Minimal Truncation of the c-myb Gene Product in Rapid-Onset B-Cell Lymphoma†

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Embryonic infection of chickens with EU-8 virus, a recombinant avian leukosis virus (ALV), results in the rapid induction of B-cell lymphoma, which can cause death as early as 4 to 5 weeks after infection (50). These rapid-onset lymphomas all contain proviral integrations within, or near, the c-merb proto-oncogene (24). This distinguishes them at the molecular level from classic, long-latency B-cell lymphomas, which contain ALV integrations within c-myec and frequently also at the c-bic locus (the latter previously in metastatic tumors) (5, 19; reviewed in references 4 and 28). Rapid-onset lymphomas, involving integration into the c-merb locus, have also been observed following infection of 10- to 14-day chicken embryos with other ALV strains, albeit at much lower frequencies (incidence of approximately 15%, compared to 70 to 80% for EU-8) (5, 39, 40, 43).

Myb is a sequence-specific DNA binding protein, which can either activate or repress transcription of different promoters (reviewed in references 15, 22, 31, 48, and 54). This oncogene is implicated in regulation of cell growth and differentiation, especially in hematopoietic cells (13, 15, 29, 47). The down-regulation of myb is associated with terminal differentiation in many hematopoietic cell lines (8, 55). Protein truncation of Myb is thought to be the major mechanism of its oncogenic activation (17, 48). The involvement of c-merb in B-cell lymphoma was unexpected, since the myb gene had previously been associated with myeloid leukemia (48, 56).

Proviral integrations in EU-8 ALV-induced lymphoma map to two regions of the c-merb locus: a 700-nucleotide (nt) region within the 5′ end of c-merb, upstream of c-merb sequences, and a second region upstream of c-merb coding sequences (24). This raised the possibility that insertional activation by EU-8 might involve little or no disruption of the c-merb coding region, in contrast to previously reported examples of myb activation in which extensive truncations occur (48, 56). The proviruses in the rapid-onset lymphomas are integrated in the same transcriptional orientation as c-merb (24). All of the proviruses analyzed appear to be intact, except for one with a solo long terminal repeat (LTR) (18, 24), whereas proviruses are extensively deleted in long-latency ALV-induced lymphomas (14).

In the present study we have attempted to elucidate the mechanism by which c-merb is oncogenically activated in EU-8-induced B-cell lymphoma. We have characterized the tumor-specific myb mRNAs and proteins to determine the effects of both types of integrations on the structure of the c-merb gene products. Integration within either region leads to an identical N-terminal truncation of the Myb protein, a consequence of aberrant splicing events that juxtapose the same viral and c-merb sequences in the processed mRNAs. In this rapid-onset lymphoma, the Myb protein is truncated by only 20 amino acids at its N terminus, which is less extensive than truncations observed in most other myb-induced neoplasms. Retroviral vectors expressing this minimally truncated Myb protein were highly oncogenic. In contrast, similar vectors carrying intact c-merb or c-merb mutated at serines 11 and 12 (casein kinase II [CKII] phosphorylation sites) were much less oncogenic.

MATERIALS AND METHODS

**Virus and chickens.** The construction of the EU-8 recombinant virus, which contains the subgroup A env gene of UR2AV and the rest of the genome from ring-necked pheasant virus, was described previously (50). Inoculation of 10-day chicken embryos from the SC line of White Leghorn chickens (Hyline International) with EU-8 ALV via a choriosallantoic membrane vein was as described previously (50). Characteristics of the resulting tumors have been described previously (24, 25).

cDNA cloning. Total RNA from tumor samples or normal thymus was isolated by the guanidinium isothiocyanate method (32). Poly(A)⁺ RNA was selected by two cycles of chromatography on oligo(dT)-cellulose. CDNA was prepared from poly(A)⁺ RNA from EU-8-induced tumors 21 and 186, using EcoRI linkers and a CDNA-synthesis kit from Boehringer Mannheim according to the manufacturer’s instructions but with the addition of RNase H and T4 DNA polymerase.
treatments. After EcoRI digestion, cDNAs were purified away from excess linkers by passage over CL-4B columns and ligated into λGT10 or λZAP vectors (Stratagene). The λGT10 library was first screened with the BamHI-EcoRI fragment of the chicken c-myb cDNA clone of Gerondakis and Bishop (11), which represents the 5' end of c-myb mRNA, and then rescreened through several cycles with the entire c-myb cDNA clone. High-specific-activity probes (ca. 107 Cerenkov cpm/μg of DNA) were prepared by the incorporation of [α-32P]dCTP (3,000 Ci/mmol) into double-stranded DNA by random priming (10), using labeling-grade Klenow fragment (Boehringer Mannheim) and the d(ΔN)6 primer (Pharmacia). Positive clones were isolated, and the inserts were subcloned into the EcoRI site of Bluescript SK– (Stratagene).

