JOURNAL OF VIROLOGY, Oct. 1984, p. 1–8
0022-538X/84/100001-08$02.00/0
Copyright © 1984, American Society for Microbiology
Vol. 52, No. 1

Mechanisms of Oncogenesis by Subgroup F Avian Leukosis Viruses

M. CELESTE SIMON,[1,2]* RALPH E. SMITH,[3] AND WILLIAM S. HAYWARD[1]

Memorial Sloan-Kettering Cancer Center[1] and The Rockefeller University,[2] New York, New York 10021, and Department of Microbiology and Environmental Health, Colorado State University, Fort Collins, Colorado 80523[3]

Received 19 March 1984/Accepted 29 June 1984

Subgroup F avian leukosis viruses, such as RAV-61 and ring-necked pheasant virus, are recombinants between exogenous chicken retroviruses and endogenous pheasant viruses and contain new envelope (*env*) genes. Chickens infected as 10-day-old embryos with subgroup F viruses develop fibrosarcomas, nephroblastomas, osteopetrosis, B-cell lymphomas, and a high incidence of a proliferative disorder involving the lung. Fibrosarcomas, nephroblastomas, and lymphomas appear after long latent periods (3 to 12 months). They contain discrete virus-cell junction fragments and are therefore clonal outgrowths of a single infected cell. Two ring-necked pheasant virus-induced B-cell lymphomas and an adenocarcinoma of the abdomen contained proviruses integrated at the c-*myc* locus and elevated levels of *myc* mRNA. At least four of the fibrosarcomas appeared to contain proviruses integrated at a common site, suggesting that a specific cellular gene may be involved in these tumors. The host gene has not been identified, however; 16 different oncogene probes failed to hybridize to fibrosarcoma junction fragments. In contrast to these neoplasms, lung lesions appeared rapidly (4 to 5 weeks), showed no evidence of clonality, and lacked long terminal repeat-initiated transcripts other than viral 35S and 21S mRNA. We conclude, therefore, that subgroup F retroviruses induce the proliferative disorder of the lung by a different mechanism.

Avian leukosis viruses (ALVs) cause a variety of disorders in chickens. Diseases range from debilitating conditions such as anemia, stunting, or immunosuppression to neoplasms such as B-cell lymphoma and erythroleukemia (4, 6). The avian retroviruses have been classified into seven subgroups lettered A through G based upon properties (host range, interference, and antigenicity) specified by the viral envelope glycoprotein. Viruses belonging to subgroup F were first isolated after passage of the Bryan strain of Rous sarcoma virus in ring-necked pheasant cells (7, 13, 33). Several virus isolates from pheasant cells have been reported: RAV-61 (13), ring-necked pheasant virus (RPV) (7), and W8-1-1B (33). These independent isolates are indistinguishable by liquid hybridization (16) and appear to be recombinants between endogenous pheasant viruses and the infecting Bryan Rous sarcoma virus (16, 31, 32). Approximately 75% of the recombinant virus genome consist of nucleotide sequences highly related to other avian retroviruses (16, 31). The remaining 20 to 25% are derived from pheasant DNA and share little or no homology with sequences present in viruses of subgroups A, B, C, D, or E (16). Presumably this new information provides the subgroup F envelope (*env*) gene, since the parent virus (Bryan Rous sarcoma virus) lacks *env*. It is not clear, however, whether the pheasant-derived sequences encode envelope information exclusively (16). The recombinants do not contain detectable *src* sequences.

Purchase and coworkers (27) examined the oncogenicity of subgroup F ALV after injection into 1-day-old chickens. RAV-61 caused a low incidence of lymphoma, nephroblastoma, and osteopetrosis; RPV caused no disease at all. It was questioned whether the inbred chickens used were susceptible to RPV, as viremia was not observed 18 days after inoculation (27). In a more recent study (6), RPV was inoculated into 10-day-old chicken embryos and had a surprisingly high oncogenicity. RPV caused localized foci or proliferating cells in lung blood vessels in a high percentage of infected birds. The proliferating foci were diagnosed as angiosarcoma and could be detected as early as 2 weeks after hatching. Many infected chickens died rapidly, with their lungs filled with proliferating cells. However, metastasis of these cells to other tissues was rare. Chickens living 6 weeks or longer had smaller lung lesions, if any, and developed other diseases, including nephroblastoma, fibrosarcoma, osteopetrosis, and B-cell lymphoma (6).

ALVs do not transform tissue culture cells, require 4 to 12 months for oncogenesis in vivo, and have somewhat lower efficiencies for disease induction (20 to 60%) than the rapidly transforming viruses, which contain oncogenes (10, 12). ALV proviral integration within specific regions of the host chromosome resulting in transcriptional activation of cellular oncogenes has been established for two different types of neoplasm (9, 14, 24, 26). In ALV-induced bursal lymphoma, the activated gene is c-*myc* (14). Erythroblastosis in ALV-infected chickens involves the activation of c-*erb*^{B} (9). Subgroup F viruses have many of the properties of other ALVs: they are replication competent, do not transform fibroblasts in culture, and appear to lack oncogenes. Therefore, neoplasms induced by subgroup F viruses may be caused by a similar activation of host c-*onc* genes. The present report describes some of the events of RPV transformation in both the acute lung disease and in long-latency neoplasms (fibrosarcoma, lymphoma, and nephroblastoma). We found that long-latency tumors are clonal and some involve viral integration adjacent to known cellular oncogenes. However, lung lesions showed no evidence of clonality or unique integrations. RPV and RAV-61 cause angiosarcomas rapidly (1 month) and with high incidence (88% of the infected chickens in one study). This is atypical of leukosis viruses and suggests a different mechanism of neoplastic conversion.

MATERIALS AND METHODS

Cells and viruses. Chicken cells were from C/O embryos obtained from SC birds (Hyline International, Dallas Center, Iowa). C/O fibroblasts lacking endogenous viruses (2) were cultured from eggs generously provided by L. B. Crittenden

* Corresponding author.

(U.S. Department of Agriculture Regional Poultry Research Laboratory, East Lansing, Mich.). Primary cultures were prepared by standard techniques and maintained at 37°C in F-10 medium supplemented with 5% calf serum and 1% chicken serum (11). RPV, obtained from P. K. Vogt, was plaque purified three times on C/ABE fibroblasts and one time on turkey embryo fibroblasts (29). RAV-61 was obtained from H. Hanafusa.

