RAV-1 Insertional Mutagenesis: Disruption of the *c-myb* Locus and Development of Avian B-Cell Lymphomas

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Infection of young chickens with RAV-1, a subgroup A isolate of avian leukosis virus, results in the development of lymphoid leukosis, a B-cell lymphoma characterized by provirus insertion into the *c-myc* locus. We report here that when 12- to 13-day-old embryos rather than 1-day-old chickens were infected with RAV-1, a novel B-cell lymphoma developed in which proviral insertions had activated expression of the *c-myb* gene. These tumors expressed elevated levels of a 4.5-kilobase *myb*-containing mRNA transcript that contained *c-myb* sequences not found in *v-myb*. The *c-myc* locus in these tumors appeared normal. The biological properties of the activated *myb* lymphoma were distinct from those of lymphoid leukosis. Metastatic disease developed within 7 weeks of infection. Distinct intermediate pathogenic stages with preneoplastic and primary neoplastic lesions were not detected. Although bursal tissues appeared to be nonmalignant on gross examination, Southern analyses of bursal DNA revealed the presence of tumor with the same clonal origin as abdominal lymphoma masses. The dependence on embryonic infection for development of activated *myb* lymphoma suggests that the target cells in which *c-myb* is activated are found only in embryos and are distinct from those cells that give rise to lymphoid leukosis.

The avian leukosis viruses (ALVs) are so named because they induce a well-studied lymphoma in chickens called lymphoid leukosis. Lymphoid leukosis is a tumor of immunoglobulin M (IgM)-expressing B cells that arises in the bursa of Fabricius, the primary organ of B-cell maturation in avian species, after a latency of 15 weeks or longer (9, 33). In 70 to 90% of the tumors, the cells are clonally marked by provirus integration into the *c-myc* locus (17, 25, 26, 30). The deregulation of *myc* expression which results from this insertion is a critical event in the development of the tumor. Integration of a provirus into the *c-myc* locus is believed to be the first of several stochastic events required for the eventual emergence of the lymphoma. Preneoplastic bursal follicles appear during the viremic period and probably contain the progeny of individual cells activated by proviral insertion within *c-myc* (3, 27). There is some evidence that the target cell in which the *c-myc* locus is initially disrupted is one of the small subset of bursal lymphocytes which normally function as bursal stem cells (42). The development of this tumor has been studied in our laboratory with RAV-1, a subgroup A isolate of ALV (3).

There are many infectious agents that cause different diseases when the infection protocol is varied. Among the avian retroviruses, RAV-1 infection of $15I_5 \times 7_1$ chickens induces lymphoid leukosis with an incidence of 70 to 100%, while RAV-1 infection of 15_1 chickens induces erythroblastosis in 70 to 80% (14). In studies designed to define the development of lymphoid leukosis, it was observed that chemical bursectomy of chickens prior to infection with RAV-1 shifted the incidence of disease observed from primarily lymphoid leukosis to exclusively nonlymphoid neoplasms, including osteopetrosis, erythroblastosis, and nephroblastomas (33). The MAV-2(0) strain of ALV produces an accelerated development of osteopetrosis following infection of chicken embryos, while infection of 10-day-old chickens produces aplastic anemia without osteopetrosis (10). In our

laboratory, we have demonstrated that reticuloendotheliosis virus infection induces two very different tumors depending on the companion helper virus that replicates the defective transforming viral genome (4). It appears that such differences occur in part because target cells for the initiation of disease are accessible to virus in one protocol but may be less abundant, less proliferative, or shielded from virus infection by host defenses in another protocol.

Our interest in the target cell for insertional activation of *c-myc* by RAV-1 led us to inoculate 12- to 13-day-old chicken embryos rather than newly hatched chickens, anticipating that more abundant bursal stem cells would provide an increase in the incidence of preneoplastic lesions (42). We report here that chickens which were infected with RAV-1 as embryos developed a novel B-cell lymphoma distinct from lymphoid leukosis. In these tumors, proviral integrations activated expression from the *c-myb* locus rather than *c-myc*. Activated *myb* lymphomas expressed IgM and were phenotypically similar to lymphoid leukosis in other respects, but the biological features of their development differed significantly. The most striking difference was that tumor development was not delayed; metastatic disease was evident within 4 to 6 weeks and probably earlier. Since activated *myb* lymphomas only develop in birds infected as embryos, our results suggest that the target cell for this disease is a very early cell in the B-lymphoid lineage. It would appear that this target cell is distinct from that which gives rise to activated *myc* lymphomas. A recent report by Kanter et al. describes a very similar lymphoma that developed following infection with a different avian retrovirus but also required embryonic infection for tumor induction (20).

MATERIALS AND METHODS

Virus stocks and cell lines. SC chicken embryo fibroblasts were cultured in plastic dishes (Nunc, Roskilde, Denmark) in Dulbecco modified Eagle medium (Flow Laboratories, Inc., McLean, Va.) containing 10% tryptose phosphate broth and 10% calf serum (Hazelton, Lenexa, Kan.) with

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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 alternate low multiplicity of infection (0.01) and cloning by endpoint dilution. BM2, a nonproducer avian myeloblastosis virus (AMV)-transformed myeloblast cell line (24), was provided by C. Moscovici (Veterans Administration Hospital, Gainesville, Fla.). LS CC-DT40 was isolated in this laboratory following several in vivo transfers of a B-cell lymphoma obtained from RAV-1-induced lymphoid leukosis in an SC chicken (1).

