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FAETL Motif Required for Leukemic Transformation by v-Myb

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The nuclear protein v-Myb, encoded by the avian myeloblastosis virus (AMV), can induce acute monoblastic leukemia in vivo and transform chicken myelomonocytic cells in culture. The N terminus of v-Myb functions as the DNA-binding domain, and multiple central and C-terminal regions of this protein have been reported to function in transcriptional activation of model reporter genes. We showed previously that a C-terminal domain (amino acids 296 to 371) is required for transcriptional activation and transformation of primary chicken myelomonocytic cells. In this study, we have now analyzed a series of C-terminal mutants of v-Myb to further investigate this domain. A strong correlation was observed between transcriptional activation and leukemic transformation by this series of mutants. Furthermore, deletion analyses demonstrate that the C-terminal 41 amino acids of v-Myb^{AMV} (amino acids 331 to 371 of the Myb portion) are nonessential whereas further deletion of amino acids 321 to 330 (EFAETLQLID) results in a nonfunctional protein. Hence, we defined a 10-amino-acid subregion (the "FAETL" motif) required for transcriptional activation and oncogenic transformation by v-Myb^{AMV}. The FAETL region is part of a putative leucine zipper structure and lies near a cluster of phosphorylation sites. Our analysis of mutants with substitutions of the zipper leucines or multiple adjacent phosphorylation sites demonstrates that the function of the FAETL motif is not dependent on an intact leucine zipper structure or adjacent phosphorylation sites. The study of GAL4-Myb fusions suggests that this region is important in maintaining a fully functional conformation of v-Myb. The putative leucine zipper structure has previously been proposed to exert inhibitory effects on c-Myb because its mutation caused increased transcriptional transactivation and transformation. Interestingly, our results show that this region is essential for the functions of v-Myb without requiring a heptad leucine repeat.

The nuclear proto-oncogene c-*myb* is a regulator of hematopoietic development (for a review, see reference 22). Elevated expression of c-*myb* occurs in normal early-stage immature cells of hematopoietic origin and leukemia cell lines, whereas its level decreases dramatically upon spontaneous or chemically induced differentiation (9, 14, 56). Furthermore, mice with a homozygously disrupted c-*myb* gene die in utero from deficient hematopoietic development (37). Structural alterations of c-*myb* cause oncogenic transformation of hematopoietic cells (13, 18, 19). Activation of c-*myb* by proviral insertional mutagenesis had been found in myeloid leukemias (38, 51, 52) and B lymphomas (24, 43). In both cases, truncation at the N or C terminus activates the oncogenic potential of c-Myb.

Virally transduced *myb* genes (v-*myb*) were originally identified as the transforming principle in two naturally occurring avian acute leukemia viruses: avian myeloblastosis virus (AMV) and erythroleukemia virus E26 (reviewed in references 4, 22, and 49). AMV transforms myelomonocytic cells in vitro and induces acute monoblastic leukemia in vivo. E26 transforms progenitors with both myeloid and erythroid potential and causes erythroblastosis in vivo. In AMV, v-Myb is encoded as a 48-kDa fusion protein with 6 and 11 additional terminal amino acids provided by the viral Gag and Env, respectively, whereas in E26, v-Myb is expressed as a part of the 135-kDa Gag-Myb-Ets fusion protein. Myb residues in both AMV and E26 (designated v-Myb^{AMV} and v-Myb^{E26}, respectively) are doubly truncated forms of c-Myb but also contain amino acid substitutions (for a review, see reference 22). v-Myb^{AMV} still contains the DNA-binding and transactivation domains of c-Myb, is a nuclear sequence-specific DNA-binding protein, and functions as a transcriptional activator (for a review, see reference 22). v-Myb^{E26} comprises an internal region of v-Myb^{AMV} (Fig. 1), lacking 9 and 79 additional N- and C-terminal amino acids, respectively. In animal cells, a v-Myb^{AMV} mutant (LX3) truncated at the C terminus to a similar extent to that in v-Myb^{E26} (Fig. 1) is strongly reduced in its transactivation and transformation ability, although the DNA-binding domain and previously mapped central transactivation domain are intact (20). Interestingly, this mutant can function as a transactivator in *Saccharomyces cerevisiae* (11). C-terminally truncated c-Myb proteins resulting from the loss of similar C-terminal sequences to those missing in LX3 as a result of retroviral insertions are also found in several interleukin-3-dependent murine myeloid leukemia cell lines (51, 55). This evidence suggests that the sequences deleted in LX3 contain a crucial subregion required for oncogenic transformation.

The region missing in LX3 contains a previously mapped multiply phosphorylated peptide (3, 7) and a putative leucine zipper structure (5). No publications describe the function(s) of the multiple phosphorylation sites in this region of v-Myb. In c-Myb, the "leucine zipper" negatively regulates transactivation and transformation, presumably by interacting with other cellular proteins (16, 23). Hence, it was of interest to define the features in the subregion within the C terminus of v-Myb that are necessary for oncogenic transformation. In this study, a series of mutants have been constructed and analyzed, leading to the definition of a 10-amino-acid subregion required for oncogenic transformation. Neither the putative leucine zipper structure within this region nor the upstream multiple phosphorylation sites are required for its function in v-Myb.

MATERIALS AND METHODS

Plasmid constructions. The deletion mutants (N-1114, N-1120, N-1151, and N-1183) truncated within the C-terminal region of v-Myb^{AMV} (amino acids 296

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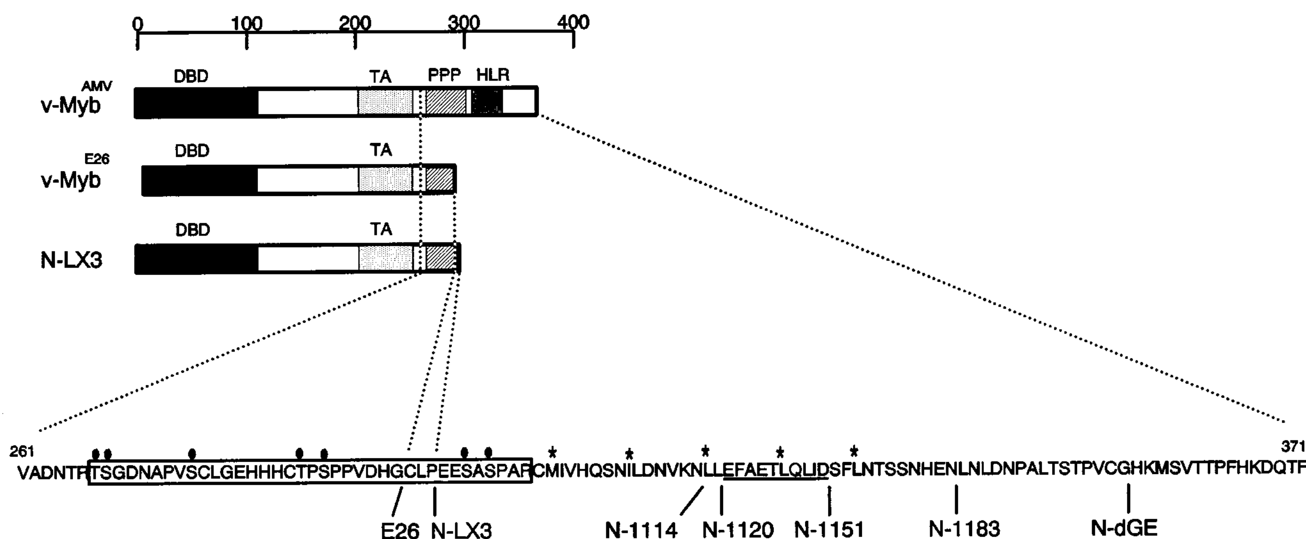