Inoculation of chickens. The chickens used were outbred white leghorns from Hyline or SPAFAS, Inc. (Norwich, Conn.) and an inbred flock having no endogenous avian leukosis viruses (ev^-). After a 10-day incubation period, fertile eggs received 0.1 ml of a virus suspension (10^5 PFU/ml) intravenously. Infected embryos were incubated another 14 days and then transferred to a separate room of an isolation facility for hatching and maintenance. Postmortem examinations were performed, and tissues were placed in 10% buffered Formalin and processed for histopathological analyses (6).

DNA extraction and endonuclease restriction digests. High-molecular-weight DNA was prepared from 0.5 to 1.0 g of normal or tumor tissue (24). Tissues were homogenized in 0.1 M NaCl–0.01 M Tris-hydrochloride (pH 8.0)–0.01 M EDTA, digested with pronase (final concentration, 0.5 mg/ml), extracted with phenol, and then digested with 100 μg of RNase A per ml in 0.01 M Tris-hydrochloride (pH 8.0)–0.01 M EDTA. After phenol-chloroform extraction and ethanol precipitation, the DNA concentration was determined by measuring the absorbance at 260 nm.

All DNA samples were digested with restriction endonucleases obtained from New England Biolabs (*Sac*I, *Pvu*I, *Bgl*II, *Hin*dIII, *Kpn*I, and *Sma*I) or Bethesda Research Laboratories (*Eco*RI, *Bgl*I, *Hha*I, *Hpa*I, *Hin*cII). DNAs (200 μg/ml) were digested overnight at 37°C with 3 to 5 U of enzyme per μg. Reactions were monitored for completeness of digestion by mixing a portion of the digestion mixture with lambda DNA at a ratio of 3:1 and analyzing products on ethidium bromide-stained agarose gels.

Nucleic acid blot hybridization. Genomic DNA samples were electrophoresed in 0.8% agarose in Tris-acetate buffer and transferred to nitrocellulose paper (Schleicher & Schuell Co.). ^{32}P-labeled restriction fragments of lambda DNA digested with *Hin*dIII were used as molecular weight markers. Blots were pretreated with solutions containing 1× Denhardt solution (0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidine) and hybridized in 50% formamide–5× SSC (5× SSC is 0.75 M NaCl plus 0.075 M sodium citrate)–1× Denhardt solution–0.5 mg of yeast RNA per ml–50 mM Tris-hydrochloride (pH 7.4)–0.2% sodium dodecyl sulfate. Filters were hybridized at 37°C for 48 h and washed twice at room temperature in 2× SSC–0.1% sodium sulfate (5 min), followed by two 30-min washes at 37°C in 0.1× SSC–0.1% sodium dodecyl sulfate. XAR-5 film and intensifying screens were used for autoradiography.

RNA extraction and poly(A) selection. Isolation of total RNA from tissues and fibroblasts was performed by using the guanidinium isothiocyanate method (20). Sample material (1 g) was homogenized in 10 ml of 4 M guanidinium thiocyanate (Eastman Kodak Co.)–0.1 M sodium acetate (pH 5.0)–0.005 M EDTA in a Tekmar Tissuemizer for 60 s. Supernates were then layered onto a 4-ml cushion of 0.83 g of CsCl per ml in 0.1 M sodium acetate (pH 5.0) and centrifuged in a Beckman SW40 rotor at 33,000 rpm at 4°C for 18 to 20 h. Pelleted RNA was resuspended in sterile water. RNA concentration was determined by UV absorbance. Total cellular RNAs were spotted directly onto nitrocellulose that was equilibrated with 20× SSC by using a Bethesda Research Laboratories Hybridot Manifold (34).

Polyadenylate [poly(A)]-containing RNA was isolated by one or two cycles of chromatography on oligodeoxy thymidylate-cellulose T-2 (Collaborative Research, Inc.) as described (35).

Glyoxal gel electrophoresis and northern transfer. Purified poly(A)-containing RNAs (5 to 10 μg) were suspended in 16 μl of glyoxal buffer and denatured at 50°C as described (22). Four microliters of marker dyes (1× dye is 0.05% bromophenol blue, 0.05% xylene cyanol, and 10% glycerol) was added, and samples were electrophoresed in a 1% agarose gel in 10 mM sodium phosphate (pH 6.8). Denatured, ^{32}P-labeled *Hin*dIII lambda DNA restriction fragments were used as molecular weight markers. After electrophoresis, RNAs were transferred to nitrocellulose paper in 20× SSC (34), and the blots were baked at 80°C. Filters were pretreated with 50% formamide–5× SSC–1× Denhardt solution–0.5 mg of yeast RNA per ml–50 mM sodium phosphate (pH 6.8)–0.2% sodium dodecyl sulfate for 24 h at 37°C. Hybridizations were carried out with 5% (wt/vol) dextran sulfate to enhance the reaction. Blots were incubated for 1 day at 37°C, washed, and prepared for autoradiography as described.

Preparation of ^{32}P-labeled DNA probes. High-specific-activity probes (ca. 1×10^8 to 2×10^8 Cerenkov cpm/μg) were prepared by incorporation of [α-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear Corp.) into double-stranded DNA (21). Plasmid pRAV-2 (from A. Skalka), a molecular clone of an RAV-2 provirus permuted at the *Sal*I site, was used as a probe for ALV sequences. The U_5 probe in these studies was a 300-base-pair *Eco*RI-*Sac*I fragment of plasmid p5′NCR (from E. Stavnezer). A c-*myc* probe was made from a *Cla*I-*Eco*RI fragment of clone pc*myc*-1 which represents the 0.96 kilobase (kb) 3′ exon of c-*myc* (see reference 23). Fifteen different *onc* probes were used in addition to c-*myc* in hybridization analyses (c-*src*, v-*fos*, v-*myb*, v-*fps*, v-*abl*, v-*sis*, c-*mos*, v-ras^H, v-ras^K, v-*rel*, v-erb^A, v-erb^B, v-*ski*, v-*yes*, and v-*ros*).