**cDNA sequence analysis.** Double-stranded DNA sequencing was carried out with the Sequenase kit (U.S. Biochemical), according to the manufacturer's instructions, except that plasmids were first linearized by restriction enzyme cleavage and then denatured and hybridized to oligonucleotide primers by 2 min of boiling followed by 1 min on ice. After reactions were complete, the products were heated to 90°C and analyzed on 6% polyacrylamide denaturing gels. For the 5' end of c-myb, the 5' end of the cDNA extending at least 60 nt into the myb sequence.

**Protein preparation and Western blotting.** Nucleus-enriched fractions from frozen tumors were prepared by lysing cells at 4°C in 0.5% Nonidet P-40–5 mM MgCl2–25 mM KCl–Tris-HCl (pH 8.0)–2 mM phenylmethylsulfonyl fluoride (Sigma) for 5 min, followed by pelleting the insoluble material at 1,000 x g for 2 min. Pellets were solubilized in 2% SDS sample buffer (2% [w/v] dodecyl sulfate, 10% [v/v] mercaptoethanol, 200 mM Tris-HCl [pH 8.0], and 2 mM phenylmethylsulfonyl fluoride) by sonication and boiling for 5 min and electrophoresed on sodium dodecyl sulfate-polyacrylamide gels as previously described (44). The products were transferred to nitrocellulose membranes (Bio-Rad) using a Hoeffer minigel blotter at 200 V for 1 to 2 h at 4°C. Filters were blocked with 10% nonfat dry milk in Tris-buffered saline (TBS) (25 mM Tris-HCl [pH 8.2], 144 mM NaCl) with 0.1% Tween 20 for 1 h at room temperature. Incubation with anti-Myb specific monoclonal antibody 1.1 (44) was at a dilution of 1:500 in 1% nonfat skim milk in TBS overnight at 25°C, followed by washing three times with 100 ml of TBS. In some experiments hybridoma culture medium containing a 1:1 mixture of anti-Myb antibodies 2.7 and 2.2 (9) was used instead. Bound antibodies were detected with alkaline phosphatase-conjugated anti-mouse immunoglobulin G (Bio-Rad).

**Construction and testing of myb retroviral vectors.** Full-length c-myb, as well as mutated c-myb cDNA fragments, were generated by PCR amplification of the chicken c-myb cDNA clone pSG3 of Gerondakis and Bishop (11). A downstream primer from the 5' end of the c-myb gene, which contains a NotI restriction site, was used for all constructs (5' ATATCGATGCCGCGAGGATGGCCCGGAGACCCCGGCACAGCATATACG 3'). All upstream primers included a Clal site. The Cmyb primer included the 5' coding region of c-myb (5' ATATCGATGCCGCGAGGATGGCCCGGAGACCCCGGCACAGCATATACG 3') as defined by Gerondakis and Bishop (11), whereas the T20myb upstream primer began 60 nt downstream of the first AUG (5' GCATCGATTGTTGAGATGTACGACCA 3'). The upstream primer used to generate (5' ATATCGATGCCGCGAGGATGGCCCGGAGACCCCGGCACAGCATATACG 3') was identical to the Cmyb primer at its 5' end and encoded a double mutation of serines 11 and 12 to alanines (the mutation is underlined). PCR was performed with a Geneamp Kit (Perkin-Elmer/Cetus); the reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 35 mM MgCl2, 0.001% gelatin, 0.2 mM deoxynucleoside triphosphate, 20 pmol of primers, and 1.5 U of Taq polymerase (Perkin-Elmer). The reaction mixture was subjected to 30 cycles of amplification, each consisting of 700-nt region of c-myb. The upstream primer used to generate (5' ATATCGATGCCGCGAGGATGGCCCGGAGACCCCGGCACAGCATATACG 3') was identical to the Cmyb primer at its 5' end and encoded a double mutation of serines 11 and 12 to alanines (the mutation is underlined). PCR was performed with a Geneamp Kit (Perkin-Elmer/Cetus); the reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 35 mM MgCl2, 0.001% gelatin, 0.2 mM deoxynucleoside triphosphate, 20 pmol of primers, 0.1 ng of DNA template, and 2.5 U of Taq polymerase (Perkin-Elmer). The reaction mixture was subjected to 30 cycles of amplification, each consisting of 700-nt region of c-myb. The upstream primer used to generate (5' ATATCGATGCCGCGAGGATGGCCCGGAGACCCCGGCACAGCATATACG 3') was identical to the Cmyb primer at its 5' end and encoded a double mutation of serines 11 and 12 to alanines (the mutation is underlined).

