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Overexpression of C-Terminally but Not N-Terminally Truncated Myb Induces Fibrosarcomas: a Novel Nonhematopoietic Target Cell for the *myb* Oncogene

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The *myb* oncogene encodes a DNA-binding transcriptional transactivator which can become a hematopoietic cell-transforming protein following the deletion of amino acid sequences from either its amino or carboxyl terminus. Although a number of hematopoietic tumors express terminally deleted variants of Myb, the involvement of truncated Myb in nonhematopoietic tumors has not been adequately investigated. To assess the full spectrum of Myb's oncogenic capability, a replication-competent retroviral vector (RCAMV) was used to express a full-length protein (C-Myb), an amino-terminally truncated protein (VCC- or ΔN-Myb), a carboxyl-terminally truncated protein (T-Myb), or a doubly truncated protein (VCT-Myb) in vivo. These viruses were injected intravenously into 10-day chicken embryos, and the infected chicks were monitored for tumors. Approximately 4 to 8 weeks after hatching, the majority (30 of 39 [77%]) of animals infected with the T-Myb retrovirus (without 214 carboxyl-terminal residues) developed nodular muscle tumors which could be identified by both morphologic and immunohistochemical criteria as fibrosarcomas. Identically appearing tumors could also be found in the kidney of some T-Myb-infected animals. The T-Myb-induced fibrosarcomas expressed the appropriately sized T-Myb protein, contained an unaltered proviral T-*myb* gene, and showed clonal proviral integration sites. In comparison, no sarcomas were observed in any of the animals infected with the amino-terminally truncated (VCC- and ΔN-Myb) or doubly truncated (VCT-Myb) viruses. A loss of carboxyl-terminal but not amino-terminal sequences can thus convert Myb into a potent in vivo transforming protein for nonhematopoietic mesenchymal cells. In comparison, a truncation of either or both ends of the protein can activate Myb into a hematopoietic cell-transforming protein.

c-*myb*, the cellular homolog of the v-*myb* transforming gene from avian myeloblastosis virus (AMV), encodes a nuclear DNA-binding transcriptional transactivator involved in regulating normal and neoplastic hematopoietic cell development. c-*myb* is expressed predominantly in undifferentiated hematopoietic progenitor cells, and as the cells mature, c-*myb* expression disappears (7, 19, 67). Constitutive overexpression of c-*myb* in immature erythroid or myeloid cells prevents their differentiation implying a crucial functional role for c-*myb* in hematopoietic cell development (5, 35, 58). Alternatively, inhibition of c-*myb* expression with antisense c-*myb* oligonucleotides causes growth arrest not only in hematopoietic cells (2, 16) but also in neuroectoderm-derived cell lines (50), epithelial cell-derived colonic adenocarcinoma cell lines (36), and mesenchymally derived smooth muscle cells (61, 62). Finally, before dying, mouse embryos homozygous for a c-*myb* knockout gene show significant hematopoietic hypoplasia, confirming an essential in vivo role for c-*myb* in fetal hematopoietic development (40).

The transcriptional transactivator protein encoded by c-*myb* has at least three known effector domains. The amino terminus of the protein contains three imperfect tandem repeats of 51 to 52 amino acids which constitute a functional DNA binding domain (30, 56). Structurally, this DNA binding domain resembles the well-characterized helix-turn-helix DNA binding domain of the homeodomain family of transcription factors (13, 54). Once bound to its specific recognition site (PyAACG/TG), Myb is able to activate the transcription of nearby genes by an undetermined mechanism which requires the presence of a well-conserved 50-amino-acid transcriptional transactivation domain located in the middle of the protein (68). Immediately C terminal to this *myb* transcriptional transactivation domain lies a negative regulatory domain that leads to inhibition of transactivation activity (56). The leucine zipper motif within this negative regulatory domain is essential for transcriptional inhibitory activity, perhaps by allowing the binding of an associated inhibitory factor (26).

The conversion of Myb into a hematopoietic cell-transforming protein requires a loss of amino acid sequences from either the amino (N) or carboxyl (C) terminus (or both). For example, compared with full length c-Myb, the AMV v-Myb oncoprotein has extensive deletions at both its amino and carboxyl termini (28, 29). In addition, virus-induced murine myeloid tumors often contain a truncated c-*myb* gene with proviral integration either at the 5′ or 3′ end of the *myb* coding sequence (41, 59, 60, 66). Similarly, virally induced avian B-cell lymphomas often contain a proviral integration in the 5′ end of c-*myb* and produce an amino-terminally truncated protein (27, 45). These naturally occurring amino-terminally truncated Myb proteins maintain a functional DNA binding domain but have deletions of an upstream regulatory casein kinase II phosphorylation site (34). In comparison, the naturally occurring carboxyl-terminally truncated Mybs have deletions that remove some of the transcriptional inhibition domain (often including the leucine zipper). The overexpression of Myb proteins with similar amino- or carboxyl-terminal deletions results in hematopoietic cell transformation in vitro, confirm-

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ing the requirement for terminal sequence losses in the oncogenic activation of Myb (20). The transforming capability of the C-terminally truncated Myb variants correlates with their enhanced DNA binding and/or transcriptional transactivational activity (21, 49).

The focus of the present line of investigation was to delineate the relationship between terminally truncated Myb proteins and their tumorigenic activity in vivo. In particular, Myb proteins with amino- and/or carboxyl-terminal truncations analogous to those within Myb-induced tumors were overexpressed in whole animals, and these animals were then monitored for neoplastic disease. This approach allowed not only a determination of the structure-function characteristics of Myb but also an evaluation of the spectrum of cell types susceptible to Myb-induced oncogenesis in vivo. We have found that the in vivo overexpression of a carboxyl-terminally (but not an amino-terminally) truncated Myb protein efficiently induces aggressive fibrosarcomas. In comparison, Myb proteins with truncations of either or both termini transform hematopoietic cells (20). Our findings not only confirm that carboxyl-terminal truncation oncogenically activates the Myb protein but also define a new nonhematopoietic mesenchymal target cell for Myb-induced cell transformation.

MATERIALS AND METHODS

Retroviral constructs. The various Myb retroviral constructs were each subcloned as *Cla*I fragments into the unique *Cla*I site of the replication-competent retroviral vector RCAMV (48). To allow Myb expression in its natural target cell, the regulatory long terminal repeats of this vector were from the oncogenic Myb-expressing AMV. *myb* cDNAs with *Cla*I ends were generated by subcloning each construct into the adaptor plasmid Cla12-Nco (23). This plasmid contains two *Cla*I sites flanking a multienzyme polylinker cloning region.

The full-length 2.8-kb thymic c-*myb* cDNA (53) was subcloned as a *Hin*dIII-*Xho*I fragment, first into the *Hin*dIII-*Sal*I site of Cla12-Nco and then as a *Cla*I fragment into RCAMV. This c-*myb* cDNA (derived from normal chicken thymus) encodes a protein with 58 additional N-terminal amino acids relative to another c-*myb* cDNA cloned from a lymphoma cell line (15). We chose the thymic and not the lymphoma c-*myb* cDNA as our normal full-length c-*myb*, as it is the only c-*myb* cDNA derived from a nontransformed cell source.

The ΔN-*myb* cDNA insert was generated by an initial PCR amplification of the 295- to 611-nucleotide region of the thymic c-*myb* cDNA, using 5′-GATCAAGCTTGAGAGATGGAC GACCACGA-3′ as the upstream primer (note the *Hin*dIII site and the ATG initiator codon) and 5′-CTTCCCTTCAAAT GCTTAGC-3′ as the downstream primer. After digestion with *Hin*dIII and *Age*I, the 190-bp PCR fragment (nucleotides 295 to 477 of c-*myb*) was used to replace the 480-bp *Hin*dIII-*Age*I fragment from the full-length c-*myb* cDNA (nucleotides 1 to 480). This 2.5-kb ΔN-*myb* cDNA has an open reading frame beginning with the ATG at nucleotide 298 of the thymic c-*myb* cDNA clone and has thus lost 78 amino-terminal amino acids.

The VCC-*myb* cDNA was generated by replacing the *Hin*dIII-*Age*I fragment of c-*myb* (nucleotides 1 to 480) with a shorter 70-bp *Hin*dIII-*Age*I fragment from a subgenomic v-*myb* cDNA clone (48, 52). The resulting 2.4-kb VCC-*myb* cDNA has an open reading frame beginning with six amino acids from the viral *gag* gene (MEAVIK) followed by c-*myb* sequences beginning at nucleotide 451. VCC-Myb has thus lost 129 amino-terminal amino acids. None of the 11 amino acid changes of v-Myb are present in VCC-Myb.

