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Activation of the c-*myb* Locus Is Insufficient for the Rapid Induction of Disseminated Avian B-Cell Lymphoma

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We have previously reported that infection of 9- to 13-day-old chicken embryos with RAV-1 results in rapid development of a novel B-cell lymphoma in which proviral insertion has activated expression of the c-*myb* gene (E. Pizer and E. H. Humphries, J. Virol. 63:1630–1640, 1989). The biological properties of these B-cell lymphomas are distinct from those associated with the B-cell lymphomas that develop following avian leukosis virus proviral insertion within the c-*myc* locus. In an extension of this study, more than 200 chickens, infected as 10- to 11-day-old embryos, were examined for development of lymphomas that possess disrupted c-*myb* loci. Fourteen percent developed disseminated B-cell lymphoma. In the majority of these tumors, the RAV-1 provirus had inserted between the first and second exons that code for $p75^{c\text{-}myb}$. However, insertions between the second and third exons and between the third and fourth exons were also detected. In situ analysis of *myb* protein expression in tumor tissue revealed morphological features suggesting that the tumor originates in the bursa. Within the bursa, the lymphoma appeared to spread from follicle to follicle without compromising the structural integrity of the organ. Tumor masses in liver demonstrated heterogeneous levels of *myb* protein suggestive of biologically distinct subpopulations. In contrast to the morbidity data, immunohistological analysis of bursae from 4- to 6-week-old chickens at risk of developing lymphomas bearing altered c-*myb* loci revealed lesions expressing elevated levels of *myb* in 16 of 19 birds. The activated *myb* lymphoma displayed very poor capacity to proliferate outside its original host. Only 1 of 33 in vivo transfers of tumor to recipient hosts established a transplantable tumor. None of the primary tumor tissue nor the transplantable tumor exhibited the capacity for in vitro proliferation. Similar experimental manipulation has yielded in vitro lines established from avian B-cell lymphomas expressing elevated levels of c-*myc* or v-*rel*. The dependence on embryonic infection for development of activated-*myb* lymphoma suggests a requirement for a specific target cell in which c-*myb* is activated by proviral insertion. It is likely, moreover, that continued tumor development requires elevated expression of *myb* proteins within a specific cell population in a restricted stage of differentiation.

The avian leukosis viruses (ALV) are a family of retroviruses that cause, among other diseases, two distinct avian B-cell lymphomas (21, 41). One tumor, lymphoid leukosis, develops following ALV infection of hatchling chickens, proviral insertion within the locus encoding the cellular proto-oncogene c-*myc*, and deregulation of c-*myc* expression (20, 29, 36). Microscopic examination of bursal tissue 2 to 4 weeks after infection reveals the presence of pyroninophilic preneoplastic lesions designated transformed follicles (3, 12, 32). It is believed that these lesions originate following clonal expansion of a specific target cell in which proviral disruption of the c-*myc* locus has led to unregulated proliferation. Support for this model can be found in the observation that infection of early bursal stem cells with HB-1, a defective acute oncogenic virus that encodes v-*myc*, results in formation of transformed follicles when the infected cells are used to repopulate chemically ablated bursae (31). Preneoplastic lesions persist during a latent period lasting between 10 and 15 weeks. In typical cases of lymphoid leukosis, a large tumor nodule is found in the bursa of Fabricius within 2 to 3 months (12, 41). Such nodules are also found in members of the infected cohort that do not develop disseminated disease and therefore represent the primary tumor. Like the disseminated lymphoma, these bursal nodules have clonal proviral integrations within the c-*myc* locus (30, 33). Progression of the preneoplastic lesion to a primary nodule and later to disseminated disease requires additional genetic changes, occurring over a period of 3 to 6 months. One such change may involve a locus designated c-*bic* (11). The development of lymphoid leukosis, therefore, resembles a multistep, stochastic process.

The development of activated-*myb* B-cell lymphoma following embryonic infection with ALV is fundamentally different from development of the activated-*myc* tumor (23, 39). Most strikingly, the course of the disease is fulminant. Most affected chickens acquire a lethal tumor burden within 4 weeks after hatch, or 5 weeks after infection with the virus. Within a period of 3 to 4 weeks, clonally derived lymphoma infiltrates the abdominal viscera and bone marrow as well as the bursa of Fabricius. Tumor development is associated early with the ability to proliferate widely in the chicken. The rapid development of activated-*myb* lymphoma following viral infection suggested that, in contrast to the series of rate-limiting changes during activated-*myc* lymphomagenesis, a single limiting event was sufficient to confer a fully malignant phenotype on the affected cell. It appeared, therefore, that insertional mutagenesis of c-*myb* produced a B-cell lymphoma with very aggressive growth behavior. We undertook a more extensive analysis of the biological properties of this tumor, making use, in part, of immunohistochemical analysis. Our results revealed several unique properties of the activated-*myb* tumor. Perhaps of greatest significance, the incidence of elevated expression of c-*myb* is far greater

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than the proportion of animals that succumb to disease. Our results suggest that additional factors modulate the tumor process.

MATERIALS AND METHODS

Virus stocks and cell lines. SC chicken embryo fibroblasts were cultured in plastic dishes (Nunc, Roskilde, Denmark) in Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories, Inc., McLean, Va.) containing 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.) and 10% calf serum (Hazelton, Lenexa, Kans.) with antibiotics. The RAV-1 strain of ALV used throughout these studies was obtained in 1978 from L. Crittenden (Regional Poultry Research Laboratory, East Lansing, Mich.). The virus has been maintained through successive passages on chicken embryo fibroblasts by alternating passage at low multiplicity of infection (0.01) and cloning by endpoint dilution.

Chickens and virus infection. Embryonated SC eggs (Hyline International Hatcheries, West Des Moines, Iowa) were incubated with humidity at 39°C. Infection of 10- to 11-day-old embryos was achieved by inoculation of 10^5 IU of RAV-1 into a chorioallantoic vein. Newly hatched chickens were inoculated with 10^5 IU into the jugular vein. Animals were housed by the Animal Resource Center, University of Texas Southwestern Medical Center at Dallas, in rooms isolated for control or ALV-infected chickens. All infected animals except those in hatches 89 and 95 were assayed for viremia at hatch or 1 week after inoculation. Establishment of viremia was assayed by collection of plasma, which was diluted and used to infect duplicate cultures of chicken embryo fibroblasts. These cultures were transferred twice, and the culture supernatants were assayed for reverse transcriptase activity (2). More than 97% of inoculated animals became viremic. Nonviremic animals were excluded from analysis.

Evaluation of gross pathology at necropsy. Chickens in cohorts at risk for lymphoma were sacrificed and examined grossly and/or histologically. All abdominal organs and the bursa of Fabricius were removed, inspected, and palpated. Tissues that had grossly detectable evidence of tumor were collected for further analysis. Samples for histological evaluation were snap frozen in 2-methylbutane that was precooled to −70°C and were stored at −70°C. All animals that died prior to scheduled sacrifice were similarly examined.

Histology. Frozen tissue was embedded in OCT medium (Lab-Tek Products, Naperville, Ill.) and serially sectioned on a cryostat at 8 μm. Sections were dried and fixed in ice-cold acetone for 5 to 10 min. Once dried, slides were stored at −20°C until used. Sections to be stained with methyl green pyronin (MGP) (Sigma Chemical Co., St. Louis, Mo.) were additionally fixed in 10% formalin (pH 7.4) for 10 min and in 50% ether–50% ethanol for 10 min and then extracted with xylene for 10 min. Sections were immersed in MGP stain for 20 to 30 min, rinsed, air dried, and mounted.

