. Neither of the polymorphic variations seems to affect the coding region of c-*erbB*, although conclusive evidence awaits the direct DNA sequence comparison of the two alleles. In an extensive survey of the inbred chickens maintained at the United States Department of Agriculture Regional Poultry Research Laboratory, we found that most lines (e.g., 15I$_5$, 15$_B$, 7, and 6$_3$) carry α alleles. β alleles were identified in line 15$_1$ and RLC (and in K28; H. Robinson, personal communication). Among the 15$_1$ birds surveyed, 65% are homozygous for α, 10% are homozygous for β, and the remaining 25% are heterozygous for α and β.

Activation of the α Allele by ALV Proviral Insertion. We previously have shown that a c-*erbB* structural alteration correlates with the development of erythroblastosis (9). One fragment from an altered c-*erbB* locus was molecularly cloned, and it was shown by direct sequencing that the alteration is due to the insertion of an ALV LTR about 1.6 kb upstream from the VB1 exon. To determine whether LTR insertion near the VB1 exon is a general activation mechanism, we have analyzed 37 additional ALV-induced erythroblastosis samples. The VB1 exon is located inside the 4.5-kb *Eco*RI fragment of the α allele (and the 2.3-kb fragment of the β allele). Thus, to determine whether ALV provirus insertion occurs in this region, the 4.5-kb *Eco*RI fragment of the α allele was subcloned and used as a probe (the R4.5 probe, Fig. 2*A*). The strategy of this experiment is illustrated in Fig. 2*A*. If the ALV proviral insertion occurs near VB1 as depicted, we should see an interruption of the *Eco*RI 4.5-kb fragment by the provirus, resulting in two fragments (X and Y) detectable with probe R4.5. Since there is an *Eco*RI site present in the ALV LTR, fragment X should contain a portion of the LTR, and fragment Y should contain the complementary part of the LTR. As a result, the sum of X and Y should be equal to 4.5 kb plus the size of an ALV LTR, which is 0.34 kb. Thus, one would anticipate seeing two altered fragments, with their sum being ≈4.8 kb. (This calculation was based on the α allele, but the same argument holds for the β allele, except that the sum should be 2.6 kb.) The following data (Fig. 2*B* and Table 1) clearly demonstrate that this is indeed the case. The left panel shows *Eco*RI-digested

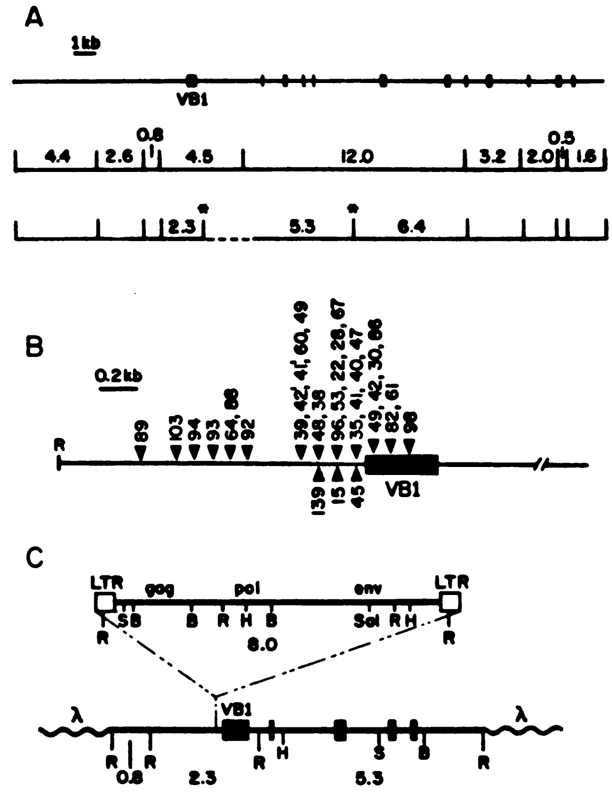

FIG. 1. (*A*) *Eco*RI restriction map of the α and β alleles of c-*erbB*. The α-allele map is according to that reported by Vennstrom and Bishop (10) and Sargeant *et al.* (15). The β-allele map was established by the isolation and restriction enzyme mapping of overlapping clones of this allele (data to be published elsewhere). Solid boxes show regions homologous to v-*erbB*. Size and approximate location of exons are based on previously reported heteroduplex analysis (10, 15). The location of the first exon homologous to v-*erbB*, designated VB1, is defined more accurately by fine restriction-enzyme mapping of the 4.5- and 2.3-kb *Eco*RI fragments. The vertical bars denote the *Eco*RI cleavage sites; ---, deleted sequences; *, *Eco*RI sites present only in the β allele. (*B*) Proviral integration sites in the c-*erbB* gene of erythroblastosis samples. Positioning of the integration sites in different samples (indicated by arrowhead with corresponding sample number) is based on the sizes of *Eco*RI restriction fragments as described in the text. The integration sites in α or in β are placed according to their relative distances from the 5′ *Eco*RI site of the 4.5- or 2.3-kb fragment, respectively. (*C*) Restriction enzyme map of clone λ139. Clone λ139 was isolated from a genomic library derived from leukemia sample 139. Restriction fragments were ordered based on their single- and double-digestion patterns as well as on their hybridization to specific cellular and viral probes. *Eco*RI, R; *Bam*HI, B; *Hin*dIII, H; *Sac* I, S; *Sal* I, Sal. The bottom line represents cellular sequences of the β allele. Solid boxes denote exon sequences. Wavy lines indicate the arms of the λ vector. Dotted line indicates the point of insertion of the ALV provirus (top line). LTRs are shown as boxes; gag, group-specific antigens; pol, polymerase; env, envelope glycoproteins.

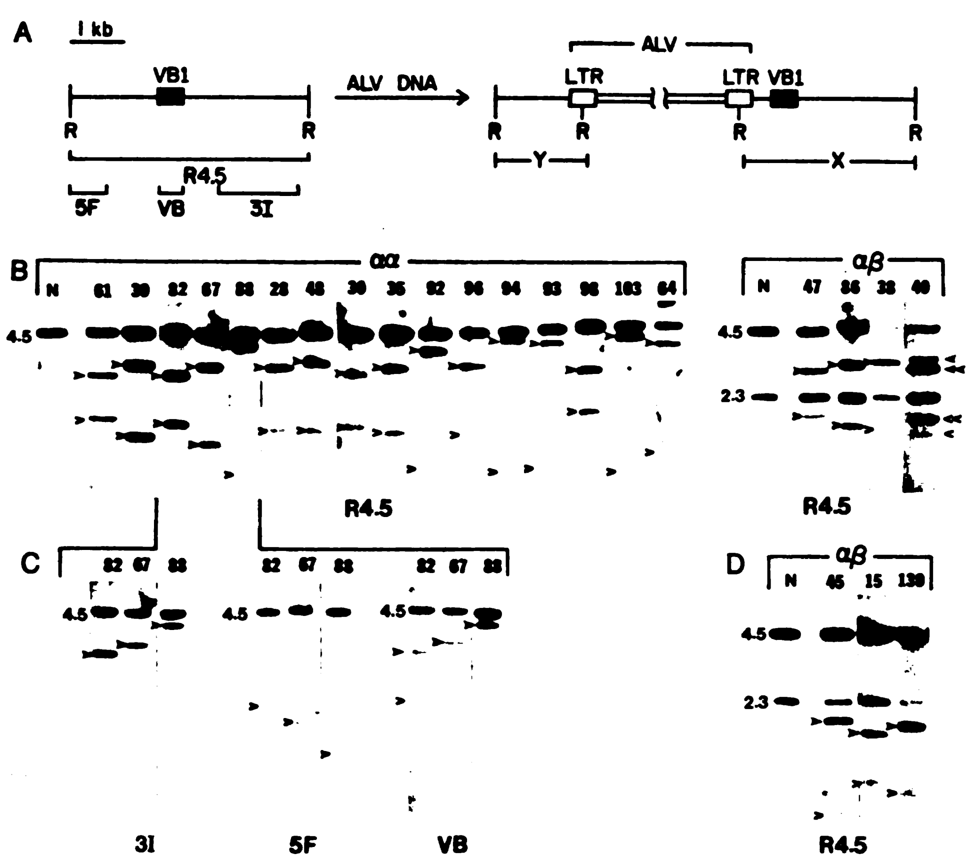

FIG. 2. *Eco*RI-digestion analysis of the proviral integration sites. (*A*) Schematic diagram of proviral insertion upstream from VB1. Also shown are probes used in this study, and the regions they detect. Probe R4.5 represents the 4.5-kb *Eco*RI fragment of the α allele. Probe 5F is a 0.8-kb *Eco*RI–*Pst* I fragment derived from the 5′ end of R4.5. Probe 3I is a 1.6-kb *Pvu* II fragment located in the 3′ intron region of R4.5. A 0.7-kb *Bam*HI–*Sac* I fragment specific for the 5′ end of v-*erbB* was used as the VB probe. This probe recognizes the 4.5- and 12.0-kb *Eco*RI fragments of the α allele (9); for clarity, only hybridization to the 4.5-kb fragment is included in *C*. (*B–D*) Southern blot analyses of *Eco*RI-digested DNA from normal uninfected (N) and erythroblastosis samples (numbers above lanes). Filters were hybridized with the probes indicated at the bottoms of the autoradiograms. Leukemia-specific bands that show rearrangements within the 4.5-kb *Eco*RI fragment (*B* and *C*) or the 2.3-kb *Eco*RI fragment (*D*) are indicated by solid or open arrowheads (X and Y fragments, respectively; see *A*). Sizes of the rearranged bands in *B* are summarized in Table 1. *B–D* are composites of five gels, among which the migration properties of the fragments differ slightly.

DNA samples from chicks of the αα type. In the normal control (lane N) probe R4.5 hybridizes to the 4.5-kb *Eco*RI fragment as expected. In other lanes with leukemic samples, two additional bands X (solid arrowheads) and Y (open arrowheads) can be identified (the larger fragments are arbitrarily designated X). In every case, X and Y total approximately 4.8 kb (Table 1).

Analysis of samples from chicks heterozygous for the α and β alleles are shown at right in Fig. 2*B*. The 4.5- and 2.3-kb fragments present in the normal control represent the α and β alleles, respectively. The panel shows samples in which alteration of the α allele is observed. Again, the sum of fragments X and Y is ≈4.8 kb. Sample 49 carries four rearranged fragments which pair into two sets of X and Y; presumably, this sample contains DNA from two clonal populations of leukemic cells, each harboring an ALV provirus near VB1 but at a slightly different site. It is noteworthy that the intensity of the 4.5-kb band is reduced relative to the 2.3-kb band in a few samples. Since these samples are from birds heterozygous for α and β, disruption of the α allele should correlate with the loss of the 4.5-kb band, assuming that all cells in the samples are transformed erythroblasts. Analysis of bone marrow samples that contain ≈80% erythroblasts (i.e., samples 38 and 49) does show significant reduction in the intensity of the 4.5-kb band relative to the 2.3-kb band. The residual 4.5-kb band is presumably derived from the undisrupted α allele present in the untransformed leukocytes in the bone marrow.

Table 1. Size of viral-cell junction fragments of proviruses inserted in the α allele

	Fragment size, kb			Fragment size, kb	
Sample	*Eco*RI	*Sac* I	Sample	*Eco*RI	*Sac* I
89	4.3, 0.5	12.0, 4.2	28	3.2, 1.6	10.5, 5.3
103	4.1, 0.7	11.0, 4.1*	22	3.2, 1.6	ND
94	4.0, 0.7	12.0, 4.2	53	3.1, 1.6	11.5, 5.3
93	4.0, 0.7	11.0, 4.3*	96	3.3, 1.6	11.0, 5.3
64	3.7, 1.0	12.0, 4.5	35	3.2, 1.7	11.0, 5.2
88	3.8, 1.0	11.5, 4.7	41	3.2, 1.7	ND
92	3.6, 1.1	11.5, 4.6	40	3.1, 1.7	10.5, 5.4
49	3.4, 1.4	11.5, 4.8	47	3.1, 1.7	10.5, 5.4
60	3.4, 1.4	9.6, 4.1*	49	3.0, 1.8	10.6, 5.3
41′	3.4, 1.4	11.8, 4.6	42	3.0, 1.8†	10.5, 5.4
42′	3.3, 1.4	10.5, 5.2	30	3.0, 1.8†	10.2, 5.4
39	3.4, 1.4	11.0, 5.0	86	3.0, 1.8†	10.5, 5.5
38	3.3, 1.5	11.0, 5.2	82	3.0, 1.9†	ND
48	3.4, 1.5	10.5, 5.2	61	3.0, 1.9†	11.0, 5.5
67	3.3, 1.6	11.0, 5.3	98	2.8, 2.0†	10.8, 5.4

Shown are the 30 typical cases in which direct proviral insertions into the α allele were found. Cases involving the processed *erbB* gene (see *Discussion*) and the proviral insertions in the β allele are not included. *Eco*RI and *Sac* I junction fragments are determined as described for Figs. 2 and 3, respectively. Samples are arranged in order of their integration sites relative to VB1. ND, not determined.

*Deleted provirus.

†Integration site within VB1.

The experiment described above indicates that there is a high frequency of proviral integrations near the VB1 exon and in the *Eco*RI fragment, but it does not reveal whether the proviral integration sites are located upstream or downstream from the VB1 exon. To examine this, we hybridized the same DNA blot as in Fig. 2*B* to the following region-specific probes (see Fig. 2*A*): 5F (the 5′ flanking sequence), 3I (3′ intron), and VB (v-*erbB*). Examples of such hybridizations are shown in Fig. 2*C*; probe 3I detects exclusively the longer (X) fragment, whereas probe 5F hybridizes more strongly to the shorter (Y) fragments. This indicates that the interruption due to proviral insertion is in the 5′ half of the *Eco*RI 4.5-kb fragment. Hybridization with probe VB detects only fragment X in most cases, as shown for samples 67 and 88. This result suggests that VB1 is linked to its downstream intron sequence, implying that the ALV provirus must integrate on the 5′ side of VB1. In sample 82, both fragments X and Y are detected by probe VB, suggesting that, in this case, the ALV provirus is integrated within VB1. Based on the sizes of fragment X (or Y) and the information regarding their relative positions to VB1, the individual proviral integration sites can be determined. They are summarized in Fig. 1*B*. It is apparent that the ALV proviral integration sites are clustered in a region immediately upstream from VB1. In those cases (e.g., sample 82) where proviral integration within VB1 is suspected, the sizes of fragments X and Y match very well with what is predicted if there is a disruption inside VB1. Based on these data, we conclude that in erythroblastosis samples, the ALV provirus preferentially integrates just 5′ to or within the region where homology to v-*erbB* starts.

Activation of the β Allele by ALV Proviral Insertion. Having found that the α allele is frequently mutated by proviral insertion near VB1, we were interested in determining whether the β allele could be interrupted similarly. Using the strategy described above, we were able to show for three $\alpha\beta$ heterozygous samples that insertion near the β allele occurs. As shown in Fig. 2*D*, the sum of X and Y in these cases equals 2.6 kb (as opposed to 4.8 kb for the α allele). In addition, the intensity of the unaltered β 2.3-kb band is largely reduced compared to that of the unaltered α 4.5-kb band. The locations of the three integration sites relative to VB1 map to the same region as those for the α allele (Fig. 1*B*). Since the alterations in the β allele are the only ones detectable in these leukemia samples, the data indicate that insertional activation of the β allele can also induce erythroblastosis.