The T-*myb* cDNA was generated by cutting c-*myb* cDNA with *Bal*I and *Nhe*I, blunting with Klenow enzyme, and religating the ends so as to join the *Bal*I site at nucleotide 1515 to the *Nhe*I site around nucleotide 2400. This manipulation generated a premature TAG stop codon just downstream of the ligation junction (the destroyed *Bal*I site at nucleotide 1515). The resulting 1.9-kb T-*myb* cDNA encodes a protein without 214 carboxyl-terminal amino acids.

The VCT-*myb* cDNA was generated as a chimera between the VCC-*myb* and T-*myb* cDNAs by replacing the *Hin*dIII-*Sal*I 5′ half of T-*myb* (1.2 kb) with the analogous *Hin*dIII-*Sal*I 5′ half from VCC-*myb* (0.7 kb). The resulting 1.5-kb VCT-*myb* cDNA should encode a protein without 129 amino-terminal and 214 carboxyl-terminal amino acids. None of the 11 amino acid changes of v-Myb are present in VCT-Myb.

Virus production and quantitation. Primary chicken embryo fibroblasts (CEFs) prepared from a line of chickens free of endogenous retroviruses (line EV-0) were transfected with each of the Myb-RCAMV plasmid constructs or with the RCAMV-0 control plasmid (no Myb insert), and virus-containing culture supernatants were then harvested as previously described (48). To quantitatively estimate relative infectious particles in these supernatants, serial 10-fold dilutions of each viral stock solution (0.1 ml) were used to infect fresh CEFs as previously described (48). Three days after infection, these infected cultures were fixed and stained by immunofluorescence for the cytoplasmic viral $p27^{gag}$ antigen (see below). The most dilute virus solution that still gave a predominantly positive Gag signal was considered to be the endpoint titer.

Immunoprecipitation analysis. CEFs were infected with equal volumes (containing approximately equivalent titers) of viral stock solutions, either one of the five Myb-RCAMV viruses (C-, ΔN-, T-, VCC-, or VCT-Myb), or the RCAMV-0 control virus. After 3 weeks, the infected CEFs were labeled in vivo with [^{35}S]methionine, lysed in detergent buffer, and analyzed for Myb protein expression by immunoprecipitation with Myb monoclonal antibody 4/14 (4) as previously described (48). Sarcoma cells grown in tissue culture after isolation from *myb*-infected animals were similarly analyzed for the expression of Myb protein. To prepare these cells, sarcoma nodules were trimmed free of surrounding muscle and placed into sterile culture medium (without serum). The firm nodule was then minced into millimeter-size tissue pieces with sterile scissors and was digested at 39°C for 40 to 60 min (with stirring) with collagenase (1 mg/ml) and trypsin (0.25%, 1:250). Following a low-speed spin, the supernatant was removed, and the single-cell suspension from this tissue digestion was washed in serum-containing medium and plated into the appropriately sized culture vessel in CEF culture medium (48). These fibroblastoid sarcoma cell cultures were then maintained, amplified, and in vivo labeled exactly as were the CEF cultures (48).

Chickens. The same stocks of virus used to infect the CEFs were used to infect 10-day chicken embryos in vivo. Embryonated eggs (line SC; Hyline International, Dallas Center, Iowa) were incubated in a humidified egg incubator (Petersime Incubator Co., Gettysburg, Ohio). By using a 30-gauge needle, undiluted viral stock solutions of approximately equivalent titers (0.1 ml) were injected intravenously into a chorioallantoic vein of a 10-day chicken egg. After hatching, these infected chicks were reared in the Wistar Institute Animal Facility and were monitored by both general appearance and palpation for the presence of neoplasms. Sick birds, those with palpable tumors, or those 8 weeks of age were sacrificed and necropsied immediately.

Histology and immunohistochemistry. Immediately after sacrifice, thin slices of tumor tissue and control normal tissue

were processed for histologic and immunohistochemical analysis by a number of different methods. For routine morphologic analysis, the tissue was fixed in buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin.

For most immunohistochemical procedures, the tissue was fixed in cold ethanol and processed by a modification of the method of Sainte-Marie (11). After paraffin embedding, thin sections (8 μM) on glass slides were deparaffinized (55) and rehydrated in phosphate-buffered saline (PBS). A minimal volume of primary antibody specific for a viral or cellular antigen was then reacted with the section for 30 to 60 min at room temperature in a humidified chamber. After two 5-min PBS washes, a fluorescein isothiocyanate-labeled (for Gag) or biotinylated secondary antibody reactive with the primary antibody was added to the slide and incubated for 30 min. The sections were then washed twice in PBS, and those reacted with a biotinylated secondary antibody were incubated with avidin DH-biotinylated horseradish peroxidase H complex (Vector Laboratories). Peroxidase activity was visualized microscopically after incubation with diaminobenzidine and hydrogen peroxide (Vectastain kit) as instructed by the manufacturer (Vector Laboratories). For viral Gag antigen localization, tissue-associated fluorescence was visualized with a fluorescence microscope.

Immunohistochemical localization of the Myb protein required the use of frozen tissue sections, prepared by embedding fresh tumor tissue in OCT medium (Miles Inc.) and snap-freezing the material in 2-methyl butane immersed in liquid nitrogen. Thin (8-μm) sections of frozen tissue were fixed in cold acetone, dried, and stained with Myb monoclonal antibody 2.7 as described by Pizer et al. (46). For experiments requiring serial sections stained sequentially with Myb and other antibodies, frozen tissue sections were used, and the appropriate dilution of the desired primary antibody was substituted for Myb antibody.

Antibodies. A rabbit polyclonal antibody to the $p27^{gag}$ retroviral core antigen was purchased from SPAFAS and was used at a dilution of 1:60. Rabbit anti-chicken α-keratin serum was from Loren Knapp (43) and was used at a dilution of 1:400. Mouse monoclonal antibody to human epithelial keratin (AE1/AE3) was purchased from Boehringer Mannheim and used at a dilution of 1:100. A mouse monoclonal antibody to Myb (2.7) was produced from a hybridoma purchased from the American Type Culture Collection (9). Mouse monoclonal antibodies specific for the amino-terminal propeptide of type I procollagen (SP1.D8) (12), for the sarcomere of skeletal muscle (MF-20) (3), and for the muscle-specific intermediate filament protein desmin (D3) (8) were obtained from the Developmental Studies Hybridoma Bank. HHF35, the mouse monoclonal antibody to muscle actin (65), was obtained from Enzo Diagnostics (New York, N.Y.). IA4, the mouse monoclonal antibody to alpha smooth muscle actin (63), was obtained as an ascites fluid from Sigma and was used at a 1:400 dilution. The fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G secondary antibody was obtained from Organon Teknika Corp. (West Chester, Pa.) and was used at a 1:50 dilution. The biotinylated anti-mouse immunoglobulin G secondary antibody was provided in the Vectastain kit (Vector Laboratories) and was used at a 1:200 dilution. The common leukocyte monoclonal antibody CLA3 (also called ChL3) has been described previously (44). Unless otherwise mentioned, all monoclonal antibodies were used as undiluted culture supernatants.

Genomic Southern blots. To prepare high-molecular-weight DNA, frozen tissue pieces were thawed, minced with a razor blade into fine pieces, and placed into 1 ml of lysis buffer (10 mM Tris [pH 7.5], 0.1 M EDTA, 0.5% sodium dodecyl sulfate [SDS], 0.1 mg of proteinase K per ml). After a 2- to 3-h digestion at 56°C, the lysate was extracted once with phenol, twice with phenol-chloroform (50/50), and once with chloroform. Genomic DNA was then precipitated, dissolved in Tris-EDTA, and quantitated by optical density. For Southern analysis, 10-μg aliquots of DNA were digested with the appropriate restriction enzyme as instructed by the manufacturer, subjected to electrophoresis through a 0.8% agarose gel, transferred to an Immobilon-N membrane as recommended by the manufacturer (Millipore), and hybridized to a labeled DNA probe. The 5′-*myb* probe was a 393-bp *Sac*II-*Nco*I fragment (nucleotides 125 to 518) from the chicken thymic c-*myb* cDNA clone (53). The retroviral envelope probe was a 1.1-kb *Kpn*I-*Eco*RI fragment of the Rous-associated virus-0 envelope gene from plasmid R-8 (22). The probes were ^{32}P labeled by random priming, using a kit from Bethesda Research Laboratories.