RESULTS

Tumor incidence in chickens inoculated with subgroup F viruses. Three different lines of chicken were inoculated with two independent isolates of subgroup F ALV, RPV and RAV-61 (see above). There were no apparent differences in pathogenesis among the different chicken lines or between the two virus strains. Most of the experiments reported here utilized RPV. Infected chickens appeared normal for the first 10 to 15 days after hatching, but soon showed signs of stunting, diarrhea, elevated hematocrits, and breathing difficulties. Lung tumors appeared as localized foci of proliferating cells in the lung as early as 2 weeks after hatching. Vascular capillaries were frequently occluded by hyperplastic endothelial cells. More than one type of tumor was detected in many birds. In a study with 162 birds, the proliferative disorder of the lung was detected in 88%, and ca. 80% died between 3 and 10 weeks of age. Most of the chickens that survived 6 weeks after hatching eventually developed other neoplastic conditions, such as fibrosarcoma, nephroblastoma, osteopetrosis, and B-cell lymphoma. These tumors appeared at relatively low frequencies, with the exception of fibrosarcomas, present in 27% of the chickens inoculated with RPV. A more detailed account of RPV pathogenicity has been reported elsewhere (6). Thus far, no avian retroviruses other than subgroup F have caused lung lesions. A total of eight virus strains belonging to

subgroups A through E have been tested (R. Smith, unpublished data).

Activation of c-*myc* in RPV-induced tumors. The most common neoplasm induced by most subgroups of ALV is B-cell lymphoma (24, 27). In the majority of the lymphomas analyzed, ALV caused activation of the cellular oncogene c-*myc* by proviral insertion adjacent to the *myc* locus (8, 14, 25). Bursal lymphoma was observed in only 2.5% (4 of 162) of the RPV-infected birds; two chickens, numbers 27 and 73, died of this neoplasma with liver, kidney, and spleen metastases. To determine whether RPV-induced lymphomas resulted from *myc* activation, DNA and RNA were isolated from normal and neoplastic tissues of these two birds. A preliminary restriction map of RPV proviral DNA revealed that, like other ALVs (5, 30), the long terminal repeat (LTR) and *gag* gene contain *Eco*RI sites (see Fig. 6) and that this enzyme would therefore be appropriate for DNA analyses (24). Tumors that are clonal outgrowths of a single infected cell have discrete virus-cell junction fragments detectable by *Eco*RI digestion and hybridization to a probe derived from the U_5 sequences of the LTR (24, 26).

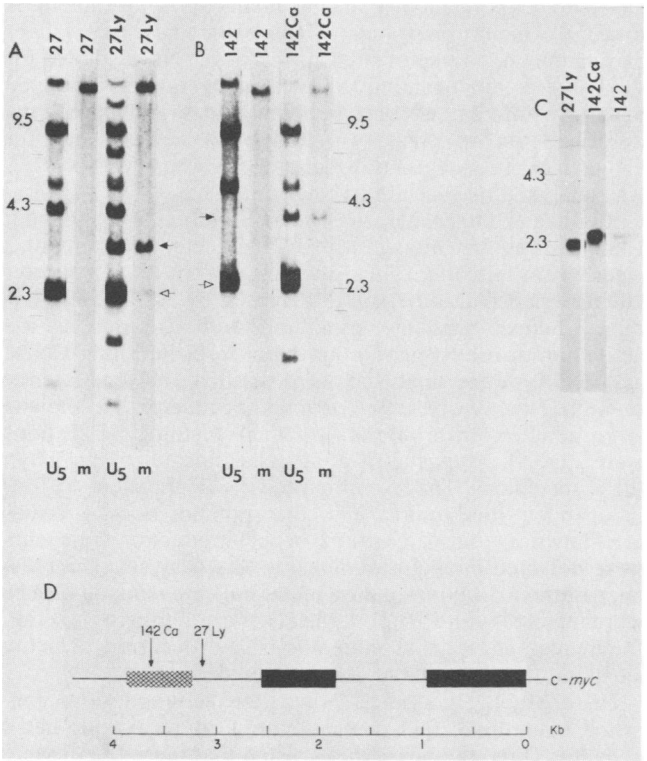

FIG. 1. Activation of c-*myc* in RPV-induced tumors. (A and B) DNAs from normal and tumor tissues were digested with *Eco*RI, transferred to nitrocellulose, and hybridized to a U_5 probe (U_5) or a c-*myc* probe (m) as indicated below each lane. (A) DNA from normal muscle (lanes 1 and 2) and a lymphoma (lanes 3 and 4) from bird 27. (B) DNA from normal muscle (lanes 1 and 2) and an adenocarcinoma (lanes 3 and 4) from bird 142. Open arrows indicate the internal *Eco*RI fragment of RPV; solid arrows indicate the positions of comigrating *myc* and U_5 restriction fragments. (C) Poly(A)-containing RNAs (5 μg per lane) from the lymphoma of bird 27, the carcinoma of bird 142, and normal liver of bird 142 were glyoxylated, blotted, and hybridized to the *myc* probe. (D) Site of proviral integrations in RPV-induced B-cell lymphoma and adenocarcinoma. The two c-*myc* coding exons are designated by solid boxes; the hatched box shows the approximate location of a 5′ noncoding exon described by Shih et al. (in press). Positions of proviral integration sites were based on the sizes of *Eco*RI tumor junction fragments and an *Eco*RI site located 150 nucleotides from the 3′ end of the viral LTR.

All DNA samples contained restriction fragments corresponding to avian endogenous viruses, which hybridize to the ^{32}P-labeled U_5 probe. The 2.5-kb fragment (indicated by the open arrow in Fig. 1A) present in all infected tissues examined represents an internal fragment of RPV. In bird 27, liver lymphoma DNA had multiple (at least six) U_5-containing restriction fragments that were absent in normal tissues such as muscle (see Fig. 1A, lanes 27 and 27Ly). Two restriction fragments were detected in lymphoma DNA with the ^{32}P-labeled *myc* probe, a 14-kb fragment (the normal c-*myc* gene) and a 3.3-kb fragment that was not present in DNA from normal tissue. The 3.3-kb fragment comigrated with a restriction fragment observed with the U_5 probe. Poly(A)-containing RNA from neoplastic tissue was tested for the presence of new tumor-specific mRNAs. A new 2.3-kb transcript was detected in liver RNA with the *myc* probe (Fig. 1C, lane 27Ly). This transcript also hybridized to the U_5 probe (data not shown), was slightly smaller than normal *myc* mRNA (2.4 kb), and was present at ca. 30 times the level of normal *myc* RNA. Lymphomatous tissue from bird 73 also exhibited proviral integration in the *myc* locus and elevated c-*myc* mRNA (data not shown). Therefore, RPV appears to induce lymphomas by activating the same cellular gene that is activated in lymphomas induced by other ALV subgroups.