FIG. 1. Schematic diagrams of v-Myb^{AMV}, v-Myb^{E26} and a C-terminal truncation v-Myb mutant, N-LX3. The numbers indicate the amino acid numbers starting at the first residue of the Myb portion in AMV v-Myb. The black boxes represent the DNA-binding domain. The transactivation domain defined by GAL4 fusions (57) is shown as stippled boxes. The hatched boxes indicate a previously mapped phosphorylated tryptic peptide. The shaded boxes illustrate the region harboring the putative leucine zipper. DBD, DNA-binding domain; TA, transactivation domain; PPP, multiple phosphorylation sites; HLR, heptad leucine repeat. The sequence of the C terminus of v-Myb^{AMV} and a series of deletion mutants is shown below the diagrams. The black dots specify all possible phosphorylated serine and threonine residues in the previously defined tryptic phosphopeptide (open box). The asterisks indicate leucines (or methionine and isoleucine) in the putative leucine zipper structure. The underlined region indicates the FAETL motif (see the text). The solid bars indicate the extent of C-terminal deletion mutants. The truncation mutants (N-1114, N-1120, N-1151, and N-1183) are named on the basis of their parental linker insertion mutants (30). N-dGE is an AMV v-Myb mutant in which the Gag, Env, and last 15 Myb-specific amino acids were deleted but which functions as well as wild-type v-Myb (13). In v-Myb^{E26}, amino acid 266 is methionine instead of arginine (42). The *env*-encoded C terminus of AMV v-Myb is not shown.

to 371 of Myb portion) were constructed from linker insertion mutants previously made in our laboratory (30). MT7-1114, MT7-1120, MT7-1151, and MT7-1183 were created by isolation of the *Bst*EII-*Apa*I fragments from corresponding linker insertion mutants and ligation to the large *Bst*EII-*Apa*I fragment of MT7-dGEAX. MT7-dGEAX was made by ligating a synthetic double-stranded linker, SL23 (Table 1), which contains an *Apa*I site followed by stop codons in all three reading frames, into the *Xba*I site in MT7-dGE. The swapping of *Kpn*I-*Xba*I fragments between MT7-dGEAX and N-LX3 (see below) gave rise to MT7-LX3 (20). Mutants with point mutations were generated from MT7-1151 by site-directed mutagenesis as described by Kunkel et al. (28). All oligonucleotides used for mutagenesis and the resulting mutants are described in Table 1. The double mutant MT7-1151L34P was made by using both L3P and L4P in site-directed mutagenesis. The double mutant MT7-1151ASAS was built from MT7-1151TSDA by applying both S2A and TSAA in site-directed mutagenesis. A multiple mutant, MT7-1151TS7A, in which seven potential phosphorylation sites (two threonines and five serines) between amino acids 267 and 303 have been changed to alanines, was created by replacing the *Sal*I-*Bsu*36I fragment of MT7-1151SSDA with that of MT7-1151ASAS. In each case, the mutated regions were sequenced and recloned into wild-type MT7-1151 to avoid unexpected point mutations created during site-directed mutagenesis. Each mutant in the MT7 vector was then cloned into an avian retroviral vector by swapping the *Kpn*I-*Cla*I fragment with that of N-dGE (13). N-LX3 was created by swapping the *Bst*EII fragment of pAMV-LX3 (20) into N-dGE.

GAL4-myb fusion genes were constructed with pSG424Cla, a variant of the pSG424 vector which expresses the GAL4 DNA-binding domain (45). A *Cla*I linker was inserted at the *Hin*dIII site of pSG424 to create pSG424Cla. The *Sma*I-*Cla*I fragments of MT7-LX3, N-1120, N-1151, and N-dGE were cloned into the polylinker region of pSG424Cla to produce GAL4-LX3, GAL4-1120, GAL4-1151, and GAL4-dGE, respectively. The PolyA-EW5(−)Luc reporter plasmid, which harbors a simian virus 40 poly(A) site and five *mim-1* A Myb-binding sites upstream of a minimal E1b TATA box and the *luciferase* gene, was built from E1bCAT (31). E1bCAT was digested with *Hpa*I, and an *Apa*I linker was added to create a unique *Apa*I site. The *Bam*HI-*Apa*I fragment from the resulting plasmid was then replaced with corresponding fragment from pT81luc (41) to produce E1bLuc, so that the *luciferase* gene was substituted for the *CAT* gene. E1bLuc was then digested with *Hin*dIII and filled in, and a *Bgl*II linker was inserted to create a new upstream *Bgl*II site. A *Bam*HI fragment from pLXC(d 1/9) (kindly provided by Paul Godowski, Genentech), containing the simian virus 40 polyadenylation site, was then ligated to *Bgl*II-digested E1bLuc to produce poly(A)-E1bLuc. An *Xho*I-*Sal*I fragment from EW5(−)CAT (11), containing five Myb-binding sites, was then cloned into the *Sal*I site of poly(A)-E1bLuc to give rise to PolyA-EW5(−)Luc. The PolyA-GAL4Luc reporter plasmid (13a) contains five GAL4-binding sites instead of Myb-binding sites upstream of the E1b TATA box.

TABLE 1. Oligonucleotides for mutagenesis

Name	Sequence	Product
SL23	5′CTAGGGGGCCCTAGACTAGTCTAGT3′ 3′CCCCGGGATCTGATCAGATCAGATC5′	MT7dGEAX
L3A	5′CTGCGAATTCTAAGGCATTCT3′	MT71151L3A
L4A	5′TCTATTAACTGGGCTGTTTCTGC3′	MT71151L4A
L3P	5′CTGCGAATTCTAAGGGAT3′	MT71151L3P
L4P	5′GTCTATTAACTGGGGTGT3′	MT71151L4P
TSDA	5′GTGGAGCTGGAGCACAGTGGTGAT3′	MT71151TSDA
S2A	5′GTGGAGCTGGAGCACAGTGGTGAT3′	
TSAA	5′CATTGTCACCCGCGGCCCTG3′	MT71151ASAS
SSDA	5′GTGCGGGGGCTGCAGCTTCCTCAG3′	MT71151SSDA

Cell culture. QT6 quail fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with glucose (4.5 g/liter), 1× minimal essential medium (MEM) nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, streptomycin (100 μg/ml), penicillin (100 U/ml), and 5% fetal calf serum. Yolk sac cells isolated from day 12 to 13 chicken embryos were maintained in Iscove's medium plus 10% fetal calf serum, 5% heat-inactivated chicken serum (56°C for 1 h), 1× MEM vitamins, and the same concentrations of the other supplements as described above. QT6 cells were grown in a humidified 10% CO_2–90% air 37°C incubator, and yolk sac cells were maintained in a 5% CO_2–95% air 37°C incubator.

Transcriptional activation assay. Transient transfections were performed by a modified calcium phosphate precipitation method (8, 21). *myb*-expressing plasmid (3 μg), 1 μg of reporter plasmid, 5.5 μg of tRNA, and 0.5 μg of internal control plasmid expressing β-galactosidase (CMV–β-Gal) were cotransfected into approximately 10^6 QT6 cells per 10-cm-diameter plate. At 48 h after transfection, the cells were washed with 5 ml of phosphate-buffered saline (PBS), scraped, and resuspended in 1 ml of PBS. Each sample was then divided in half and pelleted by centrifugation. One half was resuspended in 100 μl of 0.25 M Tris buffer (pH 7.5), lysed by freezing and thawing three times, and assayed for luciferase activity (2) and β-galactosidase (β-Gal) activity (46). β-Gal activity was then used to normalize for the variation of transfection efficiency among different plates. The other half of each sample was dissolved in 100 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Normalized volumes of each sample based on β-Gal activity were then used to determine

Myb expression by immunoblot analysis (see below). All transfection experiments were repeated at least three times.

Transformation assay. To convert proviral plasmids into infectious viruses, 10 μg of each proviral construct, carrying both the *myb* and *neo* genes, was cotransfected with 1 μg of replication-competent helper virus plasmid pMAVdX into QT6 cells. G418-resistant cells were selected by growing these cells in standard QT6 medium supplemented with G418 (Gibco-BRL) at a final concentration of 200 μg/ml for 2 weeks. Mitomycin-treated virus-producing QT6 cells were then used as a feeder layer in cocultivations for 24 h with primary hematopoietic cells isolated from day 12 or 13 chicken embryonic yolk sacs. The next day, the nonadherent primary cells were transferred to fresh plates. These infected cells then were monitored by microscopy and fed with 3 ml of fresh medium every 2 to 3 days. On day 5, 10^5 cells from each plate were seeded into 4 ml of 0.8% methocel (Stem Cell Co., Vancouver, Canada; HCC-4100) supplemented with 1× DMEM, 5% fetal calf serum, and 5% heat-inactivated chicken serum, and then incubated at 37°C for 2 to 3 weeks. Each batch of cells was also infected with a virus containing an equivalent *neo*-only vector as a negative control to examine the effect of the vector itself and of endogenous viruses from chicken embryos in this assay. The viral supernatants from each plate of QT6 cells used for cocultivation were saved and used to reinfect 10^6 fresh QT6 cells; this was followed by G418 selection to determine viral titer. At least two independent transformation assays were performed for each construct.