RESULTS

Construction of retroviruses for the in vivo expression of full-length or truncated Myb protein. To assess the in vivo oncogenic capability of an overexpressed, activated Myb protein, we infected whole chicken embryos with a retrovirus expressing either a full-length c-*myb* cDNA or an amino- or carboxyl-terminally truncated *myb* cDNA. The truncated *myb* cDNAs corresponded to oncogenic versions of *myb* from virus-induced hematopoietic tumors of the chicken or mouse (27, 28, 45, 60, 66). Figure 1A shows the proteins encoded by each of the five *myb* cDNA constructs designed for in vivo expression within the chicken. These cDNAs were engineered so as to express either a full-length Myb protein (C-Myb), an amino-terminally truncated Myb protein (ΔN-Myb and VCC-Myb), a carboxyl-terminally truncated Myb protein (T-Myb), or a doubly truncated Myb protein (VCT-Myb). The full-length thymic c-*myb* cDNA used in these studies is the longer of two c-*myb* cDNAs described to date (53). It is the only c-*myb* cloned from a normal tissue source (chicken thymus), and it encodes a protein with an additional 58 N-terminal amino acids compared with the protein encoded by a lymphoma-derived c-*myb* cDNA (15). The two amino-terminally truncated Mybs (ΔN- and VCC-Myb) maintain a functional DNA binding domain but lack the casein kinase II phosphorylation site which, when phosphorylated in vivo, results in a significant decrease in DNA binding activity (34). The carboxyl-terminally truncated T-Myb protein has lost 74 of the 176 amino acids from its transcriptional inhibitory domain.

Each of these *myb* cDNAs was subcloned into the replication-competent AMV-based retroviral vector RCAMV (Fig. 1B) and transfected into CEFs (48). The virus-containing culture supernatant harvested from these transfected CEFs was then used to infect fresh CEFs. Immunoprecipitation analysis of the CEFs infected with each of the five Myb viruses showed expression of the appropriately sized Myb protein (Fig. 1C). In contrast, cells infected with an RCAMV virus without a Myb insert (RCAMV-0) failed to express Myb protein. The size of each viral Myb protein, as judged by its gel migration, was slightly larger than the predicted size of the primary translation product.

Having confirmed that each of the Myb-RCAMV viruses promoted the expression of the appropriately sized Myb protein, the same stocks of virus were used for intravenous (in ovo) injections of 10-day chicken embryos. The infected chicks from these eggs were hatched and carefully observed over the next 8 weeks for the presence of Myb-induced tumors. To

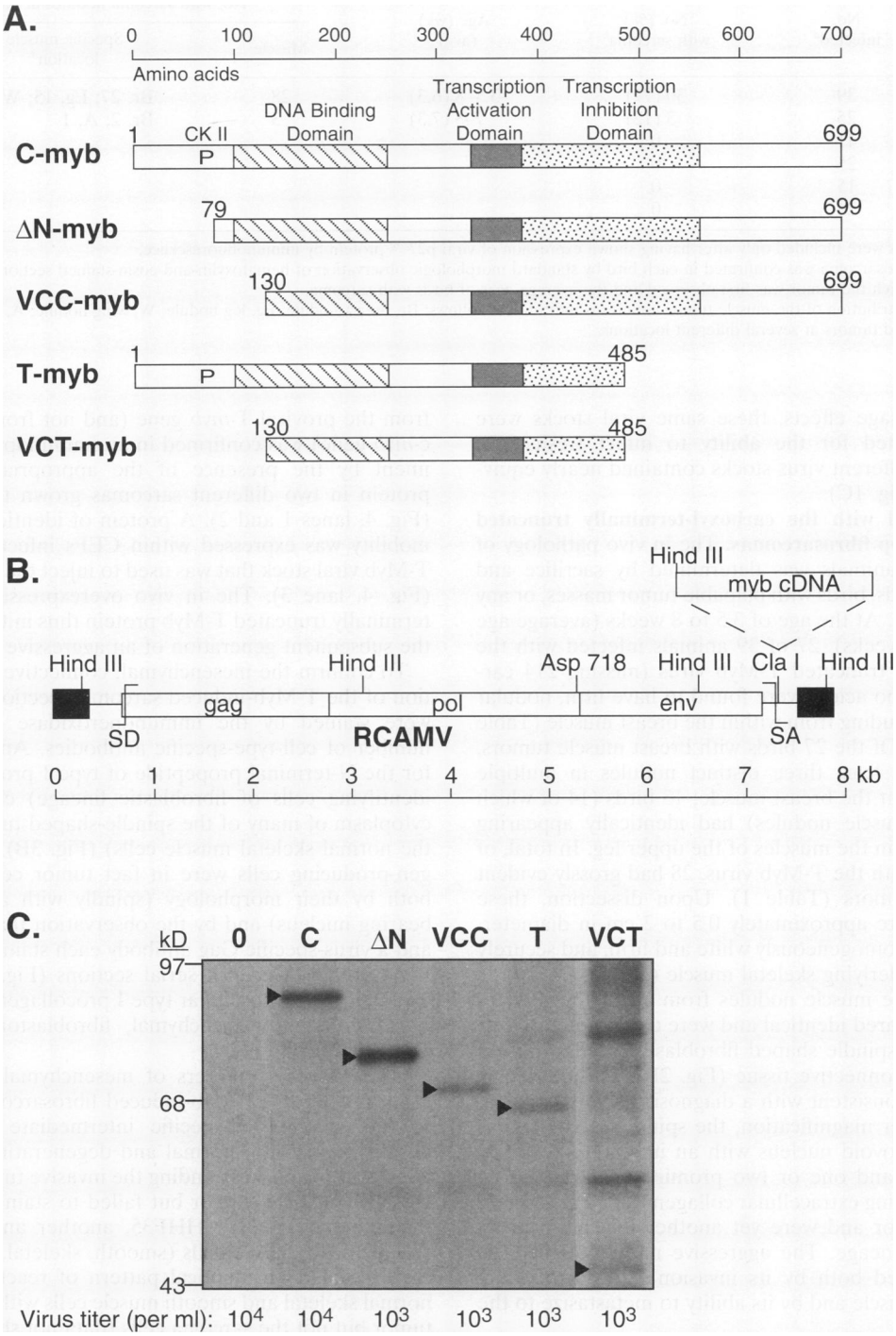

FIG. 1. Expression of full-length and truncated Myb from the RCAMV retroviral vector. (A) Map of the proteins encoded by each of five *myb* cDNA constructs, with their respective functional domains indicated (P, casein kinase II [CK II] phosphorylation site). These five cDNAs were each subcloned into the RCAMV retroviral vector (B) at the unique *Cla*I site. Relevant restriction enzyme sites and viral structural genes are indicated. The black boxes at either end represent the AMV-derived proviral long terminal repeats, and SD and SA indicate splice donor and splice acceptor sites, respectively. Each of the five Myb-RCAMV viral stocks (C-, ΔN-, VCC-, T-, and VCT-Myb) and an RCAMV-0 (vector only) control virus (0) was used to infect CEFs. These infected CEFs were in vivo labeled with [^{35}S]methionine, lysed, immunoprecipitated with Myb monoclonal antibody 4/14, and analyzed by SDS-polyacrylamide gel electrophoresis (C). Arrowheads point to bands representing each of the five Myb proteins. To show the appropriate protein band, the VCT lane required a longer autoradiographic exposure than the other lanes. The predicted sizes of the primary Myb translation products are as follows: C-Myb, 78 kDa; ΔN-Myb, 70 kDa; VCC-Myb, 65 kDa; T-Myb, 54 kDa; and VCT-Myb, 41 kDa. The infective titer of each virus stock (as determined by serial dilution) is also indicated.

TABLE 1. Incidence of sarcomas in Myb-infected birds

Virus	No. infected[a]	No. (%) with sarcoma[b]	Age (wk) (avg)[c]	No. with sarcoma nodules in indicated location		
				Muscle	Specific muscle location[d]	Kidney
T-Myb	39	30 (77)	3.5–8 (6.3)	28	Br, 27; Lg, 15; W, 1	7
C-Myb	25	3 (12)	7–8 (7.3)	3	Br, 2; A, 1	0
ΔN-Myb	41	0				
VCC-Myb	25	0				
VCT-Myb	15	0				
RCAMV-0	24	0				

[a] Birds without tumors were included only after having shown expression of viral $p27^{gag}$ protein by immunofluorescence.
[b] The diagnosis of fibrosarcoma was confirmed in each bird by standard morphologic observation of hematoxylin-and-eosin-stained sections.
[c] The age range at which the tumor was first observed and the average ages of birds with sarcoma.
[d] The anatomic subdistribution of the muscle tumor nodules is indicated as follows: Br, breast nodule; Lg, leg nodule; W, wing nodule; A, abdominal wall nodule. Note that many birds had tumors at several different locations.

minimize virus dosage effects, these same viral stocks were quantitatively titrated for the ability to infect fresh CEF cultures. The six different virus stocks contained nearly equivalent viral titers (Fig. 1C).