Chickens and virus infections. Embryonated SC eggs (Hy-line International Hatcheries, West Des Moines, Iowa) were incubated with humidity at 39°C.

Infection of 12- to 13-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 were assayed for viremia 1 week after inoculation. Nonviremic animals were excluded from analysis.

Histology. All histological analysis was performed on frozen tissue sections (8 μ m) fixed in acetone. Methyl green pyronin (Sigma Chemical Co., St. Louis, Mo.) was used according to the manufacturer's instructions to stain sections that were also fixed in Formalin and ether-ethanol (1:1) and cleared in xylene. Immunohistology was performed by incubation of tissue sections with monoclonal antibodies and peroxidase-antiperoxidase reagents (Dakopatts, Glostrup, Denmark) as described before (4). Monoclonal antibodies against four antigens found on chicken lymphoid cells have been developed in this laboratory (Huffnagle and Humphries, unpublished data). CLA-1 (common leukocyte antigen 1) is found on avian B cells in a 220-kilodalton (kDa) form and on avian T-cells in a 180- to 200-kDa form. It appears analogous to the murine T₂₀₀/B₂₂₀ antigen (18, 32). Bu-2 is a newly identified molecule with expression distribution similar to that of Bu-1 (Huffnagle, Ratcliffe, and Humphries, manuscript in preparation). It is found on B cells and at least some cells within the monocyte lineage. Cla is chicken Ia, a chicken major histocompatibility complex class II molecule. CT-1 is found on all chicken thymic T cells but not on B cells (7). The monoclonal antibody against chicken IgM has been described previously (4). The monoclonal antibody against p19^{gag} of ALV was obtained from D. Boettiger (University of Pennsylvania, Philadelphia).

Analysis of DNA and RNA. Cellular DNA was isolated from cells as described previously (2). Restriction enzymes (Boehringer Mannheim, 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 described previously (2).

Tumor RNA was isolated from frozen liver fragments which were pulverized over dry ice and dispersed into 4 M guanidine thiocyanate (Fluka, Buchs, Switzerland)-0.1 M Tris (pH 7.4)-0.2 M β -mercaptoethanol. RNA was pelleted through CsCl in 1 $\times$ SSC (0.15 M NaCl, 0.015 M sodium citrate). RNA electrophoresis, Northern transfer, and hybridization were done as described before (23).

Nucleic acid probes. Isolated DNA fragments and plasmids were labeled with [α -³²P]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/ μ g.

FIG. 1. Restriction endonuclease maps of RAV-1 and *v-myb*-derived nucleic acid probes. (A) 5' LTR of an RAV-1 provirus. Fragments used as probes are indicated. The position of the *BstEII* cleavage site is from Goodenow et al. (16). Restriction endonuclease cleavage sites are as indicated: R1, *EcoRI*; Bs, *BstEII*; Sc, *SacI*. (B) The *BamHI*-*HindIII* fragment of the AMV provirus containing the *v-myb* gene is shown with exon boundaries as described before (21). Restriction endonuclease cleavage sites are as indicated: B, *BamHI*; K, *KpnI*; R1, *EcoRI*; S, *Sall*; X, *XbaI*; H, *HindIII*. Fragments used as probes are indicated.

Procedures used to construct the plasmids listed below were described before (23).

RAV-1 probes were subcloned from pRAV-1, provided by H. E. Varmus (Fig. 1A). pUSLTR was constructed by isolation of the *EcoRI*-*SacI* fragment from pRAV-1 and directional cloning into the multiple cloning site of Bluescribe M13⁺ (Stratagene, La Jolla, Calif.). The leader probe was isolated from an acrylamide gel after double digestion of pUSLTR with *BstEII* and *SacI*.

v-myb-specific probes were derived from pVM2, which contains the 3' portion of the AMV provirus, provided by J. M. Bishop (Fig. 1B) (21). The 400-base-pair (bp) *EcoRI*-*Sall* fragment of *v-myb* was isolated and directionally cloned into the multiple cloning site of Bluescribe M13⁺. The *KpnI*-*XbaI* fragment containing *v-myb* was isolated directly from pVM2 for use as a probe. Genomic DNA from the chicken *c-myb* locus was subcloned from clone λ -121 (see Fig. 3B), provided by M. Baluda (31). The 7.5- and 5.4-kb *EcoRI* fragments from the 5' end of the *c-myb* locus were isolated and cloned into the *EcoRI* site in Bluescribe M13⁺ to construct *pmyb7.5*⁺ and *pmyb5.4*⁺, respectively. *pmyb5.4*⁺ was digested with *PstI*; fragment *b* was isolated and ligated into the *PstI* site in Bluescribe M13⁺ to construct *pmyb-b*. *pmyb7.5*⁺ was digested with *SmaI* and religated to construct *pmyb-SmR*.