Cytocentrifugation and fluorescence-activated cell sorter analyses. Approximately 5×10^4 transformed yolk sac cells were spun onto glass slides with a cytocentrifuge (Cytospin 2; Shandon), air dried, fixed with methanol, and stained with a modified Wright-Giemsa stain (Diff-Quick; Baxter). The cells were photographed under a magnification of ×1,000. For analyses of cell surface markers, we resuspended 10^6 cells in 1 ml of cold DMEM–10% fetal calf serum–25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES, pH 7.4), centrifuged the cells at 4°C, removed the supernatant, added 30 to 100 μl of the monoclonal antibody supernatant of interest, and incubated the mixture on ice for 20 min. We then added 500 μl of cold DMEM–10% fetal calf serum–25 mM HEPES (pH 7.4) to each tube, centrifuged the cells, washed them twice with cold medium, and finally washed them once with Hanks' balanced salt solution. We next added 30 μl of fluorescein-conjugated goat anti-mouse immunoglobulin G (Cappel) diluted 250-fold with Hank's balanced salt solution and incubated on ice for 20 min. Stained cells were washed with Hanks' balanced salt solution three times at 4°C and then analyzed on a FACStar^{plus} (Becton Dickinson). As a negative control, each type of cells was processed similarly except that no primary antibody was added.

Immunoblot. Extracts were prepared by lysing transfected QT6 cell lines or transformed yolk sac cells in SDS loading buffer and boiling the lysate for 5 min. Normalized volumes of lysates (based on internal control β-Gal activity for transient transfections or equivalent cell numbers for transformed yolk sac cells) were subjected to SDS-PAGE (10% polyacrylamide), and then the proteins were transferred to a nitrocellulose membrane (BA-S 83; Schleicher & Schuell). Myb expression was detected by using a Myb 2.2 and 2.7 monoclonal antibody mixture (15). Blots were developed as specified by the manufacturer by using goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase (Promega), 5-bromo-4-chloro-3-indolylphosphate (BCIP), and nitroblue tetrazolium.

Two-dimensional protein electrophoresis. For two-dimensional protein electrophoresis, a system from Pharmacia was used: Immobiline DryStrip Kit (IEF strip; pH 3.0 to 10.5), ExcelGel precast gels (acrylamide gradient, 8 to 18%), ExcelGel buffer strips, Multiphor II electrophoresis system, and power supply EPS3500XL. Nuclear extracts from transformed yolk sac cells were prepared as described previously (1). The extracts were diluted with lysis buffer (8 M urea, 2% 2-mercaptoethanol, 2% Pharmalyte 3-10, 0.5% Triton X-100, 0.04% bromophenol blue) and run from the anodic side of the DryStrip. Subsequent sample treatments and running conditions were as specified by the manufacturer.

RESULTS

Construction, expression, and oncogenic activity of C-terminally truncated v-Myb^{AMV} mutants: a subregion required for both transactivation and transformation. Previous reports suggested that the C terminus of v-Myb^{AMV} (amino acids 296 to 371) is necessary for transactivation and transformation (6, 10, 20). To further define this region, a series of progressive C-terminal deletion mutants were constructed. These mutants are described in Fig. 1 and Table 2.

We first tested the C-terminal truncation mutants for transcriptional activation and protein expression. Proviral expression vectors encoding v-*myb* mutants were cotransfected into QT6 cells with the internal control plasmid CMV–β-Gal and the Myb-responsive reporter plasmid PolyA-EW5(−)Luc, which harbors five copies of the strongest Myb-binding site of the *mim-1* promoter (A site) upstream of the *luciferase* reporter gene (39). Luciferase assays and β-Gal assays were then performed to determine transactivation ability. Compared with the positive control, N-dGE, which was previously shown to function as well as wild-type v-Myb^{AMV} (13), deletion of the last 41 amino acids or fewer (N-1151, N-1183) at the C terminus of v-Myb does not affect its transactivation activity. However, further deletion of 10 amino acids or more toward the N terminus (N-LX3, N-1114, and N-1120) strongly reduces this property (Fig. 2A). These results suggest that amino acids 321 to 330 (EFAETLQLID) serve as a positive element for transactivation. Immunoblot analyses of normalized volumes from each sample showed similar levels of Myb protein expression by the different mutants (Fig. 2B). Furthermore, a protein of the expected size was produced in each case, indicating that differential protein expression or stability was not the cause of the differences in transcriptional activation.

TABLE 2. Characteristics of v-Myb deletion mutants

Mutant	Deletion[a]	Extra amino acids at C terminus[b]	Predicted mol mass (Da)[c]
N-LX3	296-371	Ser-Arg-Ala-Gln-Leu	34,430
N-1114	319-371	Gly-Pro	36,410
N-1120	321-371	Gly-Pro	36,630
N-1151	331-371	Trp-Ala-Leu-Asp	37,950
N-1183	342-371	Gly-Pro	38,940
N-dGE	357-371	Ser-Arg-Ala-Gln-Leu	40,920

[a] The numbering of amino acids starts at the first amino acid of the Myb portion in v-Myb^{AMV} (27).
[b] In each construct, 11 additional amino acids (MAMITNSSSVP) were present at the N terminus.
[c] The predicted molecular mass was estimated as 110 Da times the total amino acid number.

The same series of C-terminal truncation mutants were tested for oncogenic transformation. To convert proviral plasmids into infectious viruses, each proviral plasmid carrying both the *neo* and *myb* genes was stably cotransfected with helper virus pMAVdX into QT6 fibroblasts. After G418 selection for *neo* expression, these virus-producing QT6 cells were cocultivated with yolk sac cells isolated from day 12 to 13 chicken embryos. Yolk sac cells at this stage are rich in myelomonocytic progenitor cells. Uninfected day 12 to 13 yolk sac cells spontaneously differentiate within 3 weeks and stop growing in tissue culture, whereas yolk sac cells infected with a v-*myb*^{AMV} virus fail to differentiate and continue to proliferate. Another property of transformed cells is the ability to form colonies in a semisolid methocel matrix. We examined the transformation activity of these C-terminal truncation mutants both in liquid culture and in methocel assays. In liquid culture, obvious outgrowth was observed 2 weeks after infection in plates with N-1151, N-1183, or the positive control N-dGE. In contrast, cells in plates with N-LX3, N-1114, N-1120, or the negative control N-Cla hardly grew. As a representative experiment shows in Table 3, when hematopoietic cells were counted on day 19 after cocultivation, the cell numbers were 120 to 1,000-fold greater for the transforming than the nontransforming mutants. After prolonged culture, no growth could be detected in cultures of N-LX3 or N-1114. N-1120-transformed cells grew slowly, and larger numbers of these cells could be obtained in the presence of commercially available growth factors (insulin-transferrin-selenium) or chicken myeloid growth factor (see Discussion). The results of methocel assays further support this observation. All transforming constructs gave rise to much larger numbers of colonies (Table 3), and the colonies were also much bigger (Fig. 3A).