For immunohistology, acetone-fixed frozen tissue sections were blocked for 10 min in 30% fetal calf serum (Sterile Systems, Logan, Utah) in TBS (20 mM Tris-HCl [pH 7.5], 150 mM NaCl); then an equal volume of hybridoma supernatant containing primary antibody was added and allowed to react for 30 min. Sections were washed three times for 5 min each in TBS and then were fixed for 5 to 10 min in 10% formalin (pH 7.4). Sections were rinsed in TBS, reacted for 20 min successively with rabbit anti-mouse immunoglobulin (Ig) and then monoclonal murine peroxidase-antiperoxidase complex (Dakopatts, Santa Barbara, Calif.), and diluted in blocking solution, with washes as described above after each reaction. Sections were developed for 10 min in 3% ammonium acetate-citrate (pH 5.5) containing 0.5 mg of diaminobenzidine (Sigma) per ml and 0.005% H_2O_2. The reaction was terminated by rinsing in water; counterstaining with 1% methyl green was performed prior to mounting if indicated. The monoclonal antibody against chicken IgM has been previously described (4). The monoclonal antibody against *myb* proteins, *myb* 2.7, was obtained from J. M. Bishop (University of California, San Francisco). Its reactivity has been described previously (15, 39).

Tumor transfer protocols and cyclophosphamide treatment. Cell suspensions were prepared from tumors and bursa by extrusion of tissue through nylon mesh into DMEM and 1% fetal calf serum. Bone marrow cells were prepared by flushing medium through femurs followed by centrifugation on Ficoll (Isolymph; Gallard-Schlesinger, Carle Place, N.Y.) gradients to remove erythrocytes. Cells were washed twice and resuspended in DMEM at between 0.5×10^7 and 2×10^7/ml. Activated tumor *myb* transfer recipients, between 6 days and 4 weeks of age, received between 0.1 and 0.5 ml of cell suspension by injection into the jugular vein. Most recipients were injected intraperitoneally with 3 mg of cyclophosphamide (Cytoxan; Mead Johnson, Syracuse, N.Y.) daily for 4 days after hatching to eliminated resident B lymphocytes. Activated *myc* tumor transfer recipients, 1 day to 2 weeks old, received approximately 10^7 cells injected into the leg vein. All recipients were normal.

Culture conditions for tumor explants. Single cell suspensions from bursae, bone marrow, and tumors from livers of lymphomatous birds were diluted into Hahn's medium (1) and cultured at 37°C with 10% CO_2. Cultures were monitored daily for growth and fed at 6-day intervals by removal of medium and replacement with fresh medium. Cultures that contained no growing cells after 3 weeks were discarded.

Analysis of DNA. Cellular DNA was isolated from cells and tissues as previously described (2). Restriction enzymes (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) were used in digestion conditions specified by the manufacturer. Analysis of DNA by agarose gel electrophoresis, Southern transfer, and hybridization was done as previously described (2).

Nucleic acid probes. Isolated DNA fragments and plasmids were labeled with [α-^{32}P]dCTP (ICN, Lisle, Ill.) by nick translation (Bethesda Research Laboratories, Bethesda, Md.) to a specific activity of 1×10^8 to 2×10^8 cpm/μg. The RAV-1 U5 long terminal repeat (LTR), c-*myc* exon 3, and germ line c-*myb* probes have been described elsewhere (39).

Polymerase chain reaction. Amplification of DNA from RAV-1–c-*myb* junctions by polymerase chain reaction was performed in a thermal cycler in standard reaction conditions (Perkin Elmer-Cetus, Norwalk, Conn.). Thirty cycles between 94°C for 1 min and 72°C for 6 min were used. Primers for DNA synthesis were 32-base oligonucleotides. One, 5′-TGACGACTACGAGCACCTGCATGAAGCAGA AGG-3′, was from the 3′ end of the sense strand of RAV-1 U5 LTR. The other, 5′-CTCTTCACGGGTCCACCTGGTT TTCCCTAGGTG-3′, was from the 3′ end of the antisense strand of c-*myb* exon UE2. Specificity of amplification products was confirmed by Southern transfer and hybridization to internal c-*myb* intronic sequences and by restriction endonuclease digestion with *Pst*I to detect the recognition sequence at the 5′ end of UE2.