The λ probe used to detect avian immunoglobulin light-chain rearrangements contains a 1.2-kb *EcoRI*-*Sall* fragment which encodes the C λ region exon (35). It was provided by J. C. Weill.

The *c-myc* exon 3 probe used as a 685-bp *ClaI*-*SphI*

fragment from the chicken *c-myc* locus isolated from pcmc *Bam*HI (purchased from American Type Culture Collection). The *Sph*II site was converted to an *Xba*I site in an intermediate subcloning step (E. Filardo, unpublished data), and then the *Cla*I-*Xba*I fragment was directionally cloned into the *Acc*I and *Xba*I sites in Bluescribe M13⁺.

RESULTS

Novel B-cell lymphoma results from infection of chicken embryos with RAV-1. RAV-1, a standard laboratory strain of ALV, was used to infect chicken embryos at 12 to 13 days of incubation. These chickens were sacrificed at 4 to 7 weeks posthatch. Sixteen of 53 birds had obvious or abundant tumor masses in the liver. Bursae from tumor-bearing chickens appeared healthy on gross inspection, with no visible neoplastic involvement. In contrast, readily identifiable bursal primary tumors are typical of lymphoid leukosis, and metastatic disease is only rarely observed prior to 15 to 16 weeks postinfection.

Tumors from liver were analyzed histologically. Immunoperoxidase staining was performed with several monoclonal antibodies against cell surface antigens of the chicken hematopoietic system. The tumors were found to express IgM, Bu2, CLA-1, and ClA but not the T-cell antigen CT-1. This antigenic profile was found on both bursal B cells and ALV-induced long-latency B-cell lymphomas (data not shown). Within these tumors, methyl green pyronin staining revealed an elevated RNA content, often found in blasting lymphocytes (9). There was no obvious phenotypic variation among the tumors.

During their residence in the bursa, avian B lymphocytes undergo a process of somatic diversification at the λ light-chain locus in order to acquire the ability to develop a mature humoral immune response. As further confirmation of the B-cell nature of these liver tumors, we examined the configuration of the λ locus by using *Sca*I endonuclease digestion and Southern analysis. Under these conditions, hybridization with a C λ probe detects germ line, rearranged, and diversified loci (35, 42, 43). The result of this analysis (Fig. 2) indicates that one allele of the light-chain locus in each of the tumors was rearranged and somatically diversified at the *Sca*I site. The expression of IgM in cells containing rearranged and diversified light-chain alleles indicates that these tumors have a mature B-cell phenotype.

The presence of disseminated tumor in the livers of RAV-1-infected chickens at 5 to 6 weeks after infection was novel. However, in a recent study, infection of chicken embryos with molecular recombinants constructed in vitro between UR2AV and ring-necked pheasant virus induced the rapid development of similar tumors (20). Therefore, to ensure that the tumors that we observed developed following infection with RAV-1, the virus used in this study was characterized by several methods (data not shown). Proviral structures derived from virus prior to inoculation and from virus reisolated from tumor-bearing chickens were mapped by restriction endonuclease digestion and Southern analysis and shown to be indistinguishable from RAV-1 at 11 restriction sites. The locations of 15 restriction endonuclease sites in RAV-1 (44) were confirmed in virus reisolated from tumor-bearing chickens. Pre- and postinfection virus were scored as subgroup A by the standard viral interference assay (11). Furthermore, virus from tumor-bearing chickens did not demonstrate focus formation in the MC29 focus assay on quail embryo fibroblasts. Finally, hybridization with *v-myb* and *c-myc* probes following Southern analysis

FIG. 2. Somatic diversification of the λ light-chain immunoglobulin locus in B-cell lymphomas. (A) Tumor DNAs, 66-15 through 66-43, were doubly digested with *Sca*I and *Sal*I, analyzed by Southern transfer, and hybridized to a C region λ locus probe. Erythrocyte DNA (RBC) and bursal DNA from a 2-week-old chicken served as controls and provided marker DNA fragments for each form of the C λ locus. Bands derived from germ line (G), methylated germline (G*), and rearranged, undiversified (R) and somatically mutated (M) alleles are indicated. (B) Restriction endonuclease map of the C λ locus showing *Sca*I (Sc) and *Sal*I (Sa) sites detected in the analysis of germ line (G), rearranged (R), and somatically mutated (M) λ light-chain loci. Somatic mutation within the V region eliminates the *Sca*I site and is represented as a hatched box. —*, The location of the 1.2-kb *Eco*RI-*Sal*I λ C probe.

performed on tumor DNAs and on proviral structures from infectious virus reisolated from lymphomatous chickens enabled us to exclude the possibility that *c-myc* or *c-myb* had been transduced during infection and tumor development. These data, together with the functional criterion that post-hatch inoculation with this virus resulted in typical lymphoid leukosis (see below), allowed us to conclude that the virus used in these experiments was RAV-1.