Overall, the transactivation activity correlates well with transformation ability for these C-terminal truncation mutants

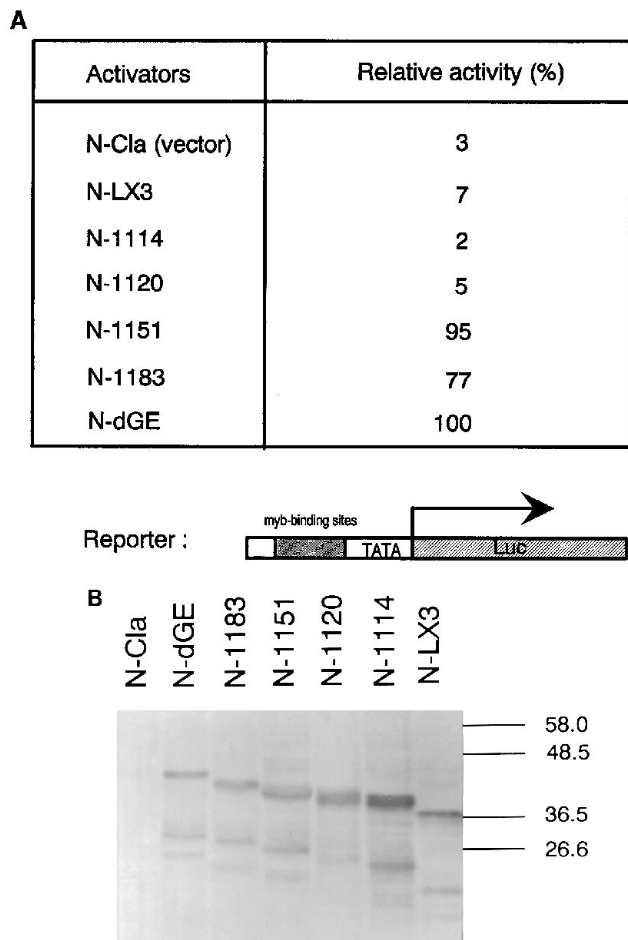


FIG. 2. Transcriptional activation by different C-terminal deletion mutants of v-Myb. (A) Relative transactivation by different mutants. Each Myb protein was tested for its ability to activate transcription from a reporter plasmid containing five Myb-binding sites (shaded box) shown at the bottom of the figure. TATA, TATA box; Luc, *luciferase* gene (hatched box). Reporter and activator plasmids were cotransfected into QT6 fibroblasts, and activities were determined as described in Materials and Methods. Activities are shown relative to that of N-dGE, which is assigned a value of 100. Data are means from four experiments. (B) Immunoblot analysis of Myb proteins in transfected cell extracts. Normalized volumes of samples were analyzed by SDS-PAGE, and then Myb proteins were detected with a mixture of anti-Myb-2.2 and Myb-2.7 antibodies as described in Materials and Methods. Molecular weight markers are indicated on the right in thousands.

of v-Myb. The same region (EFAETLQLID) important for transactivation is also required for transformation. The phenotype of cells transformed by N-1151 and N-1183 is indistinguishable from that of cells transformed by the positive control N-dGE (Fig. 3B). Analysis of molecular markers by FACS and immunoblotting (Table 3) showed that these cells are positive for HLO72 (monoblast specific) (32), but negative for IC3 (granulocyte specific) (33) and Mim-1 (promyelocyte/granulocyte specific) (39). This pattern is typical of v-Myb^{AMV}-transformed monoblasts. Similar levels of Myb expression were detected in cells transformed by these viruses, and the expression of proteins with the expected sizes confirms that transformation was not due to viral rearrangement or contamination (Fig. 4A). The Myb^{AMV}-transformed cell line BM2 was used as a positive control for immunoblotting. To estimate the viral titer and confirm Myb expression, equal amounts of viral supernatant collected from each type of virus-producing QT6 cell were used to reinfect fresh QT6 cells, and G418 selection was performed. Colonies were then counted, and equal numbers of cells from pooled colonies of each construct were used to examine Myb expression by immunoblotting. As shown in Fig. 4B, Myb expression levels are similar in virally infected QT6 cells with either transforming or nontransforming constructs, and corresponding proteins of the correct size were produced in all cases. In addition, the titers of all viruses were similar (data not shown). In summary, we have defined a 10-amino-acid region (EFAETLQLID) in v-Myb that is required for transcriptional activation and oncogenic transformation. In the remainder of this paper, we refer to this region as the FAETL region.

As shown in Fig. 1, the FAETL region (underlined) constitutes part of a putative leucine zipper motif and is located downstream of a multiply phosphorylated peptide mapped previously (3, 7). Leucine zipper structures have been shown to mediate protein-protein interactions (29), and phosphorylation can be important in regulating the function of transcription factors (reviewed in references 25 and 26). Hence, we decided to test whether the deletion of the FAETL region disrupts a critical leucine zipper structure or phosphorylation pattern.

The heptad leucine repeat of v-Myb is not essential for leukemic transformation. The putative leucine zipper motif located at amino acids 305 to 333 (**M**IVHQSN**I**LDNVKN**LL** EFAET**L**QLIDSF**L**) (Fig. 1), is distinguished by a predicted amphipathic α-helical structure with hydrophobic residues (leucine, methionine, or isoleucine) at every seventh position. The leucine zipper in c-Myb plays an inhibitory role in terms of transactivation and transformation ability (23). However, linker insertion mutants of v-Myb which are predicted to disrupt the α-helical nature of this motif still retain their trans-

TABLE 3. Summary of transformation assays of C-terminal deletion mutants

Mutant	Transformation ability		Expression of molecular markers in transformed hematopoietic cells[a]		
	Liquid culture outgrowth (no. of cells/ml on day 19)[b]	Methocel assay (no. of colonies)[c]	Mim-1[d]	IC3[e]	HLO72[f]
N-LX3	$<10^4$	5	ND	ND	ND
N-1114	$<10^4$	4	ND	ND	ND
N-1120	5.5×10^4	11	ND	ND	ND
N-1151	10^7	216	−	−	+
N-1183	7.7×10^6	125	−	−	+
N-dGE	6.6×10^6	134	−	−	+
N-Cla	$<10^4$	5	ND	ND	ND

[a] Different molecular markers were assayed by immunoblot (Mim-1) or FACS analyses (IC3 and HLO72) with corresponding antibodies. ND, not determined; −, not detectable; +, detectable in immunoblot or more than 90% cells were stained positively in FACS analyses.

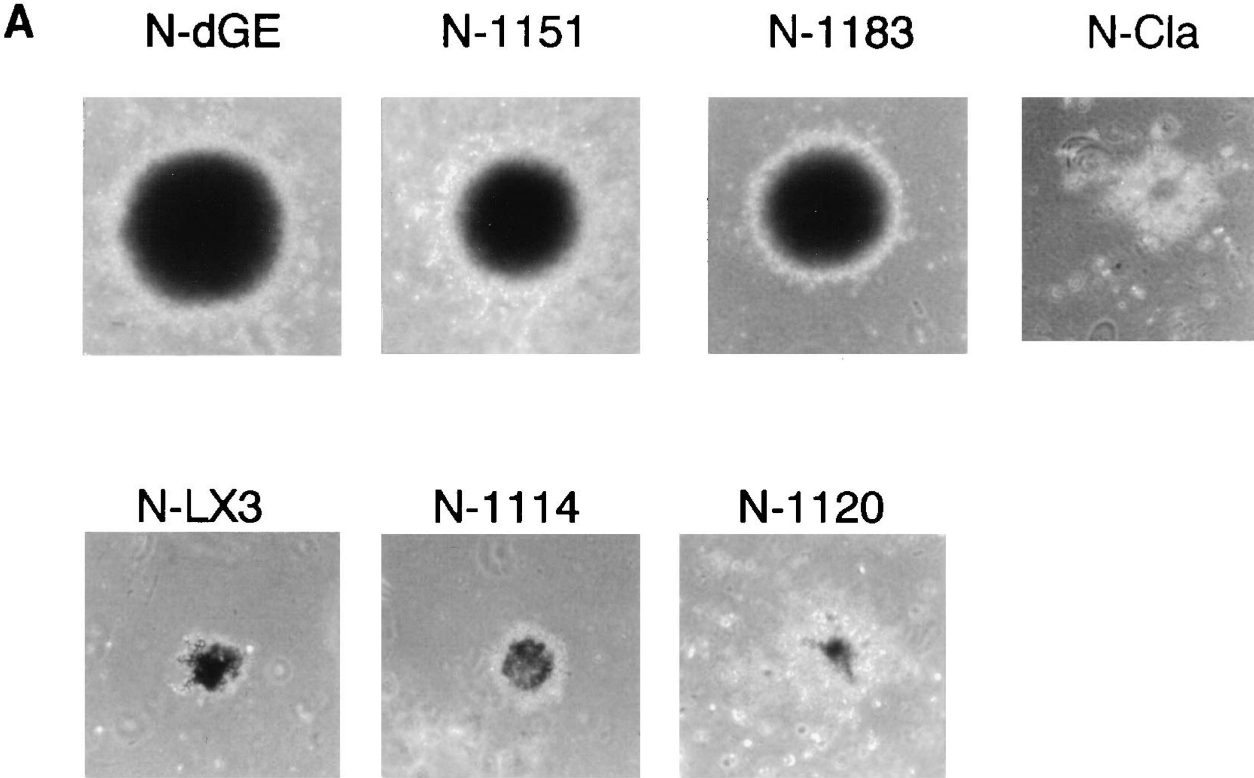
[b] Hematopoietic cells were counted on day 19. Data from one representative experiment are shown.