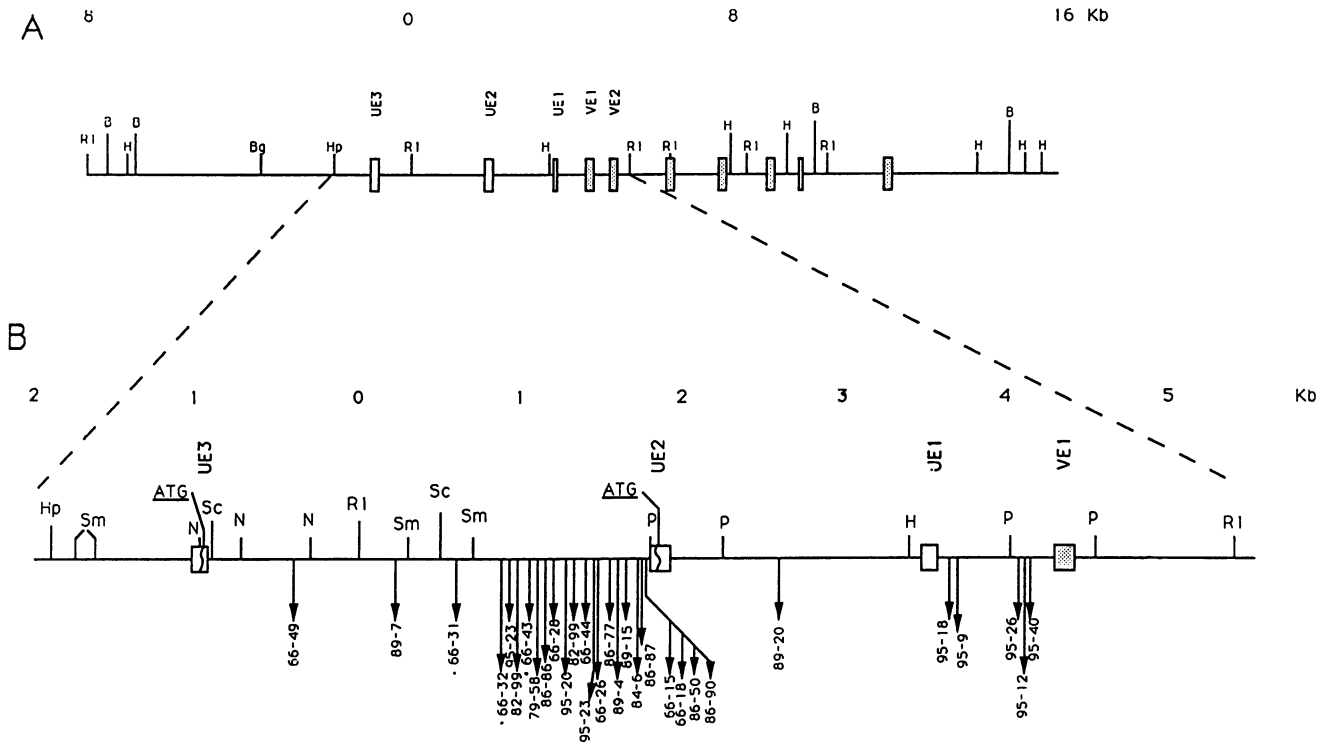

FIG. 1. Restriction endonuclease map of the chicken c-*myb* locus and locations of RAV-1 proviral insertion sites in lymphomas. (A) Most restriction sites were taken from a published map (37). Of 13 sites examined in this study, all were confirmed in SC strain chicken DNA by Southern analysis with v-*myb*-derived probes. Additional restriction sites were mapped in clone 121 provided by M. Baluda (37). v-*myb* homologous exons are indicated by shaded boxes; c-*myb* exons 5′ of v-*myb* homologous sequences are indicated by open boxes. RI, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; Bg, *Bgl*II; Hp, *Hpa*I. (B) A 7-kb region of the locus is enlarged. Methionine codons defining the amino terminus of the c-*myb* open reading frame in exon UE3 and providing a potential second translational start site in exon UE2 are indicated. Splicing from the RAV-1 splice donor to c-*myb* exon UE2 would join *gag* and *myb* out of frame. The splice acceptors of exons UE1 and VE1 are in frame with *gag*. Additional restriction endonuclease sites were taken from a study of Dvorak et al. (13) or were mapped in clone 121: Sm, *Sma*I; N, *Not*I; Sc, *Sac*I; P, *Pst*I. Proviral insertion sites are depicted as arrows below the line, with the hatch cohort and chicken numbers of the animals from which tumors were collected.

RESULTS

RAV-1 insertional mutagenesis occurs in three different locations within the c-*myb* locus. In our previous study, 11 tumors bearing RAV-1 proviral insertions within the c-*myb* locus were described. All insertions were located 5′ of UE2 (39). An additional 19 tumors containing disrupted c-*myb* loci have been analyzed. Consistent with our previous findings, 13 contained proviral insertions 5′ of UE2 (Fig. 1). In contrast, one insertion was located between UE2 and UE1, while another five were located between UE1 and VE1. These insertions were mapped by both Southern and polymerase chain reaction analysis. Five of seven tumors in hatch 95 contain RAV-1 insertions 5′ of VE1. We have no experimental data that provide an explanation for the high frequency of these distinct integrations occurring in a single experiment. As presented earlier, the bursae in all of these additional tumor-bearing birds appeared normal in size and follicular structure, including the cortical medullary border. Southern analysis revealed, however, that a major fraction of the constituent tissue was clonally derived lymphoma as defined by an altered c-*myb* locus. Histological analysis with MGP revealed that the majority of bursal lymphocytes were pyroninophilic and similar to cells within the preneoplastic lesion, the transformed follicle, observed during development of lymphomas expressing elevated levels of *myc*. In contrast to the activated-*myb* lymphoma, the transformed follicle expands locally into a primary tumor that disrupts the follicular architecture. Histological examples of a normal bursa, a transformed follicle from a posthatch RAV-1-infected chick, as well as primary c-*myb*-activated and c-*myc*-activated lymphomas are shown in Fig. 2.

In situ analysis of *myb* protein expression in tumor tissue reveals nondestructive metastatic growth within the bursa and modulation of *myb* expression in the periphery of liver nodules. On the basis of the MGP analysis of the bursal lymphocytes in activated-*myb* tumor-bearing chicks, we undertook an immunohistological analysis of bursal tissue from these chicks. Immunoperoxidase staining of tissue with a monoclonal antibody against *myb* protein provided a more sensitive technique for examination of tumor development. In this in situ analysis, the elevated expression of *myb* protein functions as a marker of the tumor. Comparison of abnormal staining in tumor tissue with the appearance of normal lymphoid tissues stained with the anti-*myb* antibody *myb* 2.7 provided a context for interpreting bursal and hepatic pathology. While in situ staining is not quantitative, the staining detected a broad range of *myb* expression in both normal and tumor tissue and provided evidence of variation in the relative abundance of *myb* product. As expected with a protein localized in the nucleus, examination at high magnification revealed that all anti-*myb* staining was nuclear. Staining of normal thymus (Fig. 3A and B)