To conclusively document that ALV proviral insertion indeed occurs in the β allele, we have isolated a c-*erbB* clone, λ139, from a genomic library of partially *Eco*RI-digested DNA from leukemia sample 139. This clone carries a 16.4-kb insert. A battery of enzymes was used to construct a restriction map, which is summarized in Fig. 1*C*. The map is in complete agreement with the insertion of an intact ALV provirus in the 2.3-kb *Eco*RI fragment of the β allele, with the provirus oriented in the same transcriptional direction as the c-*erbB* gene. That the ALV provirus is intact was further substantiated by *in vitro* transfection of chicken embryo fibroblasts with the λ139 DNA, resulting in the release of infectious virus (unpublished data). The integration site of the provirus fits exactly that determined by the Southern analysis [Fig. 2*D* (lane 139) and Fig. 1*B*].

The Structure and the Orientation of the Provirus. The finding that an intact ALV provirus is present near the c-*erbB* gene deviates from the previous observations that the ALV proviruses linked to the c-*myc* gene in B-lymphomas frequently show large deletions, especially near and including the 5′ LTR (4–6). It was postulated that active transcription of an upstream promoter (in this case, the 5′ LTR) may significantly affect the strength of the downstream promoter (3′ LTR)—a phenomenon described as promoter occlusion (16, 17). Therefore, removal of the 5′ LTR appears to be necessary for efficient utilization of the 3′ LTR for downstream promotion of the oncogene. It was therefore of interest to find that λ139 carries a full-length ALV provirus. To see whether this is generally true for other leukemic-cell DNA, *Sac* I digestion was conducted. As shown in Fig. 3, *Sac* I has a single cleavage site near the 5′ terminus of ALV DNA. The *Sac* I map of c-*erbB* surrounding the proviral integration sites is also shown. For the undisrupted α allele, probe R4.5 should detect two fragments, 8.0 and 3.5 kb long. Upon proviral integration, the 8.0-kb fragment is disrupted into two fragments, due to the presence of the additional *Sac* I site in ALV DNA. If the provirus is full-length (8 kb), the sum of the two new *Sac* I fragments should approximate 8.0 + 8.0, or 16.0 kb. This appears to be the case for the majority of the leukemic samples (Fig. 3 and Table 1). It is also noteworthy that *Eco*RI digestion analysis presented above indicates that both LTRs might be intact, since the *Eco*RI sites of the LTRs appear to be present in all cases. Although rigorous proof that the proviruses are intact has to come from transfection studies such as those described above for λ139 clone, the preponderance of full-sized proviruses in erythroblastosis samples indicates that the presence of an intact provirus may not be unique to the DNA of sample 139. This data suggests that promoter occlusion, if it occurs in this case, is not absolute and that its effect is not sufficient to block the activation of c-*erbB* by a 3′ LTR. Alternatively, the provirus may utilize the 5′ LTR as the promoter to activate the c-*erbB* gene.

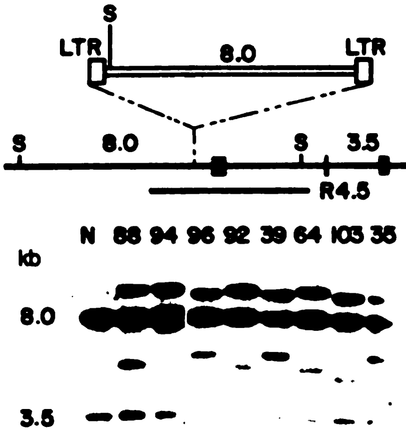

FIG. 3. *Sac* I digestion analysis of proviral DNA structure. (*Upper*) *Sac* I (S) restriction map of an intact ALV provirus integrated 5′ to VB1 and in the same transcriptional orientation as the α allele. Solid boxes represent exons. Sizes of the full-length provirus and the two *Sac* I fragments of the uninterrupted allele are given (in kb) above the provirus diagram and the restriction map, respectively. The region detectable by R4.5 is shown. (*Lower*) Southern hybridization of *Sac* I-digested normal (N) and erythroblastosis DNA with R4.5 probe. Erythroblastosis sample numbers are above lanes. The sizes of the rearranged bands in leukemia samples are listed in Table 1.

Sac I analysis also provides important information regarding the orientation of the proviruses. For example, sample 88 gives two *Sac* I, viral-cell junction fragments of about 11.5 and 4.7 kb. The relative intensity of the two bands suggests that the 11.5-kb band is the downstream fragment and the 4.7-kb band, the upstream one. Hybridization to probe 3I (Fig. 2) invariably detects the larger of the two tumor-specific bands, confirming this assignment (data not shown). We know from the data in Table 1 the sizes of the *Eco*RI junction fragments and, hence, the location of the integrated provirus (Fig. 1*B*). These data together allow the viral *Sac* I site to be unambiguously placed near the 5′ end of the inserted provirus; the provirus therefore is oriented in the same transcriptional direction as c-*erbB*. The calculated distances from the viral *Sac* I site to the LTRs agree very well with the intact ALV map, further confirming this alignment. All the proviruses surveyed by *Sac* I analysis in this study are oriented in the same direction as c-*erbB*.

DISCUSSION

The studies described here suggest that ALV activates c-*erbB* in erythroblastosis by a mechanism very similar to its activation of c-*myc* in B-lymphomas: the proviruses are oriented in the same transcriptional direction as the host oncogene and are clustered either at or immediately 5′ to a region (∼1.5 kb) corresponding to the start of the viral oncogene. Although exceptions to this general activation scheme exist

(7), this mechanism, referred to as promoter insertion, represents the predominant one used by the ALV provirus. Reticuloendotheliosis virus, another avian retrovirus, also uses promoter insertion as the major mechanism of c-*myc* activation in B-lymphomas caused by this virus (ref. 18 and unpublished results). In contrast, almost all the proviruses in mouse mammary tumor virus-induced mammary carcinomas are arranged either in the opposite orientation or downstream from the putative oncogene. Furthermore, the integration sites are spread over a large (20-kb) region (19). In this case, presumably, the LTR enhancer is involved in activation. Clearly, in different systems, different activation mechanisms are favored. What dictates the mode of viral integration as well as oncogene activation is unclear, but it may be related to the intrinsic properties of the virus (e.g., the strengths of the enhancer and promoter), the oncogene in question (the local conformation and the structural requirements for activation), or a combination of both. In the case of c-*myc* activation, most of the ALV and reticuloendotheliosis virus DNA integrations result in truncation of the c-*myc* transcript and removal of the first noncoding exon (ref. 20 and unpublished results). It was postulated that the first noncoding exon may contain a negative-controlling element that inhibits either the transcription or translation of the gene (20–22). The situation with c-*erbB* is less clear, since the coding capacity of the gene has not been defined fully. However, recent evidence strongly suggests that the c-*erbB* product is closely related or identical to the epidermal growth factor (EGF) receptor (23–25). Since the region of homology involves the carboxyl-terminal portion of the EGF receptor and v-*erbB*, one would expect the c-*erbB* coding sequences to extend significantly further upstream from VB1, where the proviral integration sites are concentrated. All the activated c-*erbB* products would, therefore, represent truncated versions of the normal protein. It is, then, interesting that the starting points of all the activated c-*erbB* genes studied here map very close to the point of insertion of v-*erbB* sequences in $\mathrm{AEV_R}$ and $\mathrm{AEV_H}$. This suggests that interruption in this region of c-*erbB* probably is important for activation. The requirement to interrupt the c-*erbB* locus and generate a truncated product perhaps also imposes a need for the promoter-insertion (as opposed to enhancer-insertion) type of proviral activation, since, in this region, no cellular promoter is present to be activated by the LTR enhancer.

Whether c-*erbB* is identical to EGF receptor or not, the chicken c-*erbB* locus is a complex one; the region homologous to v-*erbB* spans more than 20 kb and contains at least 12 exons. Added to this complexity is the presence of structural polymorphisms in different lines of chickens. Using cloning and hybridization studies, we have identified a second allele, β, which differs from the previously known allele, α, primarily by a deletion in the intron region. The α and β alleles can account for the majority but not all of the polymorphic *erbB* elements observed in chickens and must constitute the major c-*erbB* locus, since we have not found any chickens that lack both alleles. Among the few $\alpha\beta$ heterozygotes studied, the β allele appears to be as susceptible to proviral insertion as the α allele (3/7 vs. 4/7; Fig. 3 *A* and *B*).

The present communication is primarily concerned with the typical promoter-insertion mechanism of c-*erbB* activation, which accounts for 90% (34/38) of the cases examined. We previously reported an atypical case where a single altered c-*erbB* fragment contains an LTR and v-*erbB* related exons but no intron sequences, as if the activated c-*erbB* message had been reverse-transcribed and reinserted into the host genome (9). We have again observed this phenomenon in the present study; the altered c-*erbB* fragment of the four remaining cases possesses these features (unpublished data). One such fragment was cloned, and structural analysis confirms the processed nature of the *erbB* gene. The linkage point between the provirus and the processed *erbB* gene again maps near VB1. The generation of such a processed gene can occur either intracellularly, as has been suggested for the formation of other pseudogenes (26), or via virus intermediate (ref. 27 and H. Robinson, personal communications). In either case, promoter-insertion probably was involved in the initial activation of the oncogene (4, 5). When we include these cases in the category of promoter-insertion activation, we find a striking statistic for c-*erbB* activation: In the 37 chicks with erythroblastosis (38 proviruses) analyzed, there is a 100% correlation between c-*erbB* alteration and erythroblastosis development. Furthermore, virtually all proviruses found linked to c-*erbB* are clustered in a small chromosomal region and are uniformly aligned in a configuration compatible with the promoter-insertion type of activation.

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APPENDIX B:

C-ERB B ACTIVATION IN ALV-INDUCED ERYTHROBLASTOSIS: NOVEL RNA PROCESSING AND PROMOTER INSERTION RESULT IN EXPRESSION OF AN AMINO-TRUNCATED EGF RECEPTOR

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c-*erb*B Activation in ALV-Induced Erythroblastosis: Novel RNA Processing and Promoter Insertion Result in Expression of an Amino-Truncated EGF Receptor

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Summary

ALV-induced erythroblastosis results from the specific interruption of the host oncogene, c-*erb*B, by the insertion of an intact provirus. Integrated proviruses are oriented in the same transcriptional direction as c-*erb*B, and expression of truncated c-*erb*B transcripts is observed. Evidence, including sequence analysis of cDNA clones, indicates that transcription of truncated c-*erb*B mRNA is initiated in the 5′ LTR of the integrated provirus. This transcript is processed through a series of remarkable splicing reactions to yield viral *gag* and *env* sequences fused to *erb*B sequences. These results establish a novel pathway of promoter insertion oncogenesis that stands in contrast to the pathways used in the activation of c-*myc* in B lymphomas.

Introduction

Avian leukosis virus (ALV) is a replication competent retrovirus that lacks a transforming gene but causes a variety of neoplasms in chickens, including B lymphomas and erythroblastosis, usually after a long latent period (Weiss et al., 1982; Crittenden and Kung, 1983). Analysis of genomic DNA from neoplastic tissue has provided a molecular explanation of tumor induction by ALV. In particular, ALV induces B lymphomas by activating the cellular proto-oncogene c-*myc* (Hayward et al., 1981; Payne et al., 1982). This activation results from the insertion of an ALV provirus, which carries strong promoter/enhancer functions, near the c-*myc* gene. Several lines of evidence indicate that a similar mechanism may activate the cellular proto-oncogene c-*erb*B in ALV-induced erythroblastosis (Fung et al., 1983; Raines et al., 1985).

The c-*erb*B locus was initially defined by homology to the transforming gene v-*erb*B identified in avian erythroblastosis virus (AEV) (Weiss et al., 1982; Venstrom and Bishop, 1982). Subsequent comparison of the predicted amino acid sequence of v-*erb*B with portions of the human epidermal growth factor (EGF) receptor amino acid sequence revealed striking homology and raised the possibility that c-*erb*B was, in fact, the gene coding for the EGF receptor (Downward et al., 1984b). The EGF receptor contains three domains, an extracellular EGF binding domain, a short transmembrane domain, and a cytoplasmic domain that possesses protein kinase activity (Hunter, 1984). The v-*erb*B gene is homologous to sequences encoding a small portion of the extracellular domain, the transmembrane domain, and the entire cytoplasmic domain excluding a short sequence that codes for the 32 carboxy-terminal amino acids of the human protein (Yamamoto et al., 1983; Ullrich et al., 1984; Lin et al., 1984; Xu et al., 1984). Both the c-*erb*B gene and the EGF receptor gene map to the same chromosome in humans (Spurr et al., 1984; Davies et al., 1980; Shimizu et al., 1980), and our own recent evidence (see below) indicates that, in chickens, the EGF receptor and c-*erb*B appear to be derived from a single locus.

In 37 cases of ALV-induced erythroblastosis examined, most of the proviral integration sites were clustered within a few hundred bases upstream from the first c-*erb*B exon with homology to v-*erb*B (Raines et al., 1985). Of those proviruses analyzed, all were inserted in the same transcriptional orientation as c-*erb*B, and elevated expression of c-*erb*B-related RNA was consistently observed (Fung et al., 1983; Raines et al., 1985). While these studies revealed important similarities between the mechanisms whereby ALV induces erythroblastosis and B lymphomas, the activation of c-*erb*B is not directly analogous to the activation of c-*myc*.

Whereas proviruses integrated near c-*myc* frequently carry deletions near or encompass the upstream or 5′ LTR (Neel et al., 1982; Neel et al., 1981; Payne et al., 1981; Fung et al., 1981; Pachl et al., 1983), most proviruses inserted into the c-*erb*B locus appeared to be full-length (Raines et al., 1985). One such provirus was recovered by molecular cloning and was shown to be completely intact (Raines et al., 1985). Furthermore, proviral insertion does not directly disrupt or alter the protein coding sequence of the c-*myc* gene (Hayward et al., 1981; Payne et al., 1982; Fung et al., 1981; Shih et al., 1984). Thus, enhanced or inappropriate expression of the intact c-*myc* protein may be directly oncogenic. In the case of erythroblastosis, however, proviral insertion disrupts the coding sequence of the EGF receptor gene. The fact that all of the proviral insertions observed in ALV-induced erythroblastosis map to a small region in the middle of the EGF receptor gene coinciding with the point of transduction of cellular sequences into AEV strongly suggests that specific truncation of the EGF receptor gene is required for oncogenesis (Raines et al., 1985).

In an attempt to define the mechanism of c-*erb*B activation in ALV-induced erythroblastosis, we have begun a detailed analysis of *erb*B-specific transcripts in leukemic samples. We find that two prominent size classes of *erb*B transcripts are expressed. The nucleotide sequence of *erb*B-specific cDNA clones indicates that at least one of these transcripts is initiated in the 5′ LTR of the inserted

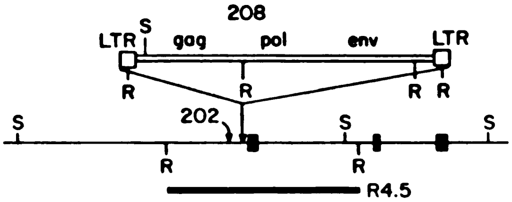


Figure 1. Position and Structure of the Integrated ALV Provirus in Leukemic Samples 202 and 208

The diagram illustrates the c-*erb*B locus containing an integrated ALV provirus. The approximate proviral integration sites determined for leukemic samples 202 and 208 are indicated by arrows. The assignment of integration sites was based upon blot hybridization of restriction digests of leukemic DNA using a 4.5 kb fragment of chicken DNA, which contains the first exon of c-*erb*B with homology to v-*erb*B (Raines et al., 1985). This fragment, designated R 4.5 is indicated on the diagram.