FIG. 3. Restriction map of the *c-myb* locus and the location of specific hybridization probes. (A) Most restriction sites were taken from a published map (31). 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 λ 121, provided by M. Baluda (31). *v-myb*-homologous exons are indicated by open boxes. *c-myb* exons 5' of *v-myb*-homologous sequences are indicated by shaded boxes. The locations of these exons are derived from our unpublished data and those of Dvorak et al. (13). While it has been possible to determine the positions of these two exons, their exact sizes are approximate. Restriction endonuclease abbreviations are as follows: B, *Bam*HI; H, *Hind*III; R, *Eco*RI; Sm, *Sma*I; S, *Sal*I. (B) The 5' end of the *c-myb* locus was mapped in more detail by using published data (13) and mapping the λ 121 clone. Additional restriction endonuclease abbreviations not used in panel A are as follows: Bgl, *Bgl*II; Hpl, *Hpa*I; N, *Not*I; P, *Pst*I; Sc, *Sac*I; A, *Ava*I. Three additional *Pst*I sites between -3 and -0.7 kb have not been precisely located. Exons are indicated as in panel A. Locations of RAV-1 insertion sites in lymphomas are indicated below the line. The asterisks indicate individuals which demonstrated the same *c-myb* integration event in both tumor and bursal DNA. Three proviruses which have not been precisely mapped are indicated as having probable integration sites within the region bounded by the brackets. (C) Restriction fragments were subcloned from λ 121 to construct plasmids *pmyb*5.4⁺, *pmyb*-b, and *pmyb*-SmR and used as probes in the studies presented here. Designation of fragment *b* was as published before (13).

Identification of proviral integrations in the 5' end of the *c-myb* locus. In the initial analyses of these tumors, DNA was isolated from tumor masses within the livers of nine chickens infected as embryos, and the configuration of the *c-myc* locus was examined by Southern analysis. With the exception of tumor 66-44 (see below), all the *c-myc* loci appeared normal, suggesting that these B-cell lymphomas had developed in the absence of RAV-1 insertion within this locus.

We then examined the *c-myb* loci within these tumors. The 5' end of the chicken *c-myb* locus, including the six 5'-most exons transduced by AMV, was contained within a *Bam*HI fragment of approximately 17 kb (Fig. 3A). Integration of an RAV-1 provirus within the vicinity of the *c-myb* sequences contained in this fragment would insert additional *Bam*HI sites into this locus. We analyzed tumor DNAs by *Bam*HI digestion, Southern transfer, and hybridization to the *Eco*RI-*Sal*I fragment from AMV, which contains *v-myb* sequences. All nine tumors possessed the 17-kb germ line fragment and an additional smaller *c-myb*-containing *Bam*HI fragment, indicating the presence of a new *Bam*HI site within one allele of the *c-myb* locus (data not shown).

Several presumptive integration sites were mapped more precisely by digestion of tumor DNA with *Eco*RI and hybridization to the cloned 5.4-kb *Eco*RI fragment from the *c-myb* locus designated *pmyb*5.4⁺ (Fig. 3C). *Eco*RI cuts the RAV-1 provirus within the long terminal repeats (LTRs), so that the sum of the sizes of the 5' and 3' junction fragments resulting from a proviral insertion, assuming no deletions within either LTR, will equal the size of the germ line *Eco*RI

fragment in which the insertion occurred plus the additional 350 bp present in one LTR. *Eco*RI digestion, Southern transfer, and hybridization of the *pmyb*5.4⁺ probe to DNA isolated from seven liver tumors identified two potential *myb*-containing junction fragments as well as the uninterrupted *c-myb* allele (Fig. 4A and data not shown). For four of these tumors, determination of the sizes of the tumor-specific *myb* fragments was accurate enough to estimate that the sum of their sizes was between 5.6 and 5.8 kb. The larger fragment of each pair also hybridized to the *Kpn*I-*Xba*I *v-myb* probe, demonstrating that it contained the 3' portion of the 5.4-kb fragment (data not shown). Figure 4B shows a parallel analysis of the tumor DNAs hybridized with the U5 LTR probe from RAV-1. In all cases, this probe hybridized to the 3' junction fragments (arrows), indicating both that they are junction fragments and that all of the proviruses are in the same transcriptional orientation as *c-myb* (Fig. 4D). The additional bands seen in Fig. 4B represent the 2.3-kb RAV-1 internal and presumed junction fragments derived from additional RAV-1 integrations in tumor tissue.