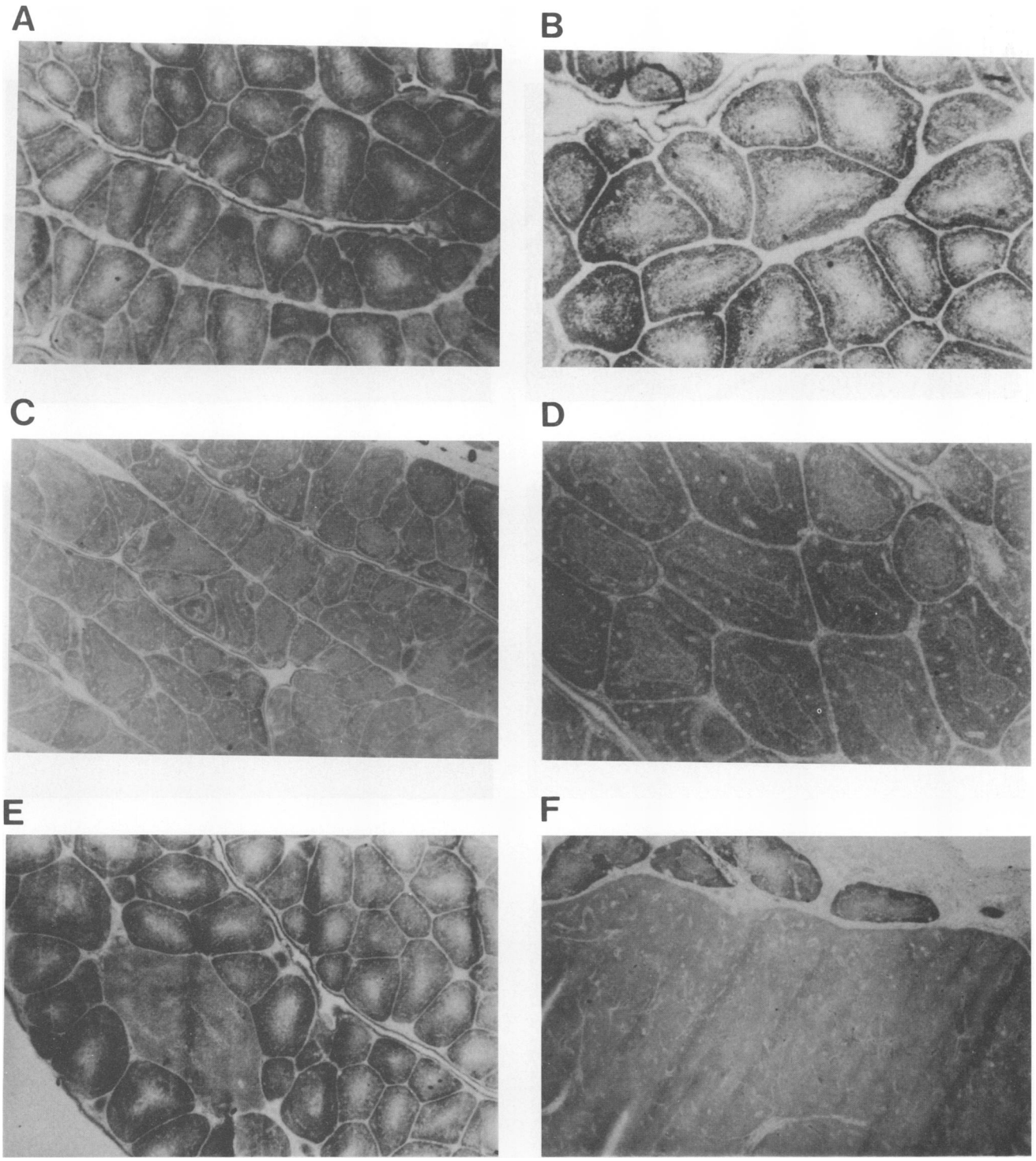

FIG. 2. Histological appearance of bursal lesions stained with MGP. (A and B) Normal bursa, ×100 and ×400 magnification. Cortices of follicles are narrow. Cells in medullae are less densely organized. (C and D) Bursa from a chicken with a primary activated-*myb* lymphoma, ×100 and ×400 magnification. The staining properties of the cells are altered. The follicular cortices are enlarged, and follicular vasculature is prominent. Medullae are compressed, and medullary cells are densely packed. All normal follicular structures remain present. The size of the bursa is normal. (E) A transformed follicle in the bursa of a 2-week-old chicken infected with RAV-1 on the day after hatching. The altered staining properties of transformed follicle cells are similar to those of lymphomatous bursal cells in panels C and D. However, follicular compartments are ablated. (F) Bursal nodule (primary tumor) from a 20-week-old chicken with lymphoid leukosis. A few normal bursal follicles remain at the nodule periphery. Cells in nodule have altered staining properties with MGP stain. Vasculature in nodule is prominent. A capsule of compressed bursal stroma surrounds the nodule. These lesions often grow to several centimeters in diameter.

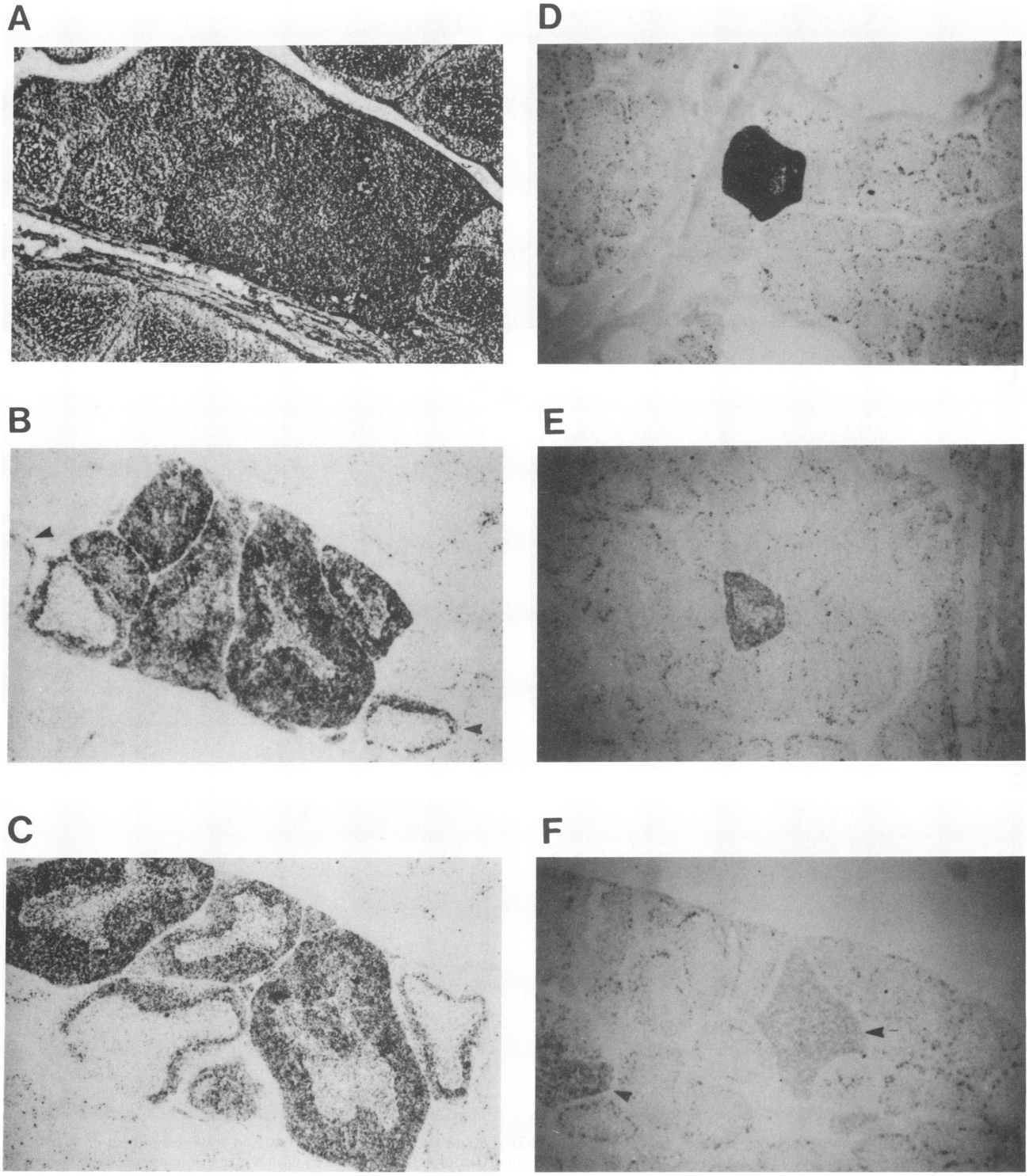

FIG. 3. Histological appearance of lymphoid organs stained by immunoperoxidase with anti-*myb* monoclonal antibody. (A and B) Normal thymus, ×100 and ×400 magnification. (C and D) Normal bursa, ×100 and ×400 magnification. Scattered cells located mostly in the cortex express high levels of *myb* antigen. (E and F) Bursa from a chicken with activated-*myb* lymphoma. Many follicles are filled with cells expressing high levels of *myb* antigen. Others contain a high fraction of intensely stained cells in cortex only, suggesting that they may have been filling with these cells at the time of sacrifice.

revealed that a large fraction of thymocytes was stained and also that the highest density of darkly stained cells was detected in the subcapsular cortex. The staining of normal bursa (Fig. 3C and D) revealed high c-*myb* expression in a small number of cells located mostly in the cortex. The relative amounts of anti-*myb* staining in normal thymus and bursa were consistent with levels of c-*myb* mRNA detected in these organs (18, 39, 45). The vast majority of normal bursal lymphocytes exhibited no detectable c-*myb* antigen. In contrast, staining of sections of bursae from activated-*myb* tumor-bearing chickens revealed elevated *myb* expression in a large fraction of cells (Fig. 3E and F). These bursae contained (i) follicles that were filled with intensely stained cells, (ii) follicles in which the cortex had expanded and filled with intensely stained cells while the medulla possessed cells expressing lower levels of *myb* antigen, and (iii) follicles apparently structurally unaltered that contained intensely stained cells only in the cortex. The appearance of these follicles suggested that they were filling with tumor at the time of sacrifice. There were also follicles that appeared degenerate, with little remaining structure and with heterogeneous lower levels of *myb* expression. Examination of the c-*myb* locus demonstrated that bursal tumor cells had a clonal origin (39; data not shown). These histology data confirm the MGP staining analysis of bursal tissue and suggest that activated *myb* lymphoma metastasizes from follicle to follicle without compromising follicular structural integrity. Furthermore, the cells are responsive to their anatomical locations, since they fill follicular but not interstitial spaces, and apparently recognize cortical and medullary microenvironments. The histology further suggests that the tumor locates first in the cortex of follicles indicating the tumor uses the same trafficking mechanisms that normal lymphocytes use to exit the bursa or that stem cells use to populate it (22, 27).