Cellular DNA (thin line) with indicated Sac I and Eco RI sites was mapped using digests of cloned DNA isolated from a genomic library of normal chicken DNA. Solid black boxes represent the 5′-most exons of c-*erb*B, as defined by homology to v-*erb*B. The restriction map of ALV DNA, represented as a double line, is identical with that reported elsewhere (Raines et al., 1985). R, Eco RI; S, Sac I.

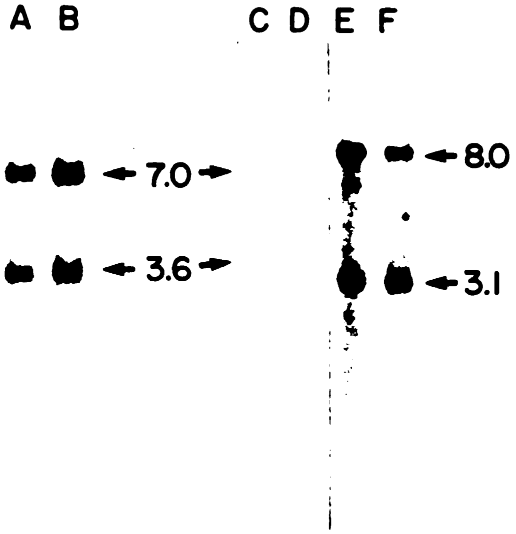


Figure 2. Northern Blot Analysis of c-*erb*B-related RNAs and Viral RNAs in Leukemic Samples 202 and 208

Total cellular RNA was extracted from frozen bone marrow samples as described in Experimental Procedures; polyadenylated RNA was prepared and fractionated on 1% denaturing formaldehyde agarose gels and was transferred to Gene Screen (NEN). Five micrograms polyadenylated RNA from leukemic sample 202 (lanes A, C, and E) or sample 208 (lanes B, D, and F) was analyzed in each lane.

Nick-translated probes used in hybridization included the 550 bp Bam HI (Vennstrom and Bishop, 1982) restriction fragment of v-*erb*B (lanes A–D) and a 300 bp Eco RI restriction fragment of RSV LTR sequences (Eco RI D in Delorbe et al., 1980) (lanes E and F).

Hybridizations and washes were as described in Experimental Procedures. The blot containing lanes A and B was exposed for 24 hr. The blots containing lanes C–F were exposed for 4 hr. Sizes of transcripts were determined relative to the mobility of ^{32}P-labeled RNAs of known molecular weight electrophoresed in parallel lanes. These transcripts were generated in vitro with SP-6 polymerase (NEN) and ranged in size from 7.3 kb to 1.4 kb.

provirus. This transcript reads through the polyadenylation signal present in the 3′ LTR and is processed via a series of remarkable splicing reactions to yield viral *gag* and *env* sequences linked to *erb*B sequences. The predicted amino acid sequence deduced from this mRNA indicates that the resultant protein contains six amino acids of the viral *gag* gene and 53 amino acids of the *env* gene fused to sequences corresponding to the entire v-*erb*B gene. In addition, sequences encoding 34 amino acids homologous to the carboxyl terminus of the human EGF receptor, which are absent in v-*erb*B, are encoded in the mRNA of the activated c-*erb*B gene. This result provides additional evidence that the c-*erb*B locus is identical with the EGF receptor gene.

Results

Proviral Integration Sites

Proviral insertion sites near c-*erb*B were mapped in two leukemic samples (designated 202 and 208) by employing a strategy previously described for the analysis of 37 other cases of ALV-induced erythroblastosis (Raines et al., 1985). In that study, all of the proviral insertions were confined to a 4.5 kb Eco RI fragment of cellular DNA, which contains the first exon of c-*erb*B with homology to v-*erb*B (Figure 1).

To determine whether provirus insertion had occurred within this region in samples 202 and 208, a subclone containing the 4.5 kb fragment was used to probe Eco RI digests of leukemic DNA, as well as DNA derived from control (nonleukemic) tissue from the same birds (data not shown). This analysis indicated that the proviruses in the leukemic samples 202 and 208 were inserted within the 4.5 kb fragment and had integrated approximately 300 and 100 bases, respectively, upstream of where v-*erb*B homology begins. The integration sites are indicated in Figure 1. Additional mapping analysis (Raines et al., 1985) revealed that both proviruses appeared to be intact, were oriented in the same transcriptional direction as c-*erb*B, and represented the only ALV provirus present in the respective leukemic cells (data not shown).

c-*erb*B Related mRNAs

Having demonstrated that the c-*erb*B locus was disrupted by proviral insertion, we proceeded to examine c-*erb*B expression in these two leukemic samples. The sizes of *erb*B-specific mRNAs in samples 202 and 208 were determined by Northern blot analysis using a fragment of v-*erb*B as a probe. The probe hybridized to two prominent size classes of mRNA, 7.0 kb and 3.6 kb, in both samples (Figure 2, lanes A and B). In light of the position and orientation of the integrated provirus, parallel blots were hybridized with an LTR-specific probe to determine whether either or both of the c-*erb*B mRNAs were transcribed from a viral promoter (Figure 2, lanes E and F). The LTR probe hybridized with two prominent mRNA species of about 8 kb and 3.1 kb. These sizes are consistent with the genomic length transcript and the subgenomic *env* mRNA of ALV. Since there is a single proviral insertion in both leukemic samples, this analysis indicated that the provirus integrated within the c-*erb*B locus was actively transcribed and provided additional evidence that this provirus was full-length. The viral transcripts appeared to be at least ten times more abundant than were transcripts detected with the v-*erb*B probe (Figure 2, cf. lanes C and D with lanes E and F). This fact, coupled with the close similarity in size between viral transcripts and *erb*B transcripts, made it impossible to determine by Northern blot analysis if the *erb*B transcripts contained LTR sequences.

Nucleotide Sequence of c-*erb*B cDNA Clones

To facilitate a detailed analysis of the sequence content of c-*erb*B mRNAs, an oligo(dT)-primed cDNA library was prepared from mRNA derived from the leukemic sample 208. Double-stranded cDNA was tailed with dC residues and was inserted into the dG-tailed Pst I site of pBR322. Ten thousand clones were screened with a ^{32}P-labeled 0.55 kb Bam HI restriction fragment of v-*erb*B (Venstrom and Bishop, 1982). A single clone, which hybridized to the probe, was purified. This clone, designated pErb-1, contained a 1.5 kb insert. Restriction digestion and Southern blot analysis using specific fragments of v-*erb*B as probes (not shown) indicated that the pErb-1 insert contained the entire 0.55 kb Bam fragment, about 700 nucleotides 5′ and about 250 nucleotides 3′ to this fragment (Figure 3).

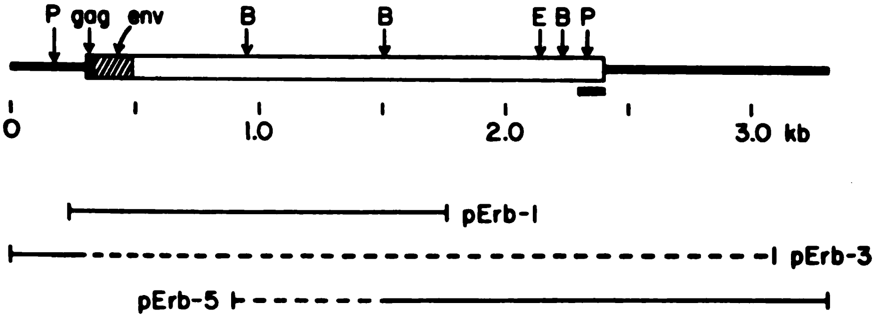
The entire insert was sequenced using the dideoxynucleotide chain termination procedure (Figure 3). The resulting sequence confirmed the presence of v-*erb*B homologous sequence, which extended approximately 450 nucleotides 5′ to the Bam fragment before diverging completely from published v-*erb*B sequences (Yamamoto et al., 1983). The sequence at the point of divergence coincided exactly with a putative splice acceptor site postulated by Debuire et al. (1984) to serve in the expression of v-*erb*B in AEV$_R$, suggesting that sequences 5′ to v-*erb*B homology were spliced to this c-*erb*B transcript. Since Southern blot analysis had indicated that the site of ALV proviral integration was immediately adjacent to the start of v-*erb*B homologous sequence (see above), we compared the unknown sequence with the published sequence of Rous Sarcoma Virus (RSV), as a comparable sequence of ALV is not available (Schwartz et al., 1983).

Surprisingly, two regions of homology between pErb-1 and RSV were revealed by this analysis; nucleotides 1–93 of pErb-1 closely matched nucleotides 324–397 of RSV and nucleotides 94–252 of pErb-1 were homologous to nucleotides 5078–5236 of RSV (Schwartz et al., 1983). The first region of homology spans a portion of the viral 5′ untranslated leader sequence, the *gag* translational initiation codon, and five additional codons of the *gag* precursor. The second region of homology includes codons 7–59 of the *env* precursor (Schwartz et al., 1983). The nucleotide sequence of the *gag-env* junction in pErb-1 (AAG/GCA) was exactly that predicted if this mRNA were processed through the same splice donor and acceptor sites thought to be used in the generation of the subgenomic viral *env* mRNA (Figure 3; Hackett et al., 1982). The sequence in pErb-1 corresponding to the *env-erb*B junction was GGG/GGC, which would be expected if the splice acceptor site (ag/GGCC) noted by Debuire et al. (1984; see above) were used in the formation of this c-*erb*B mRNA (Figure 3). Inspection of the RSV *env* sequence corresponding to the *env-erb*B junction revealed the sequence GGG/gtagg, which shares considerable homology with a consensus splice donor site (Schwartz et al., 1983; Mount, 1982). A similar sequence, GG/gtgag, is a splice donor site in the late leader of adenovirus (Mount, 1982). These observations indicate that pErb-1 was derived from a transcript that was processed using a cryptic splice donor site in the viral *env* gene and a splice acceptor site presumably used in the generation of normal c-*erb*B mRNAs (see Figure 5).

Although this sequence data indicated that at least one activated c-*erb*B transcript contained viral sequences spliced to c-*erb*B sequences, the transcriptional initiation site of the mRNA corresponding to pErb-1 was not defined by this analysis. To address this question, we screened an additional 50,000 cDNA clones with *erb*B-specific fragments of pErb-1 and obtained four clones containing *erb*B sequence. Restriction digestion and Southern blotting using fragments of pErb-1 as probes (not shown) established that one of these clones, pErb-3, contained a sequence that spanned the entire length of pErb-1, as well as additional sequences both 5′ and 3′ to the boundaries of pErb-1 (Figure 3). Sequencing of the first 300 nucleotides of pErb-3 revealed that this clone contained 233 nucleotides 5′ to the beginning of pErb-1. These nucleotides were homologous to nucleotides 91–323 of RSV (Schwartz et al., 1983) and included the last 11 bases of the viral U$_5$ sequence (Figure 3). This result indicates that leukemic cells contain transcripts with the viral LTR sequence physically linked to the c-*erb*B sequence and provides strong evidence that at least one of the activated c-*erb*B transcripts is initiated in the 5′ LTR of the integrated provirus (see Figure 5).

The transcript defined by pErb-1 and pErb-3 contains a single open reading frame that begins at the initiator ATG of *gag*, encodes 6 amino acids of *gag*, 53 amino acids of *env*, and 426 amino acids of c-*erb*B. To describe completely the portion of this transcript coding for c-*erb*B, we analyzed an additional overlapping cDNA clone, pErb-5. Restriction mapping, Southern blotting, and partial nucleotide sequencing established that this clone contained approximately 1.6 kb of sequence 3′ to the end of pErb-1 (Figure 3). This region was sequenced, and the resultant sequence as well as the sequence derived from pErb-1 were compared with the published sequence of v-*erb*B (Yamamoto et al., 1983). This analysis revealed that v-*erb*B is remarkably conserved with respect to c-*erb*B. There were only six base changes between the sequence we determined and the sequence of v-*erb*B derived from AEV$_H$. Of these, four were conservative, and the other two resulted in a single amino acid change, phenylalanine in v-*erb*B to asparagine in c-*erb*B at residue 596 of the activated c-*erb*B protein (Figure 3).

A more striking difference between c-*erb*B and v-*erb*B became evident when the carboxyl termini of these proteins were compared. The coding sequence of c-*erb*B did not terminate at the end of v-*erb*B homology, but instead included an additional 34 amino acids (Figure 3). These 34 amino acids were of particular interest, since both iso-



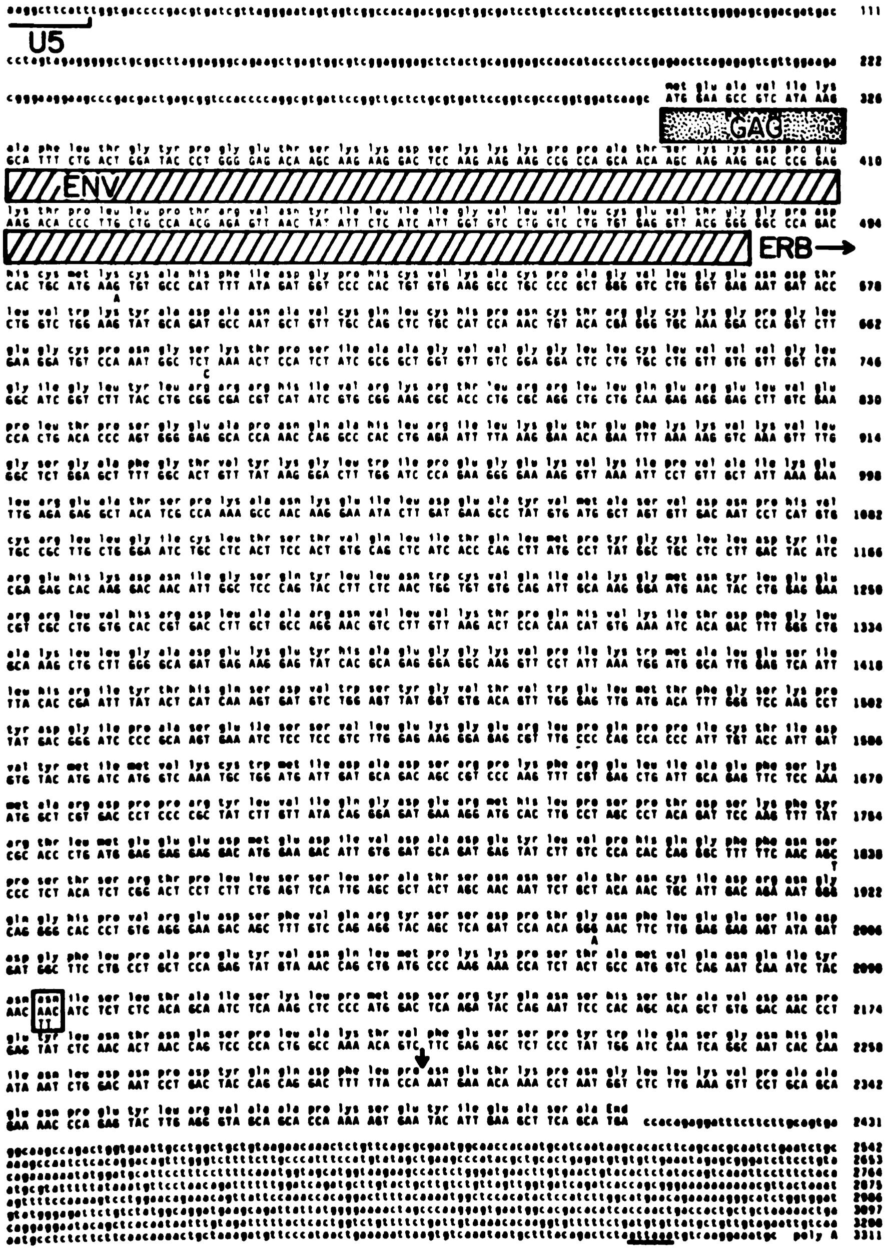


Figure 3. Composite Restriction Map and Nucleotide Sequence of cDNA Clones Derived from Activated c-*erb*B mRNAs