DNA was obtained from whole bursal tissue of chickens bearing liver tumors 66-28, 66-31, and 66-49. Figure 4C shows the results of *Eco*RI digestion of bursal DNAs 66-28 and 66-31 and hybridization with the *pmyb*5.4⁺ probe. They demonstrate that a high proportion of bursal cells contained an RAV-1 insertion within the *c-myb* locus, producing the same junction fragments that were seen in the liver tumors from these birds. This analysis indicates that the majority of the cells within the bursa had the same clonal origin as the

FIG. 4. Location of RAV-1 proviral insertions within the *c-myc* locus. (A) DNAs from erythrocytes (RBC) and from five tumors digested with *Eco*RI were hybridized with the germ line 5.4-kb *Eco*RI fragment from the *c-myc* locus (Fig. 2C). Control DNA and all tumors contain an unrearranged 5.4-kb fragment. The tumors each contained two additional smaller fragments. The larger of these two fragments hybridized with a *v-myc* probe, and this indicates that they are 3' junction fragments. The smallest fragment from tumor 66-31 is not visible in this figure but was detected in the original film. (B) Digests identical to those used for the Southern analysis in panel A were hybridized with the U5 LTR probe from RAV-1 shown in Fig. 1A. Arrowheads indicate fragments that colocalized with 3' junction fragments in panel A. The 2.3-kb *Eco*RI internal fragment of RAV-1 and the 17.5- and 8.7-kb *Eco*RI fragments of EV-1 (an endogenous provirus) are indicated. All other bands are derived from additional proviral integrations in the tumors. (C) Southern analysis of bursal DNA from chickens bearing tumors 66-28 and 66-31. Digestion and hybridization conditions were the same as in panel A. (D) Schematic representation of an RAV-1 proviral insertion within the *c-myc* locus. *Eco*RI (RI) fragments detected in this analysis are shown as striped bars. They are the 5' junction fragment (5' JF), the 2.3-kb RAV-1 internal fragment (2.3 Kb i), and the 3' junction fragment (3' JF). Regions of each fragment that hybridized to the different probes are depicted below. VEI and VEII are the two 5'-most *c-myc* exons transduced by AMV. Two other upstream *c-myc* exons (Fig. 3B) are not shown.

liver tumor. Analysis of bursa 66-49 and tumor 66-49 produced an equivalent result after double digestion with *Eco*RI and *Sma*I and hybridization to the *pmc*-SmR probe indicated in Fig. 3C (data not shown).

Southern analysis with the *pmc*5.4⁺ and *pmc*-SmR probes mapped the proviral insertions shown in Fig. 3B. Three tumors, 66-15, 66-18, and 66-26, carried apparent insertions within the 17-kb *Bam*HI fragment. While these insertions were detected as altered *c-myc*-specific fragments following digestion with *Bam*HI, altered fragments were not observed following digestion with *Eco*RI. We interpret this result to indicate that these insertions are very close to the *Eco*RI site at the 5' end of the 5.4-kb fragment in the *c-myc* locus. Precise mapping of these insertions with other enzymes proved difficult, and although possible junction frag-

ments were identified in one of these three tumors, we were unable to locate these insertions precisely. Ultimately, a shortage of tumor DNA required us to indicate the probable integration sites in these cases by brackets as shown in Fig. 3B.

Expression of *myb* RNA altered in these B-cell lymphomas. We performed Northern (RNA blot) analysis on RNA from 10 B-cell lymphomas. Hybridization with a *v-myc*-specific probe revealed a major class of tumor-associated *myb* transcripts of similar size, approximately 4.5 kb, that were larger than the 4.0-kb *c-myc* mRNA (Fig. 5A). A variety of additional *myb* transcripts were also present in individual tumors.

Hybridization of tumor RNAs to the U5 LTR probe from RAV-1 detected an mRNA at the same location as the major

FIG. 5. Northern analysis of B-cell lymphoma RNAs. (A) RNA from 10 tumors containing RAV-1 insertions within *c-myb* was hybridized to the *v-myb*-containing *KpnI-XbaI* fragment from AMV. Total tumor RNA (10 μ g) was loaded in each lane and electrophoresed on a 1.2% agarose gel. The location of normal 4.0-kb *c-myb* mRNA is indicated. The positions of ribosomal bands with apparent sizes of 4.7 and 2.1 kb are indicated by arrowheads. The major tumor-*myb* transcripts (*) migrated between the 4.7-kb ribosomal band and the 4.0-kb *c-myb* band. (B) RNA from seven tumors (10 μ g per lane) was electrophoresed on a 0.8% agarose gel. The U5 LTR probe from RAV-1 shown in Fig. 1A was used for hybridizations. RNA isolated from RAV-1-infected fibroblasts (RAV-1) was used as a positive control. Genomic (G) and envelope (E) RNAs from RAV-1 and ribosomal bands (arrowheads) are indicated. Bands that colocalized with *v-myb*-hybridizing tumor transcripts are indicated (*). Thymic and bursal RNAs isolated from an uninfected chicken were used as negative controls. (C) RNA samples electrophoresed as in panel B were hybridized with the *BstEII-SacI* leader fragment shown in Fig. 1A. Markers are as in panel B. RNA from RAV-1-infected fibroblasts (RAV-1) and from uninfected fibroblasts (SC-5) served as positive and negative controls, respectively. (D) RNA from eight tumors, from normal bursa, and from the AMV-transformed cell line BM2 were hybridized to *pmyb-b*. Ribosomal bands (4.7 and 2.1 kb), *c-myb* (4.0 kb), and tumor *myb* (*) transcripts are indicated.