Figure 1B shows the restriction pattern obtained with DNA extracted from an abdominal tumor of bird 142. This tumor was diagnosed as an adenocarcinoma of the omentum surrounding the intestine. The carcinoma DNA contained two tumor-specific junction fragments that hybridized to the U_5 probe (Fig. 1B, lane 142Ca). The same filter was eluted and then hybridized to a *myc* probe, and the 3.7-kb U_5 fragment comigrated with a *myc*-specific restriction fragment (Fig. 1B, lane 142Ca). An abundant 2.4-kb *myc* mRNA which also hybridized to the U_5 probe was present in the carcinoma (Fig. 1C, lane 142Ca). The level of mRNA was at least 20 to 30-fold higher than that in normal liver. These data are consistent with RPV integration upstream of c-*myc* and in the same transcriptional orientation. Apparently, in this tumor cellular *myc* was activated in nonlymphoid tissue, producing a different type of neoplasm (carcinoma). Multiple neoplasms were noted in bird 142, including nephroblastoma, a muscle fibrosarcoma, a liver fibrosarcoma, and lung lesions. There was no evidence of bursal lymphoma. Only the intestinal tumor contained viral integration at the c-*myc* locus.

Sites of proviral integrations in these two tumors are shown in Fig. 1D. We calculate that RPV integrated 600 base pairs upstream of the first coding exon of *myc* in the lymphoma and 1 kb upstream of this exon in the carcinoma. The carcinoma integration site would be located within or close to an untranslated exon recently described by C.-K. Shih, M. Linial, M. M. Goodenow, and W. S. Hayward (Proc. Natl. Acad. Sci. U.S.A., in press). The carcinoma *myc* mRNA has a larger size, because it contains sequences from the first exon that are absent in the lymphoma transcript (data not shown).

Integration sites in other long-latency tumors. Tumors such as nephroblastomas, fibrosarcomas, and hemangiomas also appeared in RPV-infected chickens after latent periods of 2 to 12 months. All of these long-latency tumors were found to be clonal, with at least one new virus-cell junction fragment present in each. Figure 2A shows examples of nephroblastoma and fibrosarcoma DNAs digested with *Eco*RI, blotted,

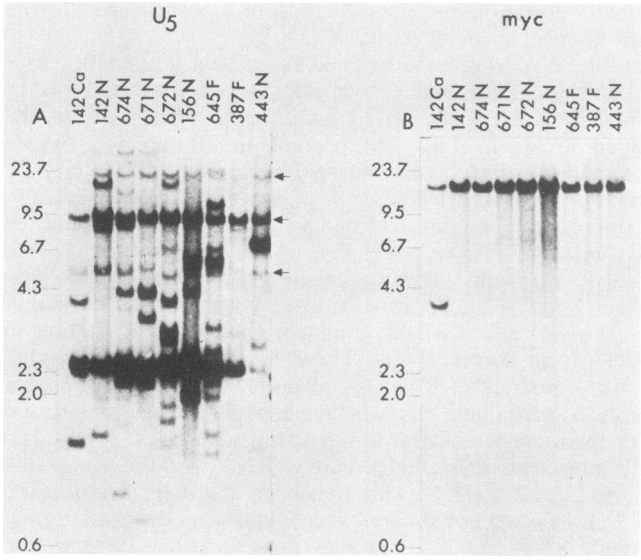

FIG. 2. Restriction analysis of RPV-induced nephroblastomas (N), fibrosarcomas (F), and adenocarcinoma (Ca). *Eco*RI digested tumor DNAs were blotted and hybridized to a U_5 probe (A) or a *myc* probe (B). Restriction fragments derived from the endogenous proviruses most frequently found in the chicken DNAs analyzed are indicated by solid arrows in panel A.

and hybridized to the U_5 probe. In addition to the 2.5-kb internal RPV fragment, several endogenous proviral restriction fragments were detected. The three endogenous provirus restriction fragments most frequently observed in this study are indicated by solid arrows in Fig. 2A. Multiple tumor-specific junction fragments were evident when tumor DNAs were compared with normal DNA of the same bird.

It was important to determine whether similar tumors from different birds have proviruses integrated within a common domain. That is, do tumor junction fragments from different samples contain the same cellular sequences? Multiple junction fragments (e.g., 10 in the DNA of a nephroblastoma [672N]; Fig. 2A, lane 5) were observed in the DNA isolated from nephroblastomas and liver tumors, making the restriction data of these neoplasms difficult to interpret. However, a number of fibrosarcoma DNAs contained only one or two tumor-specific RPV proviruses, and in many cases junction fragments were similar or identical in size. Of 27 fibrosarcomas examined, 92% had U_5-containing fragments 2.8 to 4.3 kb in size.

To determine whether comigrating or similarly sized fibrosarcoma junction fragments contained common cellular sequences, fibrosarcoma DNAs were digested with a combination of *Eco*RI and each of six other restriction enzymes: *Kpn*I, *Hin*dIII, *Bam*HI, *Hin*cII, *Pvu*II, and *Sac*I. Figure 3 shows restriction maps deduced for the cellular DNA adjacent to integrated proviruses in four independent RPV-induced fibrosarcomas (samples 444, 387, 419, and 385.) All of the *Eco*RI restriction fragments were resistant to digestion by *Kpn*I and *Hin*dIII, but contained common sites for *Sac*I, *Bam*HI, *Hin*cII, and *Pvu*II. The slight differences (ca. 0.1 kb) in the restriction maps from samples 444 and 385 are within the experimental error expected in this type of analysis. This strongly suggests that RPV proviruses are integrated at slightly different sites adjacent to the same cellular sequence. Other fibrosarcoma junction fragments were also tested with *Eco*RI in combination with several restriction enzymes. In most cases the data were consistent with integrations in the same region, but unambiguous assignments could not be made because of the large numbers of integrations in these tumors and the resultant complex restriction patterns.