Animals infected with the carboxyl-terminally truncated T-Myb virus develop fibrosarcomas. The in vivo pathology of the Myb-infected animals was determined by sacrifice and necropsy of sick birds, birds with palpable tumor masses, or any bird 8 weeks of age. At the age of 3.5 to 8 weeks (average age at sacrifice of 6.3 weeks), 27 of 39 animals infected with the carboxyl-terminally truncated T-Myb virus (missing 214 carboxyl-terminal amino acids) were found to have firm, nodular tumor masses protruding from within the breast muscle (Table 1 and Figure 2A). Of the 27 birds with breast muscle tumors, 17 animals had at least three distinct nodules in multiple locations throughout the breast muscle; 15 birds (14 of which also had breast muscle nodules) had identically appearing tumor nodules within the muscles of the upper leg. In total, of 39 birds infected with the T-Myb virus, 28 had grossly evident nodular muscle tumors (Table 1). Upon dissection, these muscle nodules were approximately 0.5 to 2 cm in diameter, nonencapsulated, homogeneously white and firm, and securely attached to the underlying skeletal muscle (Fig. 2A).

Histologically, the muscle nodules from all of the T-Myb-infected birds appeared identical and were composed of interwoven fascicles of spindle shaped fibroblastoid cells within a matrix of fibrillar connective tissue (Fig. 2B). This histologic pattern was most consistent with a diagnosis of fibrosarcoma. Under higher-power magnification, the spindly sarcoma cells had an elongated, ovoid nucleus with an immature vesicular chromatin pattern and one or two prominent nucleoli (Fig. 2C). Fibers resembling extracellular collagen were a prominent feature of the tumor and were yet another indication of its connective tissue lineage. The aggressive nature of this sarcoma was confirmed both by its invasion into surrounding areas of skeletal muscle and by its ability to metastasize to the kidney (Fig. 2D).

The tumors induced by the T-Myb virus express the T-Myb protein and are of mesenchymal origin. Expression of the Myb protein product by the T-Myb-induced sarcoma cells was initially confirmed by immunoperoxidase staining of tumor tissue sections with a Myb-specific antibody. Figure 3C and F show the characteristic nucleus-specific Myb staining within many of the tumor cells (but not the surrounding skeletal muscle cells) of a T-Myb-induced fibrosarcoma. A total of six different muscle sarcomas similarly analyzed showed the same pattern of Myb reactivity with the nuclei of spindle-shaped tumor cells. That this tumor cell Myb protein was expressed from the proviral T-*myb* gene (and not from the endogenous c-*myb* gene) was confirmed in an immunoprecipitation experiment by the presence of the appropriately sized T-Myb protein in two different sarcomas grown transiently in vitro (Fig. 4, lanes 1 and 2). A protein of identical electrophoretic mobility was expressed within CEFs infected with the same T-Myb viral stock that was used to inject the sarcomatous birds (Fig. 4, lane 3). The in vivo overexpression of a carboxyl-terminally truncated T-Myb protein thus initiated or promoted the subsequent generation of an aggressive fibrosarcoma.

To confirm the mesenchymal, connective tissue cell derivation of the T-Myb-induced sarcomas, sections of tumor tissue were stained by the immunoperoxidase technique with a number of cell-type-specific antibodies. An antibody specific for the N-terminal propeptide of type I procollagen (SP1.D8, identifying cells of fibroblastic lineage) clearly stained the cytoplasm of many of the spindle-shaped tumor cells (but not the normal skeletal muscle cells) (Fig. 3B). That these collagen-producing cells were in fact tumor cells was confirmed both by their morphology (spindly with a large nucleolus-bearing nucleus) and by the observation that a Myb antibody and a virus-specific Gag antibody each stained cells within the same area of tissue in serial sections (Fig. 3C and D). The expression of intracellular type I procollagen within the tumor cells implied a mesenchymal, fibroblastoid origin for this T-Myb-induced tumor.

Other specific markers of mesenchymal origin were also used to stain the T-Myb-induced fibrosarcomas. An antibody to the muscle cell-specific intermediate filament protein desmin stained the normal and degenerating skeletal muscle fibers within and surrounding the invasive tumor and the blood vessels within the tumor but failed to stain the sarcoma cells themselves (Fig. 3E). HHF35, another antibody specifically reactive with muscle cells (smooth, skeletal, and cardiac muscle), revealed an identical pattern of reactivity, staining the normal skeletal and smooth muscle cells within and around the tumor but not the sarcoma cells (data not shown). In contrast, an antibody specific for alpha smooth muscle actin (IA4) clearly stained the cytoplasm of many of the sarcoma cells, providing additional support for this tumor's mesenchymal origin (data not shown). As expected, the tumor cells did not express the markers characteristic of either epithelial, hematopoietic, or skeletal muscle cells, as they failed to stain with the respective marker antibodies anticytokeratin, CLA3 (reactive with a common leukocyte antigen), and MF-20 (reactive with sarcomere myosin) (data not shown). The in vivo overexpression of a carboxyl-terminally truncated T-*myb* gene thus efficiently induced an aggressive tumor which could be char-

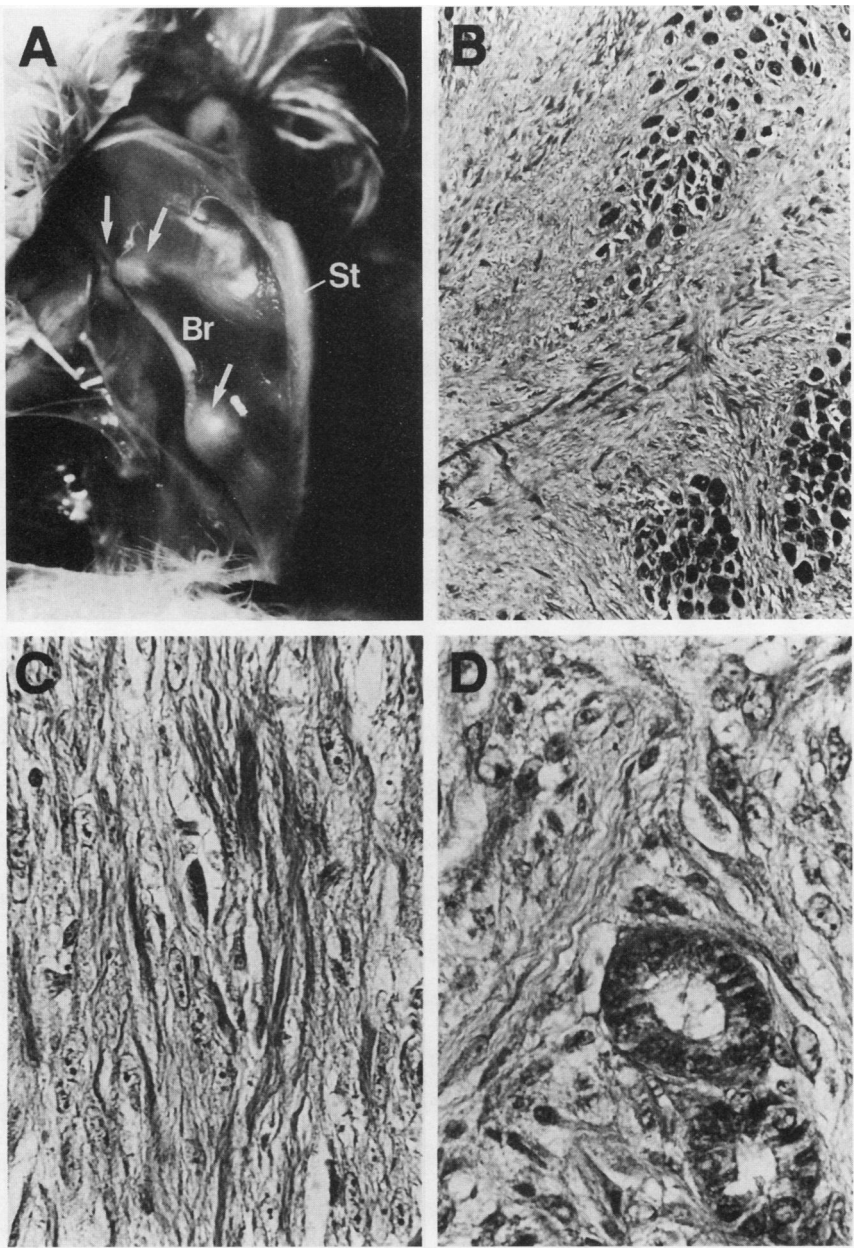

FIG. 2. Birds infected with the carboxyl-terminally truncated T-Myb virus develop fibrosarcomas. (A) Lateral view of the breast area of a representative 6-week-old T-Myb-infected bird. The sternum (St), right breast muscle (Br), and three protruding tumor nodules (arrows) are indicated. Low- and high-power photomicrographs of one of these T-Myb-induced muscle nodules are also shown (B [hematoxylin and eosin stain] and C [Gomori's trichrome stain]). A kidney metastasis in another T-Myb-infected bird is shown in panel D (hematoxylin and eosin stain).