[c] 10^5 cells from each culture were seeded into 0.8% methocel on day 5 of the transformation assays, and colonies were counted after 2 weeks. Data from one representative experiment are shown.

[d] Mim-1 is a promyelocyte/granulocyte-specific marker. The BM2 cell line was used as a positive control, although primary AMV-transformed cells do not express Mim-1 (12).

[e] IC3 is a granulocyte-specific marker. The same cells stained only with secondary antibody were used as negative controls. IC3-positive BAB-transformed cells served as the positive controls (12).

[f] HLO72 is a monoblast-specific marker. As negative controls, the same cells were processed similarly except that no primary antibody was added. BM2 cells were the positive controls for HLO72 (32).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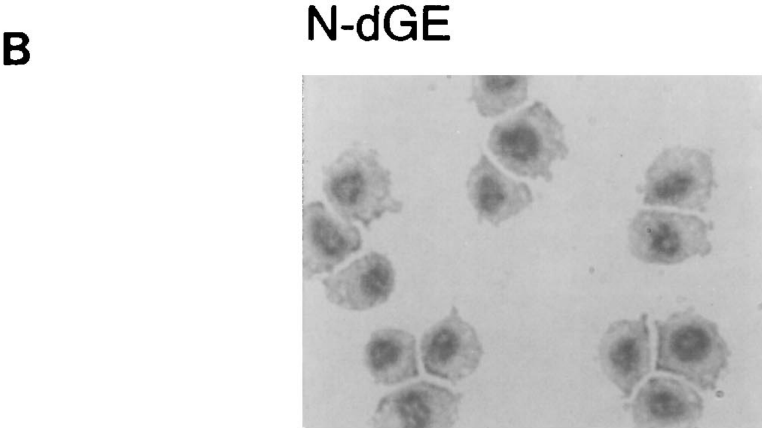
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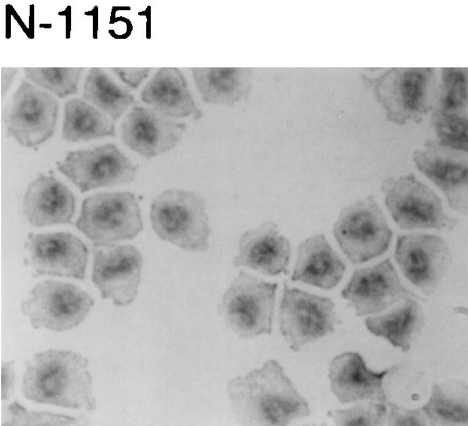
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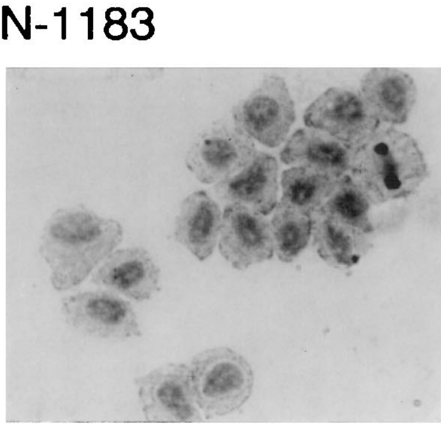
N-1183

FIG. 3. (A) Colony morphology of infected hematopoietic cells by either transforming or nontransforming viruses. Methocel assays were performed as described in Materials and Methods. Photographs were taken after 3 weeks with the phase-contrast microscope at a magnification of ×100. (B) Morphology of hematopoietic cells transformed by N-1151, N-1183, and N-dGE. The transformed cells were cytocentrifuged and stained with Diff-Quik. Photographs were taken at a magnification of ×1,000.

activation and transformation activity (30). N-1151, the shortest transforming v-Myb mutant described in this paper, contains the entire leucine repeat except the final 3 amino acids. In contrast, in N-1120, the longest nontransforming mutant, almost half of the leucine repeat is deleted. To determine if the leucine repeat is required for transformation, we further constructed a series of mutants with amino acid substitutions in the leucine repeat of v-Myb (Table 4). Since both the α-helical structure and hydrophobicity are important for the function of leucine zippers, we mutated the two Leu residues that are important in c-Myb (23) to either Ala or Pro. All mutants were built from N-1151. N-1151L3A and N-1151L3P consist of single substitutions of the central Leu in the "zipper" at position 319 with Ala and Pro, respectively. N-1151L4A and N-1151L4P carry substitutions of the next Leu in the "zipper" at position 326 with Ala and Pro, respectively. N-1151L34P is a double substitution of both leucines with prolines. All these mutants have slightly higher (two- to threefold) transactivation activity than N-1151 (Fig. 5A). They all caused leukemic transformation in culture as well. However, substitution of prolines for leucines generally reduces the transforming ability compared with substitution of alanines for leucines (Fig. 5A). For example, cultures infected with the double mutant N-1151L34P had 40- to 50-fold fewer cells than did the positive control, N-1151, when hematopoietic cells were counted on day 19 after infection (Fig. 5A). However, in contrast to N-1120-transformed cells, cells transformed by N-1151L3P, N-1151L4P, or N-1151L34P appear growth factor independent and a significantly larger number of transformed cells can be obtained after continuous culturing in our standard transformation assays. The phenotypes of cells transformed by all the leucine substitution mutants show no differences from wild-type-transformed monoblasts (data not shown). Similar levels of Myb expression by these mutants were confirmed for transiently transfected QT6 cells (data not shown) and transformed yolk sac cells (Fig. 5B). These data demonstrated that an intact "leucine zipper" motif of v-Myb is not essential for leukemic transformation by v-Myb, because replacement of leucines with alanines, which can still maintain both α-helical structure and overall amphipathicity, had no effect. However, proline substitutions did show some reduction in transforming ability. This suggests that an α-helical structure and/or hydrophobicity of this region, rather than specific leucines, is important for its optimal function in v-Myb.

Phosphorylation in the central region of v-Myb is not required for leukemic transformation. Multiple serine and threonine phosphorylation sites in the central area of v-Myb^{AMV} (amino acids 267 to 303) were mapped previously by using different antibodies which recognize different regions of Myb and by two-dimensional tryptic peptide mapping (3, 7). These phosphorylation sites are summarized in Fig. 1. It had been shown that GSK-3 (glycogen synthase kinase) can phosphorylate these sites in vitro (58). However, the functional consequences of this phosphorylation have not been reported. To examine whether the loss of function of N-1120, which lacks the FAETL region, is due to deregulated phosphorylation, we constructed a series of mutants with substitutions in potential phosphorylation sites (Table 4) and assayed their functions. In N-1151TSDA, Thr-284 and Ser-286 were converted to Ala; N-1151SSDA has substitutions of both Ser-298 and Ser-300 with Ala; in N-1151TS7A, all potential phosphorylation sites (two threonines and five serines) in the tryptic peptide between residues 267 and 303 were substituted with Ala. All of these mutants, positive control N-1151, and negative control N-Cla were tested for their transactivation and transforming activity as described above. As shown in Fig. 6A, none of the mutations of phosphorylation sites significantly affect transactivation or transformation. The transformed phenotype of all mutants is typical of AMV-transformed monoblasts. Similar levels of Myb proteins were produced from each sample in transactivation assays (data not shown). Figure 6B confirms that Myb proteins are produced in yolk sac cells transformed by each mutant. To examine phosphorylation, a two-dimensional electrophoretic analysis of nuclear extracts from transformed yolk sac cells of N-1151 or N-1151TS7A was performed. The proteins were separated according to pI and relative molecular weight. Myb expression was detected by immunoblot with a mixture of Myb antibodies (antibodies 2.2 and 2.7). Two major spots were detected in N-1151 (Fig. 7A), spot *a* closer to the anode and spot *b* closer to the cathode. Spot *a* was not present in N-1151TS7A, whereas spot *b* was present (Fig. 7B). These results suggest that the major phosphorylated form of v-Myb was eliminated in mutant N-1151TS7A. These data further demonstrate that phosphorylation within the central section of v-Myb is not required for transcription activation or leukemic transformation. Therefore, the essential function of the 10 amino acids deleted in N-1120 relative to N-1151 is not the regulation of phosphorylation.