New RNA species (ca. 3.6 kb) homologous to U_5 were detected in several fibrosarcomas (data not shown). These RNAs failed to hybridize to the RAV-2 probe (representing viral structural genes) and thus probably contain U_5 sequences linked to cellular information. These results are consistent with the hypothesis that muscle fibrosarcomas result from activation of a specific cellular gene.

To test for possible rearrangements of the c-*myc* gene in fibrosarcomas and nephroblastomas, DNA was digested with *Eco*RI (Fig. 2B) or *Sac*I (data not shown) and hybridized to ^{32}P-labeled *myc* probes. Figure 2B, lane 1 shows DNA extracted from the carcinoma of bird 142, described above, which carries a proviral integration adjacent to c-*myc*. None of the other tumors appeared to involve alterations in this gene. Sixteen different oncogene probes (see above) were hybridized to *Eco*RI- and *Sac*I-digested tumor DNAs to test for genetic rearrangements (data not shown). A total of 6 nephroblastomas, 27 fibrosarcomas, and 7 liver carcinomas have been tested in this way. No evidence for viral integration near any known oncogene was obtained with any tumors, except the lymphomas and carcinoma described above. Also, we could find no evidence for elevated expression of known oncogenes when cellular RNA was analyzed by dot blot hybridization.

Analysis of integration sites in RPV-induced lung tumors. Lung lesions first appeared in RPV-infected chickens at 2 weeks after hatching. Thus, the lung lesions have a shorter latent period than other tumors caused by ALV. To determine whether this acute, proliferative disease involves the activation of a host gene by proviral insertion, lung DNAs and RNAs were analyzed as described for long-latency tumors. Twenty-one different lung specimens that showed large numbers of proliferative foci in histological sections were studied. At least 50% of all cells appeared abnormal in these specimens. The U_5-containing *Eco*RI fragments of four independent lung tumors and corresponding normal tissues are shown in Fig. 4. No tumor-specific junction fragments were detected in angiosarcomas by this analysis. The presence of the 2.5-kb internal fragment indicates that all tissues were infected with RPV, but the absence of discrete junction fragments suggest that integrations had occurred at many sites and that the tumors are not clonal.

Histopathologicl studies shows that the lung lesions consisted of multiple foci of proliferating atypical endothelial cells (6). Thus, the possibility existed that these tumor were clusters of different clonal populations derived from independent integration events. To test this, lung tumor tissue from bird 228 was fractionated into 2 to 3-mm^3 pieces. Erythrocytes were removed by an LSM gradient, and the pieces were digested by collagenase and trypsin. Figure 5 shows an analysis of DNA samples isolated from 12 separate pieces. No tumor-specific virus-cell junction fragments were detected after digestion with *Eco*RI (Fig. 5) or with *Sac*I or *Pvu*I (data not shown). DNA from a clonal tumor, the kidney lymphoma of bird 27, is shown for comparison. Similar negative results were obtained with fractionated lung tumor tissue from a second bird (data not shown).

Poly(A)-containing RNAs were analyzed for the presence of new mRNAs initiating from the viral LTR and transcribing into contiguous cellular sequences. Although all lung tumors contained both 35S and 21S viral transcripts, no new U_5-specific transcripts containing cellular information could

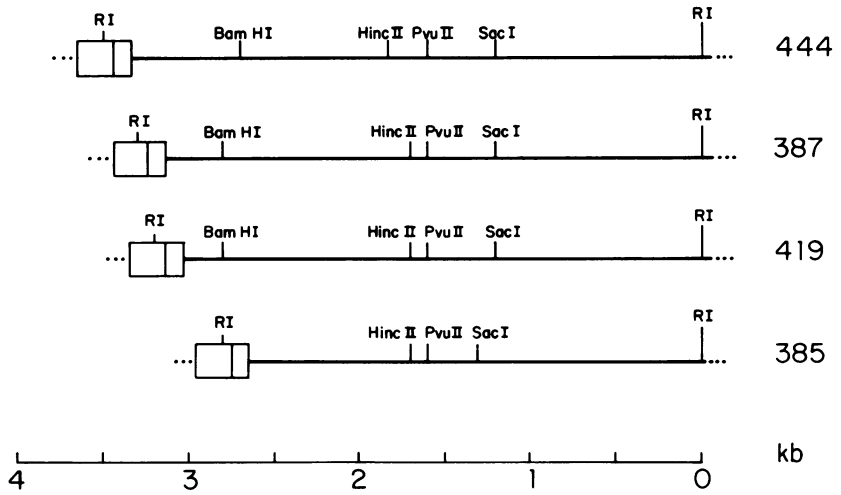

FIG. 3. Restriction maps of tumor junction fragments in DNA from four representative fibrosarcomas. Fibrosarcoma DNAs were digested with *Eco*RI in combination with each of six other enzymes (*Bam*HI, *Hin*cII, *Pvu*II, *Sac*I, *Kpn*I, and *Hin*dIII) and probed with ^{32}P-labeled U_5. The LTR sequences in the tumor junction fragments are represented by open boxes. The cleavage positions are accurate to within 0.1 kb. There were no *Kpn*I or *Hin*dIII sites in any of these junction fragments.

be detected (data not shown). It seems likely, therefore, that the induction of acute lung lesions by subgroup F viruses occurs by a different mechanism than that of the long-latency tumors.