acterized both morphologically and immunohistochemically as a mesenchymally derived sarcoma. Prior to this report, truncated *myb* was thought to be involved, as an active transforming gene, exclusively in hematopoietic tumors. This description of a novel sarcomagenic activity for T-*myb* then expands the spectrum of cell types susceptible to *myb*-induced transformation to those of nonhematopoietic mesenchymal origin.

A minority of birds infected with the T-Myb virus develop kidney sarcomas. In addition to the high incidence of muscle fibrosarcomas affecting birds infected with the T-Myb virus (28 of 39), 7 of these same animals (5 of which also had breast muscle nodules) had grossly evident kidney tumors that were morphologically indistinguishable from the muscle fibrosarcomas (Table 1 and Fig. 2D). The total number of fibrosarcoma-bearing birds was therefore 30 out of the 39 that were injected with T-Myb virus (77%). The invasive nature of these kidney sarcomas was confirmed by the presence of only small amounts of residual normal kidney tissue, the remainder having been displaced by the aggressive tumor. Immunoperoxidase staining of these T-Myb-induced kidney tumors with the same panel of antibodies used on the muscle sarcomas revealed an identical pattern of reactivity: the tumor cells expressed Myb, procollagen, and smooth muscle actin but not desmin, HHF35, cytokeratin, or common leukocyte antigen (data not shown).

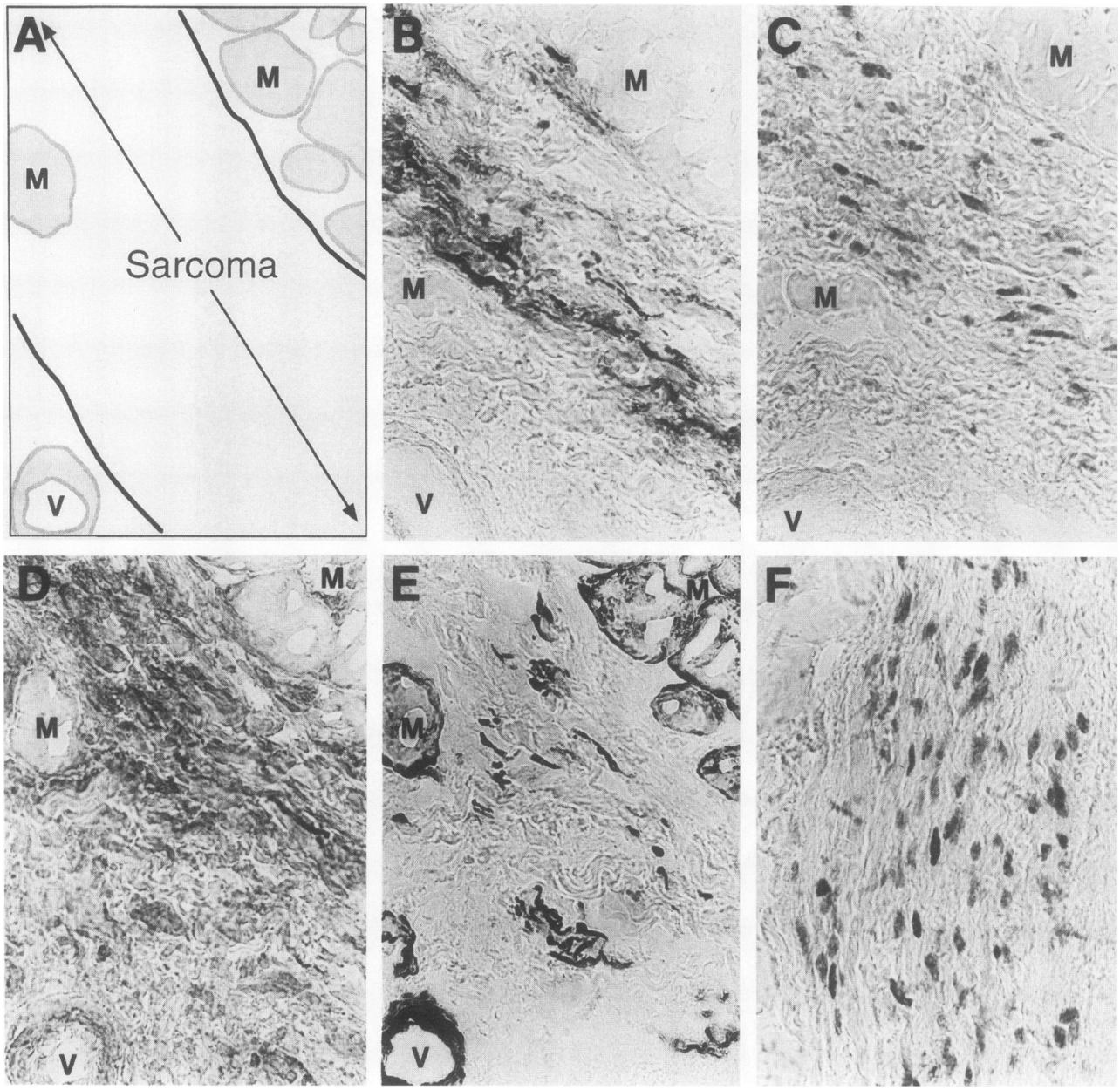

FIG. 3. The T-Myb-induced tumors express Myb and Gag and are of mesenchymal origin. Adjacent serially cut frozen sections of a muscle nodule from a T-Myb-infected bird were stained by the immunoperoxidase method with a number of different antibodies. (A) Schematic drawing of the area of tissue photographed in panels B to E. In this area, a fascicle of invasive tumor stretches from upper left to lower right. Noteworthy landmarks include two areas of normal skeletal muscle (M) and a blood vessel (V). Adjacent serial sections were stained with antibodies reactive with intracellular procollagen (monoclonal antibody SP1.D8 [B]), Myb (monoclonal antibody 2.7 [C]), viral Gag protein (rabbit polyclonal serum [D]), or desmin (mouse monoclonal antibody D3 [E]). (F) Higher-magnification view of another area of tissue (same tumor) stained with the Myb antibody. As no counterstains were used, any visible staining is antibody specific.

Although these kidney fibrosarcomas are probably metastatic lesions, it is possible that they arose from a susceptible Myb-responsive mesenchymal target cell in the kidney.

The T-Myb-induced sarcomas have clonal proviral integration sites and intact proviral T-*myb* genes. A useful marker of the malignant nature of a particular neoplasm has been the determination of its content of clonally derived cells. Highly malignant tumors often result from the outgrowth of one (or a few) aggressive clones. As retroviral proviruses insert into random locations within the host cell genome, the detection of a specific nonrandom site of provirus integration within an entire population of tumor cells is an accurate indication of the presence of a clonally derived tumor cell subpopulation.