A schematic diagram of a composite cDNA is shown at the top of the figure. Untranslated sequences are shown as a line, and coding sequences are boxed. The diagram shows the location of viral *gag* (stippled box) and *env* (hatched box) sequences followed by c-*erb*B sequences (open box). The line underneath the schematic designates the carboxyl region of c-*erb*B not present in v-*erb*B. The nucleotide sequence and predicted amino acid sequence of this composite cDNA is also shown. This sequence was derived from three overlapping cDNA clones, which are shown schematically below the diagram. Regions of these clones that were sequenced are indicated by solid lines. The untranslated regions are shown in small

```
                           ↓
tyr gln gln asp phe leu pro asn glu thr lys pro asn gly leu leu lys val pro ala ala glu asn pro glu
TAC CAG CAG GAC TTT TTA CCA AAT GAA ACA AAA CCT AAT GGT CTC TTG AAA GTT CCT GCA GCA GAA AAC CCA GAG   chicken
                  C   T   C   G     G C   G   A       C A     T   G  GC T C A     T       T G     A   human
                    phe     lys     ala                 ile phe     gly ser thr             ala

tyr leu arg val ala ala pro lys ser glu tyr ile glu ala ser ala End
TAC TTG AGG GTA GCA GCA CCA AAA AGT GAA TAC ATT GAA GCT TCA GCA TGA ccacagaggatttcttcttgcagtgaggcaa   chicken
    C A       C   G C    A   GC          TT      G  --- ---              g     agta gagc ctaa aatcc   human
                    pro gln ser  '      phe     gly
```

Figure 4. Comparison of the Carboxy-Terminal Sequence of the Chicken c-*erb*B and Human EGF Receptor cDNAs

Only those bases and amino acids of the human sequence that are not identical with the chicken c-*erb*B sequence are shown. Dashes indicate gaps introduced in the human sequence to maximize homology. The arrow designates the 3′ end of v-*erb*B (Yamamoto et al., 1983) and c-*erb*B homology. A conserved tyrosine residue (Tyr 1173 in the human EGF receptor), a major autophosphorylation site in the human protein, is boxed. 3′ untranslated sequences are shown in small letters.

lates of v-*erb*B lack not only the EGF binding domain but also the carboxy-terminal region of the EGF receptor (Yamamoto et al., 1983; Sealy et al., 1983; Ullrich et al., 1984). Recombinational events involved in the transduction of *erb*B sequences into AEV$_R$ or AEV$_H$ presumably create this "double truncation." In the present situation, however, since c-*erb*B is altered at only the 5′ end, it would be expected that activated c-*erb*B transcripts would encode the missing carboxy-terminal region if c-*erb*B were identical with the EGF receptor gene. The carboxy-terminal 34 amino acids of chicken c-*erb*B share 60% homology with the 32 carboxy-terminal amino acids of the human EGF receptor (Figure 4). Notably, the tyrosine corresponding to tyrosine 1173 of the human EGF receptor is conserved in the chicken gene. This tyrosine is a major site of receptor autophosphorylation and may be involved in receptor regulation (Hunter, 1984; Downward et al., 1984a). The presence of these amino acids at the carboxyl terminus of c-*erb*B provides additional evidence that c-*erb*B is indeed the EGF receptor gene.

The protein coding sequence of c-*erb*B terminated approximately 900 bases upstream from the end of pErb-5. Complete nucleotide sequence analysis of the 3′ untranslated region revealed no significant homology with the corresponding region of the human EGF receptor gene (Ullrich et al., 1984). A sequence that is homologous to but not identical with a consensus polyadenylation signal was present 14 bases upstream from a poly(A) tract (Figure 3). Assuming that pErb-1, pErb-3, and pErb-5 are derived from the same mRNA, and that transcription of this mRNA is initiated in the 5′ LTR (see above), the resultant c-*erb*B mRNA would contain 575 bases corresponding to viral sequence, 1917 bases coding for c-*erb*B, and a 909 base 3′ untranslated sequence. Thus, the total length of this mRNA would be 3401 nucleotides. With the addition of a poly(A) tail, this size would come very close to the 3.6 kb estimated for the smaller of the two c-*erb*B transcripts described above (see Figure 2).

Discussion

We have analyzed the expression of the c-*erb*B locus in ALV-induced erythroblastosis. Our results indicate that the c-*erb*B protein expressed in these leukemic samples is an amino-truncated form of the EGF receptor fused to portions of ALV *gag* and *env* proteins (Figure 5). These results establish a novel pathway for promoter insertion oncogenesis, which stands in contrast to the pathways used in the activation of c-*myc* in B lymphomas. (This pathway has previously been suggested as a possible mode of oncogene activation [Neel et al., 1981; Payne et al., 1981].) It seems likely that these distinct pathways reflect the unique molecular requirements that must be fulfilled to activate either c-*erb*B or c-*myc*.

Available evidence suggests that the level of expression of c-*myc* is of prime importance in ALV-induced B lymphomas. Thus, either enhancement of the normal c-*myc* promoter (Payne et al., 1982) or, more commonly, provision of a strong viral promoter appears to be sufficient to activate the c-*myc* gene (Hayward et al., 1981). The requirement for strong 3′ LTR promoter function probably explains the high frequency of deletions observed in proviruses integrated upstream from c-*myc* (Neel et al., 1981; Payne et al., 1981; Fung et al., 1981; Pachl et al., 1983). These deletions, which usually eliminate 5′ viral promoter activity, may allow efficient use of promoter elements in the 3′ LTR (Cullen et al., 1984).

In contrast, our results indicate that the presence of the 5′ LTR and additional internal viral sequences may be required to bring about activation of c-*erb*B. Furthermore, the absolute level of expression of c-*erb*B may be less important than the structure of the activated c-*erb*B protein. In this regard, the sequence content of activated c-*erb*B mRNAs raises some interesting questions. The presence of the *gag* sequence may not be surprising, since, by analogy to v-*erb*B, this sequence may be required to provide a translational start site for expression of c-*erb*B. However,

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letters, and the viral *gag* and *env* coding sequences are indicated by boxes. The 11 nucleotides that are part of the viral LTR U5 sequence are bracketed. The 6 bp differences between the c-*erb*B sequence determined here and the published sequence of v-*erb*B are indicated below the c-*erb*B sequence, and the single amino acid difference within the v-*erb*B domain is boxed. The arrow marks the end of the v-*erb*B sequence. A hexanucleotide with homology to the consensus polyadenylation signal is underlined.

The sequence of pErb-1 and pErb-3 corresponding to viral regions was not totally homologous to the published sequence of RSV. Most notably, pErb-1 contained a 14 base direct repeat 5′ to the initiator ATG. To confirm that this repeat was indeed of ALV origin, we sequenced an appropriate restriction fragment isolated from a λ clone containing an intact ALV provirus (Raines et al., 1985). This analysis indicated that the sequence in pErb-1 exactly matched the ALV sequence (not shown). P, Pst I; B, Bam HI; E, Eco RI.

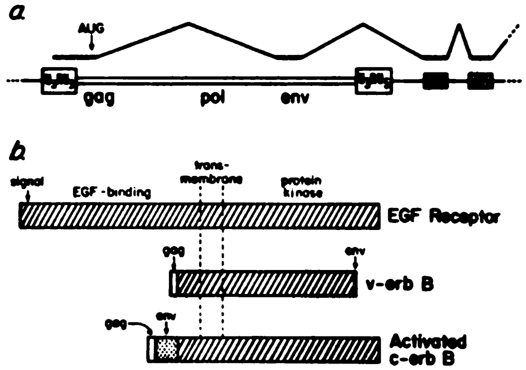

Figure 5. Schematic Representation of c-*erb*B Activation by ALV, and Comparison of the Resulting c-*erb*B Protein with the EGF Receptor

(a) Schematic diagram of an ALV provirus integrated upstream from c-*erb*B exons (hatched boxes), and the resulting c-*erb*B-containing transcript. Evidence presented in the text suggests that c-*erb*B-containing messages are initiated in the 5′ LTR of the integrated provirus and contain viral *gag* and *env* sequences fused to c-*erb*B sequences. Regions of the RNA appearing in the processed transcript are shown as thick lines, while introns are shown as thin lines. The AUG codon presumed to be used for initiation of translation of c-*erb*B-containing transcripts, as well as that used in translation of viral proteins, is indicated.

(b) Comparison of *erb*B and EGF receptor proteins. The diagram of the EGF receptor is adapted from a similar schematic of the human receptor (Hunter, 1984) and shows the three domains of the protein. Below is shown the presumed structure of the v-*erb*B protein if its mRNA is spliced as described by Debuire et al. (1984). This protein would contain 6 amino acids of viral *gag*, 605 amino acids of *erb*B, and 4 amino acids of viral *env*. Below this is shown the proposed structure of the ALV-activated c-*erb*B protein as deduced from the sequence of pErb-1 and pErb-5. Like v-*erb*B, this protein would begin with 6 amino acids of viral *gag*. However, it would also contain 53 amino acids of viral *env* between the *gag* and the *erb*B sequence. In addition, the activated c-*erb*B does not terminate with viral *env* amino acids but instead contains 34 carboxy-terminal amino acids homologous to those present in the human EGF receptor.

the presence of the *env* sequence was unexpected. The six amino acids of *gag* and 53 amino acids of *env* comprise 59 of the 62 amino acid translational signal sequence responsible for directing the viral *env* protein to the cell surface (Hunter et al., 1984). While we do not know whether this sequence is cleaved after translation from the c-*erb*B protein, it would undoubtedly serve as a signal sequence to direct c-*erb*B mRNAs to membrane-bound ribosomes. It is tempting to speculate that this sequence is required for correct intracellular transport of the c-*erb*B protein and may thus be important for oncogenic activation. Recent results of Hannink and Donoghue, (1984) indicate that the transforming activity of v-*sis* is dependent upon a viral signal sequence fused to the v-*sis* protein.

Alternatively, the presence of the *env* sequence may simply reflect accidental RNA processing resulting from efficient use of the *gag-env* splicing sequences and the presence of a fortuitous, inframe, splice donor site in the envelope gene. In this regard, v-*erb*B does not contain *env* sequence and is apparently positioned in the cell membrane (Hayman and Beug, 1984; Privalsky and Bishop, 1984). Furthermore, our data do not exclude the presence of an mRNA or mRNAs formed by direct *gag-erb*B splicing. Appropriate transfection experiments may provide the means to assess the importance, if any, of *env* sequences fused to c-*erb*B.

The finding that the activated c-*erb*B mRNA or mRNAs contain sequences that code for 34 amino acids homologous to the 32 carboxyl-terminal amino acids of the human EGF receptor further strengthens the case for the identity of c-*erb*B and the EGF receptor. Furthermore, this result indicates that the carboxyl-truncation of c-*erb*B found in both AEV_H and AEV_R, which results in the removal of a major autophosphorylation site (Hunter, 1984; Downward et al., 1984a), is probably not necessary for oncogenic activation. However, the clustering of proviral integration sites, as well as the structure of both v-*erb*B isolates, may indicate that precise amino-truncation of the EGF receptor is required. Alternatively, the apparent precision of truncation could result from the use of preferred integration sites by ALV. Transfection experiments designed to assess the transforming potential of appropriate truncations of EGF receptor cDNAs will be needed to resolve this question.

Two lines of evidence suggest that the mode of activation of c-*erb*B revealed by analysis of tumor 208 may be used in most or all cases of ALV-induced erythroblastosis. First, in all cases studied, the provirus is integrated upstream and in the same transcriptional orientation as c-*erb*B (Raines et al., 1985). Furthermore, all of the proviruses apparently retain the 5′ LTR and the bulk of viral sequence (Raines et al., 1985). Second, Northern blot analysis indicates that c-*erb*B mRNAs are of the same size, regardless of the site of proviral integration (Figure 2; unpublished results). This would be expected if a common splicing pathway were used in the generation of activated c-*erb*B mRNAs.

The requirement to maintain the 5′, rather than the 3′, LTR of ALV to activate c-*erb*B may help explain the relatively high frequency generation of *erb*B-containing transducing viruses in ALV-induced erythroblastosis. Under appropriate conditions, up to 50% of leukemic samples release such viruses (Miles and Robinson, 1985; C. Moscovici and the authors' unpublished observations). If the 5′ LTR is maintained intact, a deletion of the 3′ end of the provirus could result in the appropriate fusion of viral and *erb*B sequences such that an mRNA capable of being packaged would be produced. Recombination during reverse transcription as suggested by Swanstrom et al. (1983) could subsequently give rise to a virus containing *erb*B sequences.

The presence of correctly processed viral genomic and envelope mRNAs in the leukemic cells we have analyzed indicates that the viral polyadenylation signal present in the 3′ LTR is intact. Thus, transcripts containing c-*erb*B sequences must result from the processing of RNAs that have "read through" this polyadenylation signal. The relative abundance of mRNAs containing *erb*B and viral sequences may, to a large extent, reflect the relative efficiencies of splicing of the primary transcript versus polyadenylation at the viral site. Without knowing the stabilities of viral mRNAs and c-*erb*B mRNAs, it is difficult to estimate the frequency of "read through" transcription. However, the existence of mRNAs containing viral "exons" spliced to c-*erb*B exons may have important implications with regard to eukaryotic gene expression. While there is convincing evidence that polyadenylation site selection is important in differential gene expression (i.e., immunoglobulin synthesis [Alt et al., 1980] and calcitonin production [Amara et al., 1982]), our observations raise the possibility that stochastic processes could be involved in the generation of multiple gene products using a single promoter.

The activated c-*erb*B gene is expressed in the form of at least two mRNAs with sizes of about 7 kb and 3.6 kb. We have not established the exact relationship between these mRNAs. They appear to be expressed at about the same level (see Figure 2), and Northern blot analysis using various probes derived from c-*erb*B cDNA clones did not reveal any differences in sequence content (not shown). The presence of two mRNAs with *erb*B coding sequence appears analogous to the presence of two major transcripts of the human EGF receptor (Ullrich et al., 1984; Lin et al., 1984; Xu et al., 1984; Merlino et al., 1984) and the two normal c-*erb*B mRNAs described by Vennstrom and Bishop (1982; and our unpublished results). The structures of the overlapping cDNA clones pErb-1, pErb-3, and pErb-5 indicate that the 3.6 kb mRNA can encode the entire activated c-*erb*B protein. Thus, it seems likely that the two c-*erb*B mRNAs could have similar 5′ ends and differ by alternate splicing pathways or poly(A) addition sites such that the 7 kb mRNA carries a longer 3′ untranslated sequence. Isolation of cDNA clones containing sequences specific to the 7 kb mRNA should resolve these questions.