The FAETL region is specifically required by the Myb DNA-binding domain. To determine if the FAETL region we defined cooperates with the N-terminal DNA-binding domain of v-Myb, we constructed a series of heterologous fusion proteins in which the Myb DNA-binding domain of each mutant was replaced with the GAL4 DNA-binding domain. We then performed transactivation assays by using a reporter with GAL4-binding sites (Fig. 8A). GAL4-1120 demonstrated a much weaker transactivation activity than did GAL4-dGE. These data support the idea that the C terminus of v-Myb is required

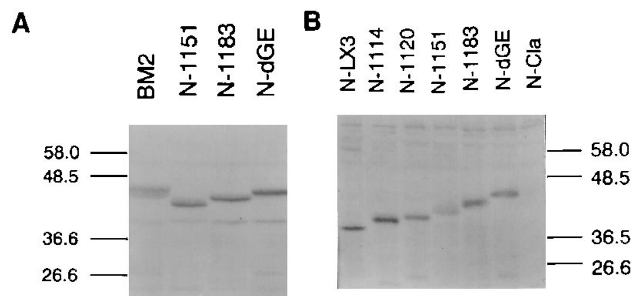

FIG. 4. (A) Myb expression in hematopoietic cells transformed by N-1151, N-1183, and N-dGE. Cells (10^6) from each sample were lysed and separated by SDS-PAGE, immunoblotted, and then detected with a mixture of Myb antibodies (2.2 and 2.7). An equal amount of v-Myb-transformed BM2 cells was loaded as a positive control. The molecular weight markers are shown on the left in thousands. (B) Myb expression by proviruses in infected QT6 fibroblasts. QT6 cells were infected with the indicated virus-containing supernatant and selected for G418 resistance as described in Materials and Methods. Equal amounts of cell lysates were analyzed by immunoblotting as described for panel A.

TABLE 4. Features of mutants with mutations in the leucine zipper structure and phosphorylation sites

Name	Amino acid substitutions based on N-1151[a]
N-1151L3P	Leu (319) Pro
N-1151L34P	Leu, Leu (319, 326) Pro, Pro
N-1151L4P	Leu (326) Pro
N-1151L3A	Leu (319) Ala
N-1151L4A	Leu (326) Ala
N-1151TSDA	Thr, Ser (284, 286) Ala, Ala
N-1151SSDA	Ser, Ser (298, 300) Ala, Ala
N-1151TS7A	Two Thr and five Ser between amino acids 267 and 303 were converted to Ala

[a] The numbering of amino acids starts at the first amino acid of the Myb portion in v-Myb^{AMV}. The amino acid(s) before the parentheses is wild type; the amino acid(s) after the parentheses is mutant; the numbers in the parentheses are amino acid positions.

for transactivation. GAL4-1151 shows twice the activity of GAL4-1120; however, it retains only half of the activity of GAL4-dGE. Hence, in the context of a heterologous DNA-binding domain, the FAETL region is important but not sufficient for maximal transactivation. Similar levels of GAL4-Myb fusion proteins were confirmed (Fig. 8B). These results with a heterologous DNA-binding domain do not completely reflect those found in the context of v-Myb itself. Therefore, these data suggest that the C terminus of v-Myb, including the 10-amino-acid region we defined, may somehow interact with the N terminus in maintaining a fully functional conformation.

DISCUSSION

In this paper, we defined a subregion (amino acids 321 to 330) near the C terminus of v-Myb that is required for transactivation and leukemic transformation. We refer to this region as the FAETL motif. This region is part of a putative leucine zipper structure and lies near a cluster of phosphorylation sites. We tested several possible functions of the region, including (i) mediation of protein-protein interaction via the leucine zipper motif; (ii) modulation of phosphorylation at multiple adjacent sites; and (iii) maintenance of a functional protein conformation. Our data from analyzing mutants with substitutions in the putative leucine zipper structure or the phosphorylation sites suggested that the requirement of the FAETL region for v-Myb is not related to an intact leucine zipper motif or phosphorylation. The data from GAL4-Myb fusions suggest that this motif is important in fully maintaining a functional conformation of v-Myb, possibly by interacting with the Myb DNA-binding domain.

Several additional observations support the notion that the FAETL motif may be crucial for Myb function. First, this motif is highly conserved among c-Myb and A-Myb proteins in different vertebrate species (Fig. 9). However, the "leucine zipper" itself is not the most conserved feature, and prolines are present within this region of A-Myb. Second, myeloid leukemia cell lines NFS-60 and VFLJ2 were found to have C-terminally truncated Myb proteins owing to retroviral insertion (51, 54, 55). These truncations are located within the FAETL motif (Fig. 9). In contrast, another myeloid leukemia cell line, FB-ML R1-4-11 (38), induced by integration of amphotropic virus Friend murine leukemia virus strain FB29, has rearranged c-Myb truncated in front of the FAETL region (Fig. 9). The transformed cells of FB-ML R1-4-11 are interleukin-3-independent promonocytes (macrophage precursors), while NFS-60 and VFLJ2 are interleukin-3-dependent myelomonocytic cells (granulocyte and macrophage precursors). The mechanism(s) conferring such differences is not known but might be due to the differential presence or absence of the FAETL region. Another possibility for the differences between these cell lines is the presence of undetected mutations in other genes in addition to the *myb* mutations. Third, additional forms of c-Myb arising from alternative splicing were found in both normal and malignant hematopoietic cells, with an extra exon inserted directly following the FAETL motif (Fig. 9) (44, 48, 50).

Our results showed that disruption of the putative leucine zipper motif of v-Myb by substitutions of leucines with alanine or proline does not abolish the functions of v-Myb. These results are consistent with a previous report that v-Myb linker insertion mutants predicted to disrupt the leucine zipper motif

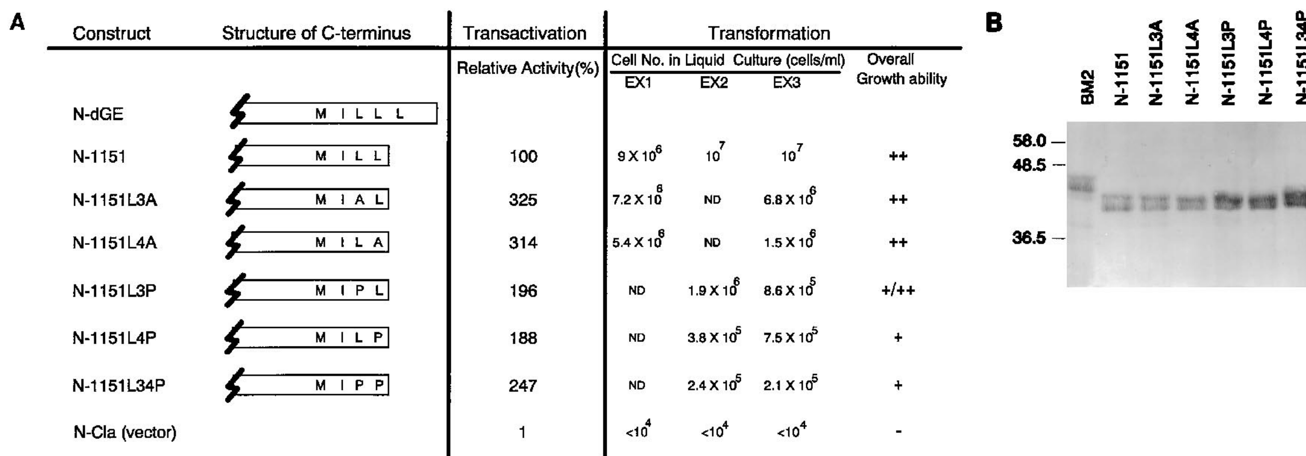

FIG. 5. (A) Transcriptional activation and transformation by mutants with a substitution(s) in the putative leucine zipper structure. Schematic diagrams illustrate C-terminal heptad leucine repeats of both the wild type and mutants. Transactivation assays and transformation assays were performed as described in the text. The transactivation activities of different mutants are shown relative to that of N-1151, which is assigned a value of 100. Data are means for five experiments. For transformation assays, hematopoietic cells were counted on day 26 (EX1) or day 19 (EX2 and EX3) of the assays. The overall growth ability indicated outgrowth status after continued culturing (see the text). −, +, and ++ represent not detectable, moderate, and robust, respectively. (B) Expression of various v-Myb proteins in transformed hematopoietic cells. Cells (10^6) of each indicated sample were lysed and detected by immunoblot as described in Materials and Methods. v-Myb-transformed BM2 cells served as a positive control.