Twenty-three individual tumors from liver were stained with *myb* 2.7. Figure 4 illustrates some characteristic features of the *myb* reactivity observed. Serial sections of tumor tissue were stained with anti-*myb* and anti-chicken IgM antibodies. In contrast to anti-*myb* staining, anti-IgM stains the plasma membranes of tumor cells (data not shown). Figures 4A and B show an abundant nodular lymphoma which appears very uniform by IgM stain but stained heterogeneously for *myb*. Cells at the periphery of each tumor nodule were intensely stained, while cells at the center were lightly stained or negative for *myb*. This pattern was characteristic of most compact nodular tumors examined. The anatomical distribution of *myb* antigen in this tumor is strongly suggestive of an ongoing process of biological diversification within the tumor cell population. Figures 4C and D show a diffuse tumor mass that infiltrated throughout the hepatic parenchyma. Roughly half of the tumor cell nuclei stained intensely for *myb*. An equal number stained less intensely. Comparison of the two stains revealed the presence of uniform anti-IgM staining throughout cells expressing both high and low levels of *myb*. Figures 4E and F show a less abundant lymphoma, again with a diffuse infiltrative pattern of growth. Variable levels of *myb* antigen detected in individual cells are clearly visible. These results are typical of the 23 lymphomas analyzed by immunoperoxidase staining. The amount of *myb* antigen in both lymphoma tissue and normal lymphoid organs is consistent with previously reported levels of *myb* mRNA in these tissues; RNA expression in lymphoma cells and thymocytes is comparable and much higher than in normal bursal B cells (10, 18, 39). Heterogeneity of staining intensity for *myb* is a feature of populations of both thymocytes and lymphoma cells. It may reflect differences in proliferative capacity or in differentiation state of the cells.

Activated-*myb* lymphoma is transplantable at low frequency and does not readily proliferate in vitro. Transplantation of tumors into recipient animals can be used as an assay to study a tumor's growth properties. Suspension of tumor cells from liver, bursa, or bone marrow were prepared from six chickens with activated *myb* lymphoma (Table 1). Cell suspensions were placed into in vitro culture. No in vitro growth was observed in any culture. Additionally, between 10^6 and 10^7 cells were injected intravenously into normal or cyclophosphamide-treated recipient chickens (14, 40). After 2 weeks of incubation, recipient chickens were sacrificed, and bursae and livers were examined grossly for evidence of tumor. Of 10 tumor cell suspensions transferred into 31 recipients, only tumor 86-86 gave rise to a successful transplant. Transplant recipient 185 was transferred into 12 secondary recipients. The tumor grew successfully only in recipient 105. A tertiary in vivo transfer into eight recipients was performed in which successful transplantation occurred in seven of the recipients, including recipients that were not cyclophosphamide treated. Six of these chickens died prior to the scheduled termination of the in vivo transfer step. Tumor from the seventh chicken, recipient 117, was transferred into five recipients, all of which succumbed to the tumor within 5 days of transfer. At each transfer, tumor reisolated from recipients was examined by Southern analysis and shown to contain the same viral *myb* junction fragments observed in tumor 86-86. At each transfer, approximately 5×10^6 cells were placed in culture for in vitro proliferation. In no case was proliferation observed. These studies indicate that the activated-*myb* tumor transplants inefficiently in vivo and may not be capable of in vitro proliferation. In contrast, a similar study has demonstrated that the activated-*myc* tumor grew successfully after most transfers and that several cell lines were successfully developed (Table 2) (1). While these studies differed in some experimental details, it appeared that the *myc* tumor was biologically distinct with respect to these properties.

Elevated expression of *myb* protein is not sufficient for development of activated-*myb* lymphoma. Activated-*myb* lymphoma develops rapidly, reaching an end-stage disease 3 to 7 weeks posthatch, or 4 to 8 weeks postinfection. This schedule suggested two corollaries: (i) activation of c-*myb* is the single rate-limiting event required for lymphoma development, and (ii) growth of the initial lymphoma is metastatic and bursa independent. However, both the apparent down modulation of *myb* antigen in peripheral tumor nodules and the very low efficiency of transplantability of the lymphoma suggested that the requirements for tumor growth might be more complex. To address this issue, we examined bursal tissue from RAV-1-infected chickens not at risk for development of activated *myb* lymphoma. These were of two types: embryonically infected chickens that did not develop lymphoma and chickens infected posthatch that are not at risk for activated *myb* lymphoma. The overall incidence of activated-*myb* lymphoma in the embryonically infected chickens in this study was 14%. Of 207 chickens surveyed, 28 had lymphoma with RAV-1 insertions in the c-*myb* locus confirmed by Southern analysis. Since tumor incidence was below 25% in every cohort, it can be assumed that in an unselected population of embryonically infected chickens, at least 75% would not develop disease. Moreover, overall tumor incidences in cohorts sacrificed at 4 and 5 weeks posthatch and in those sacrificed at 6 and 7 weeks posthatch