Experimental Procedures

Induction of Erythroblastosis

Approximately 10^3 infectious units of RAV-1, a prototype ALV, was used to inoculate 1 day old line $15_1 \times 15I_4$ chicks. This chicken line was developed at the Regional Poultry Research Lab, East Lansing, MI, for its high susceptibility to erythroblastosis and low incidence of nonspecific lesions. Over 95% of the inoculated birds developed erythroblastosis. The diagnosis of the disease and the collection of leukemic samples were as previously described (Fung et al., 1983; Raines et al., 1985). In birds with severe erythroblastosis, such as samples 202 and 208, bone marrow samples are composed primarily of leukemic erythroblasts (>80%). Livers are also heavily infiltrated with leukemic erythroblasts (>30% of the cells present).

DNA and RNA Extractions

Total cellular DNA was extracted from frozen tissue samples by homogenization, pronase digestion, and phenol extraction as previously described (Fung et al., 1983). Total cellular RNA was extracted from frozen bone marrow samples using guanidinium isothiocyanate as described (Maniatis et al., 1982).

Blot Hybridization

Southern blot analysis was carried out as described (Raines et al., 1985). For Northern blot analysis, polyadenylated RNA was prepared by oligo(dT)-cellulose chromatography, fractionated on 1% denaturing formaldehyde agarose gels, transferred to Gene Screen (NEN), and hybridized as described (Nilsen and Maroney, 1984). Washes were at 65°C with 2× SSC and 0.1% SDS.

cDNA Cloning

Polyadenylated RNA was prepared as described above. Oligo(dT)-primed double-stranded cDNA was synthesized using this RNA as template (Maniatis et al., 1982) and was size selected on a 1% agarose gel. cDNAs larger than 500 bp were tailed with dC residues using terminal transferase (Ratliff Biochemicals) and were annealed with dG-tailed pBR322 (NEN) (Maniatis et al., 1982). Recombinant plasmids were used to transform E. coli HB101 (Hanahan, 1983). Screening was as described by Hanahan and Meselson (1980). Nucleotide sequences were obtained from overlapping M13 subclones in both orientations using the dideoxynucleotide chain-termination technique (Sanger et al., 1977).

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APPENDIX C:

RAV-1 INDUCED ERYTHROLEUKEMIC CELLS EXHIBIT A WEAKLY TRANSFORMED PHENOTYPE <u>IN VITRO</u> AND RELEASE C-ERB B-CONTAINING PROVIRUSES UNABLE TO TRANSFORM FIBROBLASTS

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Rous-Associated Virus 1-Induced Erythroleukemic Cells Exhibit a Weakly Transformed Phenotype In Vitro and Release c-*erbB*-Containing Retroviruses Unable to Transform Fibroblasts

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Avian leukosis viruses induce erythroblastosis in chicks by integrating into the c-*erbB* gene and thus activating c-*erbB* transcription. We characterized Rous-associated virus 1-induced leukemic erythroblasts in vitro and showed that they mostly resemble erythropoietin-independent but otherwise normal erythroid progenitors. Some leukemic cells, however, were able to both differentiate and proliferate extensively in vitro. All 14 leukemias studied expressed high levels of *erbB*-related proteins that were 5 to 10 kilodaltons larger but otherwise very similar to the gp74^{erbB} protein of avian erythroblastosis virus ES4 with respect to biosynthesis, glycosylation, and cell surface expression. Two leukemias contained and released retroviruses that transduced *erbB*. Chicken embryo fibroblasts fully infected with these viruses expressed high levels of *erbB* RNA and protein but retained a normal phenotype. Our results suggest that certain forms of c-*erbB*, activated by long terminal repeat insertion or viral transduction, are capable of inducing erythroleukemia but unable to transform fibroblasts.

Leukemia-inducing avian retroviruses have been subdivided into two main groups (for a review, see reference 12). The acute leukemia viruses, which contain cell-derived oncogenes, rapidly transform specific types of hematopoietic cells in vivo and in vitro (6, 10, 15, 26). In contrast, the avian leukosis viruses (ALVs), which lack oncogenes, induce a wide variety of leukemias and other neoplasms in chickens but do not transform cells in vitro (22, 35).

One important step in the development of avian B-cell lymphomas is the activation of c-*myc* transcription by integration of an ALV provirus juxtaposed to the oncogene (18, 24). Similarly, erythroleukemic cells induced by ALVs in certain strains of inbred chickens carry an ALV provirus next to the c-*erbB* locus and express greatly enhanced levels of c-*erbB* RNA (9, 25a). Thus, both the broad oncogenic spectrum of ALVs and their long latency period could be explained by the hypothesis that these viruses can activate certain cellular oncogenes by integration either next to or within them (9).

Although certain molecular events leading to the activation of the c-*myc* gene by ALV promoter insertion have been elucidated, it is still unclear how this event converts a normal avian lymphoid precursor into a leukemic B-lymphoma cell. Further study of this question is difficult, since neither normal nor leukemic chicken B-lymphoblasts can be easily grown in culture and the available v-*myc*-containing avian retroviruses do not transform lymphoid cells in vitro.

Several reasons suggest that ALV-induced chicken erythroleukemia might provide a system for studying how insertional activation of the c-*erbB* gene leads to erythroblast transformation. First, both normal and leukemic erythroid precursors can be grown and analyzed in vitro (5, 28, 29). Second, avian erythroid precursors can be transformed in vitro by retroviruses that contain the v-*erbA* and v-*erbB* oncogenes or the v-*erbB* gene alone (3b, 8, 14, 20). Erythroblasts transformed by v-*erbB* or other oncogenes of the *src* family proliferate independently of the erythroid differentiation hormone erythropoietin (Epo), but also undergo spontaneous differentiation into erythrocytes with a certain frequency (3b, 3c, 5, 20). v-*erbA* acts in concert with v-*erbB* by fully arresting the differentiation of the infected erythroid progenitors and enabling them to grow in standard tissue culture media (8, 13). Finally, leukemic cells containing an activated c-*erbB* gene should express *erbB*-related proteins at their cell surface, permitting them to be distinguished from uninfected precursors at the single-cell level (3, 17).

Although molecular studies on numerous Rous-associated virus 1 (RAV-1)-induced leukemias have unraveled two possible mechanisms of c-*erbB* activation, i.e., provirus insertion and c-*erbB* transduction (9, 25a, 30a), no attempts have been made to characterize the leukemic cells in vitro to determine whether c-*erbB* activation leads to elevated expression of *erbB* gene products and causes a truly leukemic phenotype (25).

In this paper, we demonstrate that pure populations of RAV-1-induced erythroleukemic cells could be explanted and studied in vitro. These cells resemble hormone-independent erythroid progenitors and expressed *erbB*-related cell surface glycoproteins at high levels. Some leukemias contained new c-*erbB*-transducing retroviruses, which efficiently replicated in chicken embryo fibroblasts without transforming them.

MATERIALS AND METHODS

Viruses, chickens, and induction of erythroleukemia. Cloned stocks of RAV-1 (9) were obtained from L. Crittenden, East Lansing, Mich. Chickens of the inbred strain L15-1 were injected with RAV-1 in East Lansing as described previously

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(9). In a second series of experiments, L15-1 chicks were hatched from embryonated eggs shipped to Heidelberg and were injected via the leg vein with 0.1 ml of undiluted RAV-1 supernatant (10^6 to 10^7 infectious units/ml). They were monitored for leukemia development by inspection of blood smears as described previously (20). During a preleukemic phase of 40 to 60 days, low numbers of partially mature and immature erythroid cells appeared in the peripheral blood of 8 of 10 chicks. Shortly afterwards, four chicks rapidly developed lethal erythroleukemia, in which the buffy coat-containing leukemic blasts represented up to 30 to 50% of the total blood cell volume.

Purification of leukemic cells. Leukemic cells obtained from moribund chicks by heart puncture were washed twice in phosphate-buffered saline, suspended at 10×10^7 cells per ml in CFU-E medium without anemic serum (25), and centrifuged through a layer of Percoll (density, 1.072 g/cm^3 [3]). The immature cells banding at the interphase represented essentially pure leukemic cells, since more than 90% of them expressed markers of erythroid cells as well as *erbB* proteins at their surface, as revealed by fluorescent staining with *erbB*-specific antisera (3).

Cells and cell culture. For in vitro culture experiments, purified leukemic cells were seeded at 10×10^6 to 20×10^6 cells per ml in CFU-E medium with or without anemic serum and supplemented with 1 μg of insulin (Actrapid; Bayer, Leverkusen, Federal Republic of Germany) per ml. Two days later, erythrocytes and dead cells were removed by centrifugation through Ficoll (4), and cells were reseeded at 2×10^6 to 5×10^6 cells per ml in the same medium. Cultures were fed daily by the addition of fresh medium, and cell numbers were kept above 5×10^5 cells per ml by reducing culture size. Cultures were considered as nongrowing when all immature cells initially present had differentiated into erythrocytes without apparent massive cell death. Proliferating leukemic cultures were kept at cell densities between 1×10^6 and 4×10^6 cells per ml.

Chicken embryo fibroblasts were prepared from 11-day-old SPAFAS embryos as described previously (11) and cultivated in standard growth medium (Dulbecco modified Eagle medium supplemented with 8% fetal calf serum, 2% chicken serum, and 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid], pH 7.3). Normal bone marrow cells were prepared from 1- to 4-week-old chicks as described previously (11). The origin and cultivation of erythroblasts transformed by avian erythroblastosis virus (AEV)-ES4 (LSCC HD3) and by AEV S13, which contains a previously undetected oncogene rather than v-*erbB* (3a), have been described earlier (1, 3b).

Plasma clot colony assay. The plasma clot colony assay was performed essentially as described earlier (5). Briefly, 10^5 cells were seeded into differentiation medium with or without anemic serum (3) supplemented with 20% Methocel in Iscoves Dulbecco modified Eagle medium–2 mg of fibrinogen per ml–0.1 U of thrombin. After clot formation at 37°C and 5% CO_2, cultures were kept at 41°C and 2% CO_2 and processed either 3 or 6 days later as described previously (5).

Immunoprecipitation analysis. Fresh and cultivated RAV-1-induced leukemic cells (10×10^6 to 20×10^6) were labeled with [^{35}S]methionine (250 μCi) or [^{3}H]glucosamine (200 μCi) as described earlier (3). Preparation of lysates, immunoprecipitation with rat antiserum to *erbA* plus *erbB* (*erbA + B*) proteins or specific for *erbB* protein, and analysis of immunoprecipitates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography were done as described previously (16, 17). Treatment of cells with tunicamycin during metabolic labeling was done as described previously (17).

Analysis of DNA and RNA. DNA and RNA from cells and tissues was purified and analyzed essentially as described previously (8, 33). The first exon probe was made from a subcloned 4.5-kilobase (kb) *Eco*RI fragment that encodes the first exon of c-*erbB* hybridizing with v-*erbB* (33). The *erbA + B* probe was made from a subcloned 2.5-kb *Pvu*II fragment from AEV (34). The *erbB* 3' probe represents sequences present in a 570-nucleotide *Bam*HI-*Eco*RI fragment of v-*erbB* (34). The long terminal repeat (LTR) probe was made from a 1.5-kb *Eco*RI-*Bam*HI fragment of AEV that contains parts of *env*, LTR, and 5'-untranslated sequences of AEV.

Infection of chicken embryo fibroblasts with *erbB*-containing retroviruses released by leukemic cells. Cultivated RAV-1 erythroblasts (BH02, BH03) (10×10^6) were seeded into 60-mm dishes containing CFU-E medium plus insulin. After the addition of 10^6 chicken embryo fibroblasts freshly trypsinized from minced embryos (11), the cultures were incubated for 2 days at 39°C. Thereafter, the dishes were carefully rinsed with standard growth medium to remove nonadherent cells, and the adherent fibroblasts were propagated in standard growth medium with daily rinses to remove any nonadherent cells. This procedure effectively removed all leukemic erythroblasts, which do not survive in standard growth medium. After an additional five to seven passages in standard growth medium, fibroblasts were frozen in liquid nitrogen or used for analysis.

Assays for fibroblast transformation parameters. Immunofluorescent staining for actin cables and fibronectin protein network were done as described previously (23). Hexose uptake was measured as described previously (27). Undiluted, filtered supernatants from either leukemic cells or infected fibroblasts were tested for focus formation on chicken embryo fibroblasts as described previously (8).

Immunofluorescence. Staining of viable leukemic erythroblasts with fluorescent *erbB*-specific antibodies and differentiation-specific antibodies was done as described previously (3, 6). To assay *erbB* protein expression at the surface of infected fibroblasts, cells were detached from the dish by EDTA treatment and stained in suspension as described above (17).

RESULTS

Characterization of RAV-1-induced erythroleukemia cells from leukemic animals and after culture in vitro. Purified leukemic cells from moribund, RAV-1-infected chicks (see above) were first characterized for their state of erythroid differentiation. The leukemic cells consisted almost exclusively of erythroid cells at various stages of maturation, about 60% of which resembled immature erythroblasts (Table 1).

To test whether the RAV-1-induced erythroleukemic cells could be grown in tissue culture, we seeded the purified leukemic cells in CFU-E medium, since erythroid cells transformed by v-*erbB* alone proliferate well in this complex medium but quickly die in standard growth medium (8, 25). Three of eight leukemias were cultured successfully. In these cultures, total cell numbers decreased during the first 5 to 7 days. However, clumps of immature cells persisted among the increasing fraction of erythrocyte-like cells (Fig. 1B). Thereafter, the immature cells started to proliferate and continued to grow for up to 3 weeks (Fig. 1B) with doubling times of about 24 h. In contrast, the leukemic cells from the other five animals tested survived in culture for the first 4 to 7 days, but then they all differentiated into erythrocytes. As

TABLE 1. Characterization of leukemic cells

Cell type	% Cells classified as[a]:			% Cells stained with[b]:		
	Ebl	ER	LR + E	αEry	αEbl	αMbl
Fresh leukemic cells						
BH02	70	17	13	53	61	5
BH03	ND[c]	ND	ND	61	66	3
HM27237[d]	77	17	6	ND	ND	ND
Peripheral blood	0	1	>95	>95	0	5
Leukemic cells after 10 days in CFU-E medium						
BH02	83	2	15	52	70	<1
BH03	82	9	9	ND	ND	ND
HM27237	95	2	3	ND	ND	ND
AEV-ES4[e]	>99	<0.1	<0.1	<1	99	<1

[a] Cell types were defined by benzidine (at neutral pH) plus histological staining (5). Ebl, Erythroblasts; ER, early reticulocytes; LR, late reticulocytes; E, erythrocytes.

[b] Characterization of these antisera has been described elsewhere (6). αEry, Antierythrocyte; αEbl, antierythroblast; αMbl, monoclonal antibody 51/2 directed against myeloblasts.

[c] ND, Not determined.

[d] Smears prepared from frozen cells immediately after thawing.

[e] *ts*167 AEV clone E3 (3).

expected, the leukemic cells fom all animals died after 2 to 3 days in standard growth medium.

When characterized for differentiation parameters, the cultured RAV-1-induced erythroleukemic cells again consisted of a mixture of erythroblasts and more mature erythroid cells (Table 1; Fig 1C). Thus, both fresh and cultivated RAV-1-induced erythroleukemic cells resemble erythroblasts transformed by v-*erbB* or *src* (3b, 20).