To assess the extent of clonality within the T-Myb-induced fibrosarcomas, tumor DNA was digested with *Asp* 718 (an enzyme with only a single site within the Myb provirus) and subjected to genomic Southern blot analysis with a virus-specific envelope gene probe (Fig. 5A). The strongly hybridizing 4.7-kb band in every lane represents the endogenous ev-1 provirus present in all line SC birds (24). The 8.9-kb fragment

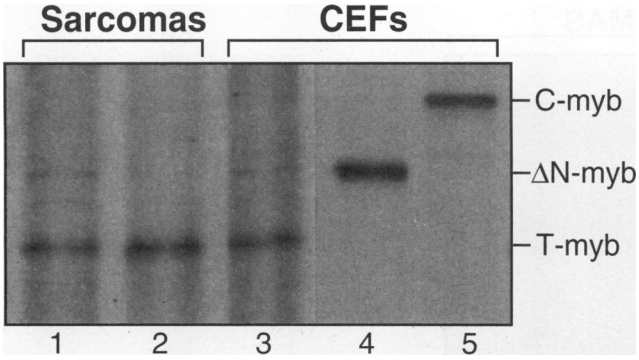

FIG. 4. The T-Myb-induced sarcoma cells express a carboxyl-terminally truncated T-Myb protein. The sarcoma cells prepared from the tumor nodules of two different birds were expanded in tissue culture for 2.5 weeks prior to in vivo labeling with [^{35}S]methionine. The lysates from these labeled sarcoma cells (lanes 1 and 2) were then immunoprecipitated with a Myb antibody (monoclonal antibody 4/14) and analyzed by SDS-polyacrylamide gel electrophoresis. The control lanes (3 to 5) show Myb immunoprecipitates of lysates prepared from CEFs that had been infected with the same stocks of virus used in the embryo injections (lane 3, T-Myb; lane 4, ΔN-Myb; lane 5, C-Myb).

seen in three of the birds then most likely represents the ev-4 endogenous provirus present in some, but not all, line SC chickens (24). In comparison, each tumor cell DNA sample (but none of the control tissue samples) contained at least one additional *env*-reactive fragment, consistent with at least one subpopulation of clonally derived tumor cells in every tumor. Every one of these same *env*-specific fragments was also detected after hybridization of this blot with a *myb* probe (Fig. 5B, arrowheads). In this *myb*-probed blot, the 7-kb band in each lane represents the endogenous c-*myb* gene and each of the other bands represents a clonal retroviral integration event. As the unique *Asp* 718 site within the Myb-RCAMV provirus is just upstream of the viral envelope gene (Fig. 1), the detection of identical tumor tissue DNA fragments hybridizing to both the *myb* and *env* probes is a clear indication of tumor cell mono- or oligoclonality. The detection of specific clonal T-Myb proviral integration fragments in a total of 12 of 13 T-Myb-induced sarcomas (from seven different birds) implies that the expression of the T-*myb* gene within an appropriate connective tissue target cell contributed to its ability to expand into a clonal population of malignant cells.

At least one bird had a kidney sarcoma and a breast muscle sarcoma which shared two identical clonally derived proviral junctional fragments (of 5.7 and 6.4 kb) (Fig. 5, lanes 5 to 7). The bursa from the same animal, in comparison, lacked any detectable clonal junctional fragment. This finding suggests that these tumors from the same animal were composed of cells from a common origin and that this kidney sarcoma originated from an aggressive subclone that had metastasized directly from the primary muscle tumor. We assume that the muscle nodule (not the kidney nodule) was the primary tumor, as the incidence of muscle tumors far exceeded that of kidney tumors. In contrast, the breast muscle and kidney sarcomas from another animal (lanes 3 and 4) did not clearly share any common clonally derived proviral integration fragments.

To assess whether the T-Myb-induced sarcoma cells were infected with a provirus with an appropriately sized T-*myb* gene, *Hin*dIII-digested tumor DNA was subjected to genomic Southern blot analysis with a *myb* probe. This enzyme cuts the T-Myb provirus once at the 5′ end of the inserted *myb* cDNA and again 2.4 kb downstream (within the 3′ long terminal repeat) (Fig. 1). Figure 6 shows the presence of the T-Myb provirus-specific 2.4-kb *Hin*dIII fragment in each of six muscle sarcomas and one kidney sarcoma from T-Myb-infected birds. The lane to lane variation in the intensity of this provirus-specific fragment most likely reflects uneven amounts of DNA loaded in each gel slot, as the intensity of the endogenous c-*myb* fragments (5.2 and 10 kb) is proportional to that of the T-*myb* fragment from the same lane. The absence of detectable bands other than those at 2.4, 5.2, and 10 kb ruled out retroviral insertion within the endogenous c-*myb* gene as a possible explanation for the expression of a truncated Myb protein in these sarcomas. Retroviral insertion within the endogenous c-*myb* gene could also be excluded as a mechanism for sarcomagenesis, since we were unable to induce

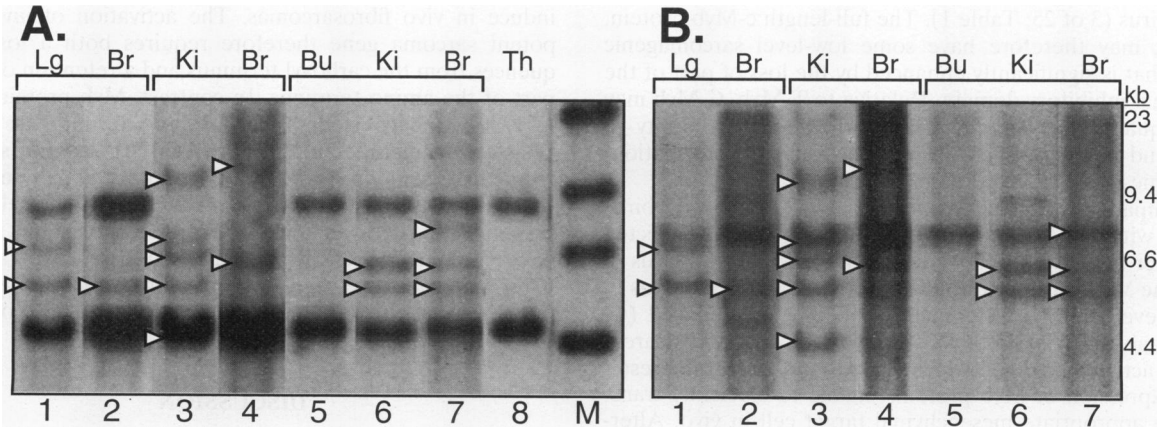

FIG. 5. The T-Myb-induced sarcomas have clonal sites of proviral integration. DNA was prepared from the normal and/or tumor tissue of three different T-Myb-infected birds (lanes 1 and 2, 3 and 4, and 5 to 7) and an uninfected control (lane 8). DNA (10 μg) digested with *Asp* 718 and its isoschizomer *Acc*65I (both are *Kpn*I isoschizomers) was electrophoresed through a 0.8% agarose gel, transferred to an Immobilon-N filter, hybridized to a viral envelope probe (A), washed, and exposed to film. In panel A, the constant bands at 8.9 and 4.7 kb represent the endogenous retroviral genomes in this line of chicken. After stripping, the same blot was rehybridized with a *myb* probe (B). The constant 7-kb band in each *myb*-probed lane represents the endogenous c-*myb* gene. The bands indicated by an arrowhead hybridized to both the envelope and *myb* probes. Lg, leg muscle tumor; Br, breast muscle tumor; Ki, kidney tumor; Bu, bursa; Th, thymus; M, *Hin*dIII-cut lambda DNA markers.

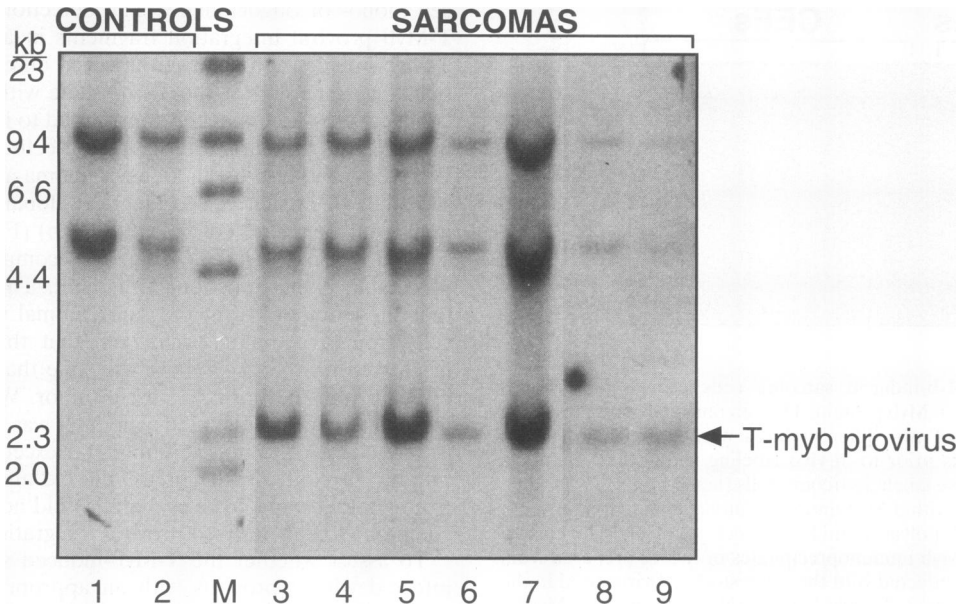

FIG. 6. The T-Myb-induced sarcomas contain an unaltered T-Myb provirus. DNA (10 μg) prepared from uninfected birds (thymus and bursa; lanes 1 and 2), and T-Myb-induced sarcomas (lanes 3 to 9) was digested with *Hin*dIII and subjected to Southern blot analysis as in Fig. 5 but with a *myb* probe. Lanes 3 is from a leg sarcoma, lanes 4 to 7 are from breast sarcomas (different birds), and lanes 8 and 9 are from a breast and kidney sarcoma of the same bird. The bands at 5.2 and 10 kb represent the endogenous chicken c-*myb* gene, while the 2.4-kb band represents the unrearranged proviral T-*myb* gene. Lane M shows *Hin*dIII-cut lambda DNA markers.