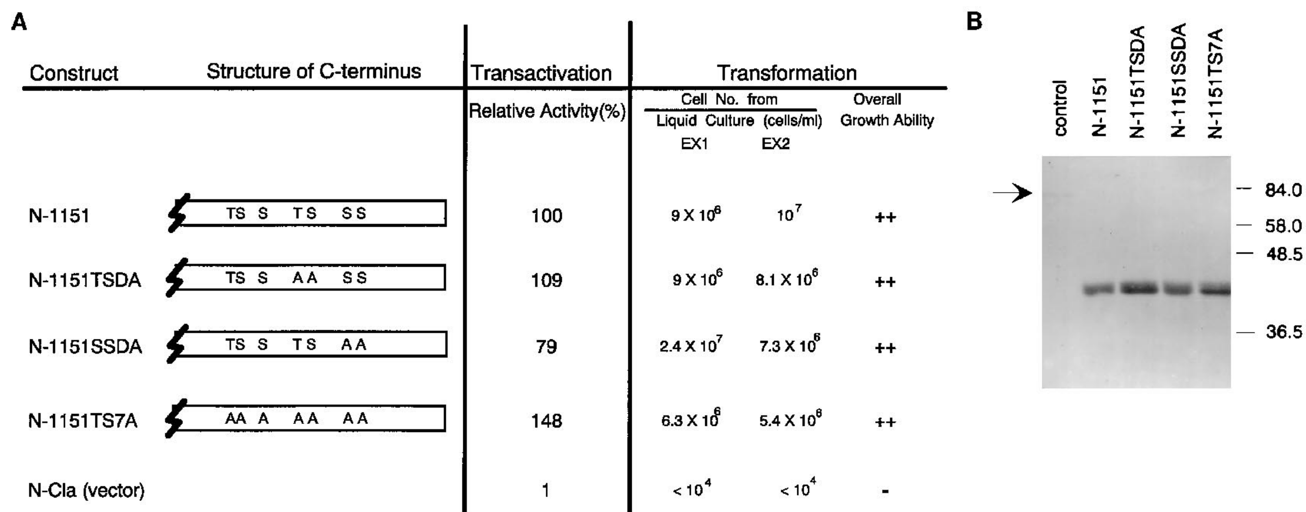

FIG. 6. (A) Summary of transactivation and transformation assays by mutants with a substitution(s) in potential phosphorylation sites. Schematic diagrams magnify the region containing possible phosphorylation sites from both the wild type and mutants. The transactivations by different mutants relative to that of N-1151 (assigned a value of 100) are shown as the mean for seven experiments. Transformed hematopoietic cells were counted on day 26 (EX1) or day 19 (EX2) in yolk sac assays. The overall growth ability is defined as −, +, or ++ by the same criteria described in the legend to Fig. 5A. Data are means for two experiments. (B) Expression of various v-Myb proteins in transformed hematopoietic cells by different phosphorylation mutants. Cells (10^6) from each indicated sample were subjected to SDS-PAGE and immunoblotting. Day 13 normal yolk sac cells, which express c-Myb, were used as a positive control for the antibody (arrow).

are still functional (30). However, LX3, a mutant lacking the C-terminal 76 amino acids of v-Myb including the FAETL motif, transactivated well in yeast but not animal cells (11, 20). This raises the possibility that the Myb-associated protein(s) required for transactivation interacts with the C terminus of v-Myb in animal cells. The present study suggests that this type of interaction, if it exists, does not depend upon the putative leucine zipper motif of v-Myb. Results from other groups suggested that the leucine zipper in c-Myb plays an inhibitory role in transactivation and transformation (23). These groups proposed that inhibition is due to either the binding of Myb-interacting proteins to leucine zipper region (16, 23) or homodimerization of c-Myb itself through the leucine zipper (40). Interestingly, our data from deletion analyses revealed that this region exerts a positive function in v-Myb. Furthermore, our results from mutants with a substitution(s) in the "zipper" leucines suggest that the function of this region does not require an intact leucine zipper motif. A unifying hypothesis is that this region serves a positive function in both v-Myb and c-Myb but can be negatively regulated by the "leucine zipper" only in c-Myb. It will be interesting to examine the effects of the mutations described in our study in the context of c-Myb.

Regulation of transcription factors by phosphorylation has been seen in several cases (25, 26). Multiple forms of v-Myb were first identified by two-dimensional gel electrophoresis, and a cluster of multiple phosphorylation sites were previously mapped to the central region of v-Myb (3, 7, 53). These sites

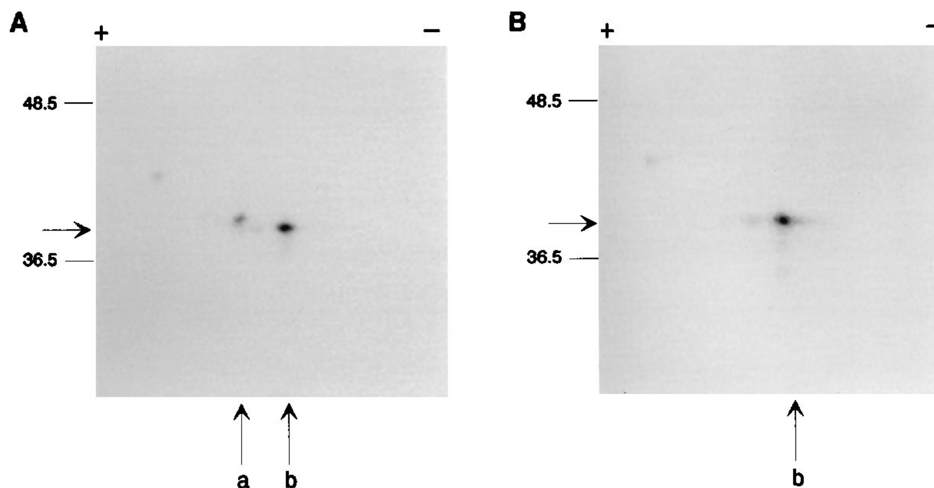

FIG. 7. Two-dimensional gel electrophoresis of Myb proteins from hematopoietic cells transformed by N-1151 (A) or N-1151TS7A (B). Equal amount of nuclear extracts from both cell types were subjected to two-dimensional electrophoresis as described in Materials and Methods. The samples were first run on isoelectric focusing gels from acidic to basic pH with an ampholine range from 3.0 to 10.5 and were then separated by SDS-PAGE with 8 to 18% polyacrylamide gradient gels. Myb proteins were identified by immunoblots with a mixture of anti-Myb-2.2 and anti-Myb-2.7 antibodies. Molecular weight markers are indicated on the left of each panel in thousands. Horizontal arrows indicate the protein sizes, and vertical arrows show the major spots in both blots.