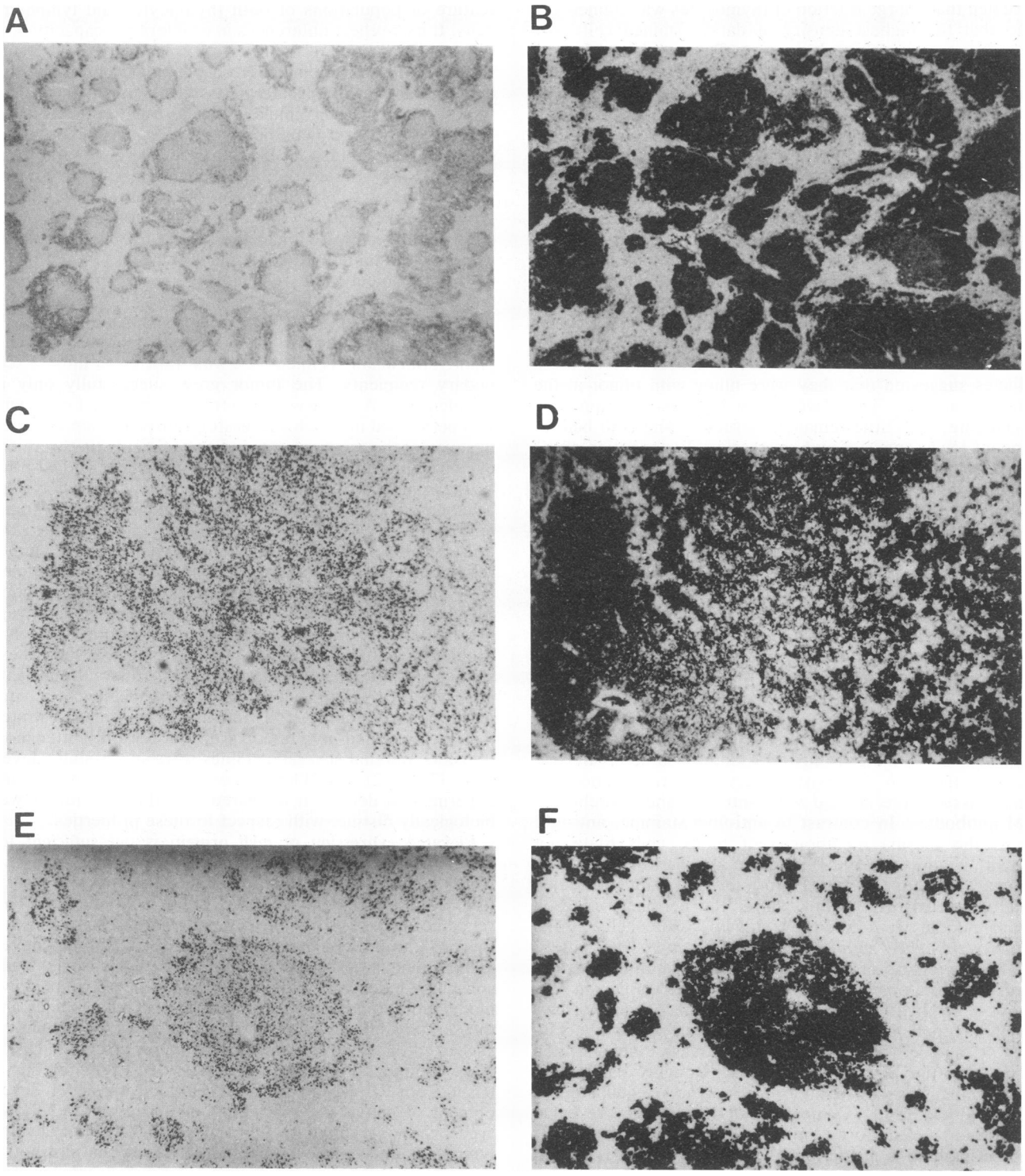

FIG. 4. In situ analysis of *myb* protein expression in metastatic lymphoma. Monoclonal antibodies *myb* 2.7 (A, C, and E) or Hy19 (anti-chicken IgM) (B, D, and F) were reacted with adjacent sections of frozen tissues. (A and B) Tumor 66-49, ×100 magnification; (C and D) tumor 89-4, ×400 magnification; (E and F) tumor 89-7, ×400 magnification.

were 11 and 10%, respectively. Apparently the tumors developed rapidly enough that incubating subclinical disease was negligible by 4 weeks. Therefore, chickens examined at 4 weeks or later could be sorted into tumor-bearing and tumor-free groups, with the tumor-free group containing very few animals that would later develop activated *myb* lymphoma. Likewise, in similar experiments, Kanter et al. report only a 10% tumor incidence for birds surviving longer

TABLE 1. Transplantation of activated-*myb* tumors

Transfer	Tumor location	No. of recipients[b]		No. of transfers/ no. of recipients (recipient no.)
		Cytoxan treated	Normal	
Primary transfer (transplant; chicken donor no.)				
66-43	Liver	0	3	0/3
82-99	Liver	2	2	0/4
86-87	Liver	2	1	0/9
86-87	Bursa	2	1	
86-87	Marrow	2	1	
86-86	Bursa	2[c]	1	1/6 (185)
86-86	Marrow	2	1	
89-7	Liver	2	2	0/5
89-7	Bursa	1	0	
89-15	Marrow	2	2	0/4
Additional transfers of tumor 86-86 (transfer no., donor no.)				
2, 185	Liver	2	2	
	Bursa	2	2	
	Marrow	2[c]	2	1/12 (105)
3, 105	Liver	2[c]	2[c]	
	Marrow	2[c]	2[c]	7/8[d] (117)
4, 117	Liver	2[c]	3[c]	5/5[e]

[a] All tumor cell suspensions that were transplanted were also put into in vitro culture. No in vitro growth occurred in any culture.
[b] Transplant recipients were necropsied 2 weeks posttransfer.
[c] Group in which successful transfer occurred.
[d] Six of these died between 8 and 14 days posttransfer.
[e] All five were found dead 5 days posttransfer.

than 48 days (23). In contrast, there was a 79% tumor incidence in those birds that died or were sacrificed in a morbid state between 27 and 48 days (between 3 and 6 weeks posthatch).

TABLE 2. Transplantation of activated-*myc* tumors

Transplant[a]		Primary transfers: no. of transfers/no. of recipients[b] (recipient no.)	Secondary transfers: no. of transfers/ no. of recipients (donor no.)	Total transfers
Donor chicken	Tumor location			
48	Bursa	0/1		
	Liver	0/1		0/2
49	Bursa	0/1		0/1
53	Bursa	2/2 (44, 48)	4/7 (44) 3/6 (48)	
	Liver	1/2	2/4[c]	12/21
58	Bursa	1/2	2/2	
	Liver	1/2	2/2[c]	6/8
63	Bursa	1/1	3/6	
	Liver	1/1		5/8
66	Bursa	1/1	1/1[c]	
	Liver	1/1		3/3
69	Bursa	0/1		
	Liver	0/1		0/2
70	Bursa	0/1		0/1

[a] All tumor cell suspensions that were transplanted were also put into in vitro culture. Those marked gave rise to in vitro B-cell lines.
[b] Transplant recipients were necropsied 8 weeks posttransfer.
[c] One of the recipients in this group gave rise to a cell line.

TABLE 3. Incidence of *myb*-expressing lesions in bursae of embryonically infected chickens without lymphoma

Determination	No.	%
Incidence of activated-*myb* lymphoma in embryonically infected chickens	28/207	14
4–6-weeks bursae analyzed from tumor-free chickens	19/19	100
Bursae containing clusters of follicles expressing *myb*	11/19	58
Bursae containing isolated follicles expressing *myb*	5/19	26
Bursae apparently free of *myb* expressing follicles	3/19	16

We analyzed 19 bursae from tumor-free, embryonically infected chickens sacrificed between 4 and 6 weeks after hatch by collection of serial frozen sections at 400-μm intervals through the entire bursa in order to sample the great majority of follicles from each bursa. Adjacent bursal sections were stained with MGP or for expression of *myb* antigen or IgM. This analysis revealed that a majority of 4- to 6-week-old bursae from tumor-free chicks contained lesions that expressed abnormal levels of *myb* (Table 3). The lesions were similar to those in bursae of chickens with activated-*myb* lymphoma. Two distinct lesions were observed. The first class consisted of follicles with high-level *myb* expression that occurred in clusters, suggesting a nascent tumor demonstrating follicle-to-follicle spread (Fig. 5A to C). The second class consisted of isolated follicles filled with cells that overexpressed *myb* antigen to various degrees (Fig. 5D to F). Only three bursae (16%) appeared not to contain any *myb*-overexpressing lesions. Eleven of nineteen bursae (58%) contained clustered *myb*-overexpressing follicles as well as numerous isolated follicle lesions. With an overall tumor incidence of 14%, we conclude that most of the lesions observed that expressed abnormally high levels of *myb* antigen would not have progressed into activated-*myb* lymphomas. It is important to note that DNA prepared from four of these bursae was analyzed by polymerase chain reaction and shown to have RAV-1 insertions within the c-*myb* locus (data not shown). It is apparent that a majority of the *myb* activation events did not result in lesions capable of clonal expansion and the development of tumors of clinically significant size.

A second and independent line of evidence indicated activation of *myb* expression was insufficient for tumor development. As suggested by the requirement for embryonic infection, activation of the expression of *myb* and the subsequent development of a B-cell lymphoma appear to require infection of a specific target cell. It was possible, therefore, that activation of *myb* expression in an inappropriate cell might occur but not progress to lymphoma. To address this issue, four bursae from chickens infected with RAV-1 on the day after hatch and sacrificed 2 weeks after infection were serially sectioned and analyzed for expression of *myb*. All four bursae contained follicles that overexpressed *myb*. These follicles were visually indistinguishable from follicles found in embryonically infected bursae. The follicles resembled those shown in Fig. 5E and F. No follicle cluster lesions were found. Most of the *myb*-expressing lesions in these bursae had lower levels of *myb* antigen than did average lesions in embryonically infected bursae.