sarcomas in any of the birds infected with the RCAMV-0 control virus (Table 1). Each of 14 muscle sarcomas (from 10 different birds) and 3 kidney sarcomas analyzed on similar genomic Southern blots contained a structurally intact, unaltered proviral T-*myb* gene. This conservation of an intact carboxyl-terminally truncated T-*myb* gene within many different tumors might suggest that expression of this gene is necessary for efficient sarcomagenesis.

The full-length, N-terminally truncated, and doubly truncated Myb viruses lack efficient sarcomagenic activity. Although the T-Myb virus was the only one capable of consistently inducing sarcomas, morphologically similar muscle tumors were rarely found in birds infected with the full length C-Myb virus (3 of 25; Table 1). The full-length c-Myb protein, by itself, may therefore have some low-level sarcomagenic activity that is significantly enhanced by the loss of part of the C-terminal inhibitory domain. Relative to T-Myb, C-Myb may have a quantitatively reduced (but still apparent) ability to either bind to or transactivate the necessary transformation-specific mesenchymal cell target genes.

In comparison with the low incidence of sarcomas in birds infected with the C-Myb virus, none of the animals infected with either the ΔN-Myb virus (0 of 41), the VCC-Myb virus (0 of 25), the VCT-Myb virus (0 of 15), or the RCAMV-0 virus (0 of 24) developed any sarcomas (Table 1). The reduced (C-Myb) or absent (ΔN-Myb, VCC-Myb, and VCT-Myb) sarcomagenic activity of these Myb viruses was most likely the result of the expression of Myb proteins unable to efficiently transform the appropriate mesenchymal target cell in vivo. Alternatively, ineffective infection by these viruses could explain the observed results. To confirm, however, that each of these oncogenically ineffective Myb viruses had successfully infected the animals in vivo, bursal tissue from sarcoma-free birds was analyzed by immunofluorescence for the presence of the virus-specific $p27^{gag}$ protein. B cells from every one of the bursas analyzed in this manner (41 from ΔN-Myb birds, 21 from VCC-Myb birds, 14 from VCT-Myb birds, 10 from C-Myb birds, 8 from T-Myb birds, and 19 from RCAMV-0 birds) expressed an abundant amount of $p27^{gag}$ protein (data not shown). In addition, not only did each of these same Myb viruses induce the expression of the appropriately sized Myb protein (Fig. 1), but several (VCC-, VCT-, and T-Myb) possessed in vitro myeloid cell-transforming activity after infection of embryonic splenocyte cultures (47). A nonsarcomagenic *myb* oncogene, not an ineffective delivery of virus, was therefore the explanation for the absence of fibrosarcomas in these infected animals. Compared with the potent sarcomagenic activity of the T-Myb protein, the ΔN-, VCC-, VCT-, and C-Myb proteins thus lack the intrinsic capacity to efficiently induce in vivo fibrosarcomas. The activation of *myb* into a potent sarcoma gene therefore requires both a loss of sequences from the carboxyl terminus and a retention of at least part of the amino terminus. In contrast, Myb proteins with a loss of sequence from either or both termini are able to transform hematopoietic cells in vitro (20). As *myb* is thought to mediate cell transformation by transcriptionally activating other stimulatory genes, the two distinct *myb* target cells clearly respond differently to the overexpression of some forms of *myb*. In some mesenchymal cells, the absence of an intact C-terminal negative regulatory domain must qualitatively or quantitatively alter the interaction of *myb* with one or several of its target genes.

DISCUSSION

Nonhematopoietic mesenchymal cells: new targets for Myb-induced cell transformation. Although activated, truncated forms of Myb have been known to possess transforming activity in hematopoietic cells, this is the first report of a role for Myb in the development of a nonhematopoietic tumor, namely, a mesenchymally derived fibrosarcoma. Other investigations into the in vivo transforming capabilities of Myb used only the

doubly truncated v-*myb* gene, both alone and in combination with the v-*ets* gene (the second oncogene within the *myb*-containing E26 leukemia virus). While the in vivo overexpression of a number of v-Myb proteins efficiently induced a diverse spectrum of leukemias in chickens (25, 38), nonhematopoietic tumors were not identified. The Myb proteins expressed in these in vivo oncogenicity studies did not, however, include any with an intact amino or carboxyl terminus (relative to c-Myb), thereby precluding a direct comparison with our observation of sarcomas induced by T-Myb.

Other investigations into the transforming activity of Myb for nonhematopoietic cells have all been done in vitro. Perbal and colleagues, for example, showed that primary fibroblasts of either chicken, hamster, or human origin could each be transformed by overexpressing a v-*myb* oncogene (31, 37, 39, 64). Other than this report, however, the fibroblast-transforming capability of Myb proteins with at least one intact terminus (C or N, relative to c-Myb) has not been analyzed. The only other report of a Myb-dependent transforming activity for nonhematopoietic cells (14) showed that chicken neuroretinal cells could be transformed by overexpression of either c-Myb or its doubly truncated v-Myb homolog. These observations of neuronal and fibroblast cell-transforming activity lend in vitro support to our hypothesis that the spectrum of cell types susceptible to Myb-induced transformation extend beyond those of hematopoietic origin. Although we have not observed any obvious morphologic alterations in CEF cultures infected with the T-Myb virus, these cells have not yet been thoroughly analyzed for anchorage independence, growth factor dependence, or enhanced growth kinetics.

Carboxyl-terminal truncation activates the sarcomagenic activity of Myb. We have shown that overexpression of a carboxyl-terminally truncated Myb efficiently induced fibrosarcomas, while overexpression of an amino-terminally truncated, doubly truncated, or full-length Myb did not. The full-length C-Myb virus did possess some sarcomagenic activity (3 tumor-laden animals out of 25), but clearly much less quantitatively than did the T-Myb virus. The absence or low prevalence of sarcomas in these animals was not the result of unequal or inefficient delivery of virus to the target cell population, as approximately equivalent viral titers were used in the infections, each tumor-negative bird expressed high levels of viral Gag protein, and some of these same viruses (VCC-, VCT-, and T-Myb) were able to transform myeloid cells in vitro (47). The loss of 214 carboxyl-terminal amino acids thus directly activated the Myb protein into a potent sarcomagenic agent in vivo.

The presence of both a normal-size T-Myb protein and a grossly unrearranged T-Myb provirus in every tumor subjected to analysis strongly suggests that the T-*myb* gene within the tumor cells is structurally intact. The T-*myb* gene has therefore not undergone any structural alteration during viral passage that may have contributed to its oncogenic activity. The high incidence of sarcomas in T-Myb-infected birds (77%) also argues strongly against the creation of an oncogenic virus by passage-induced mutation. If the generation of a virus with specific point mutations were required to activate T-Myb's sarcomagenic activity, the incidence of tumors would undoubtedly be much lower. For instance, the activation of the transforming capability of a nononcogenic c-Src retrovirus (by mutations accumulating during viral passage) was seen in only a small fraction of infected clones (33).

Several previous studies have shown that Myb C-terminal truncations analogous to our T-Myb deletion can activate Myb's in vitro myeloid cell-transforming activity. In particular, a C-terminally truncated Myb (CCd) with the same C terminus as our T-Myb construct could transform chicken bone marrow cultures, while a full-length c-Myb could not (20). Other C-terminally truncated Mybs (but not full-length Myb) have also been shown to possess transforming activity for mouse hematopoietic cells (17, 21). The transforming capability of these C-terminally truncated Mybs appears to relate directly to their enhanced ability to bind DNA and transactivate gene expression from Myb-responsive target genes (21, 49, 56). These same deletion experiments defined a Myb carboxyl-terminal inhibitory domain (residues 384 to 559 in our nomenclature) that served to inhibit all three functional activities associated with Myb, namely, DNA binding, transcriptional transactivation, and hematopoietic cell transformation. That the loss of part of this same region (residues beyond 485) is sufficient to activate *myb* into a potent sarcoma gene suggests a similar functional activity for this domain in nonhematopoietic mesenchymal cells.