FIG. 6. Comparison of *myb* expression levels in activated *myb* B-cell lymphomas and normal lymphoid cells. A 10- μ g amount of total RNA from each of four tumors and from normal bursa and thymus was examined by Northern transfer and hybridization. The *v-myb*-containing *EcoRI-SalI* fragment from AMV was used as a hybridization probe. Arrowheads indicate the positions of 4.7- and 2.1-kb ribosomal bands. Normal *c-myb* transcripts (4.0 kb) and tumor *myb* transcripts (*) are indicated.

tumor *myb* transcript (Fig. 5B). We also detected an apparent band of similar mobility in RNA isolated from RAV-1-infected fibroblasts that we believe is degraded viral RNA compressed below the 28S ribosomal band. Figure 5C presents an analysis of tumor RNA hybridized with the *BstEII-SacI* fragment of RAV-1, which contains the leader sequence. The hybridization by this probe indicates that leader sequences, as well as LTR and *myb*-specific sequences (Fig. 5A and B), were present in several distinct transcripts, of which the principal one was approximately 4.5 kb. These results indicate that synthesis of the tumor-associated *myb* mRNAs originated from the 5' RAV-1 LTR.

A *PstI* fragment containing sequences from the 5'-most exon specifically localized by Dvorak et al. was subcloned (designated *pmyb b* in Fig. 3C) and used in a Northern analysis of tumor RNA. This probe hybridized to all of the tumor *myb* transcripts as well as *c-myb* mRNA from bursal lymphocytes (Fig. 5D). As expected, there was no hybridization with BM2 *v-myb* mRNA, since the region of *c-myb* that was transduced begins two exons downstream.

The published reports of chicken *c-myb* cDNA sequences identify a *NotI* site in the sequence just upstream of the translation initiation site (15, 37). We have identified a cluster of three *NotI* sites bordering the 5' end of the proviral integration cluster, one of which may be present in an exon and detected in the *c-myb* cDNA clones. However, since the *pmyb*-SmR probe did not hybridize to bursal and thymic RNAs (data not shown), an exon in this region is probably less than 100 bp in size.

The abundance of the *myb* mRNA expressed by the tumors varied considerably, from a level similar to that observed in normal bursal lymphocytes to highly elevated levels similar to that observed in the thymus, the tissue with the highest normal expression of *c-myb* (Fig. 6). Surprisingly, the level of expression of *c-myb* in these tumors was low compared with that in normal bursal lymphocytes (Fig.

FIG. 7. Expression of *c-myc* RNA in rapid B-cell lymphomas. A 10- μ g amount of RNA from eight tumors was examined by Northern analysis and hybridization to the *Clal-SphI* fragment from the *c-myc* locus, which contains exon 3. Other RNAs are from normal bursa and from DT40, a cell line derived from a bursal lymphoma with an RAV-1 proviral integration into the *c-myc* locus. Tumor 66-44 (*) demonstrated both *c-myb* and *c-myc* proviral insertions by Southern analysis. The *c-myc* RNA expressed in DT40 is approximately 2.5 kb (1).

7). Even tumor 66-44, which contained integrations into both *c-myb* and *c-myc* (data not shown), expressed less *myc* mRNA than bursa, though more than the other activated *myb* tumors did (Fig. 7, lane 9).

Histological analysis of the *myb*-expressing B-cell lymphoma. Histological analysis revealed striking changes in the bursal lymphocyte populations from these tumor-bearing birds. While many bursae appeared normal in size and healthy at necropsy, they contained a significant fraction of follicles (greater than 50%) which stained uniformly for IgM and ALV p19^{rag} (Fig. 8). In contrast, analysis of normal RAV-1-infected bursal follicles detected intense IgM and p19^{rag} staining in the medulla, with relatively light staining in the cortex. The distinctive staining observed in these aberrant follicles suggests that they contain a more uniform cell population. Furthermore, the cells of these follicles retained excessive pyronin Y on staining with methyl green pyronin, indicating an elevated RNA content typically associated with blasting populations. The aberrant follicles occurred in clusters throughout these bursae, suggesting that they were generated in a nonrandom fashion (Fig. 8). While they were not as a rule altered in size, their internal architecture was compromised. Medullary epithelial and dendritic structures were compressed and disrupted, and IgG-expressing medullary cells were decreased in number (data not shown). Southern analysis of DNA from these bursae revealed that they contained a majority of bursal lymphocytes with a clonally altered *c-myb* locus, indicating that, consistent with the staining described above, these aberrant follicles contained tumor cells (Fig. 4C).