A primary bursal tumor containing both disrupted c-*myb*

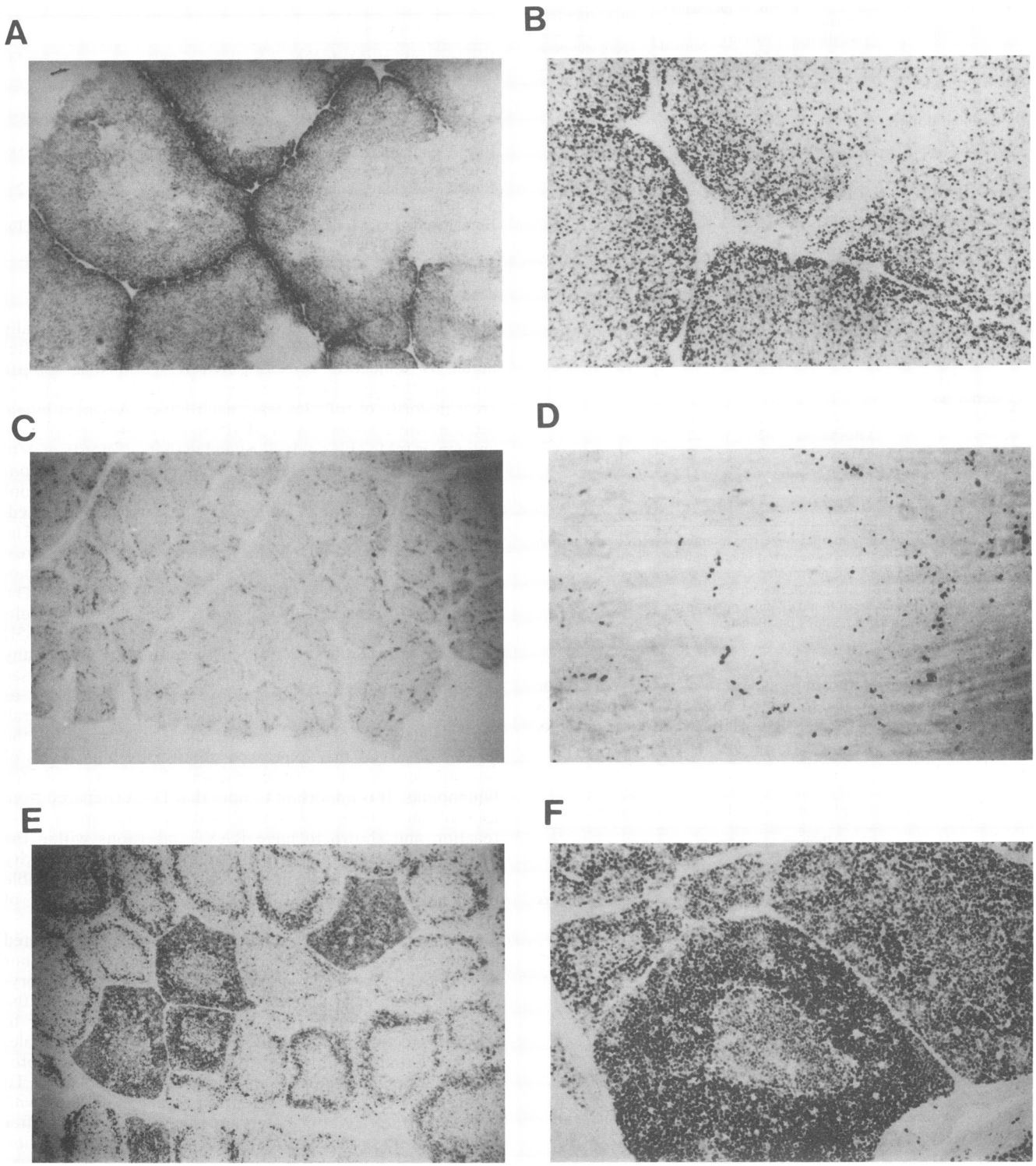

FIG. 5. Histological appearance of *myb*-overexpressing lesions in bursae of embryonically infected chickens that did not develop lymphoma. (A) Anti-IgM stain; (B to F) anti-*myb* stain. (A and B) Serial sections of the same lesion. Clustered follicles in panel A have aberrantly high levels of IgM stain in cortex, similar to the previously described staining of lymphomatous bursae (23, 39). Corresponding follicles in panel B are filled with *myb*-overexpressing cells. (C) Lesion similar to the one in panel B. (D to F) *myb*-overexpressing follicles representative of the variety observed. Staining intensity of *myb* antigen, intactness of follicular architecture, and cellularity varied among lesions.

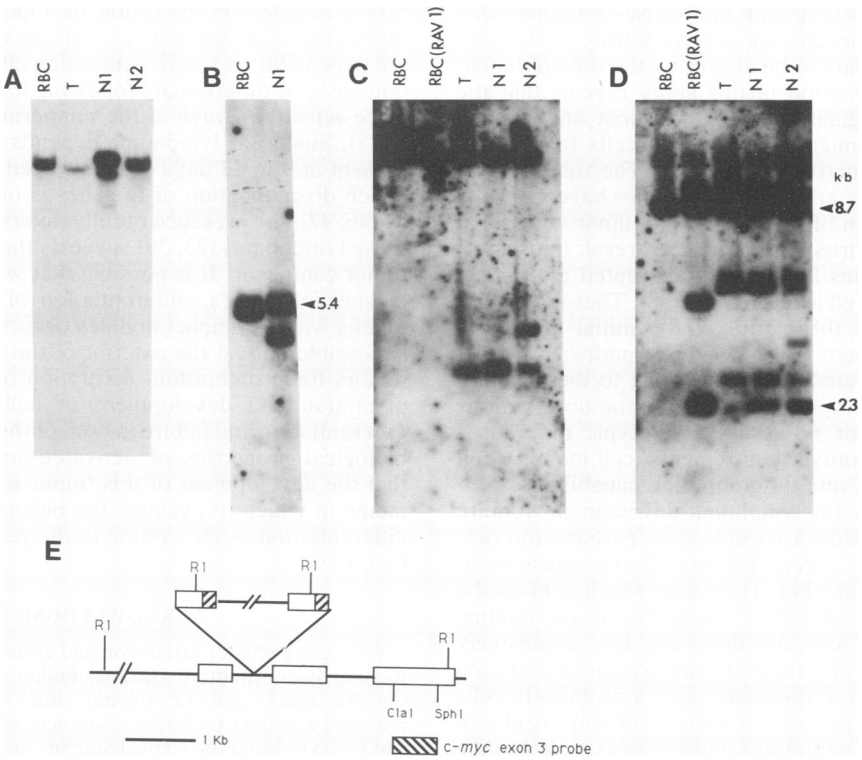

FIG. 6. Analysis of proviral integrations in tumor DNA samples from chicken 70-63. Lanes in panels A to D: RBC, uninfected erythrocyte DNA; RBC(RAV-1), erythrocyte DNA from a RAV-1-infected chicken; T, tumor DNA from a lymphoma in the liver of chicken 70-63;, N1, DNA from bursal nodule 1; N2, DNA from bursal nodule 2. (A) Samples were digested with *Bam*HI and hybridized to the 5.4-kb *Eco*RI fragment of the germ line c-*myb* locus containing exons UE2 through VE2. (B) Samples were digested with *Eco*RI and hybridized to the same probe. The germ line band is 5.4 kb. The shorter than germ line bands detected in DNA from N1 in panels A and B derive from an insertionally mutated allele as previously described (39). (C) Samples digested with *Eco*RI and hybridized to the c-*myc* exon 3 probe shown in panel E. (D) Samples parallel to those in panel C, hybridized to a RAV-1 U5 LTR probe. The 2.3-kb RAV-1 internal fragment and the 8.7-kb fragment from the *ev-1* locus (2) are indicated. (E) Schematic representation of altered c-*myc* *Eco*RI fragments generated as a result of RAV-1 proviral insertion into intron I.