Restriction map of the RPV provirus. The ability to cause lung foci rapidly and efficiently seems to be peculiar to subgroup F ALV. Other leukosis viruses, such as RAV-1 (subgroup A), MAV-1 (subgroup A), RAV-2 (subgroup B), MAV-2N (subgroup B), MAV-2O (subgroup B), and RAV-7 (subgroup C), failed to elicit the disease spectrum of RPV (R. Smith, unpublished data). A detailed restriction map for RPV was constructed and compared with those of other ALV subgroups to determine whether the genetic difference of this virus is confined to the *env* gene. Figure 6 shows maps for RAV-2 (subgroup B) and RPV proviruses. The RPV map was generated by using cellular DNA isolated from ev$^-$ chickens infected with this virus so that the pattern of internal RPV restriction fragments was not complicated by the presence of fragments derived from endogenous proviruses. DNA was digested with a combination of *Eco*RI and other enzymes (*Sac*I, *Hin*dIII, *Hin*cII, *Bam*HI, *Bgl*I, *Bgl*II, etc.), blotted, and probed with ^{32}P-labeled RAV-2 DNA. A molecular clone of RPV provirus in the λ gtWES · λB vector was also prepared and analyzed by restriction enzymes to confirm the map obtained from Southern blots (data not shown). The restriction map of RAV-2 was prepared by digesting cloned RAV-2 DNA. Restriction sites were conserved in the *gag* and *pol* genes of these two viruses; variations were localized in the envelope gene. Furthermore, restriction sites conserved in the *env* genes of subgroups A, B, and E viruses (15, 25) were absent in RPV (Fig. 6). Whereas U_5, *gag*, and *pol* probes hybridized to RPV proviral DNA, a probe specific for subgroup A envelope sequences did not hybridize appreciably to *env* fragments of the RPV clone. It now seems probable that the pheasant-derived sequences are localized exclusively within the envelope region. Although we cannot exclude the possibility that RPV contains limited regions of nonhomology in the LTR or in the *gag* and *pol* genes (too small to be detected by restriction mapping or hybridization), the data are consistent with the interpretation that the subgroup F envelope gene is responsible for the unique pathological properties of these viruses.

DISCUSSION

Avian leukosis viruses typically induce tumors (predominantly B-cell lymphomas) after prolonged latent periods (24, 26, 27). Like other ALVs RPV probably does not carry a

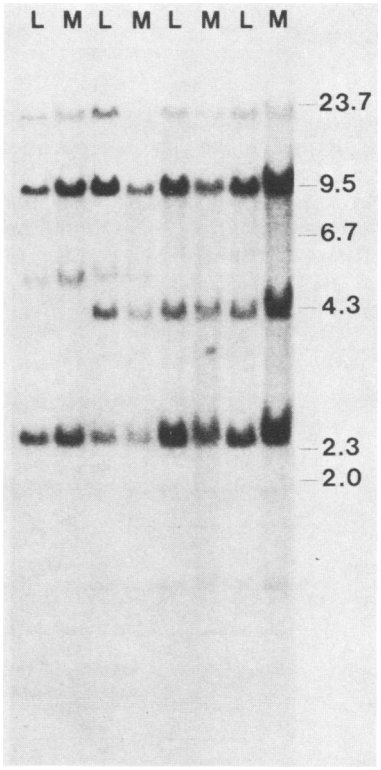

FIG. 4. Restriction analysis of DNAs isolated from chickens with lung lesions. DNA from lung lesions (L) and normal muscle (M) of four RPV-infected birds (numbers 142, 28, 115, and 228, left to right) were digested with *Eco*RI and hybridized to a U_5 probe. All restriction fragments revealed by U_5 hybridization correspond to endogenous proviruses, with the exception of the 2.5-kb internal fragment of RPV (not detected in uninfected tissue).

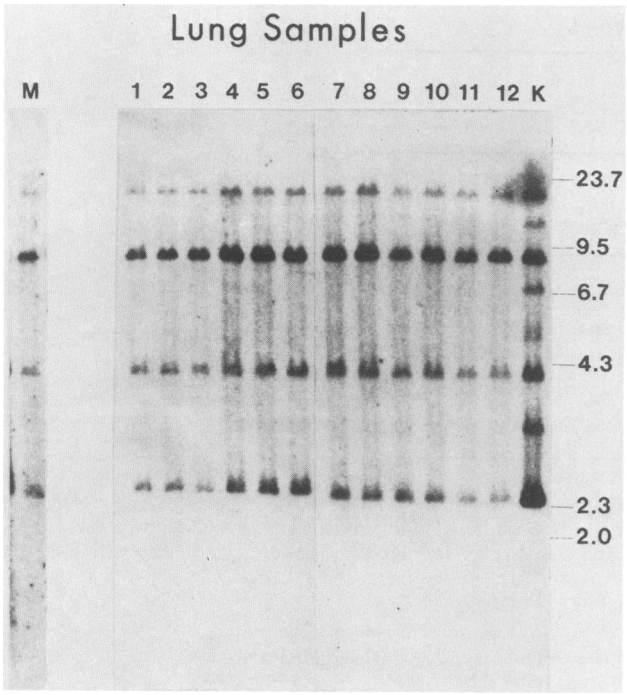

FIG. 5. Restriction analysis of DNAs isolated from subfractions of a single lung tumor. DNAs from 12 different 2- to 3-mm^3 pieces from a single lung (lanes 1 through 12) were treated as described in the legend to Fig. 4. Lane M contains DNA from normal muscle tissue of the same bird (number 228); lane K contains DNA from a kidney lymphoma from bird 27 for comparison (see Fig. 1).

transforming gene because: (i) RPV does not transform tissue culture cells and (ii) the virus is replication competent, and its genome is the same size as ALVs which lack oncogenes. However, the oncogenic spectrum of RPV differs from other leukosis viruses. Some malignant diseases induced by RPV appear only after long latent periods (fibrosarcoma, nephroblastoma, lymphoma, and carcinoma). These neoplasms are clonal and apparently arise by the same mechanism described for other ALVs: activation of host c-*onc* genes. The activated gene in RPV-induced B-cell lymphoma is c-*myc*. We also found proviral integration near c-*myc* in a nonlymphoid neoplasm, a carcinoma of the omentum. Levels of c-*myc* mRNA were elevated 20 to 30-fold in this tumor. It is interesting that c-*myc* has also been implicated in certain human nonlymphoid neoplasms (1, 18).

Restriction analyses revealed that integrations had occurred at similar sites in fibrosarcomas from different birds. It seems likely, therefore, that induction of fibrosarcomas results from proviral integration adjacent to a specific cellular gene. This putative host gene remains unidentified, as all oncogene probes tested thus far have failed to hybridize to these junction fragments. It is possible that the sarcoma gene is only weakly homologous to a known oncogene(s) or that sites of proviral integrations are distant from known genes. Alternatively, this gene may be a previously unidentified cellular gene with oncogenic potential. Cloning and further characterization of this locus should help to determine the nature of these sequences.