Histological analysis of the activated *myb* lymphoma indicated that its development differed significantly from that of the activated *myc* lymphoma (Table 1). The data presented are derived from several experiments in which three cohorts of chickens were infected with RAV-1 as embryos and two cohorts were infected as hatchlings. A contrast of the pathology that emerged, including analysis of both

FIG. 8. Histological analysis of B-cell lymphomas that contain activated *myb*. Tumor and control tissues were stained by immunoperoxidase with monoclonal antibodies against chicken IgM (A–D) or ALV p19^{gag} (E, F). Magnification, $\times 400$. (A) Control liver has undisrupted hepatic architecture. (B) Lymphomatous liver is abundantly infiltrated, with IgM-expressing B-cell masses displacing the hepatic parenchyma. In several cases the liver was more than 90% replaced by tumor. (C) Control bursa has a regular array of follicles. Cortex and medulla are demarcated by epithelium. Cortical lymphocytes stain uniformly. Medullas are more darkly stained. Bursal mucosal epithelium is unstained (arrow). (D) Bursa from lymphomatous bird contains clusters of follicles with aberrant morphology. Follicular cortices are expanded and medullas are diminished, with apparent compression of the epithelial boundary. Cortical lymphocytes stain uniformly but more intensely than surrounding tissue. (E) ALV p19^{gag} stain. Control infected bursa displays normal follicular structure. ALV p19^{gag} antigen is expressed in cortex but is most abundant in medullas and at follicular membranes (arrow). (F) Similar cluster of aberrant follicles from lymphomatous bird, as in panel D. Expanded cortical compartments contain cells that stain uniformly but differently than surrounding follicles. Increased follicular vascularity is visible in aberrant follicles.

TABLE 1. RAV-1 infection can induce two biologically distinct B-cell lymphomas^a

Chicken group and expt no.	Age at sacrifice (wk)	No. of birds with IgM ⁺ tumor/no. infected	No. of tumors/no. examined			
			Identifiable primary tumor in bursa ^b	Metastatic	Disrupted <i>c-myc</i> locus	Disrupted <i>c-myb</i> locus
Infected as embryos	4-7	16/53	1/16	15/16	2/12	11/12
1		13/34	0/13	13/13	1/9 ^c	9/9 ^c
2		1/11	0/1	1/1	0/1	1/1
3		2/8	1/2 ^d	1/2	1/2 ^d	1/2
Infected 1 day posthatch	18-20	11/21	11/11	6/11	8/10	0/10
1		10/13	10/10	6/10	7/9	0/9
2		1/8	1/1	0/1	1/1	0/1

^a All infections were carried out by intravenous injection of 10⁵ IU of RAV-1.

^b Microscopic examination of bursal tissue taken from chickens infected as embryos revealed aberrant staining of follicles but no tumor mass. In contrast, a similar examination of chickens infected at the time of hatch revealed disruption of bursal follicular architecture and extensive tumor masses.

^c One tumor sample, 66-44, had both *c-myc* and *c-myb* loci disrupted.

^d One bursal nodule that contained a disrupted *c-myc* locus was observed. This bird showed no evidence of metastatic tumor, and the *c-myb* locus appeared normal.

primary and metastatic tumors, reveals that the two diseases are biologically distinct. A comparison of the protocols used in these experiments indicates that a single factor, the developmental age of the chicken, determined which of the two B-cell lymphomas arose.

DISCUSSION

Is activation of *c-myb* sufficient to confer tumorigenicity? Lymphoid leukosis is a well-studied B-cell lymphoma that develops when hatched chickens are infected with RAV-1. We have described a novel avian B-cell lymphoma that arises after RAV-1 infection of 12- to 13-day-old chicken embryos. This tumor is biologically distinct from lymphoid leukosis in several features. The development of lymphoid leukosis requires an extended latent period. During this time, bursa-dependent preneoplastic cell populations are most probably harbored in transformed follicles within the bursa. Subsequent genetic events, stochastic in nature, permit the expansion of this preneoplastic population into primary bursal tumors and subsequent metastasis 15 to 20 weeks postinfection (3, 9, 28). In contrast, embryonic infection induces a lymphoma that develops rapidly; abundant metastatic tumor has been found as early as 5 weeks postinfection. The short time course and single-stage pathogenesis suggest that a single rate-limiting step occurs in this tumorigenic process: proviral activation of the proto-oncogene *c-myb*.

The Southern analysis and morphological data gathered from bursae of tumor-bearing birds sacrificed at 5 weeks after infection indicated that a major fraction of normal bursal lymphocytes had already been replaced by clonally derived cells containing the tumor-specific proviral integration. Our analysis of aberrant bursal follicles in embryonically infected chickens sacrificed at 2 weeks of age supports the model that this tumor first grows within bursal follicles and spreads from follicle to follicle (Fig. 7 and 8). However, as some of the follicles we detected may have been transformed follicles containing preneoplastic cells characterized by altered *c-myc* expression, this interpretation must remain tentative until we can analyze specific aberrant follicles in situ for defined tumor markers.

What changes are required for oncogenic activation of *c-myb*? The altered expression from the *c-myb* locus in the lymphoid tumors described here predicts the synthesis of a *myb* protein distinct from that associated with myeloid

tumors. Comparison of *v-myb* genes from AMV and E26 viruses with *c-myb* cDNA sequences shows truncation of the *c-myb* open reading frame at both the 5' and 3' ends of the gene, resulting in *v-myb* proteins lacking both amino- and carboxy-terminal domains (15, 21, 29, 37). Analogous 5' and 3' deletions of *c-myb* are observed in murine leukemia virus-induced myeloid tumors that contain proviral insertions at either the 5' and 3' end of the locus (39, 46). These observations suggest that truncation of these domains might be necessary to activate the oncogenic function(s) of *c-myb*.