RAV-1-induced erythroleukemia cells proliferate and differentiate independent of exogeneous Epo. The above findings prompted us to study in more detail how RAV-1 erythroleukemic cells differ from normal, late erythroid progenitors (CFU-E cells) in their ability to self-renew and differentiate in vitro and in their dependence on exogeneously added Epo. Purified leukemic cells of chicks BH02 and BH03 were seeded into plasma clot cultures in the presence or absence of anemic serum as a source of chicken Epo (5). Normal bone marrow cells were tested as controls. After 3 days of incubation, about 14% of the leukemia-derived colonies consisted entirely of immature erythroid cells (type III, Fig. 1A), a colony type which was absent from the controls. About 30% of the colonies contained partially mature erythroid cells (type II, Fig. 1A), whereas more than half of the leukemia-derived erythroid colonies were indistinguishable from the normal CFU-E colonies (type I, Fig. 1A), obtained almost exclusively in the normal bone marrow controls (Table 2; data not shown).

Table 2 also demonstrates that RAV-1-induced erythroleukemic cells grew into undifferentiated as well as differentiated colonies with similar efficiencies in the presence and absence of anemic serum, whereas normal CFU-E colonies were stimulated more than 20-fold by the addition of anemic serum.

When the plasma clot cultures of RAV-1-induced erythroleukemic cells were incubated for 6 instead of 3 days before analysis, the mature and partially mature colonies had disintegrated. However, about 20% of the undifferentiated, type III colonies seen at day 3 had grown into large colonies containing between 2,000 and 10,000 cells. About half of these colonies were completely undifferentiated, while the other half consisted of undifferentiated cells as well as more differentiated cells. Taken together, these results indicate that the majority of the RAV-1-induced erythroleukemic cells resembled Epo-independent but otherwise normal CFU-E precursors, while a minority of the cells exhibited a sustained self-renewal capacity in vitro.

RAV-1-induced erythroblasts express high levels of an *erbB*-related glycoprotein at their surface. To study whether the RAV-1-induced erythroblasts expressed *erbB*-related proteins, fresh leukemic cells from one chicken (BH02) were labeled with [^{35}S]methionine and immunoprecipitated with anti-*erb* sera (16, 17). Antisera reactive either with both v-*erbA* and v-*erbB* or with v-*erbB* alone immunoprecipitated a group of 74- to 76-kilodalton (kDa) proteins (Fig. 2A, lanes 1 to 3) probably representing rough endoplasmic reticulum precursors of *erbB*-like proteins (16, 17). In contrast, antibodies to viral structural proteins or antibodies reacting with the *gag* or v-*erbA* domains of $\text{p75}^{gag\text{-}v\text{-}erbA}$ did not immunoprecipitate the 74- to 76-kDa proteins (Fig. 2A, lanes 4 and 5; data not shown).

When leukemic cells from 12 other RAV-1-infected chicks were analyzed, proteins of slightly differing sizes were immunoprecipitated by *erbB*-specific serum. Four of the leukemias contained 74- to 76-kDa proteins (Fig. 2B, lane 4), whereas two groups of three animals each displayed smaller proteins of 72 to 74 kDa (lane 3) and 70 to 72 kDa (lanes 1 and 2), respectively. The leukemic cells of one animal expressed two distinct protein species of 69 to 71 kDa and 74 to 76 kDa (Fig. 2B, lane 5). In all cases in which the leukemic cells could be grown in culture, the proteins detected in the fresh leukemic cells were indistinguishable from those found in the cells from the same animal after 10 days of in vitro culture (Fig. 2C, lanes 1 and 2).

Leukemic cells from chick BH02 were labeled with [^{3}H]glucosamine and immunoprecipitated to determine whether their *erbB*-related proteins were glycosylated. A protein of 83 kDa was detected which probably represented the mature cell surface form of the 74- to 76-kDa *erbB*-related proteins found in these cells after [^{35}S]methionine labeling (Fig. 3A). When the cells were labeled with [^{35}S]methionine in the presence of tunicamycin, a 72-kDa nonglycosylated form of the gp83^{erbB} protein could be detected (Fig. 3B). These results indicate that the RAV-1 erythroblasts expressed an *erbB* protein that was 9 to 10 kDa larger but otherwise closely related to the gp74^{erbB} protein of AEV-ES4 erythroblasts. This was confirmed by two-dimensional peptide-mapping studies and by immunoprecipitation of *erbB* proteins from RAV-1-induced erythroblasts with an antiserum to a peptide of the human epidermal growth factor (EGF) receptor (21; data not shown).

Finally, we tested whether fresh and cultivated erythroleukemic cells expressed *erbB*-related proteins at their surface. Virtually all immature erythroblast-like cells from both fresh and cultivated leukemic cells were strongly stained by *erbB*-specific antibodies (Fig. 4A; data not shown), while the mature cells apparently underwent down-regulation of *erbB* protein expression as seen in differentiated temperature-sensitive AEV erythroblasts (Fig. 4B) (3).

Two RAV-1-induced leukemias contain retrovirus-transduced *erbB* genes. Previous studies have shown that in a large number of RAV-1-infected leukemic chicks the ALVs had integrated close to the first c-*erbB* exon homologous to v-*erbB*, leading to highly elevated levels of c-*erbB* transcripts (9, 25a). As will be shown below, two of the ALV-induced leukemias studied here (BH02 and BH03, Table 1)

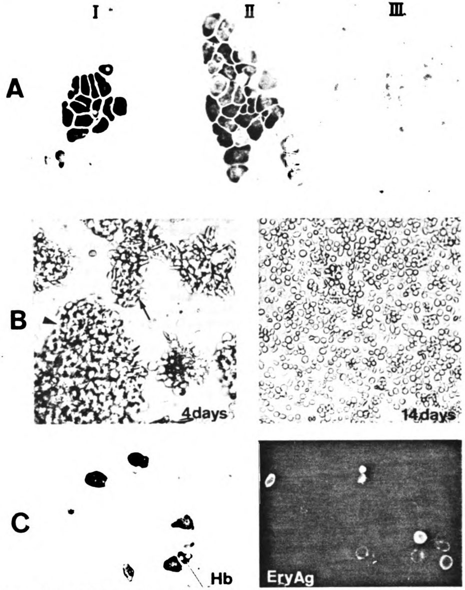

FIG. 1. In vitro characterization of RAV-1-induced leukemic cells. (A) Purified leukemic cells of chicken BH02 were seeded into plasma clot cultures without anemic serum. Three days later, clots were processed as described previously (5). Shown are photographs of mature (I), partially mature (II), and immature (III) colonies taken under blue light (5) to reveal staining for hemoglobin. (B) Photographs of leukemic cells from chicken BH02 are shown after 4 and 14 days of in vitro culture. Note clumps of mature cells (arrow) and immature cells (arrowhead) visible after 4 days. (C) Leukemic cells from chicken BH02 were cytocentrifuged onto slides and stained with neutral benzidine for hemoglobin (Hb) or stained with antierythrocyte serum (EryAg; 6) by indirect immunofluorescence. The culture consists of a mixture of immature and more mature cells, as revealed by both markers.

contained unmodified c-*erbB* genes as well as retrovirus-like elements that had transduced exons but not introns of c-*erbB*. These elements were present at multiple integration sites, suggesting an oligoclonal or polyclonal origin of the leukemias.

The first 5′ exon of the c-*erbB* gene with homology to v-*erbB* represents about 300 nucleotides encoded in a 4.5-kb (c-*erbB* α allele) or 2.3-kb (c-*erbB* β allele) *Eco*RI fragment (25a). Since ALV LTRs contain *Eco*RI sites, integration of ALV into either of these fragments would interrupt them and thus generate two smaller fragments that can be detected by using the subcloned 4.5-kb *Eco*RI fragment of the c-*erbB* α allele as a probe (first exon probe). Chick BH02 was homozygous for the α allele, and both alleles appeared to be intact in the BH02 leukemic cells (Fig. 5A). In addition, a single novel, weakly hybridizing, 3.8-kb fragment was detected with this probe. The same 3.8-kb fragment hybridized strongly with a v-*erbA* + *B* probe (which encodes all of v-*erbB*) and with a probe representative for the carboxy-terminal part of v-*erbB* (Fig. 5B and C). A second tumor-specific fragment of 2.2 kb was also detected with these probes, but it did not hybridize to the first exon probe. Finally, the additional *erbB* sequences in the leukemic cells were contained in a DNA segment similar in size to those in v-*erbB*, since double digestion with *Apa*I and *Eco*RI (which cut at the extreme ends of the AEV-ES4 v-*erbB* gene) generated a fragment of 1.7 kDa in the leukemic-cell DNA (Fig. 5D) which is of the same size as the corresponding

TABLE 2. Effect of Epo on RAV-1-induced erythroleukemic cells

Cell type analyzed	Plasma clot colonies							
	+Epo				−Epo			
	Colonies/10^5 cells	% Type[a]:			Colonies/10^5 cells	% Type:		
		I	II	III		I	II	III
Fresh BH02 Leukemic cells	2,550	58	29	13	2,580	56	30	14
Normal bone marrow	1,150	92	8	0	50	80	20	0
AEV-ES4[b]	TMTC[c]	<0.1	1	99	TMTC	<0.1	1	99

[a] Types of colonies (I, differentiated colonies; II, partially differentiated or mixing colonies; III, colonies containing immature erythroblasts) are as shown in Fig. 1.

[b] *ts*167 AEV clone E3 (3).

[c] TMTC, Too many to count.

v-*erbB* fragment. Analysis of leukemic cells after 14 days of in vitro culture gave the same results as obtained with the fresh leukemic cells (data not shown).

A parallel analysis performed on the *erbB* gene of the BH03 leukemic cells (Table 1) gave similar results as above, except that these cells were heterozygous for the α and β *erbB* alleles and that the novel *erbB* fragment, which hybridized weakly with the first exon probe but strongly with the *erbA* + *B* and *erbB* 3′ probes (Fig. 5B plus C), had a different size (3.4 kb) than in leukemia BH02.

In conclusion, the above results show that both leukemic-cell DNAs contain 5′ and 3′ v-*erbB* sequences in a fragment of similar size as in v-*erbB*, suggesting that *erbB* is present in these leukemias as a transduced sequence lacking introns. An analysis of leukemic-cell DNA from both leukemias after digestion with *Sac*I gave results supporting this hypothesis (data not shown).

We then studied whether the leukemic cells were polyclonal, as a result of spread of a c-*erbB*-containing retrovirus, or clonal, as a consequence of reverse transcription and subsequent reintegration of c-*erbB* mRNA. Leukemic-cell DNA was digested with restriction enzymes that do not

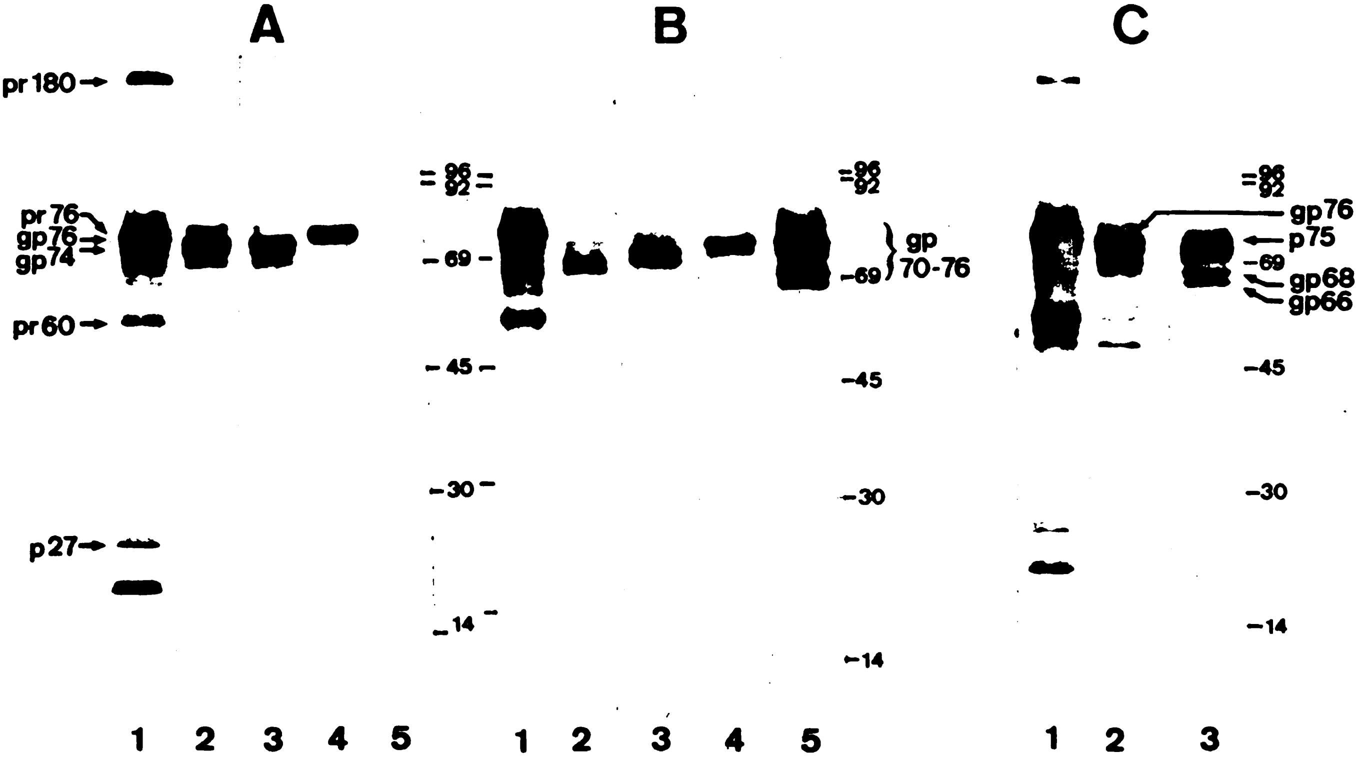

FIG. 2. RAV-1-induced erythroleukemias express *erbB*-related proteins. (A) Purified leukemic cells from chick BH02 were labeled with [^{35}S]methionine, and extracts were immunoprecipitated with anti-*erbA* + *B* serum (17) (lane 1), the same serum preincubated with virus lysate (2) (lane 2), anti-*erbB* serum (16) (lane 3), or anti-*gag-erbA* serum without (lane 4) or with (lane 5) competing virus. The positions of *erbB*-related proteins (gp74, gp76, probably representing rough endoplasmic reticulum precursors) are indicated. Small numerals indicate molecular weight markers ($\times 10^3$). (B) Leukemic cells from chicks BH03 (lanes 1 and 2), HM29393 (lane 3), HM27235 (lane 4), and HM8641 (lane 5) were immunoprecipitated with anti-*erbA* +*B* serum without (lane 1) or with (lanes 2 to 5) competing virus. (C) Leukemic cells from chick BH02 were cultivated for 14 days and then labeled and immunoprecipitated with anti-*erbA* + *B* serum without (lane 1) or with (lane 2) competing virus. The positions of *erbB*-related proteins (gp76) and of the AEV-ES4-encoded proteins (lane 3) $p75^{gag\text{-}erbA}$ (p75) and $gp74^{erbB}$ precursors (gp66, gp68) are indicated.

FIG. 3. Characterization of *erbB*-related proteins from RAV-1-induced erythroleukemia. (A) Cultivated leukemic cells from chick BH02 were labeled with [^{35}S]methionine (^{35}S Met) or [^{3}H]glucosamine (^{3}H Glu) and immunoprecipitated with *erbB*-specific antibodies. An apparently mature form (gp83) of *erbB*-related protein was immunoprecipitated from the glucosamine-labeled cells. Numbers are described in the legend to Fig. 2. (B) Cultivated BH02 cells and AEV-ES4-transformed erythroblasts (AEV-ES4) were labeled with [^{35}S]methionine in the presence (+) or absence (−) of tunicamycin (17) and immunoprecipitated with *erbB*-specific antibodies. Nonglycosylated *erbB* protein precursors of 71 kDa (p71) and 62 kDa (p62) were immunoprecipitated from the tunicamycin-treated BH02 and AEV-ES4 erythroblasts, respectively.