and c-*myc* loci behaves like a tumor expressing elevated levels of *myc*. Chicken 70-63 was infected with RAV-1 on the day after hatch and sacrificed 18 weeks later. The bursa contained two distinct encapsulated bursal nodules, each of approximately 1 cm in diameter, as well as abundant lymphoma in the liver. Southern analysis of liver tumor DNA revealed a proviral insertion in c-*myc* and unaltered c-*myb* alleles consistent with typical lymphoid leukosis. Surprisingly, when the bursa was sectioned and stained with anti-*myb* antibody, one of the nodules (N1) was found to express a low level of *myb* antigen, while the other (N2) was negative. DNA was extracted from the remnants of each nodule. Digestion with *Bam*HI and hybridization to the 5.4-kb *Eco*RI germ line c-*myb* fragment containing c-*myb* exons UE2 through VE2 as previously described (39) confirmed that the abdominal lymphoma in chicken 70-63 had unaltered c-*myb* alleles, as did the bursal nodule that did not express *myb* antigen, N2 (Fig. 6A). In contrast, N1 expressed *myb* antigen and possessed a disrupted c-*myb* allele. Digestion of this DNA with *Eco*RI (Fig. 6B) located the disruption in the vicinity of the insertion in the activated *myb* lymphoma 66-32 DNA (Fig. 1). The three 70-63 tumor DNA samples were digested with *Eco*RI and analyzed in duplicate with a c-*myc* exon 3 probe (Fig. 6C) or with the *Eco*RI-*Sac*I RAV-1 proviral fragment containing U5 LTR sequences (Fig. 6D) (39). DNA from all three tumor samples contained *Eco*RI fragments of about 2.7 kb that hybridized with both *myc* and LTR probes. N2 DNA contained an additional 3.5-kb *Eco*RI fragment that also hybridized with both probes. DNA from all three tumor samples contained two fragments of about 4.8 and 5.2 kb that hybridized with the RAV-1 LTR probe. Presumably, in the sample from N1, the altered c-*myb* fragment comigrated with one of these. These results do not suggest a simple model to explain tumor development in chicken 70-63. The presence of 2.7-, 4.8-, and 5.2-kb junction fragments in all three samples suggests a common origin, yet each sample has unique features. Despite this uncertainty, it is possible to conclude that a disruption of both c-*myb* and c-*myc* occurred in cells that gave rise to N1 but that the growth phenotype of N1 was consistent with c-*myc* activation. Consequently, the disruption of c-*myb* did not result in rapidly disseminating lymphoma and thus is similar to results for the nonprogressing *myb*-positive lesions observed in RAV-1-infected chickens.

DISCUSSION

The development of a rapidly disseminating B-cell lymphoma characterized by elevated expression of *myb* has been examined in 207 chickens. The biological properties of this lymphoma distinguish it from two other avian B-cell lymphomas as described elsewhere (21). Relevant to this discussion, three unique features of this tumor will be considered. (i) The target cell in which ALV proviral inte-

gration activates *myb* expression and causes fulminant disease is present in the developing embryo during a relatively brief interval and appears to be absent by the time of hatch. (ii) Histological examination in this study reveals that the tumor appears to originate within the bursa and expand within that organ by migration of tumor cells to adjacent follicles in a nondestructive fashion. (iii) The tumor transplants at low efficiency, and in vitro cell lines have yet to be isolated. Superinfection of primary bursal tumor with avian reticuloendotheliosis virus, however, does result in proliferation of clonal cell lines that contain disrupted c-*myb* loci and that express elevated levels of *myb* (38). These and other properties, as well as the nature of the initial oncogenic event, indicate that there exists a genetic pathway distinct from *myc*- or *rel*-activated changes leading to the development of avian B-cell lymphoma. Experimental observations indicate that the tumor possesses phenotypic differences with respect to proliferative requirements, cell mobility-cell recognition specificity, and differentiation capabilities.

The events required for lymphoma development require expression of *myb* within a specific cell, perhaps the prebursal stem cell. Three different forms of *myb* appear sufficient for tumor initiation (Fig. 1), two of which contain the full N-terminal region of $p75^{c\text{-}myb}$ responsible for binding DNA (9, 16, 19, 26, 48). All three are less extensively truncated than is typically found in tumorigenically altered *myb* alleles (24, 25, 44). Expression of *myb* proteins in cells with insertional activation of the locus probably results in several transcriptional alterations and provides a pleiotropic effect (35). Murine B-cell ontogeny is characterized by high levels of c-*myb* expression early and then down regulation to low levels during differentiation (5, 6). Presumably, c-*myb* plays a different role in early and later B cells. The nonprogression of *myb* lesions in chickens infected on the day of hatch (Fig. 5), as well as the finding of a tumor that contained an activated *myb* allele that exhibited activated-*myc* tumor properties (Fig. 6), indicates that only specific cells within the B lineage are able to respond to the *myb* product to produce neoplasia. Indeed, the isolated follicles with high-level *myb* expression (Fig. 5D to F) may parallel the transformed follicle, containing cells that lack the capacity to expand out of the follicle. It is possible that more extensive truncation of *myb* renders it tumorigenic in a wider spectrum of cell types. Regulatory factors present in most cells but not in very early B cells may inactivate normal c-*myb*.

It is curious that only 14% of the infected birds developed tumors in our study whereas 85% developed tumors in a similar study (23). Since both studies were conducted with SC chickens, it is suggested that viral proteins or genetic elements influence tumor progression. Results from our study suggest that insertional activation is frequent, but the tumorigenesis process is terminated in more than 80% of those birds at risk.

A partial explanation for the lack of tumor progression observed in this study is suggested by the heterogeneity of *myb* expression in metastatic disease. It is of interest that *myb* expression is apparently down regulated in some tumor cells, suggesting that sequences other than those in the LTR are able to regulate expression of the disrupted *myb* locus. Tumor tissue in liver was either diffuse or compact. In the former, both positive and negative cells were present. Consistent with variable expression of c-*myb* in the cell cycle (45), these cells were randomly distributed throughout the tumor. In contrast, compact tumor tissue showed *myb* expression localized at the periphery of the nodules, suggesting a nonrandom biological change within the center of tumor nodules. Proliferation of some tumors that express *myb* is factor dependent (17, 34). As well, some tumors that are dependent on v-*myb* expression either differentiate spontaneously or differentiate following inactivation of a temperature-sensitive v-*myb* at the nonpermissive temperature (7, 8, 28). Since this lymphoma depends on a target cell that is present at 9 to 13 days of embryogenesis (23, 39), a time at which diversification of Ig genes is only just beginning (42, 43, 46, 47), the presence of fully diversified light-chain loci in these lymphomas (23, 39) suggests that differentiation of the tumor can occur. It is possible that with low concentrations of specific factors, differentiation of the activated B-lymphoma within compact nodules occurs and that proliferation is possible only at the exterior of the mass. If dependent on factors for proliferation, disruption of the tumor for transplantation and development of cell lines may result in differentiation and failure in both of these assays. The unique biological properties of activated-*myb* lymphoma suggest that the development of this tumor may provide a valuable model in which to examine the balance of proliferation and differentiation as they relate to the gene expression induced by c-*myb*.

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