In contrast, the ALV integrations in the B-cell lymphomas reported here occurred upstream of two *c-myb* exons 5' of the sequences present in *v-myb*. The more 5' of these two exons is expressed as part of the major tumor-*myb* transcript. Our data suggest that transcription of the disrupted *c-myb* locus initiates in the 5' viral LTR and that viral sequences are spliced to the first downstream *c-myb* exon. This process could yield the 4.5-kb mRNA, seen in all tumors, that is larger than the normal *c-myb* transcript. While the extent to which sequences from the 3' end of *c-myb* are present in these tumor-*myb* transcripts is unknown at present, our results predict that these tumors synthesize a *myb* gene product that is less extensively truncated than *v-myb* and that might differ from p75^{*c-myb*} by as little as a few amino acids. It is important to note, however, that cDNA cloning of mRNA from myeloid tumors containing murine leukemia virus insertions in *c-myb* identified an alternatively spliced species of *c-myb* mRNA that encoded a new protein with an additional 121 amino acids and that was not detected by Northern analysis (36). This alternative form of *c-myb* has been detected as a minor fraction of the normal *c-myb* mRNA pool, although its relative abundance is much higher in the tumors in which it was identified (38). Similar perturbations of normal *c-myb* expression may contribute to the development of the chicken B-cell lymphoma.

Setting aside alteration of the primary amino acid sequence of *c-myb*, it remains possible that quantitative changes alone account for the oncogenic activity of *c-myb* in these lymphomas. While several tumors contained levels of *myb* comparable to that seen in bursal tissues, most contained significant elevation in tumor *myb* RNA in the form of the 4.5-kb mRNA. The presence of both viral and *c-myb* sequences in this mRNA indicates the presence of a splice acceptor site in the exon contained in fragment *b* (Fig. 4D).

This result suggests the presence of additional upstream *c-myb* sequences that might include 5' untranslated sequences important for transcriptional (41) and posttranscriptional regulation of *c-myb* expression. Replacement of such regulatory sequences by viral sequences may abolish normal *c-myb* function and permit tumor development without altering the primary amino acid sequence.

Target cell for activated *myb* lymphoma present only in embryos. Kanter et al. (20) have reported avian B-cell lymphomas with insertionally activated *myb* that are very similar to the ones we describe here. In their experiments, however, tumor development depended on a combination of genetic determinants from subgroup A and F parent ALVs juxtaposed in recombinant chimeric viruses. We have used a single stock of RAV-1 to induce two distinct lymphomas (Table 1). The tumor that emerged was determined by the developmental age of the chicken at the time of inoculation. Infection of hatched chickens resulted in lymphoid leukemia, while chickens infected as midgestation embryos developed the novel B-cell lymphoma described here. We infer from this result that there are two biologically distinct sets of target cells in which *c-myc* or *c-myb* is activated by RAV-1 integration, giving rise to the different tumors. At the time of infection of the embryos, 12 to 13 days of incubation, prebursal stem cells, cells not present at the time of hatch, colonize the bursal anlage (22). The prebursal stem cell, or a closely related cell, is a likely target for *c-myb* activation, while the bursal stem cell may represent the target for *c-myc* activation (42). The requisite property of a target cell may relate to accessibility of the *c-myc* or *c-myb* locus to the retroviral integration complex (40, 45).

The prebursal stem cell represents an early stage of differentiation, perhaps prior to immunoglobulin gene rearrangement, certainly prior to somatic diversification (19, 34). We found, however, that somatic diversification of the λ locus had occurred in all of these tumors, suggesting that some normal processes of B-cell differentiation may not be blocked by *myb*-mediated malignant transformation. Alternatively, if the target cell for this disease is indeed the prebursal stem cell, it is possible that elevated expression of *c-myb* may be nononcogenic in this cell and, more generally, nononcogenic in the more immature cells of other differentiation pathways. Two examples of normal, early-stage, rapidly proliferating cells that express high levels of *c-myb* are thymocytes and embryonic yolk sac myeloblasts (12, 41). A 25% decline in *c-myb* expression in total bursal RNA between day 18 of embryogenesis and the day of hatch has been reported (8). A study of murine pre-B-cell and B-cell lymphomas has correlated 10-fold higher *c-myb* expression with the pre-B-cell tumor and identified differences in transcriptional regulation that account for the expression levels (5, 6). Should the prebursal stem cell express high levels of *c-myb*, it may provide the essential property required for its role as a target cell for insertional mutagenesis of *c-myb* while at the same time being nonpermissive for *myb*-mediated oncogenesis. These considerations therefore suggest two different possibilities for development of the activated *myb* B-cell lymphoma. On the one hand, activated *c-myb* may not be oncogenic in the original target cell, so that only after it has differentiated into a more mature B cell are tumors able to develop. Alternatively, *myb*-mediated tumorigenesis may block differentiation inefficiently in the more immature target cell, so that in time, and with continued differentiation, the tumor mass consists largely of mature B cells. These models may be relevant to human chronic

myelogenous leukemia, which exhibits an analogous pattern of growth and development.

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