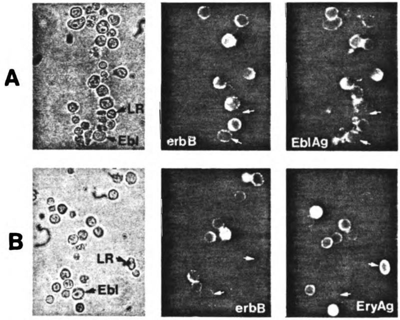

FIG. 4. *erbB*-related proteins of RAV-1-induced leukemias are expressed at the cell surface. Purified leukemic cells from chick BH02 were double labeled with anti-*erbB* serum (*erbB*) plus antierythroblast serum (A; EblAg) or antierythrocyte serum (B; EryAg) (6) in indirect immunofluorescence as described earlier (3). Photographs of the same cells viewed with bright-field illumination (left panels), fluorescein isothyocyanate-conjugated fluorescence (*erbB*, middle panels), or rhodamine fluorescence (EblAg, Ery-Ag; right panels) are shown. Erythroblasts (Ebl) and late reticulocytes (LR) are marked with arrows.

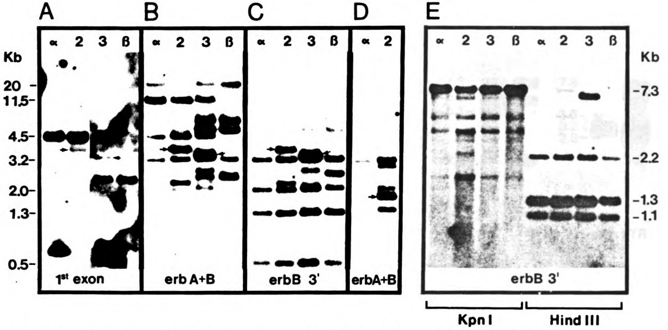

FIG. 5. Identification of transduced *erbB* sequences in two RAV-1-induced leukemias. DNA was purified from the in vitro-cultivated leukemias BH02 and BH03 and subjected to analysis by the Southern transfer technique (29a). The restriction patterns of the *erb* sequences in the leukemic cells were compared with those of normal chicken DNAs homozygous for either the α or β allele of *erbB*. The arrows indicate novel *erbB*-specific fragments. (A) The DNAs were cut with *Eco*RI and hybridized with a probe representative for the first exon of c-*erbB* homologous to v-*erbB*, as described in the text. (B and C) The *Eco*RI-digested DNAs were hybridized with probes representative for v-*erbA* + *B* and the v-*erbB* 3'-specific probe, respectively. (D) DNA from BH02 was digested with *Apa*I and *Eco*RI and hybridized with the v-*erbA* + *B* probe. (E) The DNAs were cut with *Kpn*I or *Hin*dIII and hybridized with the v-*erbB* 3'-specific probe. The sizes of the major c-*erbB*-specific fragments are indicated to the left and right.

cleave within v-*erbB* and that cut once (*Kpn*I) or twice (*Hin*dIII) in helper virus DNA. No discrete extra *erbB* fragments were detected in the DNA from either leukemia after digestion with *Kpn*I (Fig. 5E), as would have been expected if these leukemias had been of clonal origin. The polyclonal origin of the BH02 leukemic cells was confirmed by *Hin*dIII digestion, which showed a smear of *erbB*-hybridizing fragments with sizes between 5 and 7.5 kb (Fig. 5E). With BH03 leukemic cells, however, a discrete fragment of 7 kb was seen after *Hin*dIII digestion, suggesting that this *erbB*-transducing element contained two internal *Hin*dIII sites.

Last, the other 11 RAV-1-induced leukemias were analyzed for the presence of transduced c-*erbB* sequences and for a possible correlation of this event to a particular type of *erbB* protein (Fig. 2). No such correlation could be found (Table 3), because proteins of several sizes were produced both from leukemias exhibiting modified c-*erbB* alleles and from those containing transduced *erbB* sequences. Interestingly, one leukemia contained both an insertional activation and a transduction of *erbB*; consequently, these leukemic cells expressed *erbB* proteins of two different sizes (Fig. 2; Table 3).

RAV-1-induced leukemias with transduced *erbB* sequences express multiple species of *erbB* RNA at elevated levels. Polyadenylated RNA isolated from fresh BH02 leukemic cells as well as from in vitro-cultivated BH02 and BH03 erythroblasts was subjected to Northern analysis with an *erbA* + *B* probe and an LTR probe. One major 6.0-kb RNA hybridizing with both *erb* and LTR probes was seen in the fresh BH02 leukemic cells (Fig. 6A, 2f). After 14 days of in vitro culture, however, two additional *erb*- and LTR-positive RNAs of 7.8 and 5.1 kb were seen (Fig. 6A, 2c). As expected, both RNA samples contained 7.8-kb genomic and 2.8-kb subgenomic RNAs of RAV-1 virus. Similarly, the cultivated BH03 cells contained several RNA species positive with both *erb* and LTR probes (4.5, 3.6, and 3.2 kb [Fig. 6A, 3c]). Similar results were obtained with a v-*erbB*-specific probe (data not shown). This suggests that in vitro culture led to the selection of subpopulations of leukemic cells that contain *erbB*-transducing elements of similar size but have different modes of *erbB* transcription.

Next, we compared the level of *erbB* RNA transcription in RAV-1 leukemic cells with that in AEV-transformed erythroblasts and in normal chicken embryo cells. The level of *erbB* RNA in BH02 cells was about fivefold lower than in AEV-transformed erythroblasts, but still 100- to 200-fold higher than the levels of the 12- and 9-kb c-*erbB* mRNAs in chicken embryo cell RNA (Fig. 6B).

To rule out the possibility that high levels of c-*erbB* transcription are a common event in transformed erythroblasts, the RNA of erythroblasts transformed by oncogenes other than *erbB* was examined. Erythroblasts transformed by S13 virus (3a) contained no c-*erbB* RNA (Fig. 6). Erythroblasts transformed by E26 virus (25) contained low *erbB* RNA levels similar to those found in normal chicken embryo RNA, whereas RNA from normal bone marrow cells (containing erythroid precursors similar to the RAV-1-induced leukemic cells) was negative for c-*erbB* RNA (data not shown). However, c-*erbA* RNAs were found in all three samples, at levels comparable to those found in normal chicken embryonic cells (not shown).

TABLE 3. c-*erbB* activation and c-*erbB* protein expression in RAV-1-induced erythroleukemia

Leukemia	Mode of c-*erbB* activation[a]	c-*erbB* allele affected	Size of extra *Eco*RI fragments (kb)	Size of *erbB* protein (kDa)
HM27235	Proviral insertion activation	α	3.14; 1.66	76
HM8640	Proviral insertion activation	α	3.10; 1.70	76
HM8638	Proviral insertion activation	α	3.32; 1.48	74
HM27442	Proviral insertion activation	α	3.06; 1.74	74
HM29393	Proviral insertion activation	α	3.23; 1.57	74
HM27248	Proviral insertion activation	β	1.77; 0.83	76
HM27444	Proviral insertion activation	β	1.60; 1.00	74
HM8641	Two populations of cells; both insertion activation and transduced *erbB*	β	3.12; 1.68	76 and 70
HM8663	Transduced *erbB*	NR[b]		76
HM8669	Transduced *erbB*	NR		72
HM8660	Transduced *erbB*	NR		72
BH02	Transduced *erbB*	NR		76
BH03	Transduced *erbB*	NR		72

[a] Analysis of leukemic-cell DNA was performed as described previously (9, 25a).

[b] NR, Not relevant.

RAV-1-induced leukemias BH02 and BH03 produce infectious *erbB*-containing retroviruses that do not transform fibroblasts. To test whether the two leukemias containing transduced *erbB* sequences (BH02 and BH03) produce infectious, *erbB*-containing retroviruses, the leukemic cells were cocultivated with primary chicken embryo fibroblasts (see Materials and Methods). Analysis of these fibroblasts of *erbB*-specific RNA after cocultivation revealed that they expressed *erbB* RNAs of similar sizes as seen in the original erythroleukemic cells, although the relative abundance of the different RNA species seemed to be different in fibroblasts and leukemic cells (Fig. 7A). This suggests that *erbB*-containing retroviruses had been transmitted to the fibroblasts in both cases. For the virus released from BH03 cells (referred to as ERB-2 virus below) this could be confirmed by protein analysis, since the infected fibroblasts expressed the expected 70- to 72-kDa *erbB* proteins at levels that were somewhat lower than in the leukemic cells (Fig. 7B) but equivalent to levels seen in AEV-ES4-transformed fibroblasts (data not shown). In contrast, very little *erbB*-related protein could be immunoprecipitated from the fibroblasts cocultivated with BH02 erythroblasts.

To determine the proportion of virus-infected cells in the BH03 cocultivated culture, we tested the live fibroblasts for surface expression of *erbB* proteins with a fluorescent *erbB*-specific antiserum. Some 83% of the fibroblasts scored positive as compared with 86% in a control culture transformed with AEV-ES4 virus. The staining of the ERB-2-infected fibroblasts was stronger than that of the AEV-transformed control cells (data not shown), as was also observed with the corresponding erythroblasts (3).

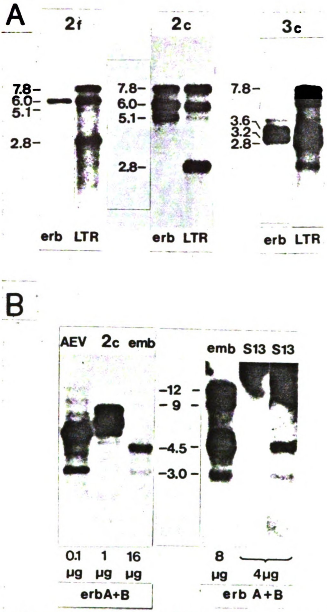

FIG. 6. Leukemias BH02 and BH03 contain abundant novel RNAs that hybridize with *erb* and retroviral sequences. (A) RNA was isolated from the fresh (f) or cultivated (c) BH02 and BH03 leukemic cells and analyzed by the Northern transfer technique. Hybridization was done with either a v-*erbA* + *B* probe (erb) or with a labeled DNA fragment from AEV-containing LTR sequences. (B) The abundance of *erb*-specific RNAs from AEV-ES4-transformed erythroblasts (AEV), BH03 leukemic cells, chicken embryonic cells (emb), and S13 virus-transformed erythroblasts (S13) was analyzed as described above. Two different exposures of the S13 RNA are shown. Hybridizing fragment sizes are shown in kilobase pairs in both panels.

By using both immunoprecipitation and immunofluorescence analysis, transmission of ERB-2 virus to fresh chicken embryo fibroblasts could also be demonstrated with filtered tissue culture supernatants from both leukemic cells and the ERB-2-producing fibroblasts generated by cocultivation (data not shown).

The ERB-2-infected cells retained a normal morphology (Fig. 7C), despite the fact that essentially all the cells

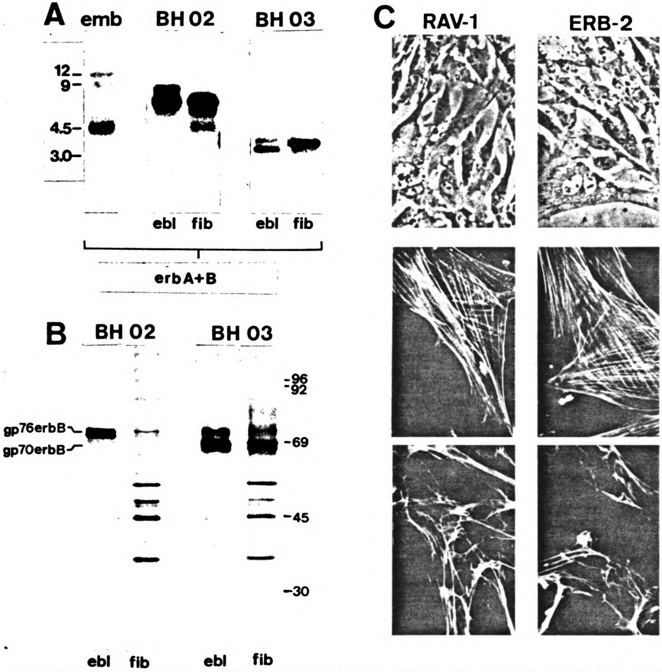

FIG. 7. Characterization of chicken embryo fibroblasts infected with *erbB*-containing virus from RAV-1-induced erythroleukemia. (A) Polyadenylated RNA from BH02 and BH03 leukemic erythroblasts (ebl) and from fibroblasts after cocultivation with the leukemic cells (fib) was analyzed by the Northern transfer technique with an *erbA* + *B* probe. Similar *erbB* RNA species are expressed in both cell types, which are clearly different from c-*erbB* RNA (9 and 12 kb) or c-*erbA* RNA (3 and 4.5 kb) present in normal chicken embryo RNA (emb). The BH03 RNA was subjected to autoradiography for 12 h and the BH02 RNA was autoradiographed for 48 h. Numbers on the left are in kilobases. (B) Leukemic erythroblasts (ebl) or fibroblasts after cocultivation with leukemic cells (fib) were labeled with [^{35}S]methionine and immunoprecipitated with *erb*-specific antibody. The same 70- to 72-kDa *erbB*-related proteins (gp70^{erbB}) are shown in BH03 erythroblasts and in fibroblasts after cocultivation. In contrast, little, if any, 76-kDa *erbB* protein (gp76^{erbB}) is detected in fibroblasts cocultivated with BH02 erythroblasts. Numbers on the right are in kilodaltons. (C) Fibroblasts cocultivated with BH03 leukemic cells (ERB-2) or infected with RAV-1 were assayed for their morphology by phase-contrast microscopy (top) or for expression of actin filament cables (middle) and fibronectin protein network (bottom) by double-label immunofluorescence as described earlier (23, 27).

expressed the *erbB* protein at their cell surface. In addition, virus harvested from the ERB-2 virus-producing BH03 leukemic cells or from the ERB-2-infected fibroblasts failed to induce foci in AEV focus assays. We therefore studied whether ERB-2-infected fibroblasts differed from normal cells in any other parameter of transformation (27).

The cells expressed actin cables (88% positive) to a similar extent as helper virus-infected control cells (92% positive) (Fig. 7C). In addition, ERB-2 cells expressed a normal fibronectin protein network (Fig. 7C) and exhibited a low rate of hexose uptake (26,000 cpm/10^6 cells as compared with 33,000 cpm/10^6 cells found with helper virus-infected control cells). AEV-ES4-transformed fibroblasts tested in parallel exhibited a typical, spindle-like morphology (25), no actin cables (13% of cells positive), a weak disordered fibronectin protein network (27), and elevated hexose uptake (80,600 cpm/10^6 cells). These results suggest that the ERB-2 virus contains a transduced c-*erbB* oncogene that does not transform fibroblasts.

DISCUSSION

RAV-1-induced leukemic cells resemble hormone-independent, late erythroid progenitors. We demonstrated that purified RAV-1-induced erythroleukemic cells exhibit a phenotype distinct from those of normal erythroid progenitors (CFU-E) and erythroblasts transformed by v-*erbB*. The leukemic cells, similar to v-*erbB*-transformed erythroblasts, were independent of exogeneous Epo for survival and differentiation, whereas normal CFU-E cells are hormone dependent for these processes. However, most of the leukemic cells differentiated into erythrocytes in a manner similar to normal CFU-E cells, while only a minority of these exhibited the sustained self-renewal characteristic of v-*erbB*-transformed erythroblasts.