A proposed mechanism by which the carboxyl terminus of Myb may function as an inhibitor of transcriptional transactivation is via a specific interaction with another nuclear factor. A prominent structural feature of the Myb carboxyl-terminal domain is a leucine zipper motif (residues 434 to 462) similar to the motif mediating the dimerization of several other nuclear transcription factors. Mutagenesis of the well-conserved leucine residues of this Myb leucine zipper was sufficient to increase both the transcriptional activation and hematopoietic cell-transforming activities of Myb (26). In addition, nuclear proteins capable of binding the wild-type (but not the mutated) Myb leucine zipper were identified (26), implying a direct functional significance for protein-protein interactions within this domain. Sequences outside the Myb leucine zipper must also be involved in determining the functional activity of the C-terminal inhibitory domain, however, as many C-terminally deleted, activated versions of Myb have an intact leucine zipper (including our T-Myb).

Amino-terminal truncation fails to activate the sarcomagenic activity of Myb. We have shown that a deletion of amino-terminal residues (either 78 or 129 amino acids) failed to activate the in vivo sarcomagenic activity of Myb. In comparison, an amino-terminally truncated Myb protein essentially identical to our VCC-Myb had potent myeloid cell-transforming activity in vitro (20, 47). This target cell-specific transforming activity for amino-terminally truncated Myb contrasts with the observed transforming activity of carboxyl-terminally truncated Myb for both types of cells. A common feature among all of the amino-terminally truncated, activated versions of Myb is a deletion of the casein kinase II phosphorylation site just upstream of the start of the DNA binding domain. Myb is phosphorylated at this site in vivo, and as a result of this phosphorylation, its sequence-specific DNA binding activity is significantly reduced (34). Truncated Myb proteins without this casein kinase II site could become oncogenically activated by being constitutively locked in the dephosphorylated, high-affinity DNA binding state. When tested, however, amino-terminally truncated Myb showed no increase in DNA binding activity relative to full-length Myb (49, 56). Cell type-specific transformation by N-terminally truncated Myb may then depend on its interaction with a qualitatively (not quantitatively) different set of target genes in the two different cell types, perhaps due to the requirement for tissue-specific DNA binding cofactors.

The correlation between in vivo sarcomagenic activity and Myb carboxyl-terminal truncation is not absolute, as the overexpression of our full-length thymus-derived c-*myb* cDNA induced fibrosarcomas in a small percentage of animals (3 of 25). In comparison, the overexpression of slightly shorter

tumor-derived c-*myb* cDNAs without the thymus-specific 58 N-terminal residues (from a chicken B-cell lymphoma [15] or a mouse myeloid leukemia [18]) was unable to induce hematopoietic cell transformation in vitro (20, 21). The potent sarcomagenic activity of T-Myb but not C-Myb (each with the same thymus-specific N terminus) confirms that the presence of these unique 58 N-terminal residues is not responsible for sarcomagenesis. Viruses expressing an N- and/or C-terminally truncated Myb both with and without this thymus-specific N-terminal sequence are currently being assayed for in vivo oncogenic activity to directly address the specific functional role of each terminal region of the Myb protein. In contrast to hematopoietic cells, in which amino-terminally truncated Mybs bind to and transactivate transformation-specific target genes, an intact amino terminus may be necessary for the optimal interaction of Myb with mesenchymal cell-specific target genes. A loss of part of the carboxyl-terminal inhibitory domain might then enhance Myb's transactivational activity enough to initiate the events leading to efficient sarcomagenesis.

Oncogenically active, doubly truncated Myb lacks sarcomagenic activity. Doubly truncated Myb proteins with terminal deletions analogous to those of our VCT-Myb are potent myeloid cell-transforming agents in vitro (20). In comparison, our doubly truncated VCT-Myb completely lacked sarcomagenic activity. The in vivo mesenchymal cell-transforming activity of C-terminally truncated Myb thus requires the presence of at least part of the amino terminus (amino acids 1 to 129 absent from VCT-Myb). As this deletion includes part of the DNA binding domain, the specificity or affinity of DNA binding may be altered. Mesenchymal cell target genes normally transactivated by T-Myb (including those controlling cell transformation) could thus be unresponsive to VCT-Myb. A similar target cell-specific alteration in *myb* DNA binding activity may explain the observed difference in phenotype between hematopoietic cells transformed by a doubly truncated Myb (myeloblasts) compared with a carboxyl-terminally truncated Myb (promyelocytes) (20).

Although we were able to induce classic myelomonocytic leukemia in chickens infected with wild-type AMV (data not shown), no animal infected with any of the Myb-RCAMV viruses (including the analogous doubly truncated VCT-Myb) developed a similar disease. Compared with the normal c-*myb* gene from which the VCT-*myb* cDNA was constructed, the AMV v-*myb* oncogene encodes a protein with 11 different amino acid point mutations, 4 of which localize to the amino-terminal DNA binding domain (29). The biological relevance of these DNA binding domain mutations has been confirmed by the observation that a virus encoding a v-Myb protein without each of these four point mutations (Myb^{77}) completely lacked in vivo leukemogenic activity (38). The mutated DNA binding domain of the AMV v-Myb oncoprotein must therefore recognize different target genes than does its wild-type counterpart. Some of these v-Myb-specific target genes may then contribute to the formation of in vivo leukemias. As observed, the doubly truncated VCT-Myb protein, without these DNA binding domain mutations, should then lack leukemogenic activity. This absence of in vivo myeloid cell-transforming activity was not the result of an inability to express the appropriate Myb protein in myeloid target cells, as myeloid cells could be successfully transformed in vitro not only by RCAMV viruses expressing v-Myb or VCT-Myb but also by those expressing VCC- or T-Myb (47, 48). Also, in addition to the sarcomas already described, about 20% of the birds infected with either VCC-, ΔN-, or T-Myb were found to harbor widely metastatic B-cell lymphomas (47). The lymphomagenic activity of these C- or N-terminally truncated Mybs lends in vivo support to the known correlation between loss of Myb terminal sequences and hematopoietic cell-transforming activity.

Myb-induced sarcomagenesis may require additional cooperating events. Although T-Myb overexpression induced fibrosarcomas efficiently, a minority of animals failed to develop this disease. As these animals expressed viral proteins in the bursa at levels comparable to those found for tumor-laden animals, the delivery of Myb to its appropriate target cell was probably unaffected. Assuming genetic homogeneity between individual animals (a good assumption in this partially inbred strain of chickens), the absence of Myb-induced tumors in some animals implies the existence of cooperating events which may be necessary for tumorigenesis. Despite infection of the entire population, the evolution of only a few specific target cells into clonally derived tumors further supports the necessity for Myb-independent cooperating events for full in vivo tumorigenesis. The somewhat prolonged latency of the T-Myb-induced sarcomas (4 to 6 weeks, compared with 2 weeks with Rous sarcoma virus) may also be the result of the requirement for additional genetic events. These cooperating events may be sufficiently common so as to allow tumor formation in most (but not all) infected animals.

What additional signals might be involved in the process of Myb-induced mesenchymal cell transformation? The signaling pathways regulated by genes with known mesenchymal cell-transforming activity would clearly be good candidates. The expression of c-*myc*, for example, which can directly induce transformation of CEFs, can be controlled at the transcriptional level by c-*myb* (6, 10, 69). Additional support for this hypothesis of cooperating effects between *myc* and *myb* comes from the observation that overexpression in the chicken embryo of the three oncogenes v-*myc*, v-*myb*, and v-*ets* can induce cardiac rhabdomyomas, a mesenchymally derived tumor (1). *ras*, another oncogene with known CEF-transforming activity, has also been implicated as a potential cooperating oncogene for *myb*-induced fibroblast cell transformation (37). Other potential *myb* target genes which may contribute to cell transformation when inappropriately expressed include *cdc2* (32), c-*myb* itself (42), IGF-1, and IGF-1 receptor (51). The involvement of any of these genes in Myb-induced mesenchymal cell transformation will await studies on their expression and/or regulation in T-Myb-induced sarcoma cells versus normal mesenchymal cells.

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