The lung lesions induced by subgroup F viruses (RPV and RAV-61) can be detected within 4 weeks after infection. They appear with high frequency, and the latent period is unusually short for leukosis viruses. We found no evidence for clonality in lung foci. All lung tumor specimens analyzed contained RPV proviral DNA and both genomic and subgenomic viral mRNAs. This differs from ALV-induced lymphomas, where proviruses are frequently defective and the 35S and 21S mRNAs are often absent (8, 24, 25). Unlike lymphomas, viral gene products might be required for maintenance of the proliferative state in the lung. RPV and RAV-61 have a unique set of nucleotide sequences in the *env* gene which have little homology to avian viruses of other subgroups (Fig. 6; reference 16). We conclude that RPV induces the acute lung disease by a different mechanism than that of long-latency tumors and suggest that the subgroup F envelope plays an important role in this mechanism.

The precise nature of the lung lesions requires comment. They consist of proliferating endothelial cells of vascular capillaries (6) and fit the characteristics of an angiosarcoma

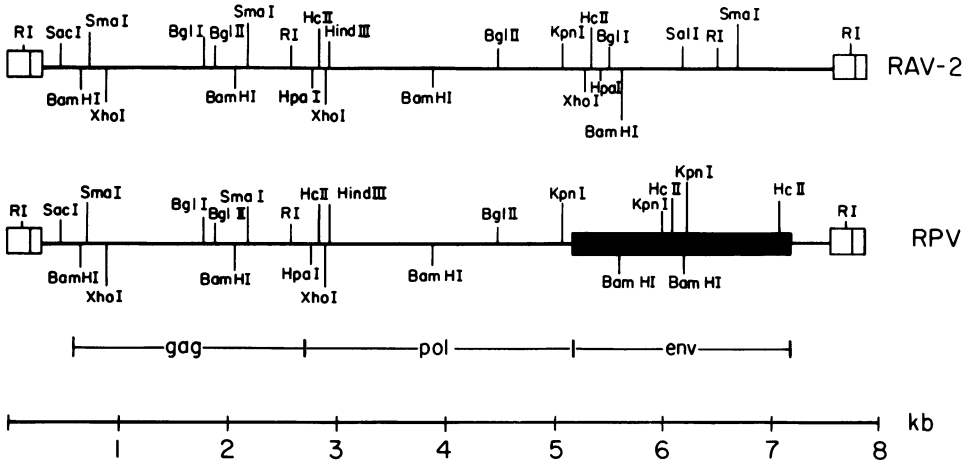

FIG. 6. Identification of unique pheasant envelope sequences in RPV. A more detailed restriction map of the RAV-2 provirus than that already described (25) was generated by digesting pRAV-2. Restriction sites in the RPV genome were determined by Southern blot analysis of RPV-infected ev$^-$ chicken cells and by digestion of a molecular clone of RPV in λgtWES · λB. The cleavage positions are accurate to within 50 base pairs. The solid box indicates the approximate boundaries of the subgroup F envelope gene of RPV.

(28). Angiosarcomas are generally found to be malignant neoplasms. There were possible examples of lung metastases to the liver in several RPV-infected birds (6), but attempts to grow lung tumors in neonatal chickens or nude mice has been unsuccessful (R. E. Smith, unpublished data). This lung disorder may therefore be an intermediate disease of a proliferative nature like osteopetrosis (29).The multiple foci in the lung may result from a premalignant or nonmalignant proliferation of blood vessel endothelial cells.

The *env* glycoproteins of other retroviruses, the leukemogenic murine leukemia viruses, have already been implicated in cellular proliferation (17). For example, the Rauscher and Friend spleen focus-forming viruses cause rapid formation of erythroid colonies in the spleens of susceptible mice (3, 17). This acute proliferative response is followed 3 to 8 weeks later by the emergence of increasingly malignant erythroleukemia cells (37). It has been suggested that the spleen focus-forming virus *env* glycoprotein performs a leukemogenic function (17, 19). The subgroup F glycoprotein of RPV and RAV-61 might provide an analogous proliferative function in the acute lung disease, possibly by interacting with endothelial cell surface receptors (36). The relationship between the subgroup F envelope and lung angiosarcomas is currently being analyzed by recombination experiments.

ACKNOWLEDGMENTS

We thank Nancy Goldberg for excellent technical assistance and Klas Wiman, Richard Baer, and Selina Chen-Kiang for critical reading of the manuscript. We also thank E. Stavnezer and A. M. Skalka for molecular clones used in this investigation and the many colleagues who provided specific oncogene probes.

This research was supported by Public Health Service grants to W.S.H. (CA34502) and R.E.S. (CA12323 and CA14236) from the National Institutes of Health.