The above phenotype of RAV-1-induced erythroleukemic cells is in accord with the idea that an activated c-*erbB* gene is less efficient in inducing erythroid precursors to self-renew in vitro than v-*erbB*. Alternatively, RAV-1-induced leukemic cells might mainly proliferate in the bone marrow, whereas progeny cells in the peripheral blood would already be committed to terminal differentiation. It is also possible that in vitro cultivation selects for a minor population of leukemic cells with an increased self-renewal ability.

c-*erb*-transducing retroviruses and their possible origin. Fung et al. (9) previously observed aa processed or transduced form of the c-*erbB* gene found in an RAV-1-induced leukemia. The majority of the RAV-1-induced leukemias studied here contained the expected c-*erbB* alleles activated by helper virus integration. However, two of the leukemias analyzed in detail (BH02, BH03) harbored c-*erbB*-transducing retroviruses which could be transmitted to other cells and were integrated in the host DNA at many different sites, suggesting an oligoclonal or polyclonal origin of these leukemias. Most likely, such c-*erbB*-transducing retroviruses arose as a secondary event by acquisition of the activated c-*erbB* sequences by the helper virus (30). Once generated, such retroviruses will continuously infect and transform new precursors, leading to rapid replacement of the primary clonal leukemia by a virus-induced polyclonal cell population. This notion is supported by our observation that the leukemic cells from one leukemia (HM8641) contained both a modified c-*erbB* allele and transduced *erb* sequences.

Owing to the small number of leukemias that can be grown into mass cultures, we do not know whether polyclonal leukemias containing transduced *erbB* sequences differ in their in vitro properties from those containing c-*erbB* alleles. Generation of such c-*erbB*-containing retroviruses, however, does not seem to grossly change the structure of the respective c-*erbB* proteins, since both types of leukemias expressed proteins of similar sizes (Table 3).

Mechanism of *erbB*-induced transformation of erythroid and fibroblastic cells. It was recently shown that v-*erbB* is homologous to part of the human EGF receptor (7, 32). RAV-1 integration into the c-*erbB*/EGF receptor gene apparently leads to increased expression of a truncated receptor molecule which lacks the ligand-binding N-terminal part but retains the C-terminal intracellular kinase domain (9, 25a). This is supported by the finding that the *erbB* RNA from an RAV-1-induced leukemia contains sequences highly homologous to the ultimate C terminus of the human EGF receptor (21a). In contrast, the v-*erbB* genes of both AEV-H and AEV-ES4 lack this sequence (36; personal communication).

In this paper, we showed that 13 RAV-1-induced leukemias expressed *erbB*-related cell surface glycoproteins that were 5 to 10 kDa larger than $gp74^{erbB}$ but which otherwise resembled the latter in their biosynthetic pathway. In addition, we have recently been able to show that the *erbB* proteins of the leukemias BH02 and BH03 possess a tyrosine kinase activity similar to $gp74^{erbB}$. However, they exhibited phosphotyrosine peptide maps similar to those of the chicken EGF receptor but distinct from those of $gp74^{v\text{-}erbB}$ (21; I. Lax, H. Beug, and J. Schlessinger, EMBO J., in press).

It is tempting to speculate that the truncated EGF receptor molecule expressed in RAV-1-induced erythroleukemic cells causes leukemia by constitutively producing a signal which mimics the signal produced by the Epo receptor in normal erythroid precursors when exposed to Epo (3c). This idea is in accord with our observation that RAV-1-induced erythroleukemic cells mostly resemble Epo-independent but otherwise normal CFU-E cells. To substantiate this notion, however, the response to EGF of erythroid progenitors containing an activated, complete c-*erbB*/EGF receptor gene would have to be tested by in vitro studies.

The c-*erbB*-containing retroviruses isolated from the BH03 erythroblasts did not transform fibroblasts, although they expressed high levels of *erbB* RNAs and proteins. These findings are in agreement with in vivo studies by others (30a) and suggest that the newly activated *erbB* genes had not yet acquired the ability to transform fibroblasts. This raises the possibility that an amino-truncated EGF receptor molecule with an intact C-terminal domain can render erythroid progenitors hormone independent by constitutively replacing the signal of the Epo receptor, but cannot induce a transformed phenotype in fibroblasts. Preliminary attempts to study this question were hampered by our inability to obtain more line 15 chickens, which appear to contain a dominant locus for susceptibility to c-*erbB*-transducing retroviruses (H. L. Robinson, submitted for publication). Although our studies on SPAFAS chickens showed that transformed erythroblast clones generated with ERB-2 virus supernatants from BH03 leukemic cells or infected fibroblasts expressed the 70- to 72-kDa *erbB* protein of the ERB-2 virus, we were unable to determine whether this protein was responsible for erythroblast transformation: all erythroblast clones expressed a second, *erbB*-transducing and erythroblast-transforming virus that was present in the original leukemia at levels too low to allow biochemical detection (ERB-2A virus; H. Beug et al., manuscript in preparation).

In this context, it is noteworthy that the v-*erbB*-containing erthyroblastosis viruses described previously caused erythroblastosis but not sarcomas when first isolated from a diseased chicken. Only after prolonged in vivo passage was a sarcomagenic potential acquired (19, 31). Since none of these earlier virus isolates is available anymore, c-*erbB*-transducing retroviruses from ALV-induced leukemias might be useful to identify discrete steps by which the normal EGF receptor is converted into an oncogenic protein.

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APPENDIX D:

MATERIALS AND METHODS

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APPENDIX D: MATERIALS AND METHODS

A. Chickens

Several inbred chicken lines were used in these studies and were provided by the Regional Poultry Research Laboratory, in East Lansing, Michigan (for description of inbred chicken lines see Stone, 1975). Most of the erythroblastosis samples were collected from used line 151 or 151 X 15I4 chickens. Both SPAFAS and line 151 X 15I4 chickens were used for sarcoma induction and testing of the oncogenic potential of leukemia extracts containing transduced erb B genes (Chapter 5).

B. Virus stocks

RAV-1 and RAV-2 strains of ALV, and the ES-4 strain of AEV were originally obtained from Dr. P.K. Vogt. ALV virus strains were further purified by limit dilution prior to inoculation. Viral extracts of tumorous tissues were made by preparing a 1:10 (w/vol) homogenate of tissue in 10% tryptose phosphate broth followed by passage through a 0.2 micron filter.

In most of our experiments approximately 10^3 or 10^4 infectious units of virus was injected into the peritoneum of one-day old chicks. AEV inoculations were identical except chicks were inoculated at two weeks of age. The newly generated erb B transducing viruses were injected into either 6 day-old embryos or 1 day-old chicks.

C. Monitoring erythroblastosis development and collection of leukemic samples

Erythroleukemia development was monitored by collecting blood samples at regular intervals. Hematocrits (the percent of packed red blood cells per total blood volume) and differential counts of stained blood smears were used to diagnose erythroblastosis. Blood smears were stained with May-Grumwald and Giemsa as recommended by Lucas et al. (1961). The number of erythroblasts and polychromatic erythrocytes were scored relative to 100 white blood cells. Leukemic birds were sacrificed based on the number of erythroid precursors in the bloodstream. Blood samples were obtained via heart puncture and were fractionated on discontinuous Percoll gradients. Gradients consisted of 5 ml steps of 1.06, 1.07, 1.08, 1.09 g/ml Percoll (Pharmacia). 10 ml of blood was used per gradient and centifuged at 2200 rpm in a swinging bucket rotor (HB-4). Fractionated cells were aspirated off with a pasteur pipet and washed with phosphate-buffered saline (PBS).

Bone marrow samples were collected by aspirating the long bones with Dulbecco's Modified Eagle's Medium (DMEM) or by quick-freezing immediately in liquid nitrogen. Particulate matter was removed by filtration through cheesecloth prior and cells were washed with PBS. Bone marrow and fractionated blood cells were frozen to -70°C in media containing DMEM 15% Fetal bovine serum (FBS, GIBCO) and 20% dimethyl sulfoxide (DMSO, Sigma). Other leukemic and non-leukemic tissues samples were excised and quick-frozen in liquid nitrogen and stored at -70^{o}C.

All birds that were sacrificed, died or were terminated were necropsied. Any gross lesions indicative of erythroblastosis or other neoplasms were recorded. Histological sections of tissues were made to verify macroscopic evidence of leukemia. Bone marrow smears were made from the aspirated cell suspensions.

D. Virus and cell culture

QT-6 and line 0 chicken embryo fibroblasts (CEF) were obtained from the Regional Poultry Research Laboratory in East Lansing, Michigan and were maintained in DMEM-high glucose (GIBCO) supplemented with 5% fetal bovine serum and 1% chicken serum, 10 u/ml penicillin, 0.5 mg/ml streptomycin, and 1 ug/ml amphotericin B. Cells were infected with virus by seeding in 60 mm dishes, within 5 hours after seeding, media was aspirated off and replaced by 1.0 ml of virus containing media. Polybrene (Sigma) was added to 2 ug/ml to enhance infection. Cells were incubated for two to three hours before adding fresh media and passed for two weeks prior to virus collection. Virus stocks were collected 8 to 12 hours after media change and stored at 70^{o}C. Prior to use cell debris was removed from the virus stocks by centrifugation at 10,000 rpm for 15 min.

Virus production was verified by pelleting virus and extracting viral RNA. Cell debris was removed from the virus containing media and layered onto a cushion of 20% sucrose, 10 mM Tris pH 7.5, 0.1 M NaCl (1 to 4 ml depending on the amount of media used). Virus is pelleted by centrifuging two hours at 25,000 rpm, 4°C. Viral pellets are resuspended in 0.4 ml of 0.1M NaCl, 10 mM Tris pH 8.0, 5 mM EDTA, 50ug/ml proteinase K (Boerhinger-Manheim), 40 ug/ml tRNA (Sigma) and incubated for 30 minutes at 37°C. Viral RNA is extracted twice with equal volume of phenol/chloroform (1:1 mixture), precipitated with two volumes of absolute ethanol, resuspended in 15 ul of water. The viral RNA is denatured by adding 5 ul formaldehyde and heating 15 minutes at 65°C, and neutralized by adding 130 ul of 20X SSC. The RNA can be directly spotted onto nitrocellulose (50 ul/spot) or serially diluted (usually 1:4 with 16X SSC). The filter is baked and hybridized similar to other DNA and RNA blots (see below).

Soft agar colonies from virus infected cells were selected by seeding various dilutions of cells in growth media containing 0.3% bacto-agar (soft agar). A 2.0 ml suspension of cells was plated onto a 4.0 ml hard base containing growth media supplemented with 0.6% bacto-agar. After one week the cultures were fed with an additional 2.0 ml of soft agar twice a week. After two to three weeks, discrete colonies were removed with a drawn out pasteur pipet and seeded into 35 mm tissue culture dishes. Uninfected CEF were added as cells started to senesce.

QT-6 cells were transfected with 20 ug of total plasmid DNA using the calcium phosphate technique (Graham et al., 1973, Vennstrom et al., 1980). Approximately 15 ug of test DNA was cotransfected with 2 to 3 ug of pSV2-neo (approximately 3:1 molar ratio; Southern et al., 1982).

Cells were split 1:4 18 hours prior to transfection and the media was changed 4 to 8 hours before addition of DNA. To precipitate DNA, 0.1 volumes of 1.25 M $CaCl_2$ was added to a 40 ug/ml DNA solution containing 20 mM HEPES pH 7.10, 150 mM NaCL, 0.7 mM $NaPO_4$ and incubated for 30 minutes. 0.5 ml of $CaPO_4$/DNA mix was added per 100 mm dish. DNA was removed and fluid media replaced after 18 hours. Growth media containing 1.0 mg/ml of G418 (GIBCO) was added 36 hours after transfection. After two weeks in selective media the remaining cells were trypsinized, replated, and grown up for further analysis.

E. DNA analysis

DNA was extracted from cell suspensions and tissues by homogenization, cell, lysis, pronase digestion, and phenol-chloroform extraction as previously described (Radinsky et al., 1985). RNA was removed by digestion with 50 ug/ ml of DNase-free RNase (Sigma) and subsequent dialysis against 10 mM Tris pH 8.0, 1.0 mM EDTA. DNAs were digested according to the recommendations of the suppliers. Digested DNAs were separated by electrophoresis on agarose gels, transferred to nitrocellulose, and hybridized to nick translated DNA probes as described by Raines et al., (1985). Filters were washed in 0.2X SSC and 0.1% SDS at 68°C and autoradiographed (2X SSC = 0.3 M sodium chloride, 0.03 M sodium citrate).

F. RNA analysis

Total RNA was extracted by pulverizing frozen tissue to a fine powder and lysing using the guanidinium/hot phenol method (Maniatis et al., 1982). Poly (A)+ RNA was selected by oligo d(T)-cellulose chromatography (Maniatis et al., 1982). RNA was selected two times, aliquoted and stored in ethanol at -70°C. Northern analysis was performed similar to that described by Radinsky et al., (1987). Hybridization probes for both southern and northern analysis were synthesized by nick translation of gel purified fragments, using ^{32}P-deoxyribonucleotide triphosphates (^{32}P-dNTPs) as label.

G. S1 analysis

Appropriate restriction enzyme sites were radiolabelled at their 5' ends by dephosphorylating with calf intestinal phosphatase and treating with polynucleotide kinase in the presence of gamma-^{32}P ATP as described in Maniatis et al. (1982). Fragments labeled at their 3' ends were synthesized by treatment of Klenow in the presence of ^{32}P-dNTPs. Gel purified fragments were used for most of the labeling reactions and increased the yields of probe considerably. 0.02 to 1.0 pmole of probe and either 1 ug of poly (A)+ RNA or 30 ug of total cellular RNA were used in each hybridization reaction. Optimal hybridization temperatures were determined empirically and gave minimal reannealing of the probe. Hybridizations were done overnight under standard conditions (see Maniatis et al., 1982 or Berk, et al., 1977). Unhybridized probe was digested with 200 units of S1 nuclease (Sigma) and the resistant fragments precipitated and run on a 5% polyacrylamide, 7 M urea gel.

H. Molecular cloning

Genomic DNA libraries were synthesized according to Maniatis et al., 1982. Partial digested Eco Rl or Mbo I genomic DNA was size-selected on sucrose gradients and used to ligate to either EMBL-4 or EMBL-3 bacteriophage vectors (Lehrach et al., 1982). Recombinant phage were packaged and screened using the appropriate hybridization probes. Recombinant clones were restriction mapped by single and double digestion and by southern blot analysis.

A cDNA library was made using the Gubler and Hoffman (1983) method. A synthetic oligonucleotide was used to prime first strand cDNA synthesis by reverse transcriptase instead of oligo dT. Except for this one difference the procedure is identical to that reported. Eco RI linkers (P-L Biochemicals) were added to the double stranded cDNA and ligated to lambda gt-10 arms (Vector Cloning System).

I. DNA sequence analysis

Restriction enzyme fragments for sequence analysis were subcloned into bacteriophage M13 vectors, transformed into JM109 and single-strand phage DNA isolated. The nucleotide sequence was determined using ^{35}S-dATP and the dideoxynucleotide chain-termination method of Sanger et al., (1977). Only one strand was sequenced when comparing to known sequence, any ambiguities were resolved by sequencing overlapping clones and the opposite strand. Both strands were sequenced if newly identified sequences (ie., viral recombination sites) were present.

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