**RESULTS**

**Proviral integrations in the c-myb locus.** All rapid-onset lymphomas examined in our previous study contain EU-8 ALV proviral integrations at the c-myb locus (24). Since that study, the ALV integration sites of several additional EU-8-induced tumors have been mapped, and all of the integration sites were localized on the c-myb gene map (Fig. 1). The integration sites of several tumors are identified by number in Fig. 1; these tumors were used as a source of RNA or protein for the studies discussed below. The structure of the 5' end of c-myb (upstream of exon 4, which is present in v-myb) was determined (18, 52) after the initial identification of integration sites.

**The alignment of integration sites in Fig. 1 showed that 76% (26 of 34) of all tumors had integrations clustered within a 700-nt region of c-myb intron 1 directly upstream of exon 1. One additional integration was near the center of the 2.85-kb intron 1. In addition, a solo LTR was integrated in c-myb exon 2, between codons for Myb amino acids 22 and 23 (18). The remaining 18% of integration sites (6 of 34) were located 1.5 to 3 kb upstream of c-myb exon 1 (Fig. 1).

**Cloning and sequencing of chimeric ALV-myb cDNAs.** Both classes of EU-8-induced tumors, bearing proviral integrations in intron 1 and upstream of exon 1, express 4.0- to 4.1-kb chimeric mRNAs as determined by Northern analysis (24). These mRNAs are slightly larger than the normal 3.7-kb c-myb mRNA (11). They appear to contain the 5' U5 part of the viral LTR and the viral leader sequence linked to c-myb sequences, suggesting that transcription initiates in the ALV 5' LTR and reads through into c-myb (24). This would result in truncation of exon 1 coding sequences for those tumors with integrations in intron 1. To determine more precisely the structures of these chimeric transcripts, we constructed cDNA libraries with poly(A)+ RNAs from two different lymphomas: tumor 21, which contained a provirus in the intron 1 cluster approximately 250 bp upstream of exon 2, and tumor 186, which contained a provirus approximately 2.5 kb upstream of exon 1 (Fig. 1).

**The ALV-myb chimeric cDNA clones from tumor 21 were of...
two types, represented by pBS21-7 and by pBS21-5 and pBS21-8. The sequence of pBS21-7 began in the proviral LTR and continued through the viral leader and the 5′ end of gag, which was juxtaposed to c-myb exon 2. The viral and host sequences were joined at the 5′ splice site of ALV gag to the 3′ splice site of c-myb exon 2. In contrast, cDNAs 21-5 and 21-8 did contain a portion of c-myb intron 1. Exon 1 of c-myb is not present in these cDNAs. (B) mRNA 186-dT1 from tumor 186. In this tumor, EU-8 ALV was integrated approximately 2.5 kb upstream of exon 1. Nevertheless, the spliced mRNA contains the ALV leader sequence spliced to c-myb exon 2. Exon 1 sequence was not present in the cDNA. Thus, the splice junction is the same for both classes of EU-8 integrations: those in intron 1 (A) and those upstream of exon 1 (B). E, EcoRI. (Only sites present in cDNA are shown.)

FIG. 2. Splicing patterns of ALV myb chimeric mRNAs in EU-8-induced lymphomas. (A) mRNAs 21-7, 21-5, and 21-8 from tumor 21, in which EU-8 integration is in the major cluster in intron 1. c-myb exons downstream of exon 2 were spliced correctly. cDNA 21-7 did not contain intron sequences. Splicing occurred from the 5′ splice site of ALV gag to the 3′ splice site of c-myb exon 2. In contrast, cDNAs 21-5 and 21-8 did contain a portion of c-myb intron 1. Exon 1 of c-myb is not present in these cDNAs. (B) mRNA 186-dT1 from tumor 186. In this tumor, EU-8 ALV was integrated approximately 2.5 kb upstream of exon 1. Nevertheless, the spliced mRNA contains the ALV leader sequence spliced to c-myb exon 2. Exon 1 sequence was not present in the cDNA. Thus, the splice junction is the same for both classes of EU-8 integrations: those in intron 1 (A) and those upstream of exon 1 (B). E, EcoRI. (Only sites present in cDNA are shown.)

FIG. 3. Determination of the N-terminal amino acid sequence of Myb protein in EU-8-induced lymphomas. The splice junction sequence between ALV and c-myb exon 2 in the chimeric mRNAs is shown. The gag ATG initiation codon is underlined, and gag amino acids encoded are shown below the sequence. Three ATG termination codons in myb in the gag reading frame are overlined. Three ATG initiation codons in reading frame 3 are underlined, and the encoded amino acid sequence is shown below the gag sequence. The ATG initiation codon in the myb reading frame is underlined, and the amino acid sequence encoded, beginning at myb codon 21, is shown on the bottom line. Translation presumably initiates at the ATG in gag, terminates at a downstream TGA, and reinitiates at myb codon 21.

all EU-8-induced lymphomas, suggesting that it is not necessary for tumorigenesis (23).

The cDNA clones from tumor 186, in which the provirus is integrated upstream of exon 1 (p186-dT1, p186-BSmyb1, and p186-dT5), were identical in sequence to pBS21-7 from tumor 21, except for their 5′ endpoints. The virus-myb junctions in these cDNAs were identical to those in tumor 21 (Fig. 3), despite the fact that the provirus in this tumor was located upstream of exon 1 rather than within intron 1 (Fig. 2B). myb exon 1 sequences were presumably removed from the primary transcript by splicing, due to the absence of a 3′ splice site in exon 1, as illustrated in Fig. 2B.

Sizes of Myb proteins in EU-8-induced lymphomas. Analysis of cDNA sequence data suggested that the majority of ALV-myb chimeric mRNAs in lymphomas were derived by splicing from the ALV 5′ splice site to myb exon 2, regardless of whether the provirus was integrated upstream or downstream of exon 1 (Fig. 2). The cDNA sequence surrounding the ALV-myb junction is shown in Fig. 3. Because the initiation codon for the viral gag gene is 18 nt upstream of the 5′ splice site, it is present in the chimeric tumor mRNAs, thus providing a potential start site for translation of myb. However, Gag and Myb are encoded in different reading frames. Proteins initiated in gag would encounter three in-frame stop codons in myb exon 2, followed by an AUG in a second reading frame (the myb reading frame) at myb codon 21 (Fig. 3), which is in a good Kozak context (26). There are also three intervening AUGs in a third reading frame (Fig. 3). Kozak has presented evidence that reinitiation efficiency increases with intercistronic distance (27); thus, codon 21 would presumably be utilized most efficiently. If translation does not initiate at codon 21, the next AUG in the correct myb reading frame is codon 189, which would generate an extensively truncated Myb protein.

To determine directly the sizes of the Myb proteins in EU-8 lymphomas, we fractionated tumor cells and analyzed both nuclear and cytoplasmic extracts by Western blotting. Negligible Myb protein was detected in the cytoplasmic fractions, but Myb proteins were readily detected in the nuclear fractions of the tumor samples. Wild-type Myb protein (75 kDa) was detected in nuclei from normal bursa but not normal liver (Fig. 4), in accordance with the expected patterns of myb expression (13, 47, 48). In EU-8-induced lymphomas, the predominant Myb protein migrated slightly faster than the wild-type 75-kDa protein, with an electrophoretic mobility corresponding to ap-
proximately 73 kDa (Fig. 4), consistent with initiation at codon 21. The 73-kDa protein was detected both in a tumor with an upstream integration site (no. 184) and in tumors with integrations in intron 1 (no. 21, 178, and 193) (Fig. 4). These results are consistent with our prediction that upstream and intron 1 integrations would give rise to identical truncated Myb proteins, lacking only 20 amino acids at the N terminus.

Oncogenicities of retroviral vectors expressing minimally truncated Myb protein. Because most cases of Myb activation involve much larger truncations, we asked whether truncation of the 20 N-terminal amino acids of Myb was sufficient to activate its oncogenic potential. For this purpose, we constructed retroviral vectors expressing either wild-type c-myb or truncated c-myb \( T_{(-20)} \) (Fig. 5A) and tested their oncogenicities in vivo by injection of chicken embryos.

Constructs were prepared by using the helper-independent retroviral vector RCASBP (Fig. 5B), which was derived from RSV by Hughes and colleagues (20, 36). The insert is expressed under control of the RSV LTR, as a spliced sub-genomic mRNA, by using the gag 5' splice site and the 3' splice site of the original v-src gene. In the case of the \( T_{(-20)} \) insert, translation presumably initiates in gag, terminates at a termination codon in the vector, and reinitiates at c-myb codon 21. This pattern of splicing and translation is similar to that in EU-8 ALV-induced tumors, except that the alternative translational initiation sites in myb are not present (Fig. 3). Because the retroviral vector is also potentially oncogenic as an insertional mutagen, parallel control animals in each experimental group were infected with the RCASBP vector alone. Virus was injected into either 12- or 16-day-old chicken embryos or into 1-day-old chicks. Chickens were monitored for 10 weeks after hatching, at which time all surviving birds were sacrificed and their tissues were removed for necropsy.

The chickens infected with the \( T_{(-20)} \) virus as 12-day-old embryos suffered the highest mortality, with 75% of the birds dying before 10 weeks after hatching, when the experiment was terminated. In contrast, only 10% of the birds infected with the Cmyb virus died during this same time period, and most of those deaths were from nonneoplastic causes. The incidence of neoplasia resulting from infection with either of the myb viruses is summarized in Table 1. Over 90% of the birds infected with RCASBP/\( T_{(-20)} \) developed tumors by 10 weeks. Surprisingly, the tumors included not only lymphoma (44% of birds) but also sarcoma (40%), adenocarcinoma (29%), and myeloid

![FIG. 4. Western blot showing Myb protein expression in EU-8-induced lymphomas. Proteins are from nucleus-enriched fractions of normal chicken bursa (NB) and four EU-8-induced B-cell lymphomas from different birds (numbered) and were detected with anti-Myb monoclonal antibody 1.1 (44). Black arrow, wild-type p75myb; gray arrow, truncated p73myb. No Myb protein was detected in normal liver (NL). The positions of molecular mass markers (in kilodaltons) are shown on the left.](http://jvi.asm.org/)

![FIG. 5. Retroviral vectors expressing Myb. (A) myb inserts include full-length c-myb cDNA (Cmyb), c-myb cDNA with an N-terminal truncation of 20 amino acids \( T_{(-20)} \), and c-myb cDNA with mutation of serines 11 and 12 (CKII phosphorylation sites) to alanines (11/12A). The 5' end of c-myb is as defined by Gerondakis and Bishop (11) and Hahn et al. (18) and lacks the alternative N-terminal myb exon sequences observed by Rosson and Reddy (46). (B) Each of the myb fragments shown in panel A was inserted into the RCASBP retroviral vector (20, 36) at the Cla I site. The Myb proteins are expressed from spliced mRNAs, using the 5' splice site in gag and the 3' splice site upstream of the deleted src gene.](http://jvi.asm.org/)
leukomas induced by T(20)myb (three of three) but not in infected birds of chickens infected with T(20)myb, Cmyb showed little tumorigenicity compared with RCASBP vector control at 10 weeks after hatching. Results are the sum of those from two separate experiments.

The birds with the RCASBP/T(20)myb-induced lymphomas exhibited enlarged and heavily infiltrated livers and occasionally affected kidneys and spleens. Immunoglobulin rearrangements were detected in all lymphoma samples tested, indicating that they are of B-cell origin (data not shown). However, extensive involvement of the liver was rare, as observed also in rapid-onset B-cell lymphoma induced by EU-8 (24). Histologic analysis showed that the majority of liver cells in sections of lymphomatosus liver were displaced by immature lymphoblastic cells. While sarcomas and adenocarcinomas were found primarily in the kidney, they occasionally appeared in the liver. Southern blot analysis of DNA from T(20)myb-induced tumors showed that the provirus was present, without obvious rearrangement, in all malignant tissues examined (21).

In contrast to T(20)myb, Cmyb showed little tumorigenicity over that induced by the RCASBP vector control at 10 weeks after hatching. A total of 15% of the birds infected with RCASBP/Cmyb developed malignancy, including B-cell lymphoma (11%), sarcoma (2%), and carcinoma (2%) (Table 1). The RCASBP vector lacking an insert resulted in 8% of birds with malignancy, consisting of a sarcoma and a myeloid tumor (Table 1). While the incidence of lymphoma following Cmyb infection was slightly higher than control levels, the tumors observed were much less aggressive than those induced by T(20)myb; most were detected only by necropsy in birds sacrificed at 10 weeks. Furthermore, Southern blots showed rearrangements within the viral myb sequences in lymphomas induced by RCASBP/Cmyb (three of three) but not in lymphomas induced by T(20)myb (zero of five) (21).

**Effect of time of infection on oncogenicity.** Both the incidence of tumors and their oncogenic spectrum were highly dependent on the time of infection with RCASBP/T(20)myb, as shown in Fig. 6. Infection of chicken embryos at day 16 instead of day 12 resulted in a substantial drop in the incidence of lymphoma (from 44 to 3%), a smaller decrease in the incidence of sarcoma (from 40 to 24%), and no significant change in the incidence of carcinoma (from 29 to 32%). These data suggest that the target cells for T(20)myb-induced lymphoma are present for only a brief period, ending at some time between 12 and 16 days of embryogenesis. A similar narrow window for lymphoma induction was observed previously with EU-8 virus (4, 50). When birds were infected on day 1 after hatching with T(20)myb, the incidence of all types of tumors was reduced markedly (Fig. 6).

**Role of CKII phosphorylation sites in oncogenesis.** Luscher et al. (30) have identified CKII phosphorylation sites near the N terminus of the Myb protein at serines 11 and 12. Phosphorylation of these sites in vitro results in reduced binding of Myb to a consensus DNA binding sequence (30), raising the possibility that the loss of these phosphorylation sites is the key factor in activating the oncogenic potential of c-myb. To test this directly, we constructed a retrovirus carrying a mutated c-myb gene encoding replacement of serines 11 and 12 with alanines, thus abolishing these phosphorylation sites (Fig. 5).

This construct, termed RCASBP/11/12A, was very weakly lymphomagenic, inducing B-cell lymphomas in only 3% of birds infected as 12-day-old embryos (Table 1). Thus, the oncogenicity associated with the 20-amino-acid N-terminal truncation cannot be attributed solely to the loss of the serine phosphorylation sites. However, the number of birds developing malignancies of any type at 10 weeks was higher with RCASBP/11/12A (25%) than with RCASBP/Cmyb (15%), suggesting that the abolition of these sites was not without...
suggesting no appreciable difference in their rates of synthesis appeared to be present in the infected CEFs at similar levels, constructs, as expected (Fig. 7A). These different Myb proteins of approximately 75 kDa were observed with the Cmyb and 11/12A insertional activated induced tumor (184L), confirming our prediction that the in-protein comigrated with the protein expressed in an EU-8-infected carcinoma, myeloid leukemia, erythroleukemia, and nephroblastoma were also seen (Table 1).

Expression of Myb proteins. The ability of each RCASBP/myb construct (Fig. 5) to express its inserted myb gene in infected CEFs was tested by Western analysis with anti-Myb-specific antibodies. Proteins of approximately 73 kDa were observed with the Tc-20myb construct, and proteins of approximately 75 kDa were observed with the Cmyb and 11/12A constructs, as expected (Fig. 7A). These different Myb proteins appeared to be present in the infected CEFs at similar levels, suggesting no appreciable difference in their rates of synthesis or their stabilities.

Western analysis was also used to study myb expression in Tc-20myb-induced tumors (Fig. 7B). Samples from a normal chicken thymus, expressing wild-type c-Myb protein (75 kDa), and from an EU-8-induced lymphoma (184L), expressing insertionally activated c-Myb (73 kDa), were included for size comparison. HD4, an avian erythroblastosis virus-transformed chicken erythroblast cell line overexpressing normal c-myb (13, 16). NL, normal liver; NK, normal kidney; NT, normal thymus. Molecular mass markers (in kilodaltons) are shown on the left.

FIG. 7. Myb protein expression from retroviral vectors. Myb proteins were detected by Western blotting with anti-Myb antibodies 2.2 and 2.7 (9). (A) The Myb retroviral constructs shown in Fig. 5 were used to infect CEFs. (B) Myb proteins were detected in B-cell lymphomas induced by Tc-20myb virus infection of 12-day-old chicken embryos. Tumors were obtained from liver (32L and 33L) or kidney (33K and 35K). Tumor 184L is an EU-8-induced B-cell lymphoma included for comparison. HD4 is an avian erythroblastosis virus-transformed chicken erythroblast cell line overexpressing normal c-myb (13, 16). NL, normal liver; NK, normal kidney; NT, normal thymus. Molecular mass markers (in kilodaltons) are shown on the left.

consequence. The tumors induced by the 11/12A construct were very diverse. The most common type were sarcomas, which were observed in 11% of birds. A few examples of carcinoma, myeloid leukemia, erythroleukemia, and nephroblastoma were also seen (Table 1).

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While Myb is also activated in murine leukemia virus (MuLV)-induced myeloid tumors by splicing of virus-myb readthrough transcripts, a cryptic 5' splice site in MuLV gag is used (49), rather than the major viral 5' splice site used by EU-8 ALV. In ALV-induced erythroblastosis, readthrough transcripts are spliced from ALV sequences into the c-erbB gene, producing an N-terminal truncation of the ErbB protein (28, 38). However, the splicing pattern in erythroblastosis is more complex than that in EU-8 lymphomas, using a cryptic 5' splice site in env and a 3' splice site in c-erbB, as well as the normal splice sites in gag and env (38). The viral gag AUG initiation codon is necessary for translation of the activated oncogene in both the MuLV-myb and the ALV-erbB chimeric mRNAs (28, 38, 49). In contrast, the cDNA sequence data from the EU-8-induced tumors predicted that translation of the chimeric mRNA initiated at the gag AUG, terminated in myb exon 2, and reinitiated at myb codon 21. Consistent with this prediction, the predominant Myb protein in these tumors migrated slightly faster than the wild-type 75-kDa protein, with an electrophoretic mobility corresponding to approximately 73 kDa.

The majority of all integration sites in EU-8-induced lymphomas are tightly clustered in the 3' one-fourth of intron 1 of chicken c-myb. It is possible that these integrations occur downstream of a transcriptional pause site; pause sites have been reported in the middle of c-myb intron 1 in mice and humans (2, 3).

Truncated Myb lacking the N-terminal 20 amino acids is highly oncogenic. Retroviral vectors were used to test whether the Myb truncation predicted to occur in EU-8 tumors is sufficient to activate the oncogenic potential of c-myb. The myb construct with a 20-amino-acid N-terminal truncation [Tc-20myb] was found to be highly oncogenic, inducing a high incidence of rapid-onset B-cell lymphomas, sarcomas, and adenocarcinomas after infection of 12-day-old chicken embryos. In contrast, a construct expressing full-length Cmyb was only weakly oncogenic at 10 weeks after hatching.

The oncogenicity and disease spectrum of the Tc-20myb construct were highly dependent on the time of infection, suggesting the presence of specific target cells between days 10 and 14 of embryonic development. Infection of day 16 embryos resulted in a high incidence of sarcoma and carcinoma but very few lymphomas. A similar time-dependent response is observed with EU-8-induced B-cell lymphomas (50). Rous-associated virus-1 also induces rapid-onset lymphomas when introduced into 12-day-old chicken embryos (39, 40), apparently by the same mechanism as EU-8 but at a much lower incidence. Both EU-8 and Rous-associated virus-1 induce classic long-latency lymphomas when injected after hatching (19, 37).

The Cmyb viral construct induced a much lower incidence of tumors of all types than did Tc-20myb. In all three cases of RCASBP/Cmyb-induced lymphomas that were tested, the myb insert of the integrated provirus was rearranged. Since no rearrangements were found in any of the Tc-20myb tumors examined, this suggests a strong selection for rearrangement of myb in RCASBP/Cmyb-induced tumors.

The Myb protein has an N-terminal DNA binding domain consisting of three tandem repeats beginning at amino acid 38 (numbering according to reference 11), a central transactiva-
tion domain, and a C-terminal negative regulatory domain (6, 12, 22, 45) (Fig. 5). Integrations in MuLV-induced myeloid leukemias generally result in extensive N-terminal truncations, deleting a portion of the first repeat sequence, or C-terminal truncations, removing negative regulatory sequences (48, 56). Similarly, the v-*myb* products of two acute ALVs, avian myeloblastosis virus and E26, are extensively truncated at both the N and C termini (48). In contrast, neither the N-terminal repeats nor the C-terminal regulatory sequences are disrupted in rapid-onset B-cell lymphoma. Because the removal of the N-terminal 20 amino acids of Myb is sufficient to activate its transforming potential (Table 1), our results implicate this short sequence in some type of negative regulation. Interestingly, Mukhopadhyaya and Wolff (35) also observed a 20-amino-acid N-terminal truncation of Myb in one case of myeloid leukemia induced by MuLV integration in *myb* intron 1.

**Mutation of CKII phosphorylation sites does not make Myb lymphomagenic.** The N-terminal 20 amino acids of c-Myb include two CKII phosphorylation sites (serines 11 and 12), which have been proposed to be part of a negative regulatory element (30); however, conflicting evidence for this has been reported (7, 30, 45). We have observed that infection of chicken embryos with a retroviral vector expressing a mutant c-Myb protein, in which serines 11 and 12 were replaced by alanines, failed to induce lymphoma efficiently, in contrast to c-Myb truncated by 20 amino acids at its N terminus. The N-terminal 20 amino acids of c-Myb include a stretch of basic amino acids, followed by the CKII phosphorylation sites, followed by a stretch of acidic residues. Removal of some or all of these charged amino acids appears to be important for oncogenic activation of c-Myb.

**Variety of neoplasms induced by truncated Myb proteins.** Using a retroviral vector expressing T20-*myb*, we observed a broad spectrum of neoplasms, including B-cell lymphomas, sarcomas, and carcinomas. Thus, the oncogenicity of this minimally truncated Myb protein is not restricted to the B-cell lineage. The low incidence of myeloid tumors induced with these constructs may be due either to the nature of the mutation or to the vector. Retrovirus-containing either the RSV LTR or the subgroup A *env* gene, which are present in the RCASBP vector (and in EU-8 ALV), have been reported to infect myeloid cells poorly (34, 41). In contrast to our results, Press et al. (43) observed a low incidence of lymphoma, coupled with activation of endogenous c-*myb* by insertional mutagenesis, with a retroviral vector expressing a minimally truncated Myb. Their constructs were similar to ours except that their LTR was derived from avian myeloblastosis virus, whereas our vectors used an RSV LTR. These two LTRs show tissue-specific differences in expression in cultured cells (41). Press et al. (42) also observed fibrosarcoma induction by vectors expressing C-terminally truncated *myb*.

In summary, we have found that a minimal, 20-amino-acid N-terminal truncation of Myb is sufficient to activate its oncogenic potential, suggesting a new regulatory domain for Myb. Mutation of the CKII phosphorylation sites in this region did not have the same effect. B-cell lymphomas induced by both EU-8 ALV and RCASBP/T20-*myb* arose with a high incidence after a very short latent period. Lymphoma induction required infection of 10- to 14-day-old chicken embryos, suggesting that the target cells are transiently present at this time. The accompanying paper (51) describes the identification of the genetic determinants of EU-8 ALV necessary for its frequent induction of rapid-onset lymphoma. The most critical determinant was found to be a 42-nt deletion in the EU-8 *gag* gene (51). This deletion is in a region of the *gag* gene previously shown to negatively regulate viral RNA splicing (1, 33).

This deletion may lead to increased splicing of readthrough transcripts from the ALV 5′ splice site to the 3′ splice site of the downstream c-*myb* exon 2, generating the minimally truncated Myb protein.

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