LITERATURE CITED

1. **Alitalo, K., M. Schwab, C. C. Lin, H. E. Varmus, and J. M. Bishop.** 1983. Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-*myc*) in malignant neuroendocrine cells from a human colon carcinoma. Proc. Natl. Acad. Sci. U.S.A. **80**:1707–1711.
2. **Astrin, S. M., E. G. Buss, and W. S. Hayward.** 1979. Endogenous viral genes are non-essential in the chicken. Nature (London) **282**:339–341.
3. **Axelrad, A. A., and R. A. Steeves.** 1964. Assay for Friend leukemia virus: rapid quantitative method based on enumeration of macroscopic spleen foci in mice. Virology **24**:513–518.
4. **Beard, J. W.** 1963. Viral tumors of chickens with particular reference to the leukosis complex. Ann. N.Y. Acad. Sci. **108**:1057–1085.
5. **Boone, L. R., and A. M. Skalka.** 1981. Proviral DNA synthesis in vitro by avian retrovirus particles permeabilized with mellitin. II. Evidence for a strand displacement mechanism in plus-strand synthesis. J. Virol. **37**:117–126.
6. **Carter, J. K., S. J. Proctor, and R. E. Smith.** 1983. Induction of angiosarcomas by ring-necked pheasant virus. Infect. Immun. **40**:310–319.
7. **Fujita, D. J., Y. C. Chen, R. R. Friis, and P. K. Vogt.** 1974. RNA tumor viruses of pheasant: characterization of avian leukosis subgroups F and G. Virology **60**:558–571.
8. **Fung, Y. K., A. M. Fadley, L. B. Crittenden, and H. J. Kung.** 1981. On the mechanism of retrovirus-induced avian lymphoid leukosis: deletion and integration of the proviruses. Proc. Natl. Acad. Sci. U.S.A. **78**:3418–3422.
9. **Fung, Y. K., W. G. Lewis, L. B. Crittenden, and H. J. Kung.** 1983. Activation of the cellular oncogene c-*erb*^{B} by LTR insertion: molecular basis for induction of erythroblastosis by avian leukosis viruses. Cell **33**:357–368.
10. **Graf, T., and H. Beug.** 1978. Avian leukemia viruses. Interaction with their target cells *in vivo* and *in vitro*. Biochim. Biophys. Acta Rev. Cancer **516**:269–299.
11. **Hanafusa, H.** 1969. Rapid transformation of cells by Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **63**:318–325.
12. **Hanafusa, H.** 1977. Cell transformation by RNA tumor viruses, p. 401–483. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 10. Plenum Press, New York.
13. **Hanafusa, T., and H. Hanafusa.** 1973. Isolation of leukosis-type virus from pheasant embryo cells: possible presence of viral genes in cells. Virology **51**:247–251.
14. **Hayward, W. S., B. G. Neel, and S. M. Astrin.** 1981. Activation of a cellular *onc* gene by promoter insertion in ALV-induced lymphoid leukosis. Nature (London) **290**:475–480.
15. **Hughes, S.** 1982. Restriction maps of retroviral proviruses and corresponding cellular oncogenes, p. 1293–1315. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Keshet, E., and H. M. Temin.** 1977. Nucleotide sequences derived from pheasant DNA in the genome of recombinant avian leukosis viruses with subgroup F specificity. J. Virol. **24**:505–513.
17. **Linemeyer, D. L., J. G. Menke, S. K. Ruscetti, L. H. Evans, and E. M. Scolnick.** 1982. Envelope gene sequences which encode the gp52 protein of spleen focus-forming virus are required for the induction of erythroid cell proliferation. J. Virol. **43**:223–233.
18. **Little, C. D., M. M. Nau, D. N. Carney, A. F. Gazdar, and J. D. Minna.** 1983. Amplification and expression of the c-*myc* oncogene in human lung cancer cells lines. Nature (London) **306**:194–196.
19. **Machida, C. A., R. K. Bestwick, and D. Kabat.** 1984. Reduced leukemogenicity caused by mutations in the membrane glycoprotein gene of Rauscher spleen focus-forming virus. J. Virol. **49**:394–402.
20. **Maniatis, T., E. F. Fritsh, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Maniatis, T., A. Jeffrey, and D. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage lambda. Proc. Natl. Acad. Sci. U.S.A. **72**:1184–1188.
22. **McMaster, G. K., and G. G. Carmichael.** 1977. Analysis of single and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. U.S.A. **74**:4835–4838.
23. **Neel, B. G., G. P. Gasic, C. E. Rogler, A. M. Skalka, G. Ju, F. Hishinuma, T. Papas, S. M. Astrin, and W. S. Hayward.** 1982. Molecular analysis of the c-*myc* locus in normal tissue and in avian leukosis virus-induced lymphomas. J. Virol. **44**:158–166.
24. **Neel, B. G., W. S. Hayward, H. L. Robinson, J. Fang, and S. M. Astrin.** 1981. Avian leukosis virus-induced tumors have common proviral integration sites and synthesize discrete new RNAs: oncogenesis by promoter insertion. Cell **23**:323–334.
25. **Payne, G. S., J. M. Bishop, and H. E. Varmus.** 1982. Multiple arrangements of viral DNA and an activated host oncogene (c-*myc*) in bursal lymphomas. Nature (London) **295**:209–213.
26. **Payne, G. S., S. A. Courteneidge, L. B. Crittenden, A. M. Fadly, J. M. Bishop, and H. E. Varmus.** 1981. Analysis of avian leukosis virus DNA and RNA in bursal tumors: viral gene expression is not required for maintenance of tumor state. Cell **23**:311–322.
27. **Purchase, H. G., W. Okazaki, P. K. Vogt, H. Hanafusa, B. R. Burmester, and L. B. Crittenden.** 1977. Oncogenicity of avian leukosis viruses of different subgroups and of mutants of sarcoma viruses. Infect. Immun. **15**:423–428.
28. **Robbins, S. L., and R. S. Cotran.** 1979. Pathologic basis of disease, p. 638. The W. B. Saunders Co., Philadelphia, Pa.
29. **Schmidt, E. V., J. D. Keene, M. Linial, and R. E. Smith.** 1982. Association of 3′ terminal RNA sequences with avian leukosis viruses causing a high incidence of osteopetrosis. Virology **116**:163–180.
30. **Shank, P. R., and M. Linial.** 1980. An avian oncovirus mutant (SE21Q1B) deficient in genomic RNA: characterization of a

deletion in the provirus. J. Virol. **36**:450–456.
31. **Shoyab, M., and M. A. Baluda.** 1975. Homology between avian oncornavirus RNAs and DNA from several avian species. J. Virol. **16**:1492–1502.
32. **Shoyab, M., and M. A. Baluda.** 1976. Ribonucleotide sequence homology among avian oncornaviruses. J. Virol. **17**:106–113.
33. **Temin, H. M., and V. K. Kassner.** 1976. Avian leukosis viruses of different subgroups and types isolated after passage of Rous sarcoma virus-Rous-associated virus-0 in cells from different ring-necked pheasant embryos. J. Virol. **19**:302–312.
34. **Thomas, P.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. **77**:5201–5205.
35. **Wang, L.-H., P. Duesberg, K. Beemon, and P. K. Vogt.** 1975. Mapping RNase T_1-resistant oligonucleotides of avian tumor virus RNAs: sarcoma-specific oligonucleotides are near the poly(A) end and oligonucleotides common to sarcoma and transformation-defective viruses are at the poly(A) end. J. Virol. **16**:1051–1070.
36. **Weissman, I. L., and M. S. McGrath.** 1982. Retrovirus lymphomagenesis: relationship of normal immune receptors to malignant cell proliferation. Curr. Top. Microbiol. Immunol. **98**:103–112.
37. **Wendling, F., F. Morean-Gachelin, and P. Tamborin.** 1981. Emergence of tumorigenic cells during the course of Friend disease. Proc. Natl. Acad. Sci. U.S.A. **78**:3614–3618.