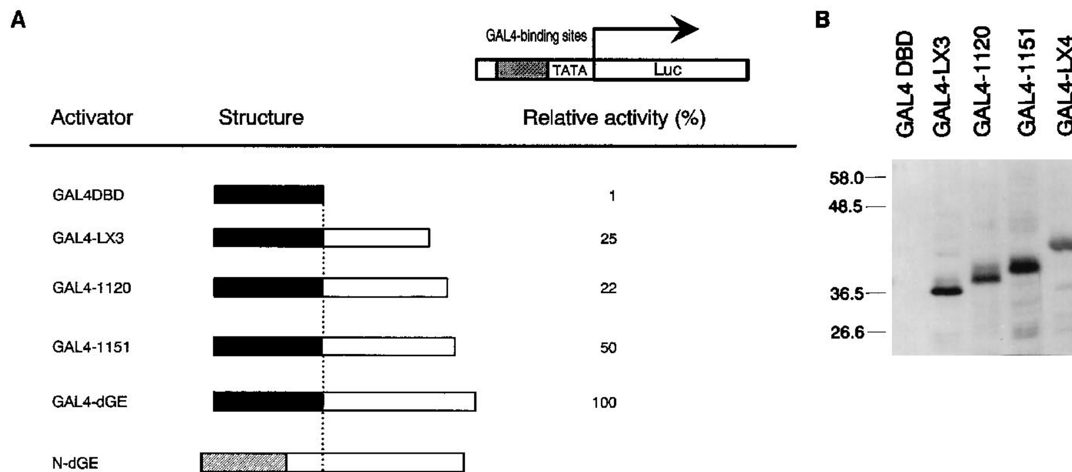

FIG. 8. Transactivation by GAL4-Myb fusion proteins in QT6 cells. (A) Relative transactivation activities of GAL4 fusion proteins. The schematic shows full-length N-dGE and various GAL4-Myb fusion proteins. GAL4DBD, GAL4 DNA-binding domain (black boxes); the Myb DNA-binding domain is shown as a hatched box; the remaining Myb sequences are indicated as empty boxes. Dashed lines indicate the locations of fusions relative to that in N-dGE. The reporter plasmid is shown at the top. GAL4-binding sites are indicated by a shaded region. TATA, TATA box; Luc, *luciferase* gene. The transactivation activities of different activators are shown relative to that of the strongest activator, GAL4-dGE (assigned a value of 100). Data are the mean for seven experiments. (B) Immunodetection of the GAL4-Myb fusion proteins in transfected QT6 cells. Normalized volumes of cell extracts from each sample were analyzed by SDS-PAGE and then detected by a mixture of Myb antibodies (2.2 and 2.7).

can be phosphorylated in vitro by GSK-3 (58). However, our data demonstrate that these phosphorylation sites are not required for transactivation or transformation by v-Myb. The role of these sites in c-Myb remains to be determined. Interestingly, in the two-dimensional electrophoretic analyses of mutant N-1151TS7A, in which all potential phosphorylation sites in the previously mapped region were mutated (Fig. 7B), there is another faint spot on the left of spot *b*. This suggests that another unknown posttranslational modification(s) of v-Myb has occurred in vivo.

From our experiments, transactivation by heterologous GAL4-Myb fusions supported the importance of the FAETL region but did not completely reflect similar studies of native v-Myb proteins. This implies that multiple regions, including the FAETL motif, are required to maintain a functional protein conformation. This idea is consistent with the observation

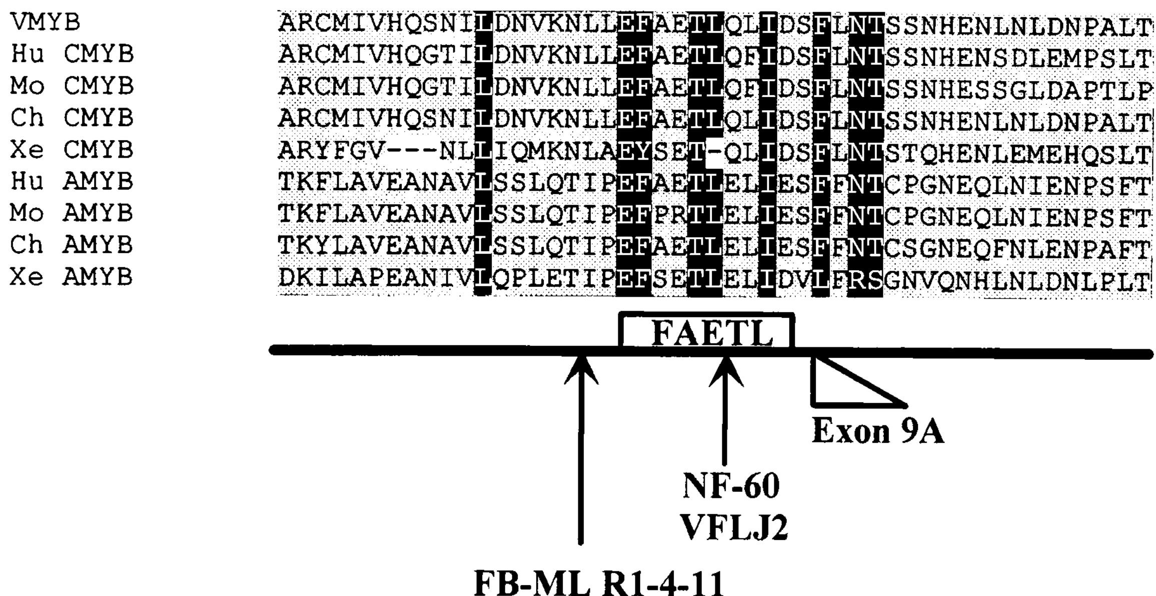

FIG. 9. Alignment of the carboxy-terminal region of AMV v-Myb with homologous regions at C-Myb and A-Myb from different vertebrate species. All sequences were obtained from GenBank. The alignments were performed with the computer program MACAW (47). The most highly conserved residues as determined by using the Blosum matrices are highlighted. Dashes indicate gaps in the alignment. VMYB, AMV v-Myb; Hu, human; Mo, mouse; Ch, chicken; Xe, *Xenopus*. The schematic diagram at the bottom shows the location of FAETL motif (open box). Arrows represent the sites of retroviral insertional mutagenesis found in mouse cell lines as indicated. The triangle indicates the location of an exon (exon 9A) found in alternatively spliced forms of c-Myb in human, mouse, and chicken sequences. The exon 9A sequences are not included in this diagram. The sequences corresponding to exon 9A in A-Myb were also excluded in this comparison.

that multiple transactivation domains are necessary for oncogenic transformation by v-Myb (10). There is additional evidence supporting the idea that the C terminus of v-Myb^{AMV} is structurally important. Others have shown that the presence of Ets in the Myb-Ets fusion of E26 which lacks the FAETL motif is required for diverse transformation ability in vitro and also for leukemogenicity in vivo (34, 35). Another study suggests that Ets might also provide a structural substitution for the C-terminal 79 Myb^{AMV}-specific amino acids missing in v-Myb^{E26} (17).

To further understand the role of Ets in the E26 fusion protein, we tested the following constructs in both transactivation and transformation assays. N-1120-I–Ets coexpresses a nontransforming v-Myb mutant C-terminally truncated just before the FAETL motif and v-Ets as two separate proteins, while N-Myb^{E26}–Ets encodes a v-Myb^{E26}–Ets fusion protein. Significant cooperation in transcriptional activation and transformation was detected in N-Myb^{E26}–Ets but not in N-1120-I–Ets (data not shown). The failure of cooperation between Ets and N-1120 in *trans* suggests that v-Ets can provide a *cis*-acting function which compensates for the lack of the FAETL region in N-1120 and N-Myb^{E26}–Ets.

On the basis of the following experiments, it had been speculated that the C-terminal region of v-Myb^{AMV}, missing in v-Myb^{E26}, conferred growth factor independence: (i) retroviruses containing both v-Myb^{E26} and cMGF induce myeloid leukemias at high efficiency, in contrast to v-Myb^{E26} alone (36); and (ii) murine myeloid leukemia cell lines NFS-60 and VFLJ2, with a C-terminal truncation of c-Myb within the FAETL motif, are interleukin-3 dependent, although the requirement for interleukin-3 in VFLJ2 can be abrogated by v-Myc (55). The observations from our laboratory also support this idea. In our standard transformation assay, we do not add any exogenous growth factors. v-Myb^{AMV}-transformed cells grow well, whereas a mutant LX3 with a similar C-terminal truncation to v-Myb^{E26} does not transform in standard assays unless macrophage feeder layers are present (20). In the present study, we found that only a small number of transformed cells grew out upon infection with N-1120, a v-Myb deletion mutant with a truncation just before the FAETL motif. Interestingly, these N-1120-transformed cells are similar to AMV-transformed monoblasts (data not shown). This result implies that the FAETL motif is important for promoting the proliferation of transformed cells but not for blocking differentiation. About 10-fold-larger cell numbers were obtained with N-1120 in the presence of chicken myeloid growth factor than in its absence over 3 weeks, but this supplement did not completely restore full proliferation ability, as seen in N-1151, which contains the FAETL motif at the C terminus (data not shown). These data suggest that the FAETL motif may enhance the growth of transformed cells by interacting with cytokine-responsive pathways. Interestingly, in our standard transformation assay, the cells transformed by N-Myb^{E26}–Ets (see above) are growth factor-independent like AMV-transformed monoblasts (data not shown). Hence, the role of the FAETL motif in transactivation, transformation, and growth factor independence in AMV can be substituted by Ets residues in a v-Myb^{E26}–Ets fusion protein.

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