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

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Oncogenic activation of *c-myb* by insertional mutagenesis has been implicated in rapid-onset B-cell lymphomas induced by the nonacute avian leukosis virus EU-8. In these tumors, proviruses are integrated either upstream of the *c-myb* coding region or within the first intron of *c-myb*. Tumors with either type of integration contained identical chimeric mRNAs in which the viral 5' splice site was juxtaposed to the 3' splice site of *c-myb* exon 2 and *myb* exon 1 was eliminated. Both classes of integrations generated truncated Myb proteins that were indistinguishable by Western analysis. In contrast to most other examples of *c-myb* activation, the truncation consisted of only 20 N-terminal amino acids and did not disrupt either the DNA binding domain near the N terminus or the negative regulatory domain near the C terminus of Myb. The significance of the 20-amino-acid Myb truncation to tumorigenesis was tested by infection of chicken embryos with retroviral vectors expressing different *myb* gene products. While virus expressing either wild-type *c-myb* or *c-myb* mutated at the N-terminal casein kinase II sites was only weakly oncogenic at 10 weeks, the minimally truncated *myb* virus induced a high incidence of rapid-onset tumors, including B-cell lymphomas, sarcomas, and adenocarcinomas.

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-myb* proto-oncogene (24). This distinguishes them at the molecular level from classic, long-latency B-cell lymphomas, which contain ALV integrations within *c-myc* and frequently also at the *c-bic* locus (the latter primarily in metastatic tumors) (5, 19; reviewed in references 4 and 28). Rapid-onset lymphomas, involving integration into the *c-myb* 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-myb* 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-myb* locus: a 700-nucleotide (nt) region within the 5' end of *c-myb*, upstream of *v-myb* sequences, and a second region upstream of *c-myb* coding sequences (24). This raised the possibility that insertional activation by EU-8 might

involve little or no disruption of the *c-myb* 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-myb* (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-myb* 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-myb* 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-myb* 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-myb* or *c-myb* 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 chorioallantoic 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 *Eco*RI 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

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† Her coauthors dedicate this paper to the memory of Wanping Jiang, whose untimely death has left us deeply saddened.

treatments. After *EcoRI* digestion, cDNAs were purified away from excess linkers by passage over CL-4B columns and ligated into λ GT10 or λ ZAP vectors (Stratagene). The λ GT10 library was first screened with the *Bam*HI-*Eco*RI fragment of the chicken *c-myc* cDNA clone of Gerondakis and Bishop (11), which represents the 5' end of *c-myc* mRNA, and then rescreened through several cycles with the entire *c-myc* cDNA clone. High-specific-activity probes (ca. 10^9 Cerenkov cpm/ μ g of DNA) were prepared by the incorporation of [α - 32 P]dCTP (3,000 Ci/mmol) into double-stranded DNA by random priming (10), using labeling-grade Klenow fragment (Boehringer Mannheim) and the p(dN)₆ primer (Pharmacia). Positive clones were isolated, and the inserts were subcloned into the *Eco*RI 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' cDNA clones, the entire sequences of both strands were determined for the region from 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–3 mM CaCl₂–Tris-HCl (pH 8.0)–2 mM phenylmethylsulfonyl fluoride (Sigma) for 5 min, followed by pelleting the insoluble material at 1,000 $\times$ g for 2 min. Pellets were solubilized in protein sample buffer (2% sodium dodecyl sulfate, 10% β -mercaptoethanol, 20 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). Proteins were transferred to nitrocellulose membranes (Bio-Rad) by using a Hoefer 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-myc*, as well as mutated *c-myc* cDNA fragments, were generated by PCR amplification of the chicken *c-myc* cDNA clone pSG3 of Gerondakis and Bishop (11). A downstream primer from the 3' end of the *c-myc* gene, which contained a *Nar*I restriction site, was used for all constructs (5'-AAGGCGCCCTTTCACATCACCAGAGT3'). All upstream primers included a *Cl*aI site. The *Cmyb* primer included the 5' coding region of *c-myc* (5'-ATATCGATGCCGCGAGGATGGCCCGGA3') as defined by Gerondakis and Bishop (11), whereas the T₁–(20)*myb* upstream primer began 60 nt downstream of the first AUG (5'-GCATCGATTGTTGAGATGTACGACCA 3'). The upstream primer used to generate insert 11/12A (5'-ATATCGATGCCGCGAGGATGGCCCGAGACCCCGGCACAGCATATACCGCCGATGAC3') 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 1 min of denaturation at 94°C, 1.5 min of annealing at 55°C, and 2 min of polymerization at 72°C. The PCR products were purified by agarose gel electrophoresis followed by GeneClean (Bio101) treatment. To facilitate cloning, the PCR products were subcloned into the TA cloning vector PCR1000 (Invitrogen), followed by digestion of the resultant plasmids with *Cl*aI and *Nar*I to excise the *myb* fragments.

After gel electrophoresis and GeneClean purification, each of the *myb* PCR fragments was ligated to the *Cl*aI-digested retroviral vector RCASBP (A) (20, 36), provided by S. Hughes. This vector is derived from the Schmidt-Ruppin, subgroup A, strain of Rous sarcoma virus (RSV). The *src* gene has been deleted and replaced with a *Cl*aI restriction site. In addition, the *pol* gene of Bryan RSV has been substituted for that of the Schmidt-Ruppin subgroup A strain to generate the BP version of the RCAS vector.

To produce virus, White Leghorn chicken embryo fibroblasts (CEFs) that were gs⁺ chf⁺ (SPAFAS, Inc.) were transfected with plasmids containing viral constructs as described previously (50). Day 12 or day 16 chicken embryos were injected with 0.2 ml of virus-containing medium (approximately 10^5 to 10^6 PFU) via a chorioallantoic vein. Day 1 chickens (approximately 24 h after hatching) were injected in the wing vein with the same amount of virus. Birds were euthanized when they appeared to be very ill or had distended abdomens; all surviving birds were sacrificed at 10 weeks after hatching. Tissues for DNA and protein extraction were flash frozen in liquid nitrogen and stored at -70°C. Tissues for histology sections were preserved in phosphate-buffered Formalin. Histologic analysis was performed by H. Nguyen, Histology Laboratory, Cornell Medical College.

FIG. 1. Location of EU-8 ALV integration sites in the chicken *c-myc* locus in B-cell lymphomas. Exons 1 to 3 were formerly termed upstream exons UE3 to UE1 (18); exons 4 to 10 were formerly termed *v-myc*-homologous exons v1 to v7 (11, 46). Integration sites in tumors 21, 178, 184, 186, and 193 used in this study are indicated by arrows. Other integrations are indicated by vertical lines on the expanded map. The integration sites shown include those in references 23 and 24. Translation of *c-myc* is initiated at an ATG at the 3' end of exon 1, which encodes only the first 7 amino acids of *c-Myb*. The open box denotes the noncoding region of exon 1; closed boxes denote coding exons. The complete *c-myc* gene has 15 exons (53). E, *Eco*RI.

RESULTS

Proviral integrations in the *c-myc* locus. All rapid-onset lymphomas examined in our previous study contain EU-8 ALV proviral integrations at the *c-myc* 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-myc* 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-myc* (upstream of exon 4, which is present in *v-myc*) 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-myc* intron 1 directly upstream of exon 2. One additional integration was near the center of the 2.85-kb intron 1. In addition, a solo LTR was integrated in *c-myc* 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-myc* 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-myc* mRNA (11). They appear to contain the U5 part of the viral LTR and the viral leader sequence linked to *c-myc* sequences, suggesting that transcription initiates in the ALV 5' LTR and reads through into *c-myc* (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

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 *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.)

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 in *gag* and the 3' splice site of *c-myb* exon 2, as illustrated in Fig. 2A. The sequence of the junction region of this cDNA is shown in Fig. 3. It appears that the chimeric mRNA was generated by splicing of a primary transcript that initiated in the viral 5' LTR and continued through the polyadenylation signals in the 3' LTR into the adjacent *c-myb* gene.

The second class of cDNAs from tumor 21, pBS21-5 and pBS21-8, contained *c-myb* intron 1 sequences between ALV 3' LTR sequences and *myb* exon 2 sequences (Fig. 2A). These viral sequences began at the *EcoRI* site (at nt -53) in the U3 region of the LTR. In contrast to the case for pBS21-7 (Fig. 2A), these cDNAs lacked viral leader sequences as well as the last 2 nt of U5. These two features identified the viral sequences as derived from readthrough of the 3' LTR of an integrated provirus. These transcripts may represent incompletely processed ALV readthrough transcripts, readthrough of spliced ALV *env* mRNA, or transcripts initiated upstream of the 3' LTR initiation site at the U3/R junction. The *myb* sequences downstream of exon 2 were correctly spliced in these transcripts (23). Northern analysis of tumor RNAs with a *myb* intron 1 probe indicated that this second class of RNAs represents a minor fraction of the total *myb* RNA in some but not

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-

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 $p75^{myb}$; gray arrow, truncated $p73^{myb}$. No Myb protein was detected in normal liver (NL). The positions of molecular mass markers (in kilodaltons) are shown on the left.

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* [$T_{(-20)}myb$] (Fig. 5A) and tested their oncogenicities in vivo by infection 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 subgenomic mRNA, by using the *gag* 5' splice site and the 3' splice site of the original *v-src* gene. In the case of the $T_{(-20)}myb$ 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 $T_{(-20)}myb$ 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 each of the *myb* viruses is summarized in Table 1. Over 90% of the birds infected with RCASBP/ $T_{(-20)}myb$ developed tumors by 10 weeks. Surprisingly, the tumors included not only lymphoma (44% of birds) but also sarcoma (40%), adenocarcinoma (29%), and myeloid

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 [$T_{(-20)}myb$], 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.

TABLE 1. Oncogenicities of *myb* viruses^a

RCASBP insert	No. of chickens infected	No. (%) with:				Total no. of birds (%) with malignancy
		Lymphoma	Sarcoma	Carcinoma	Myeloid leukemia	
T ₍₋₂₀₎ myb	48	21 (44)	19 (40)	14 (29)	3 (6)	44 (92)
Cmyb	55	6 (11)	1 (2)	1 (2)	0	8 (15)
11/12A ^b	65	2 (3)	7 (11)	2 (3)	3 (5)	16 (25)
None	26	0	1 (4)	0	1 (4)	2 (8)

^a Birds were injected as 12-day-old embryos with RCASBP vectors containing the indicated *myb* inserts or with RCASBP lacking an insert. Chickens were sacrificed at 10 weeks after hatching. Results are the sum of those from two separate experiments.

^b Birds infected with the RCASBP/11/12A virus also developed low incidences of nephroblastoma (2%) and erythroid leukemia (3%).

leukemia (6%). Many of the birds had more than one type of tumor. Although the lymphomas usually appeared earlier than sarcomas or carcinomas, all of these tumors appeared with a short latency.

The birds with the RCASBP/T₍₋₂₀₎myb-induced lymphomas exhibited enlarged and heavily infiltrated livers and occasionally had 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 bursa 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 lymphomatous 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. Phosphor-

ylation 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

FIG. 6. Effect of time of infection on oncogenicity of the RCASBP/T₍₋₂₀₎myb retrovirus. Chicken embryos were infected after either 12 or 16 days of incubation. In addition, chicks were infected 1 day after hatching. The percentage of tumors observed was determined 10 weeks after hatching; thus, there is a slight difference in latent periods assayed for the different times of infection. In addition to the data plotted, chicks infected at 1 day also developed a spindle cell tumor in the kidney. Solid bars, B-cell lymphoma; gray bars, sarcoma; hatched bars, carcinoma.

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 $T_{(-20)}myb$ 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).

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 $T_{(-20)}myb$ 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 $T_{(-20)}myb$ -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-24-transformed chicken erythroblast cell line that expresses high levels of wild-type 75-kDa c-Myb protein (13, 16), was also included as a control. The $T_{(-20)}myb$ -induced tumors of both liver and kidney (32L, 33L, 33K, and 35K) expressed a Myb protein that migrated slightly faster than the wild-type 75-kDa protein, corresponding to a molecular mass of approximately 73 kDa, consistent with the 20-amino-acid truncation encoded by this construct (Fig. 7B). Furthermore, the RCASBP/ $T_{(-20)}myb$ protein comigrated with the protein expressed in an EU-8-induced tumor (184L), confirming our prediction that the insertionally activated *myb* gene product in EU-8 lymphomas is truncated by 20 amino acids.

DISCUSSION

Aberrant splicing from EU-8 ALV generates a truncated Myb protein. The recombinant ALV EU-8 induces a high incidence of B-cell lymphoma with a short latency when introduced into chicken embryos. In the lymphomas, EU-8 proviruses were integrated into the *c-myb* locus either in the distal region of intron 1 or upstream of exon 1. Both types of integration resulted in the same chimeric mRNAs, generated by readthrough from the intact ALV genome into the *myb* gene

followed by splicing from the ALV major 5' splice site to *myb* exon 2. This mRNA encodes a Myb protein truncated by only 20 amino acids at its N terminus. In the case of proviral integration upstream of the first *c-myb* exon, Myb truncation occurred by a novel mechanism in which *myb* exon 1 was spliced out of the chimeric primary transcript, presumably because it lacks a 3' splice site.

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 [$T_{(-20)}myb$] 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 $T_{(-20)}myb$ 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 lymphoma 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 $T_{(-20)}myb$. 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 $T_{(-20)}myb$ 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 T₍₋₂₀₎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. Retroviruses 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/T₍₋₂